Studies on The Pathogenesis and Prevention of Insulin-Dependent Diabetes Mellitus (IDDM) in the Nonobese Diabetic (NOD) Mouse.

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A thesis submitted for the degree of doctor of philosophy in the Faculty of Clinical Sciences at the University of London, May 1992.

Studies described in this thesis were conducted on two sites:

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London, W1

and: Cambridge University Department of Pathology,
Immunology Division,
Tennis Court Road,
Cambridge CB2.
I would like to dedicate this thesis to my parents, Gerald and Catherine and to the memory of my godmother for their love, support and encouragement all these years. I hope you will be proud of me.

"People must understand that science is inherently neither a potential for good nor evil. It is a potential to be harnessed by man to do his bidding"

Glenn T. Seaborg, Associated Press interview with Alton Blakeslee, Sept. 29, 1964
Thesis Abstract

The Non-obese Diabetic Mouse (NOD) spontaneously develops diabetes with many similarities to the human autoimmune disease insulin dependent diabetes mellitus (IDDM). The studies outlined in this thesis attempted to a) determine the cellular constitution of the pancreatic infiltrate (insulitis) which results in beta (β) cell destruction and insulin insufficiency b) modulate the disease by a plethora of strategies either in the adoptive transfer model (irradiated NOD males reconstituted with diabetic spleen cells, producing diabetes) or by transgenesis.

Immunohistochemical analysis of transfer recipient pancreata at weekly time intervals before diabetes onset revealed a progressive influx of both the CD4⁺ and CD8⁺ T cell subpopulations with no unusual bias in T cells bearing Vβ6, Vβ8.1, Vβ8.2, or Vβ11. Class II major histocompatibility antigen (H-2) positive cells, macrophages and hyperexpression of class I MHC antigens on both pancreatic endocrine and exocrine tissues were also observed. Two subpopulations of pancreatic macrophages could be identified, one recently recruited and actively phagocytic and the other a fixed tissue population. In vivo depletion of transfer recipients with rat anti-mouse monoclonal antibodies (Mo.Ab) was used to assess the role of each particular cell type in the inflammatory process. Depletion with anti-CD4 at the time of transfer or non-depleting anti-CD4 up to 12 days after transfer was still able to arrest the infiltration of islets and prevent further β cell destruction. Administration of anti-CD8 within 2 weeks of transfer was able to prevent diabetes and halt the massive pancreatic infiltration by T cells and inflammatory macrophages. However, depletion of Vβ8-positive cells in transfer recipients showed that these T cells are not necessary for the effector phase of β cell destruction in NOD mice.

Treatment of transfer recipients with 5C6,(a Mo.Ab against the CR3 receptor which prevents macrophage adhesion and migration), was able to prevent disease. This suggested an essential contribution of macrophages either as antigen presenting cells or as effector cells in the destruction of the β cell or both, although both CD4 and CD8 T cells were necessary for disease manifestation. One of the cytocidal activities of macrophages is mediated by nitric oxide synthesised from L-arginine. However inhibition of this pathway with an analogue of L-arginine (L-NMMA), was unable to prevent IDDM in either transfer recipients or cyclophosphamide induced diabetes, suggesting that macrophage involvement in IDDM is not
through this pathway.

IDDM is under polygenic control in both man and the NOD mouse. The NOD mouse does not express the class II MHC molecule I-E and the sequence of the I-Aβ chain is unique. Introduction of an Eα^d transgene or of a modified I-Aβ chain prevented diabetes, hyperexpression of class I MHC antigens, pancreatic infiltration by T cells and macrophages but had no effect on submandibular gland infiltration.

Thymus immunohistochemical analysis showed hyperexpression of MHC class II and germinal centres composed of B220^+(B lymphocyte) CD5^- cells present in abnormally large perivascular spaces in the epithelial cell matrix of NOD mice. These thymic abnormalities were not found in the CBA mouse strain, but were present in diabetes non-susceptible NOD-E transgenics.
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<td>ACh.R</td>
<td>Acetylcholine receptor</td>
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<td>β</td>
<td>beta cell</td>
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<td>BBrat</td>
<td>BioBreeding Rat</td>
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<td>Intracellular adhesion molecule-1</td>
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<td>ICA</td>
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<td>IDDM</td>
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<td>CMJ</td>
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<td>Interferon-gamma</td>
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<td>Ig</td>
<td>immunoglobulin</td>
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<td>i.p</td>
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<td>MG</td>
<td>myasthenia gravis</td>
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<td>NO</td>
<td>nitric oxide</td>
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<td>NON</td>
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<td>PBL</td>
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<td>peritoneal exudate cells</td>
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<td>PVS</td>
<td>peri-vascular space</td>
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<td>TNF-α</td>
<td>tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TNBS</td>
<td>trinitrobenzenesulphonic acid</td>
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“In anything at all, perfection is finally attained not when there is no longer anything to add, but when there is no longer anything to take away”

Saint-Exupery, Wind, Sand and Stars (1939), tr, Lewis Galantiere.
CHAPTER 1

Concepts of Tolerance and Autoimmunity:
A Brief History.
1.1 The Concept of Tolerance

The family of antigen specific receptors on lymphocytes is unique in its requirement to discriminate between self and non-self (foreign) ligands. Recognition of the latter activates the immune system to eliminate or sequester foreign molecules. Failure to recognise self molecules is mandatory for the preservation of host integrity. The immune system acquires this knowledge during its development, accomplished by imparting to each lymphocyte a unique receptor through genetic recombination, and then selectively deleting or inactivating the subpopulation of cells bearing high affinity anti-self receptors resulting in the acquisition of self-tolerance.

The first evidence that tolerance occurred during ontogeny came from the observations of Owen et al. (1945) on dizygotic twin cattle. Such twins were chimeric, contained blood cells of both genotypes and tolerated each other’s tissue antigens.

This initial observation stimulated a series of experiments attempting to test the hypothesis of self-learning and led Burnet and Fenner (1949) to predict that exposure of the developing immune system to foreign antigens prevented the system from responding to those antigens, as it would be mistaken for self. For example Billingham et al. (1953) demonstrated that when murine spleen cells of strain B were injected into the fetuses or newborns of strain A, these mice did not reject strain B grafts although still capable of rejecting strain C (third party) grafts. Similar results were obtained in chickens with a parabiosis experiment, in which the circulatory system of the two embryos were joined and the haematopoietic chimeric adult birds did not reject each other’s skin grafts (Hasek and Hraba et al., 1955). Further support for acquisition of tolerance during ontogeny can be gained from early studies of Traub (1938) who showed that in utero infection with lymphotrophic encephalomyelitis virus (LCMV), allowed the persistence of the virus in murine blood and tissues throughout life without an immune response, whereas if the virus was first encountered in adult life a rapid antibody response ensued. Allophasic (tetraparental) mice, derived from the fusion of two heterozygous 8 cell embryos, were shown to be tolerant to grafted tissues of the parents (Mintz and Silvers, 1967). Thus it would appear that self antigens were recognised during ontogeny and that introduction of exogenous antigen at a time when tolerance to one’s own tissues was being established, resulted in tolerance whereas antigens encountered later evoked an immune response.

Interpretation of these results in terms of the clonal selection theory of Burnet and
Talmage means that if a clone of lymphocytes with a receptor for a non-self antigen is stimulated at a certain early point in development such signal transduction leads to deletion or inactivation rather than proliferation. Tolerance therefore ensues as the animal has no cells capable of responding to the antigen, reflecting the differential sensitivity of mature and immature lymphocytes.

Adult animals could also be rendered nonresponsive to foreign antigens (Mitchison, 1964) with large doses of antigen administered intravenously or orally, rather than subcutaneously or intramuscularly, and with deaggregated rather than aggregated forms of the antigen. Impairment of the immune system with irradiation, anti-lymphocyte serum, or drugs such as cyclophosphamide, at the time of antigen administration enhanced tolerance induction (reviewed Klein, 1982). Both T and B lymphocyte compartments could be affected by the treatments, and onset of nonresponsiveness was rapid. For some T dependent antigens tolerance could be induced at two dose ranges, one lower and one higher than that used for optimal immunisation (Mitchison; 1964, 1971). The signal theory of lymphocyte activation was proposed by Bretscher and Cohn (1970) to explain lymphocyte non-responsiveness. According to their proposals two inductive signals are required for lymphocytes to be activated, antigen occupancy of the receptor and a mitogenic signal provided by the Th cell. Absence of such a second costimulatory signal results in a negative signal or tolerance.

1.2 The Mechanisms of Tolerance Induction

Many models have been developed to study the mechanisms of tolerance induction (Weigle 1973, Nossal 1983, Scott 1984). Several possible mechanisms for tolerance induction exist. (i) **Clonal anergy.** In this model self reactive cells were postulated to be functionally inactivated without being physically deleted, possibly by receptor blockade, however in most experimental systems, no molecular mechanism has been uncovered. (ii) **Clonal suppression:** Tolerance was proposed to be mediated by antigen specific T suppressor cells which bear the CD8 surface marker and actively turn off T lymphocytes in an antigen-specific MHC restricted manner. These cells appeared to be part of a complex circuit of interacting cell types with the overall function of negative feedback inhibition of the immune system. (iii) **Clonal deletion:** T cells bearing self-reactive receptors were clonally deleted in the thymus. Each of these mechanisms is not exclusive and several may exist in parallel. This may occur because clonal deletion does not remove all autoreactive T
cells, especially in the case of tissue specific antigens not expressed in the thymus. It is clear from the literature that many different mechanisms of tolerance induction operate in different experimental or natural systems and indeed the mechanism of tolerance induction and maintenance may be different for T and B cells.

1.2.1 Clonal Anergy of B Cells

Recognition of the physiological imperative of avoiding autoantibody production has been appreciated since the first demonstration in 1900 of the destructive effects of isoantibodies on genetically mismatched blood cells (Ehrlich and Morgenroth, published 1957) and the possibility that “formation of autotoxins” (auto-antibodies) could constitute a dangerous threat to the organism. Thus prevention of autoantibody production and maintenance of B cell tolerance was thought to be imperative. Antigen-specific B cells need to collaborate with antigen-specific T cells to mount efficient antibody responses to many foreign antigens (Claman et al., 1966, Vitetta et al., 1989). Therefore failure of B cells to produce auto-antibodies could merely reflect deletion of antigen-specific T cells (lack of Th) rather than any change in the B cells themselves. However, T and B cells collaborate in such a way (Mitchison; 1971, Lanzavecchia; 1985) that T cells specific for foreign antigen may interact with self-reactive B cells when a foreign antigen becomes noncovalently associated with a self antigen. The absence of high-affinity auto-antibodies to self-antigens in these situations indicates that both B and T cells may be affected for the acquisition of self tolerance in the humoral compartment.

In some natural models of tolerance, the B cells appear to be fully responsive when tested with normal T cells. However in vitro studies suggest that B cells can be made tolerant. Exposure to antigens in the absence of T cells can render the B cell nonresponsive to subsequent challenge with antigen in the presence of Th cells (Metcalf and Klinman, 1976). Furthermore immature B cells appear to be more sensitive to low concentrations of antigen.

Transgenic mice in which hen egg lysozyme (HEL) is introduced into the germline (Goodnow et al., 1988) result in mice tolerant for lysozyme in both the T and B cell compartments. If T cell tolerance is bypassed by immunisation with HEL coupled to a foreign carrier (SRBC) autoantibody production is not completely prevented, although that which is generated is of a lower affinity. This would suggest that only the higher affinity B
cells become tolerant to HEL and that self-reactive B cells may need to bind a critical
threshold of self-antigen in order to be rendered tolerant (Hari and Roth, 1987). In
physiological terms the failure to induce tolerance in low-affinity anti-self B cells to many
self antigens (Benjamin et al.; 1984, Casali and Notkins; 1989, Goodnow et al.; 1990)
appears to pose little risk of autoimmune disease in most cases. Titres and affinities of such
antibodies are normally below those needed to initiate tissue destruction or to interfere with
biological functions. A further line of transgenic mice expressing the rearranged
immunoglobulin heavy and light chain genes encoding a high-affinity anti-lysozyme
antibody was created (Goodnow et al.; 1988, 1990). When mated with the previous
transgenic line the resultant double transgenic mice had 60-90% of the B cell population
expressing the anti-HEL receptors. Such receptors were functionally silent however,
resulting in almost complete absence of the anti-HEL response and down regulation of IgM
expression. Anergy in these B cells was reversible as they were capable of producing anti-
lysozyme antibody when transferred together with helper T cells into nontransgenic mice
(Goodnow et al., 1991). Therefore the inability to respond in this case would appear to be
the property of the B cell which has been functionally anergized (Nossal, 1983) rather than
clonally deleted.

However, when mice transgenic for an IgM specific for MHC class I molecules of
the K haplotype and which express the transgene on 25-50% of B cells are crossed with an
MHC K haplotype animal, the B cells in the F1 animals expressing the transgene are
deleted in the bone marrow (Nemazee and Burki, 1989). Thus, overall it would appear that
both clonal deletion and clonal anergy can operate and the mechanism employed may
depend on the antibody isotype.

1.2.2 Suppression

In contrast to clonal anergy, which has been extensively studied in the B cell
compartment, suppression remains a T cell dependent phenomenon, perhaps required to
restrain those antigen-binding B cells present in tolerant and non-responsive animals. Non-
responsiveness to high antigen doses in some models could be "infectious" (Gershon and
Kondo, 1971) in that mixtures of nonresponsive and normal cell populations gave no
response. From this finding emerged the concept that suppressor T cells which bear the
CD8 surface marker actively turn off other T lymphocytes in an antigen specific manner.
Such suppressor cells appeared to be part of a complex circuit of interacting cell types controlled in turn by contrasuppressor T cells. Control is governed by the secretion of suppressor factors, with the overall function of negative feedback inhibition of the immune system (reviewed in Germain and Benacerraf, 1981).

However the whole body of cellular experiments documenting suppression and identification of a specific lineage of Ts has recently been called into question. Although T cell clones with suppressor phenotype were obtained as hybridomas, genes encoding suppressor factors could not be generated from cell lines or soluble suppressor factors isolated from their supernatants.

Another explanation for the concept of suppression came from the observations by Parish and Liew (1972). At low antigen doses, where antibody responses were minimal DTH responses were maximal, suggesting that there were two types of T cells, one helping B cells make antibody, the other mediating DTH responses. These two cell types could negatively regulate each others growth and/ or function. Later these cells were designated by Mossmann et al (1986) TH1 and TH2 based on the observation that murine CD4+ T cell clones could be subdivided into two groups according to their cytokine profiles. Demonstration that IFN-γ produced by TH1 clones inhibited the growth of TH2 clones, could represent such an example of negative regulation (Gajewski and Fitch, 1988) and explain some of the suppressor phenomena previously described. This dual response model however, cannot account for all of the suppressor phenomenology of the 1970’s. There still remains to be explained the poorly characterised CD8+ T cell, which could be an intervening regulatory cell in the TH1 to TH2 negative feedback pathway, as CD8+ T cells produce large amounts of IFN-γ. Further IL-10 which acts on the antigen presenting cell can also inhibit cytokine production by Th1 clones (Florentino et al, 1991).

The phenomenon of suppression can be explained in several ways; it could represent the balance of cells capable of responding to a particular antigen with the potential for autoreactivity in the organism versus the presence of a regulatory cell capable of damping down the response. Alternatively suppression could represent the vital cell required in the chain of events for elicitation of an immune response.
1.2.3 Tolerance and Clonal Deletion of T Lymphocytes

It is not the purpose of this thesis to provide a detailed overview of the literature on thymic education or the mechanisms of clonal deletion, but it was considered necessary to provide some background to my own studies relating to NOD transgenic mice and the thymus, therefore only a brief discussion of relevant points is provided.

Antigen is usually recognised by T cells as a peptide embedded within major histocompatibility gene products (H-2 or HLA) (Babbitt et al, 1985; Blackman et al, 1990). The MHC is a gene cluster that encodes proteins whose function is to bind foreign peptides for presentation to T cells. In the mouse, MHC products are termed according to tissue distribution. MHC class I molecules (K, D and L) are present on nearly all nucleated cells, whereas class II molecules (I-A and I-E) are largely restricted to thymic epithelium and antigen presenting cells such as macrophages, B cells and dendritic cells.

During T cell ontogeny, precursors (prothymocytes) arise in the bone marrow (or foetal liver) and migrate to the thymus. Inductive stimuli within the thymus cause cell division and the commencement of T cell receptor (TCR) gene rearrangement. T cells bear two mutually exclusive sets of receptors αβ (Ferrick et al, 1989) and γδ (Raulet et al, 1989). The αβ-lineage represents the major developmental pathway, and little is known about the specificity and function of the γδ receptors. Each TCR gene is composed of many genetic elements V, (D), J and C, which rearrange to form the somatically functional gene. Successful rearrangement of γ and δ genes stops rearrangement of the β gene and vice versa (Adkins et al, 1987; Hugo et al, 1991). Diversity is generated from both the large number of different members of each family and from mechanisms that alter DNA at the joining regions of the V-D, D-J and V-J elements and N-region diversity. The T cell passes through a series of rearrangements, stops dividing and expresses at low levels receptors including the CD3 complex (involved in signal transduction) along with the accessory cell surface proteins CD4 and CD8 (Doyle and Strominger, 1987). Either the CD4 (reviewed Schwartz, 1989) or the CD8 (Hugo et al, 1991) molecule is first expressed as the transient intermediates CD4+CD8loTCRLOW, CD4loCD8+TCRLOW, followed by the additional expression of CD8 or CD4 to these intermediates respectively, giving rise to the
CD4^CD8^ stage. It is at this point that the cells undergo the selection events involved in tolerance induction. When cells emerge from this stage they have lost one of the accessory molecules (CD4 or CD8) and become single-positive cells. The result of this selection is two sets of mature T cells; one set that uses CD4 and recognises foreign antigen complexed to MHC class II molecules, and a second set that uses CD8 and recognises foreign antigens complexed with class I MHC molecules (Ramsdell and Fowlkes, 1990).

There are two separate mechanisms for tolerance induction in the thymus. One involves deletion of thymocytes that express T cell receptors (TCR) with high affinity for MHC molecules expressed within the thymus. The existence of such a mechanism was discovered when it was observed that the frequency of T cells reactive against a particular antigen is greatly reduced in tolerant animals (Good et al., 1983). The first understanding of mechanisms of thymic tolerance came from Kappler's observation that cells expressing the variable segment of the β chain Vβ17a which has a known specificity for I-E were deleted in I-E expressing mice. Vβ17a expressing cells were present in normal numbers in the immature thymic CD4^CD8^ double-positive subset, but diminished in the mature CD4^8^- and CD4^8^ T cells suggesting they were deleted between the double-positive and single-positive stages. This system has also been used to show that the CD4^8^ cells must recognise MHC class II molecules in order to be deleted (Fowlkes et al., 1988) as treating with a M.Ab against the I-E molecule blocked the deletion of Vβ17a^ cells from the mature CD8^ population. Further evidence for clonal deletion was obtained in other experiments involving other TCR Vβ regions (Kappler et al.; 1987, Pullen et al.; 1988, Bill et al.; 1989).

Another family of self super-antigens capable of deleting particular TCR Vβ T cells is the Mls (minor lymphocyte stimulating) antigens. The presence of self superantigens in a given mouse can be detected by the absence of mature T cells bearing receptors encoded by particular Vβ gene segments. Cells bearing these autoreactive receptors are generated in the thymus but deleted before maturation to maintain non-responsiveness or tolerance to self superantigens (Janeway, 1991). Mls antigens are encoded outside the
MHC, and were originally described by Festenstein (1973) because of their ability to initiate a vigorous MLR between MHC identical strains of mice. The stimulating cells (from Mls positive mice) are predominantly B cells, while the responding cells (from Mls negative mice) are usually CD4+ T cells. Mls antigens are derived from a number of unlinked loci, each of which encodes a diallelic system. Each locus is assigned a number and one allele is stimulatory in an Mls (a allele) and the other is not (b allele-null allele). T cell reactivity against Mls gene products is determined by the Vβ domain of the TCR. For example in mice expressing the Mls-1a gene product, T cells expressing the TCR Vβ6 (MacDonald et al., 1988), Vβ7 (Okada et al., 1990), Vβ8.1 (Kappler et al., 1988) or Vβ9 (Happer et al., 1989) are deleted. These Vβ domains are present among the immature TCRlow CD4+CD8+ (double-positive) thymocyte population but are clonally deleted from the mature T cell population as they have self reactivity for the Mls-1a gene product. Other Mls loci encode proteins that are reactive with different Vβ domains such as Mls-2a which deletes Vβ3+ T cells (Pullen et al.; 1988, Abe et al.; 1988). Presentation of the Mls proteins differs from that of conventional antigens as the requirement for a particular allelic MHC protein is not as stringent as it is for the presentation of a conventional peptide antigen but similarly results in deletion (Webb et al., 1989).

Secreted enterotoxins from a number of pathogenic bacteria such as the staphylococcal, streptococcal and mycoplasma genera are capable of acting as superantigens. These superantigens display strong binding to MHC glycoproteins and are potent T cell mitogens. Enterotoxin-pulsed APCs interact with both murine and human T cells in a way that is defined by expression of particular Vβ gene segments such that the superantigens (enterotoxins) injected into neonatal mice result in the clonal deletion of particular sets of Vβ-bearing T cells and stimulation of T cells expressing specific TCR Vβ chains in in vitro assays.

Woodland (Woodland et al., 1990, 1991) initially discovered a linkage between a gene encoding a ligand responsible for TCR Vβ5.2+ T cell deletion and the endogenous
mouse mammary tumour virus Mtv-9. Dyson (Dyson et al, 1991) subsequently showed that Etc-1 a Mls-like antigen can delete Vβ11 in addition to Vβ5-bearing T cells. This antigen could not be segregated from the mammary tumour viral pro-virus, Mtv9 and therefore Etc-1 is encoded by Mtv-9 itself.

Further data from three labs have shown that Mtv’s encoded other Mls-like gene products. Frankel et al (1991) demonstrated a perfect genetic linkage between prototypic superantigens and Mtv. Dyson et al (1991) identified 3 non-MHC loci involved in the clonal deletion of Vβ11+ T cells which map to Mtv. Finally Marrack et al. (1991) found that the C3H/HeJ mouse strain deletes most of its complement of Vβ14+ bearing T cells and that this deletion is maternally inherited and passed through C3H/HeJ milk. Thus the presence of many independently segregating Mls loci affects deletion of different subsets of T cells and reflects the presence of different unlinked endogenous MMTV (murine mammary tumour virus) proviruses, which on rare occasions integrate into the mouse genome, each producing an orf-encoded gene product of different primary sequence. The diallelic system, with a stimulatory and a null allele is explained by whether or not a mouse carries a particular MMTV integrant. It is postulated that like the enterotoxins, the viral proteins bind to MHC class II gene products with a relatively high affinity, cross-linking TCRs and MHC class II molecules. This binding of the APC and the T cell results in T cell stimulation, deletion or anergy, depending on the developmental state of the T cell or the experimental system (Janeway, 1991). It would appear that tolerance in the foregoing studies is mediated by intrathymic elimination of self reactive cells rather than by clonal anergy or suppression.

Another model by which tolerance can be achieved was discovered while studying T cell tolerance to the male specific H-Y antigen in male mice (this has been more clearly shown in T cell receptor transgenic mice). Cytotoxic T cells with specificity for the male H-Y antigen in the context of MHC Db were cloned together with the genes encoding the α and β chains of their TCR. CD4+CD8+ lymph node T cells from female mice transgenic for both α and β chains of this TCR proliferated in vitro in response to H-Y plus Db whereas very few responded in male mice. The defect was located to binding
affinity as male mice were found to have a low or undetectable level of the CD8 molecule, preventing antigen recognition. The thymus in male mice had 10-fold fewer cells than female, due to a loss of double positive cells which suggested that thymocytes specific for H-Y were either being deleted in large numbers at the double positive stage, or earlier at the CD4^+CD8^+ precursor stage (Kisielow et al, 1988).

In another transgenic model (Sha et al, 1988) mice carrying genes encoding a receptor from a T cell clone reactive against the class I molecule L^d, had T cells with diminished expression in crosses with mice expressing L^d. In this case also a deletion event took place in the thymus prior to, or at the double-positive stage, and those cells escaping negative selection had little or no CD8 expression. It is clear from these examples that deletion of T cells with the potential to respond to self antigen occurs in the thymus before they mature and are released into the periphery.

1.3 Positive Selection

In addition to the deleting process (referred to as negative selection), T lymphocytes developing in the thymus are thought to undergo a second selection event that of positive skewing in which the T cell repertoire is biased toward the recognition of the particular allelic forms of the MHC molecules expressed in the thymus (reviewed Sprent; 1988, Schwartz; 1989). Studies by Bevan on radiation-induced bone marrow chimeras, showed that irradiated strain A mice repopulated with bone marrow from (AxB)F1 mice developed T cell responses that preferentially involved recognition of antigens in association with MHC molecules of type A. Radio-resistant elements of the host must limit the potential repertoire of the T cells independent of the tolerance mechanism (Schwartz, 1989). Zinkernagel demonstrated that not only was the thymus required for this skewing but radioresistant H-2 expressing thymic epithelial cells were the critical selecting elements (Schwartz, 1989). Further evidence involving in vivo antibody blocking and T cell receptor transgenic mice, strongly support this concept (Marrack et al; 1988A, Marusic-Galesic et al; 1989).

Von Boehm (Von Boehm and Schubiger, 1984) has suggested that deoxyguanosine-sensitive, bone-marrow derived, dendritic cells are responsible for
negative selection (at least for class II molecules) while positive selection appears to be unaffected by deoxyguanosine treatment. Such findings were supported by Marrack and Sprent (Marrack et al., 1988B; Sprent et al., 1988). All data were therefore consistent with the idea of separate selecting cells.

The current model for thymic T cell positive selection suggests that prothymocytes migrate to the thymus, where they express their functional TCR along with both the CD4 and CD8 molecules (double positive stage). Interaction with MHC molecules on thymic epithelial cells results in either MHC class I recognition leading to commitment toward the CD8+ Tc/s lineage, or MHC class II recognition leading to commitment toward the CD4+ Th/i lineage (Schwartz, 1989).

Various hypotheses have arisen to explain simultaneous positive and negative selection. One model invoked receptor affinity and suggested a quantitative difference in the MHC product required to bring about positive or negative selection. Positive selection may act on thymocytes bearing receptors with a high and modest affinity for self MHC products. Negative selection however, may delete only those thymocytes bearing receptors with high affinity for self MHC, allowing low affinity receptors to escape into the periphery where they form the pool of self restricted T cells (Sprent et al., 1988). Therefore cells escape from programmed cell death with modest receptor occupancy and die with either high or no receptor occupancy.

Marrack and Kappler (Marrack et al.; 1988B, Marrack and Kappler; 1988B) have suggested an "altered ligand" hypothesis to account for the paradox of selection for, and tolerance to, self histocompatibility antigens. This hypothesis uses the discovery by Bjorkman (Bjorkman et al., 1987) that MHC proteins bind peptides in a pocket on the external surface of the molecule, and that in the absence of foreign antigen, self histocompatibility antigen contains bound peptides derived from self proteins. There is a spectrum of peptides bound to histocompatibility antigens which vary from tissue to tissue. Therefore, self MHC molecules on thymic epithelial cells do not contain the same spectrum of self peptides as MHC molecules on B cells and macrophages. Thus thymocytes may be positively selected by interaction of their receptors with self histocompatibility antigens plus epithelial cells, but clonally deleted by interaction of their receptors with self histocompatibility antigens plus peptides expressed on bone marrow derived cells such as
dendritic cells at the cortico-medullary junction. At the present time, neither model appears completely satisfactory.

1.4 Peripheral Tolerance

The concept of clonal deletion in the thymus appears to be an adequate explanation for many models of T cell tolerance, nonetheless, certain paradoxes remain. One such exception is observed when the anterior third of a Xenopus 24 hour MHC A embryo (containing the thymic stromal anlage) is grafted onto the posterior 2/3 of an MHC B embryo (containing the haematopoietic yolk sac anlage), the resulting animal harbours lymphocytes that can react against MHC A molecules in tissue culture, yet the animal does not reject its own head (Flajnik et al, 1985).

Several hypotheses have been put forward to explain how the immune system establishes and maintains tolerance to self components that are expressed extrathymically and are restricted to non-lymphoid tissues. Tissue-restricted self components may be processed and transported by circulating bone-marrow derived cells back to the thymus for tolerance induction. Alternatively, peripheral tolerizing mechanisms may include (i) clonal deletion, (ii) clonal inactivation or anergy, (iii) suppression, (iv) expression of self antigens in “privileged sites” that remain sequestered from the immune system. Finally, (v) expression of self molecules on cells exposed to the immune system that are incapable of providing appropriate signals for T cell activation and antigen presentation could also result in tolerance.

Understanding of this issue has been achieved by deliberately targeting MHC expression to particular cell types to yield precisely defined expression of desired molecules in vivo through transgenic mouse technology (Palmiter and Brinster 1986). Aberrant appearance of increased amounts of MHC class II molecules has been observed on non-lymphoid cells in diseased states, such as on thyroid cells in autoimmune thyroiditis (Bottazzo et al, 1983) and on human pancreatic β cells in IDDM (Bottazzo et al, 1985).

Evidence that T cell anergy results from expression of MHC class II molecules on cells incapable of providing appropriate signals for T cell activation was provided by Lo (Lo et al, 1988; Quill and Schwartz, 1987). Transgenic mice expressing I-E on pancreatic β cells were tolerized and failed to develop pancreatic lymphocytic infiltration, although
animals developed non-autoimmune insulin dependent diabetes. Vβ17a+ cells which were deleted normally in I-E expressing mice were not deleted in the transgenic mice. Islets retained their integrity even when the mice were primed with I-E+ spleen cells but were rejected if grafted into I-E- mice and these recipients primed with I-E+ spleen cells (Markmann et al, 1988). The tolerance mechanism in this instance involved inactivation of the T cells in the transgenic mouse and not masking of the I-E molecules on the islets as transgenic mice appeared functionally tolerant to pancreatic transgenic products in vivo. This tolerance was complete since priming of transgenic mice to the transgenic alloantigen in vivo and in vitro did not induce autoimmunity.

Similarly, transgenic mice expressing islet cell-specific self or non-self I-A (Bohme et al; 1989, Miller et al; 1990) did not develop lymphocytic infiltrates in the pancreas, yet developed diabetes in some of these cases. T cells from these mice were not tolerant to I-A in vitro. The mechanism that causes the diabetic phenotype of these mice is not known. Additionally class I MHC β cell transgenic mice (RIP-Kb), developed diabetes, but did not appear to mount an immune response against the transgene-expressing β cells even when the transgene was allogeneic with respect to the host (Allison et al, 1988). Two groups have also generated transgenic mice with I-E or I-A molecules targeted to pancreatic acinar cells. These mice also showed no evidence of lymphocytic infiltration in the pancreas. T cells of mice expressing I-E with the elastase promoter (El-I-E mice) (Lo et al, 1988) or allogeneic I-A (Murphy et al, 1989) are also functionally tolerant to the class II proteins. Further priming of El-I-E mice in vivo with subcutaneous injections of I-E+ spleen cells did not stimulate autoimmune destruction. However the I-A transgenics did show T cell reactivity to the transgene in vitro. Although all transgenic mice are functionally tolerant in vivo (because lymphocyte infiltrates are absent) Ia reactivity is detectable in vitro. The I-E reactive T cells detected by M.Abs to Vβ17a and Vβ5 were not deleted in Ins-I-E and EL-I-E mice backcrossed to I-E- strains, thus in vivo tolerance is not caused by clonal deletion of these I-E reactive populations.

Thus several independent labs have established numerous transgenic lines that have class II or class I molecules expressed specifically on pancreatic islet or acinar cells. In
these systems the transgene products were not detected in lymphoid organs, most notably
the thymus, yet T cells of these mice appear to be functionally tolerant \textit{in vivo} to the
transgenic proteins as lymphocyte infiltrates were absent.

Therefore tolerance induced by the aberrant expression of class I/II on β cells and
exocrine cells could occur by clonal inactivation. Such paralysis of T cells could result from
exposure to antigen and class II molecules in the absence of a second costimulatory signal
normally delivered by APC. Beta cells may be incapable of supplying such a signal and
therefore could functionally inactivate T cells. T cells paralysed in this manner are
subsequently unresponsive to both engagement of the TCR receptor-mediated cross-linking
and costimulatory signals. Additionally some I-E reactive T cells may be rendered anergic
\textit{in vivo}, or at least have a threshold of activation that differs between transgenic T cells and
T cells that are not tolerant to I-E. Compared to control mice, Ins-I-E transgenic T cells
respond weakly if at all to anti-Vβ17a and anti-Vβ5 and EL-I-E transgenic T cells respond
strongly with Vβ17a but poorly to anti-Vβ5.

Observations by Morahan (Morahan et al, 1989) that thymus cells but not
prediabetic spleen cells from RIP-K\textsuperscript{b} transgenic mice killed target cells bearing H-2K\textsuperscript{b}, and
that unresponsiveness by spleen cells was overcome by the addition of rIL-2, may provide
an alternative explanation for tolerance induction. The authors suggested that in older mice
responsiveness developed as pancreatic β cells were lost, and that tolerance therefore was
dependent on the continued presence of antigen, and lack of IL-2 in the local environment
of potentially reactive T cells. Their evidence pointed to an extrathymic mechanism of
tolerance induction in which the CD8\textsuperscript{+} helper cell subset for class I alloresponses (Singer
\textit{et al}, 1987) could have been inactivated by inappropriate presentation in the periphery, as
suggested for CD4\textsuperscript{+} cells in the class II β cell transgenic experiments (Markmann \textit{et al},
1988).
1.5 Biochemical Aspects of Tolerance Induction

Immunity and tolerance induction both depend on recognition events initiated by the T cell antigen receptor. The Bretscher and Cohn (1970) model suggests that two signals are required to activate a lymphocyte: one, receptor occupancy, the other, a costimulatory signal delivered by a second cell. In the case of B lymphocytes this comes from activated T cells, in the case of T lymphocytes, the second signal comes from APC such as the dendritic cell or macrophage. Tolerance induction was therefore postulated to be delivery of signal one in the absence of signal two. Activation of PKC (protein kinase C) by DAG (diacylglycerol) and IP3 (inositol triphosphate) to raise intracellular free calcium is the critical event for the induction of non-responsiveness as ionomycin, a calcium ionophore that raises intracellular Ca\(^{2+}\) levels, is sufficient to induce an nonresponsive state in vitro (reviewed in Mueller et al, 1989). These biochemical events in peripheral T cell activation could also be involved in clonal deletion in the thymus, as CD4\(^+\)8\(^+\) thymocytes are capable of responding with a rise in intracellular calcium when T cell receptors are cross-linked with anti-receptor antibodies (Havran et al, 1987). Furthermore, calcium ionophores, at concentrations that are not toxic to peripheral T cells, kill double-positive thymocytes through chromatin cleavage and apoptosis. Finally cyclosporin A a drug thought to block signal transduction through the Ca\(^{2+}\) pathway inhibits clonal deletion of V\(\beta\)17a+ and V\(\beta\)11+ T cells in mice expressing I-E (Jenkins et al, 1988)). Thus, clonal deletion could result indeed from T cell receptor occupancy in the absence of a costimulatory signal. Because dendritic cells from the spleen which are capable of delivering the costimulatory signal to mature T cells can induce antigen-specific nonresponsiveness in thymic organ cultures it is likely that the failure of the T cell to receive a costimulatory signal, rather than the failure of thymic dendritic cell to deliver a signal, would be the basis for tolerance induction (Matzinger and Guerder, 1989).
Autoimmunity may be defined as the termination of unresponsiveness to self-antigens which can be induced either by the loss of specific unresponsiveness (tolerance) or by the development of a primary immune response to an autoantigen (Sinha et al, 1990). Autoimmunity has a multifactorial aetiology. Genetic predisposition and environmental factors which interfere with the immune regulation, are among the precipitating factors leading to disease manifestation (Smith and Steinberg, 1983). The conceptual framework for understanding the basis of autoimmunity has changed drastically in the past few years (Smith and Steinberg, 1983). Ehrlich's views that autoimmunity was a "horror autotoxicus" which the organism should avoid at all costs and Burnet's theory of thymic censorship, which was supposed to prevent self reactive cells from emerging as "forbidden clones" (Burnet, 1959) have now been refined to include a physiologic rule (Beutner et al, 1979).

Grabar suggested that autoimmunity could represent the normal mechanism for the transportation of autoantigen not degraded by the body's enzyme system (Grabar, 1975). The observation that senescent red blood cells coated with specific IgG are removed by macrophages, supports this proposition (Kay, 1975). Furthermore B cells are capable of binding self components, and low levels of auto-antibodies (natural antibodies) are detectable in normal serum (Guilbert et al, 1982) with reactivity to antigens released by breakdown or alteration of tissue components. For example anti-heart antibodies are often generated as a consequence of injury caused by myocardial infarction (Liem et al, 1979), appearing as a result of, rather than being responsible for, tissue damage.

Autoimmunity can be a consequence of a normal immune response directed against antigens cross-reacting with determinants on normal cells or an abnormal immune response to a normal self antigen. It can also result following exposure to self antigens which are normally sequestered (such as the testes and lens of the eye) following trauma or infection. Aspermatogenesis is a common result of vasectomy due to formation of anti-sperm antibodies (Isahakia and Alexander, 1984) and other sequestered antigens are also capable of stimulating the formation of antibodies that can react with the corresponding antigens derived from the same tissue of other species (Shulman, 1974).
1.7 Autoimmune Disease

Ehrlich and Morgenroth (Ehrlich and Morgenroth, 1900) originally proposed the concept of autoimmunity as the phenomenon of “horror autotoxicus” when Landsteiner described a complement dependent autohaemolytic serum antibody. Autoimmunity encompasses such seemingly unrelated diseases as insulin dependent diabetes mellitus (IDDM), multiple sclerosis (MS), rheumatoid arthritis (RA), myasthenia gravis (MG) and psoriasis, all of which appear to be due to the failure of the normal mechanisms of self tolerance. Autoimmune diseases of which there are more than 40 in number affect 5 to 7% of the population often causing severe disability and thus are a major cause of chronic illness.

The mechanisms resulting in autoimmune pathology are as diverse as the disease manifestations themselves. Susceptibility to almost all autoimmune disease is strongly influenced by genes encoded within the MHC particularly class I and class II MHC molecules. In particular within the class II-associated diseases, there is a subdivision between organ-specific and multisystem (systemic) autoimmune disease. In general organ-specific autoimmune diseases are characterised by autoantibody patterns that are primarily directed to a single organ or closely related organs (reviewed Tiwari and Terasaki 1985). Systemic autoimmune disease are characterised by a variety of autoantibodies specific for nuclear and cytoplasmic molecules involved in DNA replication, DNA transcription and mRNA translation. Some of the manifestations of systemic autoimmunity are due to direct effects of these autoantibodies, whereas others are due to antigen-antibody complex disposition.

Autoimmune disease can also be classified in terms of final effector mechanism. Specifically IDDM and MS are apparently due to the action of T cells (Ogawa et al; 1985, Hafler and Weiner; 1989), whereas hyperthyroidism (Grave’s disease) and MG result from specific antireceptor antibodies (Lindstrom, 1979), and much of the pathology in systemic SLE and polyarteritis nodosa is due to the deposition of antigen/antibody complexes (Theofilopoulous and Dixon, 1981).

MHC class I-associated autoimmune diseases are few in number and fall into many categories; (i) the HLA-B27-related spondyloarthropathies, and (ii) psoriasis vulgaris associated with HLA-B13, B16 and B17.
Susceptibility to both class I and II associated autoimmune diseases is multifactorial. Autoimmunity is polygenic, and most autoimmune diseases are not inherited in a simple Mendelian segregation. For example the concordance for IDDM in monozygotic twins is less than 50% (Rotler and Landau, 1984) and is less than 5% for MS. Comparison of the concordance rate for HLA-identical monozygotic twins compared to that for HLA-identical siblings indicates that MHC genes are not the sole genetic factor in determining susceptibility to autoimmunity. However apart from relatively weak effects of immunoglobulin loci on susceptibility to hyperthyroidism and possibly MS (Davidson et al, 1987), the remaining genes determining susceptibility are unidentified.

Some autoimmune diseases show significant MHC class II association. Therefore the major aims in autoimmune disease are to characterise the self or cross-reacting environmental antigens that contribute critical peptide epitopes; the MHC alleles that are most effective in presenting these self peptides and identification of the T cell repertoire utilised for recognition of this self peptide-self MHC molecular complex. Nucleotide sequence analysis of class II genes from autoimmune patients has shown that disease is not the result of mutant MHC alleles, although it has been possible to identify short stretches of sequence or critical residues that may play a major role in susceptibility and resistance to some diseases such as IDDM (Todd et al, 1988). Clearly non-conservative changes at any of these positions could alter the structure of the peptide-binding groove (Todd et al, 1987) and affect class II function.

Genetic linkage studies have implicated the variable region gene of the TCR in the aetiology of autoimmune diseases. In murine collagen induced arthritis, mouse strains that have a genomic deletion of 50% of the V region genes of the TCR β-chain loci are resistant to RA induction despite the presence of a permissive MHC haplotype (Banerjee et al; 1988, Haqqi et al; 1989). Although genomic deletions in the human TCR Vα and Vβ region genes have not been identified, these loci do exhibit limited sequence polymorphism (Concannon et al, 1987).
1.8 Mechanisms of Autoimmunity Induction

In addition to the breakdown of tolerance following trauma or by exposure to previously sequestered antigens, further mechanisms exist by which the requirement for specific T cell help by immunocompetent B cells can be overcome. The immune mechanisms involved in the pathogenesis of autoimmunity are those described by Coombs and Gell (Coombs and Gell, 1975), anaphylactic (type I), cytotoxic (type II), antigen-antibody complexes (Type III) and cell mediated (Type IV). These specific mechanisms can be augmented by nonspecific measures: amplification cascades involving either cellular (via lymphokines), or humoral (ie. complement, coagulation, kinin and fibrinogen) systems. The overall interaction of these complex processes leads to the specific outcome of each disease.

1.8.1 Cross-Reacting Antigens Can Induce Autoimmunity.

T cell tolerance can be overcome by cross-reacting antigens (Benjamin and Weigle; 1970, Habicht et al; 1975). For example rabbits tolerant to BSA at birth, as adults make antibody responses to BSA when injected with HSA which has 15% sequence homology with BSA (Benjamin and Weigle, 1970). Novel antigenic determinants allow T helper cells to provide support for both B cells reacting specifically with HSA and those B cells recognising cross-reactive determinants on BSA. Furthermore, auto-antibodies to self thyroglobulin occur in rabbits following immunisation with cross-reacting antigens on bovine thyroglobulin (Rose et al, 1965). Similar lesions are produced in animals by immunisation with homologous thyroid extract mixed with Freund’s adjuvant (Rose and Witebsky, 1955). Exogenous microbial determinants that are sufficiently similar to cross-react with host determinants, but sufficiently different to break immunological tolerance, may also provoke autoimmunity (Oldstone, 1989). For example acute rheumatic fever initiated by group A beta-haemolytic streptococci in susceptible individuals results in an immune response, which cross-reacts to self antigens in the myocardium and joints (Kaplan and Svec, 1974). Therefore an immune reaction initially directed against a pathogen could result in an anti-self response.

The functional importance of similarities between self antigens and those of a pathogen stems from the findings that (i) the implicated pathogen can on occasions be demonstrated in, or recovered from, patients with autoimmune disease, (ii) autoantibodies
specific for a self antigen often have specificity for viral or bacterial determinants, and (iii) antigens from the organism may share sequence similarities with HLA haplotypes. This is seen in Reiter’s syndrome where there is a high incidence of class I HLA-B27, as the amino acid sequence of HLA-B27 shares a region of 5 or 6 amino acids with a *Klebsiella pneumoniae* nitrogenase protein (Schwimmbeck et al.; 1987, Bessen et al.; 1989).

### 1.8.2 Viral, Bacterial and Drug Induced Autoimmunity

Exogenous antigenic determinants including bacterial, viral or pharmaceutical may combine with self antigen and form immunogenic units recognised by Th cells prompting B cells to produce autoantibody to the self antigen and resulting in autoimmunity.

Drug induced autoimmune diseases have been observed in patients during administration of hydralazine or procainamide, as the drug or its metabolites bind to an autoantigen. These patients form antinuclear antibodies that resemble those of patients with systemic lupus erythematosus (SLE) (Weinstein, 1980; Miller and Salem, 1982; Ochi et al., 1983) or haemolytic anaemia (Worlledge, 1973; Kirkland et al, 1980)). Autoimmunity may result from stimulatory or inhibitory effects of a drug on immunoregulatory cells. It has been suggested that alpha-methyldopa inhibits suppressor cells by activating cyclic AMP and allows B cells to produce antibodies with the specificity for Rhesus antigen, thus inducing autoimmune haemolytic anaemia.

The response to viral infection may be disabling or fatal, as polyclonal activation of B cells resulting in autoimmunity may occur as a result of infection. For example B cell infection with Epstein Barr Virus (EBV) causes B cell proliferation resulting in autoantibody induction to various tissues (Garzelli et al, 1984). A positive correlation exists between the presence of EBV viral sequences in salivary gland tissue and primary Sjogren’s syndrome in humans (Whittingham et al, 1987; Saito et al, 1991). Further, several viruses share 6-10 consecutive amino acids with the encephalitogenic sites of MBP, including adenoviruses, influenza A, EBV and measles virus (Fujinami and Oldstone, 1985). Theoretically these viruses have the potential to evoke autoimmunity, however infection with such agents rarely results in such a response.

Bacterial endotoxins such as lipopolysaccharides (LPS) can themselves exert mitogenic effects on B cells. For example they can substitute for helper T cell function in the B cell antibody response to sheep erythrocytes (Moller et al, 1972). Similarly T cell
tolerance can be bypassed by immunisation with a self antigen coupled to a foreign carrier (Schmidtke and Dixon, 1972). Other agents capable of nonspecifically stimulating lymphocytes, such as immunological adjuvants, through bypassing T cell specificity are likewise prone to cause autoimmunity.

### 1.9 Target Antigens of Autoimmune Disease Responses

Certain self antigens are selected as the targets of immunity, but for many diseases, the target antigens have not been identified. Thus far, the major approach has been to isolate and determine the specificity of lymphocytes active in autoimmune responses, indeed several B cell and some T cell self-epitopes have been defined.

Although autoantibodies are a predominant feature of many diseases, the factors that lead to their production and role in pathogenesis remain largely unresolved. In many cases, autoantibodies may simply be epiphenomena. However in some diseases there is a restricted autoantibody specificity linked with pathogenesis. Antibodies that bind the TSH receptor or the AchR lead to the clinical manifestations of Graves' disease and Myasthenia Gravis (MG) (Lindstrom, 1979) respectively. In contrast, in SLE there is a generalised dysregulation of the immune system characterised by B-cell hyperactivity, which results in hypergammaglobulinemia and the production of a variety of antibodies reactive with organ-nonspecific antigens such as DNA, RNA and cell membrane structures, which may participate in immune complex formation and consequently the clinical manifestations of SLE (Theofilopoulos and Dixon, 1981). Secreted Igs can themselves be targets of an immune response. IgM antibody to IgG rheumatoid factor (RF) is found in the serum and synovial fluid of RA patients and in MRL/lpr (lymphoproliferative) mice (Fong et al., 1988). The principal role of some autoantibodies is their predictive value. Antibodies to insulin and a 64 KD islet β-cell antigen can precede the development of IDDM by several years, although there is no evidence that these autoantibodies cause β cell destruction (Baekkeskov et al., 1987).

Several antigenic targets of autoreactive T cells have been studied in animals. Disease-inducing peptides of self proteins have been defined in some cases. In EAE, amino acids 1 to 9 and 89 to 101 of MBP are the major disease-inducing determinants of the I-A^U and I-A^8 alleles respectively (Acha-Orbea et al., 1988).
A number of distinct peptides derived from the α subunit of the AChR stimulate T cells from MG patients and Lewis rats. T cells specific for thyroglobulin and P2 protein (amino acids 66 to 78) or peripheral myelin in mice can induce experimental autoimmune thyroiditis and neuritis respectively. The target antigen of T cell clones isolated from mice susceptible to collagen-induced arthritis (restricted to I-A^d) is type II collagen (Kumar et al.; 1989). Additionally induction of rat adjuvant arthritis induced by killed Mycobacterium tuberculosis in CFA (an experimental model for human RA), leads to the production of T cells specific for hsp 60, a 65KD mycobacterial heat shock protein (Van Eden et al.; 1985, Van Eden, 1988). A T cell clone CD4-CD8-γδ isolated from human rheumatoid synovium is also hsp 60 reactive (Holoshitz et al., 1989), suggesting that hsp 60 may be the autoantigen in RA. The target antigens for spontaneous models of disease in animals (such as IDDM in NOD mice or SLE in (NZB x NZW)F1 mice) and for most human autoimmune conditions are not known. The availability of clonal populations of T cells that cause disease, should facilitate the isolation of T cell target antigens in many of these diseases. Once identified, their role within the autoimmune disease must be assigned, and the targets of effector T cells distinguished from that of T cells involved in the initiation of autoimmunity.

1.10 Idiotypes and Autoimmunity

The antigenic uniqueness of a given immunoglobulin was termed its idiotype by Oudin and Michel (1969). The idiotypic nature of immunoglobulins (Igs) and T cell receptors arises as a consequence of the differences in the variable regions of the polypeptide chains. Idiotypes are therefore serologically defined determinants present in the Fab region of Igs and represent novel antigenic determinants which can be recognised in turn by idiotypic antibodies. Jerne (Jerne, 1974) proposed that the normal state of homeostasis consisted of a network of receptor interactions controlled by specific idiotypic recognition. In some cases Ab. raised to the combining site of another Ab. can act like a surrogate antigen this is called an internal image of the antigen and can lead to autoimmunity. Such idiotype anti-idiotype reactions occur in many spontaneously autoimmune diseases such as SLE (Abdou et al., 1981), MG (Dwyer et al., 1983; Dwyer et
al, 1986), and RA (Pasquali et al, 1984; Mouritsen, 1986). Some anti-idiotypes are themselves autoantibodies. This is observed with autoantibodies to neurotransmitters or hormones in which anti-idiotype gives rise to anti-receptor antibodies following immunisation with the receptor ligands. Mice immunised with bovine or porcine insulin make not only anti-insulin antibodies but autoanti-idiotypes that are anti-(anti-insulin) antibodies and are reactive to the insulin receptor on fat cells (Schechter et al, 1982; Cohen et al, 1989A). Such anti-receptor antibodies mimic the action of insulin and block binding of insulin itself leading to autoimmunity.

Cohen and Young argue against the notion that autoimmunity is caused by random mutations of lymphocytes into “forbidden clones” as suggested by Burnet (Burnet, 1959), because of the limited spectrum of autoimmune responses and that autoimmunity due to unstructured events ought to be individualised. They (Cohen et al, 1989A) proposed the alternative hypothesis that immunological dominance of selected self antigens can be explained by cellular networks. The observations that the dominant immune responses to MBP, hsp 65 and to insulin are associated with preformed sets of interacting networks allowed Cohen to formulate his theory of the immunological homunculus (Cohen, 1989B), a limited set of dominant self-antigens each encoded in a cellular network that comprises the immune system’s picture of self. In the immune network a natural autoimmune B cell (or macrophage that has bound natural autoantibody) recognises a self epitope present on a microbial antigen through the Ig receptor. Receptor-linked uptake and processing renders the conserved molecule immunologically dominant over other antigen molecules for which there are no preformed autoantibodies thus according to Cohen creating tolerance to recessive self-antigens by inattention. The antigen-presenting B cell (or macrophage) then presents both the shared epitope and the foreign microbial epitope. The response to the epitope is safely regulated by the lymphocyte network controlling the autoimmune T cells while the T cells recognising the microbe-specific epitope are free to react aggressively. Each dominant self antigen is served by an interacting set of T and B cells with receptors for the antigen (antigen-specific) and cells with receptors for the antigen-specific receptor (anti-idiotypic) (Cohen and Young, 1991). Some of the lymphocytes suppress and others stimulate, thus some lymphocytes become activated even without being driven by contact with specific antigen in an immunogenic form (Pereira et al, 1989; Cohen and Atlan, 1989B, 1989C). Thus regulatory elements channel the
autoimmune response to controlled pathways that prevent disease development. As a result the autoimmune response is graded and often transient.

Several investigators have proposed that autoantibodies, are anti-idiotypic antibodies produced against anti-microbial antibodies (Abe et al., 1984; Nepom et al., 1984). There is more than 50% sequence identity between bacterial and mammalian counterparts (Jindal et al., 1989) of microbial molecules which belong to the hsp families (proteins inducible by almost any form of cellular stress). Immunity to mycobacterial hsp 65 is associated with autoimmune arthritis in rat (Van Eden et al., 1988) and humans (Res et al., 1988) and with autoimmune diabetes in NOD mice (Elias et al., 1990) and immunity to hsp 70 (Minota et al., 1988A) and hsp 90 (Minota et al., 1988B) is associated with SLE. In these cases according to Cohen et al. (1991), the homunculus falters and the autoimmune T or B cells, channelled to respond to the dominant self antigen by the homunculus, may produce an aggressive immune reaction due to dysregulation by the suppressing control elements of the homunculus. Thus antigenic mimicry between a microbe and a host organ-specific antigen that determines immunological dominance may also result in specific autoimmune disease.

Regulatory idiotopes are thought to be antigenic determinants on immunoglobulins (or T cell receptor) which are recognised by idiotope-specific T or B cells. They can be expressed on Igs of differing antigenic specificities (the so called non-specific anti-parallel set). Administration of anti-idiotype or idiotype to an animal can result in enhancement of the immune response as well as an increase in idiotype-positive, non-antigen binding antibody, which could be pathogenic. For example an anti-idiotype from the -1,3-dextran (DEX) present on some gut commensal organisms can induce an antibody against the A. chR (Abe et al., 1984). Thus there may be an idiotypic connection between antibody responses to the AChR and certain bacterial antigens. Furthermore patients with Klebsiella infections tend to have an increased incidence of high 16/6 idiotype titres, which can activate normal PBL to secrete increased levels of 16/6 idiotype (El-Roiey et al.;1986, 1987). This idiotype which is present on both antibodies binding Klebsiella antigens in normal subjects and on anti-DNA antibodies in patients with autoimmune SLE, can result in autoimmune disease through the idiotype network’s response to endogenous or exogenous antigen. The presence of cross-reactive or shared idiotypes on CRI autoantibodies may provide some clues as to the origins of autoimmune disease state.
Cooke has proposed that some autoantibodies involved in autoimmunity may for example share regulatory idiotypes with microorganisms, or their anti-idiotypes may be antibodies to microorganisms (Cooke et al.; 1983, Cooke et al.; 1984, Cooke, 1986).

I will confine my description of human diabetes to chapter 2 and of the rodent models of IDDM to chapter 3. The purpose of this chapter was basically to provide a structured background of both tolerance and autoimmune disease mechanisms, thereby providing a backbone for my studies on how tolerance is achieved in the transgenic NOD mouse and how tolerance is broken in the first place by searching for any basic thymic abnormalities in the NOD mouse discussed in chapter 9.
CHAPTER 2

The History Of Type 1 Insulin Dependent Diabetes Mellitus (IDDM).
Diabetes Mellitus - A Historical Perspective

IDDM is due to the selective destruction of the β cells within the islets of Langerhans in the pancreas, leading to insulin insufficiency and resulting in hyperglycemia. The autoimmune aetiology of IDDM was primarily delineated by the findings of Gepts who demonstrated mononuclear cells both in and around the islets of Langerhans in diabetic patients (Gepts, 1965). Additionally anti-insulin antibodies could be detected in the sera (Atkinson et al, 1986). Before 1922 the diagnosis of diabetes was like a death sentence, diabetics died either rapidly if they were young or more slowly if they were older until the advent of the ‘miracle of insulin’.

Diabetes is unlikely to have been known in primitive cultures as the young diabetic would die before he had a chance to reproduce himself and to pass on the genes to succeeding generations. Late onset diabetes was not seen as the population as a whole did not survive long enough thus diabetes was observed infrequently. The first reference to diabetes in historical records was found at Luxor in the Egyptian Papyrus Ebers written about 1500 BC describing an illness associated with the passage of much urine (Ebbel, 1937). Later the Greek physician Aretaeus the Cappadocian (81-138 AD) related the origin of the word diabetes from the Greek word for siphon, to describe the urine “going through” from the kidneys. He described diabetes as a “wonderful affection, not very frequent among men, being a melting down of the flesh and limbs into urine”. Additionally Ayur of Susruta in a Hindu manuscript (6th century AD) described the disease as “madhumeha” or honey urine. Much later Paracelsus (1493-1541) (Parcelsus, 1926-1932) observed that evaporation of the urine of a diabetic patient yielded a white powdery residue which when dried on cloth stained like “gall”, but suggested that the kidneys themselves were thirsty because they are salty and that perhaps diabetes was due to dry salt. However it was not until a century later that the Englishman Thomas Willis (1621-1675) (Willis, 1676-1680) described the smell of diabetic urine as “wonderfully sweet as if it were imbued with honey or sugar”, thus the adjective mellitus, from the Latin for honey-sweet was added to refer to the “pissing evil”. He believed diabetes mellitus was due to acid salts in the blood, caused from “immoderate use of wine and cider” resulting in a “running through of sweet urine”.

The presence of sugar in the urine was demonstrated chemically by Mathew
Dobson (Dobson, 1776) who made the important deduction that the sugar was not formed in the secretory organ (the kidney) but previously existed in the serum of the blood. Additionally Richard Mead (1673-1754) considered diabetes as a disease of the liver which was widely believed. Kussmaul related the final stages of untreated diabetes to the failure of the body cells to utilise the carbohydrate usually broken down to carbon dioxide and water providing energy for the activities of the body. The final stages of untreated diabetes results in the failure of cells to utilise carbohydrate. Thus glucose accumulates in the blood and is passed in the urine as renal tubular resorption is saturated the overload is too great for the kidneys. Disturbed fat catabolism results in excessive ketone production. This upsets the acid/base balance in the blood leading to acidosis and coma. Beta cell destruction remains subclinical for a period of years then it is finally expressed in a clinical form by weight loss, polydipsia (constant thirst), polyuria (frequent urination), polyphagia (excessive hunger), ketoacidosis and the long term diabetic complications of microangiopathy, nephropathy, and neuropathy (Green and Houaard, 1984A; Green and Solander, 1984B).

Later Claude Bernard (Bernard, 1848) discovered that the kidney indeed did not produce sugar but excreted the excess into the urine. Additionally he demonstrated that the liver stored sugar from the alimentary tract in the form of glycogen to be broken down into sugar again when required and passed directly into the blood. Thus it was considered that diabetes resulted from an overproduction of sugar by the liver. Bernard also demonstrated that puncture of the floor of the fourth ventricle of dog brain gave rise to an increase in blood sugar level and eventually to loss of sugar in the urine and temporarily led to the belief that diabetes was a disease of the nervous system. Subsequently it was shown that this is not the mechanism which causes the increase of blood sugar, (hyperglycemia) in human diabetes.

The origin of diabetes was traced to the pancreas (from the Greek, pan-all and kreas-flesh) in 1889 by Minkowski and Von Mering (Von Mering and Minkowski 1889) who demonstrated that 24 hours after extirpation of the pancreas of a dog, severe diabetes with 5% sugar in the urine resulted. This reproduced the signs of polyuria and polydipsia normally associated with human diabetes. Prior to this discovery Paul Langerhans (1849-1888) in his doctoral thesis of 1869 showed that the rabbit pancreas contained two systems of cells (Langerhans, 1869). The acini or clusters of cells which secrete the normal
pancreatic juice were scattered throughout the organ and penetrate the acini. Floating in a sea of acinar cells were other cells, unconnected with the acini, whose function was unknown. Years later Languesse (Languesse et al, 1893) discovered similar cells in the human pancreas and named them the islands of Langerhans (Iles de Langerhans). He suggested that they were the anatomical counterpart of the internal secretory function of the organ and that if the pancreas had a secondary function besides secreting digestive juice, the islet cells were involved.

Von Mering demonstrated that ligation and/or cutting pancreatic ducts did not cause diabetes therefore the absence of pancreatic juice does not cause diabetes only complete pancreatectomy produced the disease. Therefore the pancreas was shown to have two functional secretions. First the digestive juices which pour out of the ducts into the intestine, the external secretion, and the other an internal secretion which fed directly into the blood-stream and regulated carbohydrate metabolism. The missing link was supplied by Eugene Opie (Opie, 1901) who showed a pathological connection between diabetes and damage to the islet tissue, hyalinization of the islets and intercellular fibrosis. It was then believed that the pancreas constituted an endocrine gland, the islets produced the internal secretion of the pancreas into the "milieu interieur" as there were no direct connections between these islets and the duct system of the pancreas, thus the pancreas constituted an endocrine gland as described by Dogiel (Dogiel, 1893). Sir Edward Sharpey-Schafer postulated that diabetes was due to lack of an internal secretion produced in these islets called insuline, (after the Latin insula, an island) (Schafer, 1895). Bayliss and Starling coined the term "hormone" to describe such chemical messengers and when it was realised that the pancreas controlled diabetes attempts began to treat the disease with pancreatic extracts, generally with unreproducible and mixed results.

From 1900 to 1921 several scientific workers produced various pancreatic extracts. Ludwig Zueler demonstrated that minced up, whole pancreas could suppress glycosuria and ketonuria in rabbits, and depancreatized dogs and 8 diabetic patients had their symptoms relieved by acomatol, his pancreatic extract. However there were toxic side effects such as fever and vomiting and so its use was abandoned (Zueler, 1908).

Marlin and Kramer demonstrated that injections of extracts of pancreas and duodenum caused a temporary fall in the excretion of glucose in the urine due to alkalinity of the extract. This still left the problem of how the pancreas affected sugar production and
its utilisation in the normal animal. It was still assumed that the islets of the pancreas were the source of the hyperglycemic agent which was secreted into the blood-stream, subsequently passed either to the tissues enabling utilisation of sugar, or to the liver inhibiting sugar production. Additionally Israel Kleiner demonstrated a decline in blood sugar in depancreatized dogs with ground fresh pancreas extract, similar to results obtained by Paulesco.

Treatment of diabetes before the advent of insulin mainly resulted from the studies of Fredrick Allen who claimed that diabetes was not only a problem of carbohydrate metabolism but also of proteins and fats. He instituted a totally dietary approach following on from Apollinaire Boudardat which resulted in patients either dying from their diabetes or starving from his under-nutrition regimes.

Fredrick Banting a skilled surgeon became interested in diabetes in relation to carbohydrate metabolism and persuaded Prof. Macleod of the physiology department of Toronto University to allow him to commence a research project there during the summer of 1921 with the aid of a student Charles Best aimed at isolating insulin. In May 1921 work commenced, by ligating the pancreatic ducts of dogs for 5-6 weeks to allow the gradual atrophy of the acinar tissue and elimination of the proteolytic effects of the pancreatic enzymes derived from the exocrine tissue. The degenerated pancreas was removed, sliced up in chilled Ringer's solution, partly frozen, then macerated and filtered. The filtrate was injected into diabetic dogs that had been pancreatectomized a few days earlier. The extract lowered the blood sugar of the dogs and resulted in a rapid amelioration of the diabetic state in many of these animals. They had demonstrated conclusively that something from the islets of Langerhans was missing in diabetes and this substance which they called "Isletin" could reverse the diabetic process, and also that acid/alcohol extracts were the most potent.

The need for ligation of the pancreatic ducts was avoided by using the pancreas from fetal calves which did not contain digestive enzymes such as trypsin until after the fourth month of embryonic life. Thus islet cells, more plentiful in relation to the acini, in more abundant quantities, and more potent, containing 15-20 units/ml of insulin after sterilisation were obtained. By 10th November Banting had administered over 75 doses from degenerated pancreatic tissue to 10 different diabetic animals always producing a reduction in the percentage of sugar in the blood and sugar excreted in the urine.
Additionally Collip demonstrated that the extract enabled liver of diabetic animals to form glycogen. For clinical trials, an acid/alcohol extract of normal beef pancreas was utilised, as the insulin source had to be commercially viable. Although less potent than the fetal extract, it was a suitable source of insulin for treating man.

On November 11th 1921 Banting and Best presented their discovery to the Medical Faculty of the University of Toronto and subsequently on December 30th 1921 at the Physiological Society conference at Yale university. The findings were published in an article entitled “The internal secretion of the pancreas” published in the Academy of Medicine Bulletin February 1922 and the Journal of Laboratory and Clinical Medicine (Banting, 1922A).

Word spread of their discovery when the first diabetic, a 14 year old boy Leonard Thompson was administered the acid/alcohol pancreatic extract, which reduced blood and urine sugar to normal limits, eventually allowing Thompson to lead a normal life. This was the first dramatic demonstration that insulin could produce its effects on human beings as well as diabetic dogs. Clinical trials commenced at the Toronto General Hospital in January 1922 under Drs Walter Campbell and Amon Fletcher. All demonstrated the same fall in sugar in the blood and the disappearance of sugar from the urine. Initial studies produced erratic results owing to the presence of impurities. However Collip succeeded in making an extract that was less toxic and more effective. The results were published in May 1922 in the Canadian Medical Journal describing “highly potent extracts” which were “however somewhat toxic, and they were apt to cause local abscesses at the point of injection” (Banting et al, 1922B).

Supplies of insulin dwindled as demand grew and the methods used to produce small quantities of insulin were not successful when tried on a larger scale by the Connaught Anti-toxin Labs. A patent was taken out in April 1922 and large scale production of insulin from pork pancreas followed. This was supervised by Dr Clowes, research director of the Eli Lilly company of Indianapolis, working under an exclusive one year licence granted by the insulin committee of the University of Toronto, set up to standardise all insulin preparations prior to distribution. Diabetics were literally camping at the doors of the laboratory trying to obtain insulin. Clinical trials were resumed in Indianapolis, Boston, Toronto and Morristown by Dr F. Allen. The use of insulin gradually spread throughout Europe. Produced in the UK by Burroughs Wellcome and the
joint venture between Allen and Hanburys and the British Drug Houses, and in Denmark by the Nordisk Insulin Laboratory. By mid-September 1923 Lilly's development of manufacture expanded to the point where 25,000 American diabetics were receiving insulin from 7,000 physicians. Insulin was endorsed as one of the most important medical discoveries of the modern age by doctors with impeccable credentials: Allen, Jolsin, and Geyelin. In 1923 the Nobel Prize was awarded for the discovery of insulin to F. Banting.

Separation of insulin from other proteins due to differing acidities removed inert material in the extract and prevented lump formation at the injection site. Purification of insulin in a crystalline form which contained zinc was achieved by J. Abel (Abel, 1926). This purified form produced less irritation at the injection site but was not as potent. Further, H. Hagedorn demonstrated that insulin combined with protamine, a small molecule extracted from fish sperm and a trace amount of zinc produced a stable combination that prolonged the action of insulin (PZI) which initiated a new phase in the treatment of diabetics with insulin. Additionally modified insulins, intermediate in their period of action between the quickly spent crystalline and the very long acting PZI, (namely Globin insulin, NPH insulin and lente insulin) were developed. The factor determining the promptness with which the injected zinc insulin preparation becomes effective depends upon solubility in the blood. It remains to be clarified how insulin is formed, stored and secreted by the islets. Alternatively a means of producing a glucose sensor with an insulin pump the action of which could be regulated by the blood sugar itself could solve many problems in relation to regulation.

Insulin was the first protein for which the structure was established by F. Sanger. It consists of 51 amino acids arranged in two chains of 30 and 21 amino acids.

During the 1920's it was believed that diabetes had been “cured”, however it was merely a replacement therapy which permitted a better utilisation of diet richer in calories and carbohydrate. Over-dosage of insulin leads to hypoglycemic reactions, and unless counteracted with glucose eventually to a state of unconsciousness. Therefore the amount injected had to be brought into harmony with diet and exercise. Thus the life expectancy of the diabetic taking insulin increased, as did the quality of life. In essence a miraculous change has been brought about for millions of people by the discovery of insulin and the work done to make it more effective.

In spite of adequate control of the blood sugar level with insulin an insidious
progression of degenerative changes in the walls of the blood vessels takes place continuously in the diabetic, thereby leading to the so-called “diabetic complications” due to damage of the capillary blood vessels of the retina at the back of the eye (retinopathy), the kidney (nephropathy), degenerative changes of the CNS (neuropathy) and hardening of the arteries leading respectively to blindness, kidney disease and ischaemic heart disease. After 20 years of diabetes, 80% of patients had retinopathy and of these, 20% were destined to die of kidney failure within another 10-15 years. All these conditions are believed to be consequences of metabolic disturbances and so although insulin provides an effective treatment for sustaining the life of the diabetic patient, there is threatening excess morbidity and mortality with long term insulin therapy.

Over the past 20 years a tremendous research programme has been implemented to investigate the mechanisms of the aetiology and pathogenesis of diabetes mellitus following current advances in autoimmunity and immunology in general. Significant advances in morphologic techniques such as qualitative and quantitative histochemistry, (staining with fluorescent antibodies, electron microscopy etc.), have been applied to human and experimental diabetes and contributed to a fundamental appreciation of the physiological processes occurring in the islets of Langerhans. In particular, the growing field of research in various animal species which exhibit spontaneous or hereditary disturbances of carbohydrate metabolism has remarkably widened the scope our knowledge of diabetes. The disease characterised by a common hyperglycemia has been classified into “juvenile onset” (type 1) diabetes (insulin requiring) and “maturity onset” (or type 2) (non-insulin requiring). In the 1970’s it became evident that these two hyperglycemic forms were two distinct entities since although both had an HLA association only type 1 diabetes demonstrated involvement of anti-pancreatic antibodies and cell-mediated immunity.

This thesis will deal exclusively with IDDM or Type 1 diabetes and the autoimmune nature and aetiology of the disease. Having reviewed some of the literature pertaining to autoimmunity and immunological tolerance in the previous chapter (since IDDM results from a breakdown of the latter), I will further review the relevant literature on the NOD mouse model of IDDM with reference to the other main animal model the BB rat, and human diabetes.
CHAPTER 3

The NOD Mouse as an Animal Model of Human Type 1 Insulin-Dependent Diabetes Mellitus (IDDM).
At the time of presentation with diabetes, insulitis (mononuclear cell infiltration of pancreatic islets) has progressed already to such an extent that 80% of the β cells have been destroyed by autoimmune phenomena (Gepts; 1965, Rossini et al; 1985). Thus successful treatment should commence at an earlier phase, before disease onset and involve suppression of insulitis. Therefore a twofold research campaign is required aimed at both predicting diabetes onset and prevention of further β cell destruction and diabetic complications. As it is not feasible to carry out a programme of diabetes intervention in humans, it is necessary to utilise suitable animal models of the human disease (reviewed by Castano and Eisenbarth, 1990). There are several species displaying either spontaneous or induced hyperglycemia and diabetes, including several diabetes susceptible genotypes in mice exhibiting various forms of heritable glucose intolerance syndromes (Mordes and Rossini, 1985). This thesis will describe studies on one such model: the spontaneously diabetic non-obese diabetic mouse (NOD) with reference to the BioBreeding (BB) rat model of IDDM (Nakhooda et al, 1977) and the more recently described LETL rat (Kawano et al, 1989).

### 3.1 Derivation of the Non-Obese Diabetic Mouse (NOD)

In 1966 cataract prone mice were found among the outbred ICR mice. Since cataracts are often observed in diabetic patients, selective breeding was performed in 1974 by Makino at the Shiononi Research Laboratories, Osaka, Japan. In an attempt to obtain a cataract-prone line, two lines were segregated on the basis of blood glucose. One of these lines showed normal blood glucose (later termed NOD [non-obese diabetic]) and the other, NON (non-obese normal) had a mild hyperglycemia. A female from the "normoglycemic line" exhibiting polyuria, glycosuria and severe weight loss was found and this was used to establish a selective breeding programme. At the fifth generation of selective inbreeding of the diabetogenic mice severe hyperglycemia was observed in the NOD line and after a further twenty generations insulin dependent diabetes mellitus (IDDM) was observed in 80% of NOD females. Thus in 1980 the NOD strain was derived (Makino et al, 1980).

A breeding colony of NOD mice was established at the Jackson Laboratory, Bar Harbor, USA which supplied breeding pairs to establish our own colony at the Clinical Research Centre, Harrow, (NOD/CRC). Recent worldwide distribution of NOD mice has led to the formation of different colonies with variable incidences of diabetes.
The NOD model has many desirable features as a diabetes research tool. Typically it exhibits a prediabetic period of about six months during which time exogenous insulin is not required followed by abrupt onset of overt diabetes. In addition there are many other similarities to the human diabetic: insulitis (mononuclear cell infiltration of the pancreas) with β cell destruction, insulin insufficiency leading to acute metabolic, biochemical and morphological abnormalities including susceptibility to ketoacidosis and the appearance of islet cell antibody and islet surface antibody in the plasma. In addition the NOD mouse provides a useful model for genetic and immunological studies of diabetes and is a promising model for human Sjogren's syndrome (Goillot et al, 1991). Environmental factors (Leiter, 1990) such as, diet (Coleman et al; 1990, Elliott et al; 1988) and infection (Oldstone; 1988, Dyrberg; 1990) affect the incidence of diabetes which may contribute to the observed colony-dependent variation in disease incidence.

### 3.2 General Characteristics of the NOD Mouse

NOD mice develop diabetes after 13 weeks of age with a marked excess of diabetes among female (70-80% at 30 weeks of age) in contrast with male NOD mice (below 20% at 30 weeks of age) for most colonies (Tochino, 1987). However analysis of diabetes incidence in NOD colonies suggest that the apparent gender bias is less reflective of a sex difference than of environmental factors which could modify susceptibility gene penetrance. The clinical onset of diabetes is described by the symptoms of ketonuria, glycosuria, hyperglycemia, hypercholesterolemia, polydipsia, polyuria and polyphagia. Insulin injections improve these symptoms but if left untreated the animals lose weight and death ensues (Makino et al, 1980).

Development of overt diabetes in NOD mice is under the control of sex hormones as testosterone inhibits the onset of overt diabetes in castrated males and females, while estradiol does not enhance its occurrence (Tochino, 1979). Further female ovarectomy prevents disease, the incidence reduced to that of untreated males, whereas castration increases the incidence of overt disease in males (Makino et al, 1981). More than 98% of NOD mice of both sexes have mononuclear cell infiltration of the islets of Langerhans with selective destruction of the β cells. A similar mononuclear cell infiltration of the pancreatic islets was noticed in young diabetic patients by early pathologists and designated insulitis by Von Mering (Von Mering, 1889) and reported by Gepts (Gepts, 1965) as the
characteristic pathological change in patients with juvenile-onset diabetes who died after a rapid course of the disease.

Histological changes in the pancreas of NOD mice commence at 4-6 weeks of age characterised by lymphocytic infiltration around (peri-) and later in (intra-) the islets in 82% of females and 58% of male NOD mice (Fujita et al., 1982). Consequent specific degradation and disappearance of β cells ensues in 80-90% of female, and in less than 20% of male, NOD mice resulting in overt diabetes.

Fujita (Fujita et al., 1982) produced the first detailed paper documenting the histological changes in the NOD mouse pancreas by immunohistochemical and electron microscopic observations. No insulitis and no marked changes in the number or size of pancreatic islets were observed in either male or female NOD mice until 3 weeks after birth. Subsequent histological changes were divided into three stages, the initial stage of lymphocytic infiltration occurred at 4-5 weeks of age at the post capillary venule. During the second stage of infiltration the islet was surrounded by a ring of lymphocytes of increasing size which destroyed the islet morphology. This resulted in a decrease in islet size and the gradual replacement by the massive lymphocytic infiltration which corresponded to the final stage and onset of diabetes. Only β cells were damaged by the lymphocytic infiltration. With the advance of degeneration adjacent β cells fused with each other, lost polarity and swelled with numerous secretory granules. The nuclei became pyknotic, the cisternae of rough endoplasmic reticulum became confluent and vacuolations appeared in the secretory granules. Islets free of lymphocyte invasion coexisted with those with severe insulitis, indicating that the progression of insulitis was not uniform. As the mice aged, the proportion of intact islets decreased with an increase in the number of islets showing severe insulitis. The typical manifestations of overt diabetes did not appear until most of the β cells disappeared. Glucagon and somatostatin containing cells were then the main components of the islet and formed clusters of each cell type two weeks or more after the onset of diabetes to form small islets which lack lymphocytes. Makino (Makino et al., 1982) noticed an apparent increase in the somatostatin content of the pancreas in diabetic NOD mice compared to non-diabetic. This observation has also been reported in several human and animal models (Hellman and Peterson, 1963) of diabetes although its significance is controversial.
3.3 The Role of T cells and Abnormalities of Cellular Immunity in Disease Manifestation

Recent studies with monoclonal antibodies demonstrated that the cells infiltrating the islet were predominantly T cells, most of which were of the CD4* (Th) phenotype although CD8* (Tc/Ts) cells and NK cells were also demonstrated (Tochino et al., 1987; Shimizu et al., 1987). The T lymphocytes were localised close to islet cells while Ig bearing cells appeared adjacent to blood vessels and around T cell clusters (Miyazaki et al., 1985). Definitive evidence that T cells were essential for lymphocytic infiltration not only of the pancreas but also of submandibular glands was demonstrated by Goillot (Goillot et al., 1991). Infiltration of lymphocytes into other organs is found in human type 1 diabetes combined with autoimmune abnormality (Nerup et al., 1973) and also in spontaneously diabetic BB rats (Sterniethal et al., 1981).

Neonatal thymectomy prevents the spontaneous occurrence of IDDM in both NOD mice (Ogawa et al., 1985) and BB rats (Like et al., 1982). Athymic NOD mice did not develop insulitis at 9 weeks or overt diabetes at 30 weeks in contrast to their euthymic congenic littermates. The latter also exhibited infiltration of the other organs associated with the autoimmune phenomenon (Makino et al., 1986A). Additionally, Dardenne (Dardenne et al., 1989) demonstrated that onset of diabetes in NOD mice is under thymic regulation and that T cells are essential for disease development. Their studies showed that thymectomy at weaning increased diabetes incidence in female NOD mice possibly due to the loss of some T cell dependent suppressor mechanisms, whereas thymectomy at 6-7 weeks had no effect on disease incidence. The immunosuppressive drug, cyclosporine, which is known to affect both T cell function and antigen presentation, prevents the onset of IDDM in both NOD mice and BB rats (Laupacis et al., 1983; Like et al., 1983, 1984; Jaworski et al., 1986; Mori et al., 1986). These experiments confirm that the thymus and T cells play an obligatory role in the occurrence of insulitis in the NOD mouse, although some additional factors may be necessary for the destruction of β cells.

Lymphocytic infiltration occurs not only in the pancreas of NOD mice but in other organs including submandibular glands, thyroid, adrenal glands ovaries and testes (Hanafusa et al., 1985) thus the animals appear to develop polyendocrine immunological
abnormalities. In addition autoantibodies are produced against the thyroid, submandibular glands (providing histopathological findings quite similar to Sjogrens syndrome) and red blood cells (leading to Coombs'-positive haemolytic anaemia) (Baxter and Mandel, 1991A). The immunological involvement of salivary glands in the pathogenesis of IDDM has not been extensively studied in the NOD mouse. However, I have examined such submandibular salivary glands with particular reference to transgenic animals by immunohistochemistry, which will be reviewed in chapter 8.

Many aspects of cellular immunity in the NOD mouse have been studied, often with discordant results similar to those studies in the human and BB rat. Miyazaki, Ikehara and Pontesilli (Miyazaki et al, 1985; Ikehara et al, 1985; Pontesilli et al, 1987) have shown that peripheral T cell numbers both Th (CD4) and Tc/Ts (CD8) were increased during the initial stages of insulitis and remained elevated in NOD mice of all ages and in both sexes, compared to control mice, but there was no imbalance of the two subpopulations. Also, the percentage of B cells was lower in the NOD mouse spleen compared to ICR mice during the early stages of insulitis. The number of circulating mononuclear cells in the blood of NOD mice, estimated by density centrifugation was originally reported to be significantly lower than in ICR mice (Kataoka et al, 1983). Whether these differences depend on the age of NOD mice or the method of analysis for lymphocyte subsets remains to be demonstrated. Although the later study showed that both Th number and the percentage of circulating B cells was normal, there was enhanced production of polyclonal antibodies to T cell dependent antigens. There were also selective abnormalities of cellular immunity as the antigen non-specific defence mechanisms, (namely macrophages, NK cells, CTL, ADCC and humoral factors) were decreased in the NOD mouse, and diminished resistance to herpes virus infection compared to control ICR mice. Indeed Kataoka (Kataoka et al, 1983) demonstrated that NOD mice display lymphocytopenia and impaired cellular immunity, but responsiveness to Con A was normal. It was concluded that there may be a positive association between T cell number and function in female NOD mice. This result is inconclusive as the non-inbred ICR mouse was used as the basis for comparison and because of their genetic heterogeneity they do not provide a constant reference for the comparison of in-bred strains derived from them. Leiter (Leiter et al, 1986) showed that NOD mice exhibited T-lymphocyte hyperplasia with T cells retaining strong mitogen responsiveness whereas NON mice developed T-lymphocytopenia and
functional anergy by 20 weeks. B cell functions (as measured by autoantibody production and responsiveness to lipopolysaccharide and BSA) were stimulated supporting previous findings of an exaggerated immune response in the NOD.

Interestingly, another animal model of type I diabetes the C57/KSJ mouse (homozygous for the autosomal recessive diabetogenic gene (db/db)) displays anti-islet immunity, manifests thymic dysfunction and lymphopenia. This is attributed to a marked decrease in thymic thymulin levels contributing to an excessive helper-suppressor cytotoxic ratio probably triggering anti-islet immunity (Boillot et al, 1986). The percentage of splenic lymphocytes in the BB rat has also been reported to be markedly decreased due to a reduction in both Th and Tc/s numbers (Jackson et al, 1981). Such discrepancies suggest that in spite of similarities in the clinical course of the disease, NOD mice seem to have characteristic immunological features distinct from the BB rat.

In general, studies of circulating lymphocytes in IDDM patients have yielded conflicting findings as a result of the use of different assays and selection of patients at different stages of disease. Analysis of peripheral blood mononuclear cells from such patients demonstrated that 40% had a deficiency of Ts/c and some had deficient Con A induced suppression (Gupta et al, 1982). Indeed Buschard (Buschard et al, 1983) demonstrated a high Th/Tc ratio at diagnosis but a normal distribution of T cell subsets in IDDM patients within a few weeks. Other studies have suggested that the frequency of Th cells in IDDM patients at the time of disease onset is normal, (Gupta et al; 1982, Jackson et al; 1982, Ilonen et al; 1984), or increased (De Berardinis et al, 1988B) however studies of the Tc/Ts population are inconsistent some reported normal (Jackson et al; 1982, Rodier et al; 1984), others increased (Ilonen et al, 1984) and still others decreased (Gupta et al, 1982; Buschard et al, 1983).

Cellular abnormalities in the NOD mouse were demonstrated by Maruyama (Maruyama et al, 1984). Non-MHC restricted ADCC (antibody dependent cellular cytotoxicity) activity directed towards BALB/c islets was detected, indicating that lymphocytes from NOD mice are sensitised to islet cell antigens. Non-specific cellular immunity (such as NK cells) was reduced as were spleen cell numbers compared to control ICR mice (Kataoka et al, 1983). This finding parallels that in the human where lymphocytes from IDDM patients have been shown to destroy cultured rat islet cells (Krug et al, 1991).
An increase in ADCC activity was reported in overtly diabetic but not prediabetic NOD mice compared to ICR mice (Nakajima et al, 1986). Interestingly complement-dependent antibody-mediated cytotoxicity (CAMC) was not increased and there was no correlation between ICSA levels and CAMC levels in NOD mouse sera (Suzuki et al, 1983). Spleen cells from NOD mice contained a higher percentage of Thy1.2+, L3T4+ and Lyt2+ T lymphocytes than ICR mice and were found to generate lower IL-2 production and cell proliferation following Con A stimulation (Kataoka et al, 1983; Handa et al, 1983; Yokono et al, 1989; Hatamori et al, 1990) through macrophage mediated suppression by prostaglandins. Similar suppression has also been reported in the BB rat (Prud’Homme et al, 1984) and IDDM patients (Zier et al, 1984; Kaye et al, 1986).

Diabetes may result from an alteration of the fine balance between active suppression and immunity. A defect in number or function of either of these cell types may result in the breakthrough of β cell autoimmunity. Comparisons of high and low incidence disease strains NOD/Lt and NOD/WEHI respectively showed that the latter demonstrated a heightened syngeneic mixed lymphocyte reaction (SMLR) a T cell response to self MHC class II antigen involved in the generation of a number of immunoregulatory cells including suppressor inducers, compared to the former (Baxter et al, 1989; Baxter et al, 1991C). This may reflect the generation of suppressor function in NOD/WEHI mice accounting for the low diabetes incidence. Depressed suppressor T cell activation in the NOD/Lt mice have also been reported by Serreze and Leiter (1988B), which could derange the regulatory T cell circuit allowing the generation of specific autoreactive Th and contribute to the pathogenesis of IDDM in this high incidence strain. Serreze showed that NOD mice demonstrated a depressed SMLR caused by a stimulator dysfunction and loss of Ts activity. This further abnormality could initiate an autoimmune response against the β cell (Serreze and Leiter, 1988A). Loss of Ts activity is a common finding in IDDM patients. Lohmann (Lohmann et al, 1986) has shown that the cytotoxicity of PBL from newly diagnosed IDDM patients to isolated rat islets was reduced by the addition of normal PBL indicating that a defect in suppressor function exists contributing to the imbalance of the regulatory system. The exact role of suppressor T cells or the phenomenon of suppression in IDDM awaits further clarification, but current opinion would seem to suggest that an alteration of the finely balanced network of
suppressor and inducer could lead to autoimmunity. In other autoimmune diseases such as multiple sclerosis (Morimoto et al, 1987A) and SLE (Morimoto et al, 1987B) immunoregulatory disturbances have been noted with decreased numbers of suppressor-inducer cells in the periphery. These studies suggest a predominant role for cell-mediated immunity in the pathogenesis of IDDM in the human and in both the spontaneous animal models, namely the NOD mouse and BB rat. This may indicate the prerequisite of an initial triggering stimulus prior to the activation of autoreactive lymphocytes.

3.4 The Role of Autoantibodies in Disease Manifestation

There is an indication that the humoral immune system is involved in the disease process in rodents and humans as suggested by the presence of antibody reactivity to insulin (IAA), a cytoplasmic islet cell antigen (ICA) and an islet cell surface antigen (ICSA). Such autoantibodies could be utilised to identify the autoantigen acting as the immunogen thus presenting a potential route for therapeutic immunomodulation through blocking responses to a specific autoantigen. Attempts have been made to isolate antibodies to pancreatic islets and β cells in both the human and animal models of IDDM in order to obtain reagents for the identification of islet specific antigens.

MacLaren (MacLaren et al, 1975) has described antibodies to both the surface of cultured human insulinoma cells and to the plasma membranes of islet cells from the sera of IDDM patients; islet cell surface antibodies (ICSA). ICSA-positive sera from newly diagnosed diabetics immunoprecipitated the 64KD protein from human islets (Baekkeskov et al, 1982). ICSA also develop in BB rats and can bind specifically to islet β cells. The appearance of these antibodies is related to the time of onset of disease and to the age of the animal (Pipeleers et al, 1987). Later studies demonstrated that NOD anti-islet cell antibodies bound to non diabetic islet cells, rat and human islet cells and β cell tumour lines and identified a 64Kd membrane antigen detected exclusively by sera of human diabetic individuals, in agreement with the previous theory (Supon et al, 1990). Autoantibodies to a 64,000Mr (64Kd) islet cell protein have been detected in the sera of IDDM patients years before the onset of symptoms (Baekkeskov et al, 1982, 1987; Atkinson et al, 1990). The 64,000 Mr islet cell autoantigen is a form of glutamate decarboxylase (GAD; E.C.4.1.1.15), the enzyme responsible for the synthesis of γ-aminobutyric acid (GABA)
in brain, peripheral neurons, pancreas, and other organs (Baekkeskov et al., 1990). Antibodies to the 64,000 Mr (GAD) proteins are the earliest and most reliable predictive marker of IDDM in humans and are also present in the two animal models of IDDM. It is detected in 87% of sera from newly diagnosed diabetic NOD mice (Atkinson and Maclaren, 1988). The antibody was detectable at weaning, disappeared within weeks of diabetes onset and was absent in older nondiabetic NOD mice. In the BB rat this antibody remained for 8 weeks before clinical onset of IDDM (Baekkeskov et al., 1983, 1984). An ICSA specific antigen with similar molecular weight was identified by Hari (Hari et al., 1986) from polyclonal ICSA positive sera. Interestingly upregulation of the synthesis of this 64,000Mr (64Kd) antigen was demonstrated after infection with a diabetes inducing strain of coxsackie virus B4 (CB4) in SJL/J and CD1 mice (Gerling et al.; Schemthaner et al.; 1985). Thus it is possible that an immune reaction to exogenous antigen may initiate or enhance an autoimmune reaction by increasing the 64Kd antigen expression. Kargounos (Kargounos et al., 1990) demonstrated that sera from NOD mice and 29% of IDDM patients contained autoantibodies to a 52Kd RINM5F cell specific membrane protein implying that the autoimmune response may be directed against a common epitope in the two species. Boitard (Boitard et al., 1984) has demonstrated that autoantibodies specific for a 58Kd antigen present on RIN5F insulinoma show cross-reactivity with NOD class II antigens. Common antigenic sites could be shared by the salivary gland and pancreas as antibodies to both salivary gland and islet cell proteins are detected in NOD mice (Hanafusa et al., 1985).

Other IDDM associated autoantibodies, such as those against insulin and cytoplasmic gangliosides of islet cells (ICA), appear later, possibly as a consequence of the release of these antigens from damaged islets cells. The first reports of circulating cytoplasmic islet cell antibodies (ICA) in IDDM patients were published in 1974 (Bottazzo et al., 1974; MacCuish et al., 1974). Such antibodies have a relatively high prevalence in newly diagnosed IDDM patients (Lendrum et al., 1976; Del Prete et al., 1977). However ICA have a broad cell specificity and as IDDM selectively depletes β cells, they are unlikely to play a direct role in β cell destruction. These antibodies may provide a useful marker of ongoing β cell destruction and hence have a diagnostic value in predicting future
IDDM patients since they may be present several years prior to clinical onset of disease (Bottazzo et al, 1980; Gorsach et al, 1981; Tarn et al, 1987). Antibody prevalence decreases with the duration of IDDM probably reflecting the gradual loss of the antigenic stimulus present on β cells. Insulin autoantibodies (IAA) have also been reported in 41% of nondiabetic and 46% of diabetic NOD mice (Serreze et al, 1988B) which could be secondary to β cell damage and thus represent a marker of β cell destruction. Alternatively such antibodies may participate at the onset of autoimmunity by interaction with idiotypic networks and therefore be responsible for altered glucose homeostasis. The presence of IAA did not always reflect insulitis nor was it sufficient for the development of overt diabetes and was present in the sera of low-dose streptozotocin mice (Maruyama et al, 1986). In another study IAA were present in 47-58% of 75-100 day old NOD mice, an age when insulitis is known to have already developed (Pontesilli et al, 1987). Conflicting observations have been made with regard to the significance of insulin autoantibodies. Serreze reported their appearance several weeks before the clinical onset of diabetes but at a time when β cell necrosis was already noted, suggesting that humoral autoantibody probably was not the instigator of β cell necrosis but a marker of islet cell autoimmunity (Serreze et al, 1988B). However Michel (Michel et al, 1989) detected IAA at 5 weeks in NOD mice, long before the clinical onset of disease and suggests that they may have a significant pathogenic role. Immunisation of NOD mice with p73, (a product of an endogenous retroviral [Type A intracisternal plasmid (IAP)] gene expression) which shares common antigenic sites with insulin and Ig E binding factor, (an example of molecular mimicry) results in the appearance of insulin and p73 autoantibodies. At this time there is a major decrease in pancreatic insulin content and loss of glycemic control suggesting that the appearance of IAAs reflect underlying β-cell destruction (Serreze et al, 1988B). ICSA, in contrast to ICA, can in the presence of complement, lyse cultured β cells preferentially (Dobersen et al, 1980, Dobersen et al; 1982) and thus may have some pathological importance. ICSA could mediate cytotoxicity by antibody dependent cellular cytotoxicity (ADCC) in which ICSA antibodies bind to the Fc receptors of cells such as NK, K cells and macrophages, this phenomenon has been described in vitro (Huang and MacLaren, 1976). The prognostic value of ICSA is uncertain since 25% of sera from healthy first degree relative are also positive for ICSA (Dobersen et al, 1980).
Additionally IAA have also been demonstrated in the sera of BB rats (Dean et al., 1987) and at least 18% of untreated IDDM patients and may be a superior marker of β cell damage than ICA (Palmer et al.; 1983, Wilkin et al.; 1985). Ziegler (Ziegler et al., 1989) has suggested that the appearance of IAA may be due to the abrogation of humoral tolerance to insulin during the immune destruction of insulin synthesising cells which is under genetic control. However no correlation between the presence of IAA and ICSA has been shown (Wilkin et al., 1990) suggesting that they could be independent markers for islet cell autoimmunity. IAA may provide markers for genetic susceptibility and ICA for active insulitis in man. Finally circulating IAA have been described by Palmer et al (Palmer et al., 1983) in children at the time of diagnosis of IDDM. However controversy persists regarding the prevalence and predictive value of IAA as a marker for IDDM as insulin antibodies can occur following administration of exogenous insulin, and different assay systems for detection have been utilised. Consensus of opinion states that 20-60% of IDDM patients at onset are positive for IAA (Arslanian et al., 1985, Wilkin et al.; 1985, Atkinson et al.; 1986, Karjalainen et al.; 1986) which can be detected several years prior to disease manifestation (Soeldner et al.; 1985, Dean et al.; 1986, Srikanta et al.; 1986).

Most research suggests that islet cell antibodies result from polyclonal B cell activation following release of islet-specific antigens from the damaged β cell, therefore they are not of primary pathogenic importance, but could be involved in the later stages of β cell destruction. However, the combined presence of several autoantibodies in sera, represents a higher predictive index than the presence of any single marker alone.

ICSA were also demonstrated in both NOD and control mice and in males and females regardless of subsequent diabetes onset, however no animal developed diabetes without prior appearance of both IAA and ICA. The presence of IAA has varied relative to the characteristics of the assays utilised but Ziegler (Ziegler et al., 1989) has shown that NOD mice express high, competitive insulin autoantibody levels before progressing to overt diabetes. Thus, overall current opinion would seem to suggest that the antibodies which appear after weaning in the NOD mouse are a predictive marker rather than directly involved in disease initiation or pathogenesis.

Antibodies to islet cell surface antigens (ICSA) and islet cell antigens (ICA) have
been demonstrated in the sera of NOD mice. There are few reports of ICA in the NOD mouse. However one recent study found a relatively low incidence of ICA in NOD sera (Hanafusa et al, 1985), whereas a high incidence (60-80%) was found in another study at 4 to 11 weeks of age (Toyota et al, 1984). In comparison Reddy showed that ICA appeared at day 15 after birth, preceding insulitis but the presence of such antibodies did not correlate with the expected incidence of diabetes (Reddy et al, 1988). High levels of both ICA and IAA markers prior to onset suggested a strong predisposition to clinical diabetes.

The appearance of ICSA has been reported in 10 to 50% prediabetic NOD mice at 6 to 12 weeks of age, reaching maximal prevalence (50-70%) just before the onset of overt diabetes. After disease onset, ICSA decreased (Kanazawa et al, 1984). These results complement the studies of Kida (Kida et al, 1986) who reported that the incidence of ICSA in both female and male NOD mice was approximately 70% at 9 weeks of age. Contrary to these findings Pontesilli showed that ICSA were absent in 75-100 day old NOD but 33-43% of 150-185 day old NOD expressed the autoantibodies (Pontesilli et al, 1987). ICSA and lymphocyte antibodies also precede spontaneous antibodies in the BB rat (Dyrberg et al, 1984).

Humoral abnormalities including overproduction of Ig and the presence of thymocytotoxic antibodies several weeks before diabetes onset was found to be unrelated to the pathogenesis of the disease as anti-μ suppression of NOD recipient did not abrogate the diabetogenic process induced by adoptive transfer of T cells (Bendelac et al, 1988). Bendelac (Bendelac et al, 1988) has shown in the neonatal transfer model that B cell suppressed recipients exhibited the same susceptibility to diabetes as transfer controls. Thus the NOD mouse provides a murine model whereby T cell mediated organ specific disease coexists with B cell anomalies of apparently little significance. An increased incidence of thyroid, adrenal and parietal autoantibodies has been demonstrated in children with IDDM (Nerup et al; 1973, Kokkonen et al; 1982). The presence of numerous autoantibodies in IDDM patients is in agreement with the theory of polyclonal B cell activation in recent onset IDDM.

Besides autoantibodies directed at putative islet cell antigens NOD mouse sera also contain antibodies with specificity for lymphocyte-cell-surface determinants which have similar characteristics to natural thymocytotoxic antibodies from mice undergoing
polyclonal B cell activation. The implication is that NOD mice develop manifestations of polyclonal B cell activation.

3.5 Genetics of IDDM in the NOD Mouse

Autoimmune IDDM in man is an inherited disease with MHC linked genes contributing to the genetic susceptibility. Ninety percent of diabetic Caucasians express DR3 and/or DR4 antigens compared with 60% in the total population. This association may be not with DR itself but with some other gene in linkage disequilibrium for example HLA-DQB1*0302 has been shown to be associated with disease susceptibility (Todd et al, 1987). Analysis of the BB rat model (RT1^u) has indicated that at least two independent genes or gene complexes are necessary for the inheritance of diabetes, one gene closely linked to RT1 (the rat MHC) and a second linked to the locus that controls the T cell lymphopenia (Jackson et al, 1981,1984). Buse (Buse et al, 1984) has also hypothesised that the rat's RT1 linked diabetogenic gene (RT1-DM) is linked to an I-A alpha gene. Therefore considerable effort has been expended to analyse the NOD mouse MHC, in particular the immune response genes (I-A and I-E) to assess if the diabetogenic genes map to these regions. Serological analysis of class I MHC alleles indicates that the NOD mouse is H-2K^d and H-2D^b. Hattori (Hattori et al, 1986) has also analysed the NOD mouse MHC with antibodies against class II glycoproteins, hybrid T-cell clones, mixed lymphocyte cultures and analysis of restriction fragment length polymorphisms (RFLP) and indicated that the NOD mouse has a unique class II MHC. There is no surface expression of I-E due a 650 base pair deletion in the promoter region for messenger RNA for the I-Ex similar to that seen in the H-2b haplotype and there is a unique I-A which was not recognised by any monoclonal antibodies or hybrid T-cell clones studied. RFLP analysis by Lund (Lund et al, 1990A) has shown that the H-2K gene is like the d haplotype but there appears to be mutations within and around the K region and a number of RFLP's in the class II MHC region. Fewer genes have been found in the Q region of the NOD mouse than the C57BL/10 and BALB/c mouse. NON mice showed complete identity with the b haplotype in the MHC class I region for the K1, K and Q7 and Q9 genes. The class III region in the NOD was different from that of the b- and d haplotype clearly indicating that the MHC region in the NOD and NON mouse are each unique.
Further analysis by Acha-Orbea (Acha-Orbea and McDevitt, 1987) utilising full length cDNA clones encoding I-A$^\alpha$ and $\beta$ chains demonstrated that they were identical to the H-2$^d$ haplotype except for the sequence encoding the first external domain, the leader peptide and the 5' untranslated region of the I-A$^\beta$ chain molecule. Positions 248 to 252 of the I-A$^\beta$ chain proved to be unique to NOD, these five nucleotide substitutions result in two amino acid changes at positions 56 and 57 from a proline-aspartic acid to a histidine-serine. This results in a charge difference in a region of the molecule that is conserved between I-A$^\beta$ and I-E $\beta$ and human HLA-DR$\beta$ sequences. Thus the MHC haplotype of the NOD mouse is - H-2K$^\text{g7}$ ($K^d$ I-A$^\beta$NOD I-A$^\alpha$NOD E$^\beta$NOD E$^\alpha$ 0 Ss$^h$ Slp$^0$ TNF$^\alpha$ b D$^b$) and that of NON-H-2K$^\text{b}$ ($K^b$ I-A$^{nb1}$ I-E$^k$ Ss$^h$ Slp$^0$ TNF$^\alpha$ b D$^b$). Class II MHC molecules play a central role in determining the genetic basis for immune responsiveness with the extensive allelic polymorphism located in the external domain being critical in the interaction between these molecules, antigens and the T cell receptor. Non expression of I-E in the NOD mouse and the unique sequence of the I-A$^\beta$ alleles suggests the possibility that this I-A$^\text{g7}$ (formerly known as I-A$^{\text{NOD}}$) is (one of) the MHC linked susceptibility gene(s). Breeding studies by Prochazka (Prochazka et al, 1987) confirmed Hattori's finding that homozygosity for the NOD MHC is necessary for disease susceptibility (Hattori et al, 1986) and insulitis is inherited as a recessive trait, controlled by a single recessive gene. The relative contributions of the MHC and non-MHC genes to susceptibility to type 1 diabetes have been analysed by the use of experimental crosses and congenic mouse strains. The diabetogenic trait was found to be linked to the H-2K end of the NOD MHC on chromosome 17 and termed susceptibility gene 1($Idd-1^S$ ), NON mice exhibited the resistance allele ($Idd-1^R$ ). A congenic strain NOD. B10-H-2$^b$ (NOD.H-2$^b$), in which the H-2$^g7$ of the NOD has been replaced with the H-2 region from diabetes-resistant C57BL/10SnJ (B10) strain, does not develop insulitis or diabetes demonstrating again that the NOD MHC is essential for $\beta$ cell destruction (Wicker et al, 1987). But neither
destructive insulitis nor diabetes are observed in a reciprocal congenic strain, B10.NOD-H-2\textsuperscript{\textgamma} (N6F2), showing that non-MHC genes are also required. Additionally reciprocal outcrosses between NOD and NON (not NOD H-2) were uniformly diabetes resistant. Analysis of the first backcross progeny demonstrated that disease susceptibility is determined by the polygenic inheritance of at least three functionally recessive genes or gene complexes that NON (a sister strain of the NOD mouse) shared one of the two insulitis genes and that the original diabetic mouse appeared due to a mutation of the other insulitis gene locus (Makino et al, 1986B). In addition outcross-backcross studies of NOD with diabetes resistant strains C3H, NON, B10 and SWR and screening for other polymorphic genetic markers suggested that at least two recessive genes unlinked to either Idd-1 and the MHC are necessary for disease onset (Prochazka et al, 1987). A second recessive diabetogenic gene located proximal to the Thy-1/Alp-1 (A Poa-Ib (formerly Alp-1)) cluster on chromosome 9 was found to control the frequency and severity of insulitis (Ikegami et al, 1986, Makino et al, 1986) and was therefore designated Idd-2\textsuperscript{s} in the NOD mouse. Segregation of a third diabetogenic gene Idd-3\textsuperscript{s} was suggested to explain diabetes incidence in second backcross mice (Prochazka et al; 1987, Wicker et al, 1987). Wicker suggested that Idd-3\textsuperscript{s} influences the progression from severe insulitis to overt diabetes and may control a protective suppressor T cell response to the autoimmune process. Later genetic mapping analysis of backcross to NOD from the diabetes resistant (B.10-H-2\textsuperscript{\textgamma} X NOD)F1 with microsatellite DNA markers mapped susceptibility genes for type 1 diabetes to chromosomes 3 and 11 encoding the susceptibility genes Idd-3 and Idd-4 respectively (Todd et al, 1991). Idd-3 maps near the chromosome 3 marker D3Nds1, (flanked by Il-2 and Tshb genes), affects both insulitis and diabetes and may be the susceptibility gene (or gene complex) previously proposed by Wicker et al. Idd-4 maps between Acrb and MPO near D11Nds1 on chromosome 11 and although it shows no association with insulitis in the non-diabetic progeny, it may influence the frequency of insulitis and the progression of severe insulitis to diabetes. Todd et al proposed an interaction may occur between Idd-3 and 4, such that the influence of a predisposing allele of one gene might depend on alleles of one or more unlinked genes. The human homologues of Idd-3 and Idd-4 may therefore reside on human chromosomes 1 or 4, and
17, respectively, and are candidate genes for IDDM.

A further IDDM susceptibility gene *Idd-5* maps to the proximal region of mouse chromosome 1, and was identified by analysis of the frequencies of microsatellite DNA markers on the reciprocal backcrosses (B10.H-2b7 x NOD)F1 x NOD and NOD (B10.H-2b7 x NOD)F1) (Comalli *et al.*, 1991). Three markers were associated with induced overt disease *N1Nds4*, *MIT-L2* and IL-1r1*, the gene or genes responsible for the diabetogenic effect are designated *Idd-5*, which also influences the development of insulitis. At least two candidate susceptibility genes have been proposed, the *IL-1R* gene (IL-1 is an important cofactor for T cell activation and IL-1 administration in vivo can prevent diabetes) and *Lsh/Ity/Bcg* (which encodes resistance to bacterial and parasitic infections). The homologue of *Idd-5* may map to the human chromosome 2q, suggesting that this region of chromosome 2 may contain a gene that influences human type 1 diabetes. Thus successive stages in the progression of diabetic disease appear to be controlled by distinct genes or sets of genes of which currently 5 have been identified and mapped.

### 3.6 Prevention of Insulitis and Overt Diabetes in the NOD Mouse

The pathogenesis and the precise mechanisms of destruction of pancreatic β cells in the NOD mouse are still not clear. However, many attempts have been made to prevent the onset of overt diabetes in the NOD mouse based on the knowledge of its immunological abnormalities. The establishment of the therapeutic methods for diabetes in the NOD mouse may help clarify the mechanisms of overt diabetes onset in these animals and aid in the therapy of human type 1 diabetes.

Immunoregulation is thought to play a role in controlling the development of IDDM as facilitation regimes have to be utilised for disease induction. Diabetes cannot be induced in young NOD mice by irradiation because it acts on both regulatory and effector cells, however it can act as a diabetes promoter in transfer systems. Irradiated adult mice (over 550 Rads) (Harada and Makino, 1986) receiving a diabetic cell transfer develop diabetes. The immune mechanism responsible for pancreatic β cell destruction was demonstrated to be cell mediated as adoptive transfer of splenocytes from overtly diabetic NOD mice induced diabetes within 12-22 days in 95% of irradiated mice greater than 6 weeks of age.
Acute transfer of diabetes into healthy newborns from adult diabetics also produced synchronous insulitis within 3 weeks and diabetes in 50% of mice within 10 weeks of age (Bedossa et al., 1989). Irradiation of neonatal NOD mice was not required to transfer diabetes but became mandatory for successful transfer at 3 weeks of age. Induction of diabetes in the adoptive transfer system was dependent on both the L3T4+ and Lyt2+ subsets of T cells (Bendelac et al., 1987) as neither of these T cell subsets alone mediated development of severe insulitis or diabetes when adoptively transferred to young, irradiated recipients. Spleen cells from 7 week old nondiabetic NOD mice were unable to transfer disease whereas those from 15 week old prediabetic NOD mice mediate disease transfer, indicating that sufficient effector cells were present at this stage and that the donor need not be overtly diabetic at the time of transfer (Miller et al., 1988). Similarly transfer of diabetes can also be accomplished by the injection of Con-A-activated acutely diabetic BB rat spleen cells into normoglycemic BB rats (Like et al., 1985).

Demonstration that CD4+ T cells in young NOD males are able to depress the ability of spleen cells to transfer disease has been shown by Boitard (Boitard et al., 1989). Injection of spleen cells from prediabetic (male or female) at a 24 hour interval before diabetic spleen cells to adult irradiated recipients prevented diabetes but not insulitis. The protective effect was also due to CD4+ T cells which both males and females possessed between 3 weeks and 2 months of age. This phenomenon was also illustrated by Hutchings (Hutchings and Cooke, 1990) as this population from 10-16 week old male NOD mice administered 6 days before diabetic spleen cell transfer, protect against subsequent diabetes. In the BB rat, organ specific auto-reactive cells may be present that are suppressed to variable extent in many other rat strains. Adoptive transfer could be facilitated by prior in vivo depletion of RT6.1+ regulatory T cells and in vitro mitogen activation of donor spleen cells, altering the equilibrium between autoreactive and regulatory cells which determined the expression of autoimmunity (McKeever et al., 1990).

A second form of induced diabetes is promoted by administration of 1 or 2 doses (150-200mg/Kg) of cyclophosphamide 2 weeks apart (Harada and Makino, 1984). A single higher dose (200-300 mg/Kg) (Yasunami and Bach, 1988; Charlton et al., 1989) induced clinical onset of diabetes in male and female NOD mice from 2-4 weeks after administration at an age when untreated mice do not show the disease spontaneously.
Cyclophosphamide induces a dose dependent suppression of B lymphocytes and suppression of T cells together with enhancement of DTH and antibody dependent cell mediated cytotoxicity reactions. Additionally Harada demonstrated that cyclophosphamide could not induce disease in nondiabetic mouse strains nor F1 hybrids between NOD and other mouse strains. The effect of cyclophosphamide is thought to be via immunoregulation, by abrogation of a suppressor mechanism. This is supposed to permit effector cell activation and a progression from insulitis to diabetes, rather than direct β cell toxicity or alteration of the β cell antigenic profile.

Various other immunomodulatory regimes have been utilised to prevent disease in NOD mice. Reconstitution of irradiated NOD mice with bone marrow from BALB/c nu/nu prevented development of insulitis (Ikehara et al, 1985). Lethally irradiated NOD mice reconstituted with B10.Br/cd bone marrow failed to develop disease whereas C57BL/6 or B10.Br reconstituted with NOD bone marrow developed insulitis but only 10% developed diabetes (La Face and Peck, 1989). Thus expression of NOD diabetogenic alleles in haematopoietic progenitor cells is sufficient for development of anti-β cell immunity (Serreze et al, 1988C). NOD bone marrow devoid of Thy-1+ cells was still capable of inducing diabetes indicating that haematopoetically derived immune cells from NOD mice are sufficient to induce anti-islet reactivity. Administration of cyclosporin A (25mg/Kg every 2 days) to 30-60 day old NOD mice until 160 days prevented development of insulitis and inhibited cyclophosphamide induced diabetes (Mori et al, 1986). Additionally lymphoid cells from 12 week old NOD mice cultured for 72 hours with CSA plus II-2 before reinfusion prevented diabetes. Thus ex-vivo preferential II-2 activation of splenic suppressor cells for the autoimmune process can be mediated with CSA blockade of Tc/h activities. However CSA, was ineffective in the prevention of disease recurrence in diabetic NOD mice grafted with allogenic islet tissue (Wang et al, 1987). Nicotinamide in a large dose can prevent the spontaneous expression of IDDM in prediabetic NOD mice and when given at disease onset improves the diabetic condition (Yamada et al, 1982). Daily administration of 500mg/Kg of nicotinamide also prevented development of cyclophosphamide-induced IDDM in male NOD mice (Nakajima et al, 1985A). This dose of nicotinamide given daily to female NOD mice from 14-16 weeks also prevents periductal and perivascular lymphocytic infiltration in submandibular glands (Yamada et al,
Nicotinamide inhibited the ADCC activities of splenic mononuclear cells from diabetic NOD mice (Nakajima et al., 1986) and is a potent inhibitor of mono- and poly-ADP ribosylation inhibiting DTH, ADCC and NK activities. This would seem to indicate that pyridine nucleotide metabolism in pancreatic islets and immune cells may be important in causing IDDM. In another study nicotinamide was unable to prevent islet allograft rejection except when combined with the iron chelator desferrioxamine (Nomikos et al., 1986) implying that free \( O_2 \) radicals may also be involved in islet damage.

Other methods of immunosuppression were also effective in either prevention or modulation of the diabetogenic disease process in NOD mice. Administration of Mo Abs against T cells (anti-Thy1.2) (Harada and Makino 1986) or a monoclonal antibody to the \( \alpha/\beta \) dimer of the T cell receptor for antigen protected against both spontaneous and cyclophosphamide induced diabetes (Sempe et al., 1991). Antibodies to T cell subsets such as anti-L3T4 (Koike et al., 1987) also prevented development of insulitis and diabetes. In overtly diabetic NOD mice, these antibody treatments did not normalise blood glucose levels, although they were effective in BB rats even after the onset of overt diabetes (Like et al., 1979). The essential role of this T cell subset was underlined by Wang (Wang et al., 1987) as anti-L3T4 treatment allowed pancreatic allograft survival in diabetic NOD mice. Additionally, the incidence of insulitis in T lymphocyte depleted NOD mice (B mice), which have a lower insulitis incidence, is increased when adoptively transferred with splenic cells from cyclophosphamide treated NOD mice. Lyt 2+ cell depletion from the diabetic spleen cell donor inoculum induced insulitis in irradiated NOD recipients suggesting the importance of L3T4+ T lymphocytes (Miller et al., 1988). Charlton (Charlton et al., 1988A) has also demonstrated that cyclophosphamide-induced diabetes in NOD mice could be prevented by either anti-Lyt-2+ or silica (a selective macrophage toxin) suggesting that both Lyt-2+ T cells and macrophages are necessary but not sufficient for \( \beta \) cell destruction in NOD mice. Furthermore Lee Ku has shown that silica injections prevent diabetes development in both cyclophosphamide treated and untreated NOD mice confirming that macrophages play an important role in the initiation of insulitis in NOD mice (Lee et al., 1988A). The incidence of insulitis and diabetes was reduced to less than 10% at 8 months in NOD mice given neonatal administration of anti-CD3 suggesting the
neonatal T cell repertoire is open to modulation by a single injection of a CD3 Ab
(Hayward and Schreiber, 1989). As in vivo treatment with anti-class II Mo.Ab prevents
the onset of diabetes in NOD mice and this protection can be transferred by CD4+ cells,
Timsit (Timsit et al, 1988) it has been suggested that there is adequate class II- linked
suppressor control in NOD mice. Additionally long term treatment with anti- IA Mo.Ab
prevents spontaneous development of diabetes due to elicited active immune suppression.
This Mo.Ab also prevented adoptive transfer of diabetes by splenocytes from diabetic
NOD mice into newborn irradiated mice but failed to prevent transfer into irradiated adult
NOD recipients. Passive transfer of spleen cells from anti-class II treated NOD donors
prevented diabetes in irradiated NOD mice again suggesting the induction of suppressor
cells (Boitard et al, 1988). Depletion studies indicated that CD4+ cells were responsible for
anti-class II induced protection.

Diabetes development in NOD mice can be blocked by treatment with a variety of
immunostimulants to give nonspecific immunotherapy (Table 3.1). Complete Freund’s
Adjuvant (CFA) treated NOD mice did not develop disease, thus the generation of some
type of suppressor cells in BCG vaccinated mice seems probable (McInerney et al, 1991).
Given that both TNFα and recombinant human IL-2 administration prevent IDDM (Table
3.1) then diabetes susceptibility in the NOD mouse may be due to faulty activation of
immunoregulatory cells resulting in cytokine deficiencies. Thus regulation of autoreactive
T cell clones by treatment with immunostimulatory agents could bring the cytokine levels
into harmony and prevent breakthrough of disease (Serreze et al, 1989).

Other immunomodulatory regimes prevent IDDM in NOD mice such as both the
immunosuppressive streptococcal preparation OK-432 and FK506 a novel compound
isolated from *streptomyces*. Therefore microbial immunostimulation encountered in
conventional environments may strengthen endogenous immunoregulatory functions only
marginally functional in NOD mice reared in pathogen free environments. Indeed Elias
(Elias et al, 1990) suggests that the β cell target antigen is a molecule cross-reactive with
the 65 Kd heat shock protein (hsp 65) of *mycobacterium tuberculosis*. The onset of β cell
destruction is associated with both the presence of anti-hsp65 T lymphocytes and hsp 65
cross-reactive antigen in the sera. Clones of anti-hsp 65 reactive T cells cause insulitis and
hyperglycemia in young NOD mice and the hsp 65 antigen can induce and vaccinate
against diabetes depending on the form of administration thought to be mediated by anti-
idiotypic Abs. Immunomodulation by viral infection such as with the lymphotrophic virus 
LCMV prevents diabetes. The virus affected a subset of CD4+ T cells but did not alter 
other CD4+ lymphocyte immune responses, this abortion of diabetes is maintained 
throughout the lifespan of the animal, and treated mice also fail to transfer the diabetes. 

The importance of dietary factors in either predisposition or prevention of diabetes 
has been confirmed by many researchers. DMSO may prevent diabetes in NOD mice by 
accelerating the uptake of dietary diabetogens into the β cell of genetically susceptible 
animals (Nakajima et al, 1985). The protective effect of a purified diet may be due to a lack 
of putative diabetogens in the purified diet or due to the diet itself containing factor(s) that 
protect the β cell from autoimmune attack and/or destruction. For example the 
manifestations of diabetes can be prevented by a protein-free diet (Elliott et al, 1988) 
comparable with the BB rat (Scott et al, 1985), and the immunoregulatory effects of 
monosodium glutamate may be mediated through elevation of corticosteroid levels (Table 
3.1).

3.7 Low Dose-Streptozotocin (STZ) as a Model of Induced Diabetes

In addition to the spontaneous model of IDDM in NOD mice, diabetes can be 
induced in NOD mice by cyclophosphamide and in other mouse strains by streptozotocin 
(STZ), (a metabolite of the soil fungus Streptomyces achromogenes) which was 
discovered in 1960 by Vavra while searching for new antimicrobial agents (Vavra et al, 
1960). The diabetogenic effect of STZ was reported three years later during investigation 
of its antibiotic properties. STZ is a broad-spectrum antibiotic possessing both 
antitumour and oncogenic properties (Arison and Feudale, 1967) and has widespread use 
as a method for induction of diabetes in experimental animals and treatment of malignant β 
cell tumours (Rakieten et al, 1963). Conventionally administered as a single high dose, it 
causes visible signs of β cell necrosis within 2 to 4 hours after injection and diabetes within 
24 hours in the absence of pancreatic inflammation. However in 1976 Like and Rossini 
(Like and Rossini, 1976) demonstrated that gradual elevation of plasma glucose and 
mononuclear cell infiltration in and around the pancreas resulted in diabetes after 5 
consecutive daily injections of subdiabetogenic doses (40mg/Kg) of STZ. The timing and 
appearance of inflammatory islet lesions was consistent with the hypothesis that STZ may
initiate a cell-mediated immune reaction directed against β cells, by partially damaging or modifying the β cell (Gaulton et al, 1985), thus triggering an inflammatory response ultimately resulting in further β cell loss and diabetes. B10 recombinant mice with k alleles at the K and I-A loci were particularly susceptible (Tanka et al, 1990).

The mode of action of STZ is manifold, it can directly damage the β cell plasma membrane (Cooperstein et al, 1981) and it can result in DNA strand breaks both in vivo and in vitro (Yamamoto et al, 1981). Stimulation of nuclear poly (ADP-ribose) synthetase activity is required for DNA repair resulting in intracellular depletion of its substrate, NAD, to non-physiological levels, inhibiting islet function and pro-insulin synthesis (Schein et al, 1973.).

In vivo treatment with nicotinamide, a precursor for new NAD synthesis and an inhibitor of poly (ADP-ribose) synthetase, prior to or soon after STZ had both preventative and therapeutic effects on diabetes (Gunnerson et al, 1974). Additionally studies by Yamamoto (Yamamoto et al, 1980) with picolinamide, an isomer of nicotinamide, which protects against STZ induced diabetes, demonstrated that the mechanism by which STZ decreases islet NAD content was by increased NAD degradation rather than inhibition of NAD biosynthesis. The β cell toxicity of STZ and alloxan another diabetogenic agent, has been proposed to be mediated by oxygen radical damage. This theory was suggested by the finding that SOD a widely distributed enzyme that scavenges superoxide radicals can protect against the β cell cytotoxic action of both STZ and alloxan both in vivo and in Vitro, indicating that the β cell may be particularly vulnerable to oxygen radical damage (Robbins et al; 1980, Gandy et al; 1982). Indeed combined treatment with nicotinamide and desferrioxamine, both free oxygen radical scavengers improved survival of cultured islet allografts (Mendola et al, 1989).

In addition to affecting the pancreas, STZ can also affect the immune system. Rats treated with STZ have substantial involution of the thymus and failure of normal spleen growth (Tulsiani et al, 1981). Mice treated with STZ suffer irreversible damage to bone marrow cells and important T cell precursor populations (Nichols et al, 1981). Busby (Busby et al, 1983) demonstrated that suppressor T cell activity is enhanced as there was a delay in the PFC response and serum antibody responses of diabetic mice to type III pneumococcal capsular polysaccharide.

There is a sex bias to diabetes development in the low-dose STZ model, as male
mice develop steadily increasing blood glucose levels whereas females are resistant to diabetes (Rossini et al, 1978). This can be overcome if females are treated with testosterone or castrated. Castration negates the sex difference in the hyperglycemic response to multiple-low-dose STZ. Testosterone however enhances the hyperglycemic response in castrated or non castrated females and in castrated males (Kromann et al, 1982).

In vivo administration of an antibody reactive to anti-I-A^ before low-dose STZ, suppressed diabetes development suggesting a role for I-A restricted T lymphocytes and/or macrophages (Kiesel et al, 1982,1983). However Wolf (Wolf et al, 1984) demonstrated that at least two genes, one within and one or more outside the H-2 complex determine susceptibility to multiple low-dose STZ.

The mode of action of STZ remains to be clarified. McEvoy has suggested that cell-mediated anti-β cell autoimmunity may play a role in β cell destruction as splenocytes from low-dose STZ treated mice demonstrate non-MHC restricted cytotoxicity for a rat insulinoma cell line (RIN M5F) (McEvoy et al, 1984). However transfer of spleen cell from STZ mice have been unable to induce persistent hyperglycemia, although insulitis did occur. Additionally ADCC activity is enhanced in low-dose STZ-treated animals (Marayama et al, 1986). Host B cells in contrast to host T cells are not etiologically involved in diabetes development induced by STZ. Mice rendered selectively deficient of functional B lymphocytes developed a similar disease pattern to those with normal B cell functions. However ICSA have been demonstrated in STZ treated diabetic mice and rats (Blue et al, 1984).

Immunohistochemical studies demonstrated an insulitis including Th and Tc/Ts, NK cells, macrophages and B cells similar to other experimental models of diabetes. Kantwerk has shown that treatment with monoclonal antibody against either L3T4 or Ly 2 protected STZ treated mice from diabetes implying that both cell types are involved in disease development in this model (Kantwerk et al, 1987). A role for both macrophages and Thy-1 cells in the pathogenesis of low-dose STZ has been suggested as both administration of silica particles or anti-Thy1.2 prevented β cell destruction (Oschilewski et al, 1986). Anti IL-2 receptor antibody attenuated low-dose STZ induced diabetes and insulitis. IL-2R positive cells in the periphery and in the islets themselves were
diminished, suggesting that IL-2R positive, activated T cells and macrophages are important in the development of IDDM (Hatamori et al, 1990B)

Several other treatments have been demonstrated that confer resistance to low-dose STZ, including a seven day course of whole body uv-irradiation, thought to interfere with the function of I-A positive splenic adherent cells and the induction of both disease and antigen-specific suppressor cells (Weber et al, 1986). Additionally treatment with an interferon inducer also prevented STZ induction of C-type retrovirus within B cells concomitant with the appearance of insulitis and prevented diabetes (Naji et al, 1982). The incidence of diabetes was also reduced by suppression of serotonin enhanced vascular permeability by methysergide or pargyline (Schwab et al, 1986).

Administration of CSA enhanced low-dose STZ hyperglycemia, hypoinsulinemia and B cell destruction and similarly enhanced the "toxic" diabetes produced by a single high dose of STZ. Diabetes probably resulted from the combined toxicity of CSA and STZ on the B cells (Klob et al, 1985; Sestier et al, 1985). The relevance of the STZ model of IDDM to the spontaneous disease is unresolved. Although this model demonstrates many similarities to the spontaneous disease as outlined above, in my own opinion it is a direct B cell toxin and thus the involvement of the immune system is secondary and not primary to the disease.

3.8 Conclusions With Respect to Research Objectives

Development of a therapeutic approach to the treatment of insulin dependent diabetes mellitus requires: (i) formal identification of the cell(s) involved in disease pathogenesis, (ii) elucidation of the precise sequence of events leading to B cell destruction, (iii) intervention by immunological means at the appropriate time points and (iv) examination of the outcome of such therapeutic regimes. Certainly such a protocol would be difficult practically and unethical to carry out in man. Furthermore man has an outbred genetic background, which can complicate the precise dissection of the disease. The NOD mouse, which is inbred, provides a homogeneous genetic background. Thus there is a need for studies using animal models of the disease to both precede and complement those involving the human IDDM patient. There are many remarkable similarities between animal models and the human disorder, including genetics, islet
pathology (insulitis), autoantibodies, defects in T cell activity and humoral immunity, and sensitivity of disease to immunosuppression. However as IDDM is multifactorial no single animal model is sufficient to study all aspects of the disease. The BB rat for instance being a larger rodent allows sequential pancreatic biopsy to be performed which would be difficult in a smaller animal. Genetic analysis of the disease is more appropriate in the NOD mouse as transgenic technology is more advanced in the mouse than the rat.

Utilisation of the multiple low-dose STZ-induced diabetes model has its limitations as it does not represent the spontaneous diabetes that occurs in other models, however it is useful to study the immune responses to mild β-cell cytotoxicity.

This thesis will describe research carried out on the NOD model of spontaneous IDDM: (1) I have attempted initially to describe the normal course of events occurring in the pancreas of both spontaneous IDDM and induced IDDM by transfer of diabetic spleen cells. Once the diabetes is produced within a defined and reproducible time frame the immune response can be manipulated resulting in β-cell damage. Immunohistochemical characterisation of the T cell and macrophage subpopulations in the pancreatic infiltrate set a precedent from which to study the effects of various immunological regimes. The expression of MHC antigens and cellular adhesion molecules throughout the course of β-cell destruction was also analysed.

(2) Having established the precise time course of events leading to β-cell destruction in the transfer model, I attempted to prevent the onset of diabetes by a plethora of strategies each designed to delineate which cell type was responsible for β-cell destruction.

(3) Further investigation of the role of macrophages in the disease process both in vivo and in vitro and methodologies was aimed at either preventing macrophage migration or their capacity to produce free oxygen radicals.

(4) Transgenic NOD mice which either expressed I-E or a modified I-A\textsuperscript{nod} allowed another insight into the pathogenesis of the disease particularly in relation to the development of tolerance. Pancreas from transgenic NOD mice were observed histologically to establish the effects of the transgenes on the insulitis. In relation to tolerance, a study on the thymus of NOD and transgenic NOD was carried out to observe any phenotypic thymic abnormalities which would predispose to subsequent development of autoimmunity and thus diabetes.
Table 3.1 Immunomodulatory Regimes Used to Prevent IDDM in the NOD Mouse.

<table>
<thead>
<tr>
<th>Immunomodulatory Regime</th>
<th>Protocol for Administration</th>
<th>Effect on Disease</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete Freund's Adjuvant</td>
<td>single dose at 5 weeks of age</td>
<td>prevents diabetes and transfer of diabetes</td>
<td>Michel et al, 1990</td>
</tr>
<tr>
<td>Live BCG</td>
<td>0.25-1mg as a single dose to recipients 5-10 weeks of age</td>
<td>suppression of insulitis</td>
<td>Michel et al, 1990</td>
</tr>
<tr>
<td>recombinant (r) TNF</td>
<td>long term chronic treatment</td>
<td>supresses diabetes and diabetic transfer</td>
<td>Satoh et al, 1989</td>
</tr>
<tr>
<td>r human IL-2</td>
<td>commencing 6 weeks of age</td>
<td>prevents glycosuria</td>
<td>Jacob et al, 1990</td>
</tr>
<tr>
<td>r human IL-2 + poly [I:C] (IFN α/β inducer)</td>
<td>continuous administration</td>
<td>suppressed diabetes</td>
<td>Serreze et al, 1989</td>
</tr>
<tr>
<td>OK 432</td>
<td>administered 4-24 weeks of age</td>
<td>inhibits insulitis and diabetes</td>
<td>Toyota et al, 1986</td>
</tr>
<tr>
<td>FK506</td>
<td>0.1mg, 3 times weekly</td>
<td>prevents diabetes and insulitis</td>
<td>Murase et al, 1990</td>
</tr>
<tr>
<td>hsp 65 Antigen</td>
<td>administered to young animals</td>
<td>induces or vaccinates against diabetes according to form of administration</td>
<td>Elias et al, 1990</td>
</tr>
<tr>
<td>LCMV</td>
<td>administered to newborn or adult continuous</td>
<td>prevents diabetes</td>
<td>Oldstone, 1988</td>
</tr>
<tr>
<td>DMSO (hydroxyl radical scavenger)</td>
<td>administering continuous</td>
<td>prevents diabetes</td>
<td>Klandorf et al, 1989</td>
</tr>
<tr>
<td>Monosodium Glutamate</td>
<td>administered to neonatal mice</td>
<td>attenuates insulitis and reduces diabetes incidence</td>
<td>Nakajima et al, 1985</td>
</tr>
<tr>
<td>protein-free diet</td>
<td>continuous</td>
<td>prevents diabetes</td>
<td>Elliott et al, 1988</td>
</tr>
</tbody>
</table>

DMSO : dimethylsulphoxide  
hsp65 : 65Kd heat shock protein  
LCMV : lymphotrophic choriomeningitis virus
CHAPTER 4

Materials and Methods
4.1 MICE.

A breeding nucleus of NOD mice designated (NOD/CRC) was established at the CRC, Northwick Park from mice provided by Dr. E Leiter, Jackson Laboratory, Bar Harbour, USA from the original colony at the Shiongoni Research Laboratories, Osaka, Japan. The incidence of diabetes in our colony is 70% for females, and less than 10% for male NOD mice at 30 weeks of age, when kept under conventional conditions with free access to food and water.

4.2 ASSESSMENT OF DIABETES.

The clinical onset of diabetes was judged by the presence of glycosuria (glucose in the urine) and hyperglycemia (excess glucose in the blood). Urine was tested frequently by "Diastix" reagent sticks (Mill Laboratories Ltd, Ames Division, Slough, UK) and blood samples were tested weekly, using a Glucometer (Ames, Slough, UK). A consistent reading of >10mMols/Litre coupled with a positive test by Diastix was considered to be an indication of overt diabetes. All positive animals eventually displayed weight loss which progressed to death unless sacrificed earlier.

4.3 CELL TRANSFER

Spleen cells from overtly diabetic NOD mice, male or female were prepared as single cell suspension in Hanks balanced salt solution (HBSS) and 2x10^7 cells injected intravenously into each disease free male recipient aged 2-4 months of age following irradiation (650 rads from a cobalt source, or 750 rads from an X-ray source). After transfer recipients were monitored for the development of overt diabetes (Figure 4.1). (The initial cell transfers were carried out by P. Hutchings, to whom I am grateful and subsequently by myself).

4.4 IMMUNOHISTOCHEMISTRY.

4.4.1 Tissue preparation

Freshly excised tissue was snap frozen in isopentane at -70°C for 5-7 minutes. The isopentane was contained in a glass beaker, placed in a metal kidney dish surrounded by dry ice pellets and acetone to cool the mixture. Frozen tissue was stored at -
70°C until required. Tissues were mounted in O. T. C. mounting medium (Raymond Lamb, London, UK) and sections of 5μm thickness were cut in a cryostat (Bright Instrument Company Ltd, Huntingdon, UK) onto gelatinised slides, allowed to air dry for 2-20 hours and fixed in 100% acetone at room temperature for 10 minutes. Sections were then allowed to air dry for 10 minutes and either stained or stored at -70°C until required.

4.4.2 Subbing solution- Gelatin coating of slides

Gelatin (5g) (Difco) was dissolved in 1 litre of distilled water, heated to 65°C, allowed to cool to room temperature and 0.5g of chromic potassium sulphate (KCr(SO₄)₂) (BDH Ltd) added. The mixture was then used to cover PTFE coated multispot microscope slides (Hendley (Essex) Ltd, UK). The slides were air dried in an oven and cooled before use. The subbing solution was kept at 4°C and remained fresh for 10 days.

4.4.3 Slide Mountant

2.5g of 1,4 Diazabicyclo (2,2,2) octane (Aldrich Chemical Company Ltd, Dorset, UK) was dissolved in 30 mls of Tris HCl buffer pH 8.0. The pH was then adjusted to 8.6 with concentrated HCl. 30mls of this solution was added to 70mls of glycerol (Analar, BDH Ltd, Poole, UK) mixed and stored at 4°C.

4.4.4 Antisera

Table 4.1 lists the monoclonal and polyclonal antibodies used in these studies and their specificity. Gratitude is extended to Prof. H. Waldmann (Dept. Pathology, Cambridge University) who supplied hybridomas and cloned supernatants of many rat anti-mouse T cell surface markers (CD4, CD8, Thy-1, Ly-1) and to Drs. Siamon Gordon, Paul Crocker and Hugh Rosen (Sir William Dunn School of Pathology, Oxford) who supplied me with supernatants of Mo. Abs to mouse macrophage cell surface markers (F4/80, Mac-1, SER-4 and 7/4). A Mo.Ab against the mouse homologue of ICAM-1 (YN1/1.7.4.) was a kind gift from Dr. F Takei (Terry Fox Laboratory, BC Cancer Research Centre, Vancouver, Canada) and the anti-V811 monoclonal Ab supernatant was a kind gift of Dr. Tomonari (Clinical Research Centre, Harrow, London).
4.4.5 Immunoperoxidase.

Cryostat sections were removed from storage, allowed to thaw for 10 minutes, rehydrated in PBS for 10 minutes before staining commenced. Sections were incubated for the times indicated at 25°C in a humid staining tray (Ortho, High Wycombe, Bucks, UK). Following each antibody incubation slides were washed in three changes of PBS. Mouse T cell surface components were detected on frozen pancreatic tissue by a two layer peroxidase technique. Sections were blocked with 20% normal mouse serum (NMS) for 30 minutes, incubated with the rat anti-mouse monoclonal antibodies anti-L3T4 (YTS 191.1) 1/750 dilution, or anti-Ly-2 (YTS 169.4) 1/750 dilution for 30 minutes. Then a goat anti-rat IgG biotin conjugate 1/30 dilution (Seralab) was applied for 30 minutes, followed by avidin biotin horseradish peroxidase (Vectastain ABC kit, Vector Lab) for 30 minutes. The stain was developed for 5-10 minutes with 3,3'-diaminobenzidine tetrahydrochloride (Sigma) at 0.6 µg/ml in PBS containing 0.01% hydrogen peroxide and lightly counter-stained in Harris' haematoxylin (Sigma) and mounted on DPX.

4.4.6 Immunofluorescence.

Macrophage cell surface antigens were detected by indirect immunofluorescence technique. Briefly fixed cryostat sections were preblocked as before then incubated for 30 minutes with the rat anti-mouse monoclonal antibodies. Mac-1 (M1/70) IgG2b which binds to an epitope of the C3bi receptor (CR3) on neutrophils, macrophages and NK cells, F4/80 (specific for mature mouse macrophages), 7/4 (specific for neutrophils), and SER-4 (recognises the sheep erythrocyte receptor present on stromal macrophages from lymph node liver and spleen). This was followed by fluorescein conjugated goat anti-rat IgG (H+L) (Jackson Immunoresearch Labs Inc. USA) in NMS. To facilitate double staining of macrophages, Mac-1 was detected as before followed by rabbit anti-mouse F4/80 which was detected by rhodaminated swine anti-rabbit Ig (Dako).

T cells bearing particular Vβ chains were detected by a similar indirect immunofluorescence techniques to that described for the rat anti-mouse monoclonals. Anti-Vβ6 (44.22.1), anti-Vβ8.1,2 (KJ16), and anti-Vβ11 (KT11) were used as tissue culture supernatants.

Staining of NOD class I MHC antigens was achieved using the rat anti-mouse
monoclonal M1/42.3 (rat IgG2a, Stallcup et al, 1981, kind gift from Dr G. Butcher, AFRC, Babraham, Cambridge) by the methodology previously described. Staining of class II of NOD (I-A<sup>NOD</sup>) was achieved using the mouse monoclonal OX-6 which was directly FITC conjugated (Serotec). This antibody reacts against a monomorphic determinant of the rat RTI-B antigen and cross reacts with I-A encoded molecules of certain mouse strains including NOD. The mouse equivalent of the human lymphocyte adhesion molecule ICAM-1, was observed by preblocking pancreatic sections as before with NMS followed by the rat anti-mouse M.Ab YN1/1.7.4 detected by goat anti-rat FITC. IL-2R staining was achieved using the rat anti-mouse M.Ab. 3C7 IgG2b (HPLC purified 5.19mg/ml, gift from Celltech, Slough, UK), (Ortega et al, 1984), again detected with rat anti-mouse Ig FITC.

Pancreatic beta cells were detected by preblocking sections with NMS followed by guinea pig anti-porcine insulin in NMS (ICN Immunobiologicals) and detected by rhodaminated goat anti-guinea pig IgG in NMS (Cappel).

Double immunolabelling was achieved by first staining sections with one set of primary and secondary reagents then incubating with a second set of primary and secondary antibodies. All reagents were added sequentially with three PBS washes in between as for single staining and were titrated before use. For dual immunofluorescence with rat M.Abs and rabbit polyclonal antibodies, species specific secondary reagents were used. After the final wash slides were mounted using glycerol based anti-fading mountant prepared as detailed above. Sections were examined using a Zeiss Axiophot photomicroscope (Carl Zeiss, West Germany) fitted with transmitted light and incident-light fluorescence with filter blocks for revealing fluorescein and rhodamine/Texas red fluorochromes. Photographs were taken on Kodak Ektachrome ASA 200/400 film with either automatic or manual exposure using the integrated camera system.
4.5 In Vivo Depletions

4.5.1 In Vivo Depletions With Depleting Anti-CD4 and Anti-CD8.

Depleting monoclonal antibody to mouse cell surface antigens, L3T4 (YTS 191.1) and Lyt 2 (YTS 169.4) (anti-CD4 and anti-CD8 respectively; Cobbold et al, 1984) and the non-depleting anti-CD4 monoclonal (YTS 177.9.6.1) were prepared as ascites in Lou x DA rats (Harlan OLAC Ltd) pretreated with pristane. The ascites was ammonium sulphate precipitated and analysed by SDS Page for Ig content. The depleting antibodies were of the IgG2b,k subclass and were diluted in phosphate buffered saline such that each mouse received 400μg i.v. in a final volume of 200μl on the first day of treatment and 400μg intraperitoneally on the two following days. Control mice were either treated with YTH 3.2.6 a rat monoclonal antibody which recognises human CD7 (Benjamin et al, 1986A) or with PBS alone.

4.5.2 In Vivo Depletion With Non-Depleting Anti-CD4

Non-depleting anti-CD4 monoclonal (YTS 177.9.6.1) was prepared as detailed previously. The non-depleting anti-CD4 was of the IgG2a subclass and 2mg was administered i.v, i.p, i.p. on consecutive days prior to transfer of spleen cells from a diabetic donor. Recipient mice were then injected with YTS 177.9.6.1 three times weekly during the course of the experiment. The antibody does not lead to depletion of CD4+ T cells, but specifically binds to this cell population. Control mice were similarly treated with YTH 3.2.6.

4.5.3 In Vivo Depletion With The Monoclonal Antibody 5C6.

Groups of 4-7 male NOD/CRC mice of 2-3 months were injected on day-1 with 500μg of 5C6 a monoclonal antibody specific for the myelomonocytic adhesion-promoting type-3 complement receptor (CR3 or CD11b/CD18), which does not bind to T cells (Rosen and Gordon, 1987). The initial injection was intravenous and all subsequent injections were intraperitoneal. On day 0, all mice were irradiated (650 rads from a cobalt source) and received 2x10^7 spleen cells from diabetic donors or non-diabetic syngeneic age-matched males intravenously. On day 1 and then three times every week, 5C6 or isotype matched control YTH3.2.6 antibody was administered. Mice were killed at 4 weeks for histological
examination of the pancreata. A further group of mice were given 5C6 for 10 days only
and another group was not started on this antibody treatment until day 10. Blood glucose
was assessed using a glucometer and urine tested with Diastix.

4.5.4 In Vivo Vβ8 Depletion.

Mouse monoclonal F23.1 (Staerz et al, 1985) which recognises Vβ8.1, 8.2, 8.3.
was used to deplete the Vβ8+ cells in the donor spleen cell preparation and also in the
recipient following diabetic spleen cell transfer. Donor animals were injected with 500μg of
F23.1 i.p. three days before transfer to deplete VB8+ cells. 24 hours after transfer of the
diabetogenic spleen cells into 650 rad irradiated recipients these animals were also injected
with 500μg F23.1 to ensure depletion. Control mice (donors and recipients) were injected
with a comparable volume of phosphate buffered saline [PBS].

4.6 Cyclophosphamide.

200mg/Kg of Cyclophosphamide (Faritalia Carlo Erba Ltd. St. Albans, Herts),
was administered i.p. twice 14 days apart to male or female NOD mice to induce the onset
of diabetes.

4.7 Construction of NOD-E, NOD-ASP and NOD-PRO Transgenic

NOD mice.

I-E transgenic NOD mice were produced by isolating a 7.5 Kb Bg/1 fragment
containing the Ecrd gene, 2 Kb 5' and 2 Kb 3' flanking sequences from Cos Id-2, which
was originally isolated from a genomic cosmid library together with Cos Id-1 (Hyldig-
Nielsen et al, 1983). These fragments were subcloned using cla I linkers into psk+ and
used for injection into fertilised mouse embryos after removal of bacterial sequences
(Figure 8.1A). Progeny were used to establish transgenic lines and were screened for the
presence of the transgene by Southern blot analysis of tail DNA using 32P labelled cDNA
probes to I-Erd (performed by Drs T. Lund, D. Kioussis and J. Picard). The progeny of
two founders NOD-E-3 and NOD-E-9, both of which expressed the transgene at the RNA
level were analysed to assess both that the insertion of Eαd restored expression of I-E on lymphoid cells and its influence on IDDM.

NOD-1-3 clone was isolated from a genomic cosmid constructed from NOD/CRC liver DNA by screening with a 1.5 kbp BamH I-Hind III fragment from the Aβ gene containing exon III and IV. For in vitro mutagenesis single stranded template of the Aβ exon II subclone was isolated following superinfection with the VCM13 helper phage (stratagene). The mutagenesis was carried out using PRO primer or ASP primer and a mutagenesis system (Amersham) as recommended by the manufacturer (Figure 8.1 B).

The Aβ NOD-PRO and the Aβ NOD-ASP transgenes were subcloned in three steps from the cos NOD-1-3. In the first step, a 11.0 kbp EcoR V fragment containing the structural gene for Aβ NOD was subcloned using cla I linkers into psk+ deleted for the sac II site. For in vitro mutagenesis the 330 bp sac II fragment containing exon II was subcloned into psk+ and used to change the amino acid residue 56 from histidine to proline or the amino acid residue 57 from serine to aspartate followed by recloning into the Aβ gene. The second step was to ensure sufficient 5' flanking sequences for tissue specific expression of the transgene a 4.2 kbp 5'BamH-1 kpn I flanking fragment was modified by replacing the BamH-1 site with a SaL-1 site and inserting it into the SaL I-kpn I site of pTCF. In the third step the 10.4 kbp kpn I-cla I fragment of the Aβ NOD-PRO/ASP genes were inserted into the kpn I cla I site of the 5' flanking subclone. The reconstituted Aβ NOD-PRO and NOD-ASP transgenes devoid of plasmid sequences was injected into fertilised mouse embryos. (Figure 8.1C). Insertion of the two point mutations into the Aβ gene to generate the sequences encoding a Pro 56 instead of His or an Asp 57 instead of Ser destroyed a restriction site for DdeI at this position in the AβNOD gene. When spleen DNA from transgenic mice was digested with DdeI an extra band was detected (as the DNA could only partially be digested by the restriction enzyme) on an electrophoresis gel, which allowed assessment of those progeny that had inherited either of the transgenes. Several founder transgenic NOD mice carrying either one of the mutated Aβ genes were generated and the progeny of one of each, NOD-A-PRO-3 (NOD-PRO) and NOD-A-ASP-2 (NOD-ASP) were analysed in more detail. (These procedures were carried out by Drs T. Lund and D. Kioussis assisted by Dr E. Simpson, P. Chandler and J. Picard).
4.8 **In Vivo and Vitro Inhibition of the L-Arginine Metabolic Pathway.**

4.8.1. **In Vivo Treatment of NOD Mice With L-NMMA, L-Arginine or D-NMMA.**

Groups of male NOD mice were irradiated (750 Rads) and given a diabetic spleen cell transfer following the protocol outlined above. Further groups of female NOD mice were administered cyclophosphamide. These mice were then administered 5mg of either L-Arginine (L-Arg) or a competitive inhibitor of the L-Arginine-dependent pathway for the synthesis of inorganic nitrogen oxides, namely L-NG monomethyl arginine (L-NMMA) or the inactive inhibitor DG monomethyl arginine (D-NMMA) to act as control. All drugs were given in 0.2mls PBS i.p. on the days required as indicated in chapter 7. Quantities of the D-NMMA enantiomer were restricted, in the later experiments it was substituted by 0.2mls PBS i.p. Mice were then monitored twice weekly for the presence of glucose in the blood and urine. L-arg, L-NMMA and D-NMMA were a kind gift from Prof. E. Liew (Department of Bacteriology and Immunology, University of Glasgow).

4.8.2 **In Vitro Inhibition of Nitrite Release from LPS and IFN-γ Stimulated Macrophages From NOD and B10.L-LshF Mice by L-NMMA.**

4.8.2. (i) **Media**

RPMI 1640 (Dutch Modification, ICN Flow Laboratories, Irvine, Scotland), with 20 mM L-glutamine, 100 U of penicillin per ml, 100mg streptomycin per ml and 2-mercaptoethanol (referred to hereafter as RPMI) was supplemented further with fetal calf serum (Northumberland Biologicals).

4.8.2. (ii) **Elicited Peritoneal Macrophages.**

Elicited peritoneal macrophages were obtained from 12 Week old NOD/CRC mice (14 weeks of age) and N10 congenic B10. L-LshF mice (kindly supplied by Prof. J. Blackwell and bred at the LSHTM, London) by administration of 0.1mls of a 1% Biogel P 100 solution in PBS (Biorad) i.p. 4 days previously. Peritoneal macrophages were harvested by peritoneal lavage with 5mls of RPMI 1640-2% FCS on ice into polypropylene tubes (Falcon 2070; Becton Dickinson). Cells were spun at once (260 x g, 12 minutes) and resuspended in RPMI 1640-10% FCS and counted. The concentration of
cells from both mouse strains was adjusted to $5 \times 10^6$/ml. 150μl of this cell suspension was added as a ‘bubble’ to 13-mm-diameter thermolux coverslips (Miles Scientific) in 24 well tissue culture plates (Nunc, Gibco, Paisley, United Kingdom). Liquid bubbles were left for 45 minutes to allow cell adherance, after which the wells were flooded with 0.5ml of RPMI 1640-10% FCS and left standing for an additional 2 h at 37°C in 5% CO₂. Wells were then washed three times with RPMI 1640 before the addition of 0.5ml of supplemented RPMI 1640-10% FCS to each well. Recombinant rat IFN-γ (final concentration 0, 10, 50, 100 u/ml) (TNO, Primate Centre, Netherlands) was added to the appropriate wells and/or LPS (S. typhimurium phenolic extract, tissue culture grade, L6143 [Sigma]) to a final concentration of 0, 0.1, 1.0 ng/ml, with or without 200mM D- or L-NMMA. All assay conditions were in duplicate. Cultures were incubated for 48 h at 37°C in 5% CO₂ and supernatants were collected for nitrite assays at 24 and 48 h.

4. 8.3. Measurement of Nitrites

Griess reagent (1% sulfanilamide [Sigma], 0.1% naphthylenediamine hydrochloride [Sigma], 2.5% orthophosphoric acid [Analar B.D.H., Dagenham, Essex, United Kingdom]) was freshly made prior to use. Griess reagent was added 1:1 with supernatant and left standing for 5 minutes at room temperature. Standards prepared by using sodium nitrite (Sigma) (0.7035 to 180 mM) were included on each assay plate. A₅₇₀ values were read on a spectrophotometer (Dynatech).

4.9. Biotinylation of Monoclonal Antibodies

Purified antibody was concentrated to 1mg/ml in PBS. The antibodies were dialysed against 0.1M sodium bicarbonate (Sigma) pH8.4 for 24 hours at 4°C. Biotin-X-NHS ester (Calbiochem) was dissolved in Dimethyl Sulfoxide (DMSO) to a concentration of 1mg/ml. To 0.5ml of 1mg/ml of antibody was added 50μl of Biotin-X-NHS dissolved in DMSO. This material was allowed to react with the antibody for 24-48 hours at 4°C. After conjugation, unbound biotin ester was removed by extensive dialysis against PBS. Finally the antibody was stored at 4°C either sterile or with 0.1% Sodium
Azide (Sigma).

4.10 FACscan Analysis of Thymic Cell Suspensions

Fresh thymus was digested in 0.5mg/ml collagenase (Sigma) and 0.02mg/ml deoxyribonuclease I (DNase I) (Boehringer Mannheim, Germany) for several minutes, washed in BSS and frozen in 90% FCS / 10% DMSO and stored at -70°C.

For analysis vials were rapidly thawed, washed and resuspended at 10^5-10^6 cells/sample and stained by one of the following methods (Table 9.1, Chapter 9, gives an itemised list of reagents used for thymus analysis).

4.10.1. CD4/CD8/MRC OX-6 or Mac261/CD4/CD8

Cells were resuspended in 50µl biotinylated OX6 (mouse IgG1, detects I-A^k,s,NOD class II MHC)(1µg/ml) in 1% normal mouse serum or (5µg/ml) Mac261 (restricted to the recognition of I-A^d/I-Ab^NOD MHC class II molecules, mouse IgG2a, kind gift of Dr. G Butcher, Babraham, Cambridge) in 1% normal mouse serum and incubated for 30 minutes on ice. They were then washed three times in 100µl changes of PBS/1% BSA, followed by a 30 min. incubation with the triple label cocktail anti-CD4 Phycoerythrin (clone YTS191.1, IgG2b, 1/40, Coulter Clone, U.K.), anti-CD8 FITC (clone 53-6.7, IgG2a, 1/40, Becton Dickinson) and streptavidin R613 (1/100, Gibco BRL) which binds to the biotinylated OX6 or Mac261. The cells were then washed a further three times and fixed in 1% formaldehyde.

4.10.2. OX6/B220 or Mac261/B220

Cells were labelled with biotinylated OX6 (1µg/ml), or biotinylated Mac-261 (5µg/ml), washed and incubated with B220 FITC (clone RA3-6B2, identifies only the B cell restricted determinant [Holmes and Morse, 1988], IgG2a, 1/40, Coulter Cytometry) together with avidin P.E. (1/80) (Biogenesis) and then stored as above.

4.10.3. B220/CD4/CD8

Cells were labelled with biotinylated anti-CD8 (1µg/ml) in 1% normal mouse serum for 30 minutes, washed and incubated with a cocktail of, B220 FITC, anti-CD4 P.E. and streptavidin R613. They were then washed and stored as before.
4.10.4. OX6/B220/CD5, Mac-261/B220/CD5, TIB120/B220/CD5

Mononuclear cells obtained from peritoneal lavage from NOD mice and spleen cells from NOD (I-A\textsuperscript{nod}) and C57BL/10 (I-A\textsuperscript{b}) were stained with biotinylated OX6, biotinylated Mac-261 or biotinylated TIB120 (1\(\mu\)g/ml; clone M5/114.152, rat IgG2b, defines an allotypic determinant shared between A\textalpha\textsuperscript{b}A\beta\textsuperscript{b} and E\textalpha\textsuperscript{k}E\beta\textsuperscript{k} [Bhattacharya et al, 1981]) in 1% normal rat serum for 30 minutes on ice, and washed as before. This was followed by a further 30 minutes incubation with a cocktail of B220 FITC, streptavidin R613 and CD5 P. E. (clone 53-7.3, rat IgG2a which reacts with the Ly-1 differentiation antigen found at high densities on T cells of all mouse strains and at low levels on a small population of B cells [Hardy and Kayakawa, 1986; Ledbetter et al, 1980] Pharmingen, U.S.A.). Cells were washed three times and fixed in 1% formaldehyde.

All samples were analysed by FACscan (Becton Dickinson), with fresh NOD spleen cell suspension stained with the above protocols to define the FACscan gate settings as described in chapter 9.

4.11 Immunohistochemistry of NOD and Transgenic NOD Thymus

One thymic lobe from NOD or transgenic NOD mice was snap frozen for immunohistology and the remainder used for FACscan analysis. Sections of thymus were prepared for immunohistology as described previously. Immunofluorescent staining of thymus cryostat sections permitted observation of all cell types in situ, without disruption of morphology, including cells that do not readily form suspensions, such as epithelial cells. Thymi were stained with the panel of antibodies contained in Table 9.1. Anti-keratin (rabbit anti-human [Dako]) was used to define the thymic epithelial cell network. The medullary epithelial cell network was defined with 4F1E4 and cortical epithelial cell network with IVC4 (detected by tetramethylrhodamine isothiocyanate [RITC]-labelled goat antibody against IgM (H chain specific) [Southern Biotechnology Assoc. Inc]. B cells were detected with B220 directly conjugated to FITC (Coulter Clone) and surface Ig with rabbit Igs to mouse Ig directly conjugated to FITC (Dako). MHC class II was detected by MRC-OX-6 as outlined for FACscan analysis.
Diagram of the protocol of diabetic spleen cell transfer, whereby $2 \times 10^7$ spleen cells from overtly diabetic male or female NOD mice are administered i.v. to disease free young (~2 months) male NOD recipients given 650 rads the same or previous day. This procedure produces diabetes within the reproducible time frame of approximately 4 weeks.
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<td>H81-20-8-22-6</td>
<td>I-E&lt;sup&gt;k&lt;/sup&gt;</td>
<td>mouse IgG2a</td>
<td>All I-E&lt;sup&gt;k&lt;/sup&gt; bearing cells</td>
<td>Mineta &lt;em&gt;et al&lt;/em&gt;, 1990</td>
</tr>
<tr>
<td>CD5 (clone 53-7.3)</td>
<td>Ly-1 antigen</td>
<td>rat IgG2a</td>
<td>Mouse T cells + subpopulation B cells</td>
<td>Devaux &lt;em&gt;et al&lt;/em&gt;, 1984</td>
</tr>
<tr>
<td>B220 (clone RA3-6B2)</td>
<td>B cell restricted determinant of Ly-5</td>
<td>rat IgG2a</td>
<td>Mouse B cells</td>
<td>Hardy, Hayakawa, 1986</td>
</tr>
<tr>
<td>Anti-keratin</td>
<td>predominantly MW 56KD and 64KD CTES II</td>
<td>rabbit anti-human polyclonal</td>
<td>whole thymic epithelial cell network</td>
<td>Holmes and Morse, 1988</td>
</tr>
<tr>
<td>IVC4</td>
<td>CTES XX</td>
<td>rat IgM</td>
<td>subcapsular and medullary epithelial cells</td>
<td>Coffman and Weissman, 1981</td>
</tr>
<tr>
<td>4F1E4</td>
<td></td>
<td>rat IgM</td>
<td>Subcapsular, cortical, subset of medullary epithelial cells</td>
<td>Kanariou &lt;em&gt;et al&lt;/em&gt;, 1989</td>
</tr>
<tr>
<td>Anti-mouse Ig</td>
<td>Mouse Ig</td>
<td>rabbit polyclonal</td>
<td>All Ig bearing cell (B cells)</td>
<td>Kanariou &lt;em&gt;et al&lt;/em&gt;, 1989</td>
</tr>
<tr>
<td>MRC-OX-6</td>
<td>Class II MHC</td>
<td>Mouse IgG1</td>
<td>All MHC class II bearing cells</td>
<td>Dako</td>
</tr>
<tr>
<td>Mac 261</td>
<td>I-A&lt;sup&gt;k&lt;/sup&gt;,s,nod</td>
<td>Mouse IgG2a</td>
<td>NOD MHC class II bearing cells</td>
<td>McMaster &lt;em&gt;et al&lt;/em&gt;, 1979</td>
</tr>
<tr>
<td>TIB120 (M5/114.15.2)</td>
<td>I-A&lt;sup&gt;d&lt;/sup&gt;, I-A&lt;sup&gt;β&lt;/sup&gt;nod</td>
<td>rat IgG2b</td>
<td>MHC class II bearing cells</td>
<td>Kind gift G. Butchter (AFRC, Cambridge)</td>
</tr>
<tr>
<td>M1/42.3</td>
<td>I-A&lt;sup&gt;b&lt;/sup&gt;,d,q, I-E&lt;sup&gt;d&lt;/sup&gt;,k non-polymorphic determinants of MHC class I</td>
<td>IgG2a</td>
<td>All MHC class I bearing cells</td>
<td>Bhattacharya &lt;em&gt;et al&lt;/em&gt;, 1981</td>
</tr>
</tbody>
</table>

CTES-clusters of thymic epithelial staining patterns.
II: M. Ab. stains subcapsular and perivasual TEC (only one cell layer), in addition medullary TEC and Hassall's corpuscles (HC) are stained.
XX: Miscellaneous designation of staining pattern, eg. majority of subcapsular/ cortical TEC and minority of medullary TEC.
Rab-Rabbit, Mo.- Mouse
CHAPTER 5

Characterisation of Pancreatic Islet Cell Infiltrates in NOD Mice: Effect of Cell Transfer.
5.1 Introduction

Insulin-dependent diabetes mellitus (IDDM) is histologically characterised in its early stages by a mononuclear cell infiltration of the pancreatic islets denoted "insulitis" (Von Mering, 1889) accompanied by selective β cell destruction. The presence of such an inflammatory infiltrate suggested an autoimmune aetiology for IDDM. Furthermore cell-mediated autoimmunity may play an important role in the pathogenesis of the disease resulting in specific β cell degeneration and insulin insufficiency in both the human and animal models of IDDM. The initiating event and the role of specific immune cells in mediating β cell damage have yet to be delineated. Examination of new-onset type-1 diabetes patients has demonstrated many abnormalities in T cell subsets in both the periphery and the pancreas. Elevated killer cell (K) activity (Pozzilli et al, 1979), imbalance in T cell subsets (Gupta, et al, 1982; Jackson et al, 1982; Ilonen et al, 1984; Rodier et al, 1984) or the presence of activated T cells (Ia+) (Alviggi et al, 1984; De Berardinis et al, 1988A) have all been demonstrated in the peripheral blood of type 1 diabetic patients.

The frequency of insulitis in IDDM patients with recent disease onset observed by different studies is inconsistent. In a study by Gepts (Gepts, 1965) 16 of 23 pancreata from patients with recent onset IDDM showed insulitis, however Doniach and Morgan (Doniach and Morgan, 1973) did not observe this phenomenon in the 13 patients they examined. The extent of insulitis within the pancreata of individual IDDM patients is variable, not all islets are equally affected and insulitis is more pronounced among young patients. Such discrepancies in the frequency and extent of insulitis observed in human IDDM are reflected in the spontaneous animal model of the disease and thus appear to be due to the disease pathology. Indeed studies by Gepts (Gepts, 1965) revealed in some pancreatic lobules islets devoid of β cells and insulitis, in others many large intact islets with only a few lymphocytes peri-islet, and yet others with partial insulin content and lymphocytic infiltration. Given these findings of a very patchy histopathology it is therefore not surprising that results from different studies are not in accordance. Additionally once β cell destruction is complete i.e. at clinical presentation of type 1 diabetes about 70% of the islets contain no insulin-secreting β cells and no infiltration. This is presumably because the antigenic stimulus, the β cell, has been removed by autoimmune destruction. There is a report suggesting insulitis particularly affects those islets where residual β cells are still present (Foulis et al, 1986) suggesting that some beta cell antigen
remains.

Very few studies have been reported regarding pancreatic lymphocyte infiltrates in the prediabetic period of the human owing to the difficulty in performing pancreatic biopsies. Bottazzo found Ig bearing lymphocytes, NK cells and all subsets of T lymphocytes, although mainly Tc/s (CD8+) cells, in the pancreatic lesions of a newly diabetic child (Bottazzo et al, 1985). Analysis of the pancreata from long term IDDM patients with recurrent disease who had received pancreatic transplants from their discordant, identical twins demonstrated the presence of macrophages and T cells (mainly CD8+) in the insulitis lesions (Sibley et al, 1985).

Studies have also shown that T cells from diabetic patients can inhibit glucose and theophylline induced insulin release from murine islets and are cytotoxic to rat islet cells (Boitard et al, 1982; Charles et al, 1983; Boitard et al, 1984). Indeed many studies have indicated that T cells play a fundamental role in the development of IDDM, particularly in the spontaneous animal models, the NOD mouse and BB rat. NK cells have also been implicated in β cell destruction in the BB rat (MacKay et al, 1986). Insulitis commences at about 5-6 weeks of age in the NOD mouse and is observed in more than 90% of both male and female NOD mice at 200 days. Development of overt diabetes due to β cell destruction is observed generally in 70-80% of females and 10-20% of males at 210 days (Tochino, 1987).

Experimental evidence suggests that T lymphocytes play a major role in the development of diabetes in the spontaneous models of the disease as neonatal thymectomy prevents diabetes in both the NOD mouse (Ogawa et al, 1985) and the BB rat (Like et al, 1982). Treatment with anti-thymocyte serum or anti-Thy-1.2 antibody markedly suppresses the development of overt diabetes in NOD mice (Harada et al, 1986). Thus thymus dependent cell-mediated autoimmune mechanisms are responsible for the pathogenesis of insulitis in NOD mice, the primary change leading to the development of diabetes. In addition mononuclear cell infiltration of the lacrimal and submandibular glands is delayed by the same treatment regimes which diminish insulitis, suggesting that infiltration in various glandular tissues of NOD mice is also produced by T cell mediated autoimmunity (Hayward et al, 1988).

Conflicting data have been reported concerning the percentage of lymphocyte subsets infiltrating the pancreas. A longitudinal study by Signore of the NOD endocrine pancreas showed that CD4+, MHC class II+ and surface IgM+ cells are the predominant subsets detectable in insulitis (Signore et al, 1989). Additionally he noticed that 30% of T
cells expressed surface II-2 receptors implying that they were activated. Once all the β cells had been destroyed, lymphocytes abandoned the islets. Hanafusa also investigated longitudinal changes in lymphocyte subsets in the NOD and pancreas found a predominant infiltration by activated T lymphocytes including Th/i (helper/inducer) and Tc/s (cytotoxic/suppressor) observed in the early stages of insulitis (Hanafusa et al., 1987). NK cells were also detected in the lesion. In this study, Ig bearing cells were shown to increase in number with progression towards insulitis. Whereas T lymphocytes were localized close to islets, B cells appeared adjacent to blood vessels and around T cell clusters. The percentage of splenic T lymphocytes was also markedly increased in the initial stage of insulitis suggesting that T rather than B lymphocytes participate in the development of insulitis, accompanied by marked splenic T cell proliferation which corresponded to the beginning of T cell infiltration into islets. Like demonstrated activated T cells in the diabetic BB rat pancreas but B lymphocytes were observed infrequently (Like et al., 1979, 1986). Walker also observed mainly Th, Tc/s and macrophages in the prediabetic BB rat (Walker et al., 1988A). Although studies in the human, NOD mouse and BB rat conflict within species and between species the basic principle remains that the insulitis consists of T cells (both of the Th/i and Tc/s phenotypes), monocytes and B cells to varying degrees. It remains to be clarified what the role of each particular cell type is, and if indeed they can be assigned a role or are merely present in the lesion due to nonspecific inflammatory recruitment. Therefore it is essential to observe the disease manifestations during its early phases to eliminate the latter possibility.

The essential requirement for T cells in disease manifestations in the NOD mouse was observed by Wicker et al. (Wicker et al., 1986), who demonstrated that adoptive transfer of splenocytes from overtly diabetic NOD mice induces diabetes within 12-22 days in 95% of irradiated (650-750 rads) mice older than 6 weeks of age. If either T cell subset is depleted from the donor cell inoculum then neither severe insulitis, nor diabetes, results (Miller et al., 1988). Such an adoptive transfer system provides an excellent model, inducing diabetes within a defined and reproducible time frame to allow manipulation of the immune system aimed at regulating the onset of diabetes. The neonatal adoptive transfer system described by Bendelac is also dependent on both L3T4+ and Lyt-2+ T cell populations (Bendelac et al., 1987).

In this chapter I have characterised the normal pancreatic infiltrate in the male and female NOD mouse longitudinally by immunohistochemistry of cryostat sections with monoclonal antibodies to T cell subsets, class I MHC antigens, class II MHC antigens, macrophages and B cells. My findings for the T cell subset analysis are similar to those of
previous authors but it is essential to characterise the time course of infiltration for each NOD mouse colony as the disease incidence is so variable. Normally, pancreatic β cells of mice constitutively express low levels of class I, but do not express class II antigens (Faustman et al, 1980). However given that over-expression of class I MHC proteins on pancreatic islet cells is characteristic of autoimmune type I (insulin dependent) diabetes mellitus in humans who die soon after diagnosis (Bottazzo et al, 1984, 1985; Foulis et al, 1987A,B), it was of interest to examine if this phenomenon occurred in the NOD spontaneous animal model of IDDM. It is difficult to discover whether class I MHC antigen over-expression is due to the presence of inflammatory cells or whether both are secondary to another event such as virus infection. These are all important questions that are difficult to resolve in humans because the pathology is invariably far-advanced at clinical presentation and pancreatic tissue is rarely available. However class I MHC antigen over-expression is also present in the animal models of Type I diabetes in the BB rat (Ono et al; 1988) and in the multi-low dose STZ mouse (Campbell et al; 1988A, Cockfield et al; 1989). These observations and my own studies on the NOD mouse provide the opportunity to relate the expression of class I MHC antigens to other pathological changes associated with β cell destruction. Much controversy surrounds the expression of MHC class II on pancreatic β cells in both the human and the NOD mouse. My studies in the transfer system have hopefully, delineated more precisely the class II MHC antigen expressing cells within pancreatic infiltrates.

The incidence of diabetes at 30 weeks of age in the NOD/CRC colony is 80% for females and <10% for males. Given that the full manifestation of diabetes is not evident until 30 weeks of age the adoptive transfer model provided me with an excellent experimental model for reproducing diabetes. Therefore I characterised the pancreatic infiltrates present in the irradiated recipient pancreas at weekly intervals after the transfer of diabetic spleen cells. Having completed this study the time interval between transfer and the first evidence of insulitis was ascertained thereby providing a time point or "window" for commencement of immunogenic regimes to prevent the onset of disease. Such investigations will be discussed in the next chapter.
5.2 Results

5.2.1(i) Spontaneous Progressive Pancreatic Infiltration In Female NOD Mice.

Female NOD/CRC mouse pancreata were analysed at 1, 3 and 5 months of age by immunohistochemistry of pancreatic cryostat sections by the methods previously described. It can be seen from Table 5.1A that at 1 month of age 26% of islets from the pancreata of female NOD mice demonstrated the presence of peri-islet infiltration and 4% the presence of intra-islet infiltration when stained with MRC-OX6 which detects NOD I-A. However by 3 months of age 56% of islets from NOD female mice demonstrated moderate peri-islet infiltration and 28% moderate intra-islet infiltration with complete destruction of 23% of islets. This β cell destruction continued until at 5 months 44% of islets in female NOD mice had been destroyed with only 9% of islets being completely unaffected. MHC class II expression was not observed on β cells or the endocrine cells of female NOD pancreas at any of the time points analysed. A similar pattern of infiltration was observed following the fate of CD3+ T cells. At 5 months of age 60% of islets demonstrated moderate peri-islet infiltration and 28% moderate intra-islet infiltration with complete destruction of 23% of islets. This β cell destruction continued until at 5 months 44% of islets in female NOD mice had been destroyed with only 9% of islets being completely unaffected. MHC class II expression was not observed on β cells or the endocrine cells of female NOD pancreas at any of the time points analysed. A similar pattern of infiltration was observed following the fate of CD3+ T cells. At 5 months of age 60% of islets demonstrated moderate peri-islet infiltration and 28% moderate intra-islet infiltration with complete destruction of 23% of islets. This β cell destruction continued until at 5 months 44% of islets in female NOD mice had been destroyed with only 9% of islets being completely unaffected. MHC class II expression was not observed on β cells or the endocrine cells of female NOD pancreas at any of the time points analysed. A similar pattern of infiltration was observed following the fate of CD3+ T cells. At 5 months of age 60% of islets demonstrated moderate peri-islet infiltration and 28% moderate intra-islet infiltration with complete destruction of 23% of islets. This β cell destruction continued until at 5 months 44% of islets in female NOD mice had been destroyed with only 9% of islets being completely unaffected. MHC class II expression was not observed on β cells or the endocrine cells of female NOD pancreas at any of the time points analysed. A similar pattern of infiltration was observed following the fate of CD3+ T cells. At 5 months of age 60% of islets demonstrated moderate peri-islet infiltration and 28% moderate intra-islet infiltration with complete destruction of 23% of islets. This β cell destruction continued until at 5 months 44% of islets in female NOD mice had been destroyed with only 9% of islets being completely unaffected. MHC class II expression was not observed on β cells or the endocrine cells of female NOD pancreas at any of the time points analysed. A similar pattern of infiltration was observed following the fate of CD3+ T cells. At 5 months of age 60% of islets demonstrated moderate peri-islet infiltration and 28% moderate intra-islet infiltration with complete destruction of 23% of islets. This β cell destruction continued until at 5 months 44% of islets in female NOD mice had been destroyed with only 9% of islets being completely unaffected. MHC class II expression was not observed on β cells or the endocrine cells of female NOD pancreas at any of the time points analysed. A similar pattern of infiltration was observed following the fate of CD3+ T cells. At 5 months of age 60% of islets demonstrated moderate peri-islet infiltration and 28% moderate intra-islet infiltration with complete destruction of 23% of islets. This β cell destruction continued until at 5 months 44% of islets in female NOD mice had been destroyed with only 9% of islets being completely unaffected. MHC class II expression was not observed on β cells or the endocrine cells of female NOD pancreas at any of the time points analysed. A similar pattern of infiltration was observed following the fate of CD3+ T cells. At 5 months of age 60% of islets demonstrated moderate peri-islet infiltration and 28% moderate intra-islet infiltration with complete destruction of 23% of islets. This β cell destruction continued until at 5 months 44% of islets in female NOD mice had been destroyed with only 9% of islets being completely unaffected. MHC class II expression was not observed on β cells or the endocrine cells of female NOD pancrea...
to female NOD mice in which only 32% and 9% respectively had no peri-islet infiltration. In addition, absence of intra-islet infiltration in the male ranged from 87-98% from 3-5 months whereas in the female ranged from 71-46%. Thus the male NOD mouse demonstrated a progressive increase in peri-islet infiltration with minimal-moderate intra-islet infiltration. This is consistent with the observed low incidence of diabetes in the male of <10% compared to 70% for females which demonstrate predominantly severe intra-islet infiltration at 5 months of age. Such a pattern of predominantly peri-islet infiltration observed in the male NOD mouse could also be demonstrated at 5 months of age since only 3% of islets demonstrated moderate presence of T cells at intra-islet locations (detected with a mo. Ab to CD3 present on the surface of T cells) (Table 5.2B) and macrophages respectively (Table 5.3B). It has to be noted that there is a constitutive population of macrophages present at peri/intra-islet locations in both male and female NOD mice independent of progression to diabetes, which can accumulate to moderate numbers (70% in male NOD mice at 5 months of age) this will be described later in this chapter. However hyperexpression of class I MHC antigen could be observed in 57% of male NOD mice at 5 months of age whereas 81% of female NOD mouse islets demonstrated hyperexpression of class I MHC antigens (Table 5.4B). Thus all the elements of the immune response; T cells, macrophages and class I/II MHC antigen positive cells, are present at peri-islet locations in the male NOD mouse but in most animals do not progress to intra-islet sites.

5.2.2 Diabetic Spleen Cell Transfer-Time Course of Inflammatory Events

We utilised the adoptive transfer model of Wicker et al (1986) to facilitate a histological analysis of the temporal relationships between pancreatic lymphocytic and macrophage influx and the expression of MHC antigens and adhesion molecules in the prediabetic period before disease onset in recipient mice. Seventeen non-diabetic sublethally irradiated, male NOD mice aged 10-14 weeks were injected i.v. with 2x10^7 spleen cells from diabetic adult donors and killed randomly at weeks 1, 2, 3 and 5 for histological analysis. Four age-and sex-matched control irradiated NOD mice were injected i.v. with 2x10^7 syngeneic, non-diabetic spleen cells from adult mice and analysed 5 weeks after transfer.
Histological changes occurring in the pancreas from the time of diabetic spleen cell transfer until the onset of diabetes.

5.2.2(i) T cell sub-populations

All mice which received the non-diabetic spleen transfer remained normoglycemic with only 36% and between 9% and 18% of islets displaying peri and intra-islet infiltration respectively (Table 5.5). This minimal insulitis is that which would be expected of male NOD mice of this age.

Pancreata from mice which had received diabetic spleen cells displayed neither lymphocytic infiltration nor evidence of beta cell destruction until week 2, however class II MHC antigen expression was observed on blood vessel associated cells adjoining intact islets at week 1. Class II expression on blood vessel-associated cells was not seen in the recipients of non-diabetic spleen cells at week 5 suggesting that this observation was a result of the homing of the diabetogenic effector cells. At 2 weeks after transfer 67-75% of islets analysed had peri-islet infiltration of both L3T4+ and Ly-2+ T lymphocytes (Figure 5.1A, B). Lymphocytic infiltration of islets was rapid and 75% of recipients were diabetic 3 weeks after transfer. At this point only 19% of islets were not infiltrated with 60-65% demonstrating severe intra-islet infiltration. T cells in the inflammatory infiltrate were positive for IL-2 receptor expression. Five weeks after transfer all remaining recipients of diabetic spleen cells were diabetic and 58% of islets examined contained remnants devoid of beta cells with little remaining mononuclear cell infiltrate. Those islets retaining beta cells showed extensive intra-islet infiltration and destruction of islet morphology (Table 5.5). Residual islets were not observed in pancreata from non-diabetic spleen cell recipients and all islets analysed had full insulin content. Irradiation of recipients was found to be essential for disease transfer as non-irradiated recipients did not develop diabetes or manifest this pattern of intra-islet infiltration and beta cell destruction (Table 5.6). Few B cells were detected, those observed were at peri-islet locations.

5.2.2(ii) T cell receptor VB usage by islet infiltrating cells

To characterise in further detail, the T cell subpopulations present in the pancreatic inflammatory infiltrates in the NOD mouse, histological analysis of the pancreas was performed using the monoclonal antibodies KJ16, 44.22.1 and KT11 which recognise T cell receptors using V68.1,2, V66 and V811 chains respectively. Examination of non-diabetic 4 month old male NOD mice revealed that T cells representing all of these V8 chains were present in the pancreatic infiltrate. Such intra-islet infiltration observed was minimal with most infiltration being predominantly peri-islet.

Recipient pancreata were examined at various time points following transfer, for T
cell receptor Vβ usage. Table 5.7 summarises the data from two different transfer experiments. Initially Vβ8.1,2 bearing T cells were present in greater number in peri-islet locations than Vβ6 and Vβ11 bearing T cells which were almost undetectable at week 1. By week 2, Vβ8.1,2, Vβ6 and Vβ11 were all present peri-islet. This predominance of Vβ8.1,2 was also demonstrated at intra-islet locations at later time points. Since the heterogeneity and extent of Vβ usage also reflected the proportion of all three Vβ bearing T cells present in the peripheral blood of NOD mice, this implies that there was no unusual bias toward usage of these three Vβ chains (Table 5.7) Such a heterogeneous pattern of infiltration was also observed in islets of spontaneously diabetic female NOD mice.

5.2.2.iii. The Presence of Macrophages In The Inflammatory Infiltrate.

The best characterised of the macrophage antibodies utilised is M1/70 which defines the glycoprotein antigen, macrophage 1 molecule (Mac-1) found on the surface of macrophages, monocytes, granulocytes and NK cells. The MAb F4/80 binds to F4/80 antigen present on mouse macrophages but not detectable on polymorphonuclear leukocytes or lymphocytes.

SER-4 is a MAb which recognises the sheep erythrocyte receptor present on stromal macrophages from lymph node, liver and spleen. The MAb 7/4 defines a polymorphic neutrophil differentiation antigen expressed by neutrophils in the bone marrow, blood and inflammatory exudates. The specificities of these antibodies are described in greater detail in chapter 4.

In the normal pancreas of NOD and CBA mice, cells expressing Mac-1+ and F4/80 are found lining the exocrine pancreatic acini, connective tissues and vasculature, while SER-4+ cells have a characteristically different distribution and appear as stromal macrophages (Figure 5.2 A, B, C).

A marked change in the distribution of cells expressing Mac-1 and F4/80 was observed in the prediabetic pancreas following transfer of diabetic spleen cells. Cells positive for Mac-1 and F4/80 were present at peri-islet locations at 1 week after transfer. Within two weeks of the transfer of diabetic spleen cells a large number of class II positive cells appeared at peri-islet locations and additionally some were present at intra-islet locations. This influx of class II bearing cells coincided with the massive recruitment of cells positive for Mac-1 and F4/80 at both peri- and intra-islet sites (Figure 5.2 D, E, F, Table 5.8). Double immunofluorescence studies revealed that the majority of these cells were positive for both the F4/80 and Mac-1 antigens suggesting that the infiltrate was predominantly of mature macrophages and not NK cells as the latter cells do not bear the F4/80 antigen. At 4 weeks in this particular transfer 75% of recipients were diabetic.
(transfer A); those islets that remained were heavily infiltrated with both T cells and macrophages, and few insulin containing β cells remained.

Immunoreactive insulin was identified in some cells bearing the F4/80 antigen suggesting that these cells were scavenging dead or dying β cells. Disease progression and β cell destruction led to a reduction in the numbers of macrophages and T cells. The distribution of SER-4+ cells did not alter during this prediabetic period. If the SER-4+ cells were 'fixed' then they would not come into contact with dying cells and consequently would not show signs of phagocytosis despite perhaps having the potential. This would imply that there are two distinct macrophage subpopulations present in the NOD pancreas; the Mac-1+, F4/80+, SER-4- population which were recently recruited and actively phagocytic and Mac-1-, F4/80-, SER-4+ representing a non-migratory "fixed" tissue population. Few neutrophils (7/4 positive cells) could be demonstrated in islet infiltrates of all pancreata analysed, suggesting that these cells do not have a functional role in β cell destruction.

Age and sex matched animals receiving a syngeneic, non-diabetic, spleen cell transfer showed minimal intra- and peri-islet infiltration of Mac-1+ or F4/80+ cells.

Histological analysis of pancreata from six spontaneously diabetic NOD mice revealed a similar distribution of macrophages to that observed in the transfer studies. Cells positive for Mac-1 and F4/80 were observed both peri- and intra-islet. Double positive Mac-1+ and F4/80+ cells were also observed in the peri-islet infiltrates of four month old male NOD pancreas.

5.2.3 Expression of MHC Antigens.

Expression of class I and class II MHC antigens was assessed immunohistochemically at various times following transfer of diabetic spleen cells. Class I MHC is poorly expressed in the normal pancreas of CBA mice of any age and in young NOD mice and is mainly located on the vascular endothelium and on those macrophages which are constitutively present (Table 5.9). However, in spontaneously diabetic, female NOD mice class I MHC expression was not only observed on the pancreatic infiltrate and within infiltrated islets but it was also present on endocrine cells and adjacent exocrine tissue. Hyperexpression of class I MHC could not be demonstrated on the pancreata of age- and sex-matched recipients of normal, syngeneic, non-diabetic spleen cells. In 4 month old male NOD mice, expression was limited to the peri-islet infiltrate. Hyperexpression of class I MHC appeared only to be associated with intra-islet infiltration.
and was not seen on non-infiltrated islets. Sequential analysis of pancreata from transfer recipients demonstrated a progressive increase in the number of islets hyper-expressing class I MHC from 0% at week 1 (Figure 5.3 A) to a peak of 73% at week 2 (Figure 5.3 B) correlating with the influx of T cells and macrophages at this time.

Class II MHC antigen expression was not observed on either the endocrine or exocrine tissue of the normal pancreas. Following transfer of diabetic spleen cells class II expression was observed, but appeared to be restricted to the macrophages present in the inflammatory infiltrate and vascular endothelium. Expression of class II MHC was never seen on β cells.

5.2.4 Expression Of ICAM-1.

Utilising the monoclonal antibody YN1/1.7.4 which detects the mouse homologue of ICAM-1 the presence of this adhesion molecule was assessed in normal, prediabetic and diabetic pancreata (Table 5.10). Transfer recipients demonstrated a progressive increase in the number of islets with ICAM-1+ cells at peri-islet locations from 39% at week 1 to 60% at week 2 after cell transfer (Figure 5.3C). At this time 22% of islets demonstrated intra-islet infiltration. Double immunofluorescence staining of pancreas with insulin demonstrated that there was no evidence of ICAM-1 expression on β cells themselves. However staining with the M. Ab F4/80 suggested that the cells expressing the ICAM-1 antigen were macrophages. In control animals receiving a syngeneic non diabetic spleen cell transfer 19% of islets examined had a peri-islet infiltrate of ICAM-1+ cells and expression of ICAM-1 on 5 month old male NOD pancreas was almost exclusively limited to the peri-islet infiltrate present on 48% of the islets examined.
5.3 Discussion.

From the histological analysis of female NOD mouse pancreas at 1, 3 and 5 months of age it was apparent that there was a progressive influx of T cells, macrophages and class II positive (non macrophage) cells which in my opinion resulted in the observed hyperexpression of class I MHC and β cell destruction. The pattern of infiltration in the male NOD mouse did not precisely follow that outlined for the female. Male NOD mice developed insulitis at approximately the same age as female NOD mice and the infiltrate was not significantly different in terms of its cellular constitution. However the insulitis remained at peri-islet locations and did not progress intra-islet thus no β cell destruction was observed even at 5 months of age.

There are many hormonal factors which would obviously influence the disease in the male and female as ovarectomy prevents disease and castration increases the incidence of overt disease (Makino et al, 1981). Thus there would appear to be two separate events, 1) insulitis occurring in both male and female NOD mice at 5-6 weeks of age and 2) the progression to overt diabetes occurring predominantly in the female population. Recent genetic mapping analysis has suggested that insulitis may be under the control of many genes not linked to the MHC. Idd-2 (Prochazka et al, 1987) is thought to control the frequency and severity of insulitis, Idd-3 also affects insulitis and Idd-4 is considered to influence the frequency of insulitis and the progression of severe insulitis to diabetes (Todd et al, 1991) and may control a protective suppressor T cell response to the autoimmune process. It is possible that some of the cells constituting the peri-islet infiltrate in the male NOD mouse are suppressor cells keeping in check cells which would actively destroy the β cell. Perhaps the female pancreatic lesion lacks or has insufficient numbers of such cells to prevent β cell destruction. The requirement for such facilitation regimes as cyclophosphamide and irradiation to effect rapid disease induction in the NOD mouse would seem to suggest that immunoregulation may play a role in IDDM. There is now clear evidence from Boitard (Bach et al, 1990; Boitard et al, 1990) and Hutchings (Hutchings et al, 1991) that CD4+ T cells are capable of depressing the ability of diabetic spleen cells to transfer disease. It is therefore possible that the peri-islet infiltrates observed in normoglycemic male NOD mice which do not progress to intra-islet locations may not be entirely constituted of autoaggressive T cells but contain a regulatory population of T cells. How this suppression is effected remains to be resolved but CD4+ T cells are known to regulate a variety of autoimmune responses (Parish et al, 1988). A subpopulation of CD4+ T cells (Th1) (Gajewski and Finch, 1988) has been shown to control the expansion of another subpopulation of CD4+ T cells (Th2) by the secretion of IFN-
γ, and could represent an example of negative regulation. Additionally CD8+ T cells produce large amounts of IFN-γ, and as they are present in the insulitis lesion could represent the poorly characterised CD8+ suppressor T cell which could act as an intervening regulatory cell in the TH1 to TH2 negative feedback pathway. It would be interesting to isolate such cells from the pancreas of young male NOD mice and observe if they were able to protect female mice from diabetes or delay disease onset. Further Pankewycz has shown that T cell clones isolated from NOD mouse islet infiltrates can either promote or prevent accelerated diabetes (Pankewycz et al, 1991). Additionally in situ hybridisation could be used to investigate whether the peri-islet infiltrate in male NOD mice contained more or less γ-IFN synthesising cells than the infiltrates in female mice.

Spleen cells from diabetic NOD mice are capable of transferring IDDM to irradiated NOD recipients (Wicker et al, 1986). Although this system does not provide an insight into the initiating events leading to the development of IDDM it provides clues to the effector phase of the response.

Detailed histological analysis of recipient pancreata taken at weekly intervals after spleen cell transfer demonstrated that T cell invasion of the pancreas was not evident until two weeks after cell transfer. Thereafter, infiltration was rapid as the primed T cells were able to home to their targets. The initial peri islet infiltration rapidly progressed to an intra-islet location and increased in severity until week 3 when 75% of recipients were diabetic. T cells utilising VB8.1,2, VB6 and VB11 were detected in the peri and intra-islet infiltrates of transfer recipients, spontaneously diabetic female NOD mice and the peri-islet infiltrates of male NOD mice. Although there was a predominance of VB8.1, 2 compared to VB6 and VB11 expressing T cells, infiltrates were heterogeneous and merely reflected the proportions of T cells expressing these VB chains in the peripheral blood and spleen of NOD mice. T cells bearing these particular VB T cell receptor chains did not show any bias towards CD4+ or CD8+ T cells. Thus at the particular time points analysed, there was a rather heterogeneous use of VB genes in the T cells of the pancreatic infiltrates and a large percentage of the T cell inflammatory infiltrate are presumably antigen non-specific. Subsequent to my studies additional TCR VB specific antibodies have become available and studies with them have confirmed my original observations. One cannot exclude the possibility of a restricted use of VB genes in T cells capable of destroying pancreatic β cells, as they may be present at low numbers and at the earliest point of infiltration. Since
publication of my studies (O'Reilly et al, 1991) other researchers have demonstrated that the pancreatic infiltrates of even the youngest NOD mice are characterised by the expression of multiple Vβ gene segments as assessed by polymerase chain reaction analysis with 18 Vβ-specific oligonucleotide primers (Waters et al, 1992). There also remains the possibility that a single β cell antigen-H-2 complex interacts with a specific TCR Vα chain. The observations of Candeias (Candeias et al, 1991) and Nakano (Nakano et al, 1991) of islet-derived T cell clones which specifically recognise pancreatic islet cell antigens derived from NOD mice have demonstrated unrestricted TCR Vβ and Vα usage and thus do not support Vα repertoire restriction. These observations would indicate that there is little hope of abrogating harmful T-cell responses with anti-TCR V segment antibodies, suggesting that TCR-targeted immune therapy may not prove useful. Thus my studies and the later results of others collectively support the suggestion that insulitis culminating in the onset of diabetes is initiated by a T cell population bearing diverse TCR β chains. The use of heterogeneous TCR by islet-reactive T cells in the inflammatory infiltrates perhaps suggests that the β cell has multiple antigenic epitopes or that multiple TCR β chains are capable of recognising the same diabetogenic autoantigen. Therefore preventative therapy should be directed towards identification of such molecular features and identification of disregulation of the immune system components.

The expression of MHC class II molecules on vessel associated cells at 1 week after transfer was the earliest event observed in recipient pancreas and preceded overt T cell infiltration. This phenomenon has also been described by Bedossa (Bedossa et al, 1989) in the NOD mouse and by others in the BB rat (Dean et al, 1985). Class II MHC molecules were probably expressed by macrophages which were present at peri-islet locations at this time. Pancreatic macrophages could be divided into two distinct populations on the basis of their location at diabetes onset. Mac-1+, F4/80+ cells were recruited to the sites of infiltration and were distinct from the SER-4+ resident tissue macrophages. Peri and intra-islet infiltration of Mac-1+, F4/80+ cells peaked at 2 weeks after transfer although infiltration was patchy and some islets were more affected than others. An initial interaction in which the autoantigen is presented to transferred T cells by resident macrophages could trigger recruitment of inflammatory macrophages. This
sequence of events closely mirrors that seen in the spontaneously diabetic BB rat where it has also been demonstrated that ED1\(^+\) macrophages (equivalent to Mac-1\(^+\)/F4/80\(^+\) cells in the mouse) infiltrating the islet are distinct from the ED2\(^+\) tissue macrophages (equivalent to SER-4\(^+\) cells in the mouse) of the pancreas. The infiltration in rat is also initially not uniform, some pancreatic lobules being more severely affected than others (Walker et al, 1988A). Our finding of a resolution of the inflammatory infiltrate with the disappearance of \(\beta\) cells in NOD mice is also in accord with the findings in the BB rat. The macrophage pancreatic infiltrate arose by host recruitment rather than wholly from the donor spleen cell inoculum since if the latter are depleted of adherent cells prior to injection disease transfer still occurs (P. R. Hutchings personal communication).

Evidence that activated macrophages are present early in the disease process and data implicating them in \(\beta\) cell destruction have been documented in both human (Sibley et al, 1985) and rodent (Kiesel et al, 1986; Lee et al, 1988A; Haneberg et al, 1989; Nagy et al, 1989) diabetic pancreas. Furthermore the ability of agents such as silica or desferrioxamine (which affect macrophage function) to prevent disease onset (Oschilewski et al; 1985, Nomikos et al, 1986; Charlton et al, 1988A) suggests a key role for these cells in the development of IDDM. Since there is convincing evidence of \(\beta\) cell susceptibility to the cytotoxic effects of cytokines TNF-\(\alpha\) and IL-1 particularly when in combination with \(\gamma\)-IFN, an effector role for macrophages cannot be excluded (Bendtzen et al, 1986; Campbell et al, 1988B; Pukel et al, 1988; Appel et al, 1989).

Class I MHC antigens were expressed on the endocrine and surrounding exocrine tissue of NOD islets which contained an inflammatory infiltrate. Locally produced cytokines such as \(\gamma\)-IFN, IL-1 and TNF could effect the upregulation of class I MHC which may play a role in immunopathogenesis and render the \(\beta\) cell a better target for CD8\(^+\) T cells. Leiter observed an IFN-\(\gamma\) inducible (“occult”) expression of class I like cell surface antigen on cultured NOD pancreatic \(\beta\) cells (Leiter et al, 1989). Over-expression of MHC class I has also been documented in cyclophosphamide-induced diabetes in NOD mice. Disease in this model can be prevented by in vivo administration of anti-IFN-\(\gamma\) (Campbell et al, 1991; Debray-Sachs et al, 1991) which also prevents class I MHC over-expression. This observation is consistent with the view that class I MHC over-expression
is effected by cytokines secreted by activated inflammatory cells (Kay at al, 1991A). Indeed similar class I MHC antigen expression in the pancreas is found in both the prediabetic BB rat and the diabetic human (Schwizer et al, 1984; Campbell et al, 1986A,B,1988; Foulis et al, 1987B; Ono et al, 1988; Walker et al, 1988B). Interestingly Allison has shown that transgenic class I MHC expression on beta cells in mice carrying the same MHC haplotype resulted in disease development in the absence of lymphocytic infiltration, suggesting a non-immune role for class I molecules in the impairment of beta cell function (Allison et al, 1988). Although class I over-expression may not be required for insulitis, it may however be required for amplification and the full development of pathology, as it would enhance targeting of cytotoxic T cells to β cells bearing the autoantigen. Evidence from Shimizu (Shimizu et al, 1987) and Hayakawa (Hayakawa et al, 1991) from immunohistochemical and immunoelectron microscopical techniques suggests that Lyt-2* cells are responsible directly for selective β cell destruction in the NOD mouse. Shimizu observed pseudopodia-like protrusions of Lyt-2* cells into the β cells resulting in degeneration of those cells whereas Ly1* cells were merely in loose contact with β cells (Shimizu et al, 1987). Furthermore islet-associated lymphocytes extracted from NOD mice have been shown to recognize and destroy islet cells in vitro with MHC class I Kd as the restricting element (Nagata et al, 1989) and in vivo administration of anti-Kd prevents spontaneous and cyclophosphamide induced diabetes in NOD mice (Taki et al, 1991).

Class II MHC molecules are normally expressed on the surface of antigen presenting cells such as macrophages, dendritic cells, B lymphocytes and Langerhans cells of the epidermis. In certain autoimmune diseases aberrant expression of HLA-Dr antigens has been reported, in particular on thyroid follicles in Graves disease (Hanafusa et al, 1983). It has been reported that class II MHC antigens are expressed on the beta cells of diabetic humans (Bottazzo et al, 1984) and in the BB rat on both vascular endothelium preceding infiltration and beta cells in partially destroyed islets (Dean et al, 1985), although this finding could not be confirmed by some groups (Baird, personal communication). Previous work showed that DR, Ia and RT antigens were found not to be expressed in normal human, mouse or rat islet cell lysates (Baekkeskov et al, 1981). γ-IFN has been shown to induce class II expression on murine islet cells (Wright et al, 1986), BB rat islet
cells in vitro (Walker et al, 1986) and rat insulinoma cell lines (Varey et al, 1988) and (in combination with TNF-α) on human β cells (Pujol-Burrell et al; 1987). Furthermore (CBA/J X NOD)F1 islet cells lost IFN-γ inducibility of I-A<sup>NOD</sup> implying suppression was mediated by a transacting factor from the CBA/J genome (Leiter et al, 1989). Both experimental studies in vitro, and pancreatic in situ hybridisation studies in the BB rat which have shown the presence of cells expressing IL-1, TNF and IL-6 in the pancreatic inflammatory infiltrate, suggesting that cytokines are present and may play a role in the pathogenesis of autoimmune β cell destruction (Jiang et al, 1991). Therefore they could have the capacity to ‘up-regulate’ MHC antigens on beta cells to pathologically significant levels. However Hanafusa (Hanafusa et al, 1987) has shown class II expression from 5 days of age on both female and to a lesser extent male NOD mouse beta cells, whether or not infiltration was observed. Further Formby and Miller (Formby and Miller, 1990) have demonstrated that single cell islet cells from male and female NOD mice express class II MHC ectopically as early as four weeks of age, suggesting that this represents a mechanism for targeting class II-restricted autoreactive CD4+ T cells to beta cells and initiating pancreatic insulitis. However Signore (Signore et al, 1989) found no class II molecules on insulin containing cells of female NOD mice from 5-36 weeks of age. Additional studies have indicated that class II MHC expression in the NOD mouse pancreas was not observed on CD45<sup>−</sup> islet cells at any stage (anti-CD45 recognises the leukocyte common antigen present on cells of the leukocyte differentiation pathway and includes B lymphocytes, T lymphocytes, macrophages and dendritic cells) indicating that all the class II positive cells are lymphoid cells which infiltrate the islet (McInerney et al, 1991). The author confirmed my own observations that pancreatic endocrine cells did not express class II MHC molecules and also noted the increase in MHC class I on β cells. My own studies (O’Reilly et al, 1991) indicate no evidence for aberrant expression of class II MHC on beta cells in our transfer mice or in our analysis of spontaneous diabetic female or nondiabetic male NOD mice. Macrophages containing immunoreactive insulin were observed in our studies and those of others. Indeed In’t Veld has shown by electron microscopy that the MHC class II expressing cells containing insulin were macrophages and dendritic cells (In’t Veld et al, 1988). This may provide an explanation of the previous findings based on the detection of class II bearing cells containing immunoreactive insulin. Islet class II MHC expression could be a secondary phenomenon not preceding insulitis but present as a result of macrophage and
lymphocytic invasion and subsequent engulfment of dead or dying β cells.

Whether β cells can present self or other antigens has remained an active area of interest for some researchers and the recent finding that ICAM-1 can be detected on NOD mouse β cells following culture with TNF-α and γ-IFN (Campbell et al, 1989) and can be induced de novo on human islet β cells (Vives et al, 1991) provided some support for this thesis. We have no evidence for such expression on β cells in vivo in the NOD mouse during the prediabetic period. All ICAM-1 expression appeared to be restricted to macrophages in the inflammatory infiltrate.

This is consistent with the observations of Dougherty (Dougherty et al, 1988) who has shown ICAM-1 on activated accessory cells. These authors suggest that in the course of T cell activation, accessory cells express ICAM-1 first which facilitates interaction with LFA-1 on T cells, thus stabilising T cell-accessory cell contact resulting in T-cell activation.

Although all the elements of the immune response ie. CD4+, CD8+ T cells and macrophages were present at peri-islet locations in non-diabetic male NOD mice, no β cell destruction resulted. These T cells did not express the IL-2 receptor, suggesting that they were not activated, in contrast to the finding of IL-2R positive T cells within islets in both non-diabetic and diabetic female NOD mice. The peri-islet IL-2R negative cells in male NOD mice may represent β cell specific but anergic T cells, which are characteristically IL-2R negative (Ramsdell and Fowlkes 1990). This suggests that a critical triggering event is required for intra-islet invasion and beta cell destruction.

Having demonstrated the cellular phenotypes constituting the pancreatic infiltrates, the next stage was to assess what contribution each cell type made to the disease process. If a particular type could be eliminated from the inflammatory lesion would the disease follow a more protracted time course or be prevented altogether?
FIGURE 5.1: Cryostat Sections of the Pancreata from Diabetic Spleen Cell Transfer Recipients and Male NOD Mice.

Cryostat sections of the pancreata from diabetic spleen cell transfer recipients two weeks after transfer, stained by the indirect immunoperoxidase method for CD4⁺ T cells (A) and CD8⁺ T cells (B) using the rat anti-mouse M. Abs. YTS 191.1 and YTS 169.4 respectively, demonstrating both peri- and intra-islet infiltration of these T cell subpopulations.

Figure 5.1 (C) and (D) show pancreata from 5 month old male NOD mice stained for CD4 and CD8 respectively, using the methods described above and showing peri-islet infiltration only. Magnification 400x.
FIGURE 5.2 (A): Macrophage Expression in the Non-Diabetic NOD Mouse

Pancreata

Cryostat sections of pancreata obtained from recipients one week after diabetic spleen cell transfer stained by indirect immunofluorescence with the rat anti-mouse M. Abs. Mac-1 (A) and F4/80 (B) demonstrating the normal distribution of these macrophage subpopulations lining the pancreatic acini and tissue vasculature. Figure 5.2 (C) shows the expression of the resident macrophage subpopulation labelled with the rat anti-mouse M.Ab. SER-4 (C*) three weeks after transfer. Magnification 400x, * 200x.
FIGURE 5.2 (B)  Macrophage Expression in the Diabetic NOD Pancreas

Double immunofluorescent staining of recipient pancreata two weeks post diabetic spleen cell transfer stained with Mac-1 (D) and F4/80 (E) (green) surrounding the rhodamine labelled insulin containing β cells (red). Arrow represents residual insulin containing β cells in the partially destroyed islet. The figure shows peri- and intra-islet infiltration of the MAC-1⁺ and F4/80⁺ macrophage subpopulations in NOD mice just prior to diabetes onset. Figure 5.2 (F*) shows F4/80 positive cells present at both peri and intra-islets locations of partially destroyed islets five weeks after diabetic spleen cell transfer.

Magnification 200x, * 400x.
FIGURE 5.3: Pancreatic Expression of MHC class I in the NOD Mouse

Section of pancreata from NOD mice one week after transfer of diabetic spleen cells stained by indirect immunofluorescence for MHC class I with the rat anti-mouse M. Ab. M1/42.3 (green) and insulin (red) demonstrating normal expression of class I MHC (A). Figure 5.3 (B*) shows hyperexpression of MHC class I on pancreatic endocrine and exocrine of an NOD mouse two weeks after diabetic spleen cell transfer.

Arrow represents residual insulin containing β cells.

Figure 5.3 (C) shows a section of male NOD pancreata two weeks after transfer of diabetic spleen cells stained by indirect immunofluorescence for the expression of ICAM with the rat anti-mouse M. Ab. YN1/1.7.4, showing predominantly peri-islet infiltration of cells expression this adhesion molecule.

Magnification 400x, * 200x.
### TABLE 5.1A Percentage of Islets with Class II Positive Cells at Peri- and Intra-Islet Locations in Female NOD Mice

<table>
<thead>
<tr>
<th>Age (mths)</th>
<th>No of mice</th>
<th>No of islets counted</th>
<th>Residual</th>
<th>Peri-Islet Infiltration</th>
<th>Intra-Islet Infiltration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>%</td>
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<td>moderate</td>
</tr>
<tr>
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<tr>
<td>5</td>
<td>5</td>
<td>100</td>
<td>44</td>
<td>9</td>
<td>82</td>
</tr>
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### TABLE 5.1B Percentage of Islets with Class II Positive Cells at Peri- and Intra-Islet Locations in Male NOD Mice

<table>
<thead>
<tr>
<th>Age (mths)</th>
<th>No of mice</th>
<th>No of islets counted</th>
<th>Residual</th>
<th>Peri-Islet Infiltration</th>
<th>Intra-Islet Infiltration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>%</td>
<td>none</td>
<td>moderate</td>
</tr>
<tr>
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<td>5</td>
<td>135</td>
<td>0</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>101</td>
<td>7</td>
<td>47</td>
<td>49</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>82</td>
<td>0</td>
<td>58</td>
<td>32</td>
</tr>
</tbody>
</table>

**Peri- and Intra-Islet Infiltration**

- **Moderate**: percentage of intact islets with 0-40 class II positive cells.
- **Severe**: percentage of intact islets with >40 class II positive cells.
- **Residual**: percentage of total number of islets analysed that displayed massive disruption of islet morphology and concomitant β cell destruction with loss of immunoreactive insulin.
TABLE 5.2 A Percentage of Islets with T Cells (CD3 Positive) at Peri-and Intra-Islet Locations in Female NOD Mice

<table>
<thead>
<tr>
<th>Age (mths)</th>
<th>No of mice</th>
<th>No of islets counted</th>
<th>Residual %</th>
<th>Peri-Islet Infiltration %</th>
<th>Intra-Islet Infiltration %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>none</td>
<td>moderate</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>98</td>
<td>0</td>
<td>92</td>
<td>7</td>
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<tr>
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<td>5</td>
<td>56</td>
<td>43</td>
<td>15</td>
<td>60</td>
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</tbody>
</table>

TABLE 5.2 B Percentage of Islets with T Cells (CD3 Positive) at Peri- and Intra-Islet Location in Male NOD Mice

<table>
<thead>
<tr>
<th>Age (mths)</th>
<th>No of mice</th>
<th>No of islets counted</th>
<th>Residual %</th>
<th>Peri-Islet Infiltration %</th>
<th>Intra-Islet Infiltration %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>none</td>
<td>moderate</td>
</tr>
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</tr>
<tr>
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<td>4</td>
<td>95</td>
<td>0</td>
<td>58</td>
<td>33</td>
</tr>
</tbody>
</table>

Peri- and Intra-Islet Infiltration

Moderate: percentage of intact islets with 1-20, CD3 positive T cells.

Severe: percentage of intact islets with >20 CD3 positive T cells.

Residual: percentage of total number of islets analysed that displayed massive disruption of islet morphology and concomitant β cell destruction with loss of immunoreactive insulin expression.
Table 5.3A  Percentage of Islets With Macrophages (F4/80 positive) at Peri- and Intra-islet Locations in Female NOD Mice.

<table>
<thead>
<tr>
<th>Age (Months)</th>
<th>No. Mice Analysed</th>
<th>No. Islets Analysed</th>
<th>Peri-islet infiltration</th>
<th>Intra-islet infiltration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
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<td>Moderate</td>
</tr>
<tr>
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<td>5</td>
<td>76</td>
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<td>63</td>
</tr>
<tr>
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<td>3</td>
<td>49</td>
<td>4</td>
<td>21</td>
</tr>
<tr>
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<td>5</td>
<td>73</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 5.3B  Percentage of Islets With Macrophages (F4/80) positive at Peri- and Intra-Islets Locations in Male NOD Mice.

<table>
<thead>
<tr>
<th>Age (Months)</th>
<th>No. Mice Analysed</th>
<th>No. Islets Analysed</th>
<th>Peri-islet Infiltration</th>
<th>Intra-islet Infiltration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
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<td>Moderate</td>
</tr>
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<td>15</td>
<td>73</td>
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<tr>
<td>3</td>
<td>5</td>
<td>117</td>
<td>6</td>
<td>52</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>81</td>
<td>4</td>
<td>43</td>
</tr>
</tbody>
</table>

**Peri-islet Infiltration**
Moderate: % of intact islets with 1-5, F4/80 positive cells.
Severe: % of intact islets with >5, F4/80 positive cells.

**Intra-islet Infiltration**
Moderate: % of intact islets with 1-5, F4/80 positive cells.
Severe: % of intact islets with >5, F4/80 positive cells.
Table 5.4 A/B Expression of MHC class I in NOD Mice.

Table 5.4A Percentage of Islets Expressing Class I MHC Molecules in Female NOD Mice.

<table>
<thead>
<tr>
<th>Age (Months)</th>
<th>No. Mice Analysed</th>
<th>No. Islets Analysed</th>
<th>MHC class I Expression</th>
<th>% Normal</th>
<th>% Expression on Infiltrate only</th>
<th>% Hyperexpression on exocrine/infil/residual islets</th>
</tr>
</thead>
<tbody>
<tr>
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<td>5</td>
<td>96</td>
<td></td>
<td>84</td>
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<td>5</td>
<td>66</td>
<td></td>
<td>0</td>
<td>9</td>
<td>91</td>
</tr>
</tbody>
</table>

Table 5.4B Percentage of Islets Expressing Class I MHC Molecules in Male NOD Mice.

<table>
<thead>
<tr>
<th>Age (Months)</th>
<th>No. Mice Analysed</th>
<th>No. Islets Analysed</th>
<th>MHC Class I Expression</th>
<th>% Normal</th>
<th>% Expression on Infiltrate only</th>
<th>% Hyperexpression on exocrine/infil/residual islets</th>
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<td>4</td>
<td>69</td>
<td></td>
<td>11</td>
<td>29</td>
<td>57</td>
</tr>
</tbody>
</table>

Pancreata from male and female NOD mice were analysed by immunofluorescence for insulin (rhodamine) and the presence of class I MHC (M1/42.3) (FITC) by the methods outlined previously.

Normal: MHC class I expression at peri- and intra-islet locations was normal and infiltrate if present.

Infiltrate: MHC class I expression on islet infiltrating cells.

Hyperexpression: MHC class I expression observed on pancreatic infiltrate, endocrine and also hyperexpression over the surrounding exocrine.
**TABLE 5.5 Sequential Pancreatic Infiltration of L3T4+ and Ly-2 + T Cells after Transfer of 2x10^7 Diabetic Spleen Cells to Irradiated 10-14 Week Old Male NOD Recipients**

<table>
<thead>
<tr>
<th>Time (wks) after transfer</th>
<th>No of mice</th>
<th>Pancreas Histology (% of total islets counted)</th>
<th>% residual islet</th>
<th>% Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>no insulitis</td>
<td>peri-insulitis</td>
<td>intra-insulitis</td>
</tr>
<tr>
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<td>4</td>
<td>100(100)</td>
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<tr>
<td>5*</td>
<td>4</td>
<td>64(54)</td>
<td>36(36)</td>
<td>9(18)</td>
</tr>
</tbody>
</table>

Twenty-one non-diabetic male NOD mice aged 10-14 weeks were injected i.v. with 2x10^7 spleen cells from diabetic adult donors and killed randomly at weeks 1, 2, 3 and 5 for histological analysis.

* Control mice were age-matched and were injected i.v. with 2x10^7 non-diabetic spleen cells from adult mice and analysed 5 weeks after transfer. Data represent the percentage of islets with L3T4+ T cell infiltration, data in brackets represent the percentage of islets with Ly-2+ T cells infiltration. Twenty islets were analysed from each group. Peri-insulitis refers to lymphoid infiltration peripheral to the islet, which may also be associated with intra-islet infiltration. Intra-islet infiltration refers to lymphocytic infiltration into the islet which was either a) mild, 1-10 positively staining cells located within the islet or b) severe, >10 positively staining cells located within the islet with islet disruption. Residual refers to massive disruption of islet morphology and concomitant β cell destruction with the loss of immunoreactive insulin expression.
### TABLE 5.6 Effect of Irradiation on Diabetic Spleen Cell Transfer

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% No Infiltrate</th>
<th>% Peri-islet</th>
<th>% Intra-islet (severe)</th>
<th>% Residual</th>
<th>% Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irradiation §</td>
<td>0</td>
<td>83</td>
<td>60</td>
<td>13</td>
<td>50</td>
</tr>
<tr>
<td>No Irradiation §</td>
<td>68</td>
<td>32</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Irradiation*</td>
<td>0</td>
<td>80</td>
<td>90</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td>No Irradiation*</td>
<td>84</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

2 $\times 10^7$ diabetic spleen cells were administered i. v. to 9 two month old male NOD recipients, of which 5 had received 650 Rads of irradiation the day previously.

Animals were sacrificed 4 weeks after transfer the pancreata prepared for immunohistology and stained for CD4 and CD8 positive T cells by the immunoperoxidase method.

50% of the irradiated animals were overtly diabetic at this time point, whereas none of the non-irradiated recipients were hyperglycemic and displayed no intra-islet infiltration.

No infiltrate: % of islets that had no peri- or intra-islet infiltration.

Peri-islet infiltration: % of islets demonstrating peri-islet infiltration.

Intra-islet infiltration (severe): % of islets demonstrating severe intra-islet infiltration ie. >10 positive cells.

* pancreatic sections stained for CD8.

§ pancreatic sections stained for CD4.
**TABLE 5.7** Percentage of islets with Vβ8.1.2., Vβ6 or Vβ11 Positive Cells at Peri- and Intra-islet Locations in Transfer Recipients and 4 Month Old Male NOD Pancreata.

<table>
<thead>
<tr>
<th>Time (wks)*</th>
<th>% 1-10 positive cells peri-islet</th>
<th>% ≥6 positive cells intra-islet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vβ8.1.2.</td>
<td>Vβ6</td>
</tr>
<tr>
<td>A.2</td>
<td>36</td>
<td>18</td>
</tr>
<tr>
<td>3</td>
<td>21</td>
<td>23</td>
</tr>
<tr>
<td>4</td>
<td>23</td>
<td>8</td>
</tr>
<tr>
<td>B.1</td>
<td>22</td>
<td>5</td>
</tr>
<tr>
<td>day 10</td>
<td>38</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>42</td>
<td>35</td>
</tr>
<tr>
<td>4</td>
<td>38</td>
<td>41</td>
</tr>
<tr>
<td>4**</td>
<td>43</td>
<td>33</td>
</tr>
<tr>
<td>Male NOD 4 month</td>
<td>32</td>
<td>21</td>
</tr>
</tbody>
</table>

* Time in weeks after transfer of 2×10^7 diabetic spleen cells into irradiated male NOD recipients at which pancreata were analysed. Two transfer experiments were analysed, A and B.

** Age matched control irradiated male NOD recipients which had received 2×10^7 non-diabetic syngeneic spleen cells.
Table 5.8. The average number of macrophages detected by the markers Mac-1 and F4/80 at peri, intra or residual locations in transfer recipients. CBA mice, spontaneously diabetic female NOD mice and 4 month old male NOD mice.

| Animal* | Mac-1 | F4/80 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|---------|-------|-------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|         | No. of islets analysed | No. of mice | Peri | Intra | Residual | Peri | Intra | Residual | % Diabetes |
| Wk 1*   | 2.7 | 0.6 | 0 | 2.7 | 0.8 | 0 | 0 | 0 | 0 |
| Day 10* | 6.2 | 1.1 | 0 | 7.1 | 1.2 | 0 | 0 | 0 | 0 |
| Wk 2*   | 14.7 | 2.3 | 1.4 | 14.8 | 2.7 | 1.2 | 0 | 0 | 0 |
| Wk 4*   | 4.9 | 1.8 | 10.8 | 5.7 | 2.2 | 11.1 | 75 | 0 | 0 |
| Wk 4**  | 8.8 | 0.8 | 0.5 | 7.5 | 0.9 | 0.5 | 0 | 0 | 0 |
| CBA     | 0.7 | 0 | 0.1 | 0 | 0 | 0 | 0 | 0 | 0 |
| SPON. DIAB. NOD | 5.2 | 3.2 | 4.6 | 8.9 | 3.6 | 7.1 | 100 | 0 | 0 |
| 4 MONTH MALE NOD  | 6.9 | 1.2 | 0 | 7.9 | 2.2 | 0 | 0 | 0 | 0 |

Data expressed as the average number of positive cells per islet. It should be noted that islet infiltration was patchy initially some islets being more affected than others.

*Time in weeks after transfer of 2x10^7 diabetic spleen cells into irradiated male NOD recipients at which pancreata were analysed.

**Age matched control irradiated male NOD recipients which had received 2x10^7 non-diabetic syngeneic spleen cells.
### Table 5.9. Percentage of islets expressing class I MHC molecules in CBA, NOD and Transgenic NOD.

<table>
<thead>
<tr>
<th>Animal</th>
<th>No. mice analysed</th>
<th>No. Islets</th>
<th>Normal</th>
<th>Expression on infiltrate</th>
<th>Hyperexpression on exocrine</th>
<th>residual Islet</th>
</tr>
</thead>
<tbody>
<tr>
<td>wk1*</td>
<td>4</td>
<td>39</td>
<td>41</td>
<td>57</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Day 10*</td>
<td>5</td>
<td>63</td>
<td>8</td>
<td>38</td>
<td>56</td>
<td>0</td>
</tr>
<tr>
<td>wk2*</td>
<td>5</td>
<td>44</td>
<td>0</td>
<td>27</td>
<td>73</td>
<td>0</td>
</tr>
<tr>
<td>wk4*</td>
<td>4</td>
<td>31</td>
<td>0</td>
<td>3</td>
<td>32</td>
<td>65</td>
</tr>
<tr>
<td>wk4+</td>
<td>4</td>
<td>25</td>
<td>28</td>
<td>72</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CBA</td>
<td>2</td>
<td>12</td>
<td>42</td>
<td>58</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Spon. Diab</td>
<td>6</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>86</td>
</tr>
<tr>
<td>male NOD</td>
<td>5</td>
<td>89</td>
<td>20</td>
<td>80</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>female NOD</td>
<td>9</td>
<td>256</td>
<td>23</td>
<td>12</td>
<td>23</td>
<td>42</td>
</tr>
</tbody>
</table>

Normal:- Distribution of class I MHC expression at peri and intra-islet locations was normal.

Infiltrate:- Class I MHC expression on islet infiltrating cells.

Hyperexpression:- Class I MHC expression observed on pancreatic infiltrate, endocrine and also the surrounding exocrine tissue.

*Pancreata were analysed from irradiated male NOD mice which had received $2 \times 10^7$ diabetic spleen cells (+ or $2 \times 10^7$ syngeneic non-diabetic spleen cells) at the time stated.

Male NOD mice were 4 months old. Female NOD mice were 6-7 months old. NOD Ea3 were 10-11 months old. NOD PROL-3 were 4-5 months old.
Table 5.10 Percentage of islets infiltrated with ICAM-1 positive cells at peri, intra and residual islet locations in transfer recipients. CBA mice, spontaneously diabetic female NOD mice and 4 month old male NOD mice.

<table>
<thead>
<tr>
<th>Time (wks)*</th>
<th>No. mice</th>
<th>No. Islets analysed</th>
<th>Negative</th>
<th>Peri-islet</th>
<th>Intra-islet</th>
<th>Peri + Intra-islet</th>
<th>% Residual</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>44</td>
<td>57</td>
<td>39</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Day 10</td>
<td>5</td>
<td>35</td>
<td>40</td>
<td>51</td>
<td>0</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>37</td>
<td>8</td>
<td>59</td>
<td>22</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>30</td>
<td>43</td>
<td>3</td>
<td>0</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>4**</td>
<td>3</td>
<td>26</td>
<td>62</td>
<td>19</td>
<td>4</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>CBA spon.diab</td>
<td>2</td>
<td>16</td>
<td>94</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NOD 4 month male</td>
<td>6</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>9</td>
</tr>
</tbody>
</table>

* Time in weeks after transfer of $2 \times 10^7$ diabetic spleen cells into irradiated male NOD recipients at which pancreata were analysed.

**Age-matched control NOD recipients which had received $2 \times 10^7$ syngeneic, non-diabetic spleen cells.
CHAPTER 6

In Vivo Treatment of Diabetic Spleen Cell Transfer Recipients With Monoclonal Antibodies to T Cell Subsets: Effect on Pancreatic Infiltration
6.1 Introduction.

There are two major subsets of peripheral T cells in both man and rodents, which differ in the way they recognise foreign antigens. One responds to antigens in the context of class II MHC molecules (I-A, I-E (mouse) or HLA DP, DQ, DR (human)) and expresses the differential surface molecule CD4 (Dialynas et al, 1983A,B). This subset of mature T cells which provides help for other effector functions promoting humoral and cellular immunity is termed Th/Ti or more appropriately, CD4\(^+\) T cells. The second subset recognises antigen in the context of class I MHC molecules (H-2K, H-2D (mouse) or HLA A, B, C (human)), expresses the CD8 differentiation antigen and is classically linked with generation of cytotoxic T cells, and is called Tc/Ts or CD8\(^+\) T cells.

The previous chapter established that CD4\(^+\) and CD8\(^+\) T cells were present in the inflammatory pancreatic lesions of spontaneously diabetic male and female NOD mice and in the pancreas of diabetic spleen cell transfer recipients. It was necessary to establish the exact sequence of events in the transfer model in order to determine the optimal time point to intervene and prevent disease manifestation. Infiltration of CD4\(^+\) and CD8\(^+\) T cells was evident at two weeks after transfer in recipient pancreata (O'Reilly et al, 1991). Therefore it could be argued that any immunological regime which would prevent further destruction by deletion or tolerisation should be administered before this time point. In vivo administration of rat anti-mouse monoclonal antibodies specific for particular T cell subsets (CD4 or CD8) seemed the most logical approach, since both were clearly present in the insulitis lesions. This protocol would determine whether they were essential for disease pathogenesis or merely present due to some nonspecific inflammatory recruitment.

There are now available several mouse CD4 specific M. Abs, namely YTS 191.1 (Cobbold et al, 1984), GK1.5 G2b (Dialynais et al, 1983A,B), GK1.5 G2a (Alters et al, 1989) and YTS 177.1 (Cobbold et al, 1990B). All are rat anti-mouse CD4 antibodies of the IgG2b isotype except YTS177.1 and GK1.5 which are IgG2a. The corresponding antibody to mouse Tc/s, namely YTS169.4 is a IgG2b rat monoclonal antibody to the mouse Lyt-2 antigen (Cobbold et al, 1984). Cobbold (Cobbold et al, 1984) has demonstrated that unmodified YTS 191.1 and YTS 169.4 are effective in independently depleting CD4\(^+\) and CD8\(^+\) T cell lineages respectively in vivo. Extrathymic CD4\(^+\) and
CD8+ cells are saturated with M.Ab and are rapidly depleted from the spleen and lymph node. Anti-CD8 administered at the optimal dose (x2, 0.5mg doses i.v, 1 week apart) gave specific depletion of 96% of target cells 3 weeks after antibody administration. A similar depletion could be achieved with anti-CD4 in CBA mice (Cobbold et al, 1984). Animals remain functionally depleted for 3 months after M.Ab injection. Rat IgG2b M. Abs can therefore be very effective in vivo, they would be good candidates for human therapy, because this Ab. subclass is the only one known to fix human complement and activate ADCC in vitro (Hughes-Jones et al, 1983). Similarly, >90% CD4 depletion was achieved in both spleen and lymph node 2 days after a single i.p. injection of 0.1mg of GK 1.5G2b (Ranges et al, 1987). Depletion is variable as replenishment of depleted populations occur over a span of time that is strain dependent varying from 30 days in DBA/1 mice and 2 months in BALB/c mice with GK 1.5. (Ranges, 1987).

M.Ab therapy is generally complicated by the development of host immunity to xenogeneic immunoglobulin which has adverse side effects ranging from mild anaphylactoid reaction in humans to frank anaphylaxis in mice. However an exception is the treatment of mice with rat M.Ab to L3T4 which fails to elicit a humoral anti-globulin response (Benjamin et al, 1986). Anti-CD4 additionally blocks humoral immunity to certain other antigens that are administered concurrently and thus may facilitate the administration of other xenogeneic M/Ab given under this ‘umbrella’ of anti-CD4 (Benjamin and Waldman, 1986). Indeed such treatment could induce tolerance to constant region determinants on rat IgG2b M.Abs preventing the early anti-idiotypic response to cell-binding M.Abs.

Such effects of anti-CD4 on immune function were also noted by Gutstein, namely blockade of the primary response to SRBC, soluble protein antigens and the secondary response to T dependent antigens. Less profound effects were observed with T-independent antigens. In addition anti-CD4 delays but does not prevent allograft rejection of skin grafts and reduces but does not block Tc generation (Gutstein et al, 1986).

Carteron (Carteron et al, 1988) demonstrated that F(ab')2 anti-CD4 (2C7) has similar functional characteristics to the intact anti-CD4. Including binding to CD4+ T cells, tolerance induction to other rat Ig administered at the same time and induction of long lasting antigen-specific immune tolerance even when rechallenged 19-23 weeks after first
exposure to antigen. This form of the M.Ab is non-depleting and if antigen is presented
after, rather than during, F(ab')2 anti-CD4 treatment, the immune response to that antigen
is not impaired. This makes it possible to restore normal immune responses to new antigen
within 3 days of cessation of therapy thus allowing rapid reversal of the immune
suppression. It has also been shown that deletion may not be the only mechanism which
accounts for immunosuppression, binding of anti-CD4 could induce a down-regulation
signal to the T cell preventing immune function (Haque et al., 1987). However once the
circulating titre of inhibitory Ab. has declined, a pool of auto-antigen primed T cells
protected from deletion in lymph node would be free to reestablish the disease, thus the
need for long-term therapy for sustained immunosuppression and the attendant risks of
intercurrent infection.

The immune system is characterised by the capacity to respond to "non-self" while
remaining unresponsive to "self". Self-tolerance operates through processes which
inactivate lymphocytes either centrally in the thymus and bone marrow or peripherally for
antigens more restricted in their expression in other tissues (reviewed chapter 1). Two
main areas of research, transplantation and autoimmunity, have benefited enormously from
in vivo depletion of target T cells with M.Ab technology. It is possible to re-establish
tolerance in autoimmune diseases while maintaining responsiveness to exogenous antigens
or to guarantee tolerance to a transplanted allograft and thus dispense with such drastic non­
specific measures as long-term drug immunosuppression, irradiation, anti-lymphocyte
globulin or thoracic duct drainage.

Both CD4+ and CD8+ T cell populations participate in the allograft rejection
reactions, responsible, for example, for bone marrow rejection, can be prevented by in
vivo depletion of recipient T cells with anti-CD4 and CD8 M.Abs leading to specific
tolerance (Cobbold et al, 1986). Cobbold et al (1990A) have additionally demonstrated that
for grafts differing in multiple transplantation antigens or differing in minor transplantation
antigens plus class I MHC, the need to establish some degree of haematopoietic chimerism
can be achieved with parenteral administration of CD4 and CD8 M.Abs. This was achieved
by using a M.Ab cocktail to give maximal depletion (Qin et al, 1989). The cocktail
contained two anti-CD4 M.Abs (YTS 191.1 and YTA 3.1.2) and two anti-CD8 M.Abs
(YTS169.4 and YTS156.7) reactive to non-overlapping epitopes of the CD4 and CD8 molecules respectively. It has to be noted that although anti-CD4 is a potent immunosuppressive agent associated with the creation of a tolerogenic environment, tolerance for example to HGG given under the cover of a short course of non-depleting anti-CD4 has to be reinforced by repeated injections of HGG and is lost in the absence of any further exposure to antigen. This reversal of tolerance with time, presumably due to new T cells being exported from the thymus, is avoided with tolerance to marrow and skin grafts (Qin et al., 1990) as the grafts act as a continuous source of antigen. The tolerant state is permanent suggesting that specific peripheral T cells that are anergic participate in reinforcing this state by competing at sites of antigen presentation. Additionally Cobbold (Cobbold et al., 1990) demonstrated that skin grafts incompatible across the whole MHC could survive using a combination of depleting and non-depleting antibodies, where each alone was unsuccessful. Therefore T and B cell tolerance is maintained by the cells being in an unresponsive or anergic state. Tolerization of memory, or recently activated T cells, therefore offers new opportunities for intervention in transplantation.

Many animal models of autoimmune diseases have already been successfully treated with M.Abs to T cell surface surface antigens. EAE can be inhibited in Lewis rats immunised with purified MBP (myelin basic protein) by treatment with W3/25 an M. Ab to rat CD4 (Brostoff et al., 1984). W3/25 did not mediate its effect by clearing the blood of W3/25+ cells as this Ab. (which is of the IgG1 isotype) is non-cytotoxic. This antibody has been shown to inhibit an MLR in vitro (Webb et al., 1980) and to prevent in vivo activation of cells that mediate the transfer of EAE from sensitised donors to naive recipients. Additionally, clinical signs of EAE in the SJL/J mouse model were prevented when GK1.5 was administered before disease onset, and reversed when administered after the first signs of EAE appeared (Waldorf et al., 1985). GK1.5 not only reduced the numbers of CD4+ T cells in the spleen and lymph node, but prevented clinical and histological manifestations of EAE when the Ab. was administered after the appearance of autoimmune T cells capable of selectively transferring EAE.

Repeated injections of anti-CD4, in addition to depletion of circulating target cells, also reduced auto-antibody production, retarded renal disease and prolonged life in B/W female mice (NZB/NZW) without provoking an undesirable and potentially hazardous immune response to the antibody (Wofsy and Seaman, 1985). Type II collagen-induced
arthritis (CIA) in mice and rats is an inflammatory polyarthritis with similar characteristics
to the human autoimmune disease, rheumatoid arthritis. CIA is associated with a cellular
and humoral response to type II collagen which can be prevented if anti-CD4 is
administered before immunisation with type II collagen (Ranges et al., 1985). Antibody
administration resulted in a decline in disease incidence and delayed onset of CIA which
was associated with lower IgG Ab. titres to type II collagen. Anti-CD4 treatment after the
antibody response to type II collagen was underway, but before disease onset, did not
affect disease expression indicating that the humoral response to type II collagen is a
necessary prerequisite for CIA development but not predictive of disease.

Given the potential of in vivo M.Ab. to CD4 and CD8 subpopulations (both
depleting and non-depleting) in transplantation and various autoimmune diseases, it seemed
logical that this form of therapy could have much to offer in the treatment of autoimmune
IDDM, since it has already been established that both CD4 and CD8 populations are
essential for disease manifestation in the NOD mouse. Clearly, one major goal in
understanding autoimmunity is to characterise the self or cross-reacting environmental
antigens that generate the critical diabetogenic peptide epitopes. In the case of IDDM,
where the diabetogenic antigen has not yet been identified, anti-CD4 treatment may
specifically be able to anergize those CD4⁺ T cells which recognise such an antigen and
prevent the cellular interactions leading to beta cell destruction.

In this chapter I will demonstrate that treatment of diabetic spleen cell transfer
recipients with depleting anti-CD4/CD8 and non-depleting anti-CD4 is able to prevent
disease manifestation. It was established in the previous chapter that the Vβ8 gene family
predominated in the insulitis lesion. Therefore in vivo depletion of transfer recipients using
a M.Ab. specific for T cells using Vβ8 chains in their TCRs was additionally carried out.
This did not prevent disease, the implications of which will be discussed.
6.2 RESULTS

6.2.1 In vivo administration of depleting anti-CD4 prevents disease in diabetic spleen cell transfer recipients.

The diabetic spleen cell transfer was carried out as indicated in chapter 4, using 12 male NOD mice. The rat monoclonal antibody to the mouse cell surface antigen, CD4 (YTS 191.1) was diluted in PBS such that treated mice received 400μg in a final volume of 200 μl i.v. on the first day of treatment and the same amount intraperitoneally on the 2 days following. Four transfer recipients received anti-CD4 commencing either the day after transfer or 1 week after transfer. The control group of 4 mice received no further treatment other than the spleen cell transfer. Fluorescence activated cell sorter (FACS) analysis showed that spleen cells from Ab. untreated mice had 17% CD4+ T cells compared with 0.1% in spleen cells from mice treated in vivo with YTS191.1 (anti-CD4). All animals were sacrificed at 7 weeks after diabetic spleen cell transfer at which point 75% of the animals in the control group were diabetic compared to 25% and 0% for groups receiving anti-CD4 treatment commencing at days 1 and 8 respectively. Pancreata were snap frozen, sectioned and stained by the immunoperoxidase technique (as outlined in materials and methods) for the CD4 and CD8 T cell surface antigens. Treatment with anti-CD4 not only prevented diabetes but reduced the percentage of residual islets from 26-33% in the control group to 3-8% and 0% in the groups treated on day 1 and day 8 respectively. Intra-islet infiltration by CD4+ cells was absent in 67% and 95% of islets from animals treated with anti-CD4 on days 1 and 8 respectively, compared to 31% of islets from untreated animals (Table 6.1A). Similarly prevention of intra-islet infiltration by CD8+ T cells was evident in 63% and 95% of anti-CD4 treated islets on days 1 and 8 respectively, compared to 17% in non-treated animals. However treatment with anti-CD4 at either time point appeared to have little effect on peri-islet infiltration by either CD4 or CD8 T cells (Table 6.1A). If treatment with anti-CD4 was given directly after diabetic spleen cell transfer, not only was severe intra-islet infiltration by CD4+ and CD8+ T cells prevented but macrophage recruitment was also delayed. Seventy three to 95% of islets from anti-CD4-treated animals had no intra-islet infiltration of Mac-1+ cells, whereas only 40% of islets from untreated animals were unaffected by macrophage infiltration (Table 6.1B).
6.2.2 *In vivo* administration of non-depleting anti-CD4 prevents disease in diabetic spleen cell transfer recipients.

Spleen cell suspensions from overtly diabetic female NOD mice were prepared in Hank's balanced salt solution and injected i.v. into 18 disease free male NOD recipients 2-4 months old previously given 650 rads from a cobalt source. The non-depleting anti-CD4 monoclonal antibody (Nd-YTS177.1) was of the IgG2a subclass and 2mg was given i.v, i.p. on consecutive days and thereafter i.p. three times weekly. Groups of 4-5 mice either received no treatment after diabetic spleen cell transfer (group 1) or Nd anti-CD4 (non-depleting anti-CD4) starting at day 12 (group 2) or received no treatment after transfer but were sacrificed at day 12 (group 3). Animals in groups 1 and 2 were sacrificed 4 weeks after transfer for histological examination of pancreata by double immunofluorescent staining for insulin and OX6 (detects MHC class II positive cells) or anti-CD3 (detects T cells) (Figure 6.1 A/B). At week 4 50% of the animals were diabetic in group 1 (1 animal had already died of diabetes) but none of those receiving M.Ab. therapy were hyperglycemic. Results presented in the previous chapter have established that by 10-14 days after transfer macrophages and lymphocytes had infiltrated the pancreas and some intra-islet infiltration had occurred. These observations were confirmed in this study, as the control animals sacrificed at 2 weeks receiving no Ab. treatment had >10 class II positive cells at peri-islet sites in 40% of intact islets. Thus peri-islet infiltration was already extensive and 23% of the total islets analysed were residual and devoid of β cells (Table 6.2A).

In order to determine whether Nd CD4 could arrest the destructive process, mice in group 1 sacrificed at week 4 after transfer were analysed. All islets examined demonstrated severe peri-islet infiltration of MHC class II positive cells and 83% of the remaining intact islets showed severe intra-islet infiltration with 70% of the total islets devoid of β cells. Pancreata from animals given non-depleting anti-CD4 from day 12 onwards and killed at 4 weeks after transfer, were indistinguishable from the group analysed at two weeks after transfer having 19% islet remnants, and 36% and 7% of islets demonstrated severe peri- and intra-islet infiltration of MHC class II positive cells respectively. Similarly analysis of pancreatic sections from all 3 groups stained for insulin and T cells revealed a similar pattern of infiltration to that obtained by staining for class II
MHC antigen\(^+\) cells. Table 6.2B shows that although there was considerable peri-islet infiltration in the anti-CD4 treated group, 49% of islets had >10 T cells at peri-islet locations compared to 77% for untreated controls at week 4. Severe intra-islet infiltration was also reduced from 59% of islets (control group at week 4) to 4% for those treated with Nd CD4 which is comparable to that observed in control animals analysed at week 2 (2%). Thus Nd.anti-CD4 can both independently reduce peri and prevent intra-islet infiltration by class II\(^{+}\) cells and T cells in diabetic spleen cell recipients and thereby prevent diabetes.

6.2.3 *In vivo* treatment of diabetic spleen cell recipients with depleting anti-CD8 protects against disease induction.

The diabetic spleen cell transfer was carried out as indicated in chapter 4 on a cohort of 21, 9 week old male NOD mice. The rat monoclonal antibody to the mouse cell surface antigen Lyt-2 (anti-CD8-YTS169.4) was diluted in PBS such that each animal received 400 \(\mu\)g in a final volume of 200 \(\mu\)l i.v. on the first day of treatment and the same amount intraperitoneally on the 2 days following. Control mice were similarly treated with YTH3.2.6, an isotype-matched rat monoclonal antibody to human CD7. Fluorescence activated cell sorter (FACS) analysis showed that spleen cells from Ab untreated mice had 11.8% CD8\(^{+}\) T cells compared with 0% in spleen cells from animals treated with YTS169.4 (anti-CD8\(^{-}\)). Recipient mice of diabetic spleen cells were treated with anti-CD8, beginning on day 1 (3 mice), day 8 (5 mice), day 15 (4 mice), day 22 (4 mice) or untreated (5 mice). In another experiment a group of 5 diabetic spleen cell transfer recipients were treated with anti-CD8 commencing at day 12 after transfer (Table 6.2A/B). The pancreata were examined histologically 5 weeks after transfer. Pancreatic sections from all experimental animals were prepared and stained by immunoperoxidase previously for CD4 and CD8 or by immunofluorescence for insulin, and the macrophage markers Mac-1 and F4/80.

It can be seen from Table 6.3A that mice were afforded significant protection with anti-CD8 as 80% of untreated mice were diabetic at week 5 whereas none, 20%, 75% and 25% of mice treated with anti-CD8 on the next day, week 1, week 2 and week 3 respectively were diabetic (Figure 6.2). Some protection was observed in mice treated after two weeks. Animals treated with anti-
CD8 from day 12 onwards, although not overtly diabetic, displayed evidence of considerably more β cell destruction (51% islet remnants) (Table 6.2A). Severe peri-islet infiltration by MHC class II+ cells was observed in 60% of islets, although intra-islet infiltration was the same as in the non-depleting anti-CD4 treated animals in the same experiment (Table 6.2A, B). Histological examination at week 5 of the first experiment described showed a much reduced infiltrate in mice treated with anti-CD8 immediately after transfer. The pancreata of these mice had no severe intra-islet infiltration of either CD4+ or CD8+ T cell populations and 25-45% of islets demonstrated no peri-islet infiltration compared to untreated controls which had 33% of islets demonstrating severe intra-islet infiltration by CD4+ and CD8+ T cells. The later the anti-CD8 was administered after diabetic spleen cell transfer the less protection was afforded as administration at 1 week resulted in only 10% of islets without detectable β cells ("residual islets") whereas delaying treatment with anti-CD8 for 2 or 3 weeks resulted in 15% and 31% residual islets respectively (Table 6.3A). Additionally treatment with anti-CD8 prevented severe macrophage infiltration as detected by Mac-1 and F4/80 staining if administered on day 1 after transfer compared to later time points (Table 6.3B). Thus anti-CD8 treatment can arrest disease if administered in time to prevent total destruction of the β cells.

### 6.2.4. In vivo treatment of diabetic spleen cell transfer recipients with YTH 3.2.6.

In order to determine that the observed protection from both diabetes and insulitis by the rat anti-mouse M.Abs. to the T cell surface antigens CD4 and CD8 was not due to some non-specific effect of administration of a rat monoclonal antibody, the monoclonal antibody YTH 3.2.6. (rat IgG2b) specific for human CD7 was administered in vivo to transfer recipients using 400 µg/mouse in 200 µl PBS i.v. the day after transfer and i.p. on subsequent days. Control animals received no further treatment. Four weeks after transfer mice were sacrificed and the pancreata examined by immunoperoxidase staining as described previously. At this point 100% (4/4) of control animals were diabetic as were 83% (5/6) of animals receiving YTH3.2.6. Control animals demonstrated 36-46% islet destruction, 79% and 62% of islets showed severe intra-islet infiltration by CD4 and CD8 T cells respectively. Animals treated with YTH 3.2.6. demonstrated 49-50% islet
destruction, and 63% and 41% of islets showed severe intra-islet infiltration by CD4 and CD8 T cells respectively (Table 6.4). Thus the isotype matched antibody YTH 3.2.6. specific for human CD7 does not influence the severity of diabetes or insulitis in transfer recipients compared to non treated control animals and thus can be utilised as an appropriate control in vivo M.Ab.

6.2.5 In vivo depletion of Vβ8.1+ T cells in diabetic spleen cell transfer recipients.

The complement fixing mouse γ2a monoclonal antibody F23.1 which recognises Vβ8.1,2,3. (Staerz et al, 1985) was used to deplete the Vβ8+ cells in the donor and recipient of diabetic spleen cell transfer. Donor animals were injected with 500 µg F23.1 i.p. 3 days before transfer to deplete Vβ8+ cells. Twenty four hours after transfer of diabetogenic spleen cells into 650 rad irradiated recipients, these animals were also injected with 500 µg F23.1 i.p. to ensure depletion. Control mice (donors and recipients) were injected with a comparable volume of PBS. Two experiments were carried out, each with five F23.1-depleted recipients and five control recipients. At week 5 (experiment 1) animals were sacrificed for pancreatic histology at which point 60% of F23.1 recipients were diabetic compared to 80% in the control group. At week 4 in experiment 2, animals were sacrificed for pancreatic histology at which point 80% of F23.1 recipients were diabetic compared to 100% in the control group. Two weeks after transfer pooled PBL from experiment 2 were stained with KJ16 (anti-Vβ8.1,2), 8% of lymphocytes were KJ16+ in the untreated group and 0% positive in the treated group. At 3 weeks after transfer this number had risen to 0.43% in the F23.1 treated group but was significantly less than the 7.1% KJ16+ cells of the control group. Thus depletion of Vβ8 bearing T cells did not significantly diminish the incidence of diabetes. Analysis of blood and pancreas showed that depletion of Vβ8 bearing cells had been accomplished. Intra-islet infiltration of F23.1 treated recipient diabetic pancreas was free of Vβ8+ cells (100% of islets from F23.1 recipients in experiment 2 had no intra-islet infiltration by such cells compared with 3% in untreated animals) but contained larger proportions of Vβ6 and Vβ11 bearing cells present at peri and intra-islet locations compared to control animals (Table 6.5). Thus Vβ8 bearing T cells would appear not to be necessary for β cell destruction.
6.3 Discussion.

Considerable evidence from both human and animal studies would seem to suggest that T cells play a fundamental role in the development of IDDM. Removal of T cells at birth by neonatal thymectomy prevented the spontaneous occurrence of IDDM in BB rats (Like et al., 1982), similarly athymic NOD mice did not develop insulitis or diabetes (Ogawa et al., 1985). Furthermore, anti-thymocyte serum (ATS) and anti-Thy 1.2 M.Ab. both independently reduced the spontaneous diabetes incidence in female NOD mice (Harada et al., 1986).

Conclusive evidence for the essential role of T cells in disease development was demonstrated by Harada and Makino (1984) followed by studies by Wicker (Wicker et al., 1986) and Bendelac (Bendelac et al., 1987) with syngeneic adult and neonatal, adoptive transfer models of diabetes respectively. Furthermore both CD4+ and CD8+ T cells are necessary to facilitate transfer of adult diabetic spleen cells to irradiated young NOD male recipients (Miller et al., 1988). Spleen cells alone or in combination with smaller numbers of lymph node cells produce severe hyperglycemia in young BB rat recipients, but as is the case for some other autoimmune diseases such as EAE in rodents, spleen cells need to be activated first with the T cell mitogen Con. A (Koevary et al., 1983, 1985).

Long-term treatment with Ab. to Th (anti-CD4) prevents IDDM in the NOD mouse even though insulitis has been established (Shizuru et al., 1988). Koike (Koike et al., 1987) also demonstrated that administration of anti-CD4 could prevent insulitis and diabetes in the NOD mouse. Other studies demonstrated that BALB/c islets function and prevent disease recurrence when transplanted under the kidney capsule of NOD mice if the recipients are treated with anti-CD4 (GK1.5) (Wang et al., 1987) (providing further evidence that autoimmune diabetes and allograft rejection in the NOD mouse are CD4-dependent).

Since administration of anti-CD4 together with exogenous antigen has been shown to establish specific tolerance to that antigen (Cobbold et al., 1984), it seemed logical to investigate the possibility that this system could serve as a means of re-establishing tolerance to self-antigens, such as the putative diabetogenic autoantigen. There is some data suggesting that NOD CD4+ T cell clones are capable of destroying NOD pancreatic grafts alone (Haskins et al., 1988), a CD4+ T cell clone established from a
patient with IDDM was capable of causing lysis of haplo-identical islet cells (De Berardinis
et al, 1988). However neonatal transfer studies indicate that a form of T cell-T cell
interaction is required for β cell destruction. Thivolet (Thivolet et al, 1991) suggested a
distinct function for each diabetogenic T cell subset. He thought CD4+ T cells had the
capacity to home to the pancreas, and promote in turn the influx of CD8+ effector T cells
that do not by themselves accumulate in this organ. My own studies have shown that
CD4+ T cells are present in the islet lesion, and that in vivo treatment of transfer recipients
with depleting or non-depleting anti-CD4 can prevent diabetes. In the latter case
recruitment of T cells and macrophages was prevented without impairing the immune
response to other antigens or by immunocompromising the host (Hutchings et al, in press).
In addition, the non-depleting anti-CD4 was more effective in that administration did not
need to commence until 12 days after transfer, but was still able to arrest insulitis and
confer long lasting protection from diabetes. Clonal anergy of Vβ11+ T cells specifically
recognising the I-E+ component of a pancreatic allograft has also been documented using

In contrast, to Thivolet's model, the data presented here shows recruitment of
inflammatory cells into the islet was also dependent on CD8+ T cells (Hutchings et al,
1990A). The studies of Miller and colleagues (Miller et al, 1988) have shown that both
CD4+ and CD8+ T cells are required to transfer diabetes in the NOD mouse, Reich (Reich
et al, 1989) also suggested that both CD4+ and CD8+ NOD T cell clones are required to
transfer insulitis to NOD mice. Our studies have shown that in vivo administration of anti-
CD8 to transfer recipients even as late as two weeks after transfer prevents macrophage
accumulation in the islet lesion, intra-islet recruitment by all cell types and the development
of IDDM. The exact role of the CD8+ T cell in the development of diabetes is unknown but
they are present in approximately equal

numbers with the CD4+ T cell in the insulitis lesion. Since T cell clones are known to
secrete γ-IFN and TNFα following stimulation it is possible that the apparent specificity of
islet toxicity seen in Haskin’s and de Berardinis’ experiments is attributable to the release of these cytokines by activated T cells. Neither of these papers document specific beta cell destruction, merely a lack of destruction of pituitary or EBV-transformed haploidentical targets respectively. They failed to show that glucagon and somatostatin-containing cells were not also destroyed.

As CD8\(^+\) T cells recognise antigen in association with MHC class I it is probable that the diabetogenic autoantigen is expressed in association with class I on the beta cell surface. Over-expression of class I MHC antigens has been noted by myself (O'Reilly et al, 1991) in the prediabetic pancreas of the NOD mouse and in the pancreas of transfer recipients, in the prediabetic BB rat (Ono et al; 1988, Walker et al; 1988B) and in the diabetic pancreas of humans (Bottazzo et al; 1984, 1985, Foulis, 1987B). Although class I MHC antigens are normally expressed on all mammalian nucleated cells, expression in the pancreas is low. The change in expression which preceded the onset of disease (at least in the animals) although focal is not confined to the beta cell and extends into the exocrine pancreas. It is therefore possible that infiltrating monocytes and lymphocytes may secrete cytokines which augment the expression of class I MHC antigen on surrounding cells. The presence of high levels of expression of MHC class I antigens on beta cells may make them more susceptible to attack by cytotoxic T cells.

Evidence that MHC class I restricted CD8\(^+\) T cells may play a role as effector cells in the NOD mouse stems from the experiments of Terada (Terada et al, 1988), who assessed the effect of donor H-2 antigen and the influence of autoimmune disease on survival of pancreatic grafts from various strains of mice transplanted into diabetic NOD mice which were treated with cyclosporine A to prevent allograft rejection. Pancreatic grafts from CBA mice which are incompatible with NOD at all class I MHC loci survived while those sharing MHC class I with NOD such as C57BL/6 and BALB/c islets (share D\(^b\) and K\(^d\) respectively with NOD) were destroyed on transplantation. Similar data comes from the observation that when bone marrow cells are transferred from NOD mice into (NOD X NON)F1 (Serreze et al, 1988C) or (NOD X C57BL)F1 mice (Wicker et al, 1988), beta cells of both parental types as well as F1 are destroyed by an autoimmune process. This finding is compatible with a class I restricted killing process as parental strains share K or
D class I loci respectively. Conflicting data comes from the observation that destruction of syngeneic islet grafts by spontaneously diabetic NOD mice (disease recurrence) is CD4+ and not CD8+ T cell dependent (Wang et al, 1987), and disease recurrence in islet tissue grafted to diabetic NOD mice was not restricted by islet MHC antigens (Wang et al, 1987). This data suggests that islet destruction depends on CD4+ effector T cells that are restricted by MHC antigens on NOD antigen presenting cells. These findings argue against the CD8+ T cell as a mediator of direct islet cell damage and instead raise the possibility of a CD4+ T cell dependent inflammatory response in the NOD mouse with perhaps cytokines or reactive oxygen intermediates produced by T cells and macrophages being responsible for beta cell destruction.

Depletion of OX8+ T cells (Tc/s, NK, thymocytes) or OX19 (pan T cell) from DP BB rats prevents diabetes. As virtually all of the OX8+ cells in this strain belong to the NK subset due to the lymphopenia present the effect of OX8 may be due to an effect on NK cell activity. This finding was therefore interpreted as suggesting that NK cells contribute to the mechanism of beta cell destruction. OX19 reduced the number of Th cells suggesting that Th/i may be required to maintain NK activity possibly by lymphokine secretion (Like et al, 1986). Thus the data in the BB rat implies that both NK and Th/i cells play a role in beta cell destruction. In the human a role for CD8+ T cells in beta cell destruction has also been indicated. Analysis of sequential biopsies of pancreatic grafts between HLA identical twins or siblings and patients with IDDM (Sibley et al, 1985) revealed progressive mononuclear cell infiltration composed largely of CD8+ activated T cells together with a few CD4+ T cells and some macrophages. Our own studies (O'Reilly et al, 1990; Hutchings et al [in press])and those of others have shown that both CD4+ and CD8+ T cells are required for disease development in the NOD mouse. Other models of autoimmune disease (ie collagen induced arthritis and EAE transfer studies) indicate a need and CD8+ T cells are required for diabetes development NOD mice.

If we consider the clinical situation, depletion of CD4+ or CD8+ T cells would be
likely to render the recipient immuno-compromised, whereas if there was a particular T-
lymphocyte receptor segment implicated in disease manifestation it would be feasible to
carry out selective M.Ab therapy for people with a predisposition to IDDM.

It has been demonstrated that TCR usage by autoreactive T cells in the EAE
autoimmune disease model in the PLJ mouse strain is restricted to Vβ8.2-Dβ-Jβ2 (Zamvil et al, 1986). The
autoimmune T cell response of H-2^U mice is biased towards to the dominant
encephalitogenic NH2-terminal nonapeptide of MBP (amino acids 1-9). A M.Ab specific
for the TCR Vβ8 subfamily is effective in preventing autoimmune encephalomyelitis in
these mice (Zamvil et al, 1988). The rat model of EAE exhibits a very limited heterogeneity
in response to amino-acids 68-88 of MBP and uses a Vβ gene homologous to Vβ8 in the
mouse (Ohashi and Heber-Katz, 1988; Heber-Katz and Acha-Orbea, 1989). From the
mouse and rat studies one could conclude that EAE is a monoidiotypic autoimmune disease
conferred by the use of a particular V gene combination. Identification of separate TCR β
chain gene rearrangements does not necessarily imply phenotypic differences in
antigen/MHC specificity. T cell clones that differ in TCR gene rearrangements and mediate
EAE express the same self-antigen specificity and MHC restriction. A minority of clones
use a different Vβ gene which can produce excellent responses if T cells expressing the
preferred genes are unavailable. Thus because most of the clones use a common Vβ gene in
EAE it enables the use of a novel therapeutic approach. Such an approach cannot be used
in the NOD mouse as recent studies, including my own data, imply Vβ usage is
heterogeneous.

T cells without known antigen specificity have been isolated from brain and
cerebrospinal fluid of M.S. patients and cloned in vitro. Such T cell clones did not
rearrange their TCR Vβ chain genes in a strictly clonal manner (Hafler et al, 1987;
Rotteveel et al, 1987). However adoptive transfer studies of EAE showed that only a small
percentage of T cells within the inflammatory CNS lesions are encephalitogenic (Born et al,
1985). The extent of heterogeneity in cloned T cells derived from the synovial fluid of
autoimmune RA patients is also controversial (Van Laar et al, 1991). In addition in genetic
studies in murine CIA it has been shown that despite the presence of a permissive MHC
haplotype mouse strains that have a genomic deletion resulting in the loss of 50% of the V genes of the TCR β- chain loci are resistant to arthritis induction (Banerjee et al, 1988). Further Vβ6 bearing T cells have been implicated as being necessary for induction of disease (Banerjee et al; 1988, Haqqi et al, 1988). There would therefore appear to be a somewhat restrictive element in Vβ gene use of T cells capable of mediating CIA. However interpretation of the data has been contested and at least one other group of investigators can find no evidence for a critical role for Vβ6-bearing T cells in this disease. It remains unclear whether the findings of limited heterogeneity of self reactive T cells mediating autoimmune disease seen in animal systems will be mirrored in human disease. Identifying the relevant population of T cells may be extremely difficult (reviewed Cooke, 1991).

These results, implicating a role for particular subsets of TCR-bearing T cells in the induction and maintenance of autoimmune conditions in animal models, are provocative. However there are several points which make extrapolation to human conditions difficult. Firstly many studies on TCR usage in animals detect an involvement of a restricted T cell repertoire in the initiation or early stages of autoimmune disease. However in humans all the analyses are performed once the autoimmune response is underway. Therefore the response becomes more heterogeneous due to inflammatory recruitment and subtle shifts in repertoire expression more difficult to identify and relate to disease pathology. Secondly these animal models require immunisation with a specific antigen, often in the presence of adjuvant to elicit autoimmunity. By altering the immune response with a strong immunogen one may be favouring the preferential expansion and activation of particular clones of cells, which might not occur in normal disease pathogenesis. T cell antigenic epitopes for several autoimmune diseases have been identified, eg. the a subunit of the acetylcholine receptor in Myasthenia Gravis patients, and the P2 protein (amino-acid 66-78) which can induce autoimmune thyroiditis in mice (animal model possibly relevant to Hashimoto's thyroiditis in humans) (Acha-Orbea et al, 1989; Kumar et al, 1989).

Thus in clinical situations where autoaggressive T cells can be identified and their repertoire is limited, antibodies to the antigen / HLA or TCR may produce effective immunotherapy. I have investigated whether there was a predominance of a particular
Vβ subfamily in the insulitis lesion using some of the currently available anti-Vβ M.Abs. As large numbers of Vβ8+ T cells were found at intra-islet locations it therefore seemed worthwhile attempting to modulate disease in NOD mice by depleting T cells bearing this Vβ chain. An additional incentive for undertaking this experiment were the results of recent studies which suggested that the inflammation and destruction of pancreatic islet cells in NOD mice may be mediated by T cells expressing Vβ5 (Reich et al, 1989) or Vβ8 TCR (Bacelj et al, 1989). However depletion of our transfer recipients with anti-Vβ8 (F23.1) failed to prevent the onset of disease even though Vβ8+ T cells were eliminated successfully from the peripheral blood and pancreas of recipients. This same antibody has been reputed to be able to reduce the incidence of insulitis and diabetes in the cyclophosphamide model of NOD diabetes despite, some animals developing hyperglycemia which were depleted of Vβ8+ T lymphocytes and animals treated with an isotype control antibody being protected from disease development (Bacelj et al, 1989). This antibody also prevented the development of insulitis in NOD mice (Fukada et al, 1989).

My findings are consistent with the recent observations of Shizuru (Shizuru et al, 1991) employing backcross analysis utilising the parental strains: NOD mice (TCR Vβb with 20-50 functional Vβ gene elements) and SWR strains (Vβa haplotype with extensive deletion of approximately 10 Vβ gene segments). Included in this deletion are TCR Vβ gene products previously implicated as being involved in the pathogenesis of disease in the NOD (Vβ5 and 8). By studying second backcross intercross animals, Shizuru was able to demonstrate that congenital deletion of the TCR Vβ gene segments 5, 8, 9, 11, 12 and 13 did not prevent development of insulitis or diabetes.

Thus in addition to cell clones or anti-Vβ directed M.Ab therapy, the usage of formal genetic analysis has demonstrated that diabetes can develop in the absence of T cells expressing gene segments from these families. Our findings (O’ Reilly et al, 1990) and those of others would seem to indicate that the anti-islet T cell response may be
polyclonal at its inception and therefore, the “inductive” pathogenic T cells may not demonstrate predominant TCR VB gene usage.

It is probably just a matter of time before the diabetogenic antigen/s are isolated. One could speculate that the putative auto-antigen on β cells is not expressed until such times as pancreatic infiltration takes place. Such infiltration may be in response to an antigen absent during early ontogeny due to a combination of genetic and environmental factors. It is then expressed in such a manner by APC that it appears as non-self. Immunisation with a soluble preparation of this auto-antigen under the umbrella of non-depleting anti-CD4 would induce tolerance in NOD mice and thus when the antigen did appear naturally on the β cell it would not provoke an autoimmune response, and the constant presence of the antigen would ensure anergy was not broken.

T cell monoclonal antibody therapy represents one way of preventing disease manifestation, by functionally deleting T cell populations. But as indicated in the previous chapter macrophages were also present in the inflammatory lesion. In order to determine if their role is 1) as an antigen presenting cell, involved in presentation of the auto-antigen, 2) inflammatory macrophage, involved in β cell killing by the release of the monokines IL-1 and TNF, or 3) phagocytic macrophage, present purely in a “mopping up” capacity after the T cells have destroyed the β cell, various protocols were employed. In the next chapter I present results of experiments in which I investigate each of these possibilities and the potential to prevent disease by inhibiting one or more of the above macrophage functions.
Figure 6.1 (A) depicts a pancreatic section of a control NOD mouse which received diabetic spleen cells but no antibody and was sacrificed two weeks after the transfer. The islets present on this cryostat section of the pancreas were stained with guinea pig anti-insulin antibody which in turn was detected by rhodaminated goat anti-guinea pig IgG (red). MHC class II expressing mononuclear cells detected using FITC conjugated OX6 were located peri-islet (green).

Figure 6.1 (B) depicts a pancreatic section of a NOD mouse which received diabetic spleen cells and a course of non-depleting anti-CD4 antibody which was initiated 12 days after cell transfer. This animal was sacrificed 4 weeks after the cell transfer. The cryostat section was stained as for (A) again showing peri-islet infiltration only of MHC class II expressing mononuclear cells. Magnification x400.
FIGURE 6.2: Cryostat Sections of NOD Pancreas From Anti-CD8 Treated Diabetic Spleen Cell Transfer Recipients.

Cryostat sections of pancreata were stained with the following rat anti-mouse M.Abs. using the immunoperoxidase technique.

Figure (A) shows a section of pancreata analysed five weeks after transfer of non-diabetic spleen cells stained for CD4⁺ T cells with YTS 191.1 showing mild peri-islet infiltration.

Figure 6.2 (B) section of pancreata five weeks after transfer of diabetic spleen cells stained for T cells with the anti-Thy-1 Ab. YTS 154.7 (B) or the CD4⁺ T cell subpopulation (C) showing extensive intra-islet infiltration and destruction of β cells. ß on figures shows the position of residual insulin containing islet beta cells.

Figure 6.2 (D) shows only mild infiltration of CD4⁺ T cells and the preservation of islet morphology, in a diabetic spleen cell transfer recipient 5 weeks after transfer. This recipient had received a course of depleting anti-CD8 commencing the day after treatment which prevented β cell destruction.

Magnification 200x.
Table 6.1A - Percentage of intact islets with peri- and intra-islet infiltration of CD4+ (CD8+) T cells in the pancreata of diabetic spleen cell transfer recipients with or without anti-CD4 treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Peri-islet Infiltration</th>
<th>Intra-islet Infiltration</th>
<th>% Residual Islets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1 to 10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>None</td>
<td>8 (0)</td>
<td>15 (42)</td>
<td>77 (58)</td>
</tr>
<tr>
<td>Anti-CD4 day 1</td>
<td>13 (13)</td>
<td>37 (54)</td>
<td>50 (33)</td>
</tr>
<tr>
<td>Anti-CD4 day 8</td>
<td>43 (45)</td>
<td>38 (47)</td>
<td>19 (8)</td>
</tr>
</tbody>
</table>

Table 6.1B - Percentage of intact islets with peri- and intra-islet infiltration of Mac-1 positive (macrophages) in the pancreata of diabetic spleen transfer recipients with or without anti-CD4 treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Peri-islet Infiltration</th>
<th>Intra-islet Infiltration</th>
<th>% Residual Islets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1 to 10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>None</td>
<td>10</td>
<td>25</td>
<td>65</td>
</tr>
<tr>
<td>Anti-CD4 day 1</td>
<td>5</td>
<td>54</td>
<td>41</td>
</tr>
<tr>
<td>Anti-CD4 day 8</td>
<td>45</td>
<td>40</td>
<td>15</td>
</tr>
</tbody>
</table>

Irradiated (650 Rads) male NOD recipients were given 2 X 107 diabetic spleen cells i. v. and either given anti-CD4 (YTS 191.1) beginning on days 1 or 8 or received no further treatment. Seven weeks after transfer, animals were sacrificed for pancreatic histology. Pancreatic tissue sections were stained for the presence of both CD4+ and CD8+ T cells, Mac-1 positive macrophages and insulin. The percentage of intact islets with 0, 1-10 or >10 positive staining cells at peri or intra islet locations was recorded. Percentage residual-% of islets completely destroyed with little or no immunoreactive insulin present, expressed as a percentage of the total number of islets counted.
Table 6.2: Effect of Delaying Treatment With Non-depleting Anti-CD4 (YTS 177) and Depleting Anti-CD8 (YTS 169) on Class II Infiltration in Male NOD Recipients Receiving 2 X 10^7 Diabetic Spleen Cells i.v.

A. Percentage of islets with class II MHC positive cells at peri* and intra-islet§ locations 4 weeks after transfer.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of positive cells peri-islet*</th>
<th>No. of positive cells intra-islet§</th>
<th>% Residual</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-10</td>
<td>&gt;10</td>
<td>0-5</td>
</tr>
<tr>
<td>None wk.4 (18)</td>
<td>0</td>
<td>100</td>
<td>17</td>
</tr>
<tr>
<td>Anti-CD4 &gt; day 12 (87)</td>
<td>64</td>
<td>36</td>
<td>93</td>
</tr>
<tr>
<td>Anti-CD8 &gt; day 12 (115)</td>
<td>40</td>
<td>60</td>
<td>93</td>
</tr>
<tr>
<td>None, killed day 12 (61)</td>
<td>60</td>
<td>40</td>
<td>97</td>
</tr>
</tbody>
</table>

B. Percentage of islets with T cells (CD3 positive) at peri* and intra-islet§ locations 4 weeks after transfer.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of positive cell peri-islet*</th>
<th>No. of positive cells intra-islet§</th>
<th>% Residual</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-10</td>
<td>&gt;10</td>
<td>0-5</td>
</tr>
<tr>
<td>None wk 4 (54)</td>
<td>23</td>
<td>77</td>
<td>41</td>
</tr>
<tr>
<td>Anti-CD4 &gt; day 12 (80)</td>
<td>51</td>
<td>49</td>
<td>96</td>
</tr>
<tr>
<td>Anti-CD8 &gt; day 12 (89)</td>
<td>66</td>
<td>33</td>
<td>94</td>
</tr>
<tr>
<td>None, killed day 12 (61)</td>
<td>77</td>
<td>23</td>
<td>98</td>
</tr>
</tbody>
</table>

Cryostat sections were prepared from the pancreata of NOD mice, 4 weeks after the transfer of 2 X 10^7 diabetic spleen cells and following treatment with depleting anti-CD8 (YTS 169) or non-depleting anti-CD4 (YTS 177) started at day 12. Control mice given diabetic spleen cells but no antibody, were sacrificed at week 4 ("None") or at the same time as antibody treatment was begun ("None"-day 12). Peri-islet infiltration indicates that more than 10 class II or CD3 positive cells surrounded those islets that remained in intact and intra-islet infiltration indicates that more than 5 class II or CD3 positive cells were inside those intact islets remaining. Residual islets were those in which there was no immunoreactive insulin remaining and were calculated as a percentage of the total analysed.
Table 6.3: Effect of Delaying Treatment With Anti-CD8 (YTS 169) on CD4, CD8 and Macrophage Pancreatic Infiltration in Diabetic Spleen Cell Transfer Recipients.

A. Percentage of islets with CD4 and (CD8) at peri-islet and intra-islet (1-10, >10 +ve cells) locations.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No Infiltration</th>
<th>Peri-islet Infiltration</th>
<th>Intra-islet Infiltration</th>
<th>% Residual</th>
<th>% Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Peri</td>
<td>Intra</td>
<td>1 to 10</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>None</td>
<td>0 (0)</td>
<td>50 (50)</td>
<td>67 (67)</td>
<td>33 (33)</td>
<td>50 (44)</td>
</tr>
<tr>
<td>Anti-CD8 day 1</td>
<td>25 (45)</td>
<td>75 (0)</td>
<td>64 (55)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Anti-CD8 day 8</td>
<td>0 (21)</td>
<td>75 (10)</td>
<td>95 (79)</td>
<td>5 (0)</td>
<td>10 (11)</td>
</tr>
<tr>
<td>Anti-CD8 day 15</td>
<td>0 (0)</td>
<td>85 (15)</td>
<td>67 (85)</td>
<td>23 (15)</td>
<td>15 (15)</td>
</tr>
<tr>
<td>Anti-CD8 day 21</td>
<td>0 (0)</td>
<td>56 (31)</td>
<td>69 (98)</td>
<td>31 (12)</td>
<td>31 (31)</td>
</tr>
</tbody>
</table>

B. Percentage of islets with MAC-1+ cells (0, 1-10 or >10) at peri and intra-islet locations.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No Infiltration</th>
<th>Peri-islet Infiltration</th>
<th>Intra-islet Infiltration</th>
<th>% Residual</th>
<th>% Residual</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Peri</td>
<td>Intra</td>
<td>1 to 10</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>6</td>
<td>31</td>
<td>56</td>
<td>31</td>
</tr>
<tr>
<td>Anti-CD8 day 1</td>
<td>20</td>
<td>90</td>
<td>80</td>
<td>39</td>
<td>33</td>
</tr>
<tr>
<td>Anti-CD8 day 8</td>
<td>14</td>
<td>39</td>
<td>50</td>
<td>39</td>
<td>33</td>
</tr>
<tr>
<td>Anti-CD8 day 15</td>
<td>0</td>
<td>7</td>
<td>7</td>
<td>19</td>
<td>56</td>
</tr>
<tr>
<td>Anti-CD8 day 21</td>
<td>0</td>
<td>31</td>
<td>25</td>
<td>25</td>
<td>44</td>
</tr>
</tbody>
</table>

Mice were irradiated, given 2 X 10^7 diabetic spleen cells and treated with anti-Ly-2 (YTS 169.4), beginning on day 1, 8, 15 or 21. Control mice received no antibody. Histological examination at week 5 was carried out by the immunoperoxidase method for both CD4 and CD8 T cells or for Mac-1+ cells by immunofluorescence.

Data show the percentage of islets with either no infiltration of CD4, CD8 T cells or Mac-1+ cells, peri-islet infiltration or intra-islet infiltration: moderate (1-10 +ve cells) or severe (>10 +ve cells). Residual refers to the percentage of the total number of islets counted that had no immunoreactive insulin remaining. Forty islets were analysed at least from each group except in the group treated with anti-CD8 on day 1 as fewer mice were treated at this time point (26 islets were analysed).
Table 6.4- Effect of Administration of the Control IgG2b Antibody (YTH 3.2.6) CAMPATH-1 to Diabetic Spleen Cell Transfer Recipients.

Table indicates the percentage of islets with CD4+ (and CD8+) T cells at peri-islet and intra-islet (1-10, >10 +ve cells) locations.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No Peri-islet Infiltration %</th>
<th>Peri-islet Infiltration</th>
<th>Intra-islet Infiltration %</th>
<th>% Residual</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 (0)</td>
<td>45 (62)</td>
<td>19 (38)</td>
<td>79 (62)</td>
</tr>
<tr>
<td>Diab. Sp. Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YTH 3.2.6</td>
<td>5 (5)</td>
<td>49 (50)</td>
<td>32 (53)</td>
<td>63 (41)</td>
</tr>
</tbody>
</table>

All mice were irradiated, given 2 X 10^7 diabetic spleen cells and either treated with YTH 3. 2. 6 or given no further treatment. Histological examination at 4 weeks was carried out by the immunoperoxidase method for both CD4+ and CD8+ T cells. Data show the percentage of cells with either no infiltration of CD4+ or CD8+ T cells, peri-islet or intra-islet infiltration: moderate (1-10 +ve cells) or severe (>10 +ve cells). Residual refers to the percentage of the total number of islets counted that had no immunoreactive insulin remaining. Twenty-six- thirty-three islets from the non antibody treated group were analysed for each stain, and 36-41 islets from the YTH3.2.6 antibody treated group.

Table 6.5- Percentage of Intact Islets With Peri- and Intra-slet Infiltration of V8.1, 2, 3, V8.6 and V8.11 T Cells in the Pancreata of V8.8 depleted or Control Diabetic Spleen Transfer Recipients.

Percentage of islets with V8.1,2,3, V8.6 or V8.11 T cells at peri and intra (severe [>5 +ve cells]) in experiment 1 and in brackets experiment 2.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Staining Antibody</th>
<th>Peri-islet Infiltration</th>
<th>Intra-islet Infiltration</th>
<th>% Residual</th>
</tr>
</thead>
<tbody>
<tr>
<td>F23.1</td>
<td>V8.8</td>
<td>37 (95)</td>
<td>53 (5)</td>
<td>10 (0)</td>
</tr>
<tr>
<td>None</td>
<td>V8.8</td>
<td>7 (4)</td>
<td>36 (9)</td>
<td>58 (87)</td>
</tr>
<tr>
<td>F23.1</td>
<td>V8.6</td>
<td>6 (0)</td>
<td>23 (28)</td>
<td>61 (89)</td>
</tr>
<tr>
<td>None</td>
<td>V8.6</td>
<td>13 (21)</td>
<td>41 (32)</td>
<td>50 (48)</td>
</tr>
<tr>
<td>F23.1</td>
<td>V8.11</td>
<td>38 (33)</td>
<td>48 (30)</td>
<td>13 (37)</td>
</tr>
<tr>
<td>None</td>
<td>V8.11</td>
<td>24 (43)</td>
<td>72 (29)</td>
<td>64 (3)</td>
</tr>
</tbody>
</table>
CHAPTER 7

An Investigation of Macrophage Function in Relation to Disease Pathology in the NOD Mouse.
7.1 INTRODUCTION

Further to my studies in chapter 5 in which I described two distinct populations of macrophages present in the pancreata of NOD mice before the onset of diabetes, I decided to assess their function in relation to disease pathology. It was clearly demonstrated that macrophages were present at early points in the disease process in both the spontaneous disease and in the diabetic spleen cell transfer recipients at 1 week after transfer. Indeed two distinct populations of macrophages could be identified, namely a Mac-1⁺/F4/80⁺/SER-4⁻ population which was recently recruited, actively phagocytic and present in inflammatory infiltrates and a resident population which were Mac-1⁻/F4/80⁻/SER-4⁺ (O'Reilly et al, 1991). These two populations corresponded to the ED1⁺ and ED2⁺ macrophage populations respectively found in the prediabetic BB rat pancreas (Walker et al, 1988A).

However the function of the macrophage present in the insulitis lesion remained to be clarified. Macrophages have a unique place in the tissue response to external stimuli and have the capacity to perform a plethora of functions: a) They interact with many cellular molecules, internalise and submit them to intracellular metabolic changes during antigen processing. b) They secrete the growth regulatory factor IL-1, proteases and complement proteins, all of which are important in inflammatory reactions. c) They interact with T and B lymphocytes and thereby intervene in immunological responses. d) They are critically situated in various tissues close to the microvasculature and epithelia and e) They have surface receptors for lymphokines which upon engagement result in macrophage activation and the acquisition of novel properties (reviewed, Unanue and Allen, 1987). It is unclear whether the macrophage functions as an antigen presenting cell presenting the putative diabetogenic autoantigen to the T cell, whether it is directly involved in β cell destruction by the secretion of monokines, nitrite metabolites and free oxygen radicals or whether it is present in a purely “mopping-up” capacity, removing cellular debris after T cell-mediated destruction. The macrophage may combine two or more of these functions. In an attempt to examine these possibilities I studied the effect on insulitis and diabetes incidence in the NOD mouse of (a) preventing macrophage influx into the inflammatory site after diabetic spleen cell transfer with a M.Ab 5C6 and (b) preventing nitrite metabolite production by administration of L-NMMA to diabetic spleen transfer and cyclophosphamide treated NOD mouse recipients.

Much evidence has accumulated to suggest that macrophages are involved in the development of diabetes particularly in animal models. Kolb (Kolb et al, 1986) has shown
that early in the prediabetic period of the BB rat a "single-cell insulitis" is present, which on electron microscopy appeared to consist mainly of macrophages. Other ultrastructural studies have described macrophages containing ingested β cell debris within islets of diabetic BB rats (Kroncke et al, 1991). Walker (Walker et al, 1988A) showed that the major infiltrating cells in the islets of Langerhans during the early stage of insulitis were ED1⁺ macrophages. Hanenberg (Hanenberg et al, 1989) identified a non-random sequence of events during infiltration of pancreatic β cells in BB rats. The initial phase was defined by an influx of ED1⁺, W3/25⁺, OX3⁺/6⁺/17⁺, ED2⁻ macrophages. A secondary stage was characterised by an increased macrophage infiltration concomitant with infiltration of OX19⁺ T lymphocytes and OX8⁺ granular (NK) lymphocytes and followed by a terminal stage of additional massive infiltration of OX12⁺ B cells. Lee also suggested that macrophages precede lymphocytes as the earliest recognisable step of islet inflammation. MHC class I hyperexpression associated with macrophage infiltration of islets was reported in this study and in an earlier study by Lee (Lee et al, 1988A) who also demonstrated the preferential infiltration of macrophages during the early stages of insulitis. He suggested that there might be an initial change in the target β cells that precedes their immune destruction, although amplification of the immune response by activated T lymphocytes and NK cells at a later stage would be required for clinical expression of the disease.

It has been recently postulated that a population of intravascular monocytes may induce vascular leakage and hence allow enhanced diapedesis of lymphoid cells from the circulation into the pancreas of susceptible BB rats (Majno et al, 1987). Walker (Walker et al, 1988A) has speculated that this population of intravascular monocytes have been trapped in the vascular endothelia of Wistar rats as blood borne monocytes were present in inflamed (but not normal) islets. More concrete evidence that macrophages were necessary for β cell destruction in BB rats came from the discovery that administration of silica particles (which are selectively toxic to macrophages [Allison et al, 1960; O'Rourke et al, 1978]) completely prevented IDDM in BB rats (Ochilewski et al, 1985; Keisel et al, 1986; Lee et al, 1988B; Amano and Yoon 1990).

Macrophages are also thought to be active in the pathogenesis of diabetes in NOD mice and, as in the BB rat, appear early in the disease process. Treatment with silica from 4
weeks of age could deplete or inactivate macrophages in NOD mice that may in turn abolish

the development of β cell-specific effectors in either host pancreatic islets or transplanted pancreas. However later intervention with a short course of treatment did not affect the spontaneous destruction of β cells by pre-existing effector cells after the development of insulitis (Ihm et al, 1990). Macrophage directed immune intervention with silica particles in the NOD cyclophosphamide induced model of diabetes prevented disease in 95% of animals. This study demonstrated that macrophages are necessary but not sufficient for β cell destruction, since regimes which deplete T cells are also capable of preventing IDDM (Charlton et al, 1988A). An initial single-cell insulitis precipitated by STZ was followed by a slow development period of mostly macrophage-mediated cytotoxic events and then a massive lymphocytic insulitis which could be inhibited by administration of silica particles (Kolb-Bachofen et al, 1988; Oschilewski et al, 1986). Macrophages are therefore essential for the development of β-cell specific cytotoxic effectors in the initial phase of insulitis in NOD mice.

In all models of diabetes studied, macrophages are present at early points in the disease process, thus it seems reasonable to suggest that they may play a role in presentation, by uptake, breakdown and processing of the putative diabetogenic autoantigen for Th cell activation, and thereby activate other immunological effectors including possible β cell specific Tc.

How macrophages are attracted to islets at the initial stage of insulitis is unknown although release of antigenic stimuli from β cells may act as chemoattractants. The function of the macrophage once present is still unknown. In order to clarify this point, I aimed to investigate whether macrophages are required to be physically present in situ (ie. would be most probably performing an antigen presenting function) or whether it was sufficient for the macrophage secretory molecules only to be present (ie. suggesting a secretory cytotoxic function for the macrophage). Thus the M.Ab 5C6 which is specific for the myelomonocytic adhesion promoting type-3 complement receptor (CR3 or CD11b/CD18) and thus able to prevent macrophage migration and trafficking was employed in vivo in the NOD mouse to distinguish between these two possibilities.

It has been previously demonstrated that the M.Ab 5C6 can prevent migration of
macrophages to inflammatory sites (Rosen et al, 1987). This was achieved by inhibiting transendothelial migration without depletion of cells from the marrow or vascular compartment, and without inhibiting the secretory capacity of the macrophage. Sensitisation of T cells and antigen presentation by macrophages was also unaffected (Rosen et al, 1989). 5C6 would appear to exert its effect through blocking the adhesion and trafficking of macrophages. To determine the role of the macrophage in \( \beta \) cell destruction \textit{in vivo}, diabetic spleen cell transfer recipients were treated with 5C6. As 5C6 does not bind to T cells but to macrophages and neutrophils and neutrophils are not present in the inflammatory infiltrates the specific contribution of macrophages to diabetes can be assessed.

It has been suggested that IFN-\( \gamma \) produced by T cells activates macrophages to secrete TNF which in turn sensitises the same or other macrophages to secrete reactive oxygen species during the respiratory burst. These reactive oxygen metabolites destroy the immunogen through a process of lipid oxidation (Clark et al, 1986). However other mechanisms may also be involved and recently attention has turned to the potential role of reactive nitrogen intermediates such as the highly reactive nitric oxide (Higuchi et al, 1990; Kroncke et al, 1991) which could mediate the pathogenic effects of cytokines.

In 1987 Furchgott and Ignarro suggested that endothelium derived relaxing factor (EDRF) was nitric oxide (NO) and / or NO related molecules (Furchgott et al, 1988; Ignarro et al, 1987). Palmer (Palmer et al, 1987) demonstrated both the release of NO by mammalian vascular endothelial cells and by leukocytes following stimulation with TNF and IFN-\( \gamma \). TNF, interferons and endotoxin also stimulate NO production by macrophages, neutrophils, Kupffer cells and hepatocytes (Moncada and Higgs, 1990).

In immunostimulated macrophages the amino acid L-arginine is converted to \( \text{NO}_2^- \) and \( \text{NO}_3^- \) and L-citrulline by the nonconstitutively expressed \( \text{Ca}^{2+} \) independent enzyme NO synthase. The (nitric oxide) NO intermediate in this process is synthesised from one of the terminal guanidino-nitrogen atoms of L-arginine (Iyenger et al, 1987; reviewed Kolb and Kolb-Bachofen, 1992) (Figure 7.1). Synthesis of these nitrogen oxides is specific for the L-configuration at the \( \alpha \)-carbon atom and they have a wide range of biological activity including neurotransmission and are a major defence molecule of immune cells against parasites, tumour cells and intracellular bacteria (Hibbs et al, 1987A). Arginine metabolism
represents a pathway of cytocidal activity, effective against a wide range of targets. Large amounts of NO released for longer periods of time during cell activation in comparison to the constitutive expression. It could therefore be hypothesised that pancreatic β cells might be susceptible to NO and NO$_2$ (Hibbs et al, 1987A, B; Stuehr et al, 1989).

The mechanism of NO-mediated non-specific killing and cytostasis of target cells remains to be clarified, however Hibbs (Hibbs et al, 1987B, 1990; Lancaster et al, 1990) postulated that NO reacted with Fe-S groups, resulting in the formation of iron-nitrosyl complexes that cause the inactivation and degradation of Fe-S prosthetic groups of aconitase and complex I/II of the mitochondrial electron transport chain. This could result in inhibition of mitochondrial respiration by inhibition of NADH. Alternatively, NO could react with O$_2^\cdot$ to form the peroxynitrite anion (ONOO$^-$) which decays rapidly once protonated to form the hydroxyl radical (OH$^-$) and NO$_2$ as suggested by Beckman et al. (Beckman et al, 1990). The selectivity of pathogen destruction by NO may be explained by the fact that NO is very short lived, (6-50 seconds) and target pathogens would therefore have to be close to the source of NO synthesis (Griffith et al, 1984). However sustained, high levels of NO production would also be expected to be damaging to the host cells and tissues.

The L-arginine effector pathway can be inhibited by $^N\text{G}$-monomethyl-L-Arginine (L-NMMA) which is a competitive inhibitor of L-arginine metabolism, in particular of nitric oxide synthesis (Hibbs et al, 1987A, B; Palmer et al, 1988; Stuehr et al, 1989). The inhibitory effects of guanidino methylated derivatives of L-arginine are determined by structure, the most potent of which is the former $^N\text{G}$MMA but all are found to decrease or prevent, in a dose-dependent manner, activated macrophage-induced inhibition of mitochondrial respiration in target cells except the inactive enantiomer D-NMMA.

L-NMMA has been demonstrated to inhibit the L-arginine effector pathway both in vivo and in vitro in many systems. IFN-$\gamma$- and LPS- activated macrophages have a powerful cytostatic effect on the fungal pathogen Cryptococcus neoformans (Granger et al, 1986) and the protozoan Toxoplasma gondii (Adams et al, 1990), the microbiostatic effect being dependent on L-arginine and inhibited by the presence of L-NMMA. Additionally mouse peritoneal macrophages stimulated in vitro with IFN-$\gamma$ in the presence
of LPS are efficient at killing Leishmania and this can be abrogated with L-NMMA in a dose-dependant manner but not by its D-enantiomer (D-NMMA) (Green et al, 1990; Liew et al, 1990A). Furthermore culture supernatants of macrophages activated by IFN-γ contain significantly increased levels of NO$_2^-$ (Stuehr and Marletta, 1987; Drapier et al, 1988), the production of which is inhibited by L-NMMA (Green et al, 1990; Liew et al, 1990B). *Leishmania major* promastigotes are killed when incubated *in vitro* at room temperature in PBS containing NO (Liew et al, 1990). The importance of NO *in vivo* is demonstrated by the finding that disease in CBA mice infected with *L. major* is exacerbated when L-NMMA is injected into the lesions (Liew et al, 1990C). The ability of TNF-α to synergise with LPS in the activation of macrophages to kill intracellular *L. major* (Liew et al, 1990C; Bogdan et al, 1990) and *L. donovani* (Roach et al, 1991) correlates with NO release. TNF-α can also synergise with IFN-γ in inducing leishmanicidal activity that can be inhibited by L-NMMA but not D-NMMA (Liew et al, 1990D; Roach et al, 1991). Additionally lymphokine-activated macrophages are also cytotoxic for the larval stages of the helminth *Schistosoma mansoni* (James et al, 1990). The killing is L-arginine dependent, inhibited by L-NMMA and nitrite is detectable in culture supernatants (James et al, 1989).

It is possible that high levels of NO may not only be toxic to undesired microbes, parasites or malignant cells, but may also damage healthy tissue. Lysis of pancreatic β cells by activated macrophages is such a model of inflammatory death of healthy tissue. Evidence has accumulated from *in vitro* studies suggesting that macrophages are effector cells in islet cell lysis. Lymphocytes from BB rats (Pukel et al, 1987) or NOD mice have been shown to display non-MHC antigen restricted cytotoxic activity towards β cells. By use of a quantitative radioassay and electron microscopy Appels et al. (1989) presented the first direct evidence that activated BB rat macrophages lyse normal, syngeneic, pancreatic islets. Islet cells, notably β cells were particularly susceptible to the cell damaging effects of IL-1, TNF or the radical oxygen species. Rabinovitch et al later provided evidence for involvement of free radicals and lipid peroxidation in rat islet cell destruction (1992). *In vitro* studies have suggested that IL-1 and TNF-α, two cytokines mainly produced by macrophages, induce structural changes in β cells and suppression of their insulin-releasing
capacity (Mandrup-Poulsen et al, 1987; Spinas et al, 1987). There have been reports that administration of IL-1 and TNF have pronounced anti-diabetic effects in vivo (Del Rey et al, 1989). However recent work by Kroncke et al. (Kroncke et al, 1991) indicates that IL-1 and TNF-α do not contribute appreciably to the cytotoxic activity of macrophages towards β cells in vitro, and it has also been shown that activated macrophages kill syngeneic pancreatic islets in vitro via arginine-dependent oxide (NO) generation in vitro (Kroncke et al, 1991). Co-cultivation of islet cells with chemicals that spontaneously release NO also rapidly leads to cell death (Burkart et al, 1991). There is also now evidence to support a role for NO in diabetogenesis in vivo. Lukic has shown that treatment with L-NMMA suppresses the development of low dose STZ-induced autoimmune diabetes in CBA mice (Lukic et al, 1991). Kolb has also demonstrated that daily injections of L-NMMA which specifically inhibits NO synthase activity, suppressed diabetes development in this model (Kolb et al, 1991, reviewed Kolb and Kolb-Bachofen, 1992). Therefore it is now widely accepted that cytokines or NO or both factors contribute to β cell destruction during the development of IDDM.

Studies in this chapter were designed to investigate whether the presence of macrophages in the inflammatory lesion were essential to the development of disease in diabetic spleen transfer recipients by inhibition of macrophage migration by the M.Ab 5C6. Additionally, given that L-NMMA (which inhibits NO formation) prevented disease in the STZ model of diabetes, it was decided to investigate the role of NO in both the adoptive transfer model and cyclophosphamide models of diabetes induction in NOD mice.
7.2 Results

7.2.1 Treatment of diabetic spleen cell recipients with anti-CR3 (CD11b.5C6)

An experiment was designed to test whether 5C6 could prevent the primary initiation of insulitis following transfer of diabetic spleen cells. Seven male NOD mice 2-3 months of age were given 500μg 5C6 i.v., 3 control male NOD littermates were given 500μg Campath-1 and a further control group of 3 NOD males were given 250μl PBS i.v. All mice were sublethally irradiated with 650 rads from a cobalt source and reconstituted with 2X10⁷ spleen cells from overtly diabetic female NOD mice on the same day as the first antibody injection. The seven 5C6-pretreated mice subsequently received 500μg of 5C6 i.p. daily for 3 days after transfer, then every second or third day after transfer. One of this group died although not of diabetes. Mice from both control groups were subsequently pooled and glucose in the blood and urine was monitored in all mice. Mice were sacrificed 14 days after transfer at which point all mice, both antibody treated and non-treated were normoglycemic. Pancreata were snap frozen for immunohistology. To assess the extent of macrophage infiltration in all pancreata, a polyclonal rabbit anti-mouse antibody F4/80 was utilised which detected the F4/80 antigen on mature mouse macrophages on pancreatic sections double stained for insulin. The expression of Mac-1 (CR3) could not be detected directly with the rat monoclonal to CR3 as the goat anti-rat Ig fluorescent conjugate picked up the residual rat anti-mouse 5C6 M.Ab in the pancreas confirming that 5C6 had circulated through the pancreas. The use of a rabbit polyclonal M.Ab circumvented this problem.

Pancreata from individual mice were analysed on day 14 after the transfer of diabetic spleen cells and the data pooled to form a total for each group either 5C6 treated or not. At this time, 40% of islets from 5C6 treated mice demonstrated the absence of intra-islet infiltration, whereas none of the control mouse islets were intact and 84% of the islets from the 5C6 treated mice had mild peri-islet infiltration compared to 57% for the control group. The most striking result was that 43% of control islets had severe peri-islet infiltration compared to only 11% for treated mice (Figure 7.2, Figure 7.3). Thus it was clear from analysis of pancreata 14 days after the transfer of diabetic spleen cells that 5C6 had prevented both severe peri-islet and intra-islet infiltration although it was too early to observe if hyperglycemia could also be prevented. Interestingly the septa lining the pancreatic lobules contained large accumulations of F4/80+ macrophages, demonstrating
that 5C6 had indeed prevented them traversing the vascular endothelium and migrating towards the islets. Therefore the experiment was repeated and extended to investigate whether diabetes could be prevented.

Seven two-three month old male NOD recipient mice were injected either with 5C6 (3 mice, 4 mice) or PBS (4 mice) on day -1. On day 0 these mice and an additional 4 male NOD littermates were subletally irradiated with 650 rads. All groups were reconstituted with 2X10^7 diabetic spleen cells except 4 of the 5C6-pretreated group which were reconstituted with 2 X10^7 5C6-coated diabetic spleen cells. A further group of irradiated male NOD littermates were reconstituted with 2X10^7 spleen cells from non diabetic donors. Both 5C6 pretreated groups were administered 5C6 the following day and three times per week throughout the experiment, a similar protocol was followed for those mice receiving PBS. Serum samples taken at weekly intervals confirmed that a high level of circulating monoclonal antibody in recipients was correlated with protection. Four weeks after transfer, when all untreated mice were overtly diabetic (blood glucose 21.8-22.2mM), both the 5C6 treated groups (blood glucose 7.4-8.3 mM, except one recipient where blood glucose was 14.1mM/Litre, but where urine was negative by Diastix) and animals receiving a non-diabetic spleen cell transfer were sacrificed and the histology of the pancreata compared. It was found that administration of 5C6 throughout the experiment had preserved the insulin-containing islets. Staining for CD3, class II MHC antigen or the macrophage specific F4/80 antigen revealed only a predominantly peri-islet infiltrate in 5C6 treated mouse pancreata comparable to that seen in pancreata from age-matched, irradiated males reconstituted with spleen cells from non-diabetic NOD mice (Table 7.1, Figure 7.4). Untreated male recipients, however had very few intact islets remaining and all islets showed advanced intra-islet infiltration of class II^+ cells and CD3^+ cells (Table 7.1). Histological scoring of islet infiltration is shown in Table 7.1. Note that 84% of islets in control mice were scored as residual with intact glucagon and somatostatin but no insulin immunoreactivity, whereas only 20% of islets in 5C6-treated mice showed comparable destruction with complete loss of insulin. Staining for CD3 showed that with no macrophage infiltrate, intra-islet penetration by T cells was also inhibited. Similarly, severe intra-islet infiltration of cells expressing class II MHC antigen was reduced in 5C6 treated mice from 100% to 13%.

Two further experiments following this protocol were carried out. Twenty-two male
2-3 month NOD recipients treated on day -1 with 400µg 5C6 (11) or 400µg Campath-1 (11) were given 625 rads and reconstituted with 2X10^7 diabetic spleen cells the same day as before. Injections were repeated on day 1 and three times weekly thereafter. On day 39 all mice that remained were sacrificed and the pancreata snap frozen. 7/11 from the 5C6 treated group and 5/11 from the Campath treated group died during the course of the experiment, although not due to diabetes but probably the combined effects of the irradiation and excessive heat in the animal facility. From Table 7.2 (group *) it is clear that 5C6 administration prevented not only diabetes but also insulitis, 46% and 41% of the treated group had only minimal peri and intra-islet infiltration respectively compared to 5% and 23% of the Campath-treated group. Additionally the extent of islet cell destruction was reduced from 40% to 1% in the 5C6-treated animals.

In an additional experiment four, two month male NOD mice received 500µg 5C6 and a further group of 6 male NOD littermates received 500µg Campath-1 i.v. on day -1. All mice were given 650 rads and 2 X 10^7 diabetic spleen cells as previously described, and antibody injections given as before on day 1 and three times weekly until week 4 at which point 4/6 (67 %) of the control group were overtly diabetic. Peri-islet infiltration by F4/80+ cells was not prevented by 5C6 treatment although intra-islet infiltration was less severe and beta cell destruction was reduced from 40% to 1% in experiment 1 and from 60% to 11% in experiment 2. Infiltration by class II +ve cells was also reduced as 67% of the 5C6 treated animals had no intra-islet infiltration compared to 18% of untreated animals (Figure 7.2, group ζ). Overall in several experiments the incidence of overt diabetes in 5C6 treated animals was significantly reduced (from 93% to 8% p=0.00018, Fischer’s exact test).

7.2.2 Treatment of Diabetic Spleen Cell Transfer Recipients With an Inhibitor of Nitric Oxide Generation.

Twenty-one 8 week old male NOD mice were sublethally irradiated (750 rads, X-ray source) and reconstituted with 2X10^7 spleen cells from diabetic female NOD mice. From days 9-13 seven mice received 5mg L-NMMA i.p., 5mg L-arginine (one of the L-arginine treated group died of a wasting disease) or 5mg D-NMMA i.p. In this experiment the control group receiving the inactive enantiomer (D-NMMA) did not show 100%
diabetes until an unusually long period of time after transfer (day 48), although at day 14 after transfer the D-NMMA treated group demonstrated higher blood glucose levels compared to the L-NMMA treated group. By day 48, 71% of the L-NMMA animals and 67% of the control group receiving L-arginine were diabetic. However recipients were only treated from days 9-13 and by day 48 mice had received no treatment for 35 days. It is therefore perhaps not surprising that treatment effects could not be detected at such a late time point. The failure of this particular cell transfer could be due to insufficient diabetogenic T cells in the inoculum as some of the donor diabetic mice may not have been sufficiently diabetic for their spleen cells to cause disease in the irradiated recipients, although they were overtly diabetic (ie. blood glucose >12 mM/L and the urine positive by Diastix).

The experimental procedure was therefore repeated and twenty 11 week male NOD mice were sublethally irradiated and reconstituted with $2 \times 10^7$ diabetic spleen cells. Seven mice were each given 5mg L-NMMA, or 5 mg L-Arginine and 6 mice were given 5mg D-NMMA, daily from days 9-13 after transfer. As can be seen from Figure 7.5, 3 weeks after transfer 28% of both the L-NMMA and D-NMMA treated recipients were already overtly diabetic and by week 6-7, 71% of the L-NMMA, 67% of the L-Arginine, groups and 100% of the D-NMMA treated group were diabetic. Thus inhibition of nitric oxide formation by L-NMMA did not seem to affect the incidence of diabetes in diabetic spleen cell transfer recipients. The possibility remains that if L-NMMA treatment was not maintained throughout the experiment macrophages could proceed with this pathway of β cell destruction.

The treatment protocol was therefore extended such that sublethally irradiated transfer recipients received either 5 mg L-NMMA, 5mg D-NMMA or 0.2 mls PBS I.P. on days 7-11 and three times weekly until day 35 at which point all animals were sacrificed. From figure 7.6 it is evident that L-NMMA failed to prevent diabetes in diabetic spleen cell recipients as 6/7 (86%) of mice were diabetic at termination of the experiment compared to 4/7 (57%) of the PBS treated group and 6/7 (86%) of the L-arginine treated group. Thus even extended treatment with L-NMMA failed to prevent diabetes. To exclude the possibility that for a beneficial effect to be received treatment had to commence immediately after the transfer and continue throughout the experiment, the protocol was again modified. Treatment with either L-NMMA, L-Arginine or PBS was commenced on days 1-5 and continued three times weekly. At week 3 after transfer 3/7 of the L-NMMA
treated group were already overtly diabetic (Figure 7.7) and by day 34 all mice were diabetic.

### 7.2.3 Induction of Diabetes in NOD Mice by Cyclophosphamide and Treatment with L-NMMA

Female NOD mice aged 12-13 weeks were given 200mg/Kg cyclophosphamide i.p. on day 0 and 14. At weekly intervals from day 0, groups of 5 mice were sacrificed for pancreatic immunohistology. At weeks 1, 2, 3, and 4, respectively 0, 80, 80, and 100% (one recipient died of diabetes at each of weeks 3 and 4) were diabetic as assessed by serum and blood glucose levels. From Table 7.3 it can be concluded that 1 week after cyclophosphamide treatment infiltration was either absent or mostly peri-islet for both class II$^{+}$ (OX-6 positive cells) and T cells (CD3 positive cells). At 2 weeks the majority of islets were residual and devoid of insulin expression and of the remaining islets 43% and 33% had maximal peri-islet infiltration of class II$^{+}$ or CD3$^{+}$ cells respectively. At week 3 the tabulated values would seem to suggest that there was little peri or intra-islet infiltration by both class II$^{+}$ and T cells, this is merely due to the fact that one animal had already died of diabetes and thus was not included in the immunohistochemical analysis and a further 3 were severely diabetic with either no or very few islets present. The pancreas of one animal had virtually intact islets at this time and therefore affected the group scores. However by week 4 all islets were residual and all animals diabetic. Thus cyclophosphamide disease induction appears therefore to follow a similar time course to the diabetic spleen cell transfer and to be mediated by the same mononuclear populations such as inflammatory cells.

My previous studies showed that L-NMMA could not prevent disease induction by the diabetic spleen cell transfer. It was of interest to determine whether it could protect NOD mice from cyclophosphamide induced disease and thus to investigate if different mechanisms are involved in the two inductive models of diabetes. Thirteen female NOD mice at 9 weeks of age were given 200mg/Kg cyclophosphamide i.p. on days 0 and 14. Of these, 5 were given 5mg L-NMMA, 4 were given 5mg L-arginine and 4 were given 0.2 ml PBS. All injections were i.p. and were administered on days 1-5 and then three times weekly until day 35 when diabetic animals were sacrificed. At this time, 80% of the L-NMMA treated group, 75% of the L-arginine treated group and 75% of the PBS treated group were diabetic (Table 7.4). Thus inhibition of nitric oxide formation by L-NMMA
also failed to protect against cyclophosphamide induced diabetes, indicating that β cell destruction is probably not mediated by nitric oxides.

7.2.4 Inhibition of Nitrite Release From NOD and B10(L-Lsh^) Mouse Peritoneal Macrophages by L-NMMA.

Since *in vivo* treatment of NOD mice with L-NMMA in both the transfer and cyclophosphamide induced diabetes models was unable to prevent disease, but apparently treatment could prevent diabetes in the STZ induced model. It would appear that two different mechanisms of beta cell destruction might be operating in the different models. In order to establish that the L-NMMA was indeed functionally active it was decided to assess the activity of the L-NMMA *in vitro*. Four NOD and four B10 (L-Lsh^) female mice of 3 months of age were administered 0.1 ml of a suspension of “Biogel” i.p. (this activates and recruits monocytes to the peritoneum). PEC were harvested 4 days later and allowed to adhere to coverslips, and cultured in duplicate with increasing concentrations of IFN-γ and LPS either with 200mM L-NMMA or D-NMMA. Supernatants were harvested at 24 and 48 hours and assayed for nitrite release. It can be seen in Figure 7.8, that at the maximal LPS concentration of 1.0 ng/ml and at 25u/ml IFN-γ release of nitrite at 48 hours was inhibited from 139 mmoles with the inactive enantiomer D-NMMA to 67 mmoles with L-NMMA in B10(L-Lshr) PEC (52% inhibition). At these concentrations of IFN-γ and TNF-α inhibition of nitrite from 74 mmolar to 57 mmolar (23% inhibition) in NOD PEC was observed at 48 hours. At 24 hours cocultivation of PEC with cytokines and mitogens inhibition of nitrite release was also observed although it was not so dramatic. Thus, L-NMMA is active *in vitro* and failure to prevent disease in vivo is not due to lack of functional activity.
7.3 Discussion

From the previous data it is suggested that 5C6 exerts its protective effect on diabetes in the transfer system by suppressing the early phase of macrophage migration into the islets, upon which later T cell-mediated events depend. Monocyte recruitment is followed by bulk migration of both sensitised and non-sensitised T cells to the islets. Analysis of the histology in 5C6-treated and control mice supports such a scheme where inhibition of macrophage recruitment to the pancreas prevents later intra-islet T cell accumulation.

The mechanisms by which the macrophage promotes T cell recruitment remain to be clarified but several possibilities exist. Local production of cytokines such as TNF-α and IL-1 by macrophages may affect expression of adhesion molecule VCAM-1 on vascular endothelium thereby facilitating mononuclear migration (Osborn et al, 1989). Additionally it has been suggested that IFN-γ produced by T cells activates macrophages to secrete TNF which in turn sensitises the same, or a different, population of macrophages to secrete reactive oxygen species (Clark et al, 1986). Reactive oxygen intermediates produced by macrophages such as superoxide, cause vascular endothelial damage (Beckman et al, 1990). In particular, nitric oxide can profoundly affect blood pressure and flow (Vallance et al, 1989).

Culture in the presence of IFN-γ increases MHC class I expression on islets (Campbell et al, 1986A) which may itself play a part in β cell destruction (Allison et al; 1988, Hutchings et al; 1990). Macrophage accumulation in the pancreas may well have a major role in antigen processing and presentation to T cells (Charlton et al; 1988A, Lee et al; 1988) but in addition macrophages may themselves destroy β cells by release of cytokines or free radicals (Nomikos et al, 1986). By preventing the CR3 dependent monocyte migration 5C6 treatment also inhibits the passage of lymphocytes into the islets and thereby arrests the subsequent cascade of cellular interactions which culminate in the destruction of insulin-containing β cells. Later administration of 5C6, at a time when recruitment is already advanced, failed to affect the onset of hyperglycemia (Hutchings et al, 1990B). This is to be expected because 5C6, which lethally potentiates acute bacterial infections, fails to do so for listerial infections once granuloma formation has occurred (Rosen et al, 1989). Indeed the minimal infiltration and destruction observed in 5C6 treated
animals could be due to antigen processing and presentation by the constitutive tissue macrophages which could either be Mac-1+/F4/80+ or SER-4+ present in the normoglycemic animal. Further destruction is prevented since presentation cannot proceed as reconstituted macrophages are trapped within the vascular endothelium and cannot traverse to the site of antigen expression to potentiate the inflammatory process. These observations do not preclude a direct role for macrophages or their secretory products in β cell destruction once they have aggregated at the target site.

It is significant that 5C6 prevents some infiltration by T cells as well as macrophages, for CR3 is not expressed on T cells. The effect of 5C6 on T cell recruitment is therefore probably indirect, with the macrophage being implicated, as there is little evidence of other CR3-bearing cells (neutrophils and NK cells) in infiltrates of diabetic pancreata. After administration of 5C6 is stopped, blockade of CR3 is reversed, macrophage migration to the islets becomes possible and T cell recruitment and β cell destruction ensues. Recipients treated with 5C6 from day 10 developed disease with an incidence comparable to untreated control animals (Hutchings et al., 1990B). This indicates that CD4+ and/or CD8+ effectors in the transfer inoculum are still potentially agents of destruction once macrophage migration to the islet becomes possible. These data highlight the essential contribution of both macrophages and T cells to the initiation of a complex autoimmune disease and suggest that therapeutic strategies should consider both cell types and their diverse mechanisms of tissue destruction. Debrick also demonstrated that in vivo depletion of phagocytic cells completely inhibits CTL responses suggesting a cognate interaction between macrophages and CTL precursors initiating a class I restricted immune response (De rick et al., 1991).

Having established that macrophages are mandatory for disease induction, it was attempted to elucidate their exact role in diabetes manifestation in the NOD mouse. Numerous studies have demonstrated that macrophages are cytotoxic to β cells. Secreted products potentially involved are the monokines IL-1 and TNF-α and the ‘respiratory burst’ products: superoxide radicals and nitric oxide metabolites. As the enzymatic pathways leading to the synthesis of reactive oxygen and nitrite intermediates are distinct (Iyengar et al., 1987; reviewed Liew and Cox, 1991), it was decided to assess the contribution of nitric oxide metabolism to β cell destruction in the cyclophosphamide and
diabetic spleen cell transfer models of diabetes in the NOD mouse. In another accelerated model, STZ-induced diabetes, Lukic (Lukic et al., 1991) demonstrated that inhibition of nitric oxide formation by L-NMMA prevented diabetes and insulitis in CBA mice.

My studies have indicated that treatment of transfer recipients with L-NMMA by any of the protocols described was unable to prevent insulitis and diabetes. Both the incidence and time of onset of diabetes in the L-NMMA-treated recipients was similar to that of the control animals receiving the native analogue of L-arginine and the inactive enantiomer D-NMMA. L-NMMA neither prevented insulitis nor diabetes in cyclophosphamide induced diabetes in NOD mice. This data would seem to suggest that either macrophages do not mediate β cell destruction by nitric oxide formation or that the particular batches of L-NMMA used were inactive. However in vitro studies demonstrated that L-NMMA could inhibit nitric oxide formation from TNF-α and IFN-γ stimulated PECs from both NOD and B10LshF mice. NOD PEC appeared to secrete significantly less nitric oxide in response to IFN-γ and LPS than B10LshF mice. Although nitric oxide formation was down-regulated by L-NMMA it was not completely inhibited in NOD mice. This result was surprising given that macrophages are numerous in the inflammatory infiltrate and I would have anticipated that NOD mouse macrophages would have been hyperactive with regard to NO production. This level of NO may mediate vascular dilation and permeability which has been demonstrated in prediabetic diabetes susceptible BB rats (Walker et al., 1988) and which we found in the NOD mouse. It would be interesting to investigate the potential production of Il-1 and TNF-α by NOD macrophages compared to other nondiabetic mouse strains to investigate this possibility. Baxter (Baxter, 1991B; Baxter and Mandel, 1991) in his doctoral thesis suggested that diabetes induced by multiple low-dose STZ occurred by an alternative mechanism than that of the natural process. He suggested STZ diabetes appeared to involve the dual action of incremental toxic destruction and the induction of autoimmunity to a β cell neoantigen (reviewed Kolb et al., 1987), such as C-type retrovirus (Like and Rossini, 1976; Fujino-Kurihara et al., 1985). Since, the diabetogenic effect of STZ is not abrogated by cyclosporin, the major action of STZ in NOD mice appears to be toxic, rather than immune mediated. If this is the case then my own observations that L-NMMA could not prevent disease in both the diabetic spleen cell transfer and cyclophosphamide induced models (which are immune mediated) whereas it
could protect in the STZ model, would perhaps support this view (Kolb et al, 1991; Lukic et al, 1991).

My findings do not rule out the possibility that macrophages are potentially cytotoxic to the β cell. Shimada demonstrated that highly activated macrophages appear before the development of overt diabetes in NOD mice (Shimada et al, 1991) and their free oxygen radical scavengers PEG-SOD (Lafferty et al, 1991) and N-acetylcysteine (Shehadeh et al, 1991) inhibit development of disease recurrence following transplantation of islets to spontaneously diabetic or cyclophosphamide induced diabetic NOD mice respectively. Two studies have shown that a 1% probucol containing diet prevents diabetes in the NOD mouse and hyperglycemia in the multiple low-dose STZ model (Fukuda et al; 1991; Shimizu et al; 1991) by inhibition of free oxygen radical generation in islet cells theoretically induced by macrophages. Yamada has suggested that the hydroxyl radical scavengers nicotinamide, 3-amino benzamide and dimethyl urea protect islet cells from the cytotoxic effect of IFN-γ and TNF-α in vitro and attenuate MHC class I but not class II expression on β cells induced by these cytokines (Yamada et al, 1991). In contrast the oxygen radical scavenger glutathione which inhibits IFN-γ release from activated lymphocytes, does not protect NOD mice from developing diabetes (Williams et al, 1991).

Macrophages also secrete IL-1 which may be the major effector molecule in the initiation phase of the immune-mediated destruction of β cells preceding overt IDDM. Several recent studies have suggested that the presence of IL-1β in the circulation and the islets of Langerhans (Reimers et al, 1991A) may induce a diabetes-like condition by a time-dependent inhibition of β cell function, and may be involved in the initial steps of IDDM pathogenesis in the BB rat. However treatment of BB rats with IL-1β has an insignificant effect on the incidence of diabetes (Markholst et al; 1991, Reimers et al; 1991). Pociot (Pociot et al, 1991B) has demonstrated that differences in macrophage IL-1 response capacity is genetically determined and may be a contributory factor in IDDM pathogenesis. He identified a diallelic RFLP in the 5th exon of the human IL-1 gene with Taq 1 of which the larger fragment allele represents a "high secretor" phenotype which may be an additional susceptibility marker in these patients. It would be of interest to assess the potential secretory IL-1 capacity of NOD mice compared to other non-diabetes prone
mouse strains.

From these studies I would hypothesise that a) macrophages are essential for the development of diabetes in the NOD mouse and, b) they have to be present in the vicinity of the islet for β cell destruction to occur. This destruction appears not to be mediated by the L-arginine-nitric oxide metabolic pathway. Thus I suggest that the primary function of the macrophage is antigen presentation and that both the transferred diabetogenic T cells or T cells recognising the diabetogenic auto-antigen home specifically to the pancreas, where presumably the auto-antigen is expressed and presented on macrophages. Such T cells cannot commence β cell destruction themselves as they need to recognise antigen in the context of class II MHC antigens. Once activated they secrete IFN-γ, activating the resident pancreatic macrophage or dendritic cell population, which migrate up a concentration gradient and process the antigen which activates the Th cell which in turn activates Tc to kill β cells. Note that CD4+ T cells can induce insulitis of their own accord but for disease development CD8+ T cell are also required (Thivolet et al, 1991). During this phase activated macrophages may secrete TNF-α recruiting more monocytes to the inflammatory site and eventually scavenge dead and dying β cells.
Figure 7.1 Nitric Oxide Generation (NO) and the Possible Mechanisms of its Antimicrobial Effects.

(A) O2 → NO
   Synthase → Citrulline
   L-Arginine → L-NMMA → NO' → NO2- + NO3-

(B) NO' + enzyme [4Fe-4S] → Enzyme

(C) O2 + NO → ONOO- + H+ → ONOOH
   HO' + NO2' → NO3 + H+

Macrophages produce nitrite and nitrate (Stuehr et al, 1985) which are derived from L-arginine and their production is blocked by structural analogues such as NG-monomethyl arginine (L-NMMA) (Hibbs et al; 1987A, 1990; Iyengar et al, 1987). NO has now been shown to be synthesized from one of the terminal guanidino-nitrogen atoms of L-arginine (Hibbs et al; 1987A, B, Palmer et al; 1989). The enzyme responsible (NO synthetase) is cytosolic, NADPH-dependent and results in the formation of L-Citrulline and NO which is rapidly converted to nitrite (NO2-) and nitrate (NO3-) (Iyengar et al, 1978) (7.1A). There are two postulated mechanisms for this reaction (7.1B) NO may react with Fe-S groups forming iron-nitrosyl complexes and thus leading to inactivation plus the generation of Fe-S prosthetic groups of aconitase and complex I and complex II of the mitochondrial electron transfer chain (Hibbs et al, 1990). As an alternative (7.1C) NO could possibly react with O2 to form the peroxynitrite anion (ONOO-) which decays rapidly once it is protonated forming the highly reactive hydroxyl radical (HO') (Beckman et al, 1990)
Seven male NOD mice of 2-3 months of age were administered 500µg 5C6 i. v., six control littermates were given either 500µg of the control isotype matched antibody i. v. or 200µl PBS. All mice were sublethally irradiated with 650 Rad from a cobalt source and reconstituted with $2 \times 10^7$ spleen cells from overtly diabetic NOD mice the same day. The 5C6 treated mice subsequently received 500µg 5C6 the next day and then every second or third day until day 14. The control Ab. recipients received 500µg of Campath-1 i. p. the day after transfer and on two subsequent days or PBS. Mice were sacrificed for pancreatic immunohistology. Sections were stained for insulin and the macrophage marker F4/80. The percentage of islets with (1)-none, (2)-moderate (1-10 +ve cells), or (3)-severe (>10 +ve cells) peri-islets locations are shown above. It can be seen that 5C6 administration to diabetic transfer recipients afforded significant protection from severe peri-islet infiltration.
FIGURE 7.3: Histology of Islets From NOD Mice Treated With 5C6 For Two Weeks

Histology of islets of pancreata from diabetic spleen cell transfer recipients stained by indirect immunofluorescence with polyclonal rabbit anti-mouse F4/80 (green) and insulin (red), 14 days after transfer. Recipients were treated with the control antibody Campath-1 (A) or 5C6 (*B, C). The 5C6 treated mice showed very little peri-islet infiltration (C) or were completely protected from F4/80+ macrophage infiltration as in B. Magnification 200x, *400x.
FIGURE 7.4: Histology of islets showing protective effect of 5C6

Photomicrographs of pancreatic cryostat sections double-stained for insulin (red) and F4/80 (green) from 5C6 treated mice (A) and control mice (B) four weeks after the adoptive transfer of diabetic spleen cells. In C (5C6-treated mice) and D (control mice) sections were stained with polyclonal rabbit anti-F4/80 alone (red). Islets from the control group (B and D) showed uniform intra-islet infiltration by F4/80+ cells, with loss of insulin and normal islet morphology. The 5C6-treated mice showed very little intra-islet infiltration but had a mixture of completely intact islets (as in A) or islets showing peri-islet infiltration (as in C). This pattern of infiltration is similar to that found in age-matched male NOD mice after the adoptive transfer of normal spleen cells. Magnification in A and B, 100x; in C and D 200x.
Figure 7.5 Effect of L-NMMA, L-Arginine and D-NMMA on Diabetic Spleen cell Transfer.

Figure 7.6 Effect of Prolonged Treatment with L-NMMA, L-Arginine and D-NMMA on Diabetic Spleen Cell Transfer.

Irradiated male NOD mice (8-9 weeks) were given $2 \times 10^7$ spleen cells from diabetic donors and given either 5mg L-NMMA, 5mg L-arginine or 5mg D-NMMA (6 or 7 mice per group) from days 9-13 Figure 7.5 or from days 7-11 and then three times weekly until sacrificed or animals had a blood glucose reading > 22.2 mM Figure 7.6.

Blood glucose was measured on the days indicated. A reading of >15 mM coupled with a positive urine glucose reading (as measured by diastix) was considered to be an indicator of overt diabetes.

Two animals from both the L-NMMA and D-NMMA groups were sacrificed on day 31 as they were dying of diabetes and a further animal in the L-arginine treated group developed a wasting disease and died on day 21—Figure 7.5.
Irradiated male NOD mice (10 weeks) were given 2X10^6 spleen cells from diabetic donors and given either 5mg L-NMMA, 5mg L-Arginine or 0.2mls PBS from days 1-5 and then 3 times weekly until sacrificed. A further group were given 2X10^6 spleen cells from non-diabetic syngeneic age-matched mice. Blood glucose was measured on the days indicated. A reading of >15mM/Litre coupled with a positive urine glucose reading (as measured by diastix) was considered to be an indication of overt diabetes.
**FIGURE 7.8** Graphs Showing Nitrite Release From NOD and B10L-Lsh^ Peritoneal Exudate Cells Cultured With IFN-γ and LPS and Either L-NMMA or D-NMMA.

Graphs show peritoneal exudate nitrite release (micromoles) from NOD (A, C) and B10L-Lsh^ (B, D) mice cultured with increasing concentrations of IFN-γ and 1.0ng/ml LPS and either 200mM L-NMMA or 200mM D-NMMA.

The nitrite concentration of the culture supernatant was measured with Griess reagent at 24 hours (A, B) and 48 hours (C, D).
### TABLE 7.1 Immunohistochemical analysis of pancreatic islets from 5C6-treated and untreated NOD mice 4 weeks after transfer of diabetic spleen cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No of mice examined</th>
<th>No of islets analysed</th>
<th>Islets with + peri-islet infiltration (%)</th>
<th>Islets with ++ intra-islet infiltration (%)</th>
<th>Residual islets (%)</th>
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<tbody>
<tr>
<td>Double staining for insulin and F4/80:</td>
<td></td>
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<tr>
<td>5C6 + d.sp.</td>
<td>7</td>
<td>216</td>
<td>84*</td>
<td>1†</td>
<td>20</td>
</tr>
<tr>
<td>PBS + d.sp.</td>
<td>4</td>
<td>109</td>
<td>47</td>
<td>29</td>
<td>84</td>
</tr>
<tr>
<td>Non-d.sp. only</td>
<td>4</td>
<td>95</td>
<td>98</td>
<td>8</td>
<td>8</td>
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<tr>
<td>Double staining for insulin and CD3:</td>
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<td></td>
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<tr>
<td>5C6 + d.sp.</td>
<td>7</td>
<td>129</td>
<td>76¶</td>
<td>9§</td>
<td>19</td>
</tr>
<tr>
<td>PBS + d.sp.</td>
<td>4</td>
<td>43</td>
<td>38</td>
<td>52</td>
<td>72</td>
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<tr>
<td>Non-d.sp. only</td>
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<td>2</td>
<td>23</td>
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<td>Double staining for insulin and Class II</td>
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<tr>
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<td>172</td>
<td>88</td>
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<tr>
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<td>117</td>
<td>91</td>
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Irradiated male NOD mice were given $2 \times 10^7$ spleen cells from diabetic donors (d.sp) or from non-diabetic syngeneic age-matched males. Pancreatic cryostat sections were double-stained with guinea-pig anti-insulin antibody and either rabbit anti-F4/80 which recognises mature mouse macrophages (prepared by P. Dri) or KT3, a rat anti-mouse CD3 monoclonal antibody (prepared by K. Tomonari). Those islets that did not contain immunoreactive insulin are designated residual and calculated as a percentage of the total analysed.

* Minimal infiltration of 0-20 F4/80* cells peri-islet.
† Maximal infiltration, namely 11 or more F4/80* cells intra-islet.
¶ 0-10 CD3* cells peri-islet.
§ More than 6 CD3* cells intra-islet.

Grading of peri-islet and intra-islet infiltration was calculated as a percentage of those islets that remained. PBS, Phosphate-buffered saline.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. Mice Examined</th>
<th>No. Islets analysed</th>
<th>Islets with Peri-islet Infiltration</th>
<th>Islets with Intra-islet Infiltration</th>
<th>Residual islets (%)</th>
</tr>
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<tr>
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<td></td>
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<td>1-10</td>
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<td>5C6 treated*</td>
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<td>Campath*</td>
<td>6</td>
<td>274</td>
<td>0</td>
<td>5</td>
<td>31</td>
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<tr>
<td>Campath**</td>
<td>6</td>
<td>226</td>
<td>1</td>
<td>10</td>
<td>18</td>
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<tr>
<td>5C6 treated $\xi$</td>
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<td>87</td>
<td>32</td>
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<tr>
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<td>245</td>
<td>7</td>
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Irradiated male NOD mice were given $2 \times 10^7$ spleen cells from diabetic donors and either 5C6 or Campath-1, experiment $1^{(*)}$ and experiment $2^{(**)}$. Pancreatic cryostat sections were double-stained with guinea-pig anti-insulin antibody and either rabbit anti F4/80 which recognises mature mouse macrophages or OX6 which recognises NOD class II MHC ($\xi$, experiment $2^{(**)}$ only). Those islets that did not contain immunoreactive insulin are designated residual and calculated as a percentage of the total analysed. Grading of peri-islet and intra-islet infiltration was calculated as a percentage of those islets that remained.
Table 7.3 Immunohistochemical analysis of pancreatic islets from cyclophosphamide treated NOD mice

<table>
<thead>
<tr>
<th>Week after Treatment</th>
<th>No. of mice examined</th>
<th>No. of islets analysed</th>
<th>No. of islets with ++ peri-islet infiltration (%)*</th>
<th>No. of islets with + intra-islet infiltration (%)ζ</th>
<th>Residual Islets (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>88 (82)</td>
<td>9 (17)</td>
<td>8 (7)</td>
<td>11 (15)</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>23 (22)</td>
<td>43 (33)</td>
<td>4 (9)</td>
<td>68 (86)</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>33 (20)</td>
<td>0 (6)</td>
<td>21 (5)</td>
<td>6 (10)</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>18 (15)</td>
<td>0 (0)</td>
<td>6 (0)</td>
<td>100 (100)</td>
</tr>
</tbody>
</table>

Female NOD mice were given 200mg/Kg cyclophosphamide i.p. on day 0 and 14. At weekly intervals groups of mice were sacrificed for pancreatic immunohistology. Pancreatic cryostat sections were double-stained with guinea-pig anti-insulin antibody and either OX6 which recognises NOD class II MHC or a rat anti-mouse CD3 monolonal antibody (figures in brackets) which recognises T cells. Those islets that did not contain immunoreactive insulin are designated residual and calculated as a percentage of the total analysed.

* maximal infiltration of more than 41 positive staining cells peri-islet.
ζ minimal infiltration of 1-5 positive staining cells intra-islet.
Grading of peri-islet and intra-islet infiltration was calculated as a percentage of those islets that remained.
Table 7.4 Immunohistochemical Analysis of Pancreatic Islets From cyclophosphamide Induced Diabetes in NOD Mice Treated With L-NMMA or L-Arginine

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. Mice</th>
<th>% Diabetic</th>
<th>% of Islets with ++ Peri-Islet Infiltration*</th>
<th>% of Islets with + Intra-Islet Infiltration §</th>
<th>% Residual Islets</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-NMMA</td>
<td>5</td>
<td>80</td>
<td>36 (13)</td>
<td>14 (33)</td>
<td>43 (17)</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>4</td>
<td>75</td>
<td>59 (0)</td>
<td>12 (14)</td>
<td>53 (22)</td>
</tr>
<tr>
<td>PBS</td>
<td>4</td>
<td>75</td>
<td>32 (13)</td>
<td>12 (24)</td>
<td>42 (22)</td>
</tr>
</tbody>
</table>

Nine week old female NOD mice were given 200mg/Kg cyclophosphamide i. p. on day 0 and 14. Recipients were then administered either 5mg L-NMMA or L-Arginine or 0.2mls PBS i. p. the 5 days following the first dose of cyclophosphamide and 3 times weekly thereafter until either the animals were overtly diabetic or day 42. At this point the remaining animals were sacrificed. Pancreatic cryostat sections were double stained with guinea-pig anti-insulin antibody to locate islets and either OX6 (recognises NOD class II, I-A) or F4/80 (recognises mature mouse macrophages) figures in brackets. Islet infiltration was graded and the number of islets of each grade expressed as a % of the total number of intact islets analysed (-residuals). Those islets which did not contain immunoreactive insulin were designated residual and calculated as a percentage of the total analysed.

* Maximal peri-islet infiltration of >20 +Ve staining cells.
§ Minimal intra-islet infiltration of 1-10 +Ve staining cells.
CHAPTER 8

8.1 INTRODUCTION

Extensive research has been carried out in patients with autoimmune conditions to understand the mechanisms and genetics of such complex polygenic diseases. This has led to the observation of an increased incidence of certain serologically defined lymphoid cell-surface proteins encoded by the HLA-D region of the MHC and referred to as class II antigens in such patients. As described previously in Chapter 1 interaction between the T cell receptor, antigenic peptide and the class II molecule leads to T cell receptor activation and an immune response to the antigen. Given that the primary sequence of the amino-terminal polymorphic domain of the MHC molecule is essential for T-cell recognition, the immunological responsiveness of a particular individual is partly determined by their polymorphic amino acids present in the class II molecules.

Susceptibility to autoimmune IDDM is determined by both environmental and genetic influences. The HLA-D region in particular contributes over 59% of IDDM heritability (Todd et al., 1989). Further about 95% of IDDM patients possess either HLA-DR3 and/or -DR4, compared to 45-54% of the normal population. The HLA-DQ genes, which are in linkage disequilibrium with HLA-DR are more strongly associated with IDDM than the DR genes, however no single determinant or RFLP has been found that is common to the several HLA haplotypes (DR4, 3 and 11) positively associated with the disease (Todd et al., 1987). DNA sequencing and oligonucleotide analysis of class II genes from population and family studies indicate that susceptibility to IDDM maps to the MHC and is closely linked to the DQA1 locus and both DQB1 and DQA1 genes contribute to disease predisposition. Analysis of the nucleotide sequences of the DRB1, DQA1 and DQB1 genes showed that all IDDM patients had DRB1*0406, DQA1*0301, and DQB1*0302, compared with only 14% of controls (Uchigata et al., 1992).

Further analysis of DNA encoding the first domain of the DQB chain from selected multiple case Caucasian families of IDDM patients by PCR amplification, demonstrated that haplotypes carrying an aspartic acid at position 57 (Asp-57) of their DQB chain were significantly increased in frequency among nondiabetic haplotypes. Non-Asp-57 haplotypes were significantly increased in frequency among the diabetic haplotype (Morel et al., 1988). As amino acid 57 could determine a critical function of the DQ molecule, based on the proposed structure of class II MHC antigen, it is unknown whether Asp-57 alone will confer decreased
susceptibility. The amino acid sequence must determine the overall structure of the DQ molecule, thus suggesting that DQβ allelic polymorphisms, determine the specificity and extent of the autoimmune response against islet cell antigens. Full HLA susceptibility to IDDM, is correlated with the individual having two Asp-57 negative DQβ alleles, especially if they are from the DR4 and/or DR3 haplotypes. The fact that not all HLA identical siblings, as well as identical twins don't develop IDDM, may indicate an effect of non-HLA-linked genes, low penetrance of HLA-linked linked genes, stochastic receptor rearrangements, or differences in environmental exposure. However the use of DQβ typing would be of great value in determining susceptibility to diabetes.

As previously indicated in chapter 3, the NOD mouse does not express I-E molecules, thus the genetic control of immune responses in these mice is determined by the I-A molecules. The I-Aα chain is identical to the I-Aα of H-2d haplotype mice, therefore any abnormal immune responses in these mice are most likely to be dependent upon the unusual I-Aβ chain. Studies by Acha-Orbea and M^Devitt (1987) have shown that the first external domain of the NOD mouse I-Aβ contains histidine (His) and a serine (Ser) at positions 56 and 57 respectively, instead of proline (Pro) and aspartic acid (Asp) found in other strains. This parallels the situation with the human DQβ alleles associated with IDDM in that both the Aβ^nod and its human homologue lack Asp at position 57.

A model for class II MHC structure based on the crystal structure of the class I molecule, suggests a possible role for residue 57 in maintaining the integrity of the molecule and in binding of peptide antigens (Brown et al, 1988). Residue 57 is part of the helix that forms one side of the antigen-binding cleft. The Asp 57 side chain appears to form a salt bridge with conserved Arginine at position 80 in the α chain. One of the predicted consequences of this substitution at residue 57 is altered immune responsiveness.

These sequence differences may alter the nature of the trimolecular complex formed by antigens, TCR and MHC antigen in NOD mice. Thus, the unique Aβ^nod chain might have a higher affinity for islet antigens and efficiently present these to autoreactive T cells or conversely a low affinity interaction could affect central tolerance resulting in no deletion or
anergy or peripheral tolerance resulting deficient activation of Ts or regulatory cells. Equally it may be assumed that (Pro) 56 gives rise to a different conformation of the I-Aβ chain than does His 56 as described above.

The observation that both NOD mice treated with anti-I-A^d during the first two months of life (Singh et al; 1990) and long-term administration of anti-class II antibodies specific for NOD I-A antigen (Boitard et al, 1988) prevents IDDM is further evidence that the unique I-A^p chain contributes to disease susceptibility in NOD mice. Anti-class II monoclonal antibodies have been used to prevent disease in other animal models of diabetes including the BB rat (Boitard et al, 1985) and in C57/KSJ-db/db mice (Singh and Cliffe, 1986; Singh et al, 1986).

We therefore constructed transgenic NOD mice in which either the transgene encoded a modified Aβ_nod with Pro56 or a modified Aβ^nod with Asp 57, and studied their effects on the development of IDDM (Lund et al, 1990B). Mice carrying mutated Aβ^8 genes were generated by two point mutations introduced into the NOD Aβ to generate the sequence encoding Pro-56 instead of His or Asp-57 instead of Ser. This destroys a restriction site for Dde I at this position in the Aβ^nod gene and allowed detection of the transgene carrying progeny. NOD mice carrying the Aβ^k transgene (ie. pro-56, asp-57) still develop insulitis, which may have been due to low expression of Aα^8 Aβ^k (Uehira et al, 1989). It is therefore likely that normal I-A expression cannot inactivate or delete autoreactive T cells, whereas I-A^8 molecules may be involved in the generation of such cells. Thus the role of normal I-A antigens in the prevention of autoimmune insulitis remains to be clarified.

The NOD mouse does not express I-E molecules and no mRNA for the α-chain of I-E is visible in Northern blot analysis (Hattori et al, 1986). Selective expression of I-E molecules in NOD mice without the introduction of other genes on chromosome 17 was achieved by backcrossing the I-E expressing C57BL/6 transgenic mice [B6(Eα^d)] to NOD mice. Such I-E expressing NOD mice failed to develop autoimmune insulitis or diabetes (Nishimoto et al, 1987).
Establishment of MHC class II NOD transgenic mice by either continuously backcrossing [Eα\(^d\) B6 transgenic mice X NOD]F1 to NOD or directly by micro-injecting the Eα\(^d\) gene into fertilised NOD eggs, also prevented autoimmune insulitis (Uehira et al, 1989). Therefore expression of I-E alone is sufficient to prevent the development of insulitis (Uehira et al; 1989; Lund et al, 1990B).

To further explore the protective effect of I-E in NOD mice, we constructed transgenic NOD mice carrying an Eα\(^d\) transgene which restored expression of I-E.

In addition to the pancreas, certain exocrine glands in the NOD mice such as salivary, lacrimal, peribronchial and vaginal glands, are consistently affected by cellular infiltration (Asamoto et al; 1984; Hanafusa et al; 1985, 1989; Miyagawa et al; 1986). These inflammatory infiltrates consist of focal accumulations of lymphoid cells around vessels and secretory ducts and thus closely resemble peri-insulitis. All of these glands share common physiological and anatomical features as well as antigenic determinants with the exocrine pancreas (Ludwig et al, 1977; Oxazaki et al; 1990). The presence of antibodies to salivary duct in this mouse strain coupled with salivary gland infiltration (Hanafusa et al; 1989, Yamada et al; 1985) raised the possibility that the NOD mouse might also be regarded as an animal model (Sugihara et al, 1988; Goillot et al, 1991) of human Sjogren’s disease (Whaley et al; 1973). Although this syndrome is more commonly associated with connective tissue disease, a recent study has also suggested an association with IDDM (Binder et al; 1989). Added to the simultaneous cell-mediated transfer of sialitis and insulitis in NOD neonates, (Goillot et al; 1991) these observations could suggest that at least a partly common molecular defect underlies both extrapancreatic and pancreatic inflammatory lesions in NOD mice.

Strain combination studies have confirmed that sialitis is an important disease marker in the NOD mouse and suggest that peri-insulitis is part of the diffuse inflammatory process (Garchon et al, 1991). Both the peri-insulitis and sialitis traits were influenced by nod and b haplotypes at the Bcl-2 locus on chromosome 1 mapping between Bcl-2/D1Nds2 and D1Nds1. This locus was the major determinant of sialitis although additional genes appear to be involved in the occurrence of peri-insulitis. Thus successive stages in the progression of diabetic disease appear to be controlled by distinct genes or sets of genes.

The histological features of the cellular infiltration of the exocrine glands seen in the NOD mouse are similar to that described for human Sjogren’s syndrome: There is focal
lymphocytic infiltration in the periductal and perivascular areas, leading eventually to functional impairment and fibrotic changes. In human Sjogren’s syndrome the infiltrates consist predominantly of T cells, with the majority of these belonging to the CD4⁺ subset. Small numbers of B cells and macrophages have also been reported (Moutsopoulos et al, 1986). The spectrum of cells in the NOD mouse salivary infiltrates is comparable with that of Sjogren’s patient salivary gland (Sugihara et al, 1989; Goillot et al, 1991). Class II MHC antigen expression is also seen on salivary epithelium of both NOD mice and patients with Sjogren’s disease (Rowe et al; 1987, Johnsson et al; 1987) and in both situations corresponds to those areas of maximal infiltration perhaps indicating that such expression is induced by IFN-γ produced by the infiltrating T cells. It therefore seemed appropriate to examine in more detail the autoimmune response to salivary gland in the NOD mouse and to assess the effect of the transgenes if any on this lymphocytic lesion.

This chapter will describe the histologic analysis of three classes of transgenic NOD mice, two carrying a modified I-Aβ7 with either amino acid position 56 altered from His to Pro (NOD-Pro) or at position 57 altered from Ser to Asp (NOD-Asp) respectively, or NOD mice carrying an Eαd transgene (NOD I-E). Lymphoid tissues were assessed for the presence of I-E in NOD-E transgenic mice and all three transgenic lines assessed by histological examination for autoimmune insulitis and the presence of class I/II MHC antigens in the pancreas and MHC class II expression in the salivary gland.
8.2 Results

8.2.1 NOD-E Transgenic Mice - Demonstration of I-E<sup>d</sup> on Lymphoid Tissues and Establishment of Lines.

The genomic constructs used for in vitro mutagenesis of NOD mice to make the NOD-Eα<sup>d</sup>, NOD-ASP and NOD-PRO transgenic are shown in Figure 8.1 A, B, C. Spleen sections from NOD-E mice (but not NOD mice) were stained in the B cell area with both the monoclonal antibodies Y17 (anti-I-E<sub>β</sub>) and H81.208.22.6 (anti-I-E<sup>k</sup>) (Figure 8.2 A, B) and showed a similar expression pattern to that found on CBA mice, a mouse strain that endogenously expresses I-E. Spleen sections from all three of the above strains were stained with MRC-OX6 (anti-I-A<sup>nod/K/S</sup>). The I-E transgene product, like normal class II gene products was expressed in B cells, but not T cells of NOD-E mice. Immunofluorescent staining of NOD-E thymus with Y17 and H81 demonstrated expression of I-E on thymic epithelial cells of both the cortex and the medulla similar to the staining pattern obtained for OX6 (Figure 8.2 C, D, E). Confirmation that I-E was expressed on the epithelial cells was achieved by double staining for class II MHC and IVC4 (subcapsular and medullary epithelial cells) and 4F1E4 (subcapsular, cortical and a subset of medullary epithelial cells). The pattern of expression with I-E and I-A specific M.Abs was similar to that of the NOD mouse thymus stained with OX6. Hyperexpression of MHC class II on thymic epithelial cells and on clusters of cells in the medulla and at the cortico-medullary junction was not observed on the CBA thymus. This peculiarity of the NOD-E thymus will be discussed in greater detail in the following chapter.

Having established transgenic lines of NOD-E mice which had incorporated the Eα<sup>d</sup> transgene into the genome, characterisation of the transgenic products demonstrated normal expression on spleen and thymic tissue. This was important since a variety of transgenic mice have been produced that have incorporated the transgene but have failed to express the transgene products (Palmiter and Brinster, 1986).

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8.2.2 Expression of the I-E Transgene Prevents Insulitis in NOD Mice.

Pancreata from age and sex-matched NOD-E transgenic lines or non-transgenic littermates were analysed by immunohistochemistry. Cryostat sections were stained for insulin and the presence of the MHC class II I-A molecule by MRC-OX6 FITC (Figure 8.3 B). Class II pancreatic infiltration in NOD-E transgenics was reduced to such an extent that 10-11 and 12 month female NOD-E-3 pancreatic islets had 13% and 1% peri-islet infiltration respectively and none had severe intra-islet infiltration, compared to 61% for 9 month old female NOD mice, (Table 8.1 ). NOD-E-9 mice were similarly protected from MHC class II^+ peri-islet infiltration. Intra-islet infiltration did not exceed 20 cells per section of islet in NOD-E transgenic mice and no evidence of β cell destruction was detected. In contrast 69% of islets from 9 month female non-transgenic littermates were residual, containing no insulin.

T cell infiltration in the NOD-E transgenics was reduced to an even greater extent, 1-7% of islets from 3-12 month male and female NOD-E-3 and 1% of 3-4 month female NOD-E-9 islets showed peri-islet infiltration. No grade III (severe) intra-islet infiltration of T cells or evidence of β cell destruction was observed in these transgenic mice. By comparison 20-61% of 3-10 month male and female NOD non-transgenic littermates demonstrated peri-islet infiltration and 6-63% of islets were residual (Table 8.2) (Figure 8.3 A). Expression of the I-E transgene would therefore seem to prevent pancreatic infiltration of both class II MHC expressing cells and T cells.

8.2.3 Introduction of an Ea^d Transgene in NOD Mice prevents pancreatic Hyperexpression of class I MHC.

In NOD mice as described previously in Chapter 5, over-expression of class I MHC molecules occurs adjacent to the areas of infiltration by mononuclear cells (T cells and macrophages). In the pancreas class I hyperexpression is observed on the infiltrating cells, within infiltrated islets and extends into the adjacent exocrine tissue. The over-expression of MHC class I molecules observed in the prediabetic NOD pancreas varies from 71-100% in 6-7 and 9-10 month female NOD mice (Figure 8.4, Table 8.3) and was never observed in NOD-E-3 or NOD-E-9 transgenic mice. Expression of MHC class I in NOD-E transgenics was restricted to normal tissue macrophages and the minimal infiltrate at peri-islet locations.
8.2.4 Expression of I-E Does Not Prevent Development of Salivary Gland Infiltration in NOD Mice.

In addition to developing pancreatic infiltration, NOD mice also develop infiltration of the submandibular salivary glands. MHC class II expression was observed on focal infiltrates scattered throughout the submandibular gland and is also observed on the stratified cuboidal epithelial lining of the salivary excretory duct, located in the interlobular septa and lobules themselves in both the NOD mouse and human patients. In both situations the MHC class II expression corresponds to those areas of maximal infiltration, perhaps indicating that such expression is induced by IFN-γ produced by the infiltrating T cells. I-A and I-E expression were observed both on the ductal cells adjacent to salivary gland infiltrates and on the infiltrates themselves in NOD I-E mice, increasing in frequency and size with age. Not all transgenic mice showed infiltration of class II positive cells and both the extent and frequency of infiltration was variable, which is also characteristic of such infiltrates in the NOD mouse (Table 8.4, Figure 8.5). In comparison control CBA mice demonstrated only minor lymphocytic infiltration, with no infiltrate exceeding 20 class II positive cells and no hyperexpression of class II MHC on the tubular epithelia. Thus the transgene had no influence on salivary infiltration. Its product displayed tissue specific expression and responded normally to inductive stimuli.

8.2.5 Introduction of The I-E Transgene Prevents Diabetes in NOD Mice.

Sixty-five NOD-E-3 mice (36 females and 29 males) were examined at weekly intervals for diabetes, up to 9 months of age. None developed the disease, demonstrating complete prevention of the spontaneous disease by introduction of this transgene. Comparable results were obtained with similar numbers of NOD-E-9 mice. Although female NOD mice have a higher incidence of IDDM than male NOD mice, disease can be induced in males following administration of high doses of cyclophosphamide or transfer of diabetic spleen cells. When NOD mice were treated with 200mg/Kg cyclophosphamide (200mg/Kg administered intraperitoneally on days 0 and 14) it induced IDDM in 4/10 animals. However, none of the 11 NOD-E-3 transgenic mice treated with cyclophosphamide developed IDDM. When diabetic spleen cells from NOD mice were transferred to 5 irradiated (650 Rads) NOD-E recipient mice, none became diabetic by day 26. At this point 100% (3/3) of NOD non-transgenic littermate control mice were hyperglycemic, 2/3 had already died of diabetes and 60% of the
islets from the remaining animal were residual. Sixty-five percent of islets from the irradiated NOD-E diabetic spleen cell recipients showed mild peri-islet infiltration (1-20 cells) and 82% of islets examined had no intra-islet infiltration, although there was some evidence of β cell destruction. In comparison 80% (4/5) of irradiated NOD-E recipients which had received 2\times10^7 NOD non-diabetic spleen cells died of an apparent lethal graft versus host disease (GVHD); seventy percent of islets from the remaining animal had no peri-islet infiltration, none of the infiltrates were >20 cells per islet and there was no intra-islet infiltrate or evidence of β cell destruction. Blast cells were observed in the spleens from recipient NOD-E mice, presumably due to recognition of I-E\textsuperscript{d}.

This suggests that the transgene presence did not alter recognition of antigenic determinants on the β cell by NOD diabetic spleen cells, as the latter homed to the pancreas.

8.2.6 Construction of NOD-PRO and NOD-ASP Transgenic Mice and Establishment of Lines.

NOD mice carrying a mutated Aβ\textsuperscript{nod} gene were constructed. The transgenes were expressed in these mice, as amplification using the polymerase chain reaction (PCR) of complementary DNA generated from spleen RNA of NOD-PRO or NOD-ASP mice, gives a product which could only partially be digested by Dde I. This was detected by an oligonucleotide probe specific for sequences encoded by the mutated region of the transgenes. Any DdeI-sensitive components present in both the NOD-PRO and NOD-ASP progeny derive from the endogenous Aβ\textsuperscript{nod} gene.

8.2.7 Mutated NOD Aβ Prevents Insulitis in NOD-PRO but not NOD-ASP Mice.

Histological analysis of the pancreas stained for insulin and class II MHC with MRC-OX6 of NOD-PRO and NOD-ASP transgenics demonstrated differing effects of the transgenes on pancreatic infiltration. Twenty-two percent of intact islets of 6 month female NOD-PRO showed peri-islet infiltration with class II positive cells, with no β cell destruction, whereas 69% of age and sex matched NOD-ASP transgenic islets showed peri-islet infiltration and 9%
of islets were residual. The infiltration of cells expressing class II MHC observed in NOD-ASP transgenics was not significantly different from littermate NOD mice which had not inherited the transgene (Figure 8.3 C, D) cf. Table 8.5 with Table 8.1.

Histological analysis of pancreatic cryostat sections stained for insulin and anti-CD3 (which detects T cells), further showed that NOD-PRO but not NOD-ASP transgenics were protected from T cell infiltration. One to six percent of NOD-PRO mice at 4-10 months of age had peri-islet T cell infiltration and no evidence of β cell destruction, compared to 14-39% of NOD-ASP 4-11 month old mice which also displayed evidence of severe intra-islet infiltration and β cell destruction (Table 8.6).

Comparative analysis of the different transgenic mice revealed differences with regard to the extent of pancreatic infiltration. Although transgenic NOD-PRO mice, showed no evidence of β cell destruction, they demonstrated a more frequent and extensive peri-islet infiltrate than NOD-E mice. The PRO substitution protected against severe insulitis as there was a marked reduction in both the frequency and severity of peri-islet infiltration and none of these animals developed diabetes in over 200 mice studied. Accumulation of mononuclear cells in a peri-islet location is common in the pancreas of NOD mice from 5 weeks of age and occurs irrespectively of the subsequent development of diabetes. The presence of a mutated NOD Aβ with the Aspartate substitution did not prevent insulitis or β cell destruction and IDDM although disease development was delayed and reduced in incidence (10-12%, >200 animals studied).

8.2.8. Hyperexpression of Class I MHC is Prevented in NOD-PRO but not NOD-ASP Transgenics.

Over-expression of class I MHC was only observed on 2% of islets from 6 month male NOD-PRO mice, the total incidence from all the NOD-PRO pancreata of varying ages analysed was 0.4% (2/552 islets from 27 mice). This is in comparison to 42% over-expression in 4-6 month male NOD-ASP transgenics and a total incidence of 23% for all NOD-ASP pancreata analysed (129/554 islets from 27 mice). MHC class I expression in the pancreata of NOD-PRO mice was almost exclusively restricted to the minimal peripheral islet infiltrate (Table 8.7) in comparison to NOD mice.
8.2.9. Modified NOD-I-\(\alpha\beta\) Does Not Prevent Salivary Gland Infiltration In NOD Mice.

Analysis of the submandibular gland from both NOD-PRO and NOD-ASP transgenic mice stained for class II MHC with MRC-OX6 showed that inheritance of either of the transgenes did not prevent sialitis. Both the frequency and severity of class II MHC infiltration of salivary glands from transgenic NOD mice was comparable to that observed from both NOD and NOD-Frag mice (NOD mice which had inherited a fragment of the I-E transgene). Many infiltrates consisting of 51-100 or >100 cells were observed, although the extent of infiltration was variable within a group of mice (which represents the normal pattern of expression during the earlier stages of the disease). In comparison CBA mice which do not develop a Sjogren’s type syndrome had only minimal infiltration of 1-20 positive cells (Table 8.4, Figure 8.5). Arrow in Figure 8.5C shows induction of MHC class II antigen on salivary duct epithelium.
8.3 Discussion

At least 5 genes contribute to IDDM susceptibility in the NOD mouse. Idd-1 is located within the MHC on Chromosome 17 (Hattori, 1986) and could be a gene complex with at least one susceptibility locus, I-Aβ. Idd-1 is thought to be dominant with low penetrance and is associated with insulitis (Wicker et al., 1987). Controversy still surrounds the second susceptibility gene (Idd-2), Prochazka (Prochazka et al., 1987) claimed that Idd-2 is linked to the Thy1/Apolipoprotein A-1 gene (Alp-1) cluster but subsequent backcross studies have failed to find a significant association, although different strains were used for the backcross (Todd et al., 1991). The third susceptibility gene (Idd-3) maps to an interval on Chromosome 3. It is flanked by the IL-2 and Tshb genes and contains D3Nds1, and affects both insulitis and diabetes. It maybe the susceptibility gene (or gene complex) that was proposed previously to be essential for the development of insulitis. The fourth susceptibility gene (Idd-4) maps between the Acrb and MPO loci near DllNds1 on Chromosome 11 and is thought to influence the frequency of insulitis and may control progression to overt diabetes (Todd et al., 1991). Finally the fifth susceptibility locus (Idd-5) has been mapped to the proximal region of chromosome 1, which contains D1Nds4, MIT-L2 and at least two candidate susceptibility genes, the IL-1R gene (IL-1r1) and the Lsh/Ity/Blg gene. The latter encodes resistance to bacterial and parasitic infections and affects the function of macrophages. This latter effect may be of importance considering the results of my own studies in the previous chapter showing that macrophages are essential for disease development. Idd-5 influences the development of insulitis and may act in a predominantly additive way with Idd-3 and 4 (Cornall et al., 1991).

From the above genetic studies it is clear that pancreatic infiltration is controlled by a gene which is not linked to the MHC but the severity of infiltration is modified by an MHC linked gene. My studies in transgenic NOD-E mice would support these findings. Introduction of the I-E transgene resulted in a typical pattern of class II expression in lymphoid organs and the immune system of these mice was intact (Lund et al., 1990B). They were therefore deemed to be an appropriate model in which to study the involvement of I-E in IDDM. Introduction of the I-E transgene not only reduced the frequency of insulitis but also the severity of MHC class II and T cell pancreatic infiltration, although minimal infiltration is still seen around some islets. No islet cell destruction was observed in transgene carrying mice compared to nontransgenic littermates. This is consistent with the observations of Nishimoto (Nishimoto, 1987) that no insulitis was observed in I-E bearing NOD mice at 25 weeks of age. Thus, I-E expression
appears to prevent the onset of insulitis rather than delay it. Hyperexpression of class I MHC associated with insulitis and β cell destruction was not observed in NOD-E transgenic mice either. This finding is probably not surprising as I have previously shown that expression of MHC class I on exocrine tissue was associated with insulitis. Therefore if pancreatic infiltration was minimal one would not probably expect to observe hyperexpression of class I MHC, as upregulation of class I MHC expression is probably an epiphenomenon as a result of lymphokine production such as TNF and IFN-γ released from the infiltrating cells not the cause of infiltration per se.

The mechanism underlying the protective effect of expression of the I-E transgene remains unclear. Expression of I-E modifies the peripheral T cell repertoire by deletion of T cells expressing certain T cell receptor Vβ genes (Tomonari and Lovering; 1988, Bill et al; 1989; Tomonari and Fairchild, 1991). In particular T cells displaying Vβ5+ TCRs are negatively selected in the thymus of mice expressing I-E. Thus one suggestion is that the diabetogenic anti-islet clones would be deleted intrathymically when the wild type Eα gene was introduced into NOD mice. The inability to induce disease in NOD-E-3 transgenics with high doses of cyclophosphamid suggests protection is complete and is consistent with such a deletion mechanism by negative intra-thymic selection of autoreactive T cells. This protection afforded by the I-E gene could be the result of positive selection of a protective (suppressor) T cell or removal of an autoreactive T cell possibly by intrathymic negative selection. Evidence from fluorescence-activated cell sorting (FACS) analysis of Vβ use in peripheral T cells, does not suggest a clear candidate for Vβ deletion with the limited range of Vβ-specific monoclonal antibodies used in this study (Lund et al, 1990B). Furthermore, Bohme has described wild-type I-Eαk and promoter mutated ΔX, ΔY or Sma I-Eαk transgenes backcrossed onto the NOD genetic background and found almost complete protection from insulitis when wild type I-E was expressed on all class II expressing compartments (thymic epithelia, thymic medulla, B cells and macrophages). When expression was lacking from any one of the compartments, however, no protection was afforded. Little I-E mediated negative selection occurs on the NOD background and only moderate deletion of Vβ5-bearing T cells was observed in NOD I-Eαk.
transgenic mice. Therefore the data is inconsistent with the hypothesis that I-E protects by clonal deletion of Vβ5+ T cells (Bohme et al, 1990). No depletion of Vβ6/Vβ11+ T cells was evident in these transgenic mice or those of our own although the NOD mouse carries only Mtv 3 and 17 (Dyson et al, 1991). Although the ΔX, ΔY and Sma transgenes did not protect from insulitis they demonstrated the same moderate deletion of Vβ5 bearing cells as in the wild type I-Eαk transgenic mice which did protect. The data suggests that if expression of I-E is absent from a particular cell type required for deletion of autoreactive T cells within some compartments of the transgenic mice that (should) be positive for I-E, then autoreactive T cells would be allowed to escape.

Alternatively the mechanism of protection may not be via deletion in the thymus but may involve a peripheral presentation step such that the mechanism of protection in these mice requires I-E expression on both thymic and peripheral cell compartments such as dendritic cells (Van Ewijk et al, 1988). The I-E molecule would then have a positive influence rather than the negative one usually proposed.

Taken together, these data suggest that the protective mechanisms of these transgenes may be even more complex. It is possible the T cell receptors using other Vβ and/or Vα genes are deleted or that the introduction of new restriction elements has subtly altered the pattern of T cell responses. Kaye and colleagues suggest that the presence of the new I-E restriction element in NOD transgenic mice renders the mice more susceptible to Leishmania donovani, reflected in the relative inability of the transgenic mouse to form granulomas in the liver and clear the parasite (Kaye et al, 1992). Thus presentation of antigens (including autoantigens) on a new restriction element may alter the pattern of immune responsiveness such that a different cytokine profile is elicited. Such an alteration in the balance of cytokines may profoundly affect the inflammatory response. The presence of the new I-E restriction element might result in competition for binding of diabetogenic peptides. A higher affinity interaction between the peptide, MHC and a T cell could lead to enhanced tolerance induction to self antigens during ontogeny. The importance of the presence of I-E restricting elements was underlined by the finding that NOD-E thymus protected against diabetes when grafted into neonatally thymectomised NOD mice. But when both NOD I-E thymus and NOD thymus were double grafted into neonatally thymectomised NOD recipients, no protection from diabetes was
afforded (Kurner et al, 1989). It was therefore suggested that T cells maturing in NOD I-E thymus cannot suppress T cells maturing in nontransgenic NOD thymus, implying that protection from I-E is due to negative selection and must be imposed by thymic epithelium since the bone marrow compartment was derived from the NOD recipient system. However studies by Parish (personal communication) suggest that NOD I-E thymic grafts do not protect NOD mice from diabetes and that peripheral tolerance mechanisms are of greater importance.

Finally it is possible that I-E molecules may cross-react with an epitope formed by the interaction of the β cell antigen and MHC molecules. Thus, the expression of I-E in the early neonatal stage can make T cells tolerant to autologous β cells or the expression of I-E could induce suppressor cells which prevent the generation of auto-β-cell-reactive helper cells.

It is clear that although deletion of T cells bearing particular TCRs by I-E cannot be ruled out, it is increasingly likely that the presence of new restriction elements and peripheral monitoring mechanisms are more probable candidate mechanisms which could account for the protective effect of the I-E transgene.

The sequence of the I-AP chain in the first external domain is usually conserved, but the NOD mouse is unique having 5 consecutive nucleotide substitutions from 248-252 leading to amino acid changes at residue 56 (histidine) and 57 (serine) which in other mouse strains are proline (Pro) and aspartic acid (Asp) respectively (Acha-Orbea and McDevitt, 1987). It is conceivable that inappropriate intra-thymic presentation of antigen by NOD I-A molecules could result in failure of clonal deletion and the escape of potentially autoreactive T cells or equally that the failure of positive selection of T cells which would exert suppressive/protective effects. Alternatively extrathymic presentation of the diabetogenic antigen by NOD I-A MHC molecules could result in the influx of T cells with crossreactivity to β cells.

Intense interest has been provoked in the role the I-Aβ chain and in particular the influence of amino acid 57 on diabetes susceptibility since there are such striking similarities in the MHC region between diabetic patients and the NOD mouse. The presence of a charged residue (such as Asp) may influence the formation of a salt bridge between the two alpha-helices of the antigen binding groove affecting the binding of endogenous or exogenous peptides. Equally it could be assumed that Pro 56 gives rise to a different conformation of I-
Aβ chain than does His 56.

Although there was little evidence of intra-islet infiltration by mononuclear cells or β cell destruction in the pancreas of NOD-PRO mice, protection was not as complete as that described for NOD-E transgenics. Most of the islets in the NOD-PRO mice were free of insulitis, with only mild intra-islet lymphocyte infiltration in isolated islets of some transgenic mice. The pancreata of transgene-negative littermate controls showed massive intra-islet infiltration by T cells and macrophages and beta cell destruction, as did NOD-ASP mice. Accumulation of mononuclear cells in a peri-islet location is common in the pancreas of NOD mice from 5 weeks of age, and occurs irrespective of the subsequent development of diabetes. NOD-PRO transgenic mice showed a marked reduction in the numbers of cells present in such infiltrates and no pancreatic hyperexpression of MHC class I was observed. None of these mice developed diabetes by the age of 5 months. In contrast, NOD-ASP transgenic mice demonstrated hyperexpression of MHC class I in conjunction with islet cell infiltration and destruction and some developed diabetes. Transgene expression is occasionally associated with cellular dysfunction which may result in immunoincompentence when the transgene is expressed on cells of the immune system (Brinster et al., 1985; Allison et al., 1988; Gifillian et al., 1990). It was therefore important to establish that the NOD-PRO transgenics were immunocompetent by testing the ability of spleen cells from NOD-PRO and their transgene-negative littermates to respond to mitogens and alloantigens. No significant differences in their immune responses could be detected, showing that the peripheral T cell repertoire was essentially the same as NOD mice (Lund et al., 1990B). Indeed, the NOD-PRO transgenic mice showed no alteration in Vβ5, Vβ6, Vβ8 or Vβ11 TCR use of peripheral T cells. This absence of any gross perturbation in the T cell repertoire of NOD-PRO mice is probably not surprising, bearing in mind that although there is some evidence for intra-thymic deletion of T cells bearing specific TCR by I-E, by Mls and 'superantigens' (reviewed Ferrick, 1989), there is no direct evidence for deletion by I-A. Protection afforded by the I-Ak transgene is incomplete as diabetes can be induced in NOD I-Aβk by cyclophosphamide (Slattery et al., 1991). Thus the mechanism of protection by I-A is unlikely to be one of intrathymic deletion of all T cells reactive against the putative islet cell antigen.
The introduction of Aβ transgenes into NOD mice is not sufficient to protect from insulitis as indicated by the finding that introduction of an Aβk transgene into NOD mice does not prevent intra-islet infiltration (Uehira et al, 1989). Miyazaki et al (Miyazaki et al, 1990) and Slattery et al (Slattery et al, 1990) have also explored the role of the NOD Aβ in the development of IDDM by introducing Aαk together with Aβk. It was necessary to introduce both transgenes as NOD Aαd apparently does not pair well with Aβk. Miyazaki demonstrated that while no protection from diabetes in NOD mice was afforded by incorporation of either the I-Aα gene or I-Aβ gene alone, F1 mice incorporating both chains of I-A were protected from the development of insulitis and IDDM. Such problems relating to pairing of I-A chains were not encountered in our studies as the transgene encoding the NOD Aβ gene had been mutated in such a way that the transgenic NOD mice expressed on their cell surfaces the endogenous NOD I-A together with the mutated I-A PRO (56) or ASP (57). Results of our studies and those of other workers suggest that dominant nonsusceptibility to diabetes is conferred by the transgenic expression of a non-NOD I-A. Indeed I-Aβ may be the Idd-1 susceptibility locus located in the murine MHC on Chromosome 17.

The mechanism(s) underpinning the protective effect of these transgenes remain(s) to be elucidated but the same possibilities as those proposed for the I-E-expressing NOD transgenics also pertain. An additional explanation may be that the defect which leads to autoimmune insulitis in NOD mice may be the failure of I-A8 to present peptides which are readily bound and presented by the I-E, I-Ak or I-Ag7(Pro56) but not the I-Ag7(Asp57) allele. Such a defect in ability to present peptides may be important for tolerance induction. Indeed a model of class II structure (Brown et al, 1988) has placed this amino acid residue at position 57 within the putative antigen-binding cleft. Competition between endogenous class II MHC and the transgenic class II molecules for binding of the diabetogenic peptide might lead to a failure to present to the autoreactive T cell or to deletion or anergy of autoreactive T cells, (recently described in several induced systems in vivo: Burkly et al, 1989; Qin et al, 1989; Ramensee et al, 1989). This could occur because of a more efficient interaction between the autoreactive T cell and the APC bearing the antigen / class II MHC complex as suggested by
Blackman (Blackman; 1990) who recently created transgenic mice which express a rearranged TCR β chain with a low affinity for the superantigen Mls-1α. In Mls-1α positive animals some clonal elimination occurred but some transgene-bearing T cells escaped all forms of tolerance because the TCR α chain they bore completely prevented Mls-1α recognition. Although a significant percentage of T cells escaped elimination they were still affected by the self antigen. The I-Aβpro transgene may compete more efficiently for diabetogenic peptide than the endogenous I-Ag7. Alteration of the antigen binding groove in the I-Aβpro may affect T cell triggering such that the diabetogenic peptides are tolerated in the thymus thereby preventing autoimmunity. The Asp mutation may not have such a profound affect on T cell triggering or compete for peptide as efficiently, so that autoimmunity is not prevented. Alternatively a mutation of the I-Aβ87 may alter the pattern of immune responsiveness by changing the cytokine profile elicited as suggested previously for the I-E transgenic NOD mice.

Studies on the segregation of peri-insulitis and submandibulitis suggested that the latter was an important disease marker in the NOD mouse. Both traits showed a significant association with the Bcl-2 marker on Chromosome 1 although additional genes were involved in the occurrence of the peri-insulitis lesion (Garchon et al, 1991). Salivary gland infiltration in the NOD-I-E,-PRO and -ASP transgenics is of a similar extent and frequency as in non-transgenic NOD mice. Therefore although both the submandibular gland and pancreas share antigenic determinants, the differential inflammatory responses would appear to be influenced by presentation of different autoantigens.

It is clear from our studies and those of others that the mechanisms of protection in the I-E and I-Aβpro mice may not be identical. Peripheral mechanisms other than negative selection appear to be the best explanation for I-E protection, but this is unlikely to be the mode of protection in the I-A mice where autoantigen binding efficiency may be a more plausible explanation. Further studies are required to assess the roles of positive intra-thymic selection and the role of autoantigen presentation in susceptibility to IDDM. The findings of abnormalities in the NOD and NOD-E transgenic thymus in comparison to CBA, suggested that there may be some fundamental defect or alteration in thymic ontogeny resulting in disease susceptibility. This abnormality will be investigated in the next chapter.
FIGURE 8.1  Restriction Maps of cos NOD 1-3 and cos 1^d-2 Used in the Construction of the NOD-ASP, NOD-PRO and NOD-E Transgenic Mice.

(A). Restriction map of cos 1^d-2, isolated from a genomic cosmid library together with cos 1^d-1 and the 7.5 kbp Bg/1 fragment containing the entire Eα^d gene and 2kb 5' and ~2-kb 3' flanking sequences. This sequence was subcloned using ClaI linkers into pSK^+ and injected into fertilized mouse embryos after the removal of bacterial sequences. K, KpnI sites.

(B) Restriction map of cos NOD-1-3 containing the entire NOD Aβ gene isolated from a NOD liver genomic library.

(C) A single stranded template of the Aβ exon II subclone which was isolated following superinfection with the VCM13 helper phage. The mutagenesis was carried out using PRO or ASP primer as specified above.
RAVTELGRHSAEYYNKO
...GCCCGTGACCAGCTGCGGCACGATCGAGTACATACGAG

Dde 1

57Asp Primer: CTGCGCGCACGAGCGCGGAGTAC

56Pro Primer: CTGCGCGGGCCATCAAGCGGAGTAC
FIGURE 8.2

Cryostat sections of NOD-E spleen stained for the expression of MHC class II with H81 (detects I-E\(^k\)) and MRC-OX-6 (detects I-A\(^k,s,NOD^k\)) showing normal splenic expression of these antigens in the B cell areas of the white pulp.

Figure 8.2 C, D and E depict normal MHC expression of the MHC class II antigens, I-E stained with Y17 (C) and H81 (D) and I-A antigens stained with MRC-OX-6 (E) on the thymic epithelium of both the cortex and the medulla.

These photomicrographs show normal MHC class II expression of the I-A and I-E antigens in the spleen and thymus of NOD mice made transgenic by introduction of an E\(\alpha^{\text{d}}\) transgene.

Magnification in (A,C) 100x; in (B) 200x; and in (D) and (E) 400x.
FIGURE 8.3: Histology of Pancreata From Transgenic NOD Mice

Figure 8.3 (A) shows a cryostat section of a female NOD mouse which had inherited a fragment of the NOD Eα<sup>d</sup> transgene stained by immunofluorescence for T cells by anti-CD3 FITC (green) and insulin (red), showing peri and intra-islet infiltration and disruption of islet morphology.

Figure 8.3 (B) shows a section of pancreata from transgenic NOD-Eα<sup>9</sup> which had inherited the whole transgene showing only minimal peri-islet infiltration of MHC class II<sup>+</sup> mononuclear cells stained with MRC-OX6 FITC (green) and insulin (red).

Similarly figure 8.3 (*C) and (D) show sections of pancreata from NOD-ASP and NOD-PRO transgenic mice respectively stained for insulin (red) and MHC class II expression with MRC-OX6 FITC (green). Photomicrographs show that the Eα<sup>d</sup> and I-<sub>A</sub>βPRO transgenes but not the I-<sub>A</sub>βASP transgene protect against pancreatic insulitis.

Magnification 200x; *400x.
Cryostat sections of pancreas from nine month old female NOD-Eng (A), and eight month old female NOD (B) were stained for MHC class I with the rat anti-mouse MAb M1/42.3 (green) and insulin (red) by indirect immunofluorescence. Photomicrographs show the normal pancreatic expression of MHC class I by transgenic NOD-E (A) and hyperexpression on pancreatic endocrine and exocrine tissue in nontransgenic NOD (B) which do not express I-E. Arrow points to residual insulin containing beta cells. Magnification (A) 200x; (B) 100x.
Photomicrographs of submandibular gland stained with MRC-OX6 FITC showing MHC class II positive mononuclear cell infiltration of five month old female CBA mice (*A), three and five month old female NOD mice (B) and (C) respectively and five month old female NOD-E (D), NOD-ASP (E) and NOD-PROL (F).

These cryostat sections show the minimal infiltration of CBA submandibular glands, compared to the extensive infiltration of MHC class II + mononuclear cells and expression of MHC class II on the salivary duct epithelium in NOD and transgenic NOD mice.

Magnification *400x, 200x.

Arrow shows MHC class II on duct epithelium.
Table 8.1 Immunohistochemical Analysis of Pancreatic MHC Class II Cellular Expression From Male and Female NOD and Transgenic NOD-E mice.

<table>
<thead>
<tr>
<th>Mouse Line</th>
<th>No. of Mice Analysed</th>
<th>Sex</th>
<th>Age (Months)</th>
<th>Islet Infiltration</th>
<th>% Residual Islets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>% Islets Max. Intra</td>
<td>% Islets Peri-infil</td>
</tr>
<tr>
<td>NOD-E-3</td>
<td>6</td>
<td>F</td>
<td>3 to 4</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>NOD-E-3</td>
<td>5</td>
<td>M</td>
<td>3 to 4</td>
<td>0</td>
<td>39</td>
</tr>
<tr>
<td>NOD-E-3</td>
<td>2</td>
<td>F</td>
<td>6</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>NOD-E-3</td>
<td>11</td>
<td>F</td>
<td>10 to 11</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>NOD-E-3</td>
<td>5</td>
<td>F</td>
<td>12</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>NOD-E-9</td>
<td>5</td>
<td>F</td>
<td>3 to 4</td>
<td>0</td>
<td>46</td>
</tr>
<tr>
<td>NOD-E-9</td>
<td>4</td>
<td>M</td>
<td>3 to 4</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>NOD</td>
<td>9</td>
<td>F</td>
<td>3 to 4</td>
<td>6</td>
<td>81</td>
</tr>
<tr>
<td>NOD</td>
<td>8</td>
<td>M</td>
<td>3 to 5</td>
<td>0</td>
<td>86</td>
</tr>
<tr>
<td>NOD</td>
<td>3</td>
<td>F</td>
<td>7</td>
<td>0</td>
<td>70</td>
</tr>
<tr>
<td>NOD</td>
<td>4</td>
<td>F</td>
<td>9</td>
<td>14</td>
<td>61</td>
</tr>
</tbody>
</table>

Immunohistochemical analysis was performed on pancreatic cryostat sections double stained with guinea-pig anti-insulin antibodies and MRC-OX6 FITC, a mouse monoclonal antibody that recognises NOD class II MHC. Islets were examined, numbers of infiltrating class II positive cells counted and the severity of infiltrate graded. Severe intra-islet infiltration corresponds to >20 positive cells penetrating inside the islet. The percentage of infiltrated islets refers to intact islets (total islets minus residual islets).

Numbers of residual islets are expressed as a percentage of the total number of islets analysed. No severe intra-islet infiltration was seen in any of the transgenic animals or any islets remnants devoid of insulin (residual). The peri-islet infiltration seen in transgenic mice never exceeded 20 cells/islet in any given islet area analysed. 637 islets from NOD mice, 497 from NOD-E-3 and 152 from NOD-E-9 transgenic mice were analysed. F-female, M-male.
Table 8.2 Immunohistochemical Analysis of T Cell Infiltration In The Pancreas of Male and Female NOD and Transgenic NOD-E mice.

<table>
<thead>
<tr>
<th>Mouse Line</th>
<th>No. of Mice Analyzed</th>
<th>Sex</th>
<th>Age (Months)</th>
<th>Islet Infiltration %</th>
<th>% Residual Islets</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD-E-3</td>
<td>5</td>
<td>F</td>
<td>3 to 4</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>NOD-E-3</td>
<td>4</td>
<td>M</td>
<td>3 to 4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NOD-E-3</td>
<td>14</td>
<td>F</td>
<td>10 to 12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NOD-E-9</td>
<td>5</td>
<td>F</td>
<td>3 to 4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>NOD-E-9</td>
<td>4</td>
<td>M</td>
<td>3 to 4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NOD</td>
<td>5</td>
<td>M</td>
<td>3 to 4</td>
<td>2</td>
<td>61</td>
</tr>
<tr>
<td>NOD</td>
<td>4</td>
<td>F</td>
<td>6 to 7</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>NOD</td>
<td>4</td>
<td>F</td>
<td>9 to 10</td>
<td>16</td>
<td>20</td>
</tr>
</tbody>
</table>

Immunohistochemical analysis was performed on pancreatic cryostat sections double stained with guinea-pig anti-insulin antibodies and anti-CD3 FITC which recognises mouse T cells. Islets were examined, numbers of infiltrating class II positive cells counted and the severity of infiltrate graded. Severe intra-islet infiltration corresponds to >20 positive cells penetrating the islet. The percentage of infiltrated islets refers to intact islets (total islets minus residual islets).

Numbers of Residual islets are expressed as a percentage of the total. No severe intra-islet infiltration was seen in any of the transgenic animals or any islets remnants devoid of insulin (residual). The peri-islet infiltration seen in transgenic mice never exceeded 20 cells/islet in any given islet area analysed. 114 islets from NOD mice, 667 from NOD-E-3 and 148 from NOD-E-9 transgenic mice were analysed. F-female, M-male.
Table 8.3 Immunohistochemical Analysis of Class I MHC Expression in the Pancreas of Male and Female NOD and Transgenic NOD-E.

<table>
<thead>
<tr>
<th>Mouse Line</th>
<th>No. Mice</th>
<th>Sex</th>
<th>Age (months)</th>
<th>% of Islets with Class I MHC Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD-E-3</td>
<td>5</td>
<td>F</td>
<td>3 to 4</td>
<td>Normal (I): 74, Infiltrate (II): 26, Hyper- (III): 0</td>
</tr>
<tr>
<td>NOD-E-3</td>
<td>5</td>
<td>M</td>
<td>3 to 4</td>
<td>Normal (I): 78, Infiltrate (II): 22, Hyper- (III): 0</td>
</tr>
<tr>
<td>NOD-E-3</td>
<td>3</td>
<td>F</td>
<td>6</td>
<td>Normal (I): 78, Infiltrate (II): 22, Hyper- (III): 0</td>
</tr>
<tr>
<td>NOD-E-3</td>
<td>15</td>
<td>F</td>
<td>10 to 12</td>
<td>Normal (I): 97, Infiltrate (II): 3, Hyper- (III): 0</td>
</tr>
<tr>
<td>NOD-E-9</td>
<td>3</td>
<td>F</td>
<td>3</td>
<td>Normal (I): 92, Infiltrate (II): 8, Hyper- (III): 0</td>
</tr>
<tr>
<td>NOD-E-9</td>
<td>4</td>
<td>M</td>
<td>3 to 5</td>
<td>Normal (I): 92, Infiltrate (II): 8, Hyper- (III): 0</td>
</tr>
<tr>
<td>NOD-E-9</td>
<td>2</td>
<td>F</td>
<td>9</td>
<td>Normal (I): 89, Infiltrate (II): 11, Hyper- (III): 0</td>
</tr>
<tr>
<td>NOD</td>
<td>7</td>
<td>M</td>
<td>3 to 4</td>
<td>Normal (I): 41, Infiltrate (II): 18, Hyper- (III): 41</td>
</tr>
<tr>
<td>NOD</td>
<td>13</td>
<td>F</td>
<td>6 to 7</td>
<td>Normal (I): 19, Infiltrate (II): 10, Hyper- (III): 71</td>
</tr>
<tr>
<td>NOD</td>
<td>2</td>
<td>F</td>
<td>9 to 10</td>
<td>Normal (I): 0, Infiltrate (II): 0, Hyper- (III): 100</td>
</tr>
<tr>
<td>NOD</td>
<td>2</td>
<td>M</td>
<td>10</td>
<td>Normal (I): 9, Infiltrate (II): 30, Hyper- (III): 61</td>
</tr>
</tbody>
</table>

Immunohistochemical analysis of pancreatic cryostat sections was performed at three different levels 100-150um apart. Sections were double stained with guinea-pig anti-insulin antibodies and M1/42.3 (rat anti-mouse class I MHC) and assessed by the following scoring system as a percentage of the total islets analysed.

Grade I-Normal class I expression at peri and intra-islet positions
Grade II-Class I expression observed on islet infiltrating cells.
Grade III-Class I expression observed on pancreatic infiltrate (endocrine) and also hyperexpression over the surrounding exocrine.

799 islets from 28 NOD-E-3 mice, 112 islets from 9 NOD-E-9 mice and 542 islets from 28 NOD mice were analysed. M- Male, F- Female.
table 8.4 MHC Class II+ Cells Infiltrating NOD and Transgenic NOD Salivary Gland.

<table>
<thead>
<tr>
<th>Mouse Line</th>
<th>Age Months (No. Mice)</th>
<th>Number of Infiltrates/Salivary Gland with each grade</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Grade I 1-20 cells</td>
</tr>
<tr>
<td>NOD-PRO</td>
<td>10 F (2)</td>
<td>9,6</td>
</tr>
<tr>
<td>NOD-PRO</td>
<td>6 F (5)</td>
<td>12,15,2,6</td>
</tr>
<tr>
<td>NOD-ASP</td>
<td>5-6 F (6)</td>
<td>3,10,11,9,17,18</td>
</tr>
<tr>
<td>NOD-E-3</td>
<td>4-5 F (9)</td>
<td>1,11,6,3,0,8,9,2,4</td>
</tr>
<tr>
<td>NOD-E-3</td>
<td>4-5 M (4)</td>
<td>0,0,0,4</td>
</tr>
<tr>
<td>NOD-E-3</td>
<td>12 F (6)</td>
<td>5,5,3,10,2,1</td>
</tr>
<tr>
<td>NOD-E-9</td>
<td>3 F (2)</td>
<td>3,2</td>
</tr>
<tr>
<td>NOD-E-9</td>
<td>9-11 F (5)</td>
<td>6,4,9,6,6</td>
</tr>
<tr>
<td>NOD-E-9</td>
<td>9-11 M (3)</td>
<td>3,5,5</td>
</tr>
<tr>
<td>NOD-Frag</td>
<td>8-9 F (3)</td>
<td>3,3,5</td>
</tr>
<tr>
<td>NOD</td>
<td>3 F (4)</td>
<td>12,6,9,3</td>
</tr>
<tr>
<td>NOD</td>
<td>5 F (5)</td>
<td>1,3,18,3,0,10,8,4,15</td>
</tr>
<tr>
<td>CBA</td>
<td>3 F (5)</td>
<td>3,3,3,3,0</td>
</tr>
<tr>
<td>CBA</td>
<td>5 F (5)</td>
<td>2,2,2,0,1</td>
</tr>
</tbody>
</table>

Cryostat sections of both submandibular glands were analysed by immunohistochemistry at two different levels 150-200mm apart. Sections were stained with MRC-OX6 FITC which detects class II MHC from both NOD and CBA mice. The number of infiltrates of a particular grade for each mouse was recorded sequentially on the table. NOD mice showed extensive infiltration of the submandibular gland which increased with age, in comparison CBA mice showed either minimal or no infiltration at all ages examined. Introduction of an Ea^d transgene or a mutated NOD I-Aβ had no effect on either frequency or extent of salivary infiltration.
Table 8.5 Immunohistochemical Analysis of Pancreatic MHC Class II Cellular Expression From Male and Female NOD-ASP and NOD-PRO Transgenic NOD mice.

<table>
<thead>
<tr>
<th>Mouse Line</th>
<th>No. of Mice Analysed</th>
<th>Sex</th>
<th>Age (Months)</th>
<th>% Islets Max. Intra</th>
<th>% Islets Peri-inf il</th>
<th>% Residual Islets</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD-PRO</td>
<td>8</td>
<td>M</td>
<td>4 to 5</td>
<td>0</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>NOD-PRO</td>
<td>6</td>
<td>F</td>
<td>4 to 5</td>
<td>&lt;1</td>
<td>52</td>
<td>0</td>
</tr>
<tr>
<td>NOD-PRO</td>
<td>4</td>
<td>F</td>
<td>6</td>
<td>0</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>NOD-PRO</td>
<td>3</td>
<td>F</td>
<td>10 to 11</td>
<td>0</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>NOD-ASP</td>
<td>6</td>
<td>M</td>
<td>12</td>
<td>1</td>
<td>69</td>
<td>10</td>
</tr>
<tr>
<td>NOD-ASP</td>
<td>3</td>
<td>F</td>
<td>4 to 5</td>
<td>9</td>
<td>98</td>
<td>12</td>
</tr>
<tr>
<td>NOD-ASP</td>
<td>11</td>
<td>M</td>
<td>5 to 6</td>
<td>0</td>
<td>61</td>
<td>6</td>
</tr>
<tr>
<td>NOD-ASP</td>
<td>10</td>
<td>F</td>
<td>5 to 6</td>
<td>2</td>
<td>69</td>
<td>9</td>
</tr>
<tr>
<td>NOD-ASP</td>
<td>6</td>
<td>F</td>
<td>10</td>
<td>0</td>
<td>22</td>
<td>6</td>
</tr>
<tr>
<td>NOD-ASP</td>
<td>4</td>
<td>M</td>
<td>4</td>
<td>2</td>
<td>29</td>
<td>18</td>
</tr>
</tbody>
</table>

Immunohistochemical analysis was performed on pancreatic cryostat sections double stained with guinea-pig anti-insulin antibodies and MRC-OX6 FITC, a mouse monoclonal antibody that recognises NOD class II MHC. Islets were examined, numbers of infiltrating class II positive cells counted and the severity of infiltrate graded. The percentage of infiltrated islets refers to intact islets (total islets minus residual islets. Numbers of residual islets are expressed as a percentage of the total. Minimal severe intra-islet infiltration was seen in the NOD-PRO transgenic animals but no β cell destruction or islets remnants devoid of insulin (residual) were observed. NOD-ASP transgenics showed both severe intra-islet infiltration and β cell destruction. 699 islets from NOD-PRO mice and 852 islets from NOD-ASP mice transgenic mice were analysed.
**Table 8.6 Immunohistochemical Analysis of Pancreatic T Cell Infiltration From Male and Female Transgenic NOD-ASP and NOD-PROL mice.**

<table>
<thead>
<tr>
<th>Mouse Line</th>
<th>No. of Mice Analysed</th>
<th>Sex</th>
<th>Age (Months)</th>
<th>Islet Infiltration</th>
<th>% Residual Islets</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD-PROL</td>
<td>8</td>
<td>M</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NOD-PROL</td>
<td>5</td>
<td>F</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>NOD-PROL</td>
<td>3</td>
<td>F</td>
<td>10</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>NOD-ASP</td>
<td>7</td>
<td>M</td>
<td>4 to 6</td>
<td>1</td>
<td>39</td>
</tr>
<tr>
<td>NOD-ASP</td>
<td>10</td>
<td>F</td>
<td>4 to 6</td>
<td>0</td>
<td>36</td>
</tr>
<tr>
<td>NOD-ASP</td>
<td>4</td>
<td>M</td>
<td>11</td>
<td>3</td>
<td>31</td>
</tr>
<tr>
<td>NOD-ASP</td>
<td>5</td>
<td>F</td>
<td>10</td>
<td>0</td>
<td>14</td>
</tr>
</tbody>
</table>

Immunohistochemical analysis was performed on pancreatic cryostat sections double stained with guinea-pig anti-insulin antibodies and anti-CD3 which detects mouse T cells. Islets were examined, the numbers of infiltrating T cells counted and the severity of infiltrate graded. The percentage of infiltrated islets refers to intact islets (total islets minus residual islets).

Numbers of Residual islets are expressed as a percentage of the total. No severe intra-islet infiltration or evidence of residual islets was seen in any of the transgenic NOD-PROL animals although there was considerable intra-islet infiltration and β cell destruction in the NOD-ASP transgenic mice. The peri-islet infiltration seen in the transgenic NOD-PROL mice never exceeded 20 cells/islet in any given islet area analysed. 367 islets from NOD-PROL mice and 494 islets from NOD-ASP mice were analysed.
Table 8.7 Immunohistochemical Analysis of Class I MHC Expression in the Pancreas of Male and Female NOD and Transgenic NOD-ASP and NOD-PRO Mice.

<table>
<thead>
<tr>
<th>Mouse Line</th>
<th>No. Mice</th>
<th>Sex</th>
<th>Age (months)</th>
<th>% of Islets with Class I MHC Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Normal (I)</td>
</tr>
<tr>
<td>NOD-PRO</td>
<td>11</td>
<td>M</td>
<td>3 to 4</td>
<td>98</td>
</tr>
<tr>
<td>NOD-PRO</td>
<td>9</td>
<td>F</td>
<td>3 to 4</td>
<td>86</td>
</tr>
<tr>
<td>NOD-PRO</td>
<td>4</td>
<td>M</td>
<td>6</td>
<td>87</td>
</tr>
<tr>
<td>NOD-PRO</td>
<td>3</td>
<td>F</td>
<td>10</td>
<td>92</td>
</tr>
<tr>
<td>NOD-ASP</td>
<td>7</td>
<td>M</td>
<td>4 to 6</td>
<td>45</td>
</tr>
<tr>
<td>NOD-ASP</td>
<td>11</td>
<td>F</td>
<td>4 to 6</td>
<td>52</td>
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<tr>
<td>NOD-ASP</td>
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<td>M</td>
<td>11</td>
<td>57</td>
</tr>
<tr>
<td>NOD-ASP</td>
<td>5</td>
<td>F</td>
<td>10</td>
<td>80</td>
</tr>
<tr>
<td>NOD</td>
<td>7</td>
<td>M</td>
<td>3 to 4</td>
<td>41</td>
</tr>
<tr>
<td>NOD</td>
<td>2</td>
<td>M</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>NOD</td>
<td>13</td>
<td>F</td>
<td>6 to 7</td>
<td>19</td>
</tr>
</tbody>
</table>

Immunohistochemical analysis of pancreatic cryostat sections was performed at three different levels 100-150um apart. Sections were double stained with guinea-pig anti-insulin antibodies and M1/42.3 (rat anti-mouse class I MHC) and assessed by the following scoring system as a percentage of the total islets analysed.

Grade I-Normal class I expression at peri and intra-islet positions
Grade II-Class I expression observed on islet infiltrating cells.
Grade III- Class I expression observed on pancreatic infiltrate (endocrine) and also hyperexpression over the surrounding exocrine.

544 islets from 27 NOD-PRO mice, 552 islets from 27 NOD- E-9 mice and 434 islets from 22 NOD mice were analysed. M- Male, F- Female.
CHAPTER 9

Studies on The Thymus of NOD and Transgenic NOD Mice.
9.1 INTRODUCTION

NOD mice develop lymphocytic infiltration not only in the pancreas but also in other organs including the submandibular glands, lacrimal glands, thyroid, adrenal glands, ovaries and testes (Hanafusa et al 1989). It is therefore conceivable that the NOD mouse develops polyendocrine autoimmune disease as a consequence of disregulation of autoreactive cells. This chapter will concentrate on the possible role of the thymic microenvironment in the development of autoimmune diabetes in the NOD mouse. The thymus plays a crucial role in the development and organisation of the immune system in the vertebrate. Stem cells from the foetal liver migrate into the thymus from the 11-12th day of embryonic development, where they proliferate and mature. These T cell precursors rearrange and express TCR, followed by a series of events associated with both generation of tolerance to self MHC antigens (Marrack et al, 1988C) and other thymic self antigens (MacDonald et al, 1988; Kisielew et al, 1988). This is achieved by clonal deletion (negative selection) of T cells bearing TCR products of certain \( V\beta \) genes which have high reactivity to self-encoded determinants, or by inactivating T cells rendering them anergic (Ramsdel and Fowlkes, 1990). T cells also acquire the ability to recognise peptide fragments of antigen presented on MHC molecules (Bjorkman et al, 1987). Thus affinity for self-MHC antigens is a prerequisite for T cell responses to foreign antigen, and a failure to control or delete anti-self responses in the thymus may lead to T cell mediated autoimmunity.

The autoimmune nature of IDDM in the NOD mouse was demonstrated by prevention with cyclosporin A (Mori et al, 1986), anti-CD4 (Koike et al; 1987, Shizuru et al; 1988), anti-CD8 (Hutchings et al 1990A), anti-I-A antibodies (Boitard et al, 1988), or neonatal thymectomy (Ogawa et al, 1985). Thymectomy at 3 weeks increases the disease incidence in females (Bendelac et al, 1987) suggesting the existence of a regulatory role of late thymic derived cells.

Various accessory cell types have been implicated in the successive steps of T cell selection in the thymus. Positive selection is thought to be effected by interaction of TCR with MHC molecules on thymic epithelial cells, whereas negative selection is apparently mediated by bone marrow derived antigen presenting cells (Gao et al, 1988). Thymic hormones as well as physical interactions with non-lymphoid bone marrow derived thymic
stromal cells such as thymic epithelial cells (TEC), bone marrow derived macrophages and interdigitating dendritic cells (IDC) are thought to be instrumental in both processes. Together they represent the selective elements on which different stages of T cell development depend. At least some developmental events require close spatial interactions (if not direct contact) between migrating thymocytes and sessile stromal thymus elements. The subcapsular region, where baskets of epithelial reticular cells are located, contain most of the CD4^+CD8^- thymocytes. The outer cortex, composed of spider-shaped and sheet like epithelial cells is densely packed with small CD4^+CD8^+, TCR^{low} immature thymocytes. The inner medulla, consisting of a network of short stubbed epithelial cells, is loosely filled with CD4^+CD8^- and CD4^-CD8^+, TCR^{high} mature T cells. Other stromal cells such as interdigitating dendritic cells (IDC) are also located in this latter compartment most conspicuous at the cortico-medullary junction (CMJ) and along blood vessels (Brekelmans and Ewijk, 1991). Macrophages are present in all three compartments, mainly in the perivascular space, interlobular septa and lining blood vessels. All macrophages in the thymus express a common phenotype, as assessed by reactivity with M.Abs. Nevertheless, macrophages in the medulla and at the CMJ are morphologically distinct from each other (Brekelmans and Ewijk 1991).

Several studies have focussed on the expression of class I/II MHC antigens on frozen thymus sections in appropriate normal mouse strains. They had a reticular meshwork in the cortex between the capsule and the medulla and more confluent staining pattern in the medulla when staining with M.Ab detecting I-A, I-E and H-2D determinants. Anti-class I^k M.Ab labelled all cells in the medulla, but only weak, variable reticular staining in the cortex was reported (Rouse et al, 1979; Brekelmans and Ewijk, 1991). Anti-I-A^k M.Ab showed a delicate reticular pattern throughout the parenchyma, which in the cortex tended to be orientated perpendicular to the thymic surface and radiated towards the medulla (Farr et al, 1983). Epithelial cells were the major population of MHC class II bearing cells in the cortex. Thin lines of positive staining separate small collections of thymocytes, which generally did not appear to stain (Rouse et al, 1979). Perivascular areas of the corticomedullary area and medulla revealed a heterogeneous population of cells some of which were macrophages which expressed MHC class II (50% of macrophages both in
the cortex and the medulla and at the CMJ were positive with anti-MHC class II antibodies) (Nabarra et al., 1988). IDC also displayed MHC class II at the CMJ and in medullary areas (Farr et al., 1983; Nabarra et al., 1988). Despite these studies, the cells within the murine thymus that express MHC antigens cannot be definitively distinguished by tissue section immunofluoresence, which is limited by resolution artifacts, and reagent specificity within areas of confluent staining such as the thymic medulla. Ultrastructural electron microscopy indicated that some thymic lymphocytes did stain with anti-MHC class II reagents, however expression was heterogeneous and limited to thymocytes in contact with MHC class II+ thymic stromal cells (Nabarra et al., 1988). Farr (Farr et al., 1983) noted that 85% of thymocytes facing MHC class II+ stromal cells expressed MHC class II antigen on their membranes. Both frozen section and electron microscopy analysis by several authors indicated that MHC class II molecules are expressed in both thymic cortex and medulla, but none provided any explanation for the differential repertoire selection of mature CD4+ and CD8+ T cells that is observed, nor could they exclude the passive uptake of MHC class II antigen by thymocytes from MHC class II+ cells in the thymic milieu.

The role of class II MHC expression in the thymus and its influence on T cell maturation and differentiation remains to be clarified. Chimeric studies suggest, at least in the case of T cells recognising MHC class II, interaction with bone marrow derived radiosensitive IDC (interdigitating dendritic cell) in the thymic medulla is responsible for negative selection by clonal deletion (Von Boehmer et al., 1984; Lo and Sprent, 1986; Marrack et al., 1988B; Speiser et al., 1989; Roberts et al., 1990, reviewed Van Ewijk, 1991). This model is supported by histological analysis showing that autoreactive cells are found in the thymic cortex but not medulla and thus are deleted at the CMJ (cortico-medullary junction) (Hengartner et al., 1988). The precise location of these selection events has been investigated by mutagenesis of short conserved motifs (X and Y box) in the promoter region of the Eα gene, which influences Eα transcription in a cell-type specific fashion. Deletion of the X-box (ΔX), results in I-E expression in the medulla but not the cortex, while deletion of the Y-box (ΔY) produces the reverse pattern. Expression of I-E on
cortical TEC was essential for positive selection in T cell differentiation, and negative selection occurred in both ΔX and ΔY mice (Böhme et al, 1990). Although MHC expression in ΔX or ΔY box deletion mutants is biased, it is not complete and the precise location of negative selection is unclear.

Irradiation and cyclophosphamide result in the loss of bone marrow derived cells in the medulla thus affecting negative selection and consequently clonal deletion, implying cells such as IDC also are important in clonal deletion (Speiser et al, 1989; Roberts et al, 1990). As epithelial cells have been implicated in the developmental process leading to the generation of mature T cells, it is possible that aberrant arrangements of these cells may have a dramatic effect upon the efficiency of positive selection, which may in turn influence the generation of autoimmune phenomena.

Cytokeratin (CK) expression can be used to characterise cells of epithelial origin. CK expression is defined on the basis of molecular weight with a basic subfamily unit (CK1-8) pairing with an acidic unit (CK9-18). CK expression in the thymus is heterogeneous. Cortical TEC resemble 'simple' epithelia expressing CK8/18, while part of the medullary TEC correlates with more complex stratified epithelia using CK19/3/10 (reviewed Brekelmans and Ewijk, 1991). The entire thymic epithelium labels with polyclonal anti-keratin serum. TEC subsets might correspond to specific microenvironments which could be related to distinct functions affecting T cell maturation within the thymus. Savino (Savino et al, 1988) noticed no changes in expression of CK8/18 on cortical TEC, in normal (C57BL/6 or DBA/2) or autoimmune mice ([NZB X NZW]F1 or NOD) regardless of age. However medullary TEC subsets (CK19+3/10+) decreased in aging mice. This phenomenon occurs earlier in autoimmune mice such as the NOD (Nabarra and Andrianarison, 1991). They suggested that the thymic cortex in the mouse was derived from a different epithelial lineage than the medulla, and the CK8/18 primitive TEC subset would be the first to appear in thymic foetal development and thus probably committed to the initial steps in intra-thymic T cell differentiation.

Previous reports showed alterations in the ultrastructural morphology of the thymus in mice with genetic autoimmune diseases such as lupus (NZB, BXSB and MRL mice) (Nabarra et al, 1990) and in other strains of mice with autoimmune diabetes such as the
db/db mouse (Nabarra and Andrianarison, 1986) and the NOD mouse (Nabarra and Andrianarison, 1991). All of these mouse strains showed thymic lesions consisting of a patchwork of involuted and non-involuted zones and disorganisation of the thymic reticulum. Stromal cells without limiting membranes form a clear cytoplasmic layer where epithelial and non-epithelial cells no longer formed a distinguishable network containing thymocytes, but a large cytoplasmic layer containing cystic cavities. Such alterations appeared early in life in autoimmune prone mice (from 5 weeks) compared to 10 months in normal mice, giving the thymus an aspect of premature and permanent aging. In non-involuted areas, modifications of type 1 epithelial cells occurred, which sometimes contained crystal-like inclusions and an increase in the number of plasma cells and macrophages (Nabarra and Andrianarison, 1991). T cell dysfunction could appear secondary to a primary defect in bone marrow derived thymocyte precursor populations because of the important role of stromal cells in self recognition.

In this chapter immunofluorescent analysis of thymus cryostat sections from male and female NOD, NOD-E and CBA mice at 1, 3, 5 months of age permitted observation of all cell types within the thymus. Cells in situ could be studied without disruption of morphology including those that do not readily form suspensions such as epithelial cells. Such studies indicated that NOD thymi did indeed show evidence of premature aging. FACscan analysis of the same thymi also gave a quantitative picture of the cell populations present in these mouse strains.
9.2 RESULTS

Thymi from NOD, NOD-E, NOD-PRO, NOD-ASP and CBA mice were snap frozen and cryostat sections stained with the panel of antibodies described in Table 9.1 or prepared as single cell suspensions for FACscan analysis.

9.2.1 Immunohistological Analysis of CBA Thymus

Thymi from female CBA mice at 1, 3 and 5 months of age showed the typical MHC class II (as detected by OX6) dense staining pattern in the medulla, and enhanced staining on the fine spidery network of epithelial and IDC's throughout the cortex, (Figure 9.1A, B). Cortical expression of MHC class II was confined to the cortical epithelial cell network (4F1E4) with thin lines of positive staining separating small collections of lymphocytes which did not appear to stain with anti-class II MHC. B cells (B220+) were rare and scattered throughout the cortex and medulla. Macrophages detected by the markers MAC-1, F4/80 and SER-4 were observed in peri-vascular spaces and inter-lobular septa. Thymic epithelial cells demonstrated by the polyclonal anti-keratin antibody formed a dense network throughout the thymus both in the cortex (IVC4) and in the medulla (4F1C4) at 4 and 12 weeks Figure 9.1 C. At 5 months of age small holes in the confluent keratin staining pattern started to appear in some thymi. This observation was typical of the initial stages of thymic atrophy, and these small spaces in the epithelial layer did not contain either MHC class II positive cells or B cells (Table 9.2).

9.2.2 Immunohistochemical Analysis of NOD Thymus

Female NOD thymi demonstrated a confluent staining pattern with anti-class II MHC (OX6) both in the cortex and the medulla at 1, 3, and 5 months of age in male NOD mice. The class II MHC medullary staining pattern was both denser and the fluorescence intensity greater in NOD thymus compared to CBA (Table 9.2). Cortical staining for class II MHC was coincident with the cortical epithelial cell marker 4F1E4 (as for CBA thymus) but the intensity of staining was increased. Small lymphoid-like clusters of class II MHC positive cells were observed, (Figure 9.2 A, B, C). Hyperexpression of class II MHC observed in the NOD thymus could not be accounted for by an increase in macrophage number as the majority of these cells did not stain with the macrophage markers Mac-1, F4/80 and SER-4. They may have been dendritic cells (DC) as not all DC and Langerhans
cells express F4/80. F4/80 staining in young NOD mice demonstrated a similar
distribution to that observed in the CBA thymus although the number of positive cells
increased in older NOD mice.

Abnormally large perivascular “spaces” or “holes” in the thymic epithelium,
particularly surrounding vascular structures were detected with polyclonal anti-keratin, in
the thymus of 3-5 month old NOD mice and increased in size with age associated with a
concomitant loss of medullary epithelium, (Figure 9.2 D). These perivascular spaces
contained some class II positive cells and clusters of B cell (B220+) follicles which were
also surface Ig positive, although both the distribution and numbers of B cells did not fully
account for hyper-expression of class II MHC, (Figure, 9.2 E), B220+ cells were also
demonstrated at the corticomedullary junction.

9.2.3 Immunohistochemical Analysis of Transgenic NOD Thymus

Thymi of male and female NOD-E mice stained with anti-class II MHC showed
confluent staining in the cortex and medulla similar to those of 1, 3, 4 and 10 month old
female NOD mice and 3 and 5 month old male NOD mice, (Figure 9.3 A). A unique pattern
of MHC class II staining was demonstrated in the cortex compared to NOD and CBA. The
cortical class II MHC expression was more intense than the CBA staining on all epithelial
cells (but less so compared to the NOD) and the apparent non-epithelial class II positive
lymphoid structures (B220+/class II+, Ig−/class II+) such as thymocytes were not so readily
evident (Table 9.2). Expression of macrophage markers was similar to that of NOD mice.
B cells were scattered throughout the cortex and the medulla in younger animals but from 3
months MHC class II+ B cell clusters were evident at the corticomedullary junction and in
abnormally large holes in the thymic epithelium (Figure 9.2B). These were just as large and
numerous as those demonstrated in the NOD thymus at 5 months of age, (Figure 9.3 C).
Initial studies of 4 month old NOD-ASP females and 6 month old NOD-PRO male
suggested that these B cell clusters were also present in large holes in the thymic epithelial
layer, (Table 9.2). Thus modifications of the thymic micro-architecture such as abnormally
large perivascular spaces and the presence of class II+/B220+, B cell clusters occurs in
both diabetes prone (NOD) and protected (MHC transgenic) mice.
9.2.4 FACscan Analysis of Thymus Cell Populations.

Thymus cell suspensions from 4, 12 and 20 week, male and female CBA, NOD and NOD-E mice were stained for FACscan analysis. Table 9.3 shows the frequency of B cells (OX6+/B220+) expressed as a percentage of the total thymus cell population. The OX6+/B220+ thymic cell population showed a small increase in numbers in both male and female CBA mice from 0.01+/-0.009% in males and 0.01+/-0.00% in females at 4 weeks to 0.088+/-0.057% in males and 0.102+/-0.057% in females at 20 weeks. This population showed a greater increase in both male and female NOD mice from 1.71+/-1.76% in males and 1.155+/-1.35% in females at 12 weeks to 0.590+/-0.809% in males and 0.560+/-0.539 in females at 20 weeks of age. Thymi of NOD-E mice also showed an increase in the OX6+/B220+ thymic population from 0.02+/-0.002% in males and 0.03+/-0.03 in females at 4 weeks to 0.569+/-0.286% in males and 0.630+/-0.697 in females.

Mac-261 an additional specificity control for class II MHC staining, was compared to the staining of OX6. OX6 recognises a polymorphic mouse I-A determinant common to multiple mouse strains. Mac-261 is restricted to the recognition of I-Aα^d / I-\(Aβ^{nod}\) MHC class II molecules. Thymi from 5 month NOD, NOD-E and CBA mice were stained either with OX6 or Mac-261 and B220. The FACscan gates were set on NOD spleen cells stained with the same M.Ab. cocktail and these same parameters used to analyse the thymi. From table 9.4 it is obvious that quantitative expression of class II MHC / B220 in the thymus of NOD and NOD-E mice was similar with the NOD specific M.Ab. Mac-261 and OX6. Indeed the OX6/B220 staining would seem to be an underestimate of the B cells in NOD and NOD-E thymus. In general where large numbers of thymic B cells were detected in individual NOD/NOD-E mice with OX6, this was also reflected as a higher Mac-261 percentage. Mac-261 also acted as an excellent negative control as effectively no B cells (Mac-261^+/B220^+) were detected in CBA thymus.

No significant differences were observed in either the percentage of CD4^+, CD8^+ single positive T cells nor the percentage CD4^+/CD8^+ double positive T cells and thus no difference in the CD4/CD8 ratio in the NOD and NOD-E strains compared to CBA was recorded (Table 9.5). The immature CD4^+/CD8^+ thymocytes decreased in number in all
strains with a corresponding increase in the mature single positive CD8 T cells as a function of thymic age.

From the above observations it is clear that there is an age related increase in the OX6+/B220+ double positive population in the thymus of NOD and NOD-E mice compared to CBA (Figure 9.4- FACS profiles). These B cell populations present in the thymus of NOD and NOD-E mice are likely to correspond to the OX6+/B220+/Ig+ clusters observed by immunohistochemical analysis of thymus tissue sections of these mice. This population does not become apparent until 12 weeks of age and by 20 weeks of age there were 5 to 6 times as many OX6+/B220+ cells in NOD or NOD-E thymus compared to CBA mice of the same age. The appearance of such thymic B cell clusters was independent of sex.

9.2.5 FACscan Analysis of Thymic B Cells for CD5

In order to attribute some kind of function to the thymic B cells it was decided to assess their expression of CD5 (Ly-1) [clone 57-7.3]. The Ly-1 differentiation antigen is found at high densities on all murine T cells and also is expressed at low levels on a small population of B cells. Expression of this marker on B cells has been linked to production of autoantibodies by these cells and implicated in the initiation or perpetuation of autoimmune disease (Hardy and Hayakawa, 1986).

CD5+ B cells are more numerous in the peritoneal cavity, frequently comprising 20% of all, peritoneal exudate cells (PEC) and more than half of those B cells recoverable from the peritoneum. This population is present to a lesser extent in the spleen comprising 2% of the total splenocytes at 6 weeks of age. Female NOD PEC were used to characterise the FACS staining profile of CD5+ B cell population (Figure 9.5). FACscan gate settings used to analyse NOD PEC (Figure 9.5) were then used to determine the OX6+/B220+, CD5+ population in the spleen and subsequently to define the CD5 expressing B cells in the thymus.

Analysis of thymi from NOD, NOD-E and CBA mice at 5 months of age effectively demonstrated that thymic B cells in these mice do not express CD5 (FACS profiles Figures 9.6, 9.7, 9.8, Table 9.6). Expressed as a percentage of the total number of
thymocytes they are negligible, eg. a particular male NOD thymus had 4.55% of OX6+/B220+ cells also expressing CD5, but this percentage corresponds only to 4 positive events in 30,000 analysed and is therefore likely to be artifactual. CBA thymi effectively had no CD5+ B cells as expected.

To confirm the specificity of the OX6 label, and thereby the validity of the OX6 data, thymocyte suspensions were again stained with the positive control reagent, Mac-261 or with a control anti-MHC class II TIB 120. The thymic populations analysed in figure 9.6 were also triple labelled for Mac-261/B220/CD5 with the same result as with OX6/B220/CD5. The thymic populations were also analysed for the expression of MHC class II I-A\textsuperscript{b,d,q} and I-E\textsuperscript{d,k} using the M. Ab. TIB120 (clone M5/114.152) using the triple stain TIB120/B220/CD5. This label did not stain NOD as it does not express I-E, however C57BL/10 spleen cells and NOD-E mouse spleen and thymus did stain because the NOD-E mouse expressed the I-E\textsuperscript{d} chain.
Discussion

The generation of immunocompetent T cells requires the influence of an intact thymic microenvironment. It is now generally accepted that bone marrow derived stromal cells provide “education signals” which either support developing T cells to mature (Moller, 1988) or initiate preprogrammed cell death in the thymus (McDonald and Lees, 1990). T cells are selected during differentiation such that their heterodimeric T cell receptors recognise antigenic peptides in the context of self-MHC proteins. The contribution of stromal cells in this process is poorly understood. It seemed appropriate to undertake a morphological inspection of the thymic architecture of NOD and the transgenic NOD mice described in the previous chapter. Any apparent structural modifications could be assessed regarding their contribution to the prevention of IDDM in the NOD-E and NOD-PRO transgenic mice.

Analysis of NOD and NOD-E mouse thymi demonstrated signs of premature aging commencing around 12 weeks of age. In particular, disorganisation of thymic epithelial architecture was apparent, resulting in the formation of abnormally large perivascular spaces in the medulla and at the cortico-medullary junction. Hyperexpression of MHC class II was also evident in the NOD thymus and to a lesser extent NOD-E thymi, some of which was accounted for by the presence of OX6+/B220+ ‘B cells’ located within the perivascular spaces. These structural alterations were not observed in the non-autoimmune CBA strain thymus at any age.

During T cell differentiation, T cells are selected with TCRs biased toward MHC-associated recognition of foreign peptides (positive selection) and away from reactivity against self-peptides associated with MHC molecules (negative selection) (reviewed, Von Boehmer et al, 1989). In order to perform such a selection process, MHC antigens would have to be expressed in thymic microenvironments. Previously, several investigators (Rouse et al, 1979; Van Ewijk et al, 1990; Farr et al, 1983) have shown that class II MHC antigens are strongly expressed at the cell surface of cortical epithelial cells. The MHC pattern in the medulla is more confluent due to strong cytoplasmic staining of stromal cells. In the cortex, the staining is more restricted to a reticular network mainly composed of epithelial cells. Such a pattern of class II expression was observed in CBA mice at all ages analysed with MRC-OX6 (anti-I-A^k,s, NOD). However NOD thymi exhibited a peculiar
expression of I-A in which the staining in the medullary region was not only more confluent but the intensity of staining brighter. In the cortical region, the epithelia seemed to form a much tighter network such that thymocytes apparently were I-A positive. MHC proteins may be secreted by medullary stromal cells and picked up by surrounding thymocytes as Sharrow (Sharrow et al, 1981) has shown in the medulla occurred in chimeric mice.

The hyperexpression of class II MHC in the NOD thymus would have obvious implications for positive and negative selection as MHC-bearing radioresistant cells such as thymic epithelial cells and IDC have been implicated in these processes (Van Ewijk et al, 1988). The current view, largely based on experiments with bone marrow chimeric mice, is that the cortical epithelial network influences positive selection (Zinkernagel et al, 1978; Lo and Sprent, 1986; Marrack et al, 1989; Matzinger and Guerder, 1989; Speiser et al, 1989) whereas bone marrow derived cells regulate clonal deletion (Lo and Sprent, 1986; Schwartz, 1989; Speiser et al, 1989; Roberts et al, 1990). Negative selection may also be achieved by cortical epithelial cells inducing tolerance through clonal anergy (Roberts et al, 1990). Therefore in the NOD mouse, the positive and negative selection processes could be altered to such an extent that autoimmunity ensues. The significance of increased expression of MHC class II and its relationship to disease, if any, awaits further study. In this respect however the presence of insulitis in allophenic chimeras of the NOD/C57BL/6 strain combinations correlated with the presence of NOD H-2 on thymic cortical regions. Therefore the authors concluded that positive selection of T cells appeared to play a crucial role in the development of IDDM (Forsgren et al, 1991).

Probably of greater importance was the presence in NOD and NOD-E (and in a limited study NOD-ASP and NOD-PRO transgenic mice) of large perivascular spaces in the thymus. These holes which were not present at 4 weeks, were evident from 12 weeks and increased in size with age. They were predominantly in the medulla and at the cortico-medullary junction. These abnormal perivascular spaces were not detectable in the thymus of CBA mice even at 5 months of age. Similar structures, but on a smaller scale, have been described by Steinman (Steinman et al, 1986) in aging human thymi and by Benner et al (1981) in the mouse.

Detailed analysis of distinct cytokeratin-defined thymic epithelial cell (TEC) subsets with a panel of cytokeratin (CK) antibodies has shown that the numbers of
CK3/10^+ and CK19^+ (both medullary TEC) decrease in aging normal mice. However this phenomenon is more pronounced, and seems to occur earlier in, autoimmune mice such as [NZB X NZW]F1 and MRL/l (both of which develop a lupus-like syndrome) as well as both male and female NOD mice (Savino and Dardenne, 1988; Nabarra et al, 1990). A reduction in TEC subsets could result in the loss of specific thymic microenvironments and lead to impaired T cell selection. Savino (Savino et al, 1991) also noticed abnormally enlarged perivascular spaces in NOD thymi which increased in size with age, and contained T, and to a lesser extent, B cells. Focal ruptures of the basement membrane and rearrangement of the thymic epithelial cell network, which exhibited a rod-like shape, were also observed. Neither these structures nor the perivascular spaces were observed in NZB mice. My own histological studies and detailed flow cytometry analysis would suggest that the epithelial cell holes contained B cells (B220^- / sIg^- / class II MHC^+). Savino, however noticed no major alterations in the MHC class II bearing thymic cellular network. This discrepancy with our findings may be due to differences in the staining protocols. Savino used two different antibodies to stain NOD and control C57BL tissues whereas my studies employed a single antibody which detects both CBA and NOD I-A. The accumulation of lymphocytes in these spaces could be associated with a defect in intrathymic lymphocyte traffic, abnormal regulatory or effector T cells, and thus precipitate autoimmunity. In pathological situations it has not been elucidated whether the B cells enter the thymus from the blood or if the B cells increase in number by in situ expansion. Andreu Sanchez et al (1990) have suggested a progressive accumulation of recirculating B cells in the thymus of MLR^+/+ and MRL lpr/lpr mice. Alternatively, the above observations may reflect the premature aging of NOD and transgenic NOD-E thymus as they appear to have no bearing on disease susceptibility.

Both diabetes prone (DP) and diabetes resistant (DR) BB rats showed large areas which lacked class II MHC expression in both the cortex and medulla of their thymi from 4-5 weeks (Rozing et al, 1989). Epithelial cells were completely absent from these regions. The 'holes', in contrast to those demonstrated in the thymus of NOD mice, appeared not to be perivascular (PVS) as they are not surrounded by capsular-material. The thymocytes in the holes were almost exclusively of the CD4+CD8+ type, in contrast to the more mature
phenotypes of T cells observed in the PVS. DP BB rats have two major defects in their peripheral T cell system: The absence of a regulatory population of T cells (Greiner et al., 1986, 1987), and the presence of autoreactive effector cells (Koevary et al., 1985). The autoreactive population is the most likely candidate to be generated during inappropriate maturation in the ‘holes’ of a DP or DR thymus. In view of the current knowledge about clonal deletion of undesired autoreactive T cell clones in the thymus (MacDonald et al., 1988; Kisielow et al., 1988), one can easily imagine that the lack of particular thymic epithelia populations, as evidenced by the keratin ‘holes’ could mean a failure to negatively select, resulting in the escape of autoreactive cells in the NOD mouse, or the lack positive selection of regulatory cells to suppress or anergize autoreactive cells in the BB rat. Analysis of thymi from both the NOD mouse (whose holes contain B cells) and BB rat (whose holes contain T cells) could imply that such thymic anomalies are irrelevant to the subsequent development of disease as they are found in both disease-susceptible and protected substrains, although protection may be mediated by peripheral regulation.

The presence of large perivascular spaces in NOD and NOD-E thymi containing B cell clusters appeared not to have altered the total numbers of thymic CD4⁺, CD8⁺ mature single positive T cells subsets nor the immature CD4⁺CD8⁺ thymocyte phenotype. This is in marked contrast to the findings of Zipris (Zipris et al., 1990, 1991 A, B) who reported an altered (with respect to control BALB/cJ mice) thymic T-lymphocyte maturation process, manifest at diabetes onset as a 35% decrease of CD4⁺CD8⁺ and a reciprocal increase in CD4⁻CD8⁻ accompanied by a specific Vβ8⁺ loss of 3%. The authors suggested that a defect in the non-lymphoid thymic stromal cells caused the change in thymocyte maturation. In my own experience no phenotypic imbalance of CD4 or CD8 populations in comparison to CBA mice was observed.

Thymic B cells are a minor subset in normal and germfree mice (Benner et al., 1981), 0.2-1% of thymocytes are B cells, being present from peri-natal periods onwards. Of these, a high proportion are Ig-secreting and show no differences in VH repertoire expression when compared to their peripheral counterparts (Andreu-Sanchez et al., 1990).

There are two major roles that the B cells within the thymic perivascular spaces of NOD and NOD-E mice could perform: a) They may act as antigen presenting cells, or b)
they may produce antibody. There is some evidence that thymic B cells are capable of antigen presentation in addition to the class II expressing thymic epithelial cells, macrophages and dendritic cells. Mice treated with TNBS (trinitrobenezesulphonic acid) were effectively depleted of other class II MHC populations, but retained thymic B cells which enabled presentation of idiotypic structures (Zoller et al, 1991). Andreu-Sanchez (1990) related a physiological role for thymic B lymphocytes (TBL) in T cell ontogeny, especially as T cell hybridomas respond poorly to thymic cortical epithelium alone (Marrack et al, 1989) and T cell specificities could only be eliminated when confronted with MHC class II on B cells (Marrack et al, 1988B) but not other accessory cells. Furthermore, thymic but not spleen B cells deleted Mls-Ia reactive Vβ6+ T cells and induced tolerance when injected into neonatal mice across an Mls-1 antigenic barrier (Inaba et al, 1991). TBL are the best candidate to be involved in these phenomena as they form germinal centres at the cortico-medullary junction which subdivides unselected/selected populations of thymocytes (Hengartner et al, 1988). Many T cell selection events are dependent upon recognition of specific peptides (Abe and Hodes 1989) and since TBL express Mls antigens and T cells do not (Molina et al, 1989) and as TBL provide an excellent machinery for antigen processing and presentation they are likely to be involved in this process (Weiss et al, 1989).

Another plausible role for the TBL is the production of autoantibody, which may include ICSA, ICA and IAA all of which are predictive markers of IDDM (chapter 3). Such an expansion of thymic B cells may not be as a consequence of autoimmune disease but may result with the emergence of autoimmunity in both experimental and clinical situations. The majority of TBL present in the thymus of normal mice (Inaba, 1990) and humans (Issacson, 1987) are CD5+ (70-80% of sIgM bearing cells in the thymus) whereas these are a minor subpopulation (1-2%) in the spleen, and below detectability in lymph nodes or Peyer's patches. In some cases, increased numbers of CD5+ thymic B cells occur in the context of real thymic hypertrophy, eg in patients with the autoimmune diseases Myasthenia Gravis (MG) and SLE (MacKay, 1963; Fujii et al, 1983; Vincent et al, 1978; Williams et al, 1986). Similar findings have been reported in autoimmune prone mice such as (NZB X NZW)F1 and MRL/Mp-lpr/lpr (Mrl/lpr) (Ikehara et al, 1985) in which 60-70% of these TBL coexpressed CD5 (Inaba et al, 1988). These thymic B cells produced
autoantibodies in vitro with or without stimulants (Scadding et al, 1981; Fujii et al., 1984). Organ specific antibodies have been reported in MG (Patrick et al, 1973) and Rasedow's disease (Manley et al, 1974) which also displayed increased numbers of thymic B cells. Given the intense interest in CD5+ B cells, in particular those that reside in the thymus of autoimmune diseased patients and animals, it seemed logical to assess the CD5 phenotype of B cells in the thymus of NOD and NOD-E relative to control CBA mice. Thymic B cells from all strains did not express CD5 to any appreciable level. This finding correlates with that of Watanabe (Watanabe et al, 1991) where NOD and NOD-E TBL are CD5+, although these authors only used double labelling for B220 and CD5. This is potentially misleading because in my experience B220 labels a small percentage of CD4+CD8+ double positive thymocytes, hence the reason class II MHC was used as an additional definitive label. Watanabe has demonstrated that thymi of 12-16 week old NOD were hyperplastic with follicular B lymphocyte aggregation in the medulla and cortico-medullary junction. These B cells coexpressed sIgM and/or sIgD but not sIgG2a. Southern blot analysis of NOD thymus showed a distinct rearranged band at 3.8Kb in NOD thymus, which was not apparent in I- E expressing NOD when the extracted DNA was hybridized with a JH probe. It was concluded that a monoclonal expansion of thymic B cells occurred in NOD mice (Watanabe et al, 1991).

It would however be interesting to immortalise thymic B cells as hybridomas to assess if the antibody produced recognises NOD autoimmune targets eg. the pancreas and submandibular glands. Studies by Watanabe would suggest that NOD thymic B cells are capable of producing monoclonal antibody whereas NOD-E produce polyclonal antibody. Could this be the reason why NOD mice develop diabetes and NOD-E mice don't? (Watanabe et al, 1991).

Although NOD-E mice are protected from disease the thymi from these mice still display many abnormalities characteristic of the NOD, therefore, the bearing of these defects on disease pathology must come into question. T cell selection events taking place in the thymus may not be the only mechanism that prevents disease and events in the periphery may also be important. Further Serreze has shown that development of diabetogenic cells from NOD/Lt marrow is blocked when an allo-H-2 haplotype is expressed on cells of hemapoietic origin but not thymic epithelium (Serreze and Leiter,
1991). Parish (personal communication) has shown that chimeric NOD mice given a NOD-E thymus and NOD bone marrow still develop disease, suggesting that some kind of regulatory cell exists in the periphery such as a DC, and that the presence of a “protective NOD-E” thymus is not sufficient for disease prevention.
FIGURE 9.1: The Age Related Histology of The CBA Mouse Thymus.

One month old CBA thymus was stained for MHC class II with MRC-OX6 (green) and IVC4 (orange) which detects subcapsular and medullary epithelial cells (A) by indirect double immunofluorescence.

Figure 9.1 (B) depicts three month old CBA thymus stained with MRC-OX6 (B) and polyclonal anti-keratin which detects epithelial cells (C). Photomicrographs A and B show MHC class II expression is densely packed in the medulla and is also expressed on the epithelial cell network with the absence of peri-vascular 'holes' (C).

Magnification 200x.
Female NOD thymus at one month of age (A), and three months (B) stained by indirect immunofluorescence for MHC class II with MRC-OX6 (green) and IVC4 (orange) which detects subcapsular and medullary epithelial cells.

Figure 9.2 (C, D) depicts five month old NOD thymus stained with MRC-OX6 and anti-keratin which detects epithelial cells and further stained with B220 which detects B cells and anti-keratin (E).

These photomicrographs demonstrate hyperexpression of class II MHC in both the cortex and medulla and the presence of MHC class II positive lymphoid structures, additionally showing the presence of class II+ B cells within peri-vascular spaces (PVS). Magnification 200x.
FIGURE 9.3: The Age Related Histology of NOD-E Thymus

Female NOD-E thymus sections were stained for MHC class II with MRC-OX6 (green) and IVC4 (orange) which detects subcapular and medullary epithelial cells at one month of age (A), and at 5 months stained with MRC-OX6 and anti-keratin (orange) which detects the whole epithelial cell network (B).

Figure 9.3 (C) depicts five month old NOD-E thymus stained with B220 which detects B cells (green) and anti-keratin (orange) showing expression of MHC class II in both the cortex and the medulla and the presence of MHC class II⁺ B cells in the peri-vascular spaces (PVS).

Magnification 200x.
Fresh NOD spleen cell suspensions were stained with B220 FITC (FL-1) and biotinylated OX6 detected with avidin phycoerythrin (FL-2). The FACscan gates were set on the OX6+ /B220+ double positive population, therefore defining the 'B cells' as indicated by region R1 in the figure. These same parameters were used to analyse NOD, NOD-E and CBA thymic cell suspensions stained by the same protocol. The figure shows the typical FACscan profile of thymic cell suspensions obtained from individual 5 month old female NOD, NOD-E and CBA mice. The percentages indicate the frequency of the OX6+ /B220+ population in each thymus from the 20,000 events recorded. It is clear that NOD and NOD-E thymi have significant numbers of thymic B cells whereas age matched CBA mice, effectively have none or very few.
70% Log

NOD SPLEEN B220/OX6

R1

Region Stats —
File: 1:LOR2901001 Sam
Date: 1/29/92 Ungate
Parameters: FL1-H (LOG)
Total= 10000 Gated:
Rgn Events % Gated
1 R1 2866 28.66

Region Stats —
File: 1:LOR2901006 Sam
Date: 1/29/92 Ungate
Parameters: FL1-H (LOG)
Total= 20000 Gated
Rgn Events % Gated
1 R1 498 2.49

NOD-E THYMUS B220/OX6

CBA THYMUS B220/OX6

Region Stats —
File: 1:LOR2901014 Sam
Date: 1/29/92 Ungate
Parameters: FL1-H (LOG)
Total= 20000 Gated
Rgn Events % Gated
1 R1 186 0.93

Region Stats —
File: 1:LOR2901043 Sam
Date: 1/29/92 Ungate
Parameters: FL1-H (LOG)
Total= 20000 Gated
Rgn Events % Gated
1 R1 5 0.03

247
In order to determine whether B cells (B220+/OX6+) present in NOD and NOD-E thymus express CD5, it was important to define the normal expression of this marker as typically the B cell numbers in the thymus are at a low frequency. As CD5+ B cells are more numerous in the peritoneal cavity (usually 10-20% of total) and the spleen (2% of the total splenocytes at 6 weeks of age) these populations were used to define the CD5+ B cell population in the NOD mouse. NOD PEC and spleen cell suspensions were stained with the triple label cocktail B220 FITC (FL-1), CD5 P.E (FL-2), bio. OX6 (streptavidin R613) (FL-3). FACscan gates were set on the B220+(region 3)/OX6+ (region 2) PEC population and the percentage of this population from 30,000 events which expressed CD5 (region 1-R1 on figure) recorded. Typically for NOD PEC this was 7.8% (indicated by red dots). These same parameters were then applied to NOD spleen cell suspensions, typically 1.09% of the B220+/OX6+ population also expressed CD5 (R1). Having defined the FACscan gates for expression of CD5 on B cells, thymi from NOD, NOD-E and CBA mice were subsequently analysed in the following figures using the same parameters.
**FIGURE 9.6** FACscan profiles of 5 month NOD female Thymus Cell Suspensions Stained With OX6/B220/CD5

Fresh NOD PEC were stained with the triple label cocktail B220 FITC (FL-1), CD5 P. E. (FL-2) and biotinylated OX6 detected with streptavidin R613 (FL-3). FACscan gates were set on the B220⁺/OX6⁺ B cell population and the percentage of this population expressing CD5 recorded (shown as red dots in the figure, as a positive staining reference). Thymi from 5 month old female NOD mice were stained with the same protocol and analysed by the same FACscan parameters. The percentage of OX6⁺/B220⁺ B cells expressing CD5 from 30,000 events was recorded and expressed as red dots on the FACS profile, CD5 cells are depicted as green dots and MHC class II positive cells not bearing the CD5 marker indicated by purple dots. The FACscan profiles demonstrate that although significant numbers of B cells were present in the thymus of NOD mice (purple dots) few of these expressed CD5 (red dots), the majority of CD5 expression was on the class II⁺/B220⁻ population ie. T cells (green dots).
FIGURE 9.7 FACscan Profiles of 5 Month old NOD-E Female Thymus Cell Suspensions Stained With the Triple Label Cocktail OX6/B220/CD5.

Fresh NOD PEC were stained with the triple label cocktail B220 FITC (FL-1), CD5 P. E. (FL-2) and biotinylated OX6 detected with streptavidin R613 (FL-3). FACscan gates were set on the B220⁺/OX6⁺ B cell population and the percentage of this population expressing CD5 recorded (shown as red dots in the figure, as a positive staining reference). Thymi from 5 month old female NOD-E mice were stained with the same protocol and analysed by the same FACscan parameters. The percentage of OX6⁺/B220⁺ B cells expressing CD5 from 30,000 events was recorded and expressed as red dots on the FACS profile, CD5 cells are depicted as green dots and MHC class II positive cells not bearing the CD5 marker indicated by purple dots. The FACscan profiles demonstrate that although significant numbers of B cells were present in the thymus of NOD-E mice (purple dots) few of these expressed CD5 (red dots), the majority of CD5 expression was on the class II⁺/B220⁻ population ie. T cells (green dots).
FIGURE 9.8 FACscan Profiles of 5 Month CBA Female Thymus Cell Suspensions Stained With OX6/B220/CD5

Fresh NOD PEC were stained with the triple label cocktail B220 FITC (FL-1), CD5 P. E. (FL-2) and biotinylated OX6 detected with streptavidin R613 (FL-3). FACscan gates were set on the B220⁺/OX6⁺ B cell population and the percentage of this population expressing CD5 recorded (shown as red dots in the figure, as a positive staining reference). Thymi from 5 month old female CBA mice were stained with the same protocol and analysed by the same FACscan parameters. The percentage of OX6⁺/B220⁺ B cells expressing CD5 from 30,000 events was recorded and expressed as red dots on the FACS profile, CD5 cells are depicted as green dots and MHC class II positive cells not bearing the CD5 marker indicated by purple dots. The FACscan profiles demonstrate few B cells were present in the thymus of CBA mice (purple dots) negligible numbers of these expressed CD5 (red dots), the majority of CD5 expression was on the class II⁺/B220⁻ population ie. T cells (green dots).
Table 9.1 Panel of Antibodies used for Thymic Immunohistology

<table>
<thead>
<tr>
<th>Name</th>
<th>Molecular Specificity</th>
<th>Ig Class Species</th>
<th>Cells Recognised</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y17</td>
<td>I-E$_{\text{b}}$</td>
<td>mouse IgG2b</td>
<td>All I-E$_{\text{b}}$ bearing cells</td>
<td>Lerner et al, 1980</td>
</tr>
<tr>
<td>H81-20-8-22-6</td>
<td>I-E$_{\text{k}}$</td>
<td>mouse IgG2a</td>
<td>All I-E$_{\text{k}}$ bearing cells</td>
<td>Mineta et al, 1990</td>
</tr>
<tr>
<td>CD5 (clone 53-7.3)</td>
<td>Ly-1 antigen</td>
<td>rat IgG2a</td>
<td>Mouse T cells + subpopulation B cells</td>
<td>Devaux et al, 1984</td>
</tr>
<tr>
<td>B220 (clone RA3-6B2)</td>
<td>B cell restricted</td>
<td>rat IgG2a</td>
<td>Mouse B cells</td>
<td>Hardy, Hayakawa, 1986</td>
</tr>
<tr>
<td>Anti-keratin</td>
<td>predominantly MW 56KD and 64KD</td>
<td>rabbit anti-human polyclonal</td>
<td>whole thymic epithelial cell network</td>
<td>Holmes and Morse, 1988,</td>
</tr>
<tr>
<td>IVC4</td>
<td>CTES II</td>
<td>rat IgM</td>
<td>subcapsular and medullary epithelial cells</td>
<td>Coffman and Weissman, 1981</td>
</tr>
<tr>
<td>4F1E4</td>
<td>CTES XX</td>
<td>rat IgM</td>
<td>Subcapsular, cortical, subset of medullary epithelial cells</td>
<td>Kanariou et al, 1989</td>
</tr>
<tr>
<td>Anti-mouse Ig</td>
<td>Mouse Ig</td>
<td>rabbit polyclonal</td>
<td>All Ig bearing cell</td>
<td>Kanariou et al, 1989</td>
</tr>
<tr>
<td>MRC-OX-6</td>
<td>Class II MHC I-A$_{\text{k,s,nod}}$</td>
<td>Mouse IgG1</td>
<td>All MHC class II bearing cells</td>
<td>Dako</td>
</tr>
</tbody>
</table>

CTES-clusters of thymic epithelial staining patterns.
II: M. Ab. stains subcapsular and perivascular TEC (only one cell layer), in addition medullary TEC and Hassall’s corpuscles (HC) are stained.
XX: Miscellaneous designation of staining pattern, eg. majority of subcapsular/ cortical TEC and minority of medullary TEC.
Table 9.2 Immunohistochemical Analysis of CBA, NOD and Transgenic NOD-E, NOD-ASP, NOD-PRO Thymus.

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Age (No.)</th>
<th>MHC class II Expression</th>
<th>Keratin Expression</th>
<th>Presence of B cell Clusters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Months</td>
<td>Cortex</td>
<td>Medulla</td>
<td></td>
</tr>
<tr>
<td>CBA</td>
<td>1 (8)</td>
<td>spidery epithelial network positive</td>
<td>confluent staining on epithelial cells</td>
<td>confluent</td>
</tr>
<tr>
<td>CBA</td>
<td>3 (9)</td>
<td>spidery epithelial network positive</td>
<td>confluent staining on epithelial cells</td>
<td>confluent</td>
</tr>
<tr>
<td>CBA</td>
<td>4 (6)</td>
<td>spidery epithelial network positive</td>
<td>confluent staining on epithelial cells</td>
<td>small epithelial holes class II -ve</td>
</tr>
<tr>
<td>CBA</td>
<td>5 (5)</td>
<td>spidery epithelial network positive</td>
<td>confluent staining on epithelial cells</td>
<td>small epithelial holes class II -ve</td>
</tr>
<tr>
<td>NOD</td>
<td>1 (8)</td>
<td>strong dense staining over whole cortex + cell clusters</td>
<td>strong dense confluent staining on epithelial + cell clusters</td>
<td>confluent</td>
</tr>
<tr>
<td>NOD</td>
<td>3 (11)</td>
<td>strong dense staining over whole cortex + cell clusters</td>
<td>strong dense confluent staining +cell clusters</td>
<td>disorganisation + holes in epithelial matrix</td>
</tr>
<tr>
<td>NOD</td>
<td>5 (15)</td>
<td>disorganised strong staining</td>
<td>Strong dense staining on remaining epithelia + cell clusters</td>
<td>epithelial disorganisation + large peri-vascular spaces</td>
</tr>
<tr>
<td>NOD-E</td>
<td>1 (10)</td>
<td>dense staining of spidery network</td>
<td>strong dense confluent staining</td>
<td>disorganisation of epithelial + peri-vascular spaces</td>
</tr>
<tr>
<td>NOD-E</td>
<td>3 (13)</td>
<td>dense staining of spidery network</td>
<td>strong dense confluent staining</td>
<td>confluent</td>
</tr>
<tr>
<td>NOD-E</td>
<td>4 (5)</td>
<td>dense staining but epithelial cells disorganised</td>
<td>strong staining on remaining epithelial + cell clusters</td>
<td>disorganisation of epithelial + peri-vascular spaces</td>
</tr>
<tr>
<td>NOD-E</td>
<td>5 (18)</td>
<td>dense staining but epithelial cells disorganised</td>
<td>strong staining on remaining epithelial + cell clusters</td>
<td>large peri-vascular spaces + epithelial disorganisation</td>
</tr>
<tr>
<td>NOD-ASP</td>
<td>4 (4)</td>
<td>spidery epithelial cell network positive</td>
<td>dense strong staining of epithelial cells</td>
<td>large peri-vascular spaces + epithelial disorganisation</td>
</tr>
<tr>
<td>NOD-ASP</td>
<td>5 (3)</td>
<td>spidery epithelial cell network positive but disorganised</td>
<td>dense strong staining of epithelial cells but disorganised</td>
<td>large peri-vascular spaces + epithelial disorganisation</td>
</tr>
<tr>
<td>NOD-PRO</td>
<td>4 (5)</td>
<td>spidery network positive</td>
<td>dense confluent staining on epithelial cells</td>
<td>a few small peri-vascular spaces</td>
</tr>
<tr>
<td>NOD-PRO</td>
<td>6 (8)</td>
<td>spidery network of epithelial cells positive</td>
<td>epithelial disorganisation but strong staining</td>
<td>large peri-vascular spaces</td>
</tr>
</tbody>
</table>

Thymus cryostat sections were cut and stained by indirect immunofluorescence for the presence of keratin which detects the whole epithelial cell network and either MHC class II with OX 6 FITC or B cells detected with B220 FITC as described in Materials and Methods. From the above table it is clear that NOD and NOD-E transgenic mice hyperexpress MHC class II in the thymus. In addition NOD and transgenic NOD mouse thymus develop perivascular spaces in the epithelial cell matrix commencing around 3 months of age, which contain the major portion of the OX6+/B220+ B cells. These thymic B cell follicles increase in size as do the number of the peri-vascular spaces. Such thymic abnormalities were not observed in CBA thymus.

B cell clusters: - normal B cell expression in the thymus ie. a few scattered B cells.
+/- A few small B cell clusters (< 10 +ve cells).
+ Many small B cell clusters
++ Many large B cell clusters present in peri-vascular spaces (>50 +ve cells, frequently >100 +ve cells in each cluster).
### TABLE 9.3 Percentage of OX6+/B220+ Cells in Thymus of NOD, NOD-E and CBA Mice.

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Age (weeks)</th>
<th>No. Analysed</th>
<th>Sex</th>
<th>OX6+/B220+ (%</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBA</td>
<td>4</td>
<td>6</td>
<td>M</td>
<td>0.01+/-0.009</td>
<td>0.01-0.02</td>
</tr>
<tr>
<td>CBA</td>
<td>4</td>
<td>6</td>
<td>F</td>
<td>0.01+/-0.000</td>
<td>0.01-0.01</td>
</tr>
<tr>
<td>NOD</td>
<td>4</td>
<td>5</td>
<td>M</td>
<td>0.01+/-0.007</td>
<td>0.01-0.02</td>
</tr>
<tr>
<td>NOD</td>
<td>4</td>
<td>5</td>
<td>F</td>
<td>0.02+/-0.002</td>
<td>0.00-0.06</td>
</tr>
<tr>
<td>NOD-E</td>
<td>4</td>
<td>5</td>
<td>M</td>
<td>0.02+/-0.002</td>
<td>0.00-0.06</td>
</tr>
<tr>
<td>NOD-E</td>
<td>4</td>
<td>5</td>
<td>F</td>
<td>0.03+/-0.03</td>
<td>0.01-0.08</td>
</tr>
<tr>
<td>CBA</td>
<td>12</td>
<td>8</td>
<td>M</td>
<td>0.115+/-0.101</td>
<td>0.01-0.32</td>
</tr>
<tr>
<td>CBA</td>
<td>12</td>
<td>10</td>
<td>F</td>
<td>0.04+/-0.03</td>
<td>0.01-0.10</td>
</tr>
<tr>
<td>NOD</td>
<td>12</td>
<td>8</td>
<td>M</td>
<td>1.71+/-1.76</td>
<td>0.04-4.88</td>
</tr>
<tr>
<td>NOD</td>
<td>12</td>
<td>10</td>
<td>F</td>
<td>1.155+/-1.35</td>
<td>0.03-3.65</td>
</tr>
<tr>
<td>NOD-E</td>
<td>12</td>
<td>10</td>
<td>M</td>
<td>0.204+/-0.088</td>
<td>0.07-0.29</td>
</tr>
<tr>
<td>NOD-E</td>
<td>12</td>
<td>10</td>
<td>F</td>
<td>0.160+/-0.176</td>
<td>0.03-0.57</td>
</tr>
<tr>
<td>CBA</td>
<td>20</td>
<td>5</td>
<td>M</td>
<td>0.088+/-0.057</td>
<td>0.06-0.19</td>
</tr>
<tr>
<td>CBA</td>
<td>20</td>
<td>5</td>
<td>F</td>
<td>0.102+/-0.057</td>
<td>0.03-0.18</td>
</tr>
<tr>
<td>NOD</td>
<td>20-22</td>
<td>14</td>
<td>M</td>
<td>0.590+/-0.809</td>
<td>0.12-2.50</td>
</tr>
<tr>
<td>NOD</td>
<td>17-22</td>
<td>14</td>
<td>F</td>
<td>0.560+/-0.539</td>
<td>0.08-1.88</td>
</tr>
<tr>
<td>NOD-E</td>
<td>20-23</td>
<td>10</td>
<td>M</td>
<td>0.569+/-0.286</td>
<td>0.36-0.96</td>
</tr>
<tr>
<td>NOD-E</td>
<td>16-20</td>
<td>10</td>
<td>F</td>
<td>0.630+/-0.697</td>
<td>0.07-2.32</td>
</tr>
</tbody>
</table>

Fresh thymus was digested in 0.5mg/ml collagenase and 0.02mg/ml DNase for several minutes then washed in BSS and frozen in 90%FCS/10%DMSO at -70°C. For analysis vials were rapidly thawed, washed, resuspended and stained with biotinylated OX6 in 1% NMS for 30 minutes followed by B220 FITC and Avidin PE (1/80) pooled as the second layer. All samples were then analysed by FACscan (Becton Dickenson). The FACscan gates were set on the CD4-CD8-OX6+B220+ fresh NOD spleen cell population and these same parameters used to analyse thymus cells for expression of class II MHC (OX6) and B cells (B220). The percentage of the total thymocyte population that was positive were recorded from 20,000 events.

M-Male, F-Female
Table 9.4 Percentage of OX6+/B220+ and Mac 261+/B220+ "B cells" Present in The Thymus of 5 Month Old NOD, NOD-E and CBA Mice.

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Sex</th>
<th>No. Mice</th>
<th>OX6+/B220+ (SD)</th>
<th>Mac 261+/B220+ (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD</td>
<td>M</td>
<td>5</td>
<td>1.178 ± 0.980</td>
<td>2.017 ± 1.917</td>
</tr>
<tr>
<td>NOD</td>
<td>F</td>
<td>4</td>
<td>0.845 ± 0.370</td>
<td>0.918 ± 0.540</td>
</tr>
<tr>
<td>NOD-E</td>
<td>M</td>
<td>5</td>
<td>0.548 ± 0.425</td>
<td>1.17 ± 0.633</td>
</tr>
<tr>
<td>NOD-E</td>
<td>F</td>
<td>5</td>
<td>1.377 ± 0.790</td>
<td>1.810 ± 1.039</td>
</tr>
<tr>
<td>CBA</td>
<td>M</td>
<td>4</td>
<td>0.185 ± 0.105</td>
<td>0.008 ± 0.005</td>
</tr>
<tr>
<td>CBA</td>
<td>F</td>
<td>4</td>
<td>0.137 ± 0.122</td>
<td>0.005 ± 0.006</td>
</tr>
<tr>
<td>NOD Spleen</td>
<td>F</td>
<td>1</td>
<td>22.75</td>
<td>21.58</td>
</tr>
</tbody>
</table>

Fresh thymus was digested in 0.5mg/ml collagenase and 0.02mg/ml DNase for several minutes then washed in BSS and frozen in 90% FCS/10% DMSO at -70 C. For analysis vials were rapidly thawed, washed and stained with biotinylated OX6 (1ug/ml) or Mac261 (5ug/ml) in 1% NMS for 30 minutes followed by B220 FITC (1/40) and Avidin PE (1/80) pooled as a second layer. A freshly prepared single cell suspension of NOD spleen cells was also stained according this protocol. The spleen cell sample was used to set the FACscan gates on the OX6+/B220+ or Mac261+/B220+ cell populations and these same parameters used to analyse thymus cells for expression of class II MHC, OX6 (I-A NOD,s,k) or Mac261 (I-A NOD) and B cells (B220) and the percentage of the total thymocyte population that were positive recorded. From the above table it is clear that there was no difference in the percentage of B cells for each mouse strain using the two anti-class II M.Abs.
M-Male
F-Female.
**TABLE 9.5**
The Percentage of CD4+ and CD8+ Thymocytes in NOD, NOD-E and CBA Mice.

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Age (months)</th>
<th>Sex</th>
<th>No. Mice</th>
<th>CD8+ (SD)</th>
<th>CD4+ (SD)</th>
<th>CD4+CD8+ (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBA</td>
<td>1</td>
<td>M</td>
<td>6</td>
<td>1.90+/-0.31</td>
<td>7.70+/-0.93</td>
<td>88.14+/-1.77</td>
</tr>
<tr>
<td>CBA</td>
<td>1</td>
<td>F</td>
<td>6</td>
<td>2.18+/-0.39</td>
<td>8.62+/-1.40</td>
<td>86.52+/-1.53</td>
</tr>
<tr>
<td>NOD</td>
<td>1</td>
<td>M</td>
<td>5</td>
<td>1.68+/-0.46</td>
<td>7.19+/-0.53</td>
<td>89.08+/-1.46</td>
</tr>
<tr>
<td>NOD</td>
<td>1</td>
<td>F</td>
<td>5</td>
<td>1.27+/-0.95</td>
<td>6.16+/-2.51</td>
<td>90.89+/-3.98</td>
</tr>
<tr>
<td>NOD-E</td>
<td>1</td>
<td>M</td>
<td>5</td>
<td>1.77+/-0.30</td>
<td>8.75+/-0.73</td>
<td>86.76+/-1.68</td>
</tr>
<tr>
<td>NOD-E</td>
<td>1</td>
<td>F</td>
<td>5</td>
<td>2.02+/-0.60</td>
<td>9.66+/-1.85</td>
<td>84.52+/-2.15</td>
</tr>
<tr>
<td>CBA</td>
<td>3</td>
<td>M</td>
<td>4</td>
<td>2.02+/-0.64</td>
<td>9.19+/-2.31</td>
<td>86.08+/-2.13</td>
</tr>
<tr>
<td>CBA</td>
<td>3</td>
<td>F</td>
<td>4</td>
<td>1.84+/-0.28</td>
<td>8.30+/-1.13</td>
<td>86.72+/-1.37</td>
</tr>
<tr>
<td>NOD</td>
<td>3</td>
<td>M</td>
<td>5</td>
<td>1.52+/-0.28</td>
<td>6.08+/-0.99</td>
<td>89.23+/-1.72</td>
</tr>
<tr>
<td>NOD</td>
<td>3</td>
<td>F</td>
<td>4</td>
<td>3.08+/-1.11</td>
<td>11.23+/-3.26</td>
<td>82.37+/-4.47</td>
</tr>
<tr>
<td>NOD-E</td>
<td>3</td>
<td>M</td>
<td>5</td>
<td>1.86+/-0.51</td>
<td>9.41+/-1.84</td>
<td>85.65+/-2.35</td>
</tr>
<tr>
<td>NOD-E</td>
<td>3</td>
<td>F</td>
<td>5</td>
<td>1.62+/-0.36</td>
<td>9.15+/-1.88</td>
<td>85.37+/-2.47</td>
</tr>
<tr>
<td>CBA</td>
<td>5</td>
<td>M</td>
<td>5</td>
<td>14.18+/-1.32</td>
<td>3.53+/-0.24</td>
<td>76.35+/-0.78</td>
</tr>
<tr>
<td>CBA</td>
<td>5</td>
<td>F</td>
<td>5</td>
<td>18.88+/-4.20</td>
<td>4.66+/-1.28</td>
<td>70.19+/-6.40</td>
</tr>
<tr>
<td>NOD</td>
<td>5</td>
<td>M</td>
<td>10</td>
<td>13.77+/-3.20</td>
<td>4.34+/-1.68</td>
<td>78.90+/-6.41</td>
</tr>
<tr>
<td>NOD</td>
<td>5</td>
<td>F</td>
<td>11</td>
<td>16.42+/-7.83</td>
<td>3.86+/-1.99</td>
<td>67.84+/-22.20</td>
</tr>
<tr>
<td>NOD-E</td>
<td>5</td>
<td>M</td>
<td>10</td>
<td>16.20+/-6.11</td>
<td>3.57+/-0.82</td>
<td>74.66+/-3.44</td>
</tr>
<tr>
<td>NOD-E</td>
<td>5</td>
<td>F</td>
<td>10</td>
<td>19.06+/-7.06</td>
<td>4.11+/-2.22</td>
<td>65.04+/-2.33</td>
</tr>
</tbody>
</table>

Fresh thymus was digested in 0.5mg/ml collagenase and 0.2mg/ml DNase for several minutes then washed in BSS and frozen in 90% FCS/10% DMSO at -70°C. For analysis vials were rapidly thawed, washed, resuspended and stained with biotinylated OX6 (1 ug/ml) in 1% normal NMS followed by the triple label cocktail anti-CD4 P.E. (1/40), anti-CD8 FITC (1/40) and streptavidin R613 (1/100) which binds to the biotinylated OX6. All samples were then analysed by FACscan. The FACscan gates were set on either the CD4 single positive or CD8 single positive thymocytes (as confirmed by gating on splenic lymphocytes) or on the CD4+/CD8+ double thymocyte population. The percentage of each population was based on 20,000 events recorded. M- Male, F- Female.
Table 9.6 Percentage of OX6+/ B220+/ B cells' in The Thymus of NOD, NOD-E and CBA Mice That Express CD5.

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Sex</th>
<th>No. Mice</th>
<th>% B cells (OX6+/B220+) expressing CD5 (SD)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD</td>
<td>M</td>
<td>5</td>
<td>3.256 ± 1.643</td>
<td>1.05-5.15</td>
</tr>
<tr>
<td>NOD</td>
<td>F</td>
<td>5</td>
<td>1.554 ± 0.642</td>
<td>0.5-2.08</td>
</tr>
<tr>
<td>NOD-E</td>
<td>M</td>
<td>4</td>
<td>2.018 ± 0.288</td>
<td>1.69-2.38</td>
</tr>
<tr>
<td>NOD-E</td>
<td>F</td>
<td>5</td>
<td>2.316 ± 1.290</td>
<td>0.59-3.82</td>
</tr>
<tr>
<td>CBA</td>
<td>M</td>
<td>4</td>
<td>2.110 ± 2.623</td>
<td>0.00-5.41</td>
</tr>
<tr>
<td>CBA</td>
<td>F</td>
<td>6</td>
<td>3.320 ± 5.024</td>
<td>0.00-13.33</td>
</tr>
<tr>
<td>NOD Spleen</td>
<td>F</td>
<td>1</td>
<td>1.09</td>
<td></td>
</tr>
<tr>
<td>NOD PECs</td>
<td>F</td>
<td>1</td>
<td>7.8</td>
<td></td>
</tr>
</tbody>
</table>

Fresh thymus was digested in 0.5mg/ml collagenase / 0.02mg/ml DNase for several minutes then washed in BSS and frozen in 90% FCS/10% DMSO at -70°C. For analysis vials were rapidly thawed, washed, resuspended and stained with biotinylated OX6 (1ug/ml) in 1% NMS followed by the triple label cocktail streptavidin R613 (1/100), B220 (FITC) and CD5 P.E. (1/200) pooled as the second layer. Fresh NOD PEC and spleen cell suspensions were also stained according to the same protocol. All samples were then analysed by FACscan.

The FACscan gates were set on the NOD PEC B220+/OX6+ population and the % of this population that co-expressed CD5 from 30,000 events was recorded. Having defined the CD5+ B cell population with NOD PECs these same parameters were used to analyse thymus cells for expression of class II MHC (OX6), B cells (B220+) and CD5 and the percentage of B-cells expressing CD5 recorded.

M- Male.
F- Female.
CHAPTER 10

Concluding Remarks and Future Prospects
Human type 1 insulin dependent diabetes mellitus (IDDM) is a progressive autoimmune disease (Tarn et al, 1987) which often does not present clinical symptoms until the β cells of the pancreas are completely destroyed. At this point patients face a lifetime of dependency on insulin and the subsequent microvascular complications which accompany the disease.

The NOD mouse is considered to be a good animal model of type 1 diabetes displaying many similarities to the human disease, such as insulin insufficiency and pancreatic insulitis. Two accelerated models of the disease in the NOD were utilised in this study: a) diabetic spleen cell transfer, and b) cyclophosphamide induced diabetes.

T cells are essential for disease manifestation as neonatal thymectomy prevents IDDM in the NOD mouse (Ogawa et al, 1985) and my own studies have indicated that the diabetic spleen cell depletion of either the CD4 or CD8 subpopulation even two weeks after transfer can give partial protection from the massive inflammatory response and subsequent decline to diabetes (Hutchings et al, 1990A, Varey et al, 1991). An ideal therapy may be one with which it is possible to reestablish self tolerance to the autoantigen using a short course of therapy with a non-lytic antibody recognising CD4^+ T cells. Treatment of transfer recipients at day 12 after transfer when the autoaggressive response was well underway with such an antibody, (YTH 177.1) tolerized CD4^+ T cells which recognise the as yet unidentified diabetogenic antigens and afforded complete protection (Hutchings et al, in press). Similarly, induction of thyroiditis in situ by the transfer of primed lymph nodes or spleen, can be abrogated by this treatment regime (Dawe and Hutchings, unpublished observations). Such therapeutic regimes could therefore offer a safe approach with the minimal disruptive interruption as memory responses to exogenous antigens appear to be obtained.

The previous chapters have clearly demonstrated that the mononuclear infiltration of the pancreatic islets in type I insulin dependent diabetes in the NOD mouse is heterogeneous with respect to TCR Vβ usage of both CD4^+ and CD8^+ T cells (O’ Reilly et al, 1990; O’ Reilly et al, 1991). This suggests that there are multiple antigenic epitopes on the β cell capable of stimulating autoreactive T cells with multiple specificities or that there is rapid nonspecific recruitment once an inflammatory response is underway. Therefore specific immunotherapy targeting only those T cells responsive to the diabetogenic autoantigen, may not be a realistic alternative for this particular autoimmune disease.

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Given the massive inflammatory response present in the pancreas, it is not surprising that there exists evidence showing that vascular leakage is apparent in the pancreas of the diabetes prone BB rat and initial evidence from my own studies to suggest that this phenomenon also occurs in the NOD mouse. Theoretically if such vascular leakage occurs in the human situation, then this raises the possibility that it could be utilised as a predictive marker of disease. Nuclear magnetic resonance (NMR) which is capable of detecting such small changes in fluid flow could therefore be used as a means of detection.

The role of the macrophage in IDDM awaits clarification, the studies described in this thesis suggest that like the BB rat (Walker et al, 1988A), the NOD mouse has two populations of macrophages within prediabetic pancreata: A recently recruited and actively phagocytic population which bears the macrophage markers F4/80 and Mac-1, and a resident population with the phenotype Mac-1/F4/80'/SER-4+ (O'Reilly et al, 1991). It is clear that macrophages are essential for disease manifestation in the transfer model as prevention of their adhesion to and trafficking through the vascular endothelium by in vivo use of 5C6 (a M.Ab. which binds to the myelomonocytic receptor CR3 on macrophages) prevents IDDM. The question "what function do macrophages perform?" still remains. Do they: a) have an antigen presenting capacity presenting the diabetogenic autoantigen?, b) play a role in producing the cytokines IL-1 and TNF-α which are cytotoxic to the β cell, or c) mediate β cell damage through nitric oxide and superoxide radicals and their metabolites? Several approaches could be made to this problem. In situ hybridisation of prediabetic pancreata to assess the level of cytokine expression, or elimination of macrophages with toxic liposomes which suppresses EAE in Lewis rats (Huitinga et al, 1990) are options. The approach I used was to assess the contribution of the nitric oxide generation in IDDM by blockade of the L-arginine pathway with the competitive inhibitor L-NMMA. This was appropriate as recent studies have shown that diabetes could be prevented in the STZ model with this agent (Kolb et al, 1991; Lukic et al, 1991). However, the various regimes of L-NMMA administration had no effect on the diabetes incidence in the adoptive transfer or cyclophosphamide models.

Having investigated IDDM at the cellular level I addressed the question of genetic susceptibility. NOD mouse breeding studies and human family studies would suggest that there are predisposing genetic factors for disease development. The NOD mouse is unique among mouse strains in that it does not express I-E and has a unique I-Aβ. Introduction of an
Exαd transgene prevented insulitis and hyperexpression of MHC class I in the pancreas of the NOD mouse. Introduction of transgenic NOD I-Aβ bearing the Pro substitution at position 57 prevented IDDM, intra-islet infiltration and MHC class I hyperexpression, whereas introduction of the Asp mutation did not prevent insulitis and only slowed the progression of disease (Lund et al., 1990B). These findings have relevance to human disease as individuals with non-Asp bearing DQB chains (the human homologue to NOD I-Aβ) are predisposed to develop disease. The mode of protection of both the I-E and I-A transgenes remain to be resolved but evidence would suggest that these are dissimilar. Protection afforded by the I-A transgenes would appear to be through altered presentation of the diabetogenic autoantigen. Insertion of I-Apro56 alters the antigen binding cleft of the I-Aβ chain perhaps such that the autoantigen does not bind well during ontogeny and therefore tolerance is not established in the thymus. Alternatively, the presence of a new restriction element could alter the profile of the cytokine response elicited. Such an explanation of the mechanism of disease development could equally well be applied to the NOD-E transgenic mice but the mechanism of protection by the I-E transgene could also be more subtle. Analysis of thymi from both NOD and NOD-E mice shows many abnormalities including large perivascular epithelial cell holes and the presence of B cell germinal centres within these spaces. Such abnormalities occur irrespective of the subsequent development of diabetes. Therefore events during ontogeny which shape the peripheral T cell repertoire appear to have little bearing on the outcome of disease and events in the periphery appear to have greater importance. Evidence supporting this theory comes from the unpublished observations of Parish, who has shown that irradiated NOD mice reconstituted with NOD bone marrow under the influence of a NOD-E thymus still develop disease, suggesting that modifying events in the periphery must occur. These could be at the level of the antigen presenting cell such as the dendritic cell or macrophage which could perform a regulatory function. The recipient would therefore still have dendritic cells in the periphery of the susceptible genotype which would allow disease to ensue. I have not studied the role or distribution of dendritic cells in this disease principally because no good mouse markers for such cells were currently available. Subsequent availability would permit such a study, which is an exciting prospect, particularly in view of the principal role such cells play in transplantation rejection as “passenger leukocytes”.

What are the future prospects for both the diagnosis and treatment of autoimmune
IDDM? My studies in the NOD mouse have demonstrated that administration of depleting anti-CD4 or anti-CD8 M.Ab.s prevent IDDM, and could possibly also prevent human diabetes. Such regimes, however would be too systemically immunosuppressive to contemplate. Less radical immunotherapy with TCR M.Ab.s are not an option in this disease, as the pancreatic infiltrates in the NOD mouse are heterogeneous. There remains the possibility for NDP anti-CD4 therapy which could create tolerance to the diabetogenic autoantigen even though it remains unknown. Under this tolerogenic milieu, perhaps β cell regeneration could ensue thereby prevent the necessity for insulin therapy.

Our transgenic studies have demonstrated that the unique MHC of the NOD mouse is critical for disease development. In particular, modification of the amino acids of the I-Aβ chain which occupy critical sites in the antigen binding cleft determine the outcome of disease. Therefore, it would be useful to detect polymorphisms in the MHC and other genes associated with disease susceptibility in the human. Susceptible MHC types could be the ones presenting the pathogenic peptides. Mass screening of individuals with molecular probes to detect polymorphisms within the MHC would identify those at risk of developing disease before onset on clinical of symptoms. Once susceptible individuals were identified, the next specific immunotherapy could be applied. Cloned T cell lines capable of disease transfer have been identified in a mouse model of thyroiditis. Two distinct T cell hybridomas CH9 and ADA2 which recognise a thyroxine 9-mer epitope (T4 at position 2553) on thyroglobulin are capable of recognition in vitro and in vivo of the T4 containing peptide and can induce disease in susceptible animals (Dawe et al, in press). Peptides with the sequence of cloned TCR have indeed been used to vaccinate against the development of EAE (Wraith et al, 1989). This raises the possibility of using blocking peptide therapy for autoimmune disease. Such peptides with high affinity for the antigen-binding cleft of an MHC susceptibility allele could compete with the autoantigenic peptide stimulating the inflammatory response. Identification of the autoantigen target opens up a new realm for immunotherapy allowing a more refined manipulation of the immune response. It may be possible to specifically delete (either physically or functionally) lymphocytes with anti-self reactivity to the autoantigen or peptide administered concurrently with the Ab. regime.

Further identification of other candidate genes for both IDDM and other autoimmune diseases such as those coding for lymphokines should be studied as the genes coding for TNF
α, β map within the MHC complex of both mice and humans (Muller et al.; 1986, Gardner et al.; 1987). It is possible that a disease susceptibility allele of these genes exists in linkage disequilibrium with a class I or II disease susceptibility allele. One also cannot ignore the role that environmental factors such as diet and infections, (both viral and bacterial) play, as they could be the insult that tip the delicate balance of the immune system in susceptible individuals to initiate disease.

The exact route which leads to the breakdown of the normal mechanisms of tolerance remain to be clarified. Knowledge of the factors that predispose individuals to autoimmune disease could provide the basis for models of intervention and must await a more defined understanding of the basic mechanisms of how self tolerance is established and maintained to avoid the "horror autotoxicus" described by Ehrlich.
Bibliography


Bottazzo, G. F. and B. M. Dean. (1984). "Evidence for the expression of class II (HLA-DR) and increased presentation of class I (HLA-A,B,C) molecules in pancreatic islets in Type I (insulin dependent) diabetes." Diabetologia. 27: 259A.


288


289


300


302


Williams, C. L. and V. A. Lennon. (1986). “Thymic B lymphocyte clones from patients with myastenia gravis secrete monoclonal striational autoantibodies reacting with myosin, alpha actin, or actin.” J. Exp. Med. 164: 1043-.

Willis, T. (1676-1680). Opera Omnia (Geneva). 2:


mice: Evidence for thymic T cell anergy that correlates with the onset of insulitis.” J. Immunol. 146: 3763-3771.


Bibliography Continued
