EVALUATION OF MURINE EXPERIMENTAL AUTOIMMUNE THYROIDITIS AS AN APPROPRIATE MODEL FOR AUTOIMMUNE THYROID DISEASE

By

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ABSTRACT

Murine experimental autoimmune thyroiditis (EAT), is used as a model for human autoimmune thyroid disease. Both autoantibodies to thyroglobulin (TG) and lymphocytic infiltration of the thyroid gland are manifested in susceptible murine strains challenged with mouse thyroglobulin (MTG) and adjuvant (CFA or LPS), but little is known about the factors responsible for initiating the disease process. By studying the duration of EAT induced with either MTG/LPS or MTG/CFA I have demonstrated that the adjuvant can influence the course of the disease. Analysis of the binding specificities of the anti-TG autoantibodies established further differences. These findings indicate that the degree of qualitative differences between the two protocols is sufficient to rationalise the use of MTG/LPS induced EAT as a more appropriate model.

The main conclusion to be drawn is that finer variations in antibody specificity may, either directly or indirectly, determine the disease process. In addition, it was established that the specificity of the autoantibody response is influenced by the prior history of the animal. For example, adult mice pretreated with aminotriazole (an anti-thyroid agent) had a reduced autoantibody response to MTG/LPS challenge. These autoantibodies also had lower binding activity to rat TG. The implications of this data are discussed in relation to the structure of the pre-immune repertoire and the elements of connectivity which continuously establish a state of tolerance to specific immunogenic epitopes.

In vitro studies of the responsiveness of mouse thyroid follicular cells to the cytokine interferon-γ (IFN-γ), which is known to induce the expression of class II MHC antigens on human thyrocytes, further established the suitability of this animal model for investigating the pathogenic mechanisms which may lead to thyroiditis. IFN-γ also modulated the expression of TG in thyrocytes. Mouse thyrocytes which were stimulated with IFN-γ could present endogenously synthesized TG to MHC class II-antigen restricted TG specific T cell hybridomas.

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APC	Antigen presenting cells
ATA	3-amino 1, 2,4-triazole
CFA	Complete Freund's adjuvant
EAT	Experimental autoimmune thyroiditis
FCS	Foetal calf serum
ICAM-1	Intercellular adhesion molecule-1
IFA	Incomplete Freund's adjuvant
IFN-γ	Interferon gamma
IL-2	Interleukin-2
^{125}IUdR	^{125}I-deoxyuridine
LPS	Lipopolysaccharide
Mab	Monoclonal antibody
MHC	Major histocompatibility complex
MTG	Mouse thyroglobulin
OS	Obese strain of chickens
PBS	Phosphate buffered saline
SAT	Spontaneous autoimmune thyroiditis

TEC	Thyroid epithelial cells
TG	Thyroglobulin
TSH	Thyroid stimulating hormone

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

A persuasive explanation for the evolution and existence of the adaptive immune system is that the host needs to protect itself from potential threats in its environment (eg. viral and parasitic infections). The concept of host defence may be broadened to include resistance to malignant cells (Talal, 1978), nevertheless logistically this requires only the ability to react against non-self. Thus retaining the potential (Coutinho et al., 1987; Hooper & Taylor, 1987; Guilbert et al., 1982; Portnoi et al., 1986) to mount immune responses to autologous antigens is seemingly futile. So what is the *raison d'etre* of autoimmunity?

Wide acceptance and interest in the concept of autoimmunity was initiated by the experimental evidence which established the autoimmune aetiology of Hashimoto's disease (Roitt et al., 1956; Rose and Witebsky, 1956). This set a precedent to establish whether other idiopathic diseases had as an underlying mechanism an autoimmune response. Adopting Koch's postulates to determine if a given microorganism causes a particular disease, a set of criteria to distinguish autoimmune diseases from those conditions that are merely accompanied by autoimmune reactions have been put forward. Importantly, the accepted criteria for a disease of autoimmune aetiology is the existence of an animal model (Weetman & McGregor, 1984).

A number of animal models are available for autoimmune thyroid disease, but over the last decade the *in vitro* approach to understanding the regulatory and effector mechanisms of thyroid autoimmunity has been emphasised. This project, at the time of its inception, was therefore against the grain of research practice in that a major part of the work had an *in vivo* perspective. The murine model of experimental autoimmune thyroiditis was used to understand fundamental questions relating to the breakdown of tolerance to thyroid antigens.

1.2 CONCEPTS OF SELF-TOLERANCE

Any discussion on the mechanism by which a state of immunological self-tolerance may be maintained is inextricable from the principles of lymphocyte repertoire selection. Positive selection of the peripheral repertoire of immunocompetent T and B cells is an ongoing dynamic process. Apart from the persistent CD5+ B cell population produced in perinatal life (Hayakawa et al., 1984, 1985; Herzenberg et al., 1986), B cells are constantly renewed, primarily by bone-marrow production of new specificities from uncommitted precursors (Freitas & Coutinho, 1981; Osmond, 1986). T cells are also extensively renewed, but mostly by peripheral division of immunocompetent lymphocytes (Rocha et al., 1983; Miller & Stutman, 1984). Although it is important to note that studies in mice show that there is migration of T lymphocytes from the thymus throughout life (Scollay et al., 1980).

The fundamental difference between the antigen receptors of T and B cells invokes the need to consider that two separate, but parallel, mechanisms of tolerance and selection can operate for a defined antigen. The antigen specific receptors on B cell lymphocytes are membrane-bound immunoglobulins (Ig) (Goding et al., 1977; Vitetta & Uhr, 1976; Venkitaraman et al., 1991), which can bind soluble antigen in a manner similar to that of many well characterized receptor-ligand systems. The equivalent recognition molecule on T cells is the membrane-bound T-cell antigen receptor (TCR). The majority of T cells express the (α:β) heterodimer, but a small percentage of peripheral T cells (1-10%) and the majority of dendritic T cells in the skin express a second type of TCR heterodimer (γ:δ) (Davis & Bjorkman, 1988). In contrast to the B cell receptor for antigen, the TCR can generally recognize antigen only in association with cell surface molecules encoded by the major histocompatibility complex (MHC). This requirement, known as MHC restriction , ensures that T cell activation or effector function occurs only in an appropriate cellular context (Katz et al., 1973; Rosenthal & Shevach, 1973; Zinkernagel & Doherty, 1974). MHC molecules are polymorphic cell surface glycoproteins that occur in two distinct forms: class I MHC molecules (H-2 K and D in mice, HLA-A, B, C in humans) and class II MHC molecules (Ia in mice, HLA-D in humans) (Hood et al., 1983). It has now been established that T lymphocytes generally recognize peptide fragments of an antigen

(presumably derived from intracellular processing) that are bound to MHC molecules (Benacerraf, 1978; Shimonkevitz et al., 1983; Babbitt et al., 1985; Buus et al., 1986; Townsend et al., 1986) at what appears to be a single site (Cease et al., 1986; Guillet et al., 1986; Bjorkman et al., 1987; Brown et al., 1988). The binding of the TCR to its ligand is enhanced by CD4 and CD8 molecules expressed on the surface of T cells. These molecules bind to the nonpolymorphic portions of class II and class I MHC molecules respectively (Gay et al., 1987).

During lymphocyte development a great diversity of receptors are generated, consequently cells expressing receptors specific for self-antigens may frequently arise. Importantly, the sequence and specificity of T-cell receptors remains constant after receptor gene rearrangement (Chien et al., 1984; Ikuta et al., 1985; Fink et al., 1986). By contrast, B cell antigen receptors are further diversified after B cells have left the bone-marrow. Extensive somatic mutation of the V genes which code for the component polypeptides of the antibody combining site occurs in mature, peripheral B cells (Berek & Milstein, 1987; Rajewsky et al., 1987; French et al., 1989). Hypermutation of immunoglobulin genes not only provides the extra diversity for production of high-affinity antibodies to most foreign antigens, but also results in generation of self-reactive B-cell variants at a detectable frequency (Diamond & Scharff, 1984; Shlomchik et al., 1987).

Developments in the thymus play an important part in shaping the T-cell repertoire in the periphery. A number of selective events occur: The specific interaction of the TCR on immature thymocytes with thymic MHC antigens determines the differentiation of CD4+CD8+ thymocytes into either CD4+CD8- or CD4-CD8+ mature thymocytes (Teh et al., 1988). There is also direct evidence for positive selection of antigen-specific, class I MHC-restricted CD4-CD8+ thymocytes (Kisielow et al., 1988). In addition, at, or before the double positive CD4+CD8+ thymocyte stage, elimination of certain autoreactive T cells may occur (Sha et al., 1988; Kappler et al., 1987).

The extent to which clonal deletion limits the repertoire during induction of self-tolerance has been difficult to measure, because of both the vast heterogeneity of $\alpha\ \beta$ receptors and the complexity involved in the interaction of the receptor variable elements with

antigen/MHC ligand. Clonal elimination has been demonstrated for T lymphocytes expressing specific TCR Vβ elements depending on the expression of poorly defined autosomal dominant genes (Pullen et al., 1988; MacDonald et al., 1988). The prototype genes responsible for this effect encode the so-called "minor lymphocyte stimulatory" (Mls) antigens that induce a vigorous primary mixed-lymphocyte reaction. Recently it has been suggested that these antigens may be related to the murine "superantigens" and are encoded by mouse mammary tumour virus (Frankel et al., 1991; Woodland et al., 1991; Choi et al., 1991; Acha-Orbea et al., 1991). An important aspect of the Mls antigen is that they are presented by B cells in association with MHC class II molecules; and other class II positive cells apparently cannot present Mls (Molina et al., 1989; Webb et al., 1989). As yet there is only limited evidence to indicate that induced autoimmunity can be driven by T cells with quite restricted repertoires. For example, experimental allergic encephalitis, a model for a multiple sclerosis-like disease inducible in H-2^u mice, may be caused chiefly by T cells expressing Vβ 8.2 (Acha-Orbea, 1988). This suggests that mechanisms of self-tolerance, other than clonal elimination, may be more important.

Since the antigen dependent response of B cells is generally regulated by a complex series of interactions with helper T cells (Coffman et al., 1988; Noelle & Snow, 1990), ensuring effective deletion of self-reactive T cells would appear to be a simple solution to the problem of incomplete tolerance in B cells. However, it is unlikely that the immune system should be founded on such a precarious state of tolerance, which could be easily broken (Allison, 1971; Weigle, 1971). For example, antibody responses to many bacterial components occurs without helper T cells being involved (DeFranco, 1987). If there is a cross-reaction between such a bacterial antigen and a self-component that failed to cause efficient clonal deletion of those B cells recognizing it, then autoantibodies might be expected. Indeed, this appears to occur in rheumatic fever, in which antibody produced in response to streptococcal infection cross-reacts with heart tissue, causing tissue destruction. Other ways in which incomplete tolerance in B cells may be bypassed includes alteration of the autoantigenic structure by abnormal processing or by drugs substituting within the molecule. In cases which have been investigated, autoimmune reactions arising from drug treatments or exposure to environmental toxins appears to be linked to genetic susceptibility (Batchelor, 1984).

Self-reactive clones among mature lymphocytes have been demonstrated in several *in vitro* experimental systems (Smith & Pasternak, 1987; Battisto & Ponzio, 1981; Glimcher & Schavach, 1982). Another line of research on autoimmunity has involved the detection of autoreactive T cells in naive mice. Coutinho et al. (1987) have suggested that autoreactivity is a common property of the "normally" activated T cells. It was shown (Hooper & Taylor, 1987) that mice harbour T cells reactive with mouse erythrocytes. The fact that these mice remain healthy shows that tolerance can be maintained in the presence of autoreactive T cells and of an autoantigen that is exposed to the immune system.

Hypotheses, such as functional inhibition by other T cells (suppression/immunoregulation) (Gershon & Kondo, 1971; Sy & Benacerraf, 1988) and functional inactivation of self-reactive T cells on encounter with specific antigen (clonal anergy) (Lamb et al., 1983; Jenkins & Schwartz, 1987; Markmann et al., 1988) have been proposed to account for the unresponsiveness of such cells *in vivo*. Recent studies (Rammensee et al., 1989; Quin et al., 1989) have demonstrated that clonal anergy can account for *in vivo* tolerance. On the other hand the lack of molecular evidence for T suppressor cells has called into question the whole body of cellular experiments documenting suppression (Moller, 1988). A number of other explanations may account for the documented suppressor phenomena, nevertheless it may be that the suppressor cell will re-emerge someday as an immunoregulatory cell (Schwartz, 1989).

Since clonal deletion may not be a viable option for T cells specific for antigens not expressed on thymic haematopoietic elements, clonal anergy offers an attractive explanation in various experimental tolerance models. Burkly et al. (1989) have shown that I-E tolerant transgenic mice which express I-E on pancreatic beta cells, but not in the thymus or peripheral lymphoid organs, do not delete the I-E specific T cells. Instead, exposure to I-E on nonlymphoid cells *in vivo* apparently induces clonal paralysis in the potentially I-E reactive T cells.

In vivo models of tolerance induction to exogenous antigens administered in an appropriate form (Chiller et al., 1971; Desaymard & Waldmann, 1976; Nossal, 1983) have suggested that mature B cells can also be tolerised by a mechanism of antigen-induced anergy. More

recently Goodnow et al. (1989) using transgenic mice formally showed that mature B cells expressing receptors for a "conferred" self antigen (hen-egg lysozyme), are not deleted from the peripheral repertoire, but are functionally inactive; possibly as a result of the down-regulation of the IgM form of their antigen receptor. Importantly, this study addressed the question of whether tolerance induction and the accompanying down-regulation in surface IgM depends on exposure of anti-lysozyme B cells to a threshold concentration of serum lysozyme concentration. The findings were consistent with previous data (Chiller et al., 1971), showing that serum antigen concentration is a determining factor in tolerance induction and that the threshold for inducing unresponsiveness in B cells is higher than that required for T cells.

Expanding on the relationship between antigen concentration and tolerance induction, Gammon and Sercarz (1989) have suggested that tolerance to many self-antigens, except those at high concentrations, will not be complete. They proposed that some T cells that are specific for minor determinants on a multideterminant antigen will escape tolerance, because these determinants are only available in relatively low amounts after *in vivo* processing of the whole antigen. (A caveat to the above suggestion would be to consider that B cells specific for the minor determinants on an antigen would also escape tolerance induction.) Gammon and Sercarz suggest that autoimmune disease is a consequence of triggering a response in such T cells which have randomly escaped tolerance. However, it seems improbable that such a highly evolved system should "allow" such autoreactive cells to escape tolerance unless they serve a function in the peripheral repertoire.

It has to be accepted that activated autoreactive cells do exist in the peripheral repertoire (Guilbert et al., 1982; Portnoi et al., 1986) and classical mechanisms of tolerance (clonal deletion and its derivatives) cannot provide an adequate explanation for their persistence. Therefore other mechanisms must exist in order to down-regulate the peripheral repertoire of autoreactive cells. The counter argument is that persistence of a set of autoreactive cells in the peripheral repertoire is central to maintaining self-tolerance (Coutinho & Bandeira, 1989). Pivotal to this view of self-tolerance are the class of CD5+ B cells which may specialize in producing autoantibodies (Hayakawa & Hardy, 1988). It is suggested that this

cell type plays a primary role in selective interactions in T-cell repertoires, which recursively maintains the overall repertoire for the lifetime of the individual (Martinez et al., 1988).

If the peripheral repertoire of T and B cells is normally regulated because the immune system exists as a network, then establishing the elements of connectivity within the network is fundamental to determining how the network may be deregulated sufficiently to allow overt autoimmune disease. That ligands based on V-regions of immunoglobulins and T cell receptors are important elements in the connectivity of the immune repertoire is not in any doubt (Pereira et al., 1989), but other elements of connectivity must play a role. As Roitt et al. (1983) have pointed out, if the network is based only on idiotypic interactions, we might expect spontaneous emergence of autoantibodies without the need for triggering by the conventional autoantigen itself. At least for thyroid autoimmunity, the evidence suggests that autoantigen is obligatory for the induction and maintenance of autoimmunity. These considerations are also of practical significance: If idiotypic interactions constitute a considerable proportion of network interactions, then idiotypic manipulation (Roitt et al., 1981) may be a viable option for suppressing autoimmunity. On the other hand strategies based on peptide competition (Adorini & Nagy, 1990) may be more appropriate if the maintenance of immune networks is predominantly dependent on the continued presence of specific epitopes on a multideterminant antigen.

1.3 AUTOIMMUNE THYROIDITIS - AN EXAMPLE OF ORGAN-SPECIFIC AUTOIMMUNE DISEASE

Autoimmune thyroid disease includes two clinically disparate disorders of the human thyroid, namely, Graves' disease (GD), and Hashimoto's thyroiditis (HT) and its variants. Although the clinical expression of these diseases may be quite diverse, there are many immunological aspects common to them and interestingly, patients with a previous history of Graves' disease may develop histological Hashimoto's thyroiditis (Hirota et al., 1986). This observation may support the contention that these two conditions are merely opposite ends of a spectrum of the same disorder (Davies & Bernardo, 1983). However, as yet there are no suitable animal models for Graves' disease.

Graves' disease is currently defined clinically as a diffuse hyperplastic goitre, associated frequently with other extrathyroid manifestations such as exophthalmos (Volpe, 1984). The incidence of Graves' disease is greater in the female population and may occur at any age, but the frequency increases with age. Onset is usually insidious, occurring over a period of time. Remissions in Graves' disease may occur, however, in many cases the disease recurs; often with increased severity (Zimmerman & Hayles, 1985).Both lymphocytic infiltration and autoantibodies to thyroid specific antigens are manifested in Graves' disease (Burman & Baker, 1985).

Hashimoto's thyroiditis is a common autoimmune endocrinopathy, affecting, at least subclinically, up to 15% of the adult female population (Volpe, 1986; Gordin et al., 1979). In the most characteristic form of Hashimoto's thyroiditis, the thyroid gland is enlarged (goitre) and shows the following changes: (a) diffuse interacinar infiltration by lymphocytes and plasma cells with the formation of lymphoid follicles containing germinal centres, (b) small thyroid acini lined by abnormally large thyroid epithelial cells (variously called oxyphil, Askanazy or Hurthle cells), and (c) a variable amount of fibrosis (Anderson et al, 1967). There are several variants of Hashimoto goitre; the histological classification ranges from the mild "lymphocytic thyroiditis" through the classical "oxyphil" type, with intermediate stages up to the most destructive "fibrous" and "plasma cell" variants of the disease (Drexhage & Bottazzo, 1985). The atrophic variant of Hashimoto's thyroiditis, primary myxoedema, is a condition in which severe hypothyroidism develops in the absence of goitre (Anderson et al., 1967). In this disease there is progressive shrinkage of the gland with loss of epithelium caused by a "block" in the normal regenerative response of the follicular cells (Drexhage & Bottazzo, 1985).

Early studies showing familial aggregation of thyroid autoantibodies (Roitt & Doniach, 1967) suggested that there is a genetic predisposition to the disease. Indeed, there is a greater incidence of the MHC class II allele, HLA- DR3, in patients with Graves' disease and the atrophic variant of Hashimoto's disease, whereas the goitrous form of Hashimoto's thyroiditis is said to be associated with HLA-DR5 (Farid & Bear, 1981). However, these linkages are not absolute and may vary between different populations; for instance the incidence of HLA-DR3 is higher in Graves' disease patients from Newfoundland

compared with those from the United Kingdom. A substantial percentage (30- 50%) of patients with disease do not express an associated HLA antigen, suggesting that unique familial associations may be of primary importance (Uno et al., 1981). The suggested linkage between the MHC and autoimmune diseases appears to be complex and it is possible that a gene closely linked to these marker alleles actually specifies autoimmunity. On the other hand, it may be that conventional serologic techniques do not allow detection of minor changes in the amino acid sequence of these molecules which may confer an autoimmune state. DNA restriction analysis has revealed differences in the DNA encoding the beta chain of the DR4 molecule between diabetic patients and normal persons (Owerbach et al., 1983). The mechanisms of the MHC associations are unclear, but may reflect appropriate antigen presentation and T cell recognition.

Immune responsiveness to specific antigens has been linked to the utilization of restricted TCR genes. In the mouse, the immune response to pigeon cytochrome-c is restricted to a single TCR-Vα chain gene (Fink et al., 1986; Sorger et al., 1987). Moreover, it appears that in murine experimental allergic encephalomyelitis (EAE) induced in susceptible animals by active immunization with myelin basic protein (MBP), Vβ8 Th cells are preferentially used (Urban et al., 1988). However, such restrictions may be due to the fact that the murine T-cell receptor uses a limited repertoire of expressed Vβ gene segments (Barth et al., 1985). In humans there are few reports of restricted TCR gene usage, and in a recent study of patients with Graves' disease, no significant associations were found (Demaine et al., 1989).

A large body of evidence suggests that inheritance of a combination of genes will predispose towards autoimmunity. Recent studies of the inheritance patterns of thyroid autoantibodies have found thyroid peroxidase and thyroglobulin (TG) antibodies to be inherited as a dominant Mendelian trait in women (Phillips et. al., 1990). That one set of genes allows autoantibody formation, whilst another determines the development of lesions, has been proposed. This may explain why relatives of patients with autoimmune diseases commonly produce autoantibodies in the absence of clinical abnormalities (Burek et al., 1982).

In addition to genes affecting immunological responsiveness, geneti-

cally linked abnormalities of the thyroid gland itself may also influence the course of the disease. This is suggested by the higher incidence of non-immunological thyroid disease in the families of Hashimoto patients (Doniach et al., 1965). One of the candidate genes may be the TG gene; defects of the TG have been found in cattle (Ricketts et al., 1987) and in goats (Kok et al., 1987). In both cases the gene defect causes goitre in an autosomal recessive fashion.

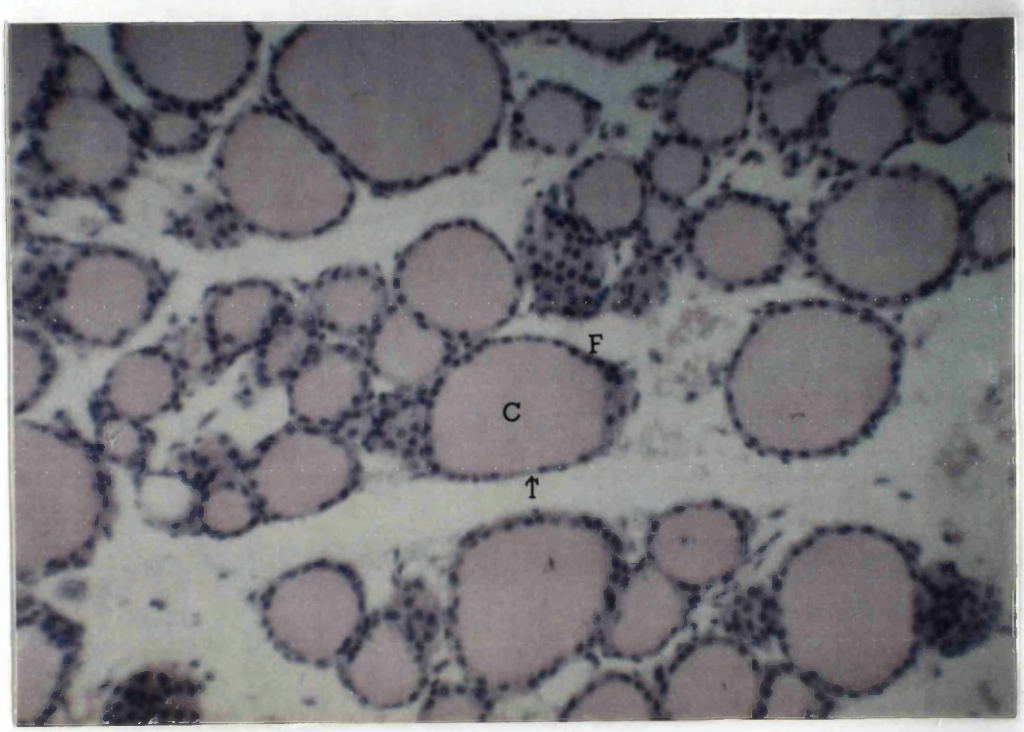
1.3.1 Autoantibodies to thyroid cell antigens

The thyroid follicular epithelial cell (DIAGRAM 1.1) is the principal cell targeted in these diseases. Autoantibodies to several thyroid specific antigens have been identified. The prevalence of these antibodies in the different disease types is variable. Antibodies against a number of thyroid antigens are present in the sera of these patients, including thyroglobulin and the thyroid microsomal antigen (Doniach et al., 1979; Weetman & McGregor, 1984). Other antigens of lesser or uncertain importance, include the second colloid antigen (Doniach et al., 1979), tubulin (Rousset et al., 1983), DNA (Katakura et al., 1987) and autoimmune thyroid disease-related antigen I (Hirayu et al., 1987). In Graves' disease, in addition to antibodies to TG and the microsomal antigen, there are autoantibodies which bind to the TSH receptors on the surface of follicular cells (Adams & Purves, 1956; Drexhage et al., 1981). These antibodies compete with TSH for interaction with its receptor binding sites and so affect the metabolic function of the thyroid cells. These TSH-receptor antibodies fall into different classes and evidence suggests that they react with different domains of the receptor (Yavin et al., 1981). Interestingly, one class of the anti-TSH receptor antibodies blocks TSH-induced hormone production and growth, and may be responsible for the progressive shrinkage of the thyroid gland observed in primary myxoedema (Drexhage & Bottazzo, 1985). Such antibodies have not been reported in goitrous Hashimoto's disease.

DIAGRAM 1.1 (a) Light microscopy of a cross-section of normal thyroid tissue. (Haematoxylin-eosin stained, magnification X 160.) The adult thyroid is composed of follicles (F), which are roughly spherical, and vary considerably in size. Each follicle is formed from a single continuous layer of epithelial cells (arrow) enclosing the lumen, which contains the thyroid "colloid" (C).

(b) Schematic represntation of the thyroid epithelial cell showing the presumed unidirectional pathways of TG secretion and degradation. The thyroid cell is normally polarised, with the apical membrane abutting on the colloid. The TSH receptors (n) are thought to be restricted to the basolateral membrane (P) pseudopod; (Mv) microvilli; (Tj) tight junction; (Ev) exocytotic vesicle; (Cd) colloid droplet; (L) lysosome; (ER) endoplasmic reticulim.

TRANSVERSE SECTION OF NORMAL MOUSE THYROID



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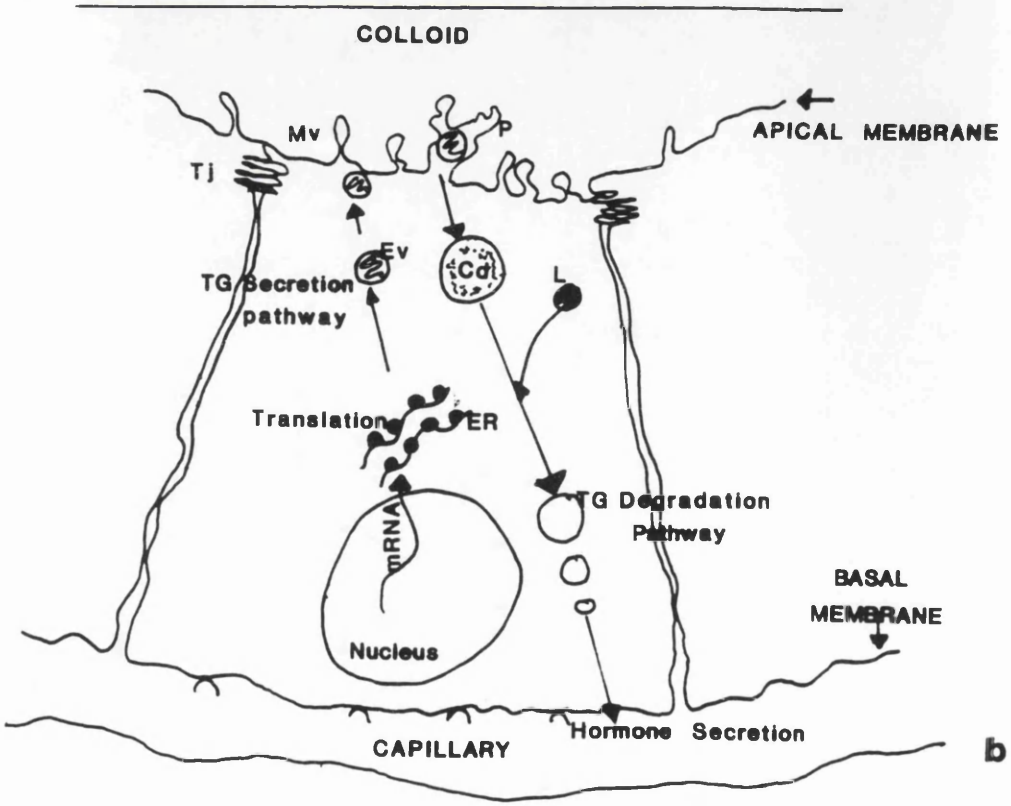

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1.3.2 Thyroglobulin

The role of anti-TG autoantibodies in the pathogenesis of disease is currently unresolved. Nevertheless it has been unequivocally demonstrated that animals challenged with TG can manifest a disease comparable to lymphocytic thyroiditis. TG plays a central role in the biology of the thyroid as an active and exclusive support for thyroid hormone synthesis (Marriq et al.,1989); a process which is dependent on appropriate iodine metabolism in the thyroid follicle (Van Herle et al., 1979). Thyroglobulin is a large glycoprotein of molecular weight 660 kD and sedimentation coefficient 19S (Edelhoch, 1965), and is the major product of thyroid protein synthesis (Seed & Goldberg, 1963). The follicle cell is polarized and integrated into an epithelial cell layer forming a closed sphere - the follicle (Mauchamp et al., 1979; Ekholm, 1981). The production of thyroid hormones by thyroid follicular cells brings into play a bidirectional transport of TG (Malthiery et al., 1989; Rousset et al., 1989; Bernier-Valentin et al., 1990), which appears to be dependent on the structural organization of the follicle (Ericson, 1983).

Newly synthesized TG progressing from the endoplasmic reticulum into the last cisternae of the trans-Goli is concentrated into secretory vesicles. These secretory (exocytotic) vesicles migrate towards the apical pole of the cell where they fuse with the plasma membrane to release their content into the intrafollicular lumen. Iodination of the tyrosyl residues of TG and coupling of a subset of iodotyrosines into 3,5,3',5'- tetraiodothyronine or thyroxine (T4) and 3,5,3'-triiodothyronine (T3) is catalyzed by thyroid peroxidase (Sugawara, 1985) bound to the apical membrane delimiting the intrafollicular lumen (Ekholm, 1981). Thyroid hormones remain part of the polypeptide chain of TG. Reabsorption and lysosomal hydrolysis represents the TG degradation pathway and involves a number of steps which ultimately results in the transfer of thyroid hormones from lysosomes into the bloodstream.

The mechanisms accounting for the polarized secretion and endocytosis have yet to be elucidated. Thyrotropin (TSH) is known to stimulate numerous steps in the intrathyroidal metabolic chain, such as iodide transport (Weiss et al., 1984a; 1984b) and TG iodination, which may be related to TSH-stimulated exocytosis (Ofverholm et al., 1985). TSH

can also stimulate the TG degradative pathway: TSH can specifically increase (via a cAMP-mediated process) intralysosomal TG hydrolysis independent of its effects on endocytosis of TG and lysosomal protease synthesis (Fouchier et al., 1987). Importantly, other extracellular signal molecules such as prostaglandins of the E type and to a lesser extent norepinephrine through beta-receptors, activate the thyroid adenylate cyclase and mimic the effects of TSH . In addition negative feedback is exerted on the TSH stimulat ory pathway by iodide (Raspe et al., 1989). Recent studies (Yamashita et al., 1989; Krogh Rasmussen et al., 1987; Nagayama et al., 1987) have shown that cytokines may also affect TG metabolism. The effects of these cytokines on the intracellular transit of TG in cultured thyrocytes would be an interesting focus of study.

Complete proteolysis of TG does not occur in the thyrocytes (Tokuyama et al., 1987), but the fate of small peptides or iodopeptides is unknown. Some TG molecules escape proteolysis and are secreted into the blood; presumably via the basolateral part of the cell (Chambard et al., 1987), but the mechanism of release is not clear. The concentration of circulating TG is of the order of 10-100 ng/ml (Roitt, 1984) which suggests that the level of TG in the serum must be controlled by its rate of secretion and metabolic clearance. However, various thyroid stimulators, including TSH, can induce an increased release of TG into the circulation (Van Herle et al., 1979; Roitt, 1984). Since TG is a heterogeneous protein containing varying amounts of iodine (Schneider & Edelhoch, 1971), iodotyrosines and iodothyronines (Corbese et al., 1976) and carbohydrates (Tarutani & Shulman, 1971), the TG in the circulation will result from a complex set of variables. An important point to note is that peripheral TG hydrolysis does not generate T3 or T4 (Izumi & Larsen, 1978), which suggests that any TG molecule retaining these epitopes are not preferentially degraded. In addition, since poorly iodinated thyroglobulin also has less sialic acid, and as sialic-acid forms appear to be preferentially degraded , it is possible that circulating TG would be enriched in highly iodinated TG (Ikekubo et al., 1980). Questions regarding the structure of circulating TG are important, because of a postulated (Romball & Weigle, 1983) role of circulating TG in maintaining a state of immunological tolerance in autoreactive cells.

1.3.3 Thyroid microsomal antigen-thyroid peroxidase

Clinical diagnosis of chronic thyroiditis is usually based on evidence of serum antibodies to TG or microsomal antigen. Until a few years ago the precise nature of the microsomal antigen, which was previously shown to be an integral component of the smooth endoplasmic reticulum (Roitt et al., 1964), was ill-defined. The human microsomal antigen, which is also expressed on the surface of thyroid cells in culture and is involved in complement mediated cytotoxicity (Khoury et al., 1981; 1984) has now been characterised as a discrete protein of 117 kD (Banga et al., 1985) or 107 kD (Hamada et al., 1985) under non-reducing conditions. Furthermore, it is now recognized that the microsomal antigen is (at least in part) the enzyme thyroid peroxidase, which is also isolated from the microsome fraction (Hosoya et al., 1971). This conclusion was based on immunologic evidence (Czarnocka et al., 1985; Portmann et al., 1985; Kotani et al., 1986; Mariotti et al., 1987) and subsequently confirmed by the molecular cloning of the cDNA for these proteins (Magnusson et al., 1987a; 1987b; Libert et al., 1987; Kimura et al., 1987) and the discovery that their derived amino acid sequences are the same (Libert et al., 1987; Seto et al., 1987). (Although it cannot be ruled out that other components in thyroid microsomes might show some antigenicity.)

It is now clear that microsomal antibodies are heterogeneous (Hamada et al., 1987). The antibodies against native TPO correspond to those detected by the standard microsomal haemagglutination test used in clinical diagnosis, but other antibodies from a proportion of patients with thyroiditis react with denatured or reduced TPO. It appears that multiple epitopes on TPO are recognized, including the catalytic sites involved in peroxidation (Kohno et al., 1986; Doble et al., 1988). In addition, there are common epitopes between TPO and TG and significantly these epitopes are recognized by anti-TG antibodies in sera from patients with chronic thyroiditis, but not from healthy subjects (Naito et al., 1990).

1.4 ANIMAL MODELS OF AUTOIMMUNE THYROIDITIS

A number of spontaneous and experimentally induced, animal models are available for Hashimoto's thyroiditis (reviewed in Weetman & McGregor, 1984). Thyroiditis can be induced relatively easily with TG and

adjuvant in a host of species; a single injection of homologous TG in an emulsion of complete Freund's adjuvant (CFA) is normally sufficient to evoke an autoimmune response in mice (Rose et al., 1981; Kong et al., 1986; Romball & Weigle, 1984). Tolerance to TG can also be abrogated if bacterial lipopolysaccharide (LPS) is used as an adjuvant (Esquivel et al., 1977). (TG in this case is administered in a soluble form). Autoantigens recognised by serum from patients with Graves' and Hashimoto's disease include TG and TPO (the microsomal antigen), however, antibodies against the microsomal antigen and other thyroid cell components are not made in the animal models of thyroiditis (Cohen & Weetman, 1990; Khoury et al., 1982).

Early attempts to induce thyroiditis with crude thyroid microsomal preparations were partly successful; chronic thyroiditis was induced in the rabbit (Mangkornkanok et al., 1972) and a transient disease has been described in monkey (Kite et al., 1966). Recently, purified preparations of TPO have been used to induce thyroiditis in mice (McLachlan et al., 1990; Kotani et al., 1990). These studies show that experimental thyroiditis induced by TPO has a different genetic restriction to that produced by TG.

Spontaneously occurring autoimmune thyroiditis has been described in several species; the Obese strain (OS) of chickens (Cole et al., 1968; reviewed by Wick et al., 1986), the Buffalo (BUF), the rat (Colle et al., 1985), and in the spontaneously diabetic BB/W rat strain (Sternthal et al., 1981). None of these models exactly represents the complex nature of the human disease. However, together they do help to elucidate some of the intricacies. For example, the polygenic inheritance of the disease has been demonstrated in the OS chicken (Wick et al., 1986). Also, the possible influence of sex hormones in the aetiology of autoimmune thyroid disease is supported by the known immunomodulatory actions of oestrogen and testosterone on experimental autoimmune thyroiditis (Gause & Marsh, 1985; Anser et al., 1986).

Data accumulated from experiments on the OS chicken suggests several possible effector mechanisms of thyroid cell damage including damage mediated by maternal autoantibodies transferred *in ovo* (Katz et al., 1981) and cell mediated cytotoxicity (Boyd et al., 1983). The relative roles of T and B cells in this process, however, remains ambiguous. Although neonatal bursectomy does not prevent T lymphocytes infiltrating the thyroid gland (Polley et al., 1981), bursectomy *in*

ovo reduces disease severity (Wick et al., 1985) thereby suggesting that B cells play a critical role in exacerbating the development of SAT. Other studies have revealed an important role for T cells in the inflammatory process: OS chicks depleted of T cells by thymectomy plus anti-thymocyte serum had less severe thyroiditis (Pontes de Carvalho et al., 1981). The controversies with regard to the relative importance of B and T cells in thyroiditis is also illustrated in murine models of EAT (reviewed in Charriere, 1989).
As in human chronic lymphocytic thyroiditis, linkage between EAT susceptibility and MHC haplotype has been inferred in inbred strains of rats (Penhale et al., 1975; De Assis-Paiva et al., 1989). However, in the murine model of EAT a close correlation between the development of thyroid lesions and MHC genes has been demonstrated. Genetic studies in mice have been facilitated by the availability of congenic inbred lines which differ only in regard to their H-2 haplotype. Mouse strains of the H-2 q, s, or k haplotypes have been classified as "good responders", and those with the H-2 b or d haplotypes as "poor responders", with lower antibody titres and little infiltration of the thyroid gland (Esquivel et al., 1977).

The predominant gene determining responsiveness to TG has been mapped to the I-A (class II) subregion of the H-2 complex (Biesel et al., 1982). That class I antigens may also be involved in regulating cellular infiltration has also been suggested. Studies of intra- H-2 recombinant strains differing only at the D-end showed that H-2K or H-2S mice had a less severe thyroiditis if the D-end genes were substituted by b or d haplotypes respectively (Kong et al., 1979). Genes in the K-end may also influence the development of thyroid lesions (Maron et al., 1980). Although there is sufficient evidence of the predominant role of H-2 in EAT in mice, an influence of non-H-2 genes has also been demonstrated (Beisel et al., 1982). Thus the polygenic influence of murine EAT may resemble that in human thyroid disease.

1.5 EFFECTOR MECHANISMS OF PATHOGENESIS

At present the predominant mechanism by which tissue damage is mediated in autoimmune thyroiditis is unresolved. Various humoral and cellular mediated mechanisms can generate pathological processes: complement-dependent, antibody-mediated cytotoxicity,

antibody-dependent cell-mediated cytotoxicity (ADCC) and direct cell-mediated cytotoxicity. All of these mechanisms have been implicated in autoimmune thyroiditis (Bogner et al., 1984; 1987; Del Prete et al., 1986). It may be that the various effector mechanisms of pathogenesis occur sequentially.

The histological feature of human autoimmune thyroid diseases, SAT and EAT, is a chronic inflammation with infiltration of lymphocytes and thyroid cell destruction to varying degrees. Phenotypic analysis of cells infiltrating the thyroid glands of HT patients shows an increase of B cells (Jansson et al., 1983; McLachlan et al., 1983; Kontiainen et al., 1987) and of activated T cells (Misaki et al., 1985; Most & Wick, 1986). Activated T cells are also observed in the infiltrate of thyroids from OS chickens (Wick et al., 1984) and rats with EAT (De Assis-Paiva et al., 1989). Importantly, a number of studies (McLachlan et al., 1979; Weetman et al., 1982; Atherton at al., 1985) demonstrated that lymphocytes isolated from Hashimoto and Graves' thyroid tissue frequently can spontaneously synthesize IgG and autoantibodies to thyroid antigens. These activated B cells may be mainly from the diffusely distributed interfollicular lymphoid population (McLachlan et al., 1986). In contrast, no thyroglobulin plaque-forming cells could be found in the thyroid of rats with EAT (Weetman et al., 1982). The lack of B cells in the thyroid infiltrate of this model was recently confirmed (De Assis-Paiva et al., 1989). This may be a peculiarity of rodent EAT, since phenotypic analysis of the infiltrate of MTG/CFA induced murine EAT showed the proportion of B cells to be consistently below 5% (Creemers et al., 1984). (Although the possibility of other determining factors, such as the immunizing protocol, cannot be ruled out.) Interestingly, these authors found that the thyroidal T cells declined between 12 and 21 days post challenge and were replaced by cells without T or B cell markers, suggesting that the effector mechanisms mediating thyroid damage may vary with time.

The question of the functional activity of the infiltrating lymphoid population has been addressed using T cell clones derived from cells invading HT thyroid glands, but a clear interpretation of this data is difficult. The cytotoxic CD8+ clones analysed were not specific to TG or other thyroid antigens, but instead showed natural killer (NK) cell activity (Del Prete et al., 1986) or lectin-dependent cytotoxicity (Bagnasco et al., 1987). MacKenzie & Davies (1987) found that the

majority of CD8+ clones derived from HT tissue lyse HLA-DR mismatched PHA blast cells, but some of these clones had cytotoxic activity directed towards autologous thyrocytes.

The use of *in vitro* systems to study the participation of different types of cells in EAT has confirmed that T cells play an important part in this disease. Thyroiditis independent of antibodies to TG has been induced by passive transfer of TG-reactive T lymphocyte lines (Maron et al., 1983). However, it was not possible to tell from these experiments whether the cells transferred were the effectors in the pathogenesis of the disease. Yeni and Charreire (1981) have demonstrated that T-cells from unprimed mice of high-responder H-2 haplotypes can be sensitized by co-culturing them with syngeneic thyroid epithelial cells. These sensitized cells are capable of eliciting thyroid lesions when injected into normal syngeneic recipients (Charreire & Michel-Bechet, 1982). Culture of *in vivo* primed lymph node cells with MTG *in vitro* also leads to the development of cytotoxic cells which kill thyroid epithelial cells (Creemers et al., 1983). It has been suggested that these cytotoxic cells interact with both TG and H-2K or D molecules on the surface of the thyroid epithelial cells. The mechanism by which thyroid cell specific cytotoxic cells are triggered *in vivo* remains unresolved. In order to devise suitable therapy it is important to know which effector mechanisms are critical to the progression of the disease. This may become evident through studies of the duration of autoimmune diseases in patients; ethical and practical reasons not withstanding. However, wide applicability of results from such studies may not be possible, because no two patients are ever the same. Therefore, corroborative use of suitable animal models of autoimmune diseases is invaluable in understanding the disease process.

1.6 OBJECTIVES

The regulation of overt autoimmunity towards thyroid antigens and the effector mechanisms which lead to pathogenesis in autoimmune thyroiditis are not clearly understood. The variability of the disease process between patients poses a problem in analysing the immune responses which may be associated with development of disease-from onset to full-blown pathology. For the purposes of interventive therapy it is clearly necessary to understand these processes and also

to know how the immune response varies with time.

1.6.1 Longitudinal studies of murine EAT

Although murine EAT is frequently used as a model to study some of the aspects of human lymphocytic thyroiditis, currently there are no prospective studies on the duration of murine EAT. Experimentally induced thyroiditis in rats is a reversible condition (Jones & Roitt, 1961), but in animals treated with pertussis vaccine as well as CFA a similar regression is not observed (Twarog & Rose, 1969), which suggests that the mode of challenge might determine the mechanisms of immune activation and pathogenesis and therefore the duration of the disease. I have analysed the effect of the adjuvants CFA and LPS on the course of EAT in high responder mice. Within this study I have also compared the specificity of the antibody response. The data from these studies are presented in chapter one. The basic distinction between the two models described obviously does not attempt to explain all aspects of thyroid autoimmunity. Its value, however, should be to serve as a conceptual framework for future studies on the mechanism of the development of autoimmunity, in which both polyclonal B-cell activation and antigen-driven responses are taken into account.

1.6.2 Regulation of autoreactivity to thyroglobulin

Self tolerance to some TG epitopes may be maintained by clonal deletion of the respective autoreactive cells, but this is not the only mechanism by which tolerance to TG is maintained since in normal mice TG-reactive cells do exist (Elrehewy et al., 1981; Charreire, 1982; Romball & Weigle, 1984; Champion et al., 1986). If, as proposed by Allison (1971) and Weigle (1971), effector self-reactive cells are rendered impotent when deprived of help because of tolerization of T helper (Th) cells, then autoimmunity may arise by a number of mechanisms which bypass the need for specific helper T cells. However, there do appear to be regulatory systems which attempt to correct autoimmune responses (Roitt, 1984). Thus the second part of this project (Chapters 4 and 5) is concerned with investigating mechanisms which may regulate immune reactivity to TG in high responder mice.

1.6.3 T cell reactivity to thyroid antigens: an *in vitro* approach

Thyroid epithelial cells constitutively express class I molecules (Salamero et al., 1983), but spontaneous expression of class II molecules can only be demonstrated on thyroid cells from patients (Hanafusa et al., 1983). Class II antigen expression is also seen in other organ specific autoimmune diseases, for example, on the beta cells of diabetic pancreases (Bottazzo et al., 1985; Foulis & Farquharson, 1986). Furthermore, encephalitogenic T-cell lines recognizing myelin basic protein (MBP) on *in vitro* cultures of Ia+ astrocytes will subsequently proceed to kill the presenting cells (Sun & Wekerle, 1986). In line with the hypothesis that the induction of different classes of immunity is determined by the nature of the antigen (Bretscher, 1981), these authors reported that the nature of the recognized antigenic epitope determines whether or not antigen recognition is followed by killing.

The aberrant expression of class II molecules has been put forward as being important in antigen presentation by target cells in a wide range of autoimmune diseases (reviewed in Bottazzo et al., 1986). Although the relative importance of this process in mediating thyroid cell destruction remains a controversial issue, there is sufficient circumstantial evidence to merit further investigation. If this is an important effector mechanism in human thyroid disease, it is necessary to investigate whether murine EAT may also share a common autoimmune pathogenesis. The aim of this study was to establish whether murine thyrocytes can present endogenously synthesized TG. The results of these investigations are presented in chapter 6.

The final chapter (chapter 7) is a general discussion of the points brought out in this project, and the overall implications of these findings in relation to our present understanding of the immune system and the cellular and molecular mechanisms involved in immunity.

CHAPTER 2

MATERIALS AND METHODS

2.1 MICE

CBA/J, CBA/Ca and BALB/c mice were obtained from the National Institute for Medical Research, Mill Hill, London. Tuck No.1 outbred mice were obtained from A.Tuck & Son Ltd., Battlesbridge, Essex, or from stock maintained in the Middlesex Hospital Medical School (MHMS) animal house. The neonatal CBA/J mice were obtained from breeding experiments carried out in the animal housing facilities of the department of Immunology MHMS.

To produce mice which were virtually devoid of iodinated TG, the thyroid peroxidase inhibitor, 3-amino 1, 2, 4-triazole (ATA), (Koch-Light Laboratories) was added to the drinking water of CBA/J mice as a 0.1% solution over a period of 4 weeks. The drinking water was also supplemented with 0.2 μg/ml thyroxine (Sigma).

2.2 THYROGLOBULINS

Thyroglobulin was prepared as previously described (De Carvalho et al.,1980). Thyroid tissue was homogenized in phosphate buffered saline (PBS). After overnight extraction and ultracentrifugation, TG was purified from the filtered supernatant by differential (37-45%) ammonium sulphate precipitation (SAS) and Sepharose 6B chromatography. Sterilized aliquots of TG were stored at -70° C until required. Mouse TG was prepared from pooled thyroids of outbred (TUCK) or inbred (CBA/Ca) strains. Rat TG was prepared and kindly provided by H. De Assis-Paiva. Bovine TG was obtained from Sigma Chemical Co., St. Louis, MO. Monkey TG was from a stock prepared by C. Shapland (1957). Other species of TG were kindly provided by B. Champion (Champion et al., 1987b).

2.3 MONOCLONAL ANTIBODIES

Monoclonal anti-human thyroglobulin antibodies (see Appendix 2) were kindly provided by P. Shepherd (Department of Chemical Pathology, Guy's Hospital Medical School, London). Monoclonal antibodies 5F6 and 1A10 are mouse anti-chicken TG. 1A10 is an IgG_1 antibody which binds to all species of TG that contain thyroxine (T4) residues (Chan et al., 1986). Monoclonals (P11, D8, P12G5, P12,C12, H3D7) are all IgG_1 antibodies which recognize only mouse and rat TG (Champion et al., 1987b). G4F6 is IgG_{2a} and recognizes many species of TG. Other anti-mouse TG monoclonal antibodies used were P4G11, P15G15, F5B3. These monoclonals were prepared by R. Quarty-Papafio.

Monoclonal antibodies to mouse MHC class II antigens were: Anti-Ia.7 (Becton Dickinson), a mouse IgG_{2a} with specificity for the I-E subregion of the $H\text{-}2^k$ and $H\text{-}2^d$ haplotypes. Anti-$I\text{-}A^k$, anti-$I\text{-}A^d$, anti-I- $E^{k,d}$, all of which were of IgG_{2a} subclass.

2.4 T-CELL LINES AND HYDBRIDOMAS

The T-cell hybridomas CH9, U3 and U10, which were originally produced and characterized by D. Rayner (Rayner et al., 1987), were maintained in RPMI-1640 (Gibco, Paisley, Renfrewshire) supplemented with 10% heat inactivated foetal calf serum (FCS) (Gibco, Myoclone), 2 mM glutamine, 10^{-5} M 2-mercaptoethanol, 100 U/ml benzylpenicillin, 100µg streptomycin, non-essential amino acids and 1 mM sodium pyruvate. CH9 is TG-specific and $I\text{-}A^k$ restricted. U3 is restricted to the class II region of k haplotypes and U10 is restricted to the $I\text{-}A^k$ region. The antigen specificities of these two hybridomas are not known. The T-cell lines MTg9B3 and MTg12B which specifically proliferate to TG in the presence of $I\text{-}A^k$ antigen-presenting cells (Champion et al., 1985) were provided by B. Champion. The IL-2-dependent indicator line CTLL (Gillis et al., 1978) (provided by M. Schreier, Sandoz, Basel, Switzerland) was maintained in complete supplemented RPMI-1640 with 5% FCS and 18% concanavlin A-stimulated rat spleen cell supernatant or 2% phorbol myristate acetate-stimulated EL-4 supernatant as a routine source of IL-2.

2.5 IMMUNIZATION PROTOCOL

To induce EAT, mice were challenged twice, seven days apart, with soluble mouse TG (MTG) (50 μg i.v) followed by 20 μg lipopolysaccharide (LPS) injected i.p. The LPS was extracted from *Escherichia coli* 055:B5 (Difco Laboratories, Detroit, MI). Alternatively, mice were immunized with MTG (50 μg) emulsified in Freund's complete adjuvant (CFA), containing heat-killed *Mycobacterium tuberculosis* (1mg/ml) (Difco) in a 1:1 ratio. Each mouse was injected in the hind foot-pads (f.p) (total volume of 25 μl MTG/CFA per foot). Unless otherwise stated, mice were challenged only once with MTG/CFA.

2.6 SOLID-PHASE RADIOIMMUNOASSAY FOR ANTI-TG ANTIBODIES

Antibody binding activity to TG was analysed by a three-layer direct binding radioimmunoassay. Titertex 96-well microtitre plates were sensitized with 100 μl of the appropriate TG (10μg/ml in PBS) at 4 °C for 24 h. All subsequent steps were carried out out at room temperature. The plates were washed three times with PBS to remove unbound TG and free binding sites were blocked by incubating with 0.5% bovine serum albumin (BSA) in PBS for 1 h. Test antibodies were diluted in PBS containing 0.5% BSA and 0.05% Tween 20 (Sigma). (Each sample was tested in duplicate). After an incubation period of 2 h, the plates were washed 3 times with PBS-Tween before adding 100 μl of ^{125} I-labelled affinity purified sheep anti- mouse $F(ab')_2$ to each well to detect the level of bound antibody. After washing 4 times with PBS- Tween, the activity (c.p.m.) in individual wells was read on a Crystal Multidetector Gamma System (United Technologies Packard).

For the inhibition assays, appropriate dilutions of the antibodies were pre-incubated for 24 h at 4 °C with various concentrations of the inhibiting TG. Each sample was tested for binding to MTG coated plates as described above. However, to minimize antibody exchange between the soluble and solid-phase antigens, the incubation at this stage was for only 30 minutes.

2.7 HISTOLOGY OF THYROID GLANDS

Mice were killed by asphyxiation with carbon dioxide (CO_2). The portion of trachea containing thyroid tissue was taken "en bloc" and fixed in 10% buffered formaldehyde. All thyroids were marked on the dorsal side prior to embedding in paraffin so that their orientation was consistent. Transverse thyroid sections were stained with haematoxylin and eosin. Sections were taken at six different levels and at each level two consecutive sections were taken. All sections were scored blind; scores were confirmed by D. Rayner, who kindly provided a second expert opinion.

For each thyroid all sections were assessed for lymphocytic infiltration to give an overall enumeration. The thyroiditis score was based on a ranking scale of 0-4:

0 = No infiltration
1 = 1-2 inflammatory foci <500 μm maximal dimension
2 = > 2 foci or one focus >500 μm maximal dimension
3 = > 40% of the thyroid interstitium infiltrated
4 = diffuse (> 70%) inflammation

2.8 PREPARATION OF SPLEEN CELLS FOR TRANSFER EXPERIMENTS

Single-cell spleen suspensions were obtained by gently teasing the spleen with flat-ended forceps in serum-free medium (RPMI-1640, Gibco, Paisley, Renfrewshire). Erythrocytes were lysed in 0.83% ammonium chloride buffer. The cell suspension was washed three times and resuspended at the required concentration in RPMI-1640. The appropriate number of cells were transferred into recipients in 0.5ml i.v.

2.9 TOLERIZATION AND IMMUNIZATION PROTOCOL

A breeding programme was set up to obtain groups of neonatal CBA/J mice which were used for the neonatal tolerance experiments. Sterilised antigen (TG or KLH (Calbiochem Ltd. (Cambridge Bioscience, Cambridge)) was injected intra-peritoneally (i.p) into mice within 2-4 h of birth. A sterilised 0.5 inch gauge needle was used to inject the

antigen in a total volume of 50μl into each mouse. Control groups of mice received PBS only.

At six weeks of age, treated and untreated controls were challenged with the respective antigens using the immunization protocols as described. Or, spleens from TG pretreated and control groups were removed and prepared for cell transfer experiments. The protocol of Parish et al., (1988) was used. Syngeneic recipients were irradiated (200 rads) in a cobalt source (courtesy of the Department of Zoology, University College London) on the day of transfer. They received 4 X 10^7 spleen cells (SC) i.v. from the appropriate donors and were immunized 24 h after SC transfer.

2.10 INDUCTION OF ANTI-ERYTHROCYTE ANTIBODY WITH PIG-TG COUPLED RAT RED BLOOD CELLS (pigTG-RBC)

The previously established (Cooke et al., 1978) regime for inducing anti-erythrocyte autoantibody was used: CBA/J mice received a total of four injections (i.p) of 2 x 10^8 pigTG-RBC at weekly intervals. Anti-erythrocyte antibody was detected by a direct Coombs test (DCT) using a rabbit antiserum to mouse immunoglobulin. A single batch of rabbit anti-mouse Ig (shown by immunoelectrophoresis to react against IgG and IgM) was stored in small aliquots at -20° C and used at a standard final dilution of 1 in 40. Mice were tail-bled at weekly intervals, red blood cells were washed three times, resuspended to 6%, and 25 μl put onto microscope slides together with 25 μl of rabbit anti-mouse Ig. After a 20 min incubation, the degree of agglutination was scored using a semi-quantitative scoring system: 1) agglutination visible only under the microscope, 2) agglutination visible with the naked eye 3) most cells agglutinated 4) massive agglutination involving all the cells. Anti-rat RBC were measured directly in microtitre plates. Starting with an initial dilution of 1 in 10, doubling dilutions of heat-inactivated mouse sera and freshly washed rat RBC (at 2% in 1% BSA/phosphate buffered saline) were incubated in microtitre wells. The results are expressed as the final well in which agglutination was visible.

To prepare thyroglobulin coupled-RBC, pig Tg was added to washed rat RBC such that the final reaction mixture corresponded to 200 μg TG/2 x

10^8 cells per mouse. The coupling reaction, mediated by chromic chloride (6.6 mg/ml), was carried out in a blocked universal by rotating at 37 °C for 40 min. The reaction was stopped by washing with PBS, pH 7.2 (400 g for 10 min). This washing procedure was repeated twice. The success of the coupling reaction was assessed by haemagglutination using rabbit anti-pig TG antisera. Whenever cell suspensions were prepared, viability was greater than 80%.

For the transfer experiments, spleens were removed from rat RBC or pig TG-RBC pretreated donor mice at 14 wk after the first injection. Spleen cells were prepared, and 40 x 10^6 cells were transferred i.v in 0.5 ml PBS to syngeneic recipients. Twenty-four hours later recipients were challenged with the first dose of rat RBC or pig TG-RBC as described above. The anti-TG antibodies were measured by solid-phase RIA.

2.11 THYROID CELL CULTURES

Mouse thyroid cells were prepared by a modification of the procedure described by Creemers et al. (1983). CBA/J (unless otherwise specified) mice were killed by asphyxiation with CO_2 and thyroids were removed asceptically and kept at 4 °C in RPMI-1640 supplemented with 100μU/ml benzylpenicillin and 100 μg/ml streptomycin. Although the thyroids of older mice are increased in size, thyroid cells obtained from them consistently failed to grow into confluent monolayers, therefore only thyroids from 6-8 week old animals were used. The thyroids were washed with fresh RPMI-1640 and then minced with fine scissors in 5ml of RPMI-1640 supplemented with 0.5% heat inactivated foetal calf serum (FCS) (Gibco, Myoclone) and 1.5 mg/ml collagenase (Worthington type IV). The suspension was incubated at 37 °C on a roller for 30-45 minutes; longer periods of incubation produced a higher percentage of single cells, however they failed to adhere and form monolayers. The enzyme treated thyroid suspension, consisting of single thyroid cells as well as complete and partially disrupted follicles, was filtered through a 150 μm pore-size nylon gauze. Cell viability was assessed by a differential staining with an acridine orange/ethidium bromide mixture under fluorescent microscopy (Lee et al., 1975). The filtrate was diluted to a final volume of 15ml with 20% FCS in RPMI-1640 and centrifuged for 10 min at 200g. The pellet was resuspended in RPMI-1640 supplemented with 10% FCS and the washing

procedure was repeated twice. After the final wash the pellet was resuspended in thyroid culture medium (see below) containing 10% FCS and dispensed (100 µl/well) into flat-bottomed 96-well microtitre plates (Sterilin, Teddington, Middlesex). Since it was not possible to calculate the cell density, thyroids from 8-10 mice were used per plate: Satisfactory monolayers were not obtained when smaller numbers of mice were used. The cultures were kept at 37°C in a 5% CO_2 humidified cell incubator. After 24hrs, nonadherent cells were removed by washing individual wells atleast 3 times with RPMI-1640 and fresh culture medium (200 µl) was added . Every second day thereafter 100 µl of supernatant was removed and replaced by fresh medium.

2.11.1 Culture medium for thyroid cells

Thyroid cells were cultured in RPMI-1640 medium supplemented with: 1 µM sodium iodide, 2mM glutamine, 8 µg/ml insulin (Novo), 5 µg/ml transferrin (Sigma) 10^{-8} M hydrocortisone (Sigma) and heat inactivated foetal calf serum (FCS) (Gigco, Myoclone) (Pujol-Borrel et al., 1983). The percentage of FCS in the medium was varied between 10-1%. Where required, the following factors were also added to the culture medium: 1 mM dibutyryladenosine 3', 5'-cyclic monophosphate (cAMP) (Sigma), 60 µU/ml bovine thyroid- stimulating hormone (TSH) (Thyrotropar, Armour pharmacutical company Ltd.), or recombinant rat interferon-gamma (IFN-γ), which was a gift from A. Cooke. The IFN-γ was produced in Chinese hamster ovary cells transfected with a chromosomal rat IFN-γ gene and was purified by antibody affinity chromatography (Van der Meide et al., 1986).

2.12 IMMUNOFLUORESCENT STAINING OF MONOLAYER CULTURES OF THYROID CELLS

Thyroid cell cultures were prepared as described above and 200 µl of the cell suspension was plated onto round glass coverslips, 13 mm in diameter, placed in 24 well culture plates (Linbro).

Human thyroid cells were kindly prepared by L. Hammond as previously described (Khoury et al., 1981). After 24 h the culture medium for both mouse and human thyroid cells was replaced with 1% FCS supplemented medium.

Viable monolayers were stained on the coverslips from day 1 to day 7 using previously described procedures (Khoury et al., 1981; Pujol-Borrell et al., 1983): Cultures were washed before and after incubations with Hank's balanced salt solution containing 1% bovine serum albumin (Sigma). All incubation and washing steps were at room temperature. Cultures were incubated with 60 µl of appropriately prediluted mouse monoclonal or polyclonal antibodies for 30 minutes followed by fluorescein-isothiocyanate (FITC) conjugated rabbit anti-mouse immunoglobulin (Dako, High Wycombe, UK) diluted at 1:20. (For cultures incubated with human sera, positive or negative for microsomal antigens, a FITC- conjugated rabbit anti-human IgG was used). Monolayers were washed and finally fixed with a chilled (- 20 °C) solution of 5% acetic acid in ethanol for 10 min. To detect intracytoplasmic antigens the monolayers were fixed with a chilled solution of 50% acetone/50% methanol for 10 minutes before the staining procedure. The coverslips were mounted in glycerol onto microscope slides and staining of cultures was analysed by phase and fluorescence microscopy using a Zeiss Photomicroscope III.

The method of analysis for these studies was qualitative rather than quantitative because of the heterogeneity of the intensity of fluorescence between the cells in culture. For example, depending on the antibody and the culture conditions, the intensity of cytoplasmic stain was variable between smaller, central cells, and large, peripheral cells (FIG. 2.1). Nevertheless, it was useful to ascribe fluorescence intensities to each section. A scale of 0-12+ was used.

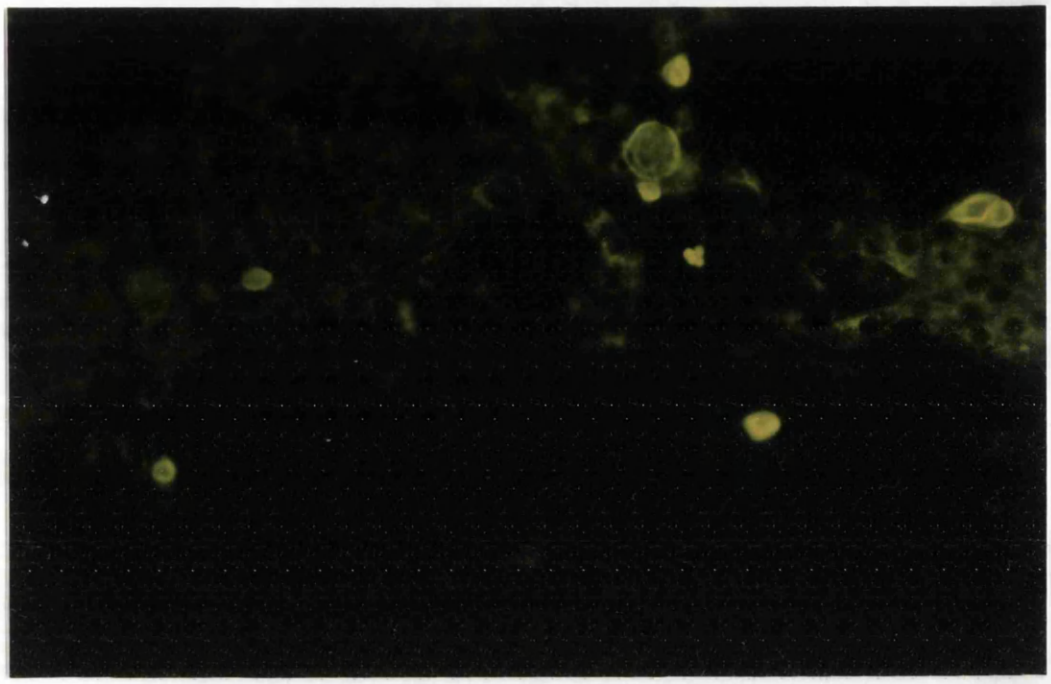
2.13 MOUSE THYROGLOBULIN (MTG) ASSAY

The concentrations of MTG in thyroid culture supernatants were determined by a solid-phase inhibition RIA. Ninety-six well round-bottomed-plates were coated as before with affinity-purified rabbit anti-mouse TG antibodies (10 µg/ml). Incubation of 100 µl of the test supernatants was carried out at room temperature for 45 min. (For the standard curve 100 µl of MTG at different dilutions was used.) The test solution was discarded before incubation with 100 µl of ^{125}I-MTG. The plates were washed four times and individual wells counted in the Gamma Counter. The concentration of MTG in the test supernants was determined from log graphs of the standard curves. The

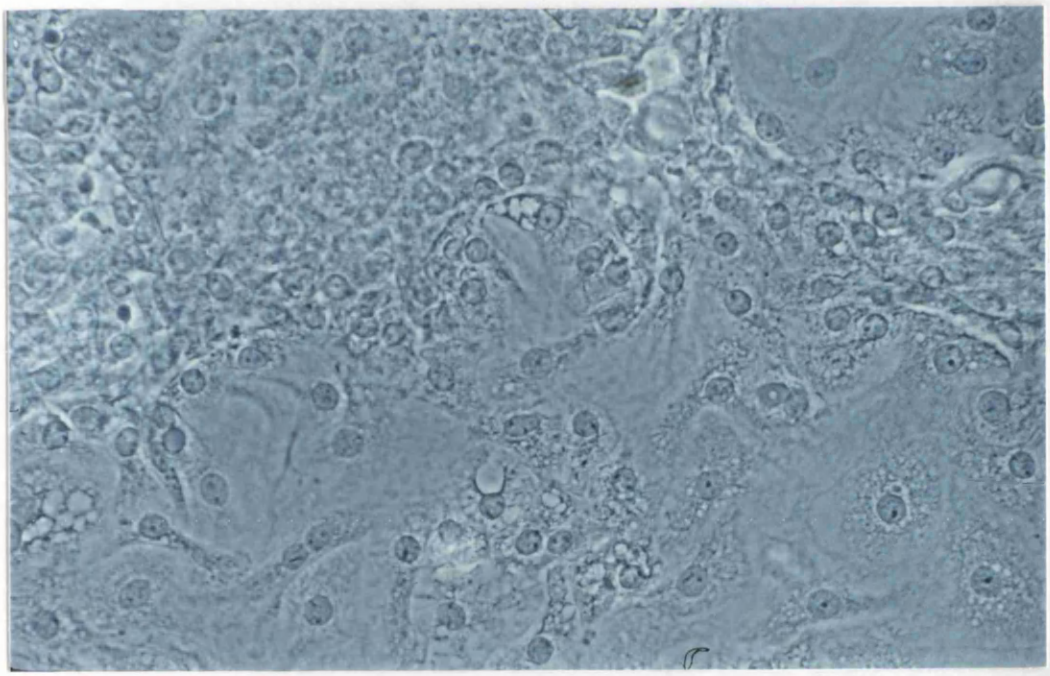
sensitivity of this assay was 0.1-0.3 μg/ml MTG.

The specificity of this assay with respect to TG was first determined by assessing the ability of different species of TG to inhibit the binding of ^{125}I-MTG. Inhibition of binding was only observed with MTG (FIG. 2.2). Not even 50 μg/ml of wallaby, pig-tail monkey, zebra or human TG could inhibit the binding activity. The non-specific antigen, human gamma globulin (HGG), also did not interfere with the specific binding.

To see whether this assay measured whole TG molecules or peptide fragments, preparations of MTG were digested with trypsin (Type XI, from bovine pancreas, Sigma) for various periods of time. Trypsin inhibitor (8 mg/ml)(Type-II-S, Sigma) was added to the the reaction mixture (2 mg trypsin/1 mg TG) to stop the digestion. These TG digests were then used to inhibit the binding of ^{125}I-MTG. A significant decrease in the inhibitory activity was observed when the TG had been treated with trypsin for only 30 minutes. With 2 or 4 h digestion, the TG preparation had only minimal inhibitory capacity (FIG 2.3). Therefore, this assay was only able to measure unfragmented MTG.



a



b

FIGURE 2.1: Expression of TG in follicular cells varies with cell size. Cytoplasmic expression of mab P11 specific epitopes in monolayer cultures of IFN-γ stimulated mouse TEC was determined by indirect immunofluorescence (a). Phase contrast of the same field (b) shows that under these culture conditions, the larger cells do not have detectable levels of TG molecules expressing this epitope. (Original magnification X 250)

INHIBITION ASSAY SPECIFIC FOR MOUSE TG

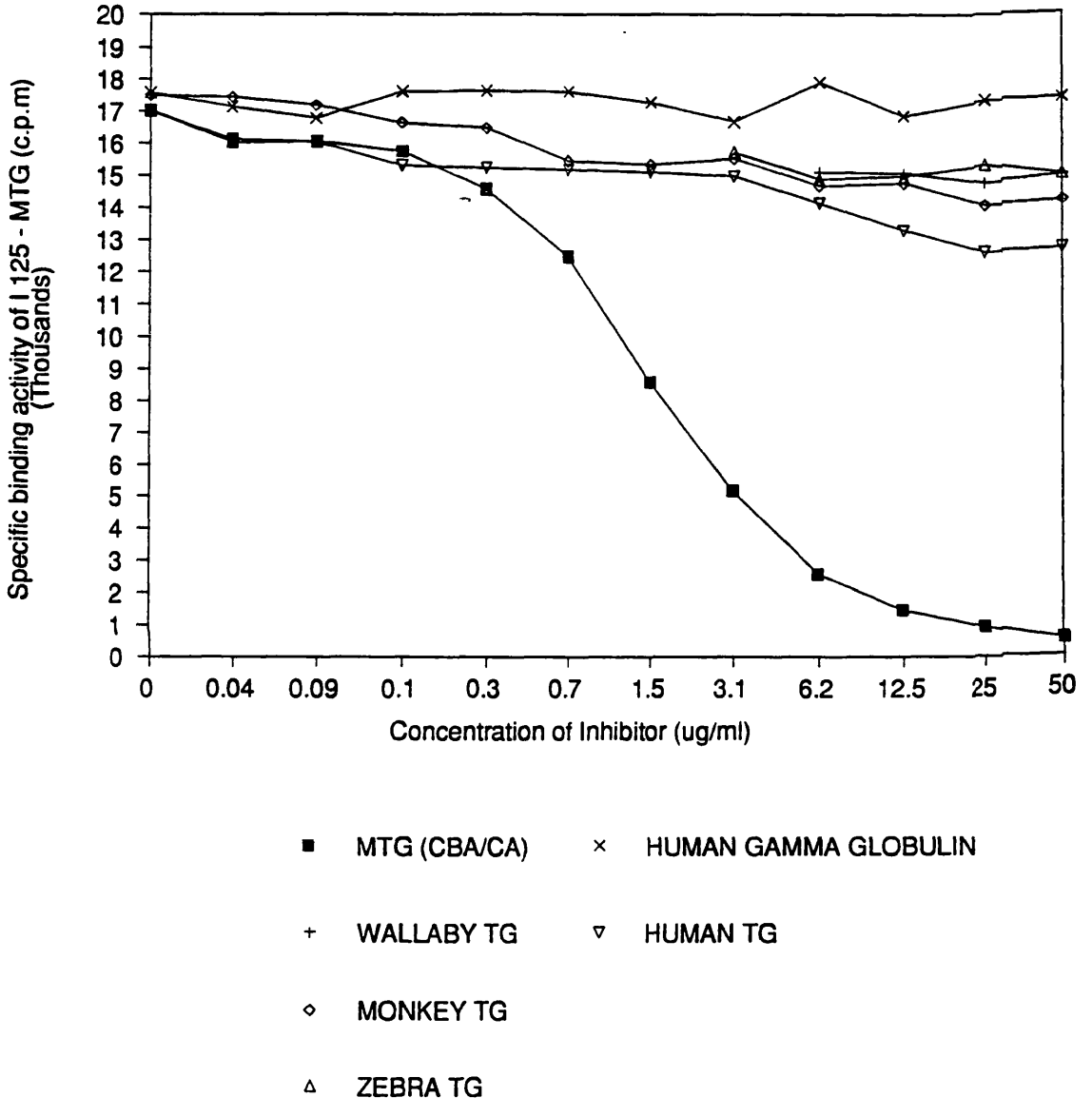


FIGURE 2.2: Only MTG effectively inhibited the binding of ^{125}I-MTG to Solid phase rabbit anti-MTG antibody.

INHIBITION ASSAY FOR MTG: PEPTIDE FRAGMENTS ARE NOT DETECTED

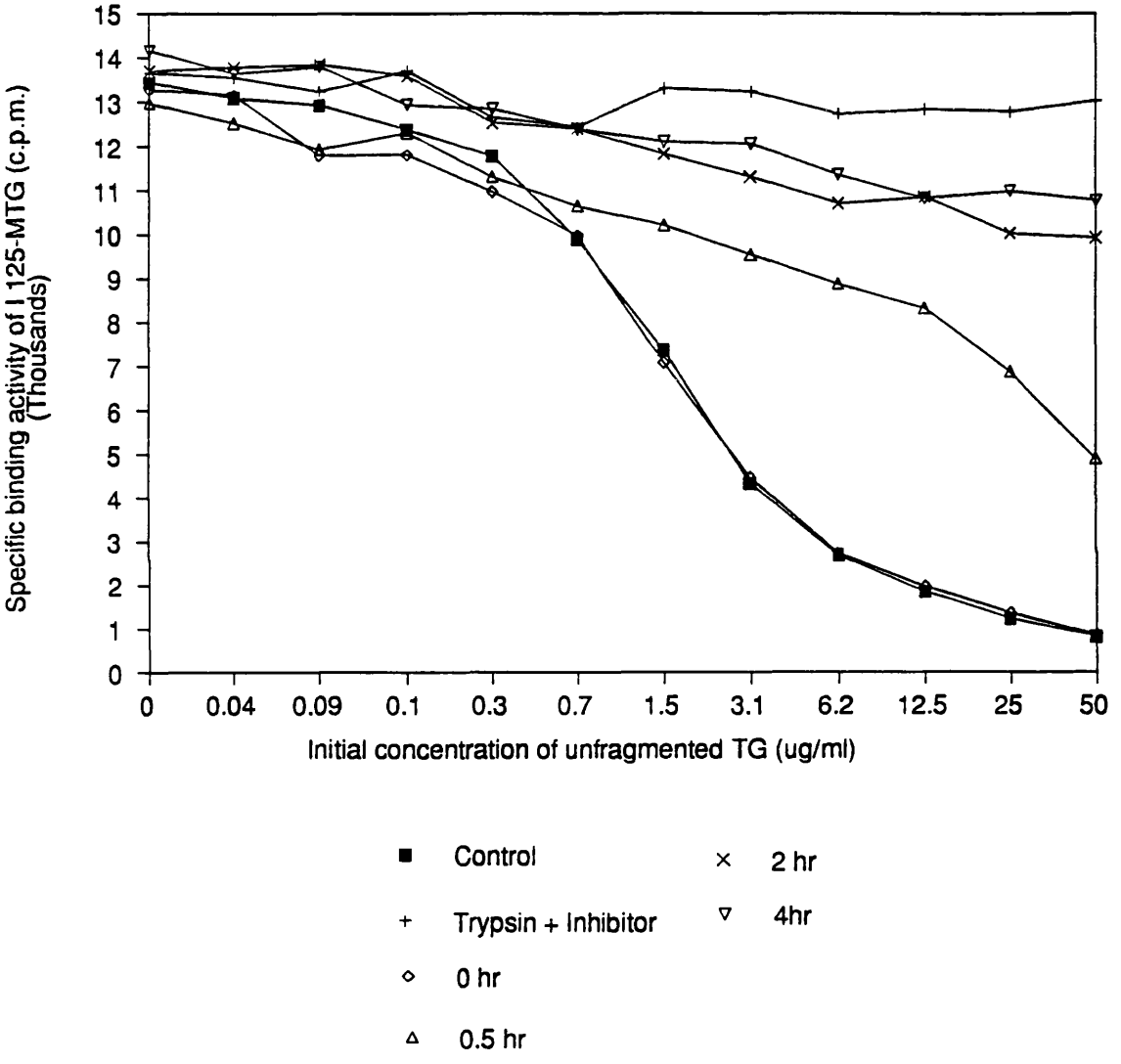


FIGURE 2.3: Serial concentrations of MTG were digested with Trypsin for 0, 0.5, 2 and 4 hours. The specific inhibition of ^{125}I-MTG binding by each sample was assayed under standard conditions. The binding activity of ^{125}I-MTG was not diminished by trypsin + trypsin inhibitor alone. The binding activity for MTG treated with Trypsin for 0 hrs was identical to that of MTG alone.

2.14 IL-2 RELEASE ASSAY

Determination of the functional activation of T-cell hybridomas by murine TEC was based on the antigen- specific release of IL-2 (Kappler et al., 1981). On day 4 or 5 of culture TEC monolayers were washed three times with RPMI-1640 and T-cell hybridomas (10^5) or lines (2 x 10^4) per well were added in a total volume of 200 µl. After culturing for 24 h, 100 µl of the supernatants were transferred to the corresponding 96-well plates. For controls, irradiated (200 rads) syngeneic spleen cells (5 x 10^5) alone, or pulsed with MTG (50µg/ml), were used as a source of antigen-presenting cells. IL-2 in test supernatants was assayed by measuring the proliferative response of CTLL cells. The sensitivity of the CTLL assay to low levels of IL-2 was variable depending on the state of the cells, but generally optimal results were obtained when 2 x 10^4 CTLL cells were used and the cells pulsed at 13 h for 6 h with 18 kBq ^{125}I-deoxyuridine (^{125}IUdR) (Amersham) prior to harvesting onto glass fibre discs with a Titertek cell harvester (Flow Laboratories). Specific IL-2 activity was expressed in terms of ^{125}IUdR incorporation (counts per minute) by the CTLL cells minus the mean c.p.m. of the background response in the absence of IL-2.

2.15 STATISTICAL ANALYSIS

Two tailed Student's t-Test was applied to the data for the antibody binding and T-cell proliferation assays. Statistical analysis of the difference between two sample means was calculated using the formula for an estimated value of variance:

$$s^2 = \frac{\Sigma (x_1 - x_1)^2 + \Sigma (x_2 - x_2)^2}{n_1 + n_2 - 2}$$

So that: $$t = \frac{x_1 - x_2}{s*(1/n_1 + 1/n_2)^{0.5}}$$

and the appropriate t-distribution is that for $\upsilon = n_1 + n_2 - 2$, the number of degrees of freedom in s^2.
The t statistic was calculated by inputting the experimental data into a computer spreadsheet.

For the thyroiditis scores, the non-parametric Mann-Whitney U Test was used to test whether two independent groups had been drawn from the same population (Siegel, 1956).

CHAPTER 3

THE INFLUENCE OF ADJUVANT ON MURINE EAT

3.1 INTRODUCTION

The initiating trigger for autoantibodies to TG in patients with Hashimoto's thyroiditis or with Graves' disease is unknown. Experimental evidence indicates that autoantibodies can be triggered (El Rehewey et al., 1981; Romball & Weigle, 1984) and maintained (Roitt, 1984) by TG. However, it is not clear whether autoantibodies are provoked because of a structural modification of the TG which makes it immunogenic, or if native TG is the immunogen giving rise to antibodies in the situation where self-tolerance mechanisms have been otherwise altered. Inhibition studies (De Carvalho et al., 1982; Sanker et al., 1983) established that there was no difference in the binding specificity of the naturally occurring TG antibodies in the sera of OS chickens towards OS, Cornell strain and normal TG. On the other hand, Romball & Weigle (1984) observed that immunization with periodic low doses of soluble homologous TG induces an antibody response that is less than 1% of the response induced by soluble heterologous TG. If altered tissue components are responsible for autoimmunity, it appears that the alteration would have to be permanent in the progressive disease (Weigle & Nakamura, 1969).

Murine experimental autoimmune thyroiditis, which is characterized by antibody production to TG and a lymphocytic infiltration of the thyroid gland, has been used as an appropriate model for Hashimoto's thyroiditis. The adjuvant most widely used to induce autoimmune responses to thyroglobulin is complete Freund's adjuvant (Rose et al., 1983), but the administration of MTG followed by LPS also induces moderate to severe thyroiditis in high responder mice (Esquivel et al., 1977). Importantly, mice challenged with CFA or LPS, without MTG, do not have detectable autoantibodies or cellular infiltrates (Esquivel et al., 1977). The mechanism of activation of adjuvant-induced EAT remains unresolved, but as the immunization protocols used with CFA and LPS are different, separate mechanisms may be involved. (An important consideration is the state of the TG molecules at the

time of challenge and whether this may directly determine which cells are activated. In the LPS induced method, the injected TG is more likely to have a native configuration, whereas with the CFA protocol, because the antigen is delivered in an oil-in-water emulsion, some of the "protruding" epitopes may be altered.)

The present experiments were designed to determine if there are qualitative differences between murine EAT induced with these two different adjuvants. Within this study, I have monitored the progression of the disease, by assaying the autoantibody response to MTG and by scoring the incidence of thyroiditis. I have also compared the epitopic specificities of the polyclonal antibody response induced with the two immunization protocols, by analyzing their cross-reactivity profiles with a large panel of TG species. This approach has proved to be a powerful aid in the examination of epitopes recognized by T and B cells (Benjamin et al., 1984) and can be used for TG, because it is a phylogenetically conserved protein and all the coding areas of known sequences reveal considerable nucleotide homology between the species (reviewed in Malthiery et al., 1989).

3.2 RESULTS

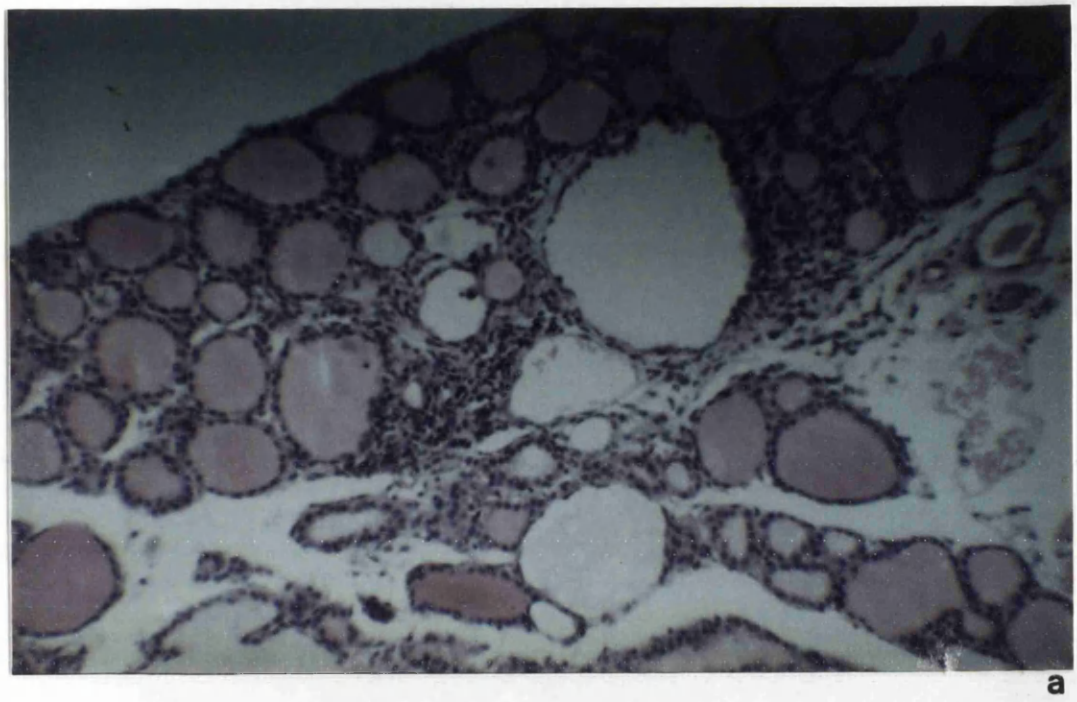
3.2.1 Longitudinal studies of murine EAT

3.2.1.1 Thyroid pathology

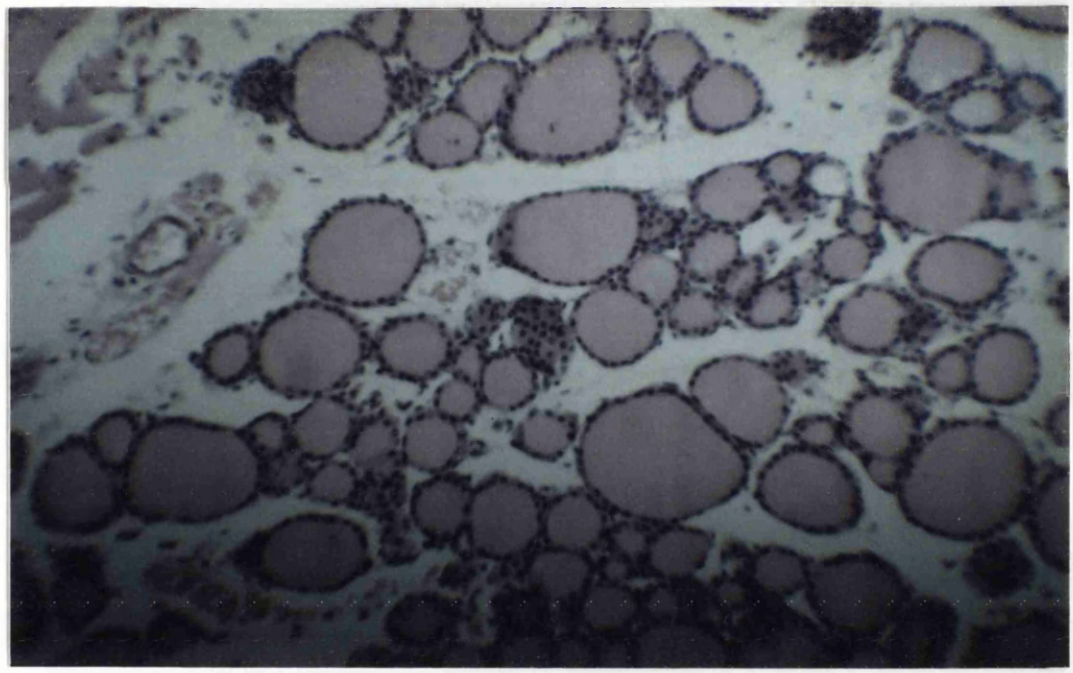
In general, at one month after immunization, thyroid sections normally had large numbers of infiltrating cells. The degree of infiltration observed was, to some extent, variable (both between different sections of the same thyroid and between different thyroids), but usually large areas of the thyroid sections were infiltrated. Fibrosis was not normally observed at this time and actual follicular damage was also variable. In the first experiment of this study, the level of mononuclear infiltration of the thyroid glands induced with MTG/CFA or MTG/LPS was comparable at 4 weeks. Thyroiditis induced with CFA as the adjuvant essentially resolved by 6 months and the glands appeared largely normal: there was minimal or no lymphocytic infiltration and the small numbers of lymphocytes which were observed, were mainly located around small blood vessels. However, a persistent

thyroiditis, with evidence of a chronic inflammatory response, was observed in mice challenged with MTG/LPS (one example section is shown in FIG. 3.1B). At 6 months, the inflammation was diffuse (covering more than 70% of the gland), but mostly interstitial. In some sections a few plasma cells were observed in the interstitium (FIG. 3.1c). Overall, there was minimal follicular damage, but fibrosis was present.

The observation that thyroiditis induced with MTG/LPS was not resolved even 6 months after challenge, was confirmed in three separate experiments. It is important to mention that the thyroiditis scores allocated (see TABLE 3.1) do not justify the qualitative differences observed. This is mainly due to difficulties with the scoring system used, which does not take into account the observed fibrotic reaction. Lymphocytic infiltration of the thyroid gland was seen to persist for as long as 11 months. Since the mean life-span of male CBA/J mice is about 24 months (Foster et al., 1981), continued lymphocitic infiltration over this period of time could realistically be taken to represent chronicity.

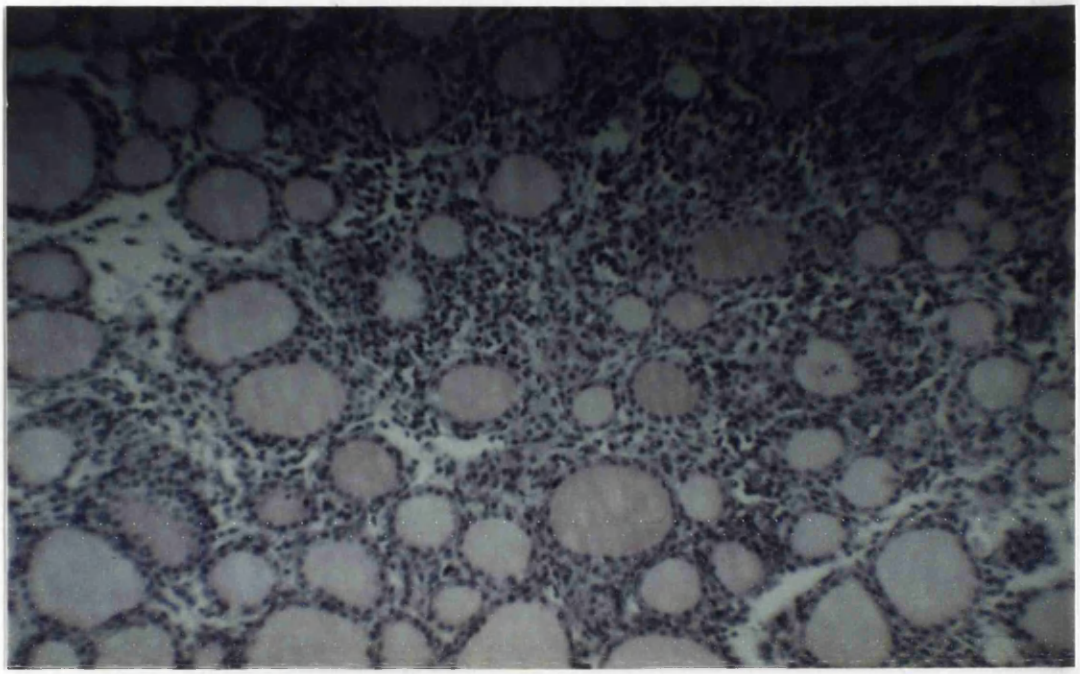


a

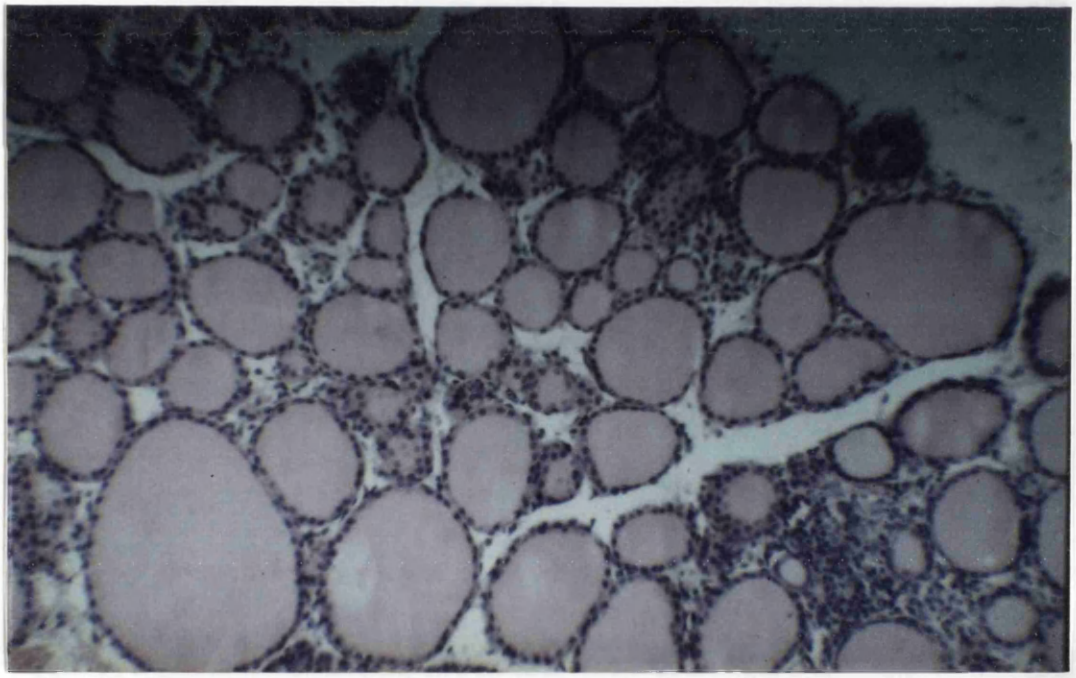


b

FIGURE 3.1A: Example of classical murine experimental autoimmune thyroiditis. Section showing typical lesion of murine EAT as seen 28 days after challenging with MTG and adjuvant (either CFA or LPS) (a). A section of normal mouse thyroid (b) is shown by comparison. Haematoxylin and eosin stain X 160.



a



b

FIGURE 3.1B: Comparison of thyroiditis at 22 weeks. Severe thyroiditis, affecting most of the thyroid gland, was seen to persist in mice challenged with MTG/LPS (a). In contrast, thyroids from mice challenged with MTG/CFA were essentially devoid of lymphocytic infiltrate (b).

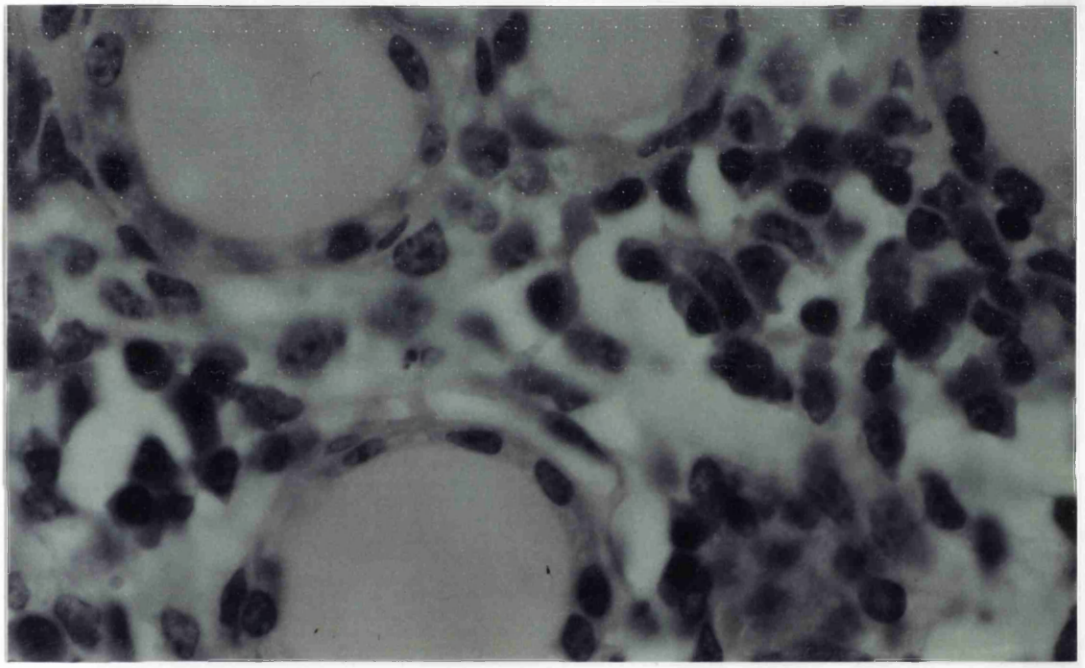


FIGURE 3.1C: Chronic MTG/LPS induced thyroiditis at 22 weeks. Section showing evidence of a plasma cell component in the inflammatory infiltrate. X 400.

TABLE 3.1

INFLUENCE OF ADJUVANT ON THE INCIDENCE OF THYROIDITIS

EXPT	Time Post-Challenge	INDIVIDUAL THYROID SCORES		STATISTICALLY DIFFERENT AT CONFIDENCE LEVEL OF :
		MTG/CFA Immunised	MTG/LPS Immunised	
1	4 wk	0 1 2	1 2 2	No Difference
	22 wk	0 0 0 0 0 1	1 2 2 3 4 4	99.6%
2	1 mo	0 0 0	0 1 1 2 2	96.0%
	3 mo	N.D.	0 1 1 2	
	6 mo	N.D.	0 1 1 1 1	
	9 mo	0 0 0 1 1	1 1 1 2 3	95%
3	1 mo	1 1 1 2	1 1 1 1 1	No Difference
	4 mo	N.D.	1 2 2 3 3	
	5 mo	1 1 1 1 1	N.D.	
	11 mo	0 0 0 1 2	0 1 1 1 1 1	No Difference

In experiments 1 and 2, the MTG/CFA immunised mice were challenged only once (d0, with 50 ug MTG). The MTG/LPS received 100 ug MTG on d0 and 20 ug MTG on d7. In experiment 3 both groups were challenged twice (100 ug d0, 20 ug MTG d7). Small numbers of mice were killed at the times shown and the thyroid pathology for each mouse was scored on a scale of 0 to 4 (see Materials & Methods).

3.2.1.2 Prolonged humoral response to MTG

The anti-MTG antibody titre was determined at several time points after immunisation (FIG. 3.2a). At 4 weeks, there was no difference in the mean antibody titres between MTG/CFA and MTG/LPS groups. By 22 weeks the MTG/CFA response was significantly down-regulated ($P = 0.001$). In contrast the MTG/LPS induced antibody titres remained at the same level as at 4 weeks, although at 16 weeks there was a small fluctuation ($P = 0.1$) in the average range of titre.

To rule out the possibility that the kinetics of the autoantibody response was determined by the higher priming dose of antigen received by the LPS challenged mice, in a second experiment the challenging regime for MTG/CFA was changed to two challenges with a 7 day interval. At one month there was essentially no difference in the antibody titres between the two groups. However, the antigen concentration did not prolong the maintenance of the autoantibody levels in MTG/CFA challenged mice (FIG. 3.2b). The mean antibody titre at 4 months was significantly ($P = 0.001$) different from the mean titre at one month and by 9 months the antibody response was down-regulated to minimal levels. In contrast, the MTG/LPS activated response at this time was shown to be at the same value as at 1 month ($P = 0.5$). Interestingly, a fluctuation in the mean titre was again observed: At 4 months the mean titre was less than the response at 1 month ($P = 0.01$). This fall in the humoral response was still apparent at 7 months after positive challenge ($P = 0.05$).

COMPARISON OF THE ADJUVANT EFFECT OF LPS AND CFA ON THE KINETICS OF ANTIBODY RESPONSE TO MTG

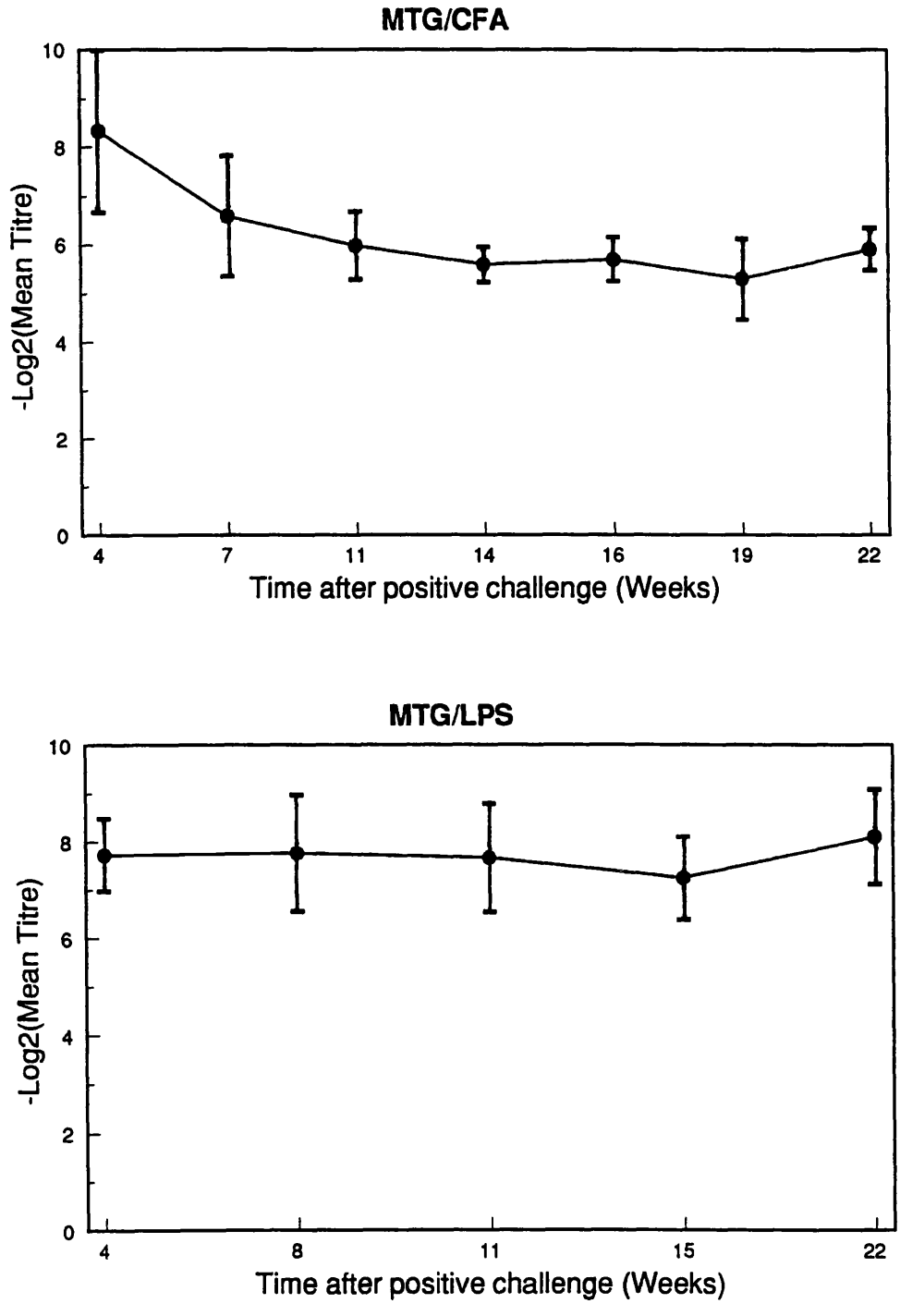


FIGURE 3.2A: Mice were challenged either with 50µg MTG/CFA (d_0), or with 100µg TG/LPS (d_0) and 20µg TG/LPS (d_7). Serum samples were taken at the points shown and the MTG antibody response analysed by RIA. Antibody titres were calculated from serial dilution curves as the intercept at 50% of the total binding activity of a standard positive control. Each point represents the Mean +/- S.D. of six or more values.

THE DOWN-REGULATION OF MTG/CFA INDUCED ANTIBODIES IS NOT ALTERED BY INCREASING THE DOSE OF ANTIGEN

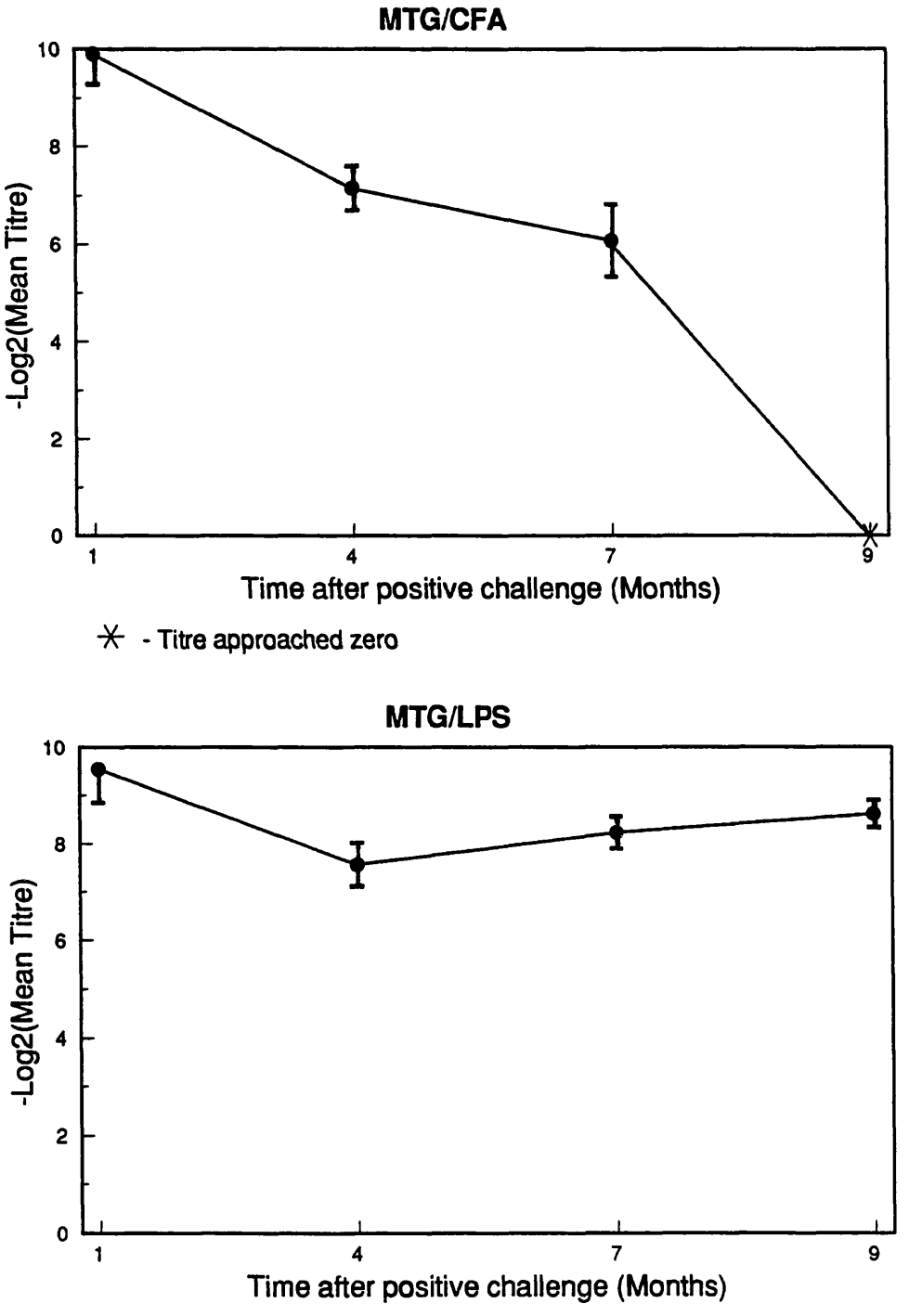


FIGURE 3.2B: Both groups of mice were challenged twice with 100μg (d_0) and 20μg (d_7) using CFA or LPS as adjuvant. Titres were calculated as before. Results shown are Mean +/- S.D values.

3.2.1.3 Qualitative differences in the anti-MTG antibody response

Since the duration of the antibody response clearly differed depending on the adjuvant used, it was of interest to examine the possibility of qualitative differences. The binding of serum antibodies to a number of TG species was tested. To rule out variation in assay conditions all sera from each group were assayed on the same day. As the level of nonspecific background binding differed for each species, the actual binding of individual sera to each species was calculated by subtracting the mean background counts from the total. For all cases, binding of serially diluted sera was determined (FIG. 3.3).

Although the total anti-MTG response at 1 month was not different between the groups, LPS activated a more restricted response than CFA (FIG. 3.4a). At one month all the MTG/CFA induced sera showed a high degree of reactivity to almost all species of TG against which they were tested. There was no apparent correlation between the number of iodothyronione residues on the various TG species (Appendix 1) and the level of cross-reactivity. Therefore, the possibility that this high level of cross-reactivity is due solely to the recognition of the conserved iodothyronine residues on the TG molecule could be ruled out. MTG/LPS immune sera showed high cross-reactivity with only five of the TG species. Interestingly, three of these (rat, Chinese hamster and Brazilian tree porcupine) belong to the order rodentia, and the other two species (pig-tail monkey and human) belong to the order primates.

Analysis of the cross-reactivity profiles at later time points (FIG. 3.4b) suggested that the observed fall in the anti-MTG titres (FIG. 3.2b) was to some extent related to the decreased ($P = 0.01$) binding activity with other species of TG. There was minimal binding activity to other species of TG in MTG/CFA induced sera at 9 months after challenge (data not shown). The fluctuations in the anti-MTG titre of MTG/LPS immune sera was also reflected in the binding activities to other TG species. For example, binding activities to Brazilian tree porcupine TG ($P = 0.01$) and Chinese hamster TG ($P = 0.02$) was decreased between 1 month and 4 months after challenge. There was no change in these specificities between 4 and 7 months. However, there was some indication ($P = 0.1$) that the binding to pig TG was reduced

EXAMPLE OF THE DILUTION CURVES OF MURINE EAT SERA AGAINST VARIOUS TG SPECIES

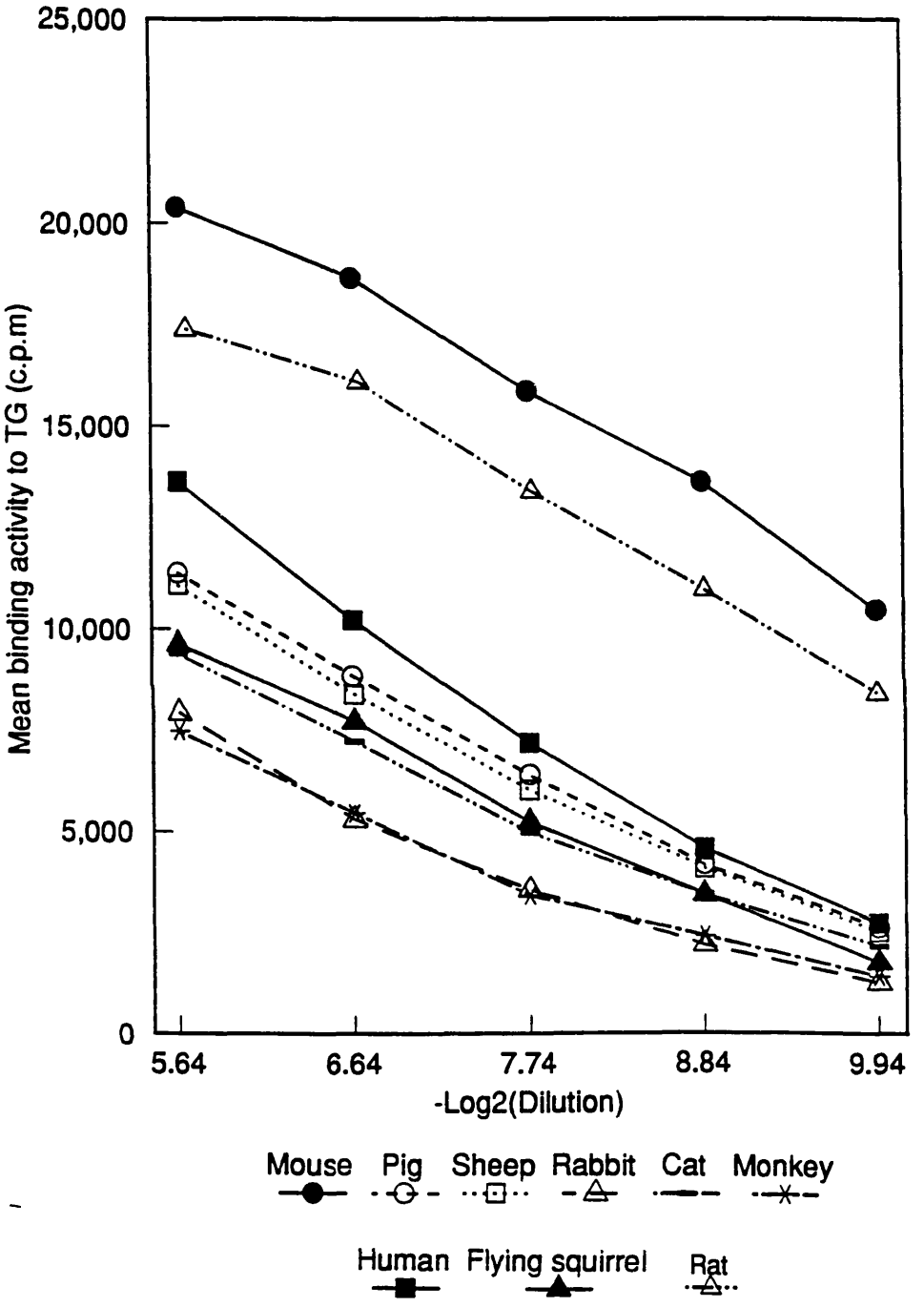


FIGURE 3.3: Serial dilution analysis (by RIA) of sera from mice challenged with MTG/CFA (d0, d7), showing that the binding activity to all species of TG tested decreases with dilution.

Comparison of the Antibody Cross-reactivity Profiles of

MTG/LPS and MTG/CFA Induced EAT Sera

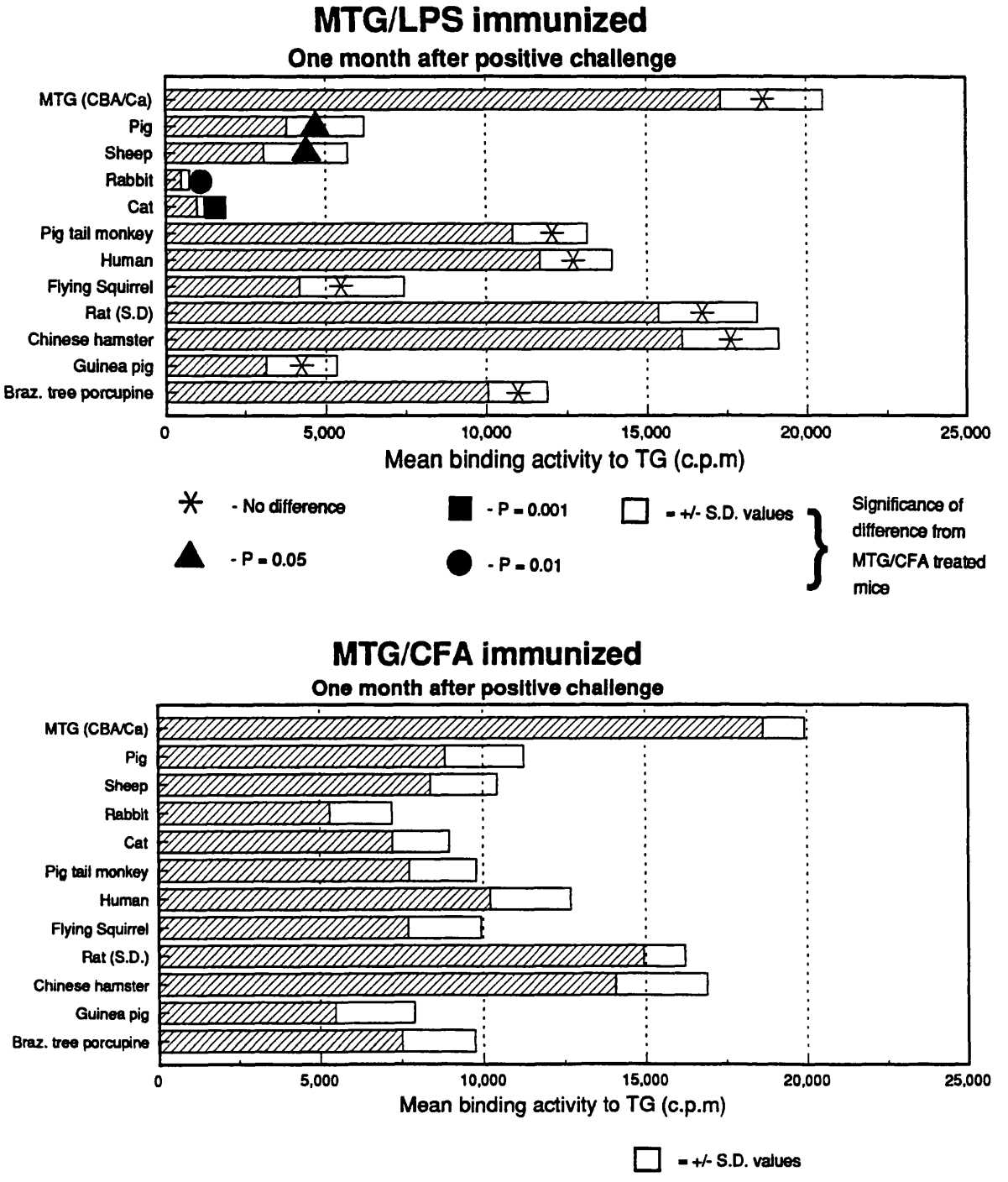


FIGURE 3.4A: The influence of adjuvant on the anti-TG response of sera at one month after positive challenge with either MTG/LPS or MTG/CFA (d_0, d_7). Shaded areas of each bar represents the mean (n=4) binding activities at serum dilutions of 1 in 100. S.D values are indicated by the unshaded portions of each bar.

Down Regulation of Cross-reactive Antibodies With Time

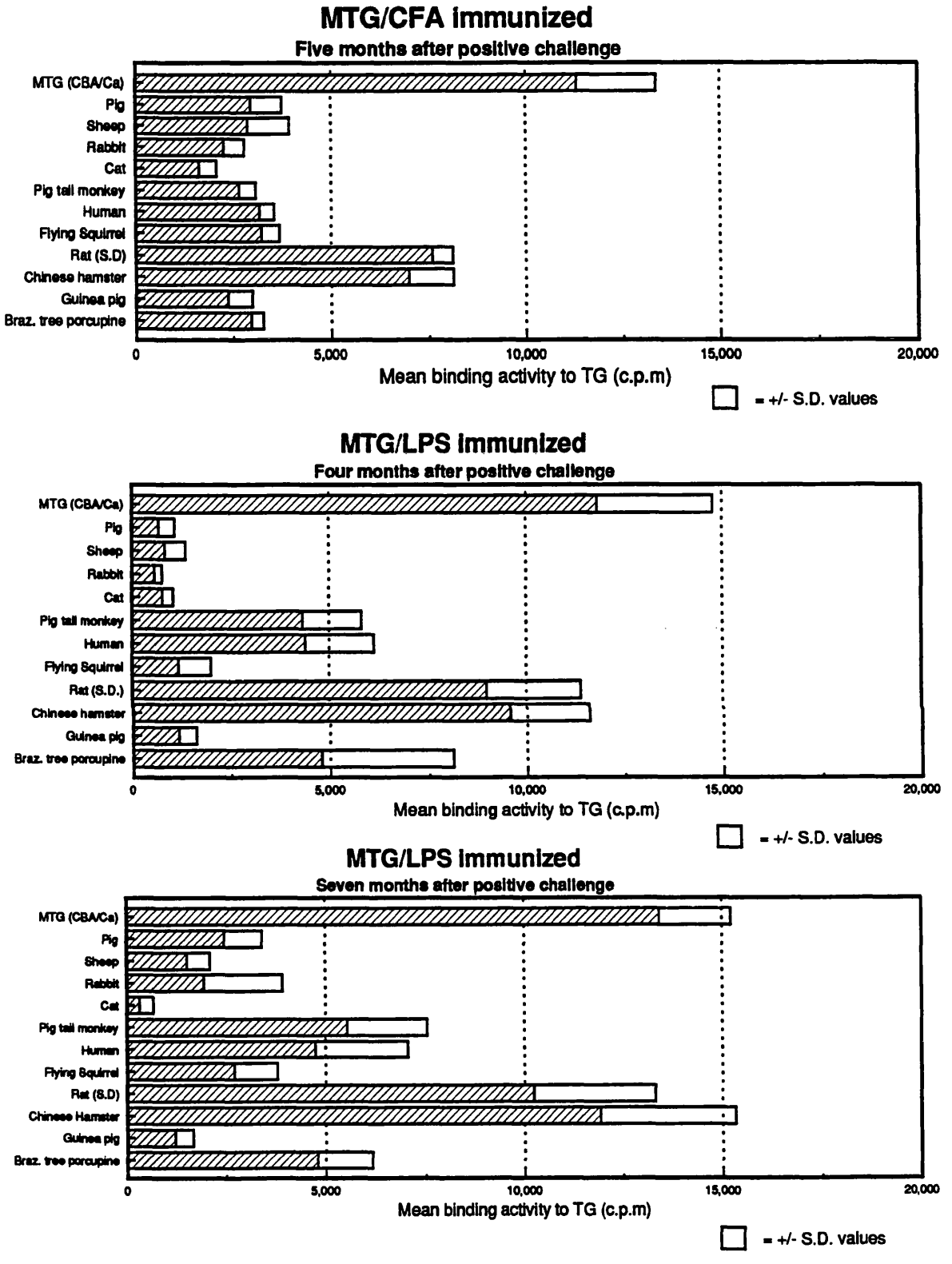


FIGURE 3.4B: Anti-TG binding activities of MTG/CFA and MTG/LPS induced sera taken several months after positive challenge. Results show the Mean +/- S.D. values at sera dilutions of 1 in 100.

between 1 and 4 months and increased again by 7 months. These qualitative differences are indicative of changes in the usage of B cell clones in the course of a prolonged humoral response.

3.2.1.4 Specificity of the autoantibody response

The rather high cross-reactivity of the MTG/CFA induced antibodies suggested that they were not MTG specific. To rule out this possibility, the binding activity to MTG was tested in a soluble inhibition assay. Eighty percent of the binding activity of both MTG/LPS, and MTG/CFA, immune sera to solid phase MTG was inhibited by soluble MTG, although MTG/CFA induced sera from later time points were less specific ($P = 0.001$) ((FIG. 3.5a). Soluble rat TG could also inhibit binding by 35-50% (depending on the source of immune sera) (FIG. 3.5b), suggesting that surface epitopes on rat TG share considerable homology with those of mouse TG. The specificity of the inhibition was demonstrated by the fact that neither rabbit or cat TG could affect the anti-MTG binding activity.

3.2.2 The Anamnestic Response to Thyroglobulin

The qualitative differences in the humoral response to MTG induced by the adjuvants CFA and LPS was investigated further by considering their effect on the anamnestic response to MTG challenge. The immunization and pretreatment protocols was as shown in diagram 3.1.

The mice which had been pretreated with PBS/adjuvant showed a very reduced immune response to positive challenge with MTG plus adjuvant (FIG. 3.6a). Interestingly, although at 22 weeks MTG/CFA and MTG/LPS immune sera showed very little cross- reactivity with sheep, rabbit and dog TG (data not shown), a second challenge with MTG and the respective adjuvant induced an antibody response which was cross-reactive with all three of these TG species. This unexpected response was corroborated by the spleen transfer experiments (FIG. 3.6b). Spleen cells from MTG/LPS, or MTG/CFA immunized mice were transferred to syngeneic recipients, which were immunized 24 hours later with MTG/LPS and MTG/CFA respectively. Control groups of mice received spleen cells from PBS/LPS, or PBS/CFA, treated donors. The

antibody response to MTG was significantly increased (P = 0.001) by adaptive transfer of spleen cells from MTG/LPS donors, compared with the response in mice which had received spleen cells from PBS/LPS treated donors. Furthermore, transfer of spleen cells from MTG/LPS primed donors increased antibody cross-reactivity with heterologous TGs, including sheep and rabbit TG. Although the transfer of spleen cells from MTG/CFA primed donors did not further increase anti-MTG antibody activity, antibody binding to sheep TG was selectively increased (P = 0.05). Within the scope of this study it was not possible to assess the *in vitro* responses of these primed cell populations. Syngeneic recipients of MTG/CFA or MTG/LPS primed spleen cells did not have any detectable anti-TG antibodies in the absence of positive challenge.

ANTI-TG ANTIBODIES IN MURINE EAT RECOGNISE "SURFACE" EPITOPES ON MTG AND RAT TG

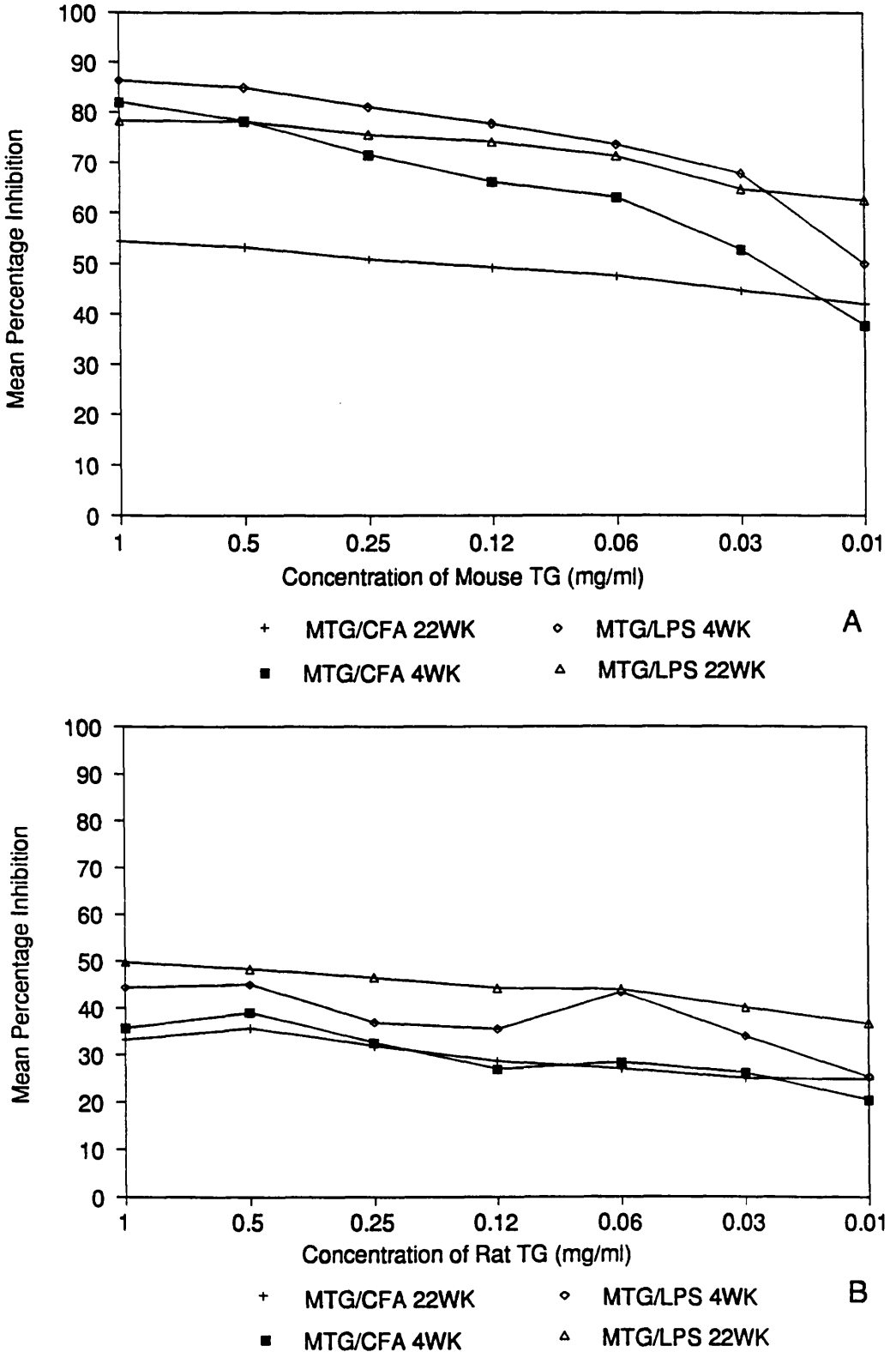


FIGURE 3.5: Soluble mouse (A) or rat (B) TG was used to inhibit the binding of individual sera (dilution at 1/100) to solid phase MTG. Binding activity was determined by RIA. There was no significant difference in the inhibition of sera by rat TG. Only the anti-MTG specificity of MTG/CFA induced antibodies altered with time (n=4, P = 0.001).

PROTOCOL FOR STUDYING THE ANAMNESTIC RESPONSE OF CBA/J MICE TO MOUSE TG CHALLENGE

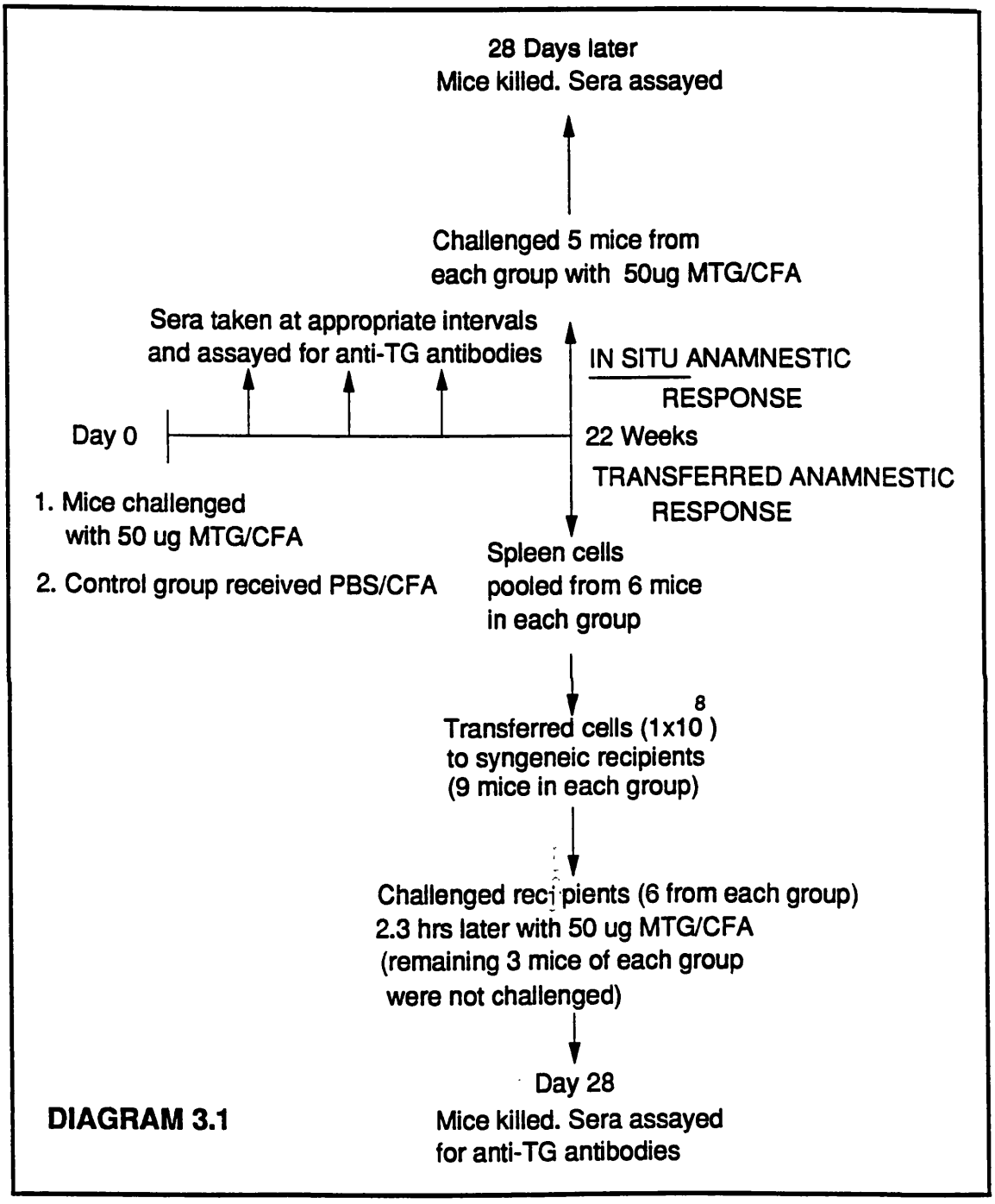
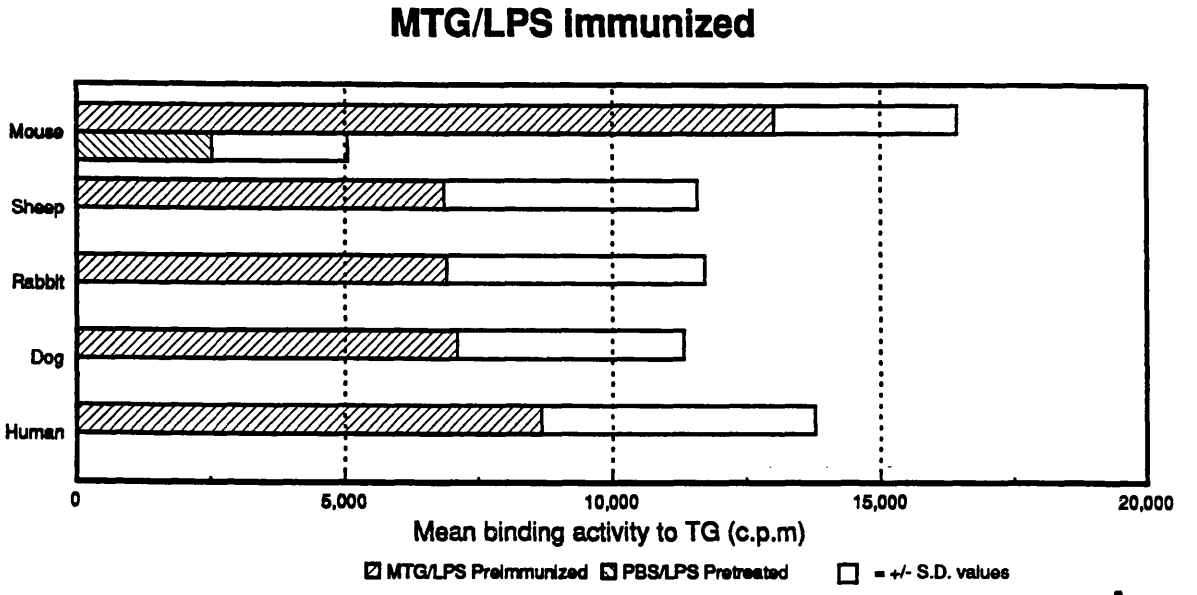


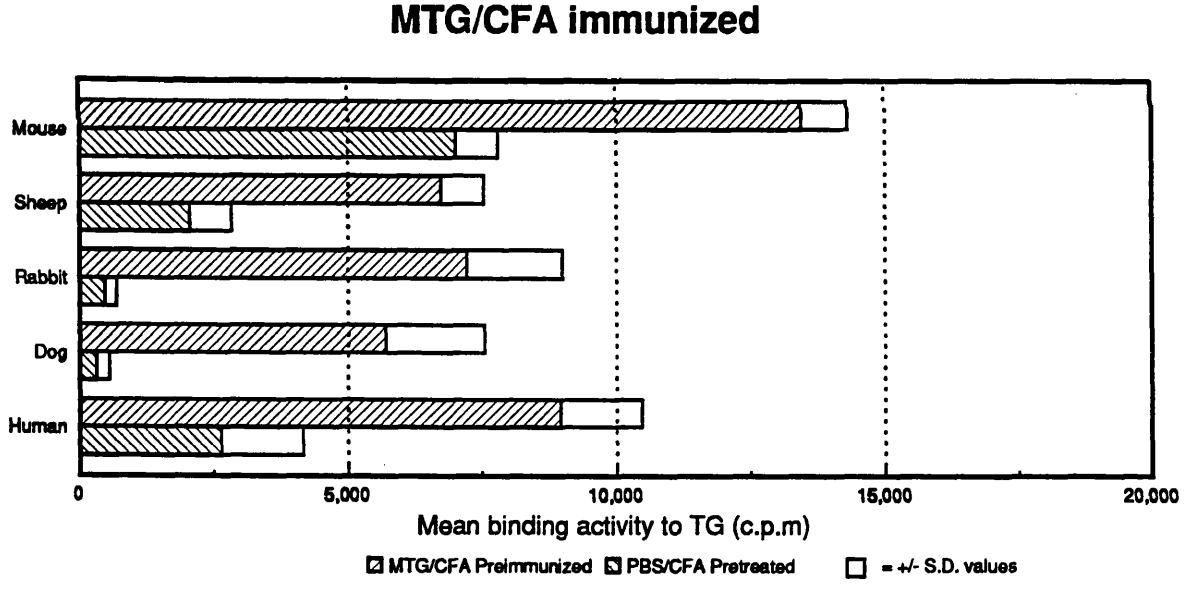
DIAGRAM 3.1

The same protocol was followed for studying the response to MTG/LPS challenge. In this case on day 0, mice (n-15) were challenged with 100 µg MTG/LPS (and 7 days later with 20 µg MTG/LPS). The control groups received PBS/LPS. At 22 weeks all groups of mice were challenged with 40 µg MTG/LPS.

Anamnestic Response In-situ



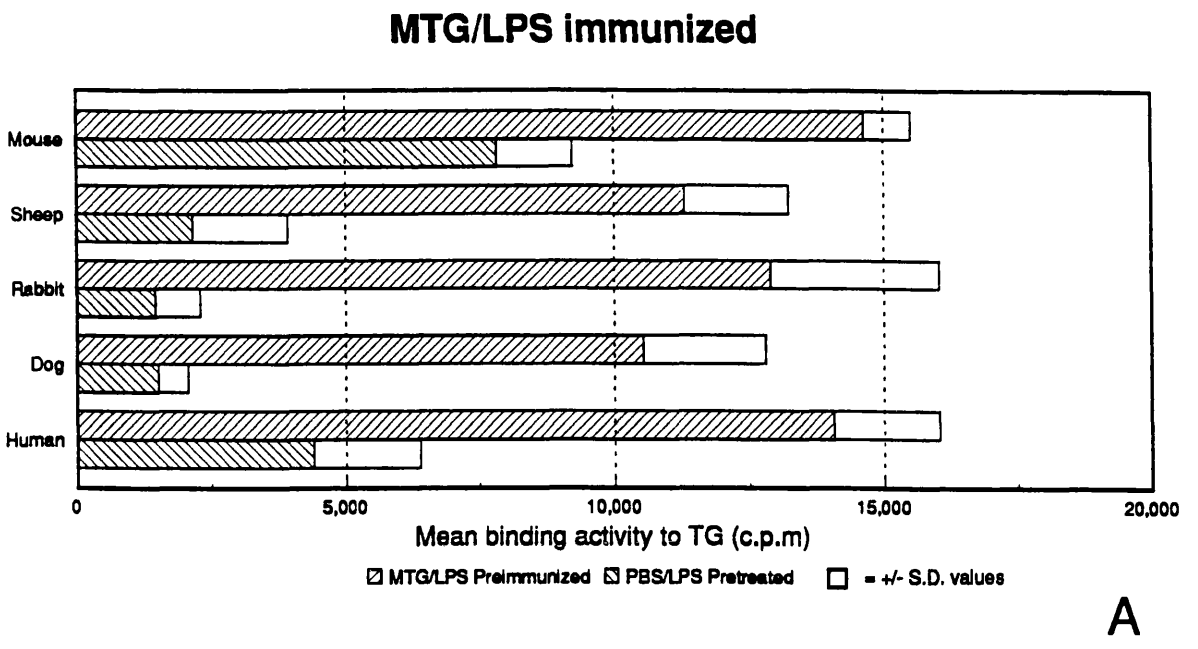
A



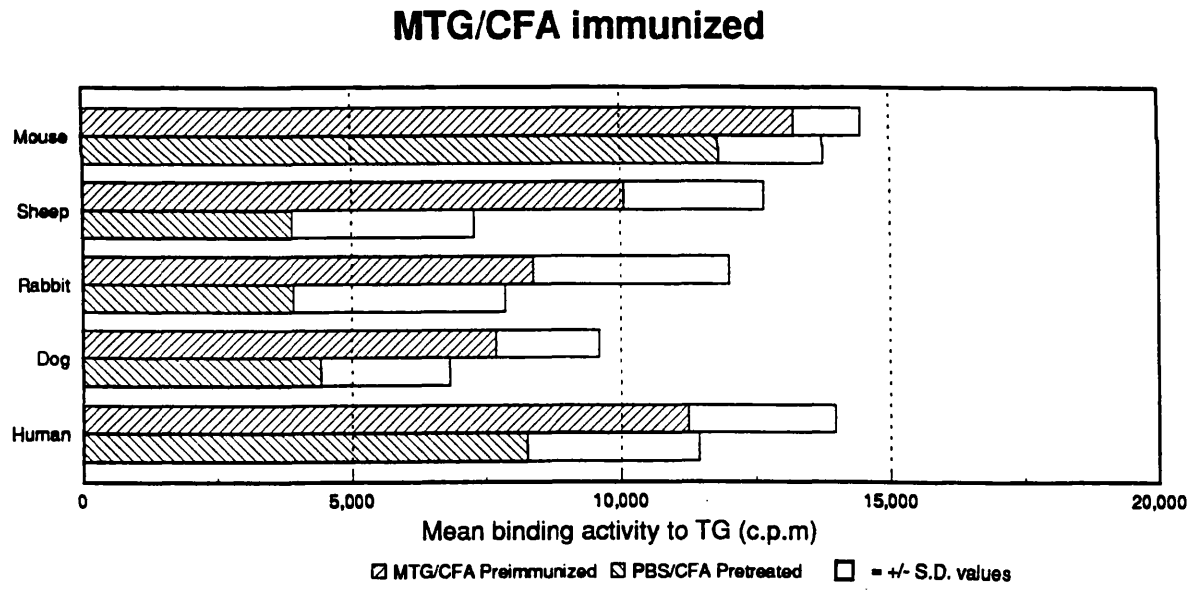
B

FIGURE 3.6A: Mice were challenged as described in Diagram 3.1. Sera collected at 28 days after immunization were assayed for anti-TG binding activities. Results shown are the Mean +/- S.D. values at serum dilutions of 1 in 100 (n=4).

Transferred Anamnestic Response



A



B

FIGURE 3.6B: The protocol for the transfer of spleen cells from MTG/LPS (A) or MTG/CFA (B) primed donors was as previously described (Diagram 3.1). The control group of mice received spleen cells from mice pretreated with adjuvant and PBS. Sera collected at 28 days after immunization were assayed for anti-TG binding activities. Results shown are Mean +/- S.D. values at serum dilutions of 1 in 100.

3.2.3 Mechanism of action of CFA and LPS

The mechanism of action of CFA and LPS has yet to be elucidated. If these models of EAT are to be studied with the view to test putative prophylactic treatments, a clearer understanding of their *in vivo* immunopotentiating mechanism is clearly desirable. The initial activated population of cells may be crucial in determining the end result and we need to determine whether, for example, these adjuvants activate the same population of antigen presenting cells.

Since appropriate antigen presentation is important for T cell activation, it may be that the route of injection will determine which cells present the antigen. Indeed the route by which MTG/CFA is given appears to be important. In an initial study, MTG/CFA given in the footpads elicited a normal and high response to MTG, but mice immunised with the same concentration of MTG and CFA intraperitonally showed a minimal reponse. A similar pattern of response was observed with MTG/IFA immunization (FIG.3.7).

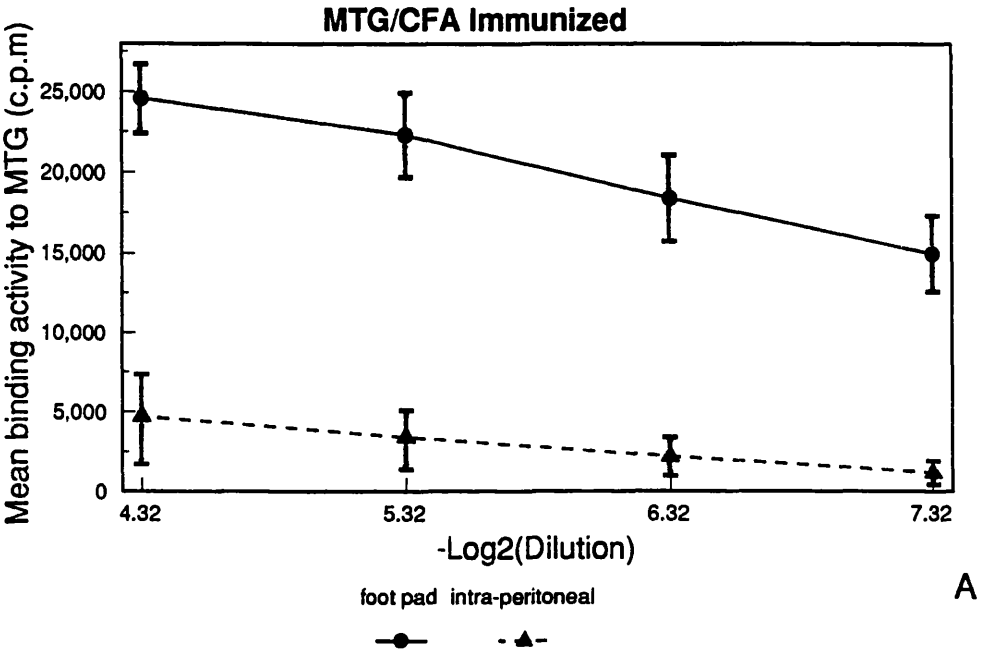
A second approach to address the above question considered the effect of short-term adjuvant pretreatment on a subsequent challenging regimen: Mice were pretreated with PBS/LPS or PBS/CFA and challenged a week later with MTG/LPS or MTG/CFA; the response was compared with non-pretreated controls. The anti-TG cross-reactivity profiles of the antibodies from these groups were analysed. Table 3.2 shows the mean binding activities of antisera from MTG/LPS and MTG/CFA immunized mice. These results confirm the observation that LPS and CFA activate different sets of antibody specificities, with LPS inducing less cross-reactive antibodies. For example, MTG/LPS induced antibodies had virtually no reactivity with reindeer, wombat, or wallabby TG. Pretreating with CFA or LPS had a variable effect on the autoantibody response induced with MTG/LPS (FIG. 3.8a).

Pretreatment with CFA significantly ($P = 0.01$) reduced the autoantibody response. LPS was less effective in down-regulating the antibody response ($P = 0.2$). By contrast neither adjuvant changed the autoantibody response to TG induced with MTG/CFA (FIG. 3.8b). This difference is partly mediated by the presence of the killed mycobac-

teria in CFA, since mice pretreated with incomplete Freund's adjuvant (IFA) and immunized with MTG/IFA had a decreased ($P = 0.01$) antibody response compared with controls (FIG. 3.8c).

Analysis of the cross-reactivity of MTG/IFA induced anti-TG antibodies showed a similar pattern to MTG/CFA induced antibodies (there was no significant difference in the binding activities to the TG species tested), which suggests that the mycobacteria in CFA did not influence the qualitative differences observed between MTG/LPS and MTG/CFA immune sera. Although, the mycobacterial component in CFA may be important in overriding the down-regulatory effect of adjuvant pretreatment, as there was no effect of CFA pretreatment on the MTG/CFA induced antibody response. Importantly, there was a selective effect of IFA pretreatment on the antibody specificities (FIG. 3.9a); of the TG species tested, the binding activity to human ($P = 0.05$) and flying squirrel ($P = 0.01$) was changed. Pretreatment with CFA also had a selective effect on MTG/LPS induced antibodies (FIG. 3.9b), in this case antibodies which were cross-reactive with Chinese hamster and human TG were down-regulated ($P = 0.02$).

THE ROUTE OF IMMUNIZATION INFLUENCES THE HUMORAL RESPONSE TO MOUSE MTG



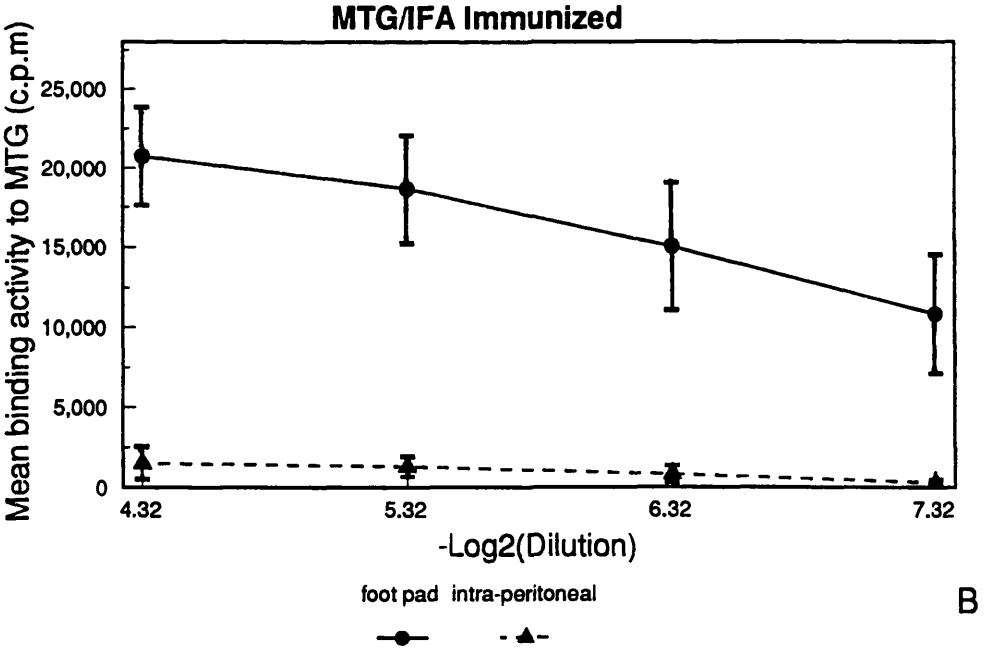


FIGURE 3.7: Mice (n=4) were challenged with either 50μg MTG/CFA (A), or (B), via the foot pads or interperitoneally. Sera taken on day 28 after positive challenge was assayed (RIA) for binding activity to mouse TG. Results shown are Means +/- S.D.

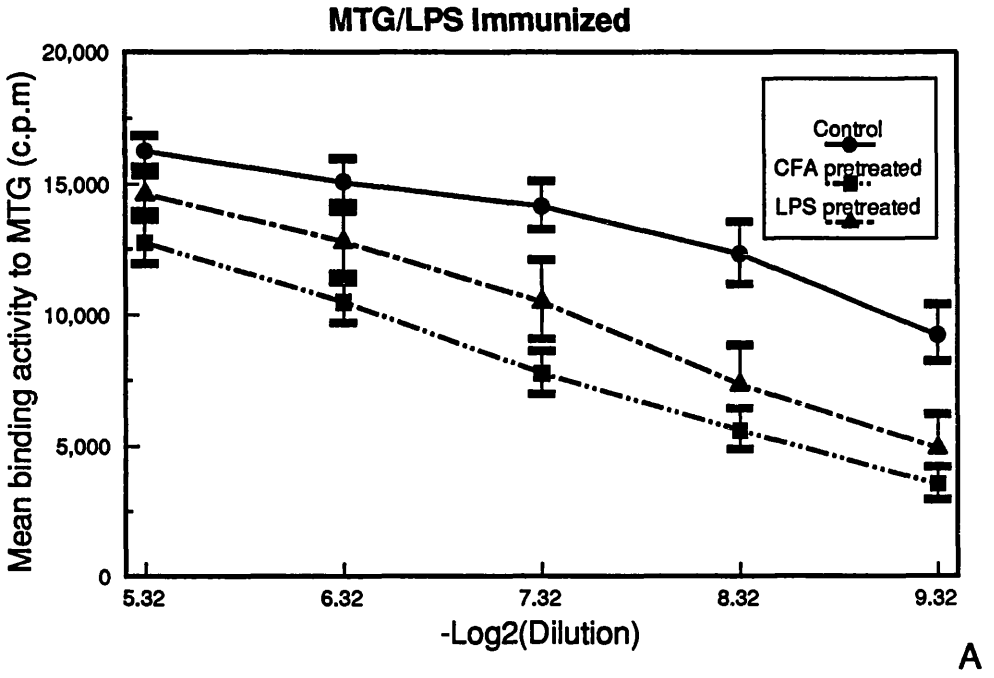
TABLE 3.2

TG CROSS-REACTIVITIES OF EAT SERA

SPECIES OF TG	IMMUNIZATION	
	MTG/LPS	MTG/CFA
Mouse	18064 +/- 139	18352 +/- 479
Chinese Hamster	14011 +/- 687	12036 +/- 762
Flying Squirrel	4913 +/- 1590	7992 +/- 1349
Braz.T Porcupine	8122 +/- 1152	8803 +/- 1331
Zebra	2933 +/- 348	7515 +/- 1355
Sheep	2872 +/- 737	7568 +/- 2148
Bovine	4938 +/- 1092	8570 +/- 1773
Reindeer	807 +/- 244	8080 +/- 1999
Pig.Tail Monkey	7168 +/- 970	9298 +/- 1269
Human	9106 +/- 1240	10289 +/- 1452
Hedgehog	588 +/- 247	2670 +/- 904
Wombat	1294 +/- 105	6863 +/- 1013
Wallaby	1318 +/- 210	5174 +/- 975

Results shown are the mean +/- s.e.m. values in c.p.m at serum dilutions of 1 in 50. Serum samples were taken at 1 month after challenging with either MTG/LPS or MTG/CFA.

SELECTIVE EFFECT OF ADJUVANT PRETREATMENT ON THE HUMORAL RESPONSE TO MOUSE MTG



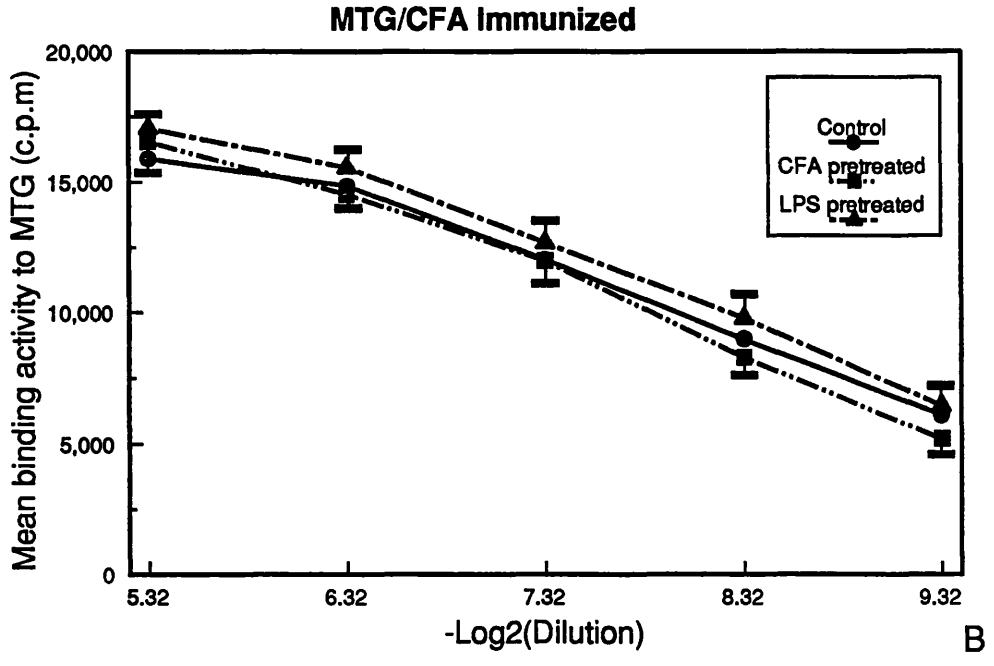


FIGURE 3.8: Groups of mice (n=5) were pretreated (i.p) with PBS/LPS (20µg) or PBS/CFA (50µl of a 1:1 emulsion containing killed *mycobacterium*). Control groups were not pretreated. All groups of mice were challenged a week later with: 50µg MTG/LPS (and again on d7 with 20µg MTG/LPS) (A), 50µg MTG/CFA (B).

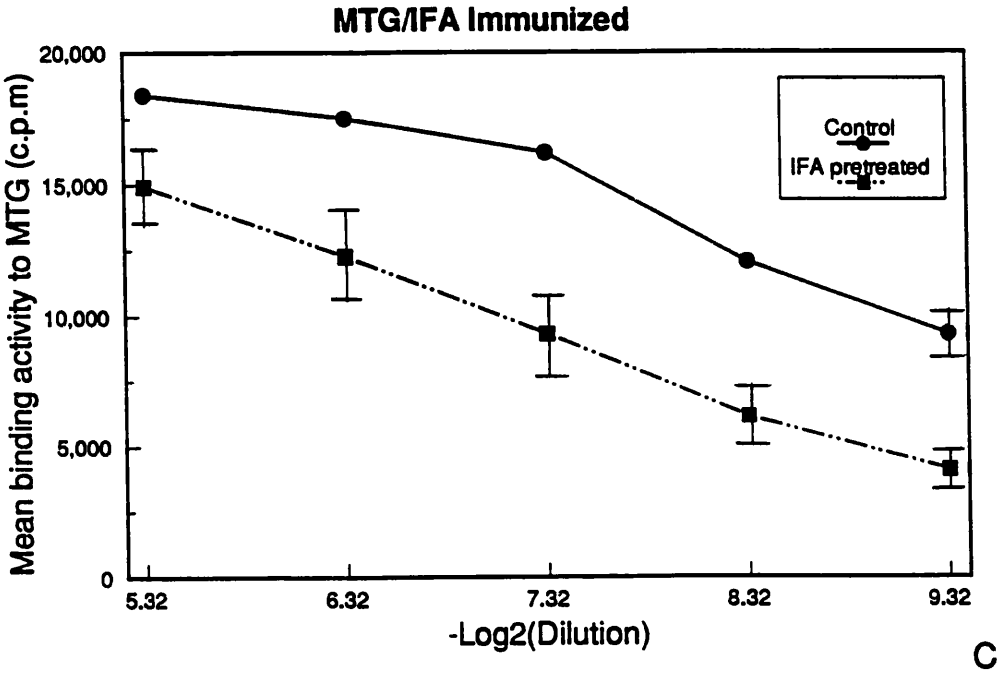


FIGURE 3.8: Mice were pretreated with PBS/IFA (50µl of 1:1 emulsion) and challenged with 50µg MTG/IFA.
Mean +/- s.e.m. values are shown, but s.e.m. < 5% have been omitted for clarity.

Specific Antibodies Are Down-Regulated By Adjuvant Pretreatment

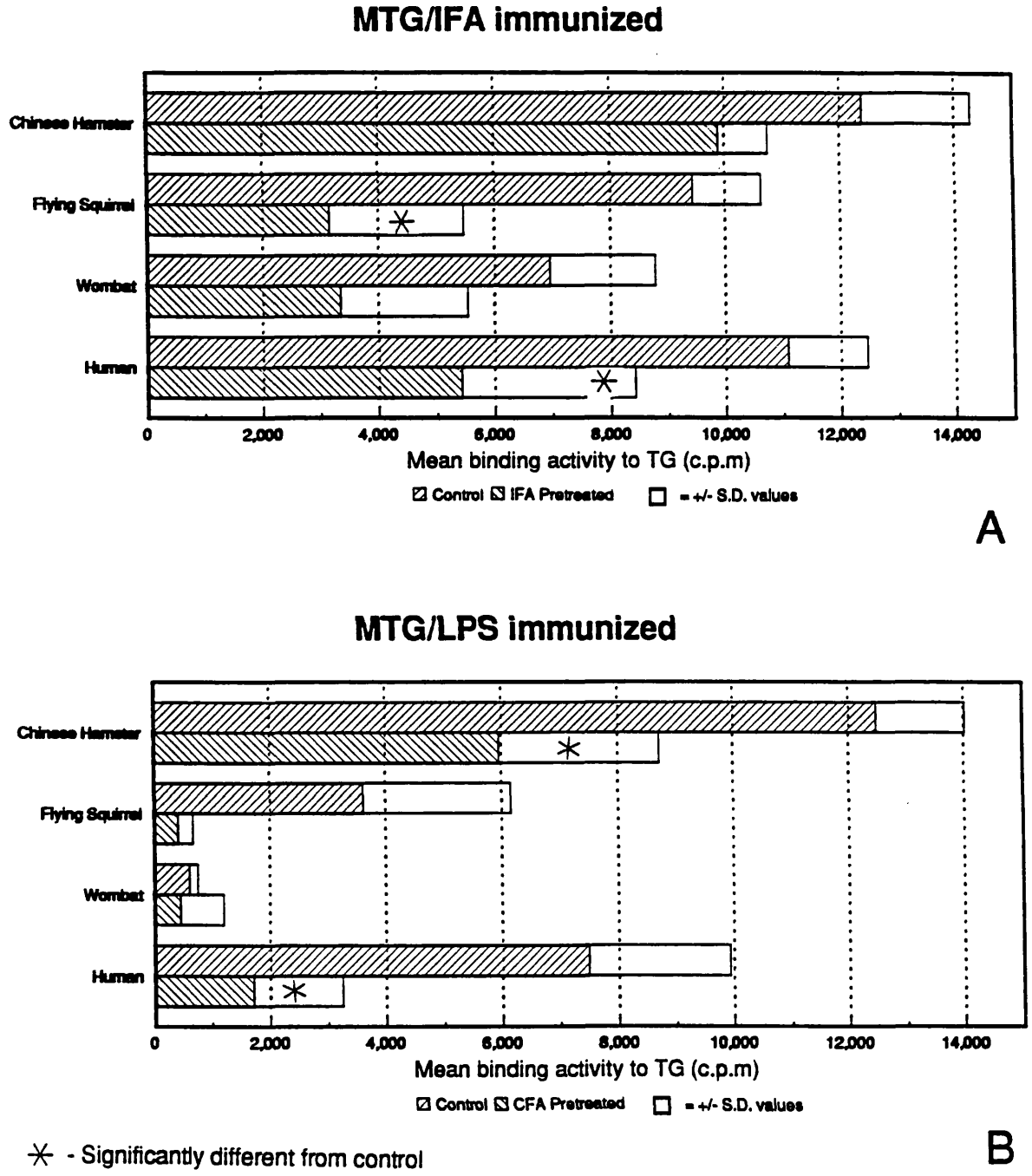


FIGURE 3.9: Adjuvant pretreatment (protocol as in FIG. 3.8) induced qualitative differences in the MTG/IFA (A) and MTG/LPS (B) activated antibody response. The cross-reactivity profiles for five TG species are shown. Bars represent the Mean +/- S.D. at serum dilutions of 1 in 100.

3.3 DISCUSSION

3.3.1 Adjuvant defined model of murine EAT

The longitudinal studies presented here show that the MTG/LPS induced model has more similarities to the human disease than the MTG/CFA model. These similarities correspond to both the humoral response and thyroid pathology, which showed that the infiltration of the thyroid was prolonged by MTG/LPS activation. Within the scope of this work it was not possible to quantify the sub-sets of infiltrating cells. However, histological examination does allow the detection of plasma cells; few plasma cells were seen in the sections examined, but notably these were observed only in thyroid sections from MTG/LPS challenged mice after prolonged disease. Creemers et al. (1984) have shown that in contrast to spontaneous thyroiditis in OS chickens and humans, the percentage of thyroidal B cells in MTG/CFA induced thyroiditis in mice is low. Phenotypic analysis of the thyroid cellular infiltrate at various intervals after MTG/LPS should determine whether, at the cellular level, MTG/LPS induced EAT is indeed a more appropriate model for autoimmune thyroiditis.

A major characteristic of Hashimoto's thyroiditis is the temporal stability of the anti-TG titre (Devey et al., 1989), spectrotype and idiotype (Delves & Roitt, 1988). However, it is difficult to know how the individual patterns are initially established. There may be an early rapid expansion, which is stabilised thereafter, as observed in the murine response to alpha-amylase (Nakashima & Kamikawa, 1984). Alternatively there may be two phases of B lymphocyte proliferation where an initial polyclonal response may be replaced by a few clonotypes which dominate the spectrotype (Stott et al., 1988). The results show that although both adjuvants stimulate a prolonged auto-antibody response, the autoantibody titres of MTG/CFA challenged mice were down-regulated. By contrast, LPS induced autoantibodies did not decline over the period of 11 months, although an initial decrease in titre was observed at 4-5 months. The processes leading to this fluctuation is not clear, but may represent the point at which some B-clones are downregulated or replaced by another set. In addition this may be associated with a switch in immunoglobulin (Ig) subclass. An understanding of events at this stage is clearly needed.

One approach to following the clonal restriction and variation in behaviour of B-cell clones during the progress of disease is to analyse the cross- reactivity profile of the sera. The specificity of anti-TG antibodies was investigated by assessing the cross-reactivity of anti-TG antibodies with different TG species: Animals immunized with MTG/CFA recognized multiple TG species, but as autoantibody titres declined, the cross-reactivity with other species of TG decreased (FIG 3.4). Studies on antibody specificity of MTG/CFA induced rat EAT also show extensive cross-reactivity (de Assis-Paiva et al., 1988). These authors were able to show that the observed cross-reactivity may be partly due to the induction of a dominant set of antibodies reactive with iodination dependent epitopes.

The cross-reactivity profile of the antisera from MTG/LPS immunized mice was more restricted. This would be more in line with the restricted recognition pattern of autoantibodies from HT patients (Nye et al., 1980; Ruf et al., 1985; Piechaczyk et al., 1987). A feature of the antibody responses is that they are almost entirely directed to "native" epitopes. This is consistent with the recognition pattern of anti-TG autoantibodies in patients' sera (Dong et al., 1989). The results presented here disagree with those of Champion et al. (1987b), who concluded that qualitative differences between TG autoantibodies are not induced when LPS or CFA are used as adjuvants. This discrepancy can be partly explained by closer examination of the immunization protocols used. In the study by Champion et al., the sera examined were from CBA/Ca mice immunized with a single dose of MTG/CFA. I have found that a single challenge of MTG/CFA is less effective in inducing cross-reactive antibodies (see Appendix 3). However, there are a number of "marker" species (eg. wombat, wallaby, reindeer, see Table 3.2) which unequivocally distinguishes MTG/CFA induced antibodies from those induced by MTG/LPS. Adjuvants do influence the isotype of antibodies produced to the same antigenic determinant (Hadjipetrou-Kourounakis & Moller, 1984; Karagouni & H-Kourounakis, 1990). Since isotype switch is intimately linked to T helper cell activation and cytokine release (Stevens et al., 1988; Finkelman et al., 1988; 1986), it is not improbable that the specificity of antibodies directed to a multideterminant antigen should also be affected by the adjuvant.

3.3.2 Adjuvant determined modes of activation

The unifying mechanisms of adjuvant action remain unresolved. The humoral response elicited in these two models of murine EAT illustrate that atleast two aspects of adjuvant action have to be considered; (i) why a distinct set of antibody specificities are induced to a given antigen and (ii) what determines the continued stimulation of one set but not the other. The first point is related to antigen structure and immune recognition, whilst the second concerns the adjuvant determining effects on immunoregulatory mechanisms.

Most, if not all, of the surface of a protein is potentially immunogenic: "Immunogenicity" indicates the ability to elicit an immune response and is the summation of a variety of influences that reflect the previous history of the animal (Benjamin et al., 1984). Since in the present study syngeneic mice from single batches were used, *a priori* the individual immune response to TG should have been uniform, but this was not the case. The mechanism by which antibody specificity is generated and selected in the mouse immune system is complex and requires the interaction of a variety of cell types: In particular the critical role played by direct cellular interactions between B cells and helper T (Th) cells (Claman et al., 1966; Mitchell & Miller, 1968; Noelle & Snow, 1990).

A restrictive factor in the immune response to a given antigen is the appropriate activation of antigen presenting cells (APC). In the past few years it has become evident that a wide variety of cells other than phagocytic macrophages can present antigen; dendritic cells (King & Katz, 1990; Kapsenberg et al., 1986; Kaye et al., 1985), Ia positive T cells (Walden et al., 1986) and resting and immune B cells (Chestnut et al., 1982; Casten et al., 1985). In this respect it is interesting to note that in an *in vitro* study by Hutchings et. al (1987), whereas the TG-specific T cell hybridoma, CH9, could only be stimulated by TG-primed B cells, its polyclonal parent line, MTg12B, proliferated equally well to normal, and primed, B cells. There are a number of reports which suggest that different APC use different pathways to process the same antigen, so it is feasible that preferential activation of a particular set of APC by different adjuvants may modulate the epitope specificities of the induced antibodies. In the MTG/LPS model of EAT it may be that antigen presentation by B cells is

favoured selectively. Interestingly, there is evidence that antigen-specific B cells present some determinants to T cells in preference to others on the same molecule (Ozaki & Berzofsky, 1987); this may explain the restricted response to TG.

As yet there is no clear understanding of which APC are preferentially activated by CFA and LPS *in vivo*. LPS is thought to act as a polyclonal activator for a large fraction of murine B cells (Andersson et al., 1972; Peavy et al., 1974). However, the stimulation of T cells (Armerding & Katz, 1974; Sciebienski & Gershwin, 1977) and macrophages (Hamilton & Adams, 1987) is included in the repertoire of LPS activities. LPS may affect antigen presentation by macrophages by increasing, or decreasing, the expression of Ia molecules on their surface, which appears to be dependent on the length of exposure to the adjuvant (Zeigler et al., 1984; Behbehani et al., 1985). Similarly, although the monocyte-macrophage system plays a major role in the adjuvant effect of CFA, muramyl dipeptide - the active component of killed mycobacteria in CFA, has a direct effect on both T and B lymphocytes (reviewed by Warren et al., 1986).

The present study was concerned with determining whether CFA and LPS activated identical populations of cells. Pretreating mice with LPS or CFA decreased the adjuvant effect of LPS on the immune response to MTG (FIG. 3.8), which suggests that CFA can affect a subset of the population of cells responsive to LPS. By contrast, neither adjuvant suppressed the autoantibody response induced by MTG/CFA. The most likely explanation for this is that CFA can activate greater functional heterogeneity of the APC than LPS, thus abortive cooperation between one set of cells would not affect the total response. Interestingly, pretreatment with IFA reduced the subsequent antibody response to MTG/IFA challenge, which suggests that pretreatment with adjuvant alone does not necessarily involve exhaustive differentiation of responsive lymphocyte populations by the mitogenic component of an adjuvant. The failure to observe reduced antibody responses by pretreating with CFA may be ascribed to enhanced IL-1 production by CFA, which may cause an augmentation of an antibody reponse (Sagara et al., 1990).

In addition to direct and indirect (via co-induction of cytokines) action of adjuvants on APC, the route of antigen presentation may also determine the population of cells activated and subsequently the

quality of the immune response. The route of immunization is clearly important in inducing an immune respose to TG with CFA, because only a minimal antibody response was observed when MTG/CFA was injected i.p. in contrast to immunization via a subcataneous route in the hind foot pads (Fig. 3.7). It has been reported that the spleen is the final destination of most antigens injected via the intraperitoneal route, whereas footpad injections are said to stimulate the inguinal and the popliteal lymph nodes (Hall, 1985). If the microenvironment plays a crucial role in determining which cells are involved in the early events in the immune response *in vivo* (van Rooijen, 1990), then this is an important conisderation. It may be that resident macrophages and dendritic cells in these two sites have different functional (phagocytic, secretory and antigen presenting) characteristics. The importance of the route of immunization has been demonstrated for other antigens. For example, H-2^k, H-2^d and H-2^s strains of mice are essentially non-responders to the male specific H-Y antigen as measured by primary skin graft rejection, but cytotoxic T cell responses occur in some strains following priming *in vivo* via the footpad but not the intraperitoneal route (Simpson, 1982). The crucial role of antigen-presentation is also evident from the fact that the same leishmanial antigen injected i.v. or s.c. leads to either protective or disease- promoting CD4+ T cells (Liew, 1989).

3.3.3 Antigen presenting cell-dependent recruitment of T cells

Mouse CD4+ helper T cell clones may be subdivided into two main groups based on differences in their pattern of cytokine production (Mosmann et al., 1986). TH1 clones synthesize IL-2, IFN-y and lymphotoxin (LT), whereas these cytokines are not detectably expressed in TH2 clones. Conversely, only TH2 clones synthesize detectable amounts of IL-4, IL- 5 and probably IL-6 (reviewed in Mosmann & Coffman, 1989). The possibility that subdivisions of T cells also exist in humans, is suggested by the evidence that CD4+ T-cell clones isolated from tissue infiltrates in different disease states may exhibit TH1- or TH2-like profiles (Romagnani, 1991). The precise relationship between the TH1-cell and TH2-cell subsets and the conditions required for the activation of one as opposed to the other is the subject of continuing debate (Bottomly, 1988; Swain et al., 1988; Gajewski et al., 1989). The APC appears to be a good candidate for the cell influencing the TH1/TH2 ratio, and it has been proposed that TH1 cells may be

selectively activated and expanded by B cells, whereas macrophages (producing IL-1) cause clonal expansion of TH2 cells (Janeway et al., 1988). The two EAT models described provide good *in vivo* models for testing these proposals.

3.3.4 Role of circulating TG in MTG/LPS induced EAT

The adjuvant action of CFA is aided by creating a depot at the site of injection, which prolongs the release and interaction of antigens with APC (Allison & Byars, 1986). In this way a prolonged antibody response could be maintained. However, in MTG/LPS induced EAT, the TG molecules enter the circulation essentially as free, native antigen. Since the measured half life of native TG in the circulation of rats is only 4.4hr (Ikekubo et al., 1980) the observed long-term maintenance of anti-TG antibodies in MTG/LPS immunized mice is difficult to explain.

One possibility is that the immune response is maintained as a result of TG being transported to the lymph node follicles and retained on follicular dendritic cells (FDC) for extended periods of time. It is now recognized that antigen persists in association with FDCs and can be presented by B cells to T cells. This alternative pathway may be responsible for maintenance of serum antibody levels and memory B cells (Gray & Skarvall, 1988; Tew et al., 1989). Since both cellular and humoral immunity declined in MTG/CFA challenged mice at earlier time points, this cannot be the only mechanism by which a prolonged immunity is maintained in MTG/LPS challenged mice.

An alternative explanation would be that the cellular and humoral immunity to TG is perpetuated in this model by specific B cells presenting circulating TG to T cells: The T cells in turn would provide help for B cell differentiation and antibody synthesis. Specific B cells, by virtue of their surface immunoglobulin molecules, are able to take up and present antigen at much lower concentrations than other antigen presenting cells (Rock et al., 1984; Lanzavecchia, 1985; Abbas et al., 1985). Such antigen presenting capacity is less effective in resting B cells (Zlotnik et al., 1983; Frohman & Cowing, 1985). In this *in vivo* model additional signals required for B cell activation in the MTG/LPS murine model of EAT could be provided by direct LPS activation, or indirectly, eg. via activation of macrophages. In

support of a role for B cells in the presentation of TG to T cells, Hutchings et al., (1987) have shown that B cells primed *in vivo* with TG could stimulate the TG specific T cell hybridoma, CH9, which in turn increased antibody production. Furthermore, some recent investigations (Ron & Sprent, 1987; Kurt-Jones et al., 1988) are indicative for a crucial role of B cells in the *in vivo* induction of T-cell help.

The ability of cytotoxic T (Tc) lymphocytes to recognize and kill B cells pulsed with antigen suggests that Tc cells elicited during an ongoing immune response against a soluble antigen may reduce the magnitude of the the antigenic stimulus by killing the APC targets (Simpson et al., 1988). An important observation is that virgin B cells are far more sensitive to lysis by Tc cells than memory B cells (Yefenof et al., 1990). If antigen presentation of circulating TG by B cells is the mechanism by which the humoral response is perpetuated in the MTG/LPS model, future studies need to establish whether this presenting capacity is primarily a property of naive or memory B cells.

3.3.5 What is the basis of cellular memory?

One of the major characteristics of the immune system is immunological memory; the ability to react more quickly and more intensely upon a second exposure to the same antigen. In the MTG/LPS model the primary response induced an antibody response to MTG which showed little cross-reactivity with heterologous thyroglobulins. However, this primary challenge had clearly induced a long-lived memory cell population which could transfer a secondary response: This anamnestic response in the syngeneic recipients could be identified because of the qualitative difference in the MTG/LPS induced antibody response (FIG. 3.6); highly cross-reactive anti-TG antibodies were demonstrated. What are the implications of this important observation for (i) the mechanisms by which immunological memory is established and (ii) the initial trigger for autoimmune thyroiditis?

Memory is thought to reside among long-lived cells, which through clonal expansion, are present at higher frequencies compared with their naive precursors (Celada, 1971). Both B and T cells participate in the establishment of immunologic memory. A major advance in

understanding memory has been the definition of numerous cell surface molecules that distinguish memory T cells from naive T cells (Sanders et al., 1988; Springer, 1990; Cerottini & MacDonald, 1989; Beverley, 1990). However, to date the longevity of memory T cells has not been demonstrated (Mackay, 1991). This is clearly an important issue since the mechanism by which T memory cells are maintained may provide answers for the mechanism by which autoimmune responses are generated. For example, if T-cell memory is maintained by cross- reactive stimulation (Beverley, 1990), then autoimmune responses generated by appropriate crossreactions may primarily generate many effector cells from the memory cell pool.

The basis of B cell memory is equally elusive. Recent experiments have confirmed that for some specificities long-lived B-cells do exist (Schittek & Rajewsky, 1990). The precise signal which allows this long term survival is unknown. One possibility is that programmed cell death is blocked by the increased expression of the inner mitochondrial membrane protein Bcl-2 (Vaux et al., 1988; Hockenbury et al., 1990). Evidence supporting this possibility comes from a recent study (Nunez et al., 1991) in which transgenic mice overproducing Bcl-2 have a long-term persistence of immunoglobulin-secreting cells and an extended lifetime for memory B cells. Other data from cell transfer experiments indicate that persistence of memory is dependent on the presence of antigen (Gray & Skarvall, 1988), therefore the above mechanism may not be employed by memory B cells of all specificities. In addition, the differentiation pathway that results in the generation of memory B cells is still poorly understood. In particular, it is not clear how, in the primary response, some of the B cells differentiate into terminal plasma cells and others into memory cells. A number of questions arise: Are mechanisms for establishing B cell memory linked to regulatory networks which maintain tolerance to self antigens? Regardless of the mechanism by which TG-specific memory B cells are initially triggered, are they always highly cross-reactive? These questions deserve further consideration and are discussed in more detail in chapter 7.

CHAPTER 4

REGULATION OF IMMUNE REACTIVITY TO TG

4.1 INTRODUCTION

Mice given a series of injections of rat red blood cells (RBC) produce antibodies to both rat and mouse erythrocyte antigens. Production of autoantibodies continues in these animals for a few weeks after the ending of rat RBC injections, followed by a gradual reversion to a Coombs' negative state (reviewed in Naysmith et al., 1981). This sequence is not unlike a number of human autoimmune diseases (including some forms of haemolytic anaemia) where onset is sudden and spontaneous, followed by a fairly rapid resolution of the condition. More chronic autoimmune diseases, such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), also are often characterized by bursts of increased autoantibody activity and periods of remission. This suggests the possibility that in the normal situation autoantibody responses may be limited by homeostatic mechanisms, which can be impaired in disease.

Immunoregulatory imbalances have been invoked to explain the occurrence of several autoimmune diseases. Sjogren's syndrome, RA and SLE have been found to be accompanied by a decrease of T lymphocyte functions *in vitro* (Sucio-Foca et al., 1974; Altman et al., 1981; Elder & MacLaren, 1983), while spontaneous autoimmune thyroiditis in OS chickens was shown to be associated with elevated *in vitro* T cell responses (Schauenstein et al., 1985). In addition to diminished or hyper-activity of T lymphocyte functions, defects in T suppressor cells has also been suggested as a plausible cause of autoimmune disease (Wick et al., 1982; Barthold et al., 1974; Gerber et al., 1974). Throughout the last two decades several studies have emphasized the immune regulatory role of antigen-induced suppressor lymphocytes, either T (Sy & Benacerraf, 1988; Herzenberg et al., 1973) or B (Zembala et al.,1976) cells, or their products (Tamiguchi & Miller, 1978; Theze et al., 1977). The concept of suppressor T cells emerged to explain the observation that some tolerant states could be

transferred from tolerant animals, to normal animals, by the injection of T cells (Gershon & Kondo, 1971). This is not in any doubt and occurs in a large variety of experimental circumstances. Transfer of tolerance to MTG has also been demonstrated (Kong et al., 1982; Parish et al., 1988).

The regulation of immune reactivity to TG epitopes was considered in the course of this study on murine EAT. The results from two separate types of models, which were exploited in a series of experiments designed to induce tolerance to TG, are presented and lend support to an alternative explanation for the observed suppression to MTG.

4.2 RESULTS

4.2.1 Attempts to co-suppress the autoantibody response to TG.

There is extensive evidence which suggests that a control mechanism operates in the rat RBC induced model of haemolytic anaemia in mice. When mice are re-challenged with rat RBC, either while they are still producing autoantibody, or after recovery, there is a short burst of autoantibody formation followed by a rapid specific cessation of production. Furthermore, spleen cells transferred from prechallenged mice prevent autoantibody production in recipient mice immunized with rat-RBC (Cooke et al., 1978; Cox & Howles, 1981; Naysmith et al., 1981).

The simplest hypothesis to explain the occurrence of autoantibodies in this model is that, rat and mouse erythrocytes share a cross-reacting surface antigen, to which mice are normally non-responsive because of insufficient T cell "help". Presentation of the autoantigen on the rat erythrocyte (acting presumably as a new "carrier") somehow bypasses the need for T cell help as a consequence of the normal response to the other erythrocyte antigens (Naysmith et al., 1981). Other crucial questions raised by these observations centres on the underlying cellular basis for the suppressor mechanisms which is generated at the same time and also the antigen-specificity of the

suppression involved.

This model was adapted to examine if antibodies to TG could be elicited by coupling pig TG to rat RBC, and whether this response could be concurrently down- regulated with the autoantibody response to erythrocyte antigens. This was a collaborative experiment with James Elliot.

Pig TG was used, because rat TG was not available in sufficiently large quantities. As TG is known to be a highly conserved antigen (eg. there is about 80% homology between human and bovine TG mRNAs (Malthiery et al., 1989)), it was expected that mouse and pig TG would share some cross-reacting epitopes. Coupling of pig TG to rat RBC was mediated by a chromic chloride reaction. The success of the coupling reaction was assessed by haemagglutination using rabbit anti- pig TG antisera.

Mice were challenged with either soluble pig TG (50ug), or 2×10^8 pig TG coupled rat RBC (TG-rat RBC), at weekly intervals, for four consecutive weeks. A third group of mice were similarly challenged with rat RBC. Antibody levels to erythrocyte antigens in the three groups were measured as described in the materials and methods (data not shown). The anti-TG antibody responses in these mice were measured by solid-phase RIA.

Figure 4.1 shows that even after two injections with pig TG, or TG-rat RBC, antibodies to both pig and mouse TG were induced. As might be expected, the mean binding activities to mouse TG were less than the respective binding activities to pig TG. Importantly, the concentration of pig TG which was coupled to the rat RBC, was sufficient to produce a higher anti-TG response than that induced with 50 μg of soluble pig TG.

At ten weeks after the last antigen challenge, the rat RBC and TG-rat RBC immunized mice were killed and their spleens removed. Spleen cells from the two groups were pooled separately and transferred to separate groups of syngeneic mice - with five mice in each group.

Each mouse, in three separate groups, was injected intravenously with spleen cells (40×10^6) from the rat RBC primed donors. Another three groups of mice were injected with spleen cells from the TG- rat RBC primed mice. Twenty-four hours later these recipient mice were immunized with either TG-rat RBC, rat RBC, or soluble pig TG. A further three groups of mice, which had not received any spleen cells, were similarly challenged. The transfer and immunization regimes of these nine groups of mice are shown in Table 4.1.

Mice were tail-bled at weekly intervals and the levels of anti-TG and anti-erythrocyte antibodies were assayed. Antibodies to rat RBC were detectable in all except group 3 mice, which were challenged only with pig TG. Low levels of anti-rat RBC antibodies were detected in TG immunized recipients of spleen cells from either rat RBC, or TG-rat RBC, primed donors. Four weeks after the first injection, only groups 1 and 2, which had been immunized with TG- ratRBC and rat RBC respectively, had autoantibodies to mouse RBC (proved positive on a Coombs'test) (Table 4.1). These autoantibodies were shown to increase with successive immunizations upto week four (FIG. 4.2). Spleen cells transferred from rat RBC primed donors completely suppressed the autoantibody response to erythrocyte antigens, regardless of the immunization regime. However, spleen cells from TG-rat RBC primed donors could not totally suppress an autoantibody response induced by TG-rat RBC challenge (group 4 mice) - a low peak of autoantibody was detected after the second injection.

In contrast to the observed suppression of autoantibodies to erythrocyte antigens, transferred spleen cells could not down-regulate the autoantibody response to TG. Interestingly, anti-MTG antibodies were significantly enhanced ($P = 0.01$) in TG-rat RBC immunized mice which had received spleen cells from TG-rat RBC primed donors (group 4). The antibody levels after the third immunization are shown in figure 4.3. (The restricted amount of serum samples available precluded us from establishing whether these antibodies could be absorbed out with pig TG.) Furthermore, an early, secondary-type anti-pig TG antibody response was observed in this group (FIG. 4.4). Comparison of the antibody titres at day 16 with those of the TG-rat RBC immunized control group of mice (group 1) showed a very significant increase ($P = 0.001$).

ANTIBODIES TO TG ARE ENHANCED IN CBA/J MICE BY COUPLING TO RAT RBC

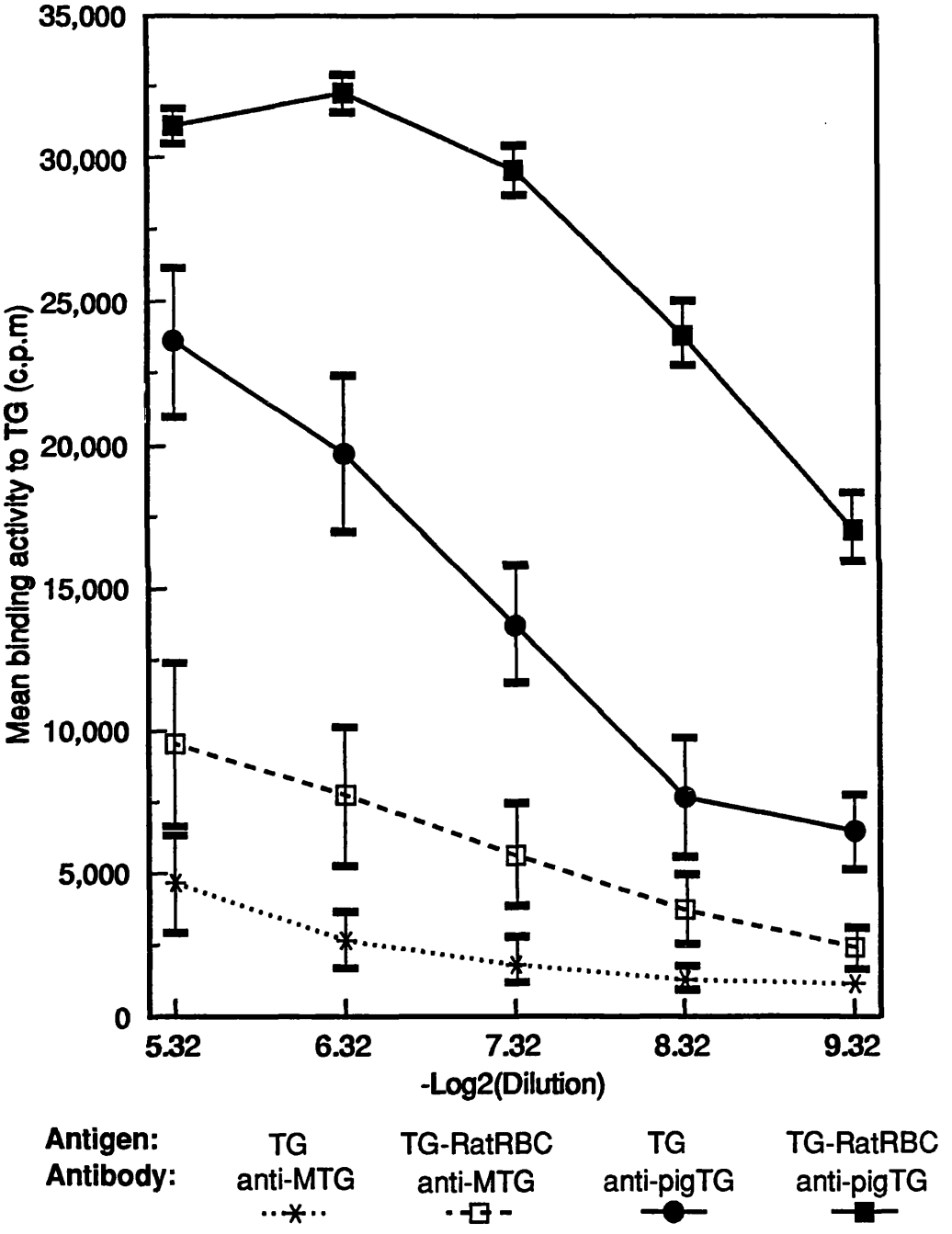


FIGURE 4.1: The antibody binding activity to mouse and pig TG of sera from CBA/J mice (5 mice per group). Mice were tail-bled 7 days after two intraperitoneal challenges (d0, d7) with either 50µg pig TG (TG), or 2 X 10^8 pigTG-coupled rat RBC (TG-ratRBC). Results are plotted as mean +/- s.e.m.

TABLE 4.1

LEVELS OF ANTIBODIES TO RBC ANTIGENS

GROUP	Donor Priming	Immunization of recipient	Antibodies to rat RBC	Antibodies to mouse RBC
1	None	TG-rat RBC	11.8 +/- 1.6	+
2	None	rat RBC	12.2 +/- 0.5	+
3	None	TG	0.0 +/- 0.0	-
4	TG-rat RBC	TG-rat RBC	13.0 +/- 1.0	-
5	TG-rat RBC	rat RBC	13.4 +/- 0.9	-
6	TG-rat RBC	TG	3.4 +/- 2.2	-
7	rat RBC	TG-rat RBC	13.2 +/- 0.3	-
8	rat RBC	rat RBC	13.8 +/- 0.9	-
9	rat RBC	TG	1.8 +/- 0.5	-

Sera assayed on day 28.

Antibodies to rat RBC were measured by direct agglutination assay in microtitre plates. Results were scored as the final well with visible agglutination. Successive wells had doubling dilutions of individual serum samples - the intial dilution was 1 in 10.

Serum antibodies to mouse RBC were measured by direct Coomb's test (DCT) - the data is shown in FIG 4.2.

Donors received four i.p. injections of either $2x10^8$ pig TG coupled rat RBC, or 50μg of pig TG. $4x10^7$ spleen cells were transferred i.v. to the recipients.

SUPPRESSION OF AUTOANTIBODY RESPONSE IN RECIPIENTS GIVEN SPLEEN CELLS FROM SYNGENEIC MICE PREVIOUSLY IMMUNIZED WITH RAT ERYTHROCYTES

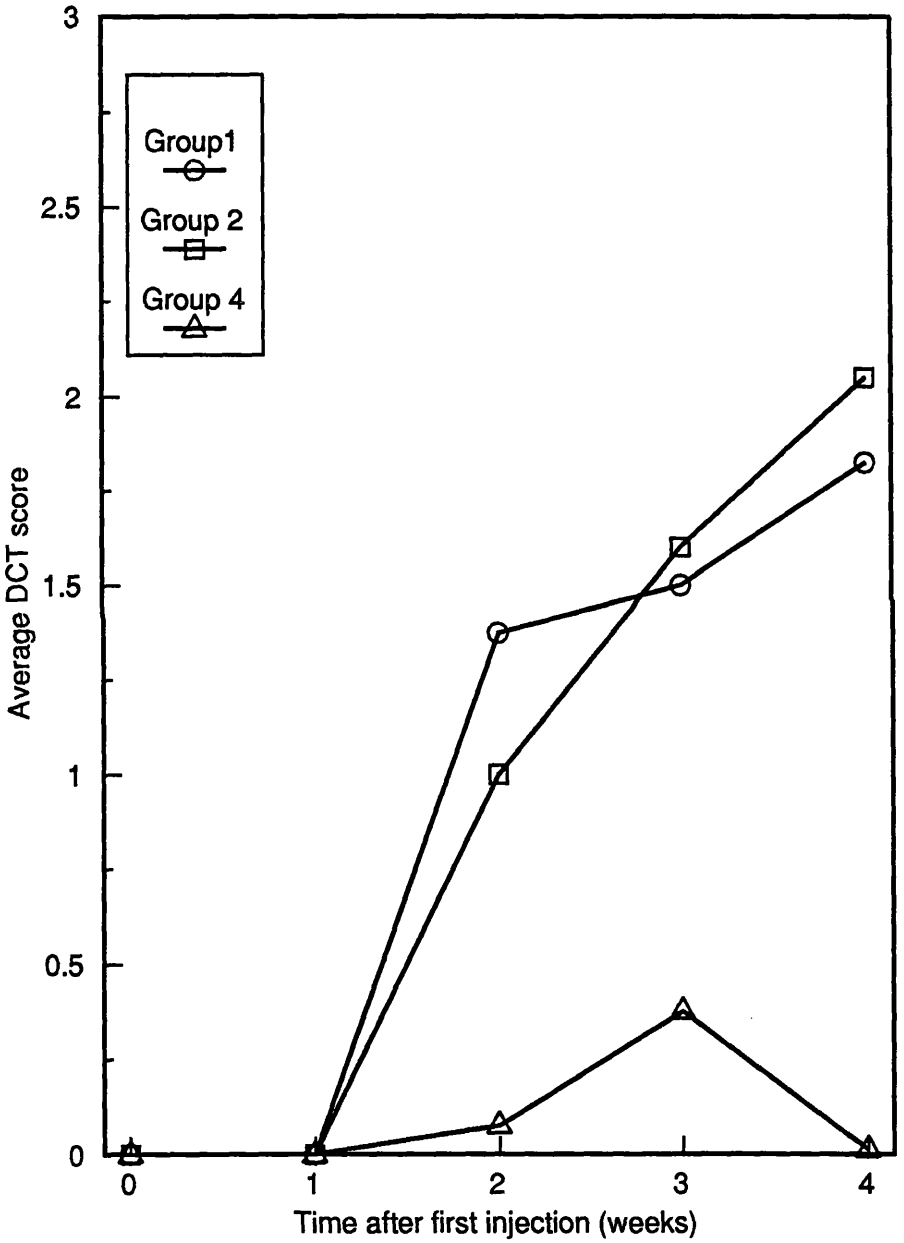


FIGURE 4.2: The DCT scores are the average of groups of 5 mice. Individual scores for the degree of agglutination was allocated as: (1) visible only under the microscope (2) visible with the naked eye (3) most cells agglutinated. Groups as Table 4.1. Note that Groups 3, 5, 6, 7, 8, and 9 remained Coomb's negative at all time points studied.

ANTIBODIES TO THYROGLOBULIN ARE NOT SUPPRESSED BY COUPLING TO RAT RBC

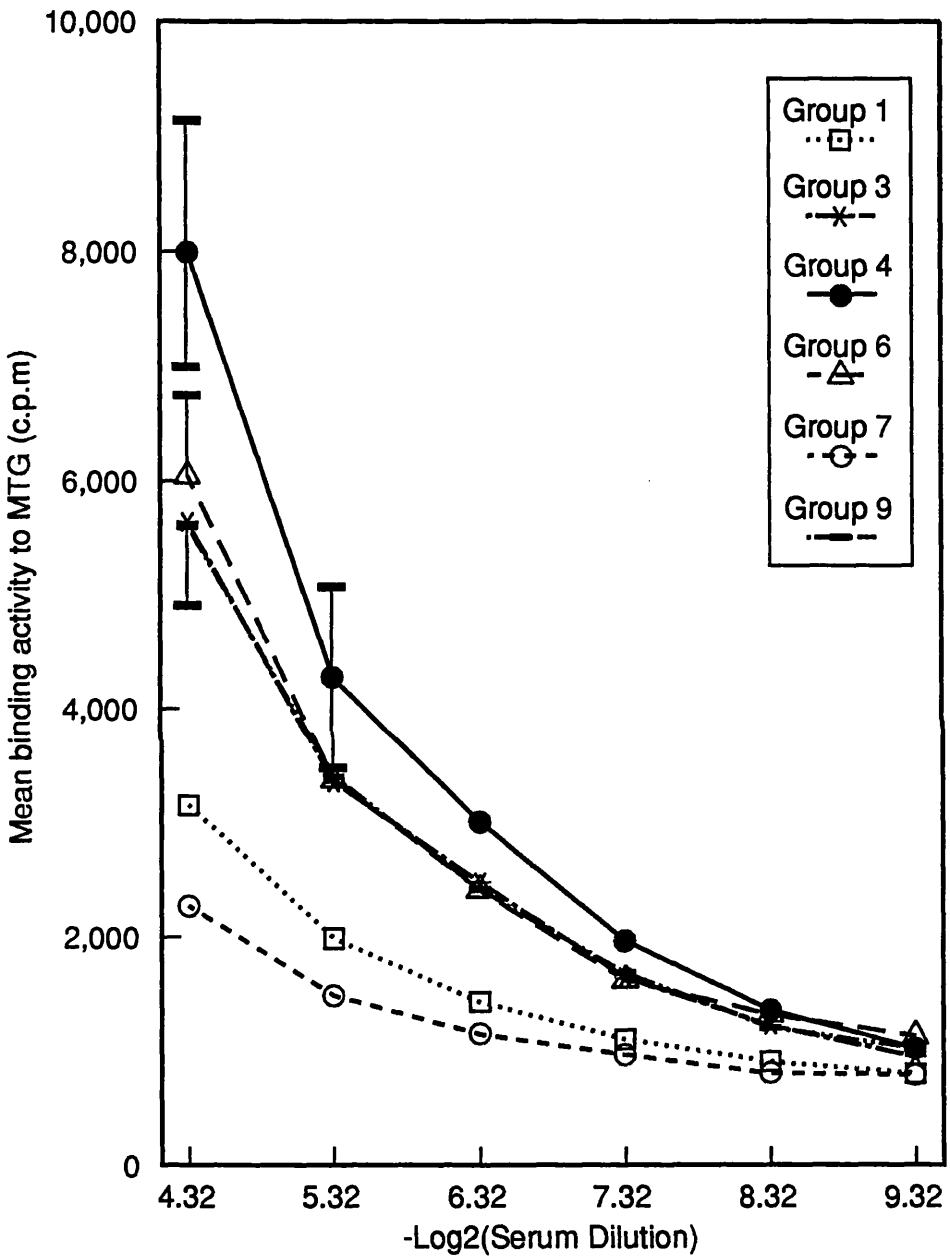


FIGURE 4.3: The groups are as described in Table 4.1. Serum samples were tested 23 days after the first challenge. Results are plotted as mean +/- s.e.m. (Only s.e.m. > 500cpm are shown.)

TG SPECIFIC HELP PROVIDED BY TRANSFERRED SPLEEN CELLS FROM DONORS PRE-IMMUNIZED WITH TG-COUPLED RAT RBC

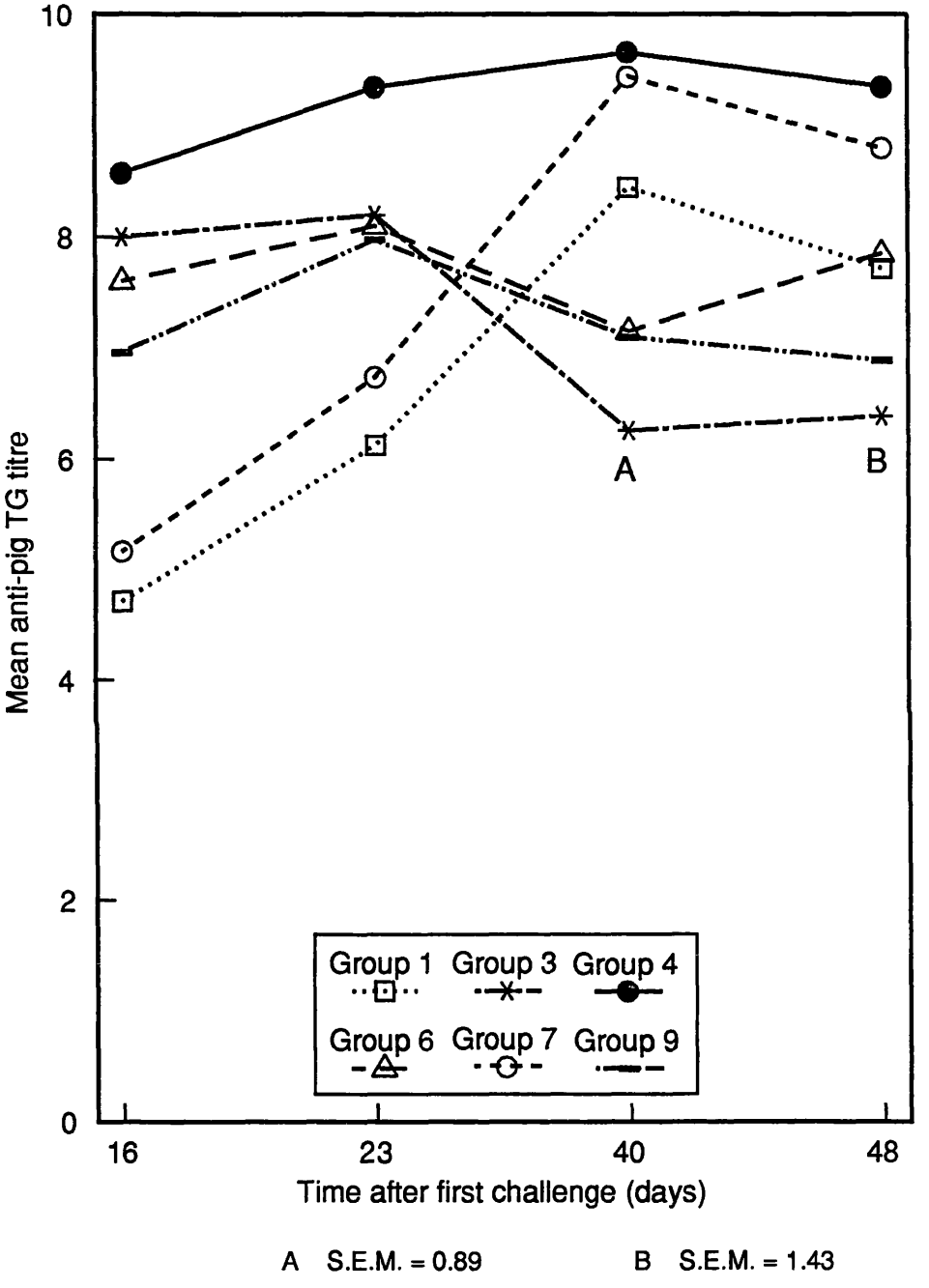


FIGURE 4.4: Groups are as previously described (Table 4.1). Anti-pig TG titres ($-\log_2$) were calculated for individual serum samples as the dilution which had equivalent binding activity to 50% of the maximum response of a positive control. S.e.m. < 0.5, except at marked points as shown.

4.2.2 Attempts to induce tolerance to TG in neonates

Neonatal mice were exposed to a large dose of TG to examine the possibility that tolerance to TG may be more readily induced in an immunologically immature environment. Difficulties in raising large litters of neonatal CBA/J mice, meant that the use of animals of different gender in these experiments was unavoidable. In a preliminary experiment it was established that there was no detectable difference in the autoantibody response to TG between male and female mice (FIG. 4.5). Neonatal mice pretreated with soluble MTG showed a reduced response to positive challenge with MTG/CFA ($P = 0.05$) (FIG. 4.6). By contrast, neonatal pretreatment with human TG did not decrease the anti-MTG antibody response induced with MTG/CFA immunization (FIG. 4.7).

The possibility that, a "masked" effect of neonatal pretreatment with human TG (HTG) could be exposed within a cell transfer system, was examined. Spleen cells (4×10^7) from mice neonatally exposed to HTG were transferred to syngeneic recipients. These mice were challenged 24 hours later with either MTG/LPS or HTG/LPS. Control groups of mice, which had received spleen cells from donors neonatally treated with PBS, were also challenged with either MTG/LPS or HTG/LPS. The anti-MTG and anti-HTG binding activities of these groups were analysed by solid-phase RIA. Spleen cells from mice neonatally treated with HTG, down- regulated the anti-MTG response in recipients challenged with MTG/LPS ($P = 0.01$) (FIG. 4.8A), but not HTG/LPS (data not shown). On the other hand, immunization with HTG/LPS did activate an increased binding to HTG in mice which had received spleen cells from donors neonatally treated with HTG ($P = 0.05$) (FIG. 4.8B). This increased binding was not observed in mice challenged with MTG/LPS (data not shown). In a separate transfer experiment mice were immunized with MTG/CFA or HTG/CFA, but the above effects were not observed (data not shown).

COMPARISON OF THE AUTOANTIBODY RESPONSE TO MTG IN FEMALE AND MALE CBA/J MICE

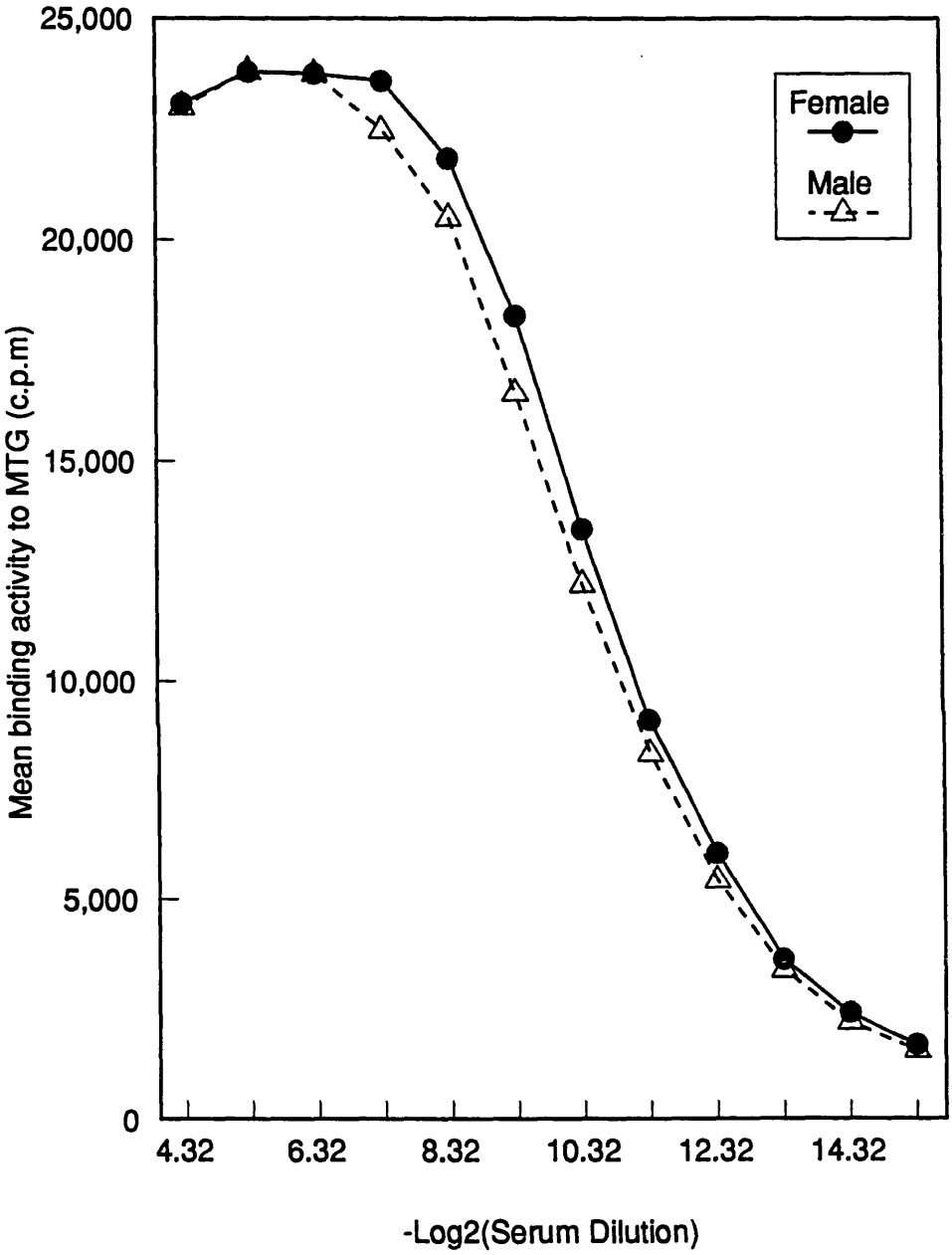


FIGURE 4.5: Female (n=7) or male (n=8) mice from mixed litters of the same age group were challenged with 50μg MTG/CFA. Mice were bled 28 days later. Serum samples were assayed for anti-MTG antibody activity.

NEONATAL PRETREATMENT WITH MTG DOWN-REGULATES THE AUTOANTIBODY RESPONSE

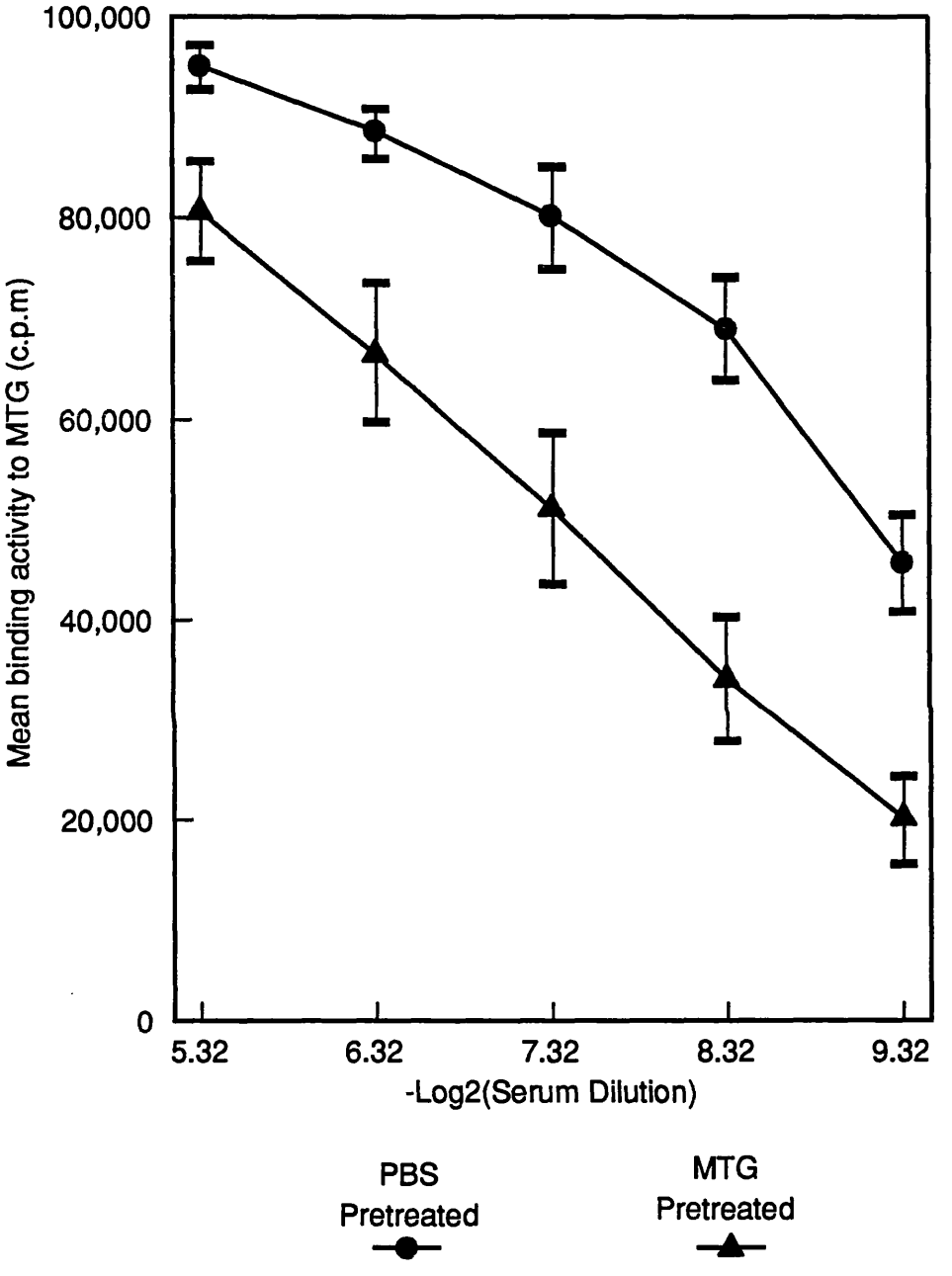


FIGURE 4.6: CBA/J mice were pretreated with 500µg MTG (n=6) or PBS (n=5) at birth. Both groups were challenged with 50µg MTG/CFA f.p. 6 weeks later. Serum samples were collected 28 days after positive challenge. Values shown are means +/- s.e.m.

THE AUTOANTIBODY RESPONSE IS NOT SUPPRESSED BY NEONATAL PRETREATMENT WITH SOLUBLE HTG

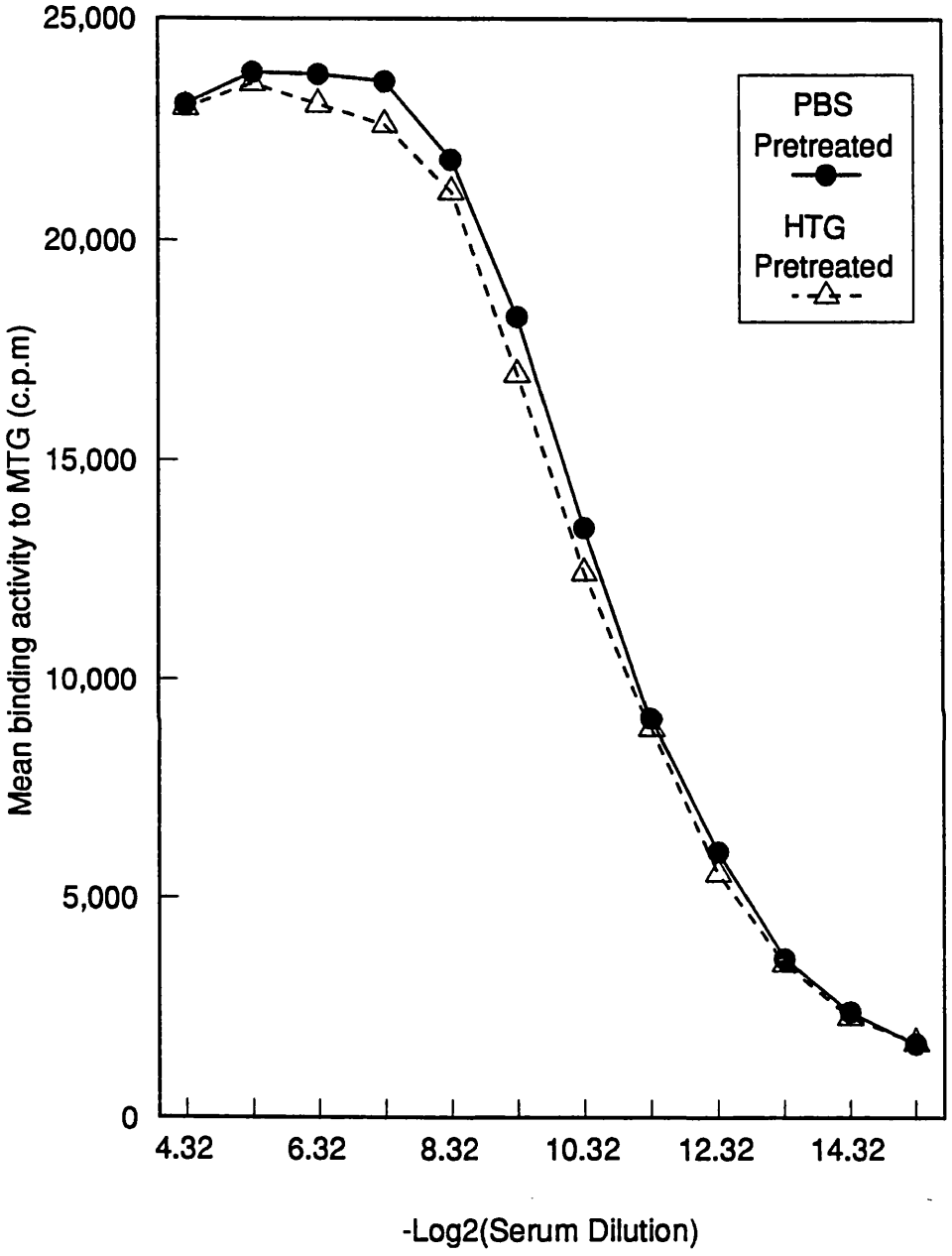


FIGURE 4.7: CBA/J mice were pretreated neonatally with either 500μg human TG (n=5) or PBS (n=7). Both groups were challenged at 6 weeks with 50μg MTG/CFA f.p. Serum samples taken at 28 days after positive challenge were assayed for binding activity to mouse TG.

TRANSFERRED SPLEEN CELLS FROM NEONATALLY TREATED MICE DOWNREGULATE THE ANTIBODY RESPONSE TO MTG

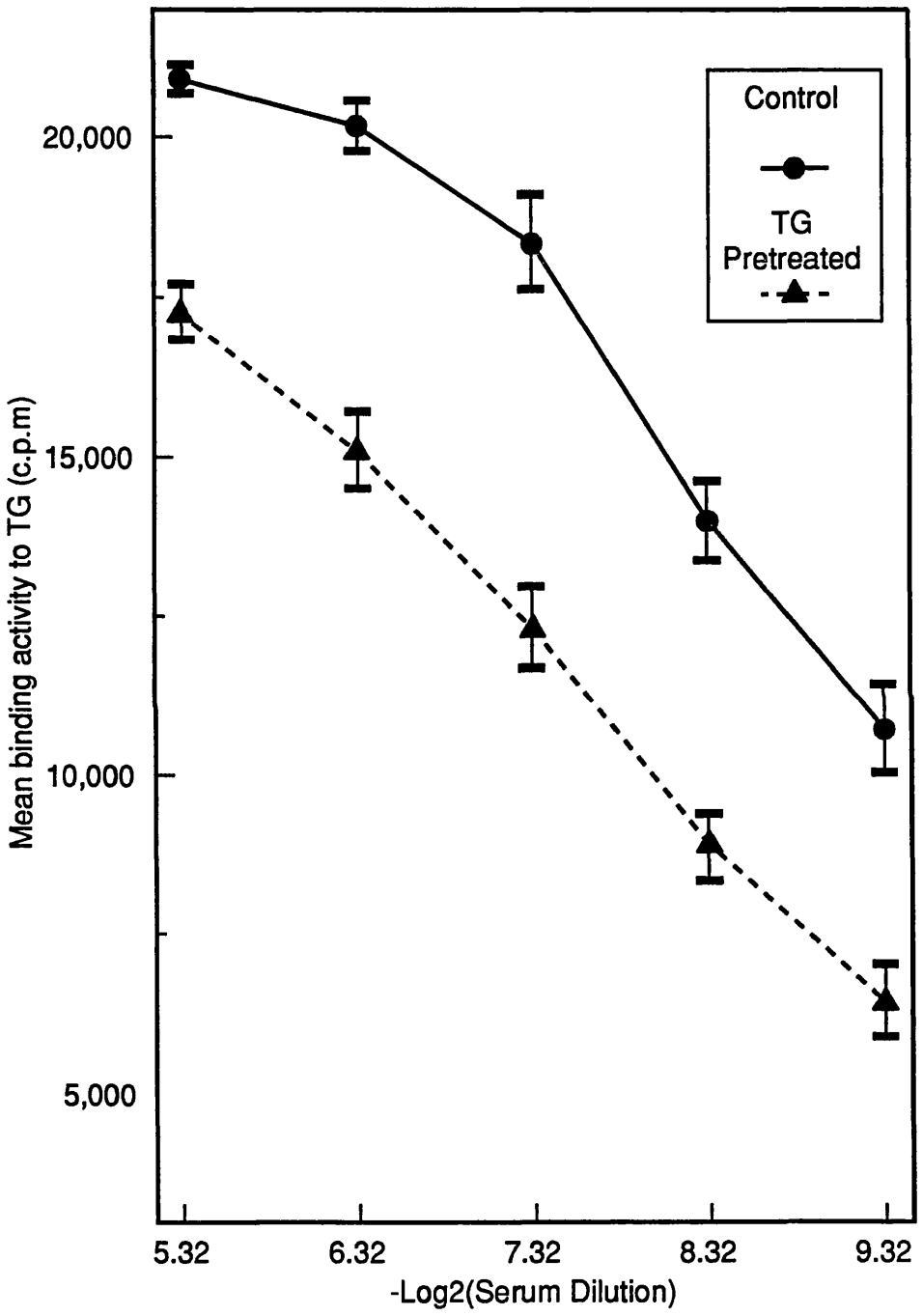


FIGURE 4.8A: Spleen cells, from 6wk old CBA/J mice were neonatally treated with PBS, or human TG, were transferred to syngeneic recipients. Both groups of mice were challenged with MTG/LPS. Serum samples were collected 21 days later. Spleen cells from HTG pretreated donors reduced the binding activity to mouse TG ($P = 0.01$).

SPLEEN CELLS FROM ANIMALS NEONATALLY TREATED WITH TG ALTER THE ANTIBODY RESPONSE TO HUMAN TG

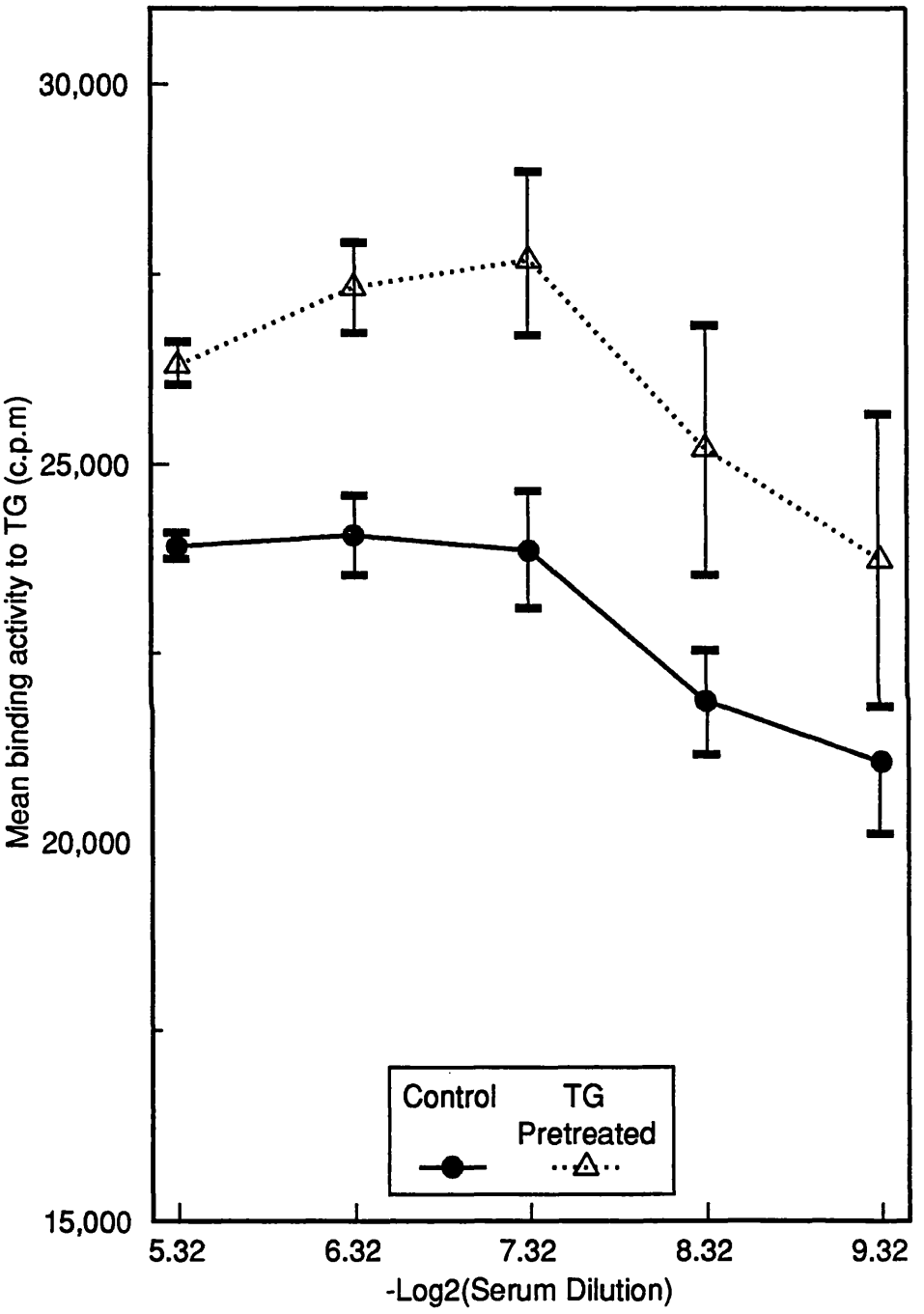


FIGURE 4.8B: Spleen cells, from 6wk old CBA/J mice neonatally treated with PBS, or human TG, were transferred to syngeneic recipients. Recipients were subsequently challenged with 50µg human TG/LPS (d0, d7). Serum samples were collected 21 days after the first challenge. The mean binding activity to human TG was increased (P = 0.05) in recipients of human TG primed spleen cells.

4.3 DISCUSSION

The precise mechanism(s) of immunological tolerance to both self-and non-self antigens remains unclear, despite substantial advances in the understanding of cellular and molecular immunology. The two broad categories of hypotheses advocate either, direct antigen-induced clonal inactivation of specific lymphocytes or, antigen-specific (or in some cases idiotype-specific) suppressor cells.

Early studies on suppressor mechanisms suggested that suppressor cells directed to one set of determinants on protein antigens commonly suppressed the response to the other determinants on the antigen. If antigen-specific suppressor cells are held to be responsible for initiating and maintaining tolerance, then, given the known homology between various species of TG, we might expect different species of TG to induce a similar state of unresponsiveness. However these results show that, when neonatal mice were exposed to MTG their total antibody response to MTG/CFA challenge was reduced (FIG. 4.6) and the same level of tolerance could not be demonstrated if mice were neonatally treated with HTG (FIG. 4.7). The initial interpretation of this data would be that the conserved TG epitopes are not the "suppressive" determinants. Alternatively, it may that the HTG-specific epitopes prevent effective tolerance induction.

In attempting to assess the states of tolerance induced in immature mice, Waters and Diener (1983) have suggested that the nature of the antigenic signal to the developing B-lymphocyte system played the most critical role in determining the outcome of such signals. These authors assessed the states of tolerance induced in immature mice by exposure to the same hapten (TNP) coupled to different carriers and found that the nature of the carrier significantly affected the extent of unresponsiveness. Determining the validity of this theory for a true self-antigen such as TG (which has many potentially immunogenic epitopes) obviously presents many problems. However, theoretically, the conserved epitopes might be viewed as the "haptens" and the rest of the molecule as the carrier. Thus the different species of TG would represent different carrier molecules and we might conclude that the nature of the antigen does affect the outcome.

By using an *in vivo* adaptive transfer system to assess the effects of neonatal treatment with HTG, it was possible to show that the neonatal immune repertoire responds differently to the various epitopes on HTG. From these studies it was apparent that neonatal treatment with HTG generated a population of cells which could be activated by HTG (but not MTG) to produce antibodies with enhanced anti-HTG binding activity (FIG. 4.8B). On the other hand, the same population of cells depressed the anti-MTG antibody response activated by MTG/LPS (FIG. 4.8A).

These interesting observations suggest that there may be both suppressor (or tolerising) and helper determinants on HTG, which interact with the pre-immune repertoire sufficiently to affect a subsequent TG-specific response. Many protein-systems have been characterized according to their helper (HD) and suppressor (SD) determinants (reviewed in Sercarz & Krzych, 1991). In the majority of cases studied these determinants do not overlap, and in the cases where overlap is reported, in each instance, the determinants are not identical. It is tempting to suggest that the helper and suppressor determinants on HTG correspond to unique and shared epitopes respectively.

The reasons why SD and HD are different remain unknown, but the current view is that in order to activate suppressor mechanisms, both HD and SD have to be present on the same molecule. Several possible mechanisms of recognition in intracellular suppression have been put forward, one of which requires that the SD and HD must be presented by the same antigen-presenting cell in a multi-cell cluster (Sercarz & Krzych, 1991).

A simple interpretation of the data for the TG-rat RBC transfer experiments is more difficult, because more than one antigen is involved, nevertheless, by immunizing adult mice with TG coupled to rat RBC, both suppressor and helper functions appeared to be activated. The transfer of spleen cells from TG-rat RBC immunized mice mediated specific help for TG antibodies (FIG. 4.4) and this function was activated by TG specific epitopes. If the argument for

the presence of helper and suppressor determinants on HTG is true, then it may be that pig TG does not have the same set of suppressor determinants (this could be verified by studying the effects of neonatal tratment with pig TG). In this respect it is interesting to note that cow and sheep insulin are each nonidentical to pig insulin in loop A and do not induce Ts cells, but all three insulins induce cross-reactive Th cells.

The specificity of the transferred suppression observed in the TG-rat RBC model is in agreement with Howles and Cox (1984). These authors showed that although antibody responses to trinitrophenyl (TNP) could be induced by TNP-coupled rat erythrocytes, they could not be co-suppressed in this system. Previous depletion studies suggested that the cells responsible for suppression in the rat RBC model are T cells (Cooke et al., 1978; Naysmith et al., 1980) and that T cells are required in recipients to become effector-suppressor cells (Hutchings & Cooke, 1981). The involvement of T cells is not doubted in this model, but in the light of recent scepticism about the very existence of Ts cells, the question centres on the mechanism by which specific suppression and specific help might be mediated.

At a time when the concept of T suppressor cells was in vogue, both Mitchell (1974) and Bretscher (1981) alluded to the possibility that T suppressor and T helper cells were one class of cells, although their proposed mechanisms for the function of these cells was less clear: Mitchell suggested that suppressor T cells may well be hyperactive "helper" T cells which, by eliminating antigen (by T cell and phagocyte activity), reduce the number of B cells which receive the "first signal". While Bretscher's proposal was that suppressor and helper activity are two properties of one class of regulatory T cell and that the number of inductive complexes, formed under different conditions, determine the class of response induced.

The experiments described above are more easily explained by a mechanism which invokes the activation of specific T helper cells. This naturally begs several questions: How would such specific activation occur? What type of Th cells are activated and how does a high dose of soluble or particulate antigen selectively inactivate a

set of lymphocyte specificites? A plausible theory is that self-reactive lymphocytes are tolerised and at the same time memory Th cell populations, which have specificity for the non-self determinants, are generated.

In the tolerance model described by Parish and co- workers (1988), although the specific autoantibody response to MTG was suppressed, the response to xenogeneic TG was not abrogated. Challenge with pig TG also induced antibodies reactive with MTG in both MTG tolerised mice and saline treated controls. The binding activity to MTG was equivalent in these two groups. However, binding to MTG in the tolerized group could be mostly absorbed out with soluble pig TG, but not in the controls. These experiments might be interpreted as showing that the tolerizing regime had induced subtle changes in the specificity of the lymphocyte repertoire. That is, while anti-MTG antibodies in the control group have greater specificity for MTG epitopes, the antibodies induced in the tolerized group are more cross-reactive with pig TG.

The switch from one set of specificities to another, on encounter with a tolerising dose of antigen, suggests that some form of interacting regulatory mechanism normally exists, which limits the reactivity of the second set of specificities. It is clear that the murine immune system is normally selective in its reponse to most antigens. Thus, even in high responders, relatively few determinants of the insulin (Talmon et al., 1983) or lysozyme (Goodman & Sercarz, 1983) molecules activate T lymphocytes. The limited responses however can be overcome. For example, low and high responder mice respond to one and five sites respectively on the myoglobin molecule, but low responders primed with higher doses of myoglobin will respond to additional antigenic sites (Young & Atassi, 1982). Manipulations with different adjuvants also appears to have a similar effect on the responsiveness to TG (see Chapter 3). It may be that persistence of a sub-set of self-reactive lymphocytes is necessary for this finer regulation of specificity.

In the above examples of models of tolerance, epitopes on the foreign antigen which are homologous with self, may induce clonal anergy in

the respective subpopulations of self-restricted cells. In addition, lymphocytes with non-self specificities are preferentially primed, either because they are no longer subject to negative regulation mediated by self-reactive clones, or simply because they compensate for the inactivation of one set of specificities. There is now sufficient evidence in support of the first stipulation: clonal anergy can occur in mature T (Qin et al., 1989; Rammensee et al.,1989) and B (Chiller et al., 1971; Desaymard & Waldman, 1976; Goodnow et al.,1988 and 1989) cells.

A second proviso has to be set to overcome the objection that those lymphocytes with specificity for foreign determinants might also be subject to functional inactivation by clonal anergy. One suggestion is that the two subpopulations are normally maintained in different maturational stages, however, the stage of B-cell development does not seem to be critical for tolerance induction (Metcalf et al., 1979). Scott and Klinman (1987), have proposed the interesting hypothesis that tolerance is induced by binding of a B cell's surface Ig at a critical point in early G_1 phase of the cell cycle. Interestingly, Portnoi et al. (1986) have shown that B cells reacting with autologous TG and erythrocytes are not only present in peripheral tissues and competent to proliferate and secrete antibodies, they are also cycling cells.

Preferential inactivation of an equivalent set of self-reactive T cells may also occur for the same reasons. T cell tolerance might occur because of antigen presentation of self epitopes in the absence of co-stimulator (reviewed in Weaver & Unanue, 1990), although this seems less probable, because the non- self reactive T cells would be subject to the same inhibition. A third possibility is that direct inactivation of self-reactive T cells is not necessary, because the functional activity of these cells is normally dependent on the presence of self- reactive B cells. The dependence of T-cell reactivity on B-cell development has been recognised for some time (Gray, 1984). T cells from mice treated with anti-IgM antibodies show a reduced capacity to proliferate in response to various antigens (Ron et al., 1983;1981) and have an impaired ability to help normal B cells produce IgG responses in general (Rosenberg & Asofsky, 1981). The role of T suppressor cells as a cause of reduced T cell help in these animals was excluded. It is therefore tempting to suggest that a

subset of B cells to self epitopes are normally maintained in the adult animal in an activated state and therefore are subject to clonal anergy. The role of such cells may be to regulate other lymphocyte specificities.

CHAPTER 5

THE INFLUENCE OF AMINOTRIAZOLE ON MURINE EAT

5.1 INTRODUCTION

It is now accepted that clonal deletion does not remove all autoreactive T cells (especially in the case of tissue-specific antigens not expressed in the thymus) (Schwartz, 1989) and there is experimental evidence (Weigle, 1981; Yeni & Charreire, 1981) which suggests that both T and B cells with receptors specific for TG are present in the peripheral repertoire. However, the exact mechanism through which these cells are controlled to maintain a natural state of tolerance remains unresolved. Several years ago it was suggested that tolerance is maintained because the concentration of circulating TG is sufficient to maintain a state of tolerance at the T cell level (thereby dispensing with the necessity to advocate a mechanism for tolerizing the autoreactive B cells) (Roitt & Torrigiani, 1967; Romball & Weigle, 1983). Given our current understanding of T cell activation, the plausibility of this idea still stands, with the addenda that these T cells might be controlled by recognition of TG peptide/MHC complexes.

Intriguingly, despite the fact that at least nineteen different epitopes can be mapped on human TG (Bresler et al., 1990), anti-TG autoantibodies found in the serum of patients with thyroiditis recognize only four to six epitopes on the molecule (Nye et al., 1980). Thus it appears that when tolerance is broken, the immune response is consistently skewed towards a limited number of epitopes; the reason for this is currently unknown. Gammon and Sercarz (1989) have suggested that tolerance to many self-antigens will not be complete, because these determinants are only available in relatively low amounts after *in vivo* processing of the whole antigen. So far it has not been possible to characterize the immunodominant TG epitopes, partly because they appear to be dependent on the tertiary and quaternary structure of TG (Dong et al., 1989). However, a number of studies have focussed on the immunogenicity of the

iodine-dependent determinants of TG (Bagchi et al., 1985; Allen et al., 1986; Champion et al., 1987; Sundick et al., 1987).

If we accept that circulating self-antigen is in some way important for maintaining tolerance, then it becomes necessary to consider how a transient period of altered TG synthesis (eg. as a result of environmental or dietary effects on thyroid function) might influence a subsequent immune response to the native antigen. It has been well established that a number of compounds (such as sulfonamides and thioureas) have the property of inhibiting the thyroid gland from reacting with inorganic iodide to form thyroid hormones (reviewed in Pitt-Rivers & VanderLaan, 1984). Many foods, including cabbage, turnips, peas, beans, strawberries and milk, also contain "antithyroid" substances (Jukes & Shaffer, 1960). Antithyroid compounds can interfere with one or more steps in the synthesis of the thyroid hormones, but the theory that many antithyroid compounds act by inhibiting thyroid peroxidase gained acceptance in the 1950's (Strum & Karnovsky, 1971). One such compound is 3-amino-1,2,4-triazole (ATA), which was shown to inhibit peroxidase activity in homogenates of the thyroid and salivary gland (Alexander, 1959). Although the covalent binding of ATA to the enzyme catalase is irreversible, cytochemical staining evidence indicates that the inhibition of thyroid peroxidase by ATA is reversible (Strum & Karnovsky, 1971). Importantly, the thyroid glands of rats fed for 17 weeks on a diet containing ATA were found to be normal in appearance two weeks after ATA was withdrawn from their diet (Jukes & Shaffer, 1960). Similar observations were made in White Leghorn chicks (Wishe et al., 1979). These authors also reported that thyroxine (T4) levels were significantly greater in the controls than in ATA treated birds. The antithyroidal effect of ATA in the mouse has also been reported (Steinhoff et al., 1983) and Champion et al. (1987) reported that TG extracted from mice given ATA (as a 0.1% solution in their drinking water) was virtually devoid of iodine content. The reversible action of ATA on thyroid function was exploited to determine whether ATA treatment can alter the characteristics of experimentally induced thyroiditis in CBA/J mice.

5.2 RESULTS

5.2.1 The effect of ATA treatment on the antibody response to TG

Age and sex matched high responder CBA/J ($H\text{-}2^k$) mice were separated into three groups (B, C and D). ATA was added to the drinking water of mice in groups C and D. After 1 month of ATA treatment group C mice were returned to normal drinking water; at this time point all groups of mice were split into sub-groups of 5 animals and challenged as shown in diagram 5.1. Group D mice were maintained on ATA throughout the course of the experiment. For comparison of the antibody response to TG challenge, low responder Balb/C ($H\text{-}2^d$) mice were included as a fourth group (A).

As it has been reported that feeding with aminotriazole significantly affects the the synthesis of thyroid hormones (Strum & Karnovsky, 1971), while ATA was administered, the drinking water was also supplemented with T4, to provide a steady supply of active hormone to the tissues. The control groups of mice were not administered T4, since Weetman et al. (1982) have demonstrated that T4 supplementation has no significant effect on TG antibody production in adjuvant-induced thyroiditis in rats.

The anti-MTG binding activity of sera taken at day 28 was measured by solid-phase RIA (FIG. 5.1). In agreement with previous work (Rose & Kong, 1983), the results showed that low responder Balb/C mice had lower autoantibody levels than the high responder CBA/J mice. By including a comparison of the antibody response of these low responder mice it was possible to have a more positive perspective of the effect of ATA treatment on the autoantibody response in the high responder animals. With MTG/CFA immunization, in comparison to the CBA/J control, the anti-MTG binding activity of CBA/J mice maintained on ATA throughout the experimental period was significantly reduced ($P = 0.01$): The level of response being essentially the same as that of the Balb/C mice immunized with MTG/CFA (FIG. 5.1B,). The down-regulatory effect of ATA treatment on anti-MTG antibodies was not observed if the drug treatment was stopped prior to the animals being challenged (ie. ATA pretreated). However, the

level of anti-MTG antibodies induced by MTG/LPS immunization was significantly affected ($P = 0.01$) even when the animals were pretreated with ATA (FIG. 5.1A).

To further analyse these differences, the cross-reactivity of these antisera with six different TG species was determined. As might be expected, the effect of ATA treatments on the anti-rat TG binding activities followed a similar pattern to that observed for the binding to MTG. Figure 5.2 shows the mean antibody binding activity to rat TG (at a serum dilution of 1 in 50) for each group of mice studied. The effect of ATA on the cross-reactivity with other species of TG, however, was variable. For example, ATA treatment had no effect on the cross-reactivity of MTG/LPS induced antisera with human TG; at serum dilutions of 1 in 50 the mean binding activities for the control, ATA pretreated and ATA maintained groups were 7438, 7594 and 6590 c.p.m. respectively.

The anti-TG cross-reactivity of each serum sample was calculated as a ratio of the binding activity to MTG, using the formula:

$$\% \text{ ratio} = \frac{\text{binding to heterologous TG (c.p.m.)}}{\text{binding to mouse TG (c.p.m)}} \times 100$$

(Where, c.p.m. = total binding activity minus the background c.p.m. for the respective TGs)

Representation of the cross-reactivity data in this form showed a number of interesting patterns. For example, when the MTG/LPS induced response of mice maintained on ATA was analysed, the ratio of reactivity with rat TG did not alter with serum dilution (FIG.5.3A), but the ratio of binding to human TG did decline with dilution (FIG. 5.3B): The difference between the lowest and highest dilutions studied was significant ($P < 0.01$). If the ratio of binding remains constant with serum dilution, it is reasonable to suggest that a single population of antibodies contributes to the cross-reactivity with the foreign TG. Therefore, in this experimental group it would appear that one major population of antibodies cross-reactive with rat TG were induced. This represents approximately

60% of the total antibody response to mouse TG. In contrast, more than one set of antibodies may have been contributing to the cross-reactivity with human TG. A similar pattern of reactivity was observed for the ATA pretreated group.

Antisera from the CBA/J control group of mice which were immunized with MTG/LPS, also bound to human TG, and the ratio of binding was similar to that observed for the two ATA treated groups; there was no difference between the three groups at a 1 in 50 dilution. However, at this dilution the anti-rat TG ratios for this control group was greater than both the ATA treated groups ($P = 0.05$). At higher dilutions there was no difference in the ratios between these three groups. This suggests that MTG/LPS immunization normally induces a second anti-rat TG reactive antibody population which can be diluted out: With ATA treatment this population appears to be suppressed.

This possibility was emphasized further by comparing the MTG/CFA induced antibody responses between the control and ATA treated groups and also with the respective MTG/LPS immunized groups. The anti-rat TG ratios of the three CBA/J groups immunized with MTG/CFA did not differ at serum dilutions of 1 in 400 ($-\log_2=8.64$). As with MTG/LPS induced antibodies, the average anti-rat TG ratio of MTG/CFA sera was 60%. At dilutions of 1 in 50 ($-\log_2 = 5.64$) the control and ATA pretreated groups had mean ratios of 83% and 77% respectively, and these values were significantly ($P < 0.05$) higher than that of the ATA maintained group (FIG.5.4A). Compared with the MTG/LPS induced response, the ratio of binding to human TG was also increased with MTG/CFA immunization in both the control and ATA pretreated groups ($P < 0.02$ and < 0.05 respectively) (FIG. 5.4B). The ratio of antibody binding to human TG was not affected by the different immunization regimes if the mice were maintained on ATA.

Comparison of the MTG/CFA immunized ATA treated groups with the CBA/J controls showed a variable effect of ATA on the cross-reactivity ratios. With the exception of the reactivity with wombat TG (FIG. 5.4E), ATA treatment significantly influenced the binding reactivities against the

other TG species studied. Interestingly, although there was no difference in the anti-rat TG reactivity between the ATA pretreated group and the control (FIG. 5.4A), the response to zebra, human, pig and dog TG was higher in the ATA pretreated groups. At serum dilutions of 1 in 50, statistical analysis of the ratios of antibody binding showed that these differences were significant: $P = 0.05$ for the response to zebra TG and $P < 0.1$ for dog, human and pig TG. Analysis of the data for serum dilutions of 1 in 400 showed that for dog, human, and pig TG, the difference between the control and ATA pretreated groups increased with serum dilution ($P < 0.02$ for dog TG and $P < 0.05$ for human and pig TG).

The increased cross-reactivity of MTG/CFA induced antibodies as a result of pretreatment with ATA was not observed if ATA treatment was maintained. With the exception of the reactivity against rat TG, there was essentially no difference in the ratios of binding with the other TG species when compared with the response of the control group (FIG. 5.4 A-F).

To further analyse the relationship between antibody cross-reactivities and immunization regimes, anti-TG binding patterns of sera from MTG/CFA immunized groups of mice were compared with those of the corresponding groups challenged with MTG/CFA twice. At serum dilutions of 1 in 50 the mean binding to MTG for the control groups challenged with MTG/CFA (x1) and MTG/CFA (x2) were 22677 +/- 495 cpm and 22887 +/- 133 cpm respectively, yet the ratios of reactivity with some of the species of TG was increased in the group challenged with MTG/CFA twice. There was no significant difference in the reactivity with rat or dog TG, but the ratios of binding to the other TG species were increased. The most prominant effect was on the reactivity with zebra TG ($P < 0.001$), followed by human TG ($P < 0.05$), wombat TG ($P < 0.02$) and pig TG ($P = 0.1$).

In ATA pretreated mice there was also no difference in the anti-MTG binding activities of sera at dilutions of 1 in 50: 22940 +/- 239 cpm versus 22602 +/- 176 cpm of MTG/CFA (x1) and MTG/CFA (x2) immunized

groups respectively. However, at this dilution the binding ratios to rat TG ($P = 0.01$) and human TG ($P = 0.1$) were increased. Analysis of these values in relation to the data for the MTG/CFA (x2) immunized CBA/J controls showed that except for a small difference in the binding to wombat TG ($P < 0.2$) (FIG. 5.5A), there was no significant difference in the reactivity with the other TG species.

Comparison of the mean anti-MTG activity (at serum dilutions of 1 in 50) of mice maintained on ATA and challenged with MTG/CFA, with that of the ATA maintained group challenged with MTG/CFA twice, showed a small difference between the two values ($P < 0.2$). The effect of the second challenge on the ratio of antibody binding to heterologous thyroglobulins was also correspondingly small: There was no difference between the values of the two groups for the binding to pig, dog or wombat TG and small increases were observed for the reactivity with rat and human TG ($P < 0.2$). The response to zebra TG was however substantially ($P < 0.05$) increased in the group immunized with MTG/CFA twice. This data compared with the corresponding CBA/J control group showed that at serum dilutions of 1 in 50, the anti-MTG binding activities of sera from the mice maintained on ATA was significantly less ($P < 0.001$). Interestingly, differences in the ratio of binding to the other thyroglobulins was quite variable: The anti-dog TG ratios were essentially unchanged and the difference in the values for human, pig and zebra TG were small ($P < 0.2$). Only the ratios of binding with wombat and rat TG were significantly higher in the control group ($P < 0.02$ and < 0.01 respectively).

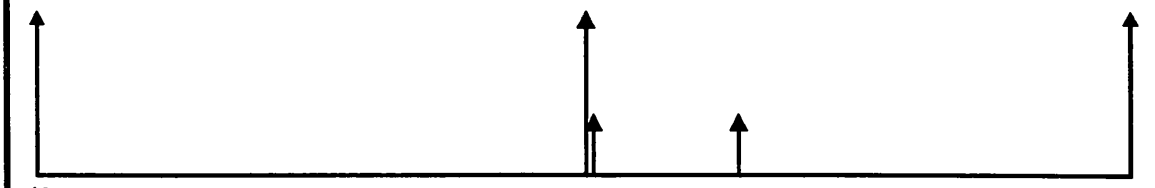
DIAGRAM 5.1

PROTOCOL FOR DETERMINING THE INFLUENCE OF AMINOTRIAZOLE ON EXPERIMENTALLY INDUCED THYROIDITIS IN CBA/J MICE

GROUP C & D MICE GIVEN THE THYROID PEROXIDASE INHIBITOR, ATA, IN THEIR DRINKING WATER.

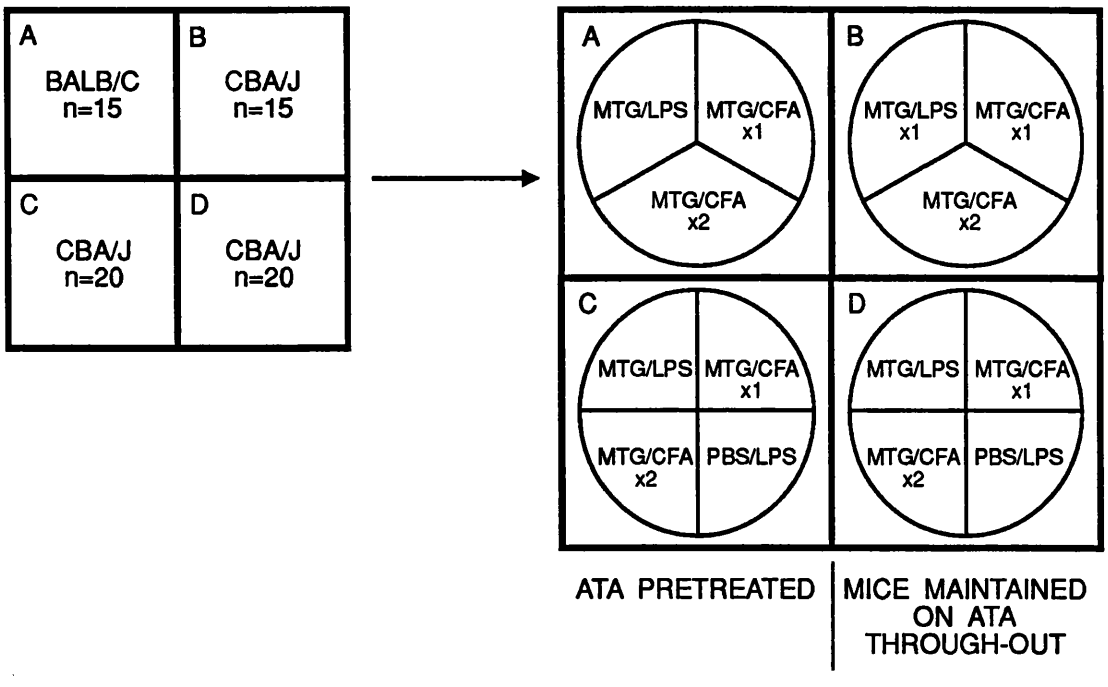
GROUP C MICE RETURNED TO NORMAL DRINKING WATER

MICE KILLED. THYROIDS TAKEN FOR HISTOLOGY, SERA COLLECTED FOR ANALYSIS OF ANTI-TG ANTIBODIES



4 GROUPS OF MICE, AGE 6-8 WEEKS, KEPT IN THE SAME HOUSING CONDITION AT THE START OF THE EXPERIMENT

EACH MAIN GROUP OF MICE WAS SPLIT INTO SUBSETS OF 5 MICE & CHALLENGED AS SHOWN BELOW



THE IMMUNIZATION PROTOCOLS WERE:

1. 50ug MTG/CFA fp ON d0
2. 50ug MTG/CFA fp INJECTED ON d0 & AGAIN ON d7
3. 50ug MTG iv/20ug LPS ip d0, d7
4. PBS iv/20ug LPS ip d0, d7

THE INFLUENCE OF AMINOTRIAZOLE (ATA) ON TG ANTIBODY LEVELS IN MURINE EAT

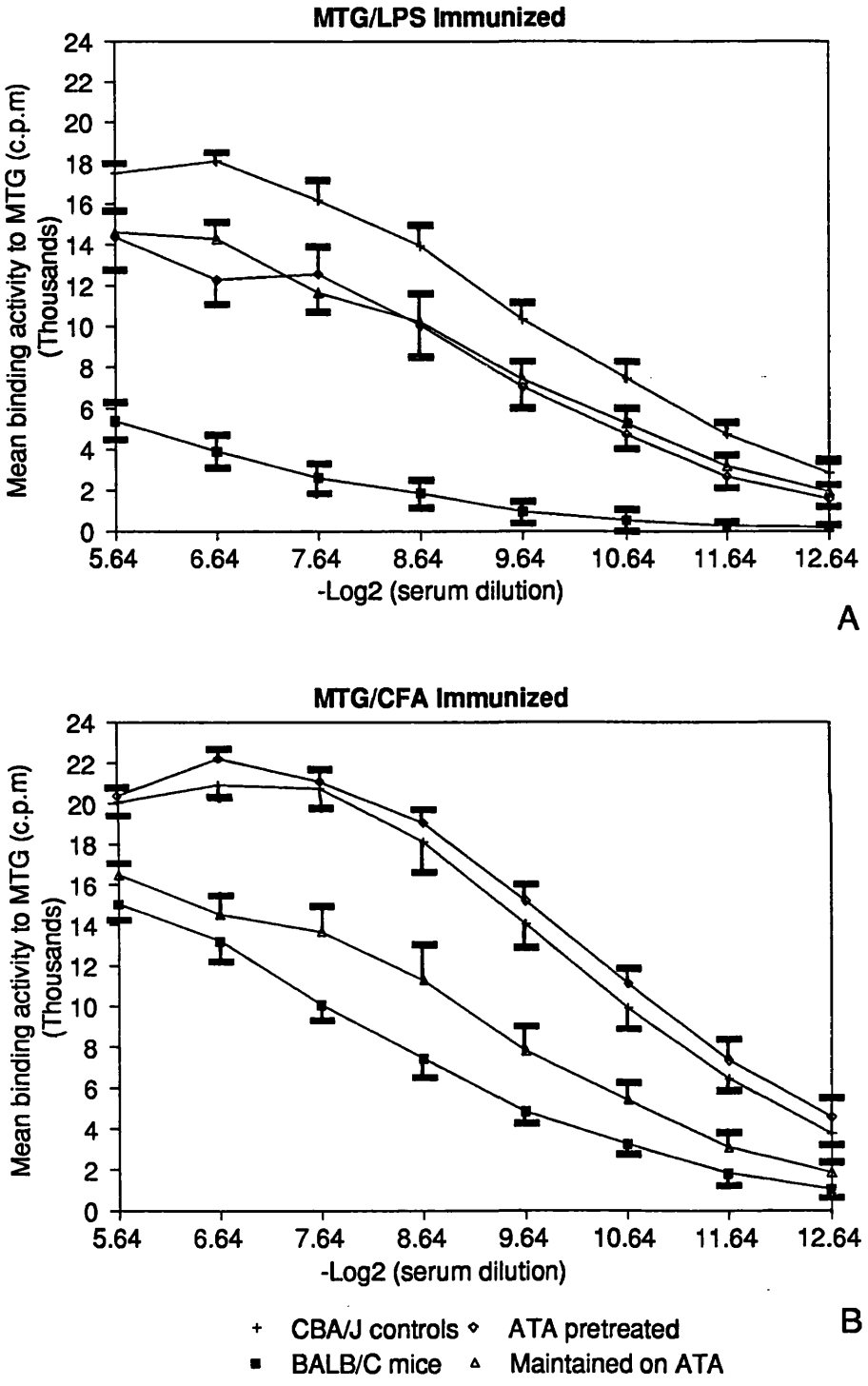


FIGURE 5.1: The anti-MTG response on day 28 in the groups of animals studied (mean +/- s.e.m.). (A) Mice were immunized with MTG/LPS (d_0, d_7) or, (B) with MTG/CFA (d_0).

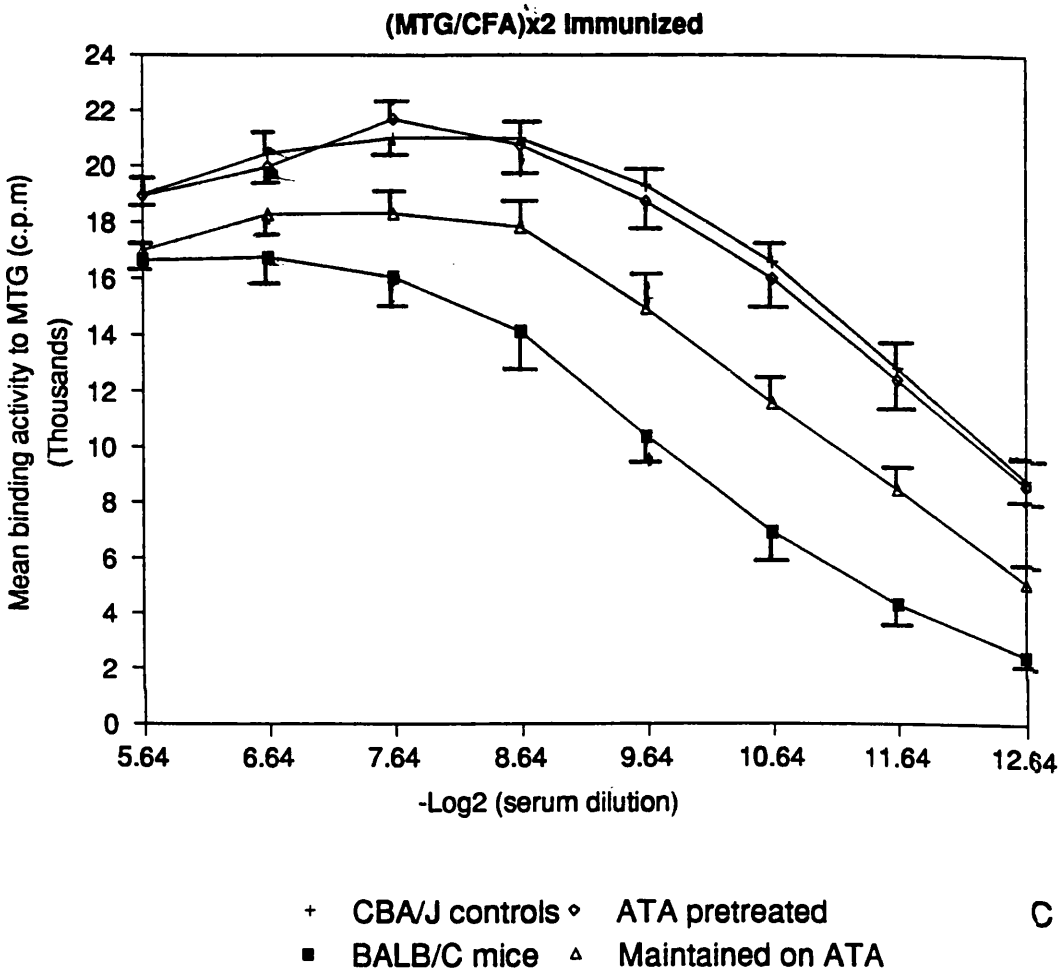


FIGURE 5.1C: The anti-MTG response in the groups of animals studied (mean +/- s.e.m.). Mice were immunized with MTG/CFA (d_0, d_7).

THE EFFECT OF ATA ON THE CROSS-REACTIVITY OF AUTOANTIBODIES WITH RAT-TG

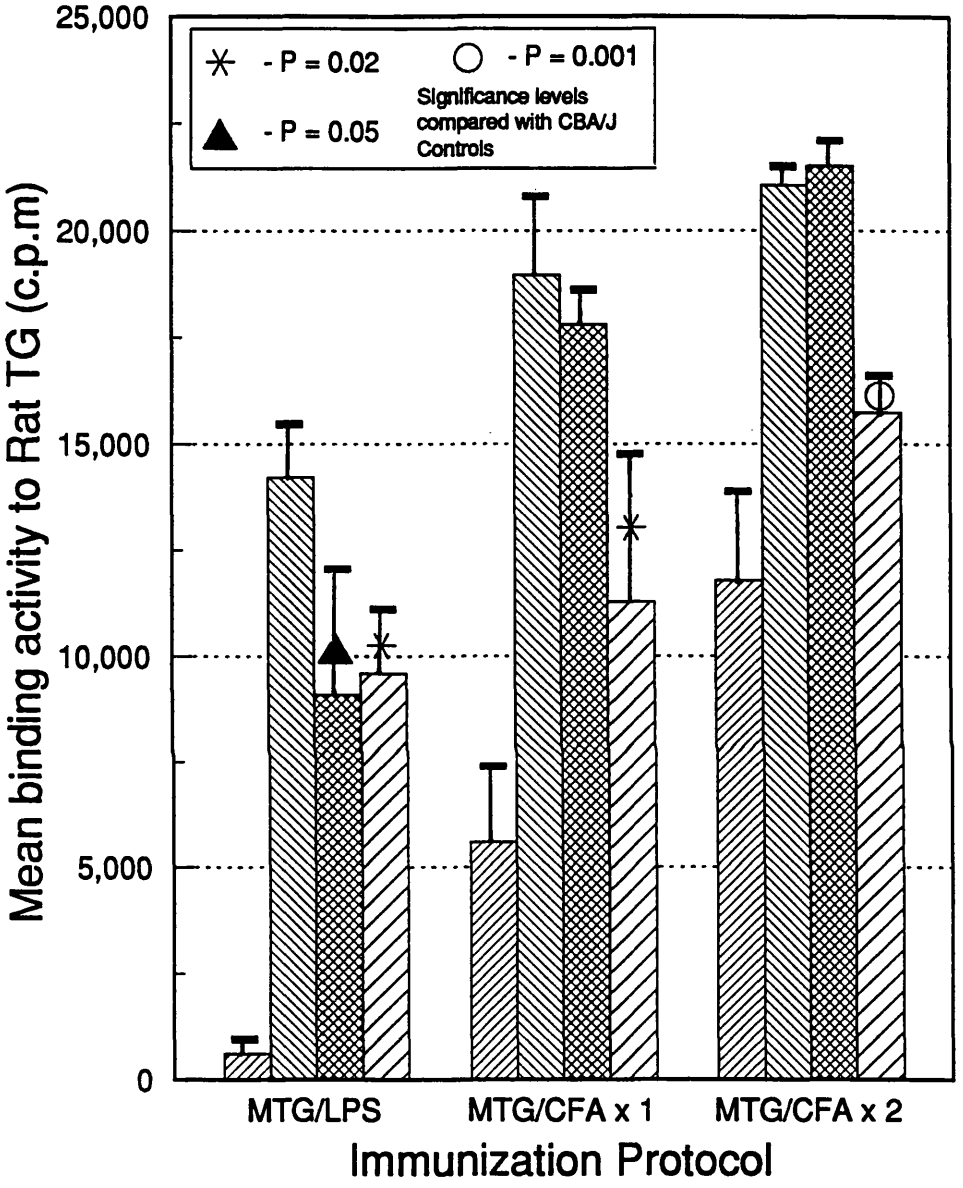


FIGURE 5.2: Each bar represents the mean value (+/- s.e.m.) for the antibidy binding activity to rat TG at a serum dilution of 1/50 for each group of animals studied:

- Balb/C;
- CBA/J (Control);
- CBA/J (ATA pretreated);
- CBA/J (maintained on ATA).

ATA TREATMENT DOES NOT ALTER THE CROSS-REACTIVITY OF MTG/LPS INDUCED ANTIBODIES

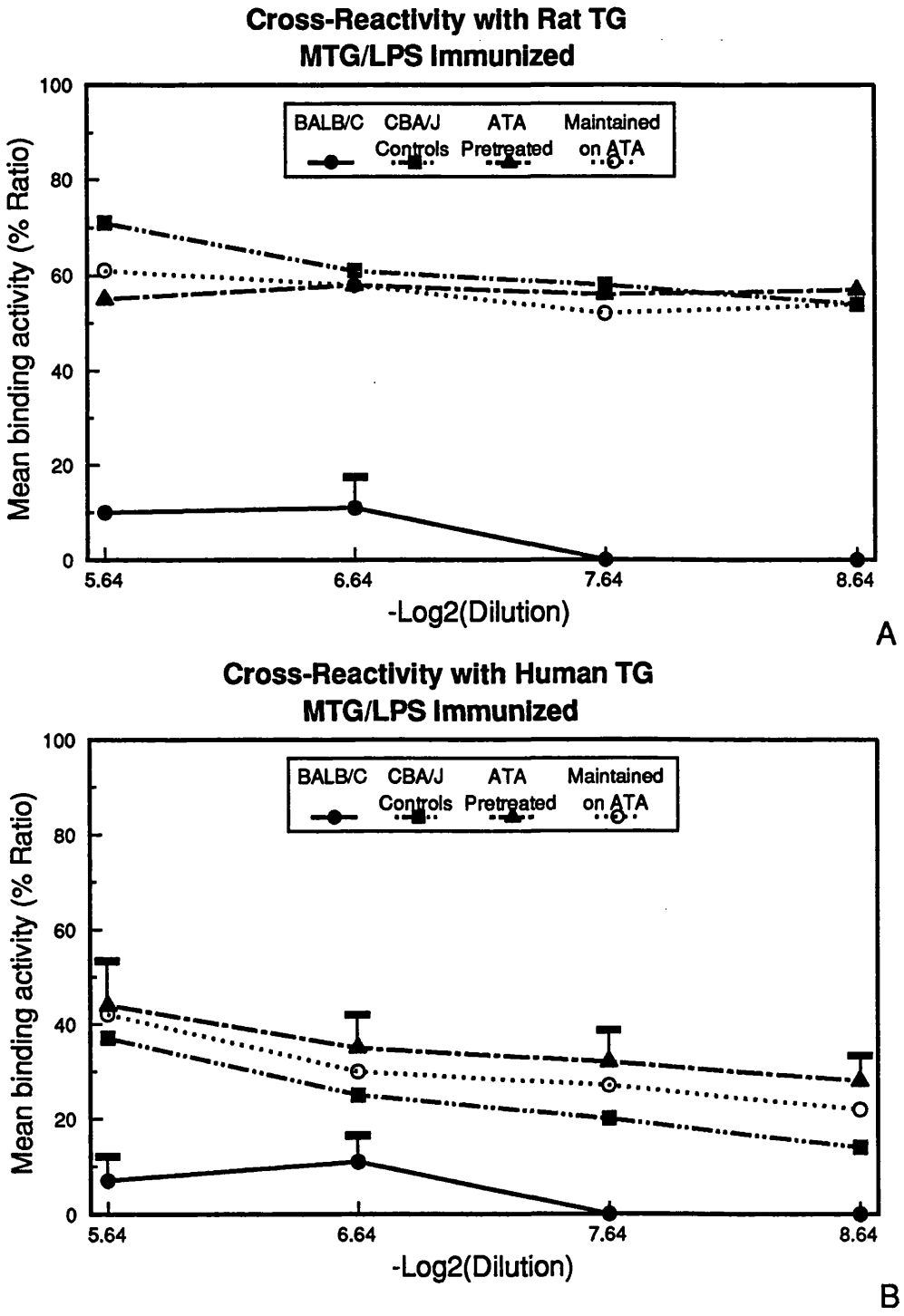
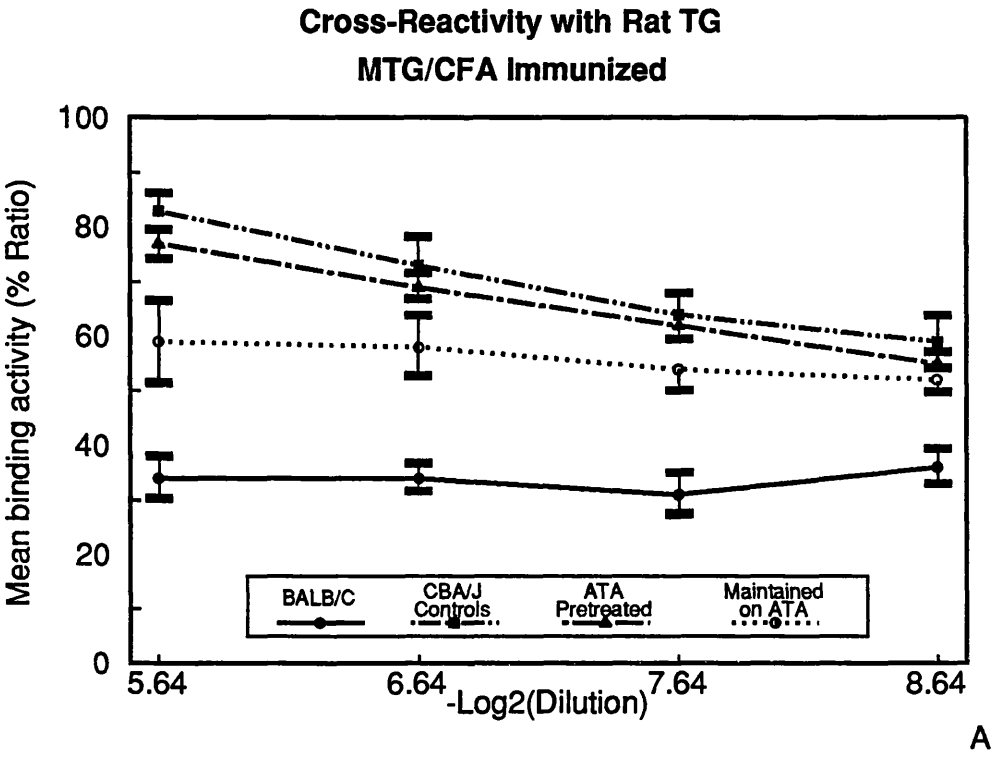


FIGURE 5.3: Results are shown for the ratio of cross-reactivity with rat (A) and human (B) TG of anti-mouse TG antibodies from MTG/LPS immmunized mice. Each point of the serum dilution curves represents the mean(+/- s.e.m.) value obtained for the groups of animals studied. (note that s.e.m. < 5% have been omitted for clarity of presentation).

ANTIBODIES IN CBA/J MICE IMMUNIZED WITH MTG/CFA



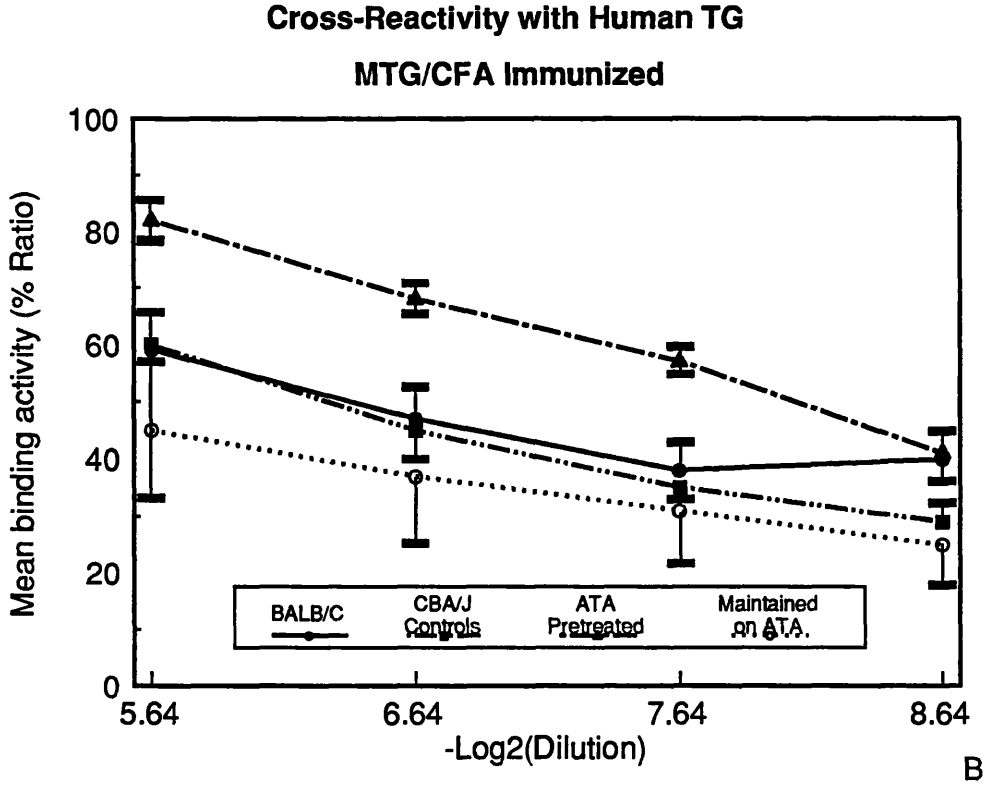
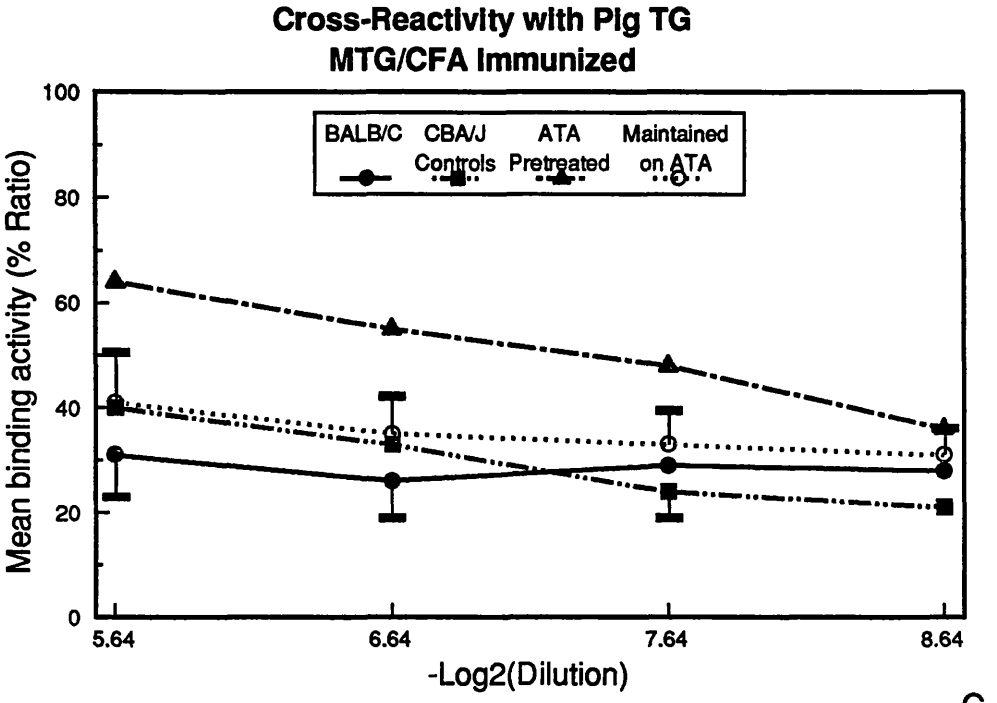


FIGURE 5.4 (A-F): Each point of the serum dilution curves represents the mean(+/- s.e.m.) value obtained for the groups of animals studied. (Note that s.e.m. < 5% have been omitted for clarity of presentation). Results are shown for the ratio of cross-reactivity with rat (A) and human (B) TG of anti-mouse TG antibodies from MTG/CFA immunized mice.



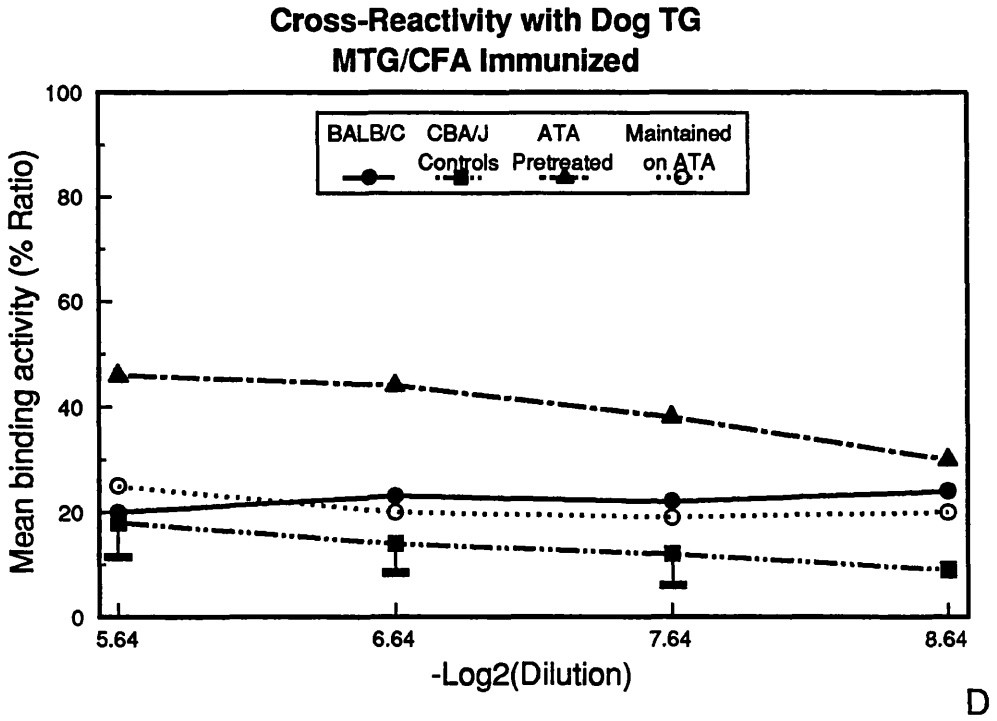
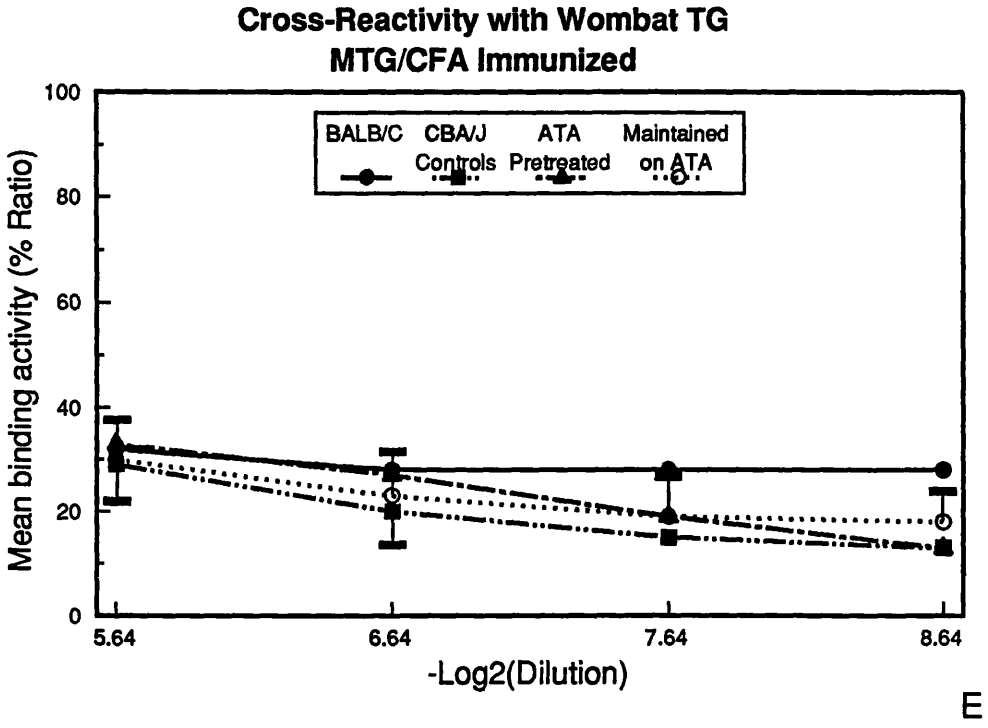


FIGURE 5.4: Results are shown for the ratio of cross-reactivity with pig (C) and dog (D) TG of anti-mouse TG antibodies from MTG/CFA immunized mice.



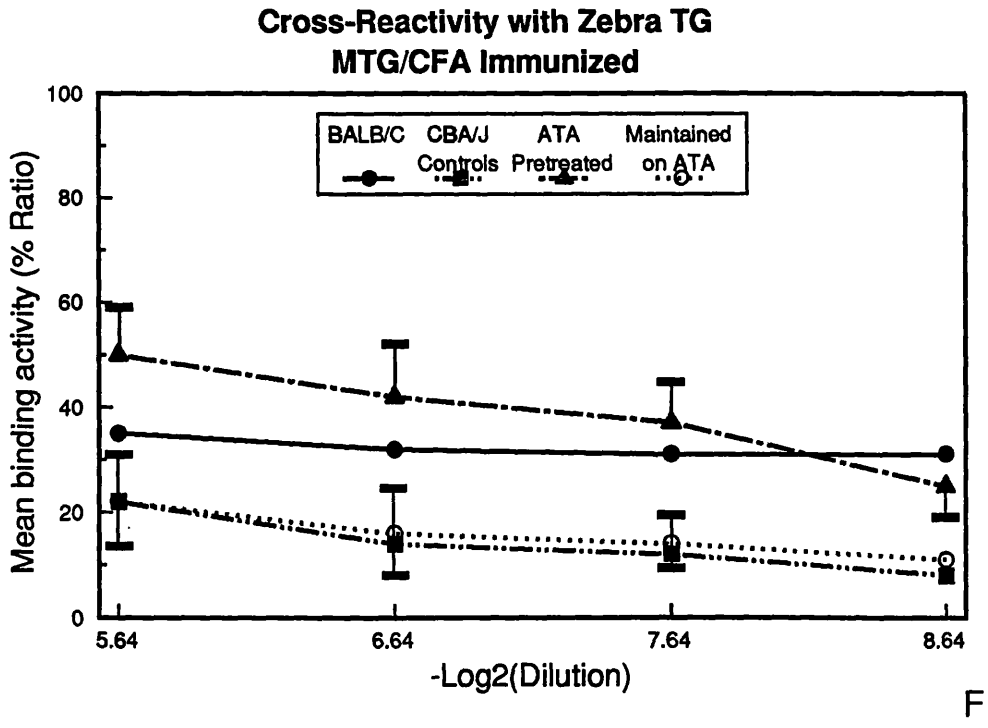


FIGURE 5.4: Ratio of cross-reactivity with wombat (E) and zebra (F) TG of anti-mouse TG antibodies from MTG/CFA immunized mice.

THE EFFECT OF ATA TREATMENT IS EVIDENT IN THE SPECIFICITY OF ANTI-TG ANTIBODIES INDUCED IN CBA/J MICE AFTER TWO CHALLENGES WITH MTG/CFA

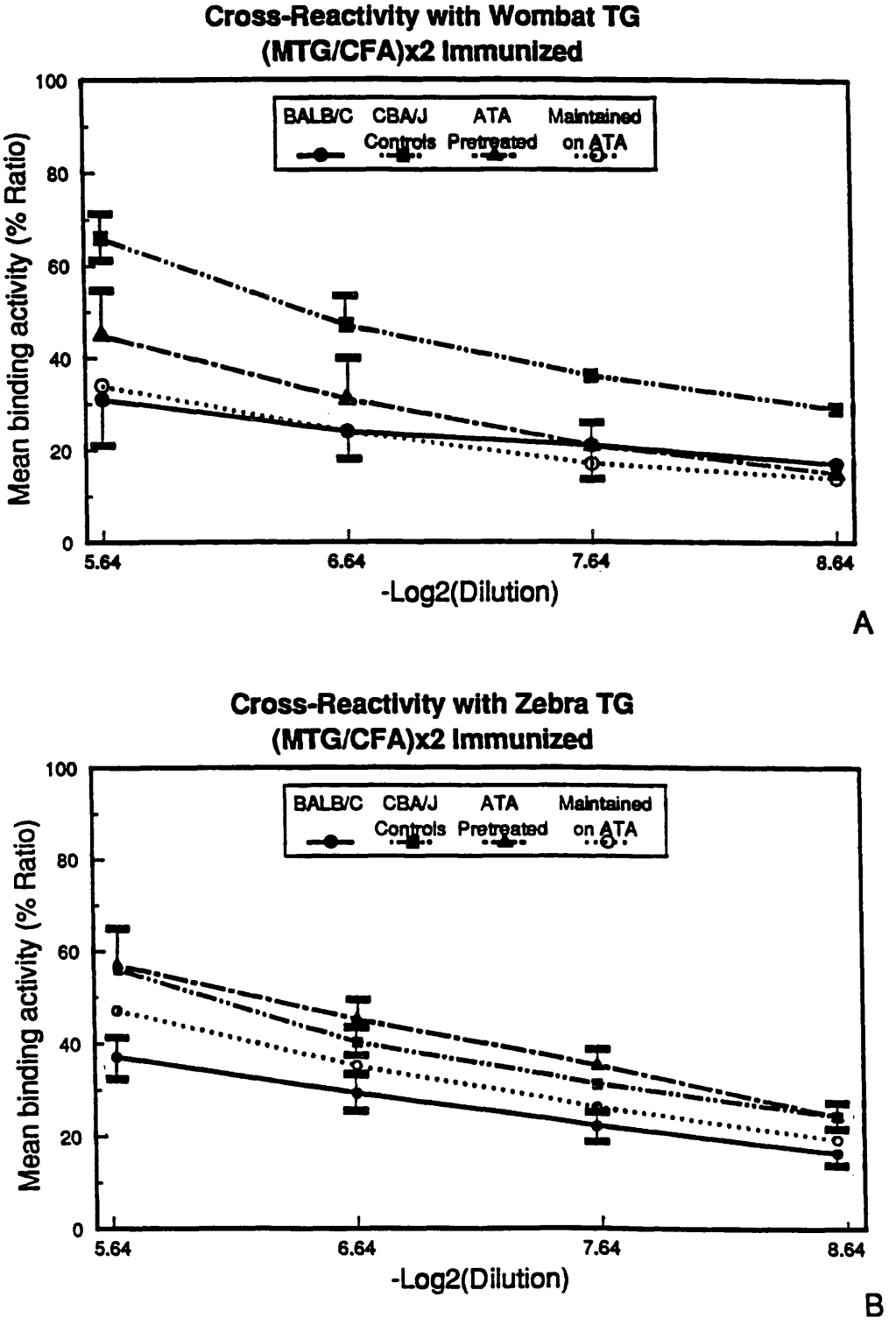


FIGURE 5.5: Results are shown for the ratio of cross-reactivity with wombat (A) and zebra (B) TG of anti-mouse TG antibodies from mice challenged twice (d_0, d_7) with MTG/CFA.
Each point of the serum dilution curves represents the mean(+/- s.e.m.) value obtained for the groups of animals studied. (Note that s.e.m. < 5% have been omitted for clarity of presentation).

TABLE 5.1

INCIDENCE OF THYROIDITIS IN ATA TREATED MICE

GROUP	INDIVIDUAL THYROID SCORES		
	MTG/LPS Immunised	(MTG/CFA) x 1	(MTG/CFA) x 2
BALB/C	00011	00001	00000
CBA/J Control	01122	00012	00111
CBA/J ATA Pretreated	11122	00112	01112
CBA/J Maintained on ATA	00112	00001	00013

The pretreatment and challenging regimes was as shown in DIAGRAM 5.1. Thyroids were taken 28 days after positive challenge. Individual thyroids were scored on a scale ranging from 0 to 4.

5.2.2 Incidence of thyroiditis

Thyroids were taken for histological analysis on day 28 after the first challenge. These results showed that the incidence and severity of thyroiditis induced in Balb/C mice was minimal (Table 5.2). In control CBA/J mice, maximum lymphocitic infiltration was obtained by using LPS as the adjuvant. Athough pretreatment with ATA for 28 days induced both vascular and parenchymal changes associated with goitrogen, these changes were not apparent 28 days after removing the drug treatment. ATA pretreated mice challenged with PBS/LPS did not have thyroiditis. One mouse in this group had necrotizing arteritis, but this is often observed with exposure to LPS and in fact this may even occur spontaneously in CBA mice (D.Rayner, personal communication). The same mouse also had minimal lymphocytic infiltration. The incidence of MTG/LPS induced thyroiditis in ATA pretreated mice did not decrease compared with the controls. There was also no significant effect of ATA pretreatment on MTG/CFA induced thyroiditis.

Those mice left on ATA throughout the experiment had enlarged thyroids which were hyperplastic to varying degrees. Lymphocytic infiltration was not observed in any of the thyroids from mice maintained on ATA and challenged with PBS/LPS. The thyroids from mice challenged with MTG/CFA were difficult to score because of the superimposition of varying degrees of two separate processes - inflammation, as well as the goitrogen induced changes. The thyroid given a score of 3 was grossly hyperplastic. Despite the structural changes within the thyroids of this group of mice, thyroiditis was not increased when compared with controls.

5.3 DISCUSSION

Circulating TG is a heterogeneous mixture and present techniques cannot differentiate between surface epitopes, expressed on circulating TG, and membrane bound TG, in the thyroid gland. Recent studies show that proteolytic cleavage of TG transferred to lysosomes is selective. This

early step leads to the formation of hormone-depleted TG molecules containing limited structural alterations (Rousset et al., 1989). The question remains as to whether all the TG molecules in circulation are depleted of hormonogenic epitopes, or if a small percentage escapes even this first processing step. Such considerations are important if the maintenance of unresponsiveness in TG-reactive cells in the peripheral repertoire is a major function of the circulating TG.

As antigen-induced tolerance is concentration dependent (Goodnow et al., 1989; Chiller et al., 1971), the relative concentration of particular epitopes on a protein may well determine whether or not epitope- specific lymphocytes escape tolerance induction. Gammon and Sercarz (1989) have studied T cell tolerance to peptide determinants of hen egg white lysozyme (HEL) and found that the T cells which escaped tolerance induction, were specific for minor determinants on the antigen, and proposed that for the same reason, these T cells are not normally activated but can be stimulated under special circumstances to circumvent tolerance.

By analogy, if a constant level of specific TG epitopes is required to maintain tolerance in adult mice, exposure to an anti-thyroid substance, such as ATA, may alter the balance of the immune repertoire. That is, more cells with specificity for the reduced, or altered, epitopes would escape tolerance. If this does occur, an increased response to a subsequent challenge with TG should be seen. The results shown in figure 5.1A does not support this contention: ATA treatment reduced the total anti-MTG binding activity of MTG/LPS induced antibodies ($P = 0.01$).

In support of this effect of ATA on TG antibody levels is a recent study by Brown et al. (1991) on the Obese strain (OS) chicken. These authors showed that OS chickens treated with ATA (0.2%) had significantly reduced incidence of TG antibodies and thyroid infiltration. They confirmed that the suppressive effect of ATA was most likely due to direct action on the thyroid (the treated birds had significantly decreased T3 and T4), and not because of a generalized suppression of the immune system. (It is important to note that the mechanism of thyroid hormone production

occurs in birds in the same manner as that noted in mammals (Wishe et al., 1979).)

The present study therefore provokes further questions regarding the role (and structure) of circulating TG. Is it possible that circulating TG maintains the expansion of epitope-specific B and/or T cells? In order to answer this question it is necessary to consider the mechanism(s) by which the peripheral repertoire of immune cells is selected. Portnoi and co-workers (1986) have put forward the notion that the antibody repertoires in the naturally activated B-cell pool are positively selected for complementarities to self-molecules and for connectivity with other variable regions. Furthermore, Martinez et al. (1988), have proposed that the pool of activated B cells producing soluble circulating antibodies have selective effects on T-cell repertoires. This hypothesis thus considers that there is positive selection of clonal specificities into a mature repertoire before exposure to external antigens.

If the peripheral repertoire of T and B cells reactive with TG is selected by such a mechanism, it would seem that in order to maintain the selected repertoire a continuous source of circulating TG is critical. This leads to questions regarding the sensitivity of the cells involved to structural, or concentration, changes in the circulating TG. Within this hypothetical model, depletion of selective epitopes would effectively abrogate some of the reactive cells and so reduce the level of response to an antigen-specific challenge. (Alternatively, increases in antigen concentration beyond a critical level may induce clonal anergy.) This hypothesis provides an attractive explanation for the observed decrease in the total antibody response to MTG as a result of ATA treatment. The sub-population of TG reactive cells affected recognise epitopes which are conserved between rat and mouse TG, as suggested by the proportional decrease in antibody binding activity with rat TG after ATA treatment. Since the available literature on the anti-thyroid effects of ATA suggests that iodine organification is inhibited by this drug, the ATA sensitive cells may recognize some of the iodination dependent epitopes on the TG molecule.

It is perhaps pertinant to mention at this point that it has recently been brought to my attention that ATA treatment does not inhibit thyroid hormone synthesis in all strains of mice (P. Hutchings, unpublished observations). The action of ATA on the thyroid gland is complex and involves many factors. The compound inhibits hormone synthesis; the amount of inhibition being dose related (Brown et al., 1991). The diminished hormone output elicits an increased secretion of TSH by the pituitary which stimulates the thyroid to synthesize at a more rapid rate. An interplay between the inhibition by ATA and the stimulation by TSH is reflected in the resulting goitre (Strum & Karnovsky, 1971). It may be that in some strains of mice this homeostatic mechanism is able to offset the inhibitory effects of ATA.

Importantly, analysis of the cross-reactivity of the antibodies with various TG species showed that ATA treatment did not decrease all antibody specificities, for example the binding of MTG/LPS induced antibodies with human TG was not affected. Analysis of the TG antibody cross-reactivities under different immunization regimes also illustrated that ATA selectively affected the fine specificity of the anti-TG antibody response. Furthermore, this specificity was also influenced by the duration of ATA treatment. For example, when ATA was withdrawn prior to MTG/CFA challenge (ATA pretreated group), compared with the control group the cross-reactivity with zebra, dog, human, and pig TG was increased, yet the reactivity with rat and wombat TG was not altered compared with the control group (FIG. 5.4). In contrast, when ATA treatment was continued, except for the response to rat TG, there was no difference in the ratios of binding to the other TG species when compared with that of the control group.

Challenging with MTG/CFA twice also had a selective effect on the TG antibody profile. For instance, when the values for the two CBA/J control groups challenged with MTG/CFA (x1) and MTG/CFA (x2) were compared, it was observed that whereas the binding to dog TG was unchanged, the increase in the ratio of binding to zebra TG was significant ($P < 0.001$), the response to human and wombat TG was also

increased ($P < 0.05$ and < 0.02, respectively). In contrast, maintaining ATA treatment prevented a further increase in the ratio of binding to wombat TG, but not to zebra TG ($P < 0.05$) (FIG. 5.5). (There was also some difference ($P < 0.2$) in the response with human and rat TG.) The binding ratios to zebra and wombat TG were not affected in ATA pretreated mice.

It seems improbable that these responses are due to the random activation of TG-reactive cells. In order to put forward a reasonable explanation for this data it is useful to consider that a preimmune network of TG-reactive cells may exist. The inhibition (or enhancement) of certain antibody specificities caused by ATA treatment, suggests that these cells are sensitive to changes in the nature (either structural or in terms of concentration) of circulating TG. The emphasis then is to establish how the cells within such a network may interact.

The variations in the ratio of binding to the species of TG used in this study, under the different immunization regimes, suggest a possible network in operation as shown in diagram 5.2. This schematic represention shows only those subsets which may be regulated by epitopes abrogated (or increased) by ATA treatment. If the total MTG/LPS activated anti-MTG antibody response is considered, the data suggests that about 60% of this response is directed towards epitopes which are shared with rat TG, and ATA treatment diminishes approximately 25-30% of this response. The set of cells responsible for this fraction of the total response is symbolised as R/A. (The designation for mouse epitope specificity is omitted for all the sub-sets illustrated, since the cross-reactivity of anti-MTG antibodies with other species of TG is being considered.) This model proposes that the R/A lymphocyte population (and it may well be an activated B cell pool) requires that a constant level of circulating TG is maintained. These cells can be activated by both MTG/LPS and MTG/CFA and are primarily affected by ATA treatment; their functional activity appears not to be restored even if ATA treatment is withdrawn.

The data suggests that more than one set of cells are sensitive to the effects of ATA. For example, it appears that with MTG/CFA activation, a set of antibodies with specificity for a number of different TG species are induced, but only with ATA pretreatment. The higher binding ratios of these cross-reactive antibodies (FIG. 5.4) suggests that the cells contributing to this response are different to those contributing to the cross-reactivity with these same TG species in the control and ATA maintained groups. Furthermore, it appears that the separate recruitment of these populations as a result of ATA pretreatment prevents the connectivity with other cells, which are perhaps, only activated by a second challenge with MTG/CFA. Thus, if a pre-immune anti-TG network of cells does exist, then there may be many levels of interaction between the various cells based on their cross-reactivity with heterologous thyroglobulins. Importantly, this model implies that cells recognising epitopes conserved between rat and mouse TG have a central role in regulating the pre-immune network: Perhaps this type of network exists to ensure a diverse TG-reactive repertoire.

The two apparently contradictory roles for circulating TG - maintaining tolerance of autoreactive T cells and promoting the generation of a diverse TG reactive T/B cell repertoire - could also be explained if the density of particular epitopes on the TG molecule is fundamental in determining which pathway is activated: Most of the epitopes may be present at tolerogenic levels, whilst those at sub- tolerogenic concentrations would prime the immune system. Since circulating TG is likely to have a low T4 content, it is possible that a small percentage of cells with specificity for this epitope will be primed.

In this study the incidence and severity of thyroiditis was comparable in all groups of CBA/J mice (Table 5.1), which suggests that lymphocytes infiltrating the glands were responding to TG epitopes not affected by ATA. However, there is indirect evidence (Brown et al., 1991; Champion et al., 1987) which suggests that iodinated epitopes abrogated by ATA treatment may be recognized by infiltrating lymphocytes in experimental models of thyroiditis. Clearly more work is needed to resolve questions surrounding the specificity of the lymphocytes in thyroid infiltrates.

HYPOTHETICAL VIEW OF THE PRE-IMMUNE THYROGLOBULIN-REACTIVE REPERTOIRE

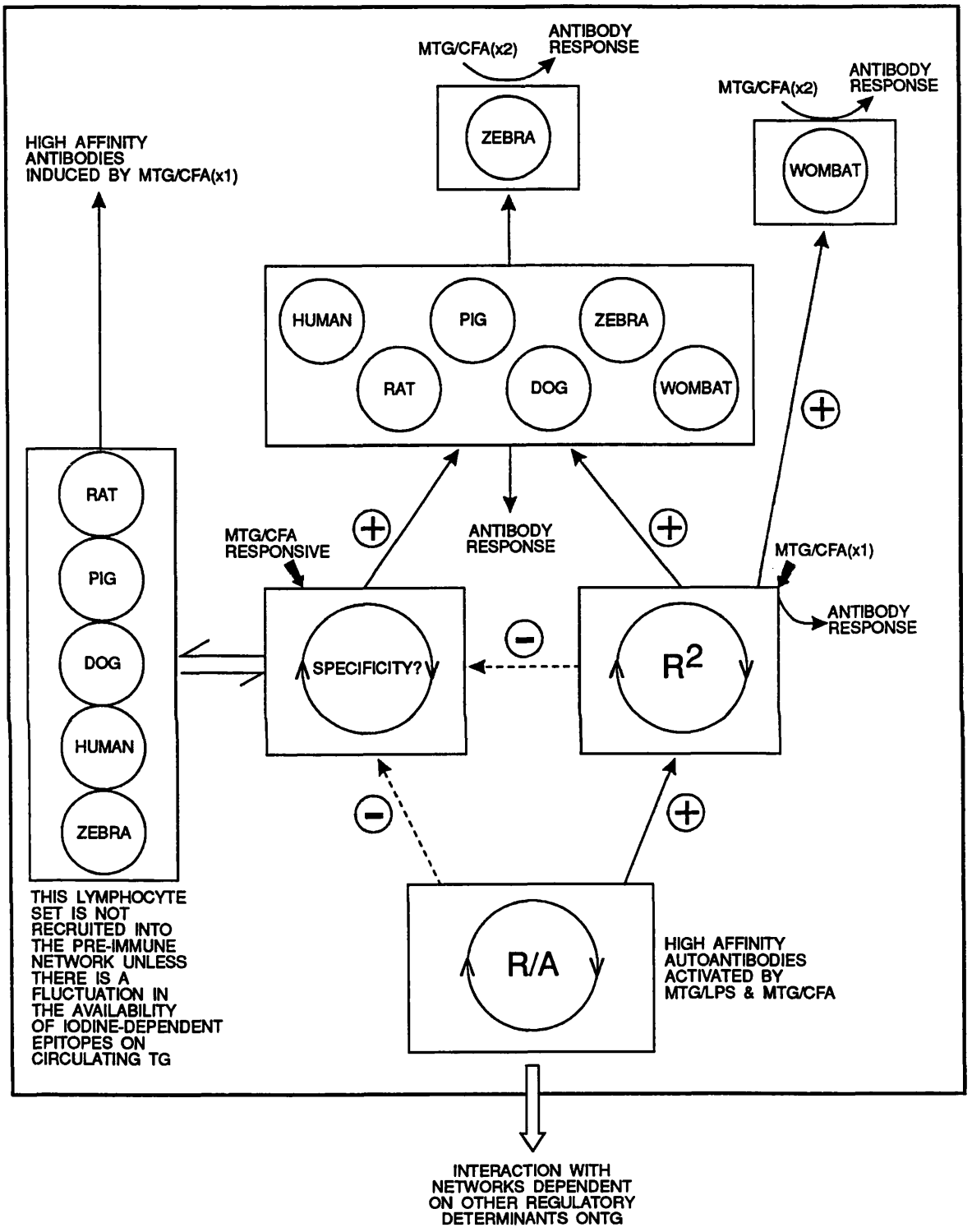


DIAGRAM 5.2: Variation of the cross-reactive profiles of induced anti-MTG autoantibodies in high-responder CBA/J mice, as a result of aminotriazole (ATA) treatment, suggests that there is a pre-formed network for the repetoire of TG reactive lymphocytes which is dependent on specific epitopes on circulating TG. (See text for explanation).

CHAPTER 6

PRESENTATION OF ENDOGENOUSLY SYNTHESIZED TG BY PRIMARY CULTURES OF MOUSE THYROCYTES

6.1 INTRODUCTION

Infiltration of lymphocytes into the thyroid gland has been known to be one of the major histological characteristics of chronic thyroiditis. Lymphocytic infiltrates are also seen in the thyroids of patients with Graves' disease. Several studies have now confirmed that lymphocytic infiltrates carry markers indicative of activated T cells (Misaki et al., 1985; Most & Wick, 1986; Wick et al., 1984; De Assis-Paiva et al., 1989). The possibility that IgG synthesis occurs within the gland (McLachlan et al., 1979, Weetman et al., 1982; Atherton et al., 1985), also suggests that activation of lymphocytes may be initiated, or maintained, within the gland. As yet, the mode of antigen presentation and activation has not been unequivocally identified.

An early breakthrough in this area of research was the demonstration that thyroid follicular cells from patients with Graves' thyrotoxicosis and Hashimoto's thyroiditis spontaneously express HLA-DR antigens (Hanafusa et al., 1983). These authors (Bottazzo et al., 1983) suggested that the inappropriate expression of MHC Class II molecules by epithelial cells might enable these cells to present their own surface molecules to autoreactive T cells. Londei et al (1985) showed that one group of CD4+ T cells cloned from the activated lymphocytes infiltrating Graves' disease thyroids, proliferated upon exposure to autologous thyrocytes, but were not stimulated by either autologous peripheral blood mononuclear cells or allogeneic thyrocytes. Furthermore, like responses induced by other antigen-presenting cells ((Lamb & Feldmann, 1984), the interaction with autologous thyrocytes could be blocked with monoclonal anti- Class II antibodies. However, the nature of the antigen presented by the thyrocytes was not determined. As a specific antigen may be differentially processed by different APC, the possibility that

thyrocytes do not present self antigens at all has to be considered. It may be that the activation of lymphocytes by syngeneic thyrocytes is due to the presentation of antigens present in the culture medium (Hirose et al., 1988). An earlier report (Londei et al., 1984) had demonstrated that thyrocytes can present a non-thyroid antigen such as the peptide p20 of the HA-1 molecule of influenza strain A to a T-cell clone (HA1.7) which is specific for this peptide. Importantly, unlike irradiated PBL, the thyrocytes were not able to present the whole formalin-inactivated A/Texas influenza virus. In the same way, definitive proof that thyrocytes can present thyroid specific antigens, such as TG or TPO, would be provided by showing that MHC compatible thyrocytes can activate thyroid antigen-specific T cell clones.

There is indirect evidence from a murine system that thyrocytes can present self-antigens. Creemers et al (1983) have shown that TG sensitized lymph node cells from MTG/CFA immunized mice develop cytotoxicity towards thyroid cells in monolayer culture. This cytotoxicity was completely inhibited by rabbit anti-MTG serum. This normally is never seen with protein antigens, but this may be a special case since T4 may be part of the cytotoxic T-cell epitope and the anti-MTG antibodies may easily contain antibodies which recognise T4. In addition to suggesting that all cytotoxic T lymphocytes induced by this method are specific for TG determinants, these results may be interpreted as evidence that there are TG determinants on the plasma membrane of mouse thyroid cells. By contrast, although the surface expression of M/TPO antigen on isolated human thyroid follicles could be demonstrated by indirect immunofluorescence (IF) using sera from patients with autoimmune thyroid disease, the surface expression of TG was not seen using this technique (Hanafusa et al., 1984). This may be due to the sparse distribution of TG rather than its total absence. (Indeed, using radiolabeled anti-TG antibodies, Fenzi et al (1982) were able to show surface binding to human thyrocytes.) Alternatively, the limited number of epitopes recognized by autoantibodies (Nye et al., 1980) may not be expressed on the thyroid cell surface.

At the outset it was important to determine if mouse thyrocytes in culture constitutively express surface TG or whether such expression can

be induced, as was shown for the microsomal/TPO antigen (Chiovato et al., 1985). A large panel of anti-TG monoclonal antibodies was used and their ability to bind to thyrocytes was determined by immunofluorescence. To corroborate these studies, the pattern of TG expression in primary cultures of human thyroid cells was also determined. In addition, the question of self-antigen presentation by thyroid cells was addressed. These studies show that primary cultures of mouse TEC can stimulate the T-cell hybridoma CH9 (which is restricted to I-A^k and TG (Rayner et al., 1987)) to secrete IL-2.

6.2 RESULTS

6.2.1 Mouse thyroid cell cultures - Morphological observations

Plated suspensions of partially disrupted follicles and single cells prepared from CBA/Ca or CBA/J mouse thyroids attached to the bottom surface of the micro- wells within 24 hours. However, repeated attempts failed to show a similar attachment of thyroid suspensions obtained from BALB/C (H-2^d) mice. Cells from goitrous (ATA induced) thyroids also failed to form monolayer cultures, suggesting that the functional state of the thyroids may also affect cell adherence. The reasons for this was not investigated further, but it was interesting to note that a reduced collagenase treatment was required to maintain the viability of both Balb/C and ATA treated CBA/J thyroid cells.

In general, most growth of thyroid epithelial cells (TEC) was observed in areas where complete or partial follicles had attached: a monolayer of cells appeared to grow outwards to form a margin around these primary follicles (FIG.6.1). By day 5 of culture approximately 80% of the surface of most of the wells was covered by thyroid cells; contamination by fibroblasts could not be detected morphologically. At later times the cells in a number of wells reached a very high density and appeared as though they were reassembling into follicles. The incidence of these structures was variable. After 5 days the number of cells did not increase, but a marked increase in the size of the cells was observed. Cultures could be maintained for almost 2 weeks, during which

time growth of the peripheral cells continued, although some cell detachment was apparent and vacuoles of variable sizes were observed in the cytoplasm of many cells.

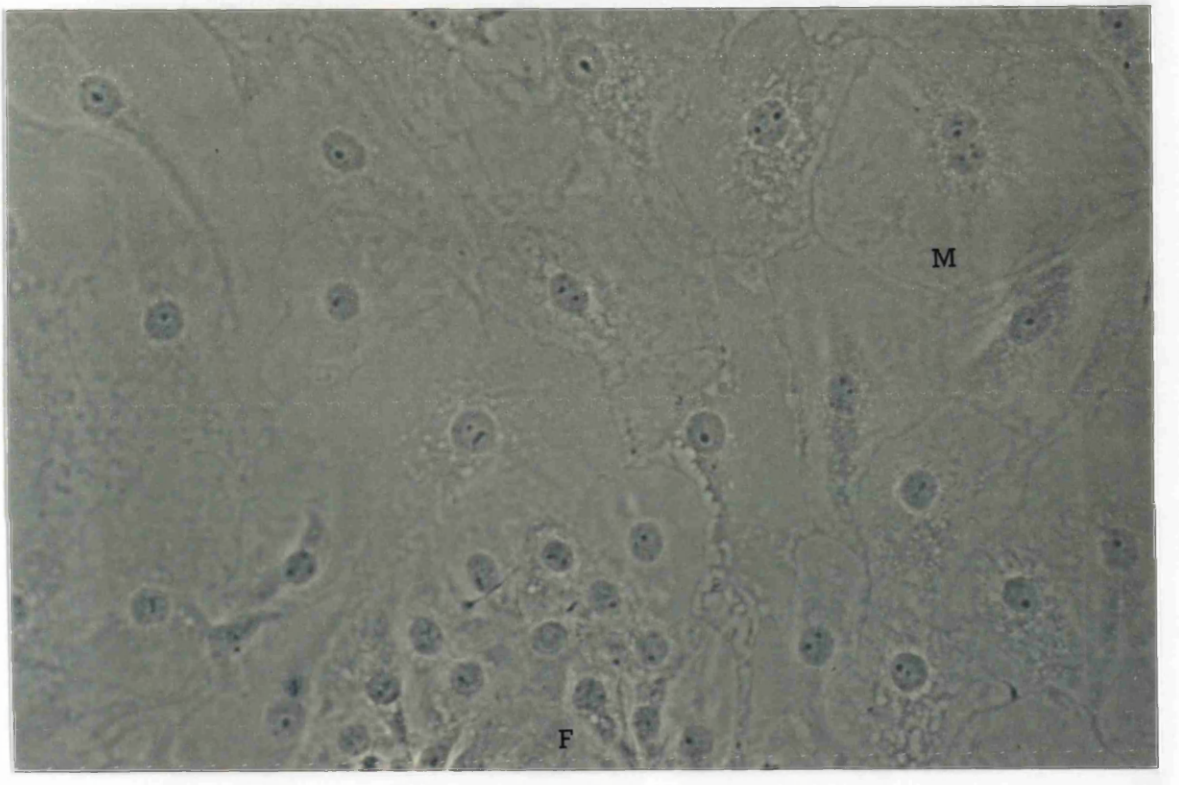
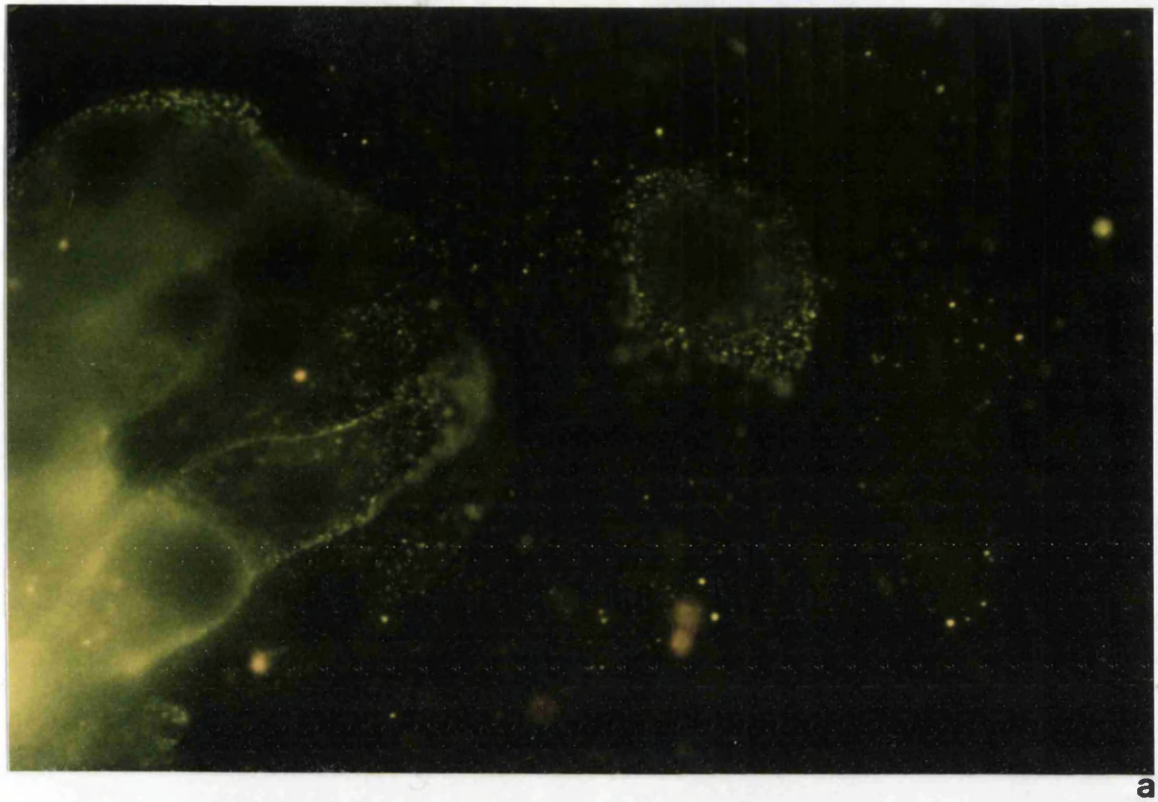


FIGURE 6.1: Morphology of monolayer culture of mouse thyroid epithelial cells on day 7. Cells were stimulated with IFN-γ for 72 hr. This frame illustrates the outward growth of cells from a central clump of small follicular cells (F), cell undergoing division (M).

6.2.2 Differentiated function of mouse thyrocytes in culture

The differentiated function of the primary cultures of mouse thyrocytes was established by demonstrating their specific responsiveness to thyrotropin (TSH). Surface expression of the microsomal\TPO antigen was specifically induced by TSH (FIG. 6.2), although the number of cells showing this expression was minimal when compared with the effect of TSH on human thyrocyte cultures (FIG. 6.3A). This level of expression was not observed if cells were stimulated with IFN-γ (FIG. 6.3B). Cytoplasmic staining of microsomal\TPO antigen was not observed at this time point.

The functional state of the thyroid epithelial cells was also assessed by measuring the concentration of TG secreted into the culture medium. After 3 days of culture there was less than 1 μg/ml TG. However, by day 5 the TG concentration was increased to greater than 3 μg/ml. The maximum amount of TG secreted was observed at 5 days post culture (FIG 6.4). Adding TSH to the culture medium did not increase the amount of unfragmented TG secreted at any time point. (This assay does not detect TG peptides (see FIG. 2.3, Materials & Methods), so the possibility that TSH stimulates secretion of fragmented TG, cannot be ruled out.) However, TG secretion was modulated by the cytokine, interferon-gamma (IFN-γ) (FIG. 6.5), although this effect was not consistently observed (TABLE 6.1).



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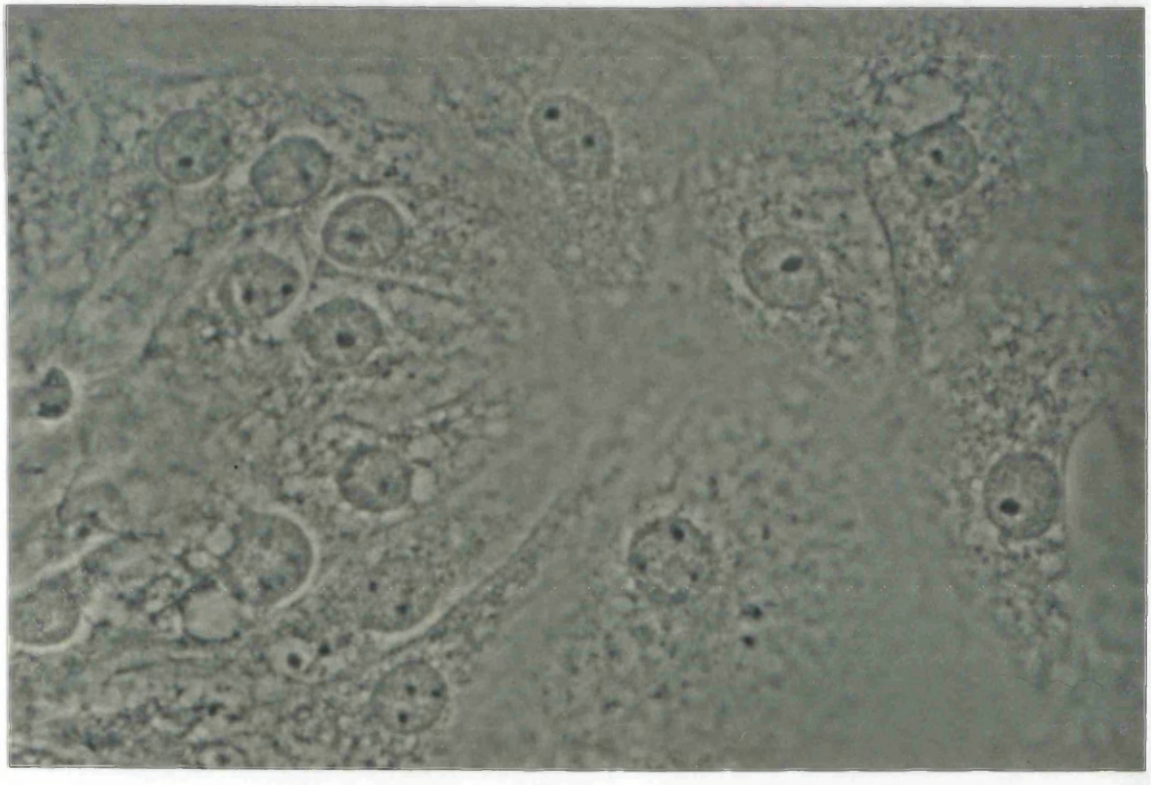
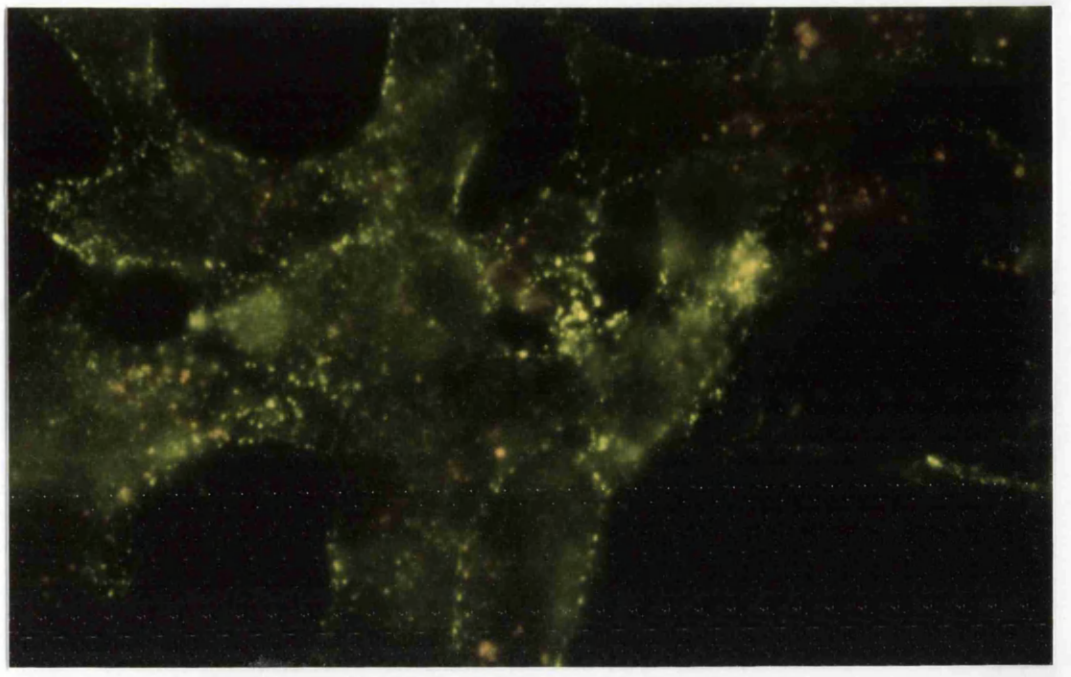


b

FIGURE 6.2: Surface expression of microsomal/TPO antigen in mouse TEC. Surface IF (a) of 5 day cultures stimulated with 60 μU/ml TSH. Phase contrast of the same field (b) shows that detection of this antigen is predominantly in the smaller cells. A human serum sample containing high levels of anti- microsomal antibodies was used. X 630. (Cells grown in the absence of TSH did not show any fluorescence.)



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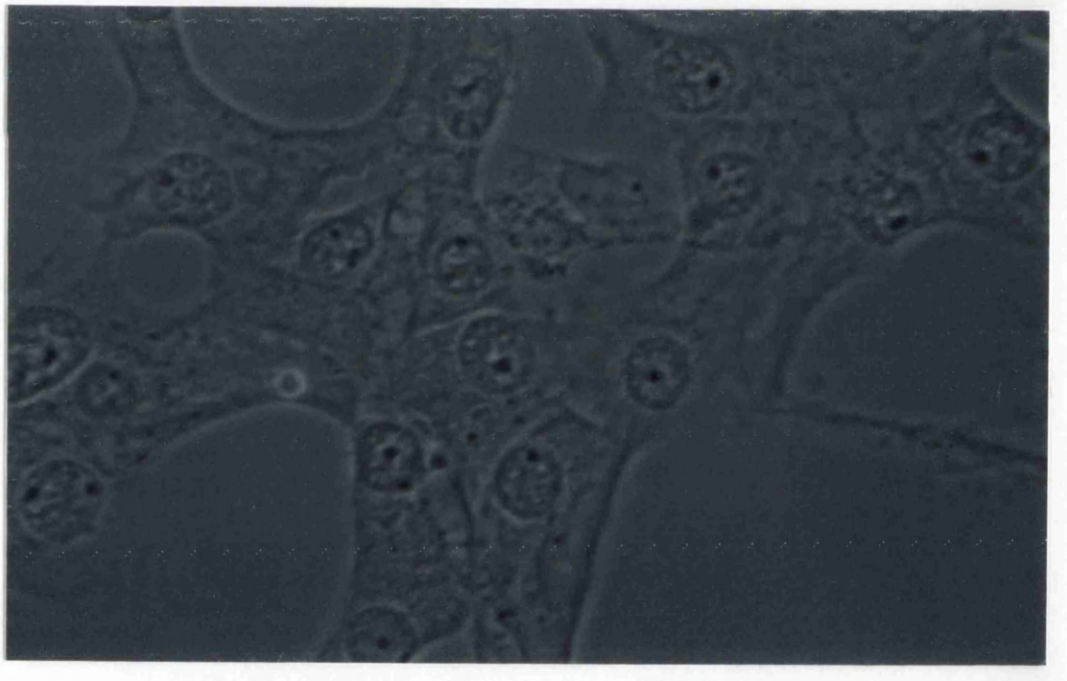
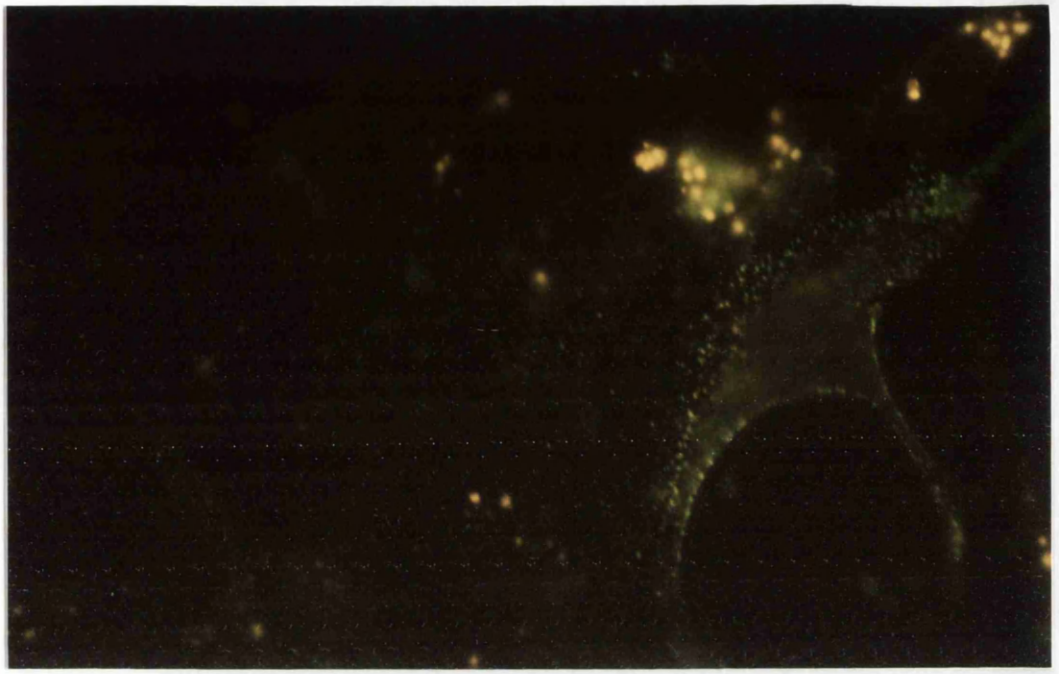


b

FIGURE 6.3A: Surface expression of microsomal/TPO antigen in human TEC. (a) 5 day cultures of human TEC stimulated with 60 μU/ml TSH were stained with anti-microsomal human serum sample. Strong dotted fluorescence was visible on the surface of the majority of cells. X 630. (b) shows the phase contrast of the same cells.



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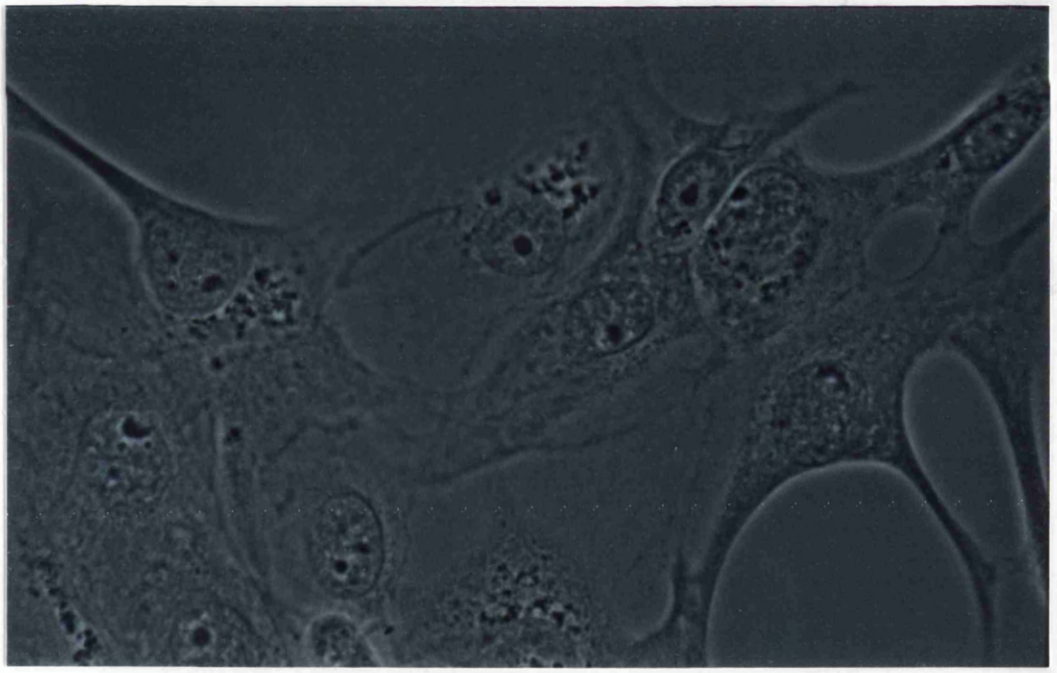


b

FIGURE 6.3B: IFN-γ does not induce microsomal/TPO antigen. Surface IF (a) of 5 day cultures of human TEC stimulated with IFN-γ. (b) Phase contrast of the same field. X 630.

FUNCTIONAL ASSAY FOR MONOLAYER CULTURES OF MOUSE TEC

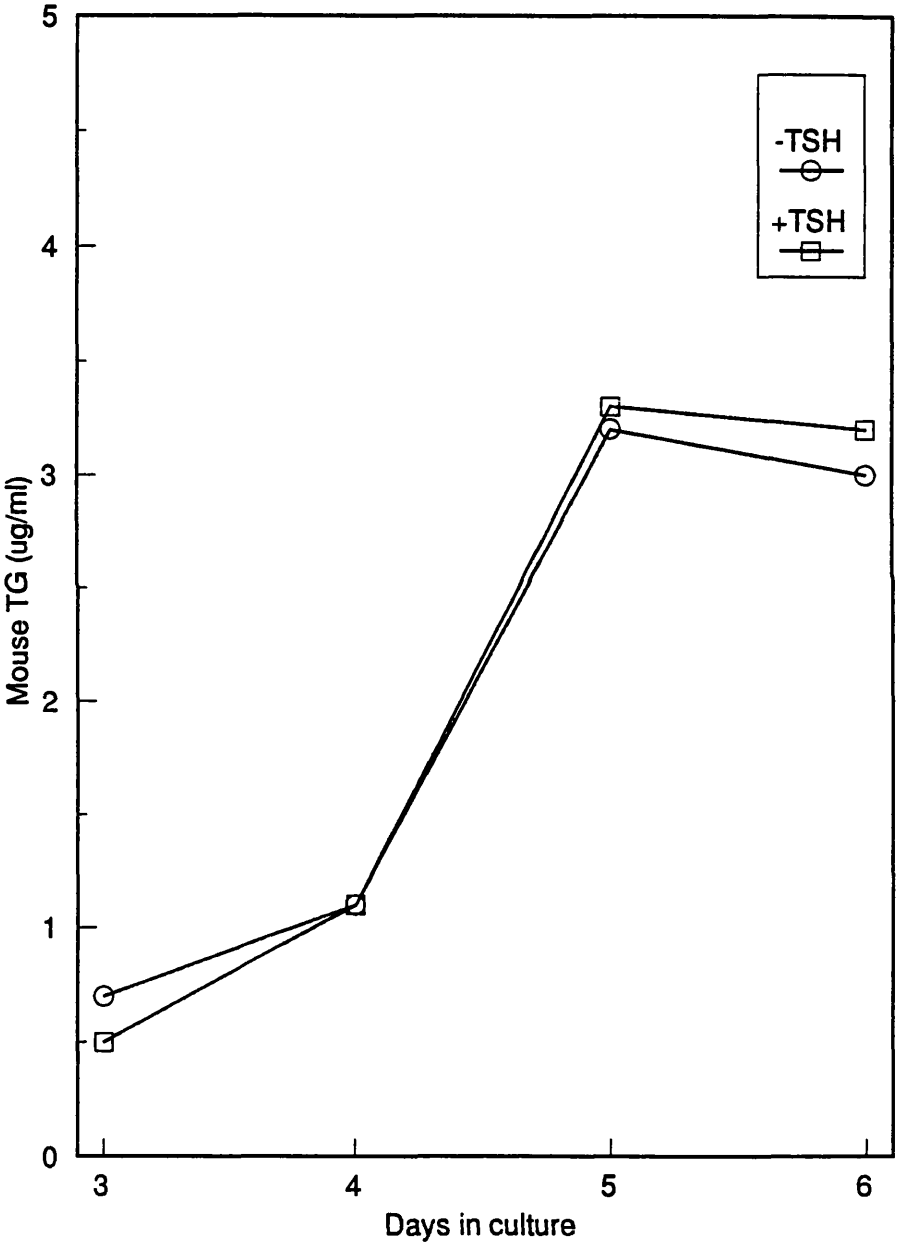


FIGURE 6.4: monolayer cultures of TEC were grown in medium supplemented with 1% FCS with, or without, TSH (60μU/ml) in 96 well micro-plates. TG release was assayed by inhibition RIA. Each point represents the mean MTG concentration determined from 6 separate wells (values for each well was calculated from duplicates). S.D values were ≤ 0.5μg/ml.

INFLUENCE OF IFN-γ ON TG SECRETION

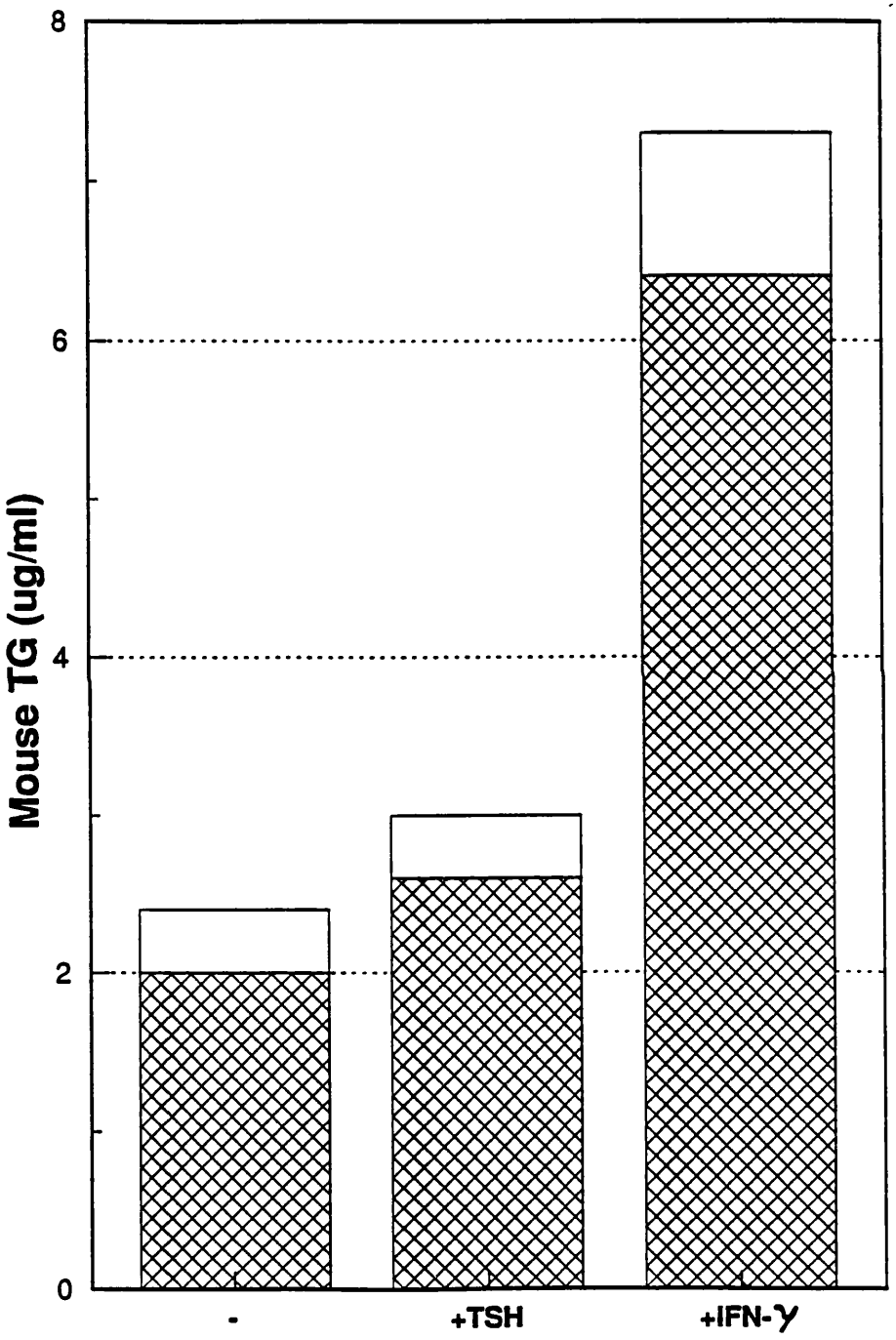


FIGURE 6.5: Supernatants were collected from 5 day old cultures and assayed for TG. There was a marked increase in TG release by cultures stimulated for 48 hrs with recombinant rat IFN-γ (150 μU/ml) compared with cultures stimulated with (+) or without (-) TSH (60 μg/ml). Shaded areas show the means. Unshaded areas of each bar represent the S.D.

TABLE 6.1

TG SECRETION BY PRIMARY CULTURES OF MOUSE TEC

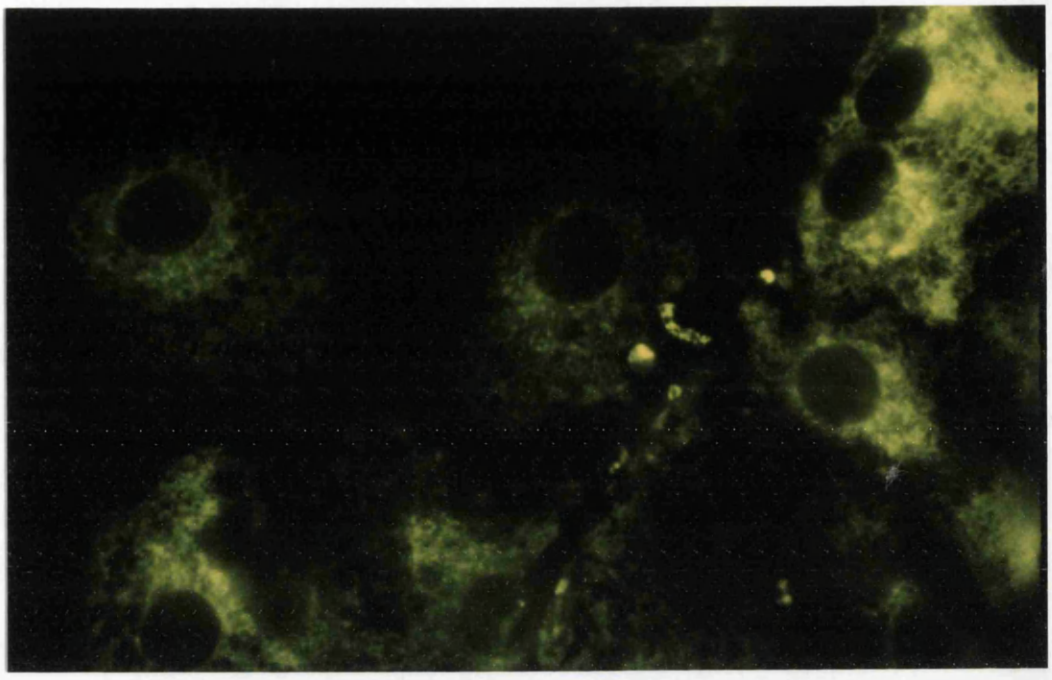
			MOUSE TG (µg/ml)			
Expt	Sample Number	Days Post Culture	—	+TSH	+IFN-γ	TSH+IFN-γ
1	6	5	2.8 +/- 0.6	2.2 +/- 0.2	1.5 +/- 0.2	1.0 +/- 0.1
2	10	5	1.6 +/- 0.2	1.8 +/- 0.4	4.0 +/- 0.5	N.D.
3	10	5	2.0 +/- 0.6	2.6 +/- 0.4	6.4 +/- 0.9	N.D.
4	6	5	3.4 +/- 0.6	3.3 +/- 0.2	2.6 +/- 0.6	4.0 +/- 0.2
5	6	6	3.0 +/- 0.2	3.2 +/- 0.5	2.5 +/- 0.2	1.4 +/- 0.2

Mouse TG concentrations (determined by inhibition RIA) in the supernatant of primary cultures of Thyroid epithelial cells. Cultures were grown in 1% FCS supplemented medium and stimulated with (+) or without (-) TSH (60 µU/ml) and recombinant IFN-γ (150 U/ml) as indicated.

6.2.3 Expression of TG in mouse thyrocytes

Initial studies showed that the majority of cells in the monolayer cultures of mouse TEC expressed TG in the cytoplasm, detectable by a sample of pooled murine EAT serum. To confirm the specificity of this stain, from the panel of monoclonal antibodies (mab) which were reactive with MTG (Champion et al., 1987), five (P12C12, P4G11, 5F6, H3D7 and 3B3) were used to study the variation in the cytoplasmic expression of TG in mouse TEC. Mab P12C12 and mab P4G11 showed a similar pattern of staining under the different culture conditions. Both of these monoclonal antibodies detected cytoplasmic TG in cells grown in the absence of TSH and, although the degree of fluorescence observed was similar in cultures stimulated with TSH, the number of cells positively stained was increased (FIG. 6.6). In addition, a number of cells cultered with TSH expressed small vesicular structures which were more intensely stained than the rest of the cytoplasm. Monoclonal antibody 3B3 specifically stained the very small central cells in the monolayer cultures (FIG. 6.7). Although the intensity of fluorescence observed with mab 3B3 did not vary, the staining observed in cells cultured in 1% FCS only, was more granular (punctate). TSH pretreatment increased the number of cells stained. In cells stimulated by IFN-γ, the fluorescence was particularly dense around the nuclei. Mab 5F6 and mab H3D7 did not not bind to cytoplasmic TG under any culture condition.

These studies were extended to determine whether surface expression of TG could also be detected. A panel of fourteen monoclonal antibodies to MTG were used and of these only two, mab H3D7 and 5F6, were able to show the surface expression of TG. Importantly, there was no detectable surface staining in the absence of TSH. In cultures stimulated with TSH, very low levels of surface stain were observed (but not always). However, in cultures stimulated with IFN-γ, intense staining was observed on the outer edges of the largest cells (FIG. 6.8). This was not due to non-specific staining, because these mabs did not bind to IFN-γ stimulated pancreatic islet cells (which were kindly provided by R. Pujol-Borrell).



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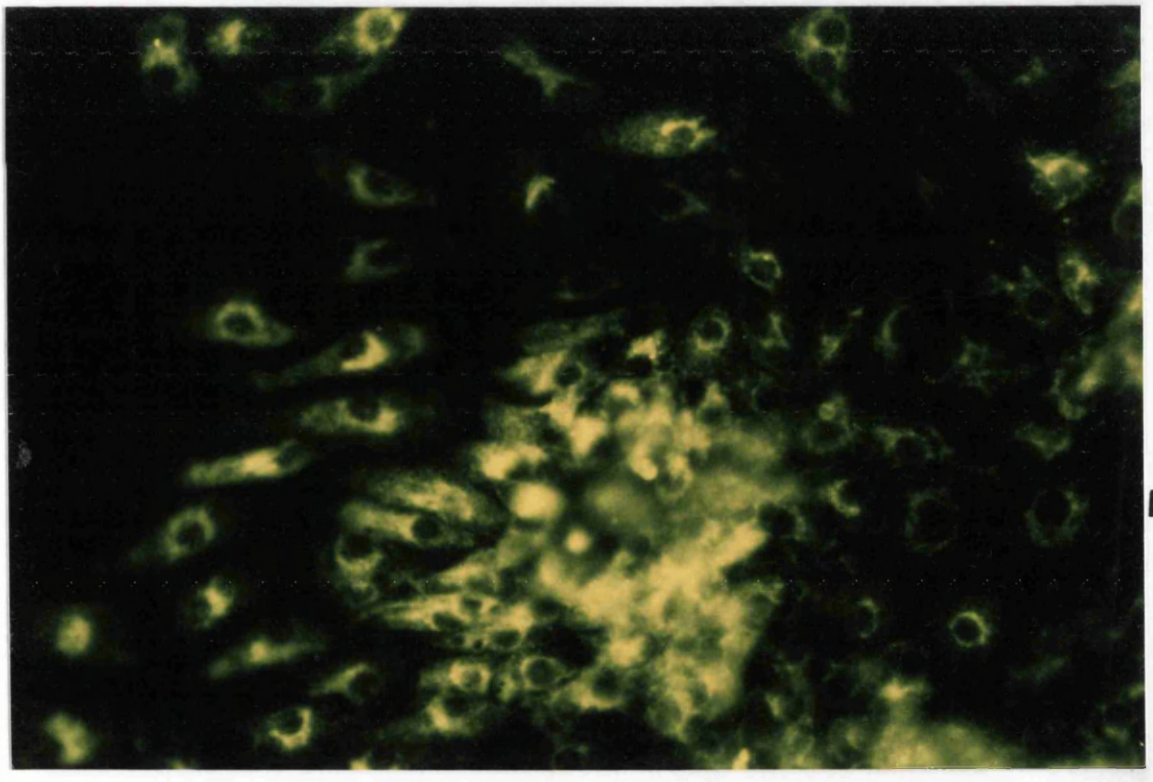
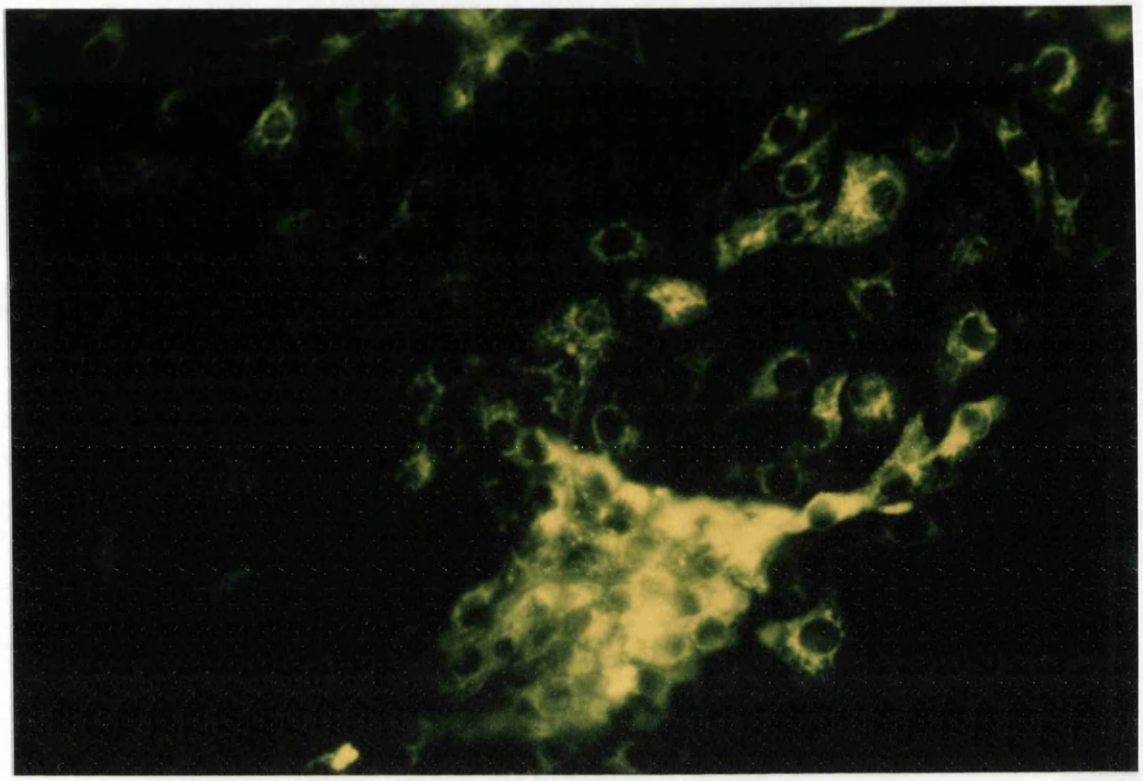


b

FIGURE 6.6: Indirect IF study of TG synthesis by primary cultures of mouse TEC. The mab P4G11 was used to detect the cytoplasmic expression of TG in 5 day cultures stimulated with (b) or without (a) TSH. The intensity of fluorescence was comparable under different culture conditions, although with TSH stimulation a more intense perinuclear stain was observed in many cells. Many of the larger cells stimulated with IFN-γ alone, were not positively stained (see FIG. 6.6c).



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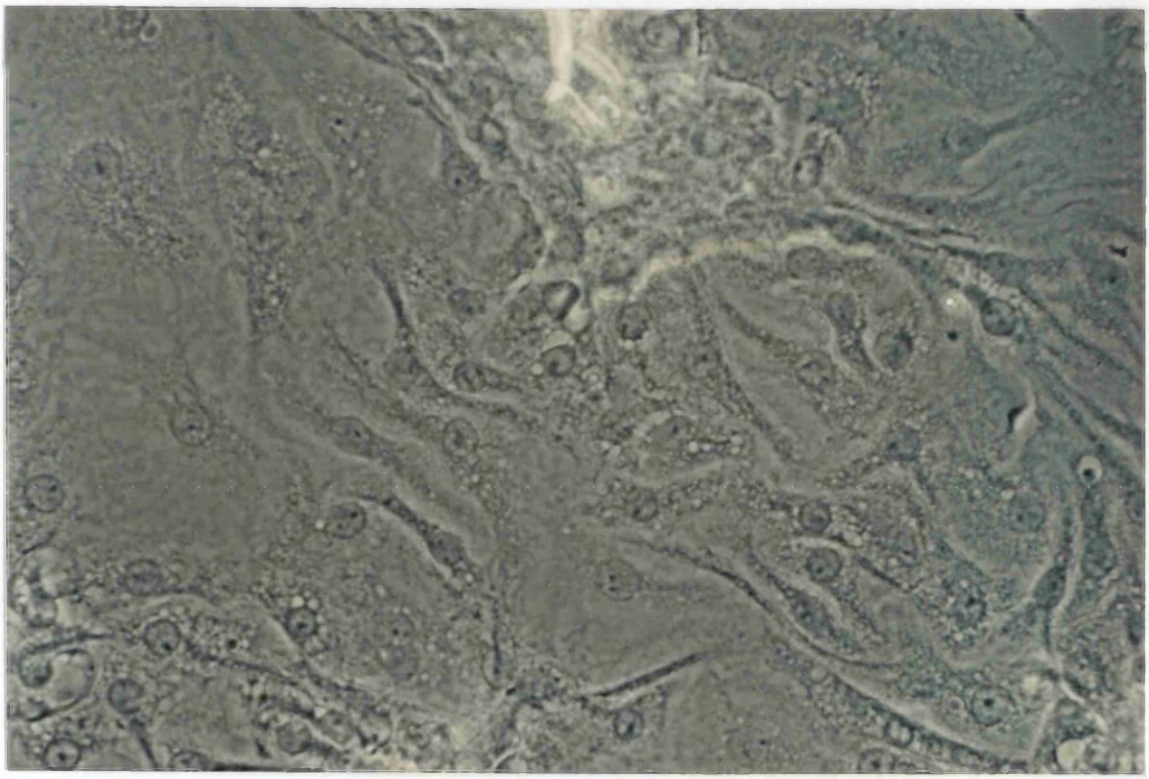


d

FIGURE 6.6: (c) 5 day cultures of mouse TEC stimulated with IFN-γ. Cytoplasmic expression of TG was detected with mab P4G11. (d) Phase contrast of the same field, shown as a mirror image, illustrates that many of the larger cells were not positively stained.

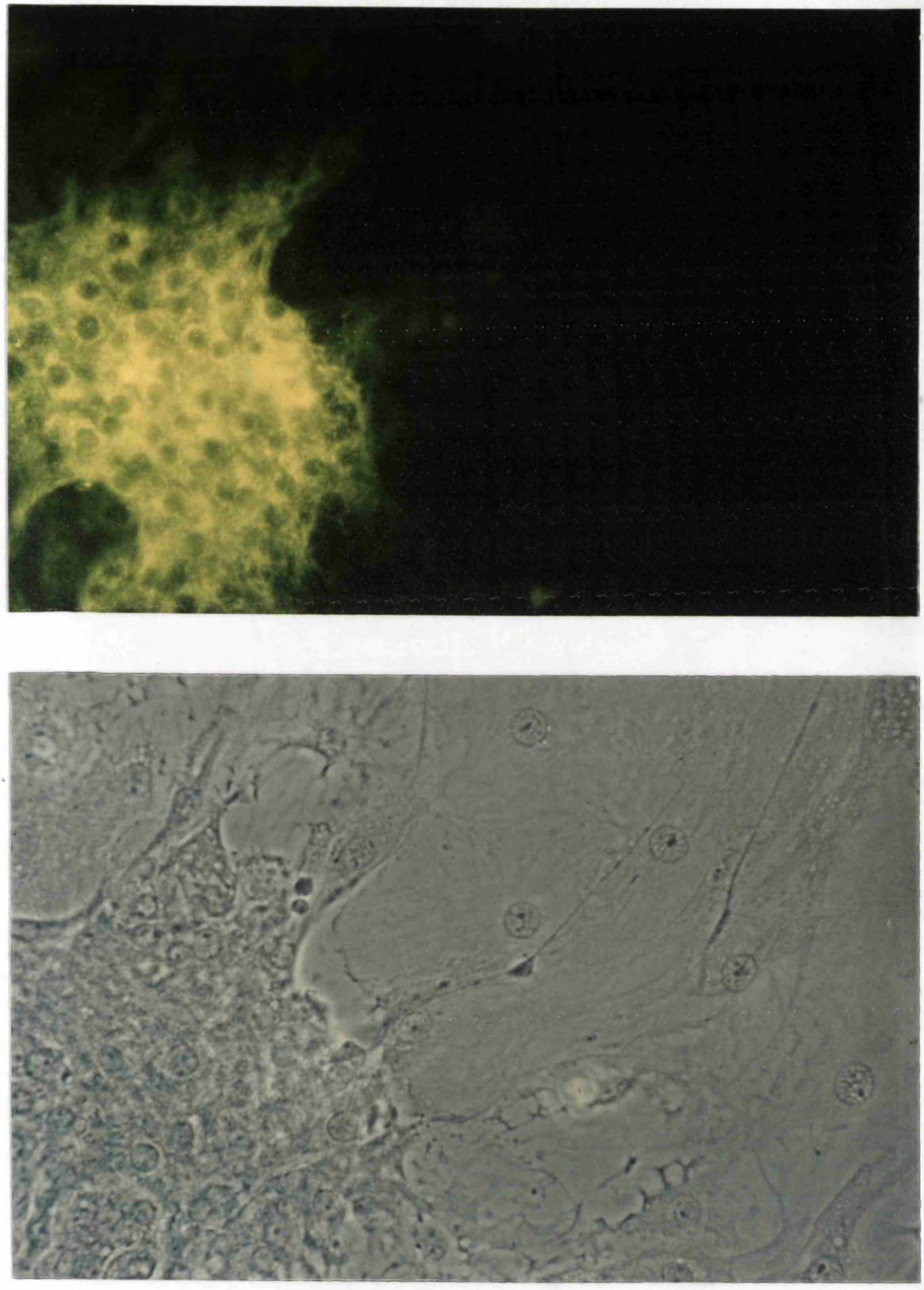


FIGURE 6.7: The relation between cell size and TG expression. 5 day cultures of mouse TEC stimulated with IFN-γ. Cytoplasmic expression of epitopes recognized by mab 3B3 was consistently shown to be restricted to the small follicular cells. Phase contrast of the same field is shown below. (magnification X 250)

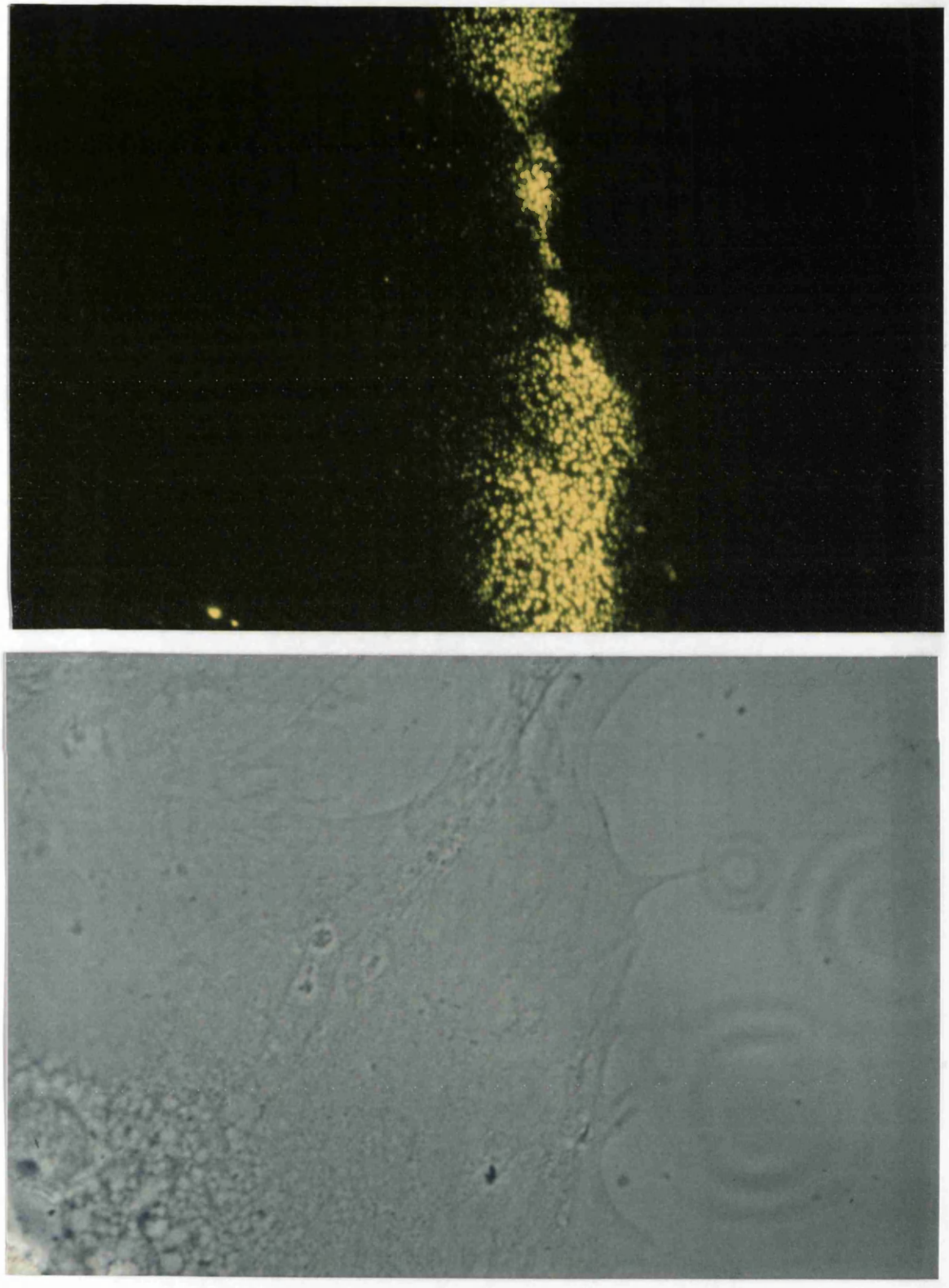


FIGURE 6.8: TG epitopes expressed on the surface of mouse thyroid follicular cells. Indirect IF surface staining of 5 day cultures of mouse TEC stimulated with IFN-γ. Mab 5F6 was used. The intense dotted fluorescence was restricted to the outer edges of the largest cells. X 630.

6.2.4 TG expression in human thyrocytes

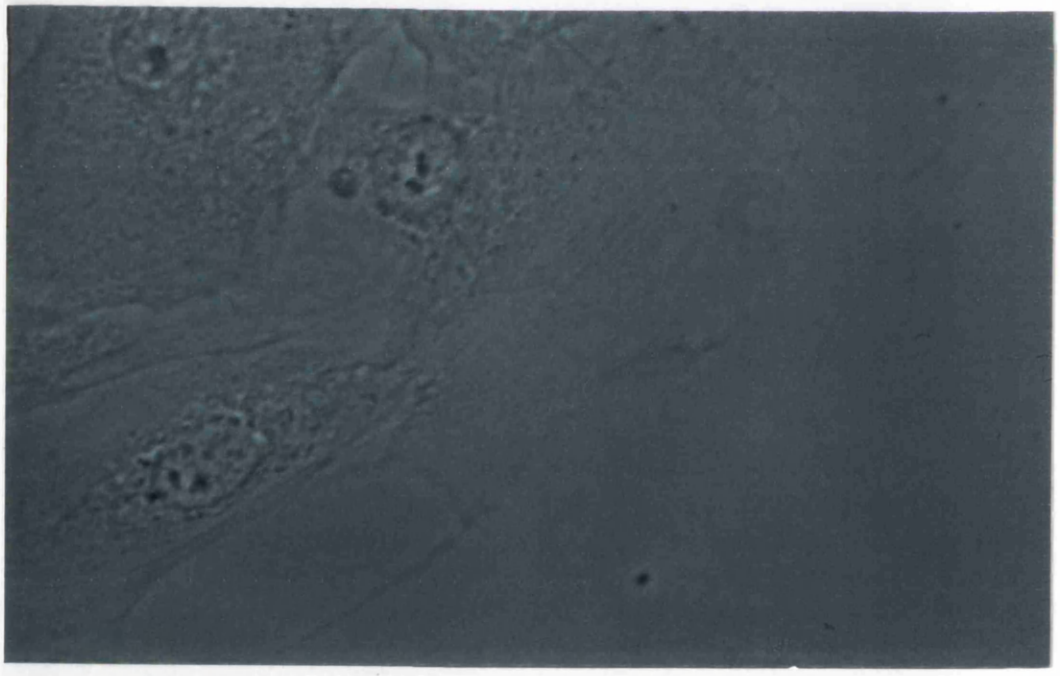
The above studies were expanded to see whether there was a similar variability in the binding pattern of anti-TG monoclonal antibodies in human thyroid cells. In all the experiments the cells used were from the thyroid of a patient presenting with colloid goitre. The anti-human TG monoclonal antibodies used in this study were kindly provided by Phil Shepherd. Of seventeen monoclonal antibodies tested, only two (mab 3B3 and mab 5F6) could unequivocally show the surface expression of TG (FIG. 6.9). Whereas mab 3B3 showed a granular surface stain, mab 5F6 seemed to stain more discreet areas of the cells. Mab 5F6 failed to detect any cytoplasmic TG in human thyroid cells, but mab 3B3 detected a population of TG molecules localised in large vesicular structures stimulated by TSH (FIG. 6.10). (Both of these monoclonal antibodies bind mouse and human TG as assessed by RIA, data not shown.)

Cytoplasmic staining of human thyrocytes cultured under different conditions, was used to determine the epitopic specificities of this panel of monoclonal antibodies. TSH caused a dramatic increase in the formation of vesiclular structures, which were detectable to variable degrees with the different antibodies. IFN-γ did not have a similar effect and cells stimulated with both IFN-γ and TSH had no, or very few (FIG. 6.11), numbers of vesicles.

Differential staining patterns with the various monoclonal antibodies suggested that the population of TG expressed in these structures was heterogeneous. Furthermore, there was some indication that these structures may contain TG moieties which are not expressed in the rest of the cell. For example, only vesicular staining was observed with mab G4F6 (FIG. 6.12). Similarly, mab 6D2 only faintly (1+) stained the cytoplasm of TSH stimulated cells, although intense (10-12+) staining of the vesicles was observed (FIG. 6.13). However, these structures also contained a TG population which was present in the rest of the cytoplasm, as determined by the mab 2C6 (FIG. 6.14). From these observations it was

possible to class these monoclonal antibodies into different groups (TABLE 6.2). There was no apparent correlation between this classification and the cross-reactivity profile (see Appendix 2) of these mabs.

c



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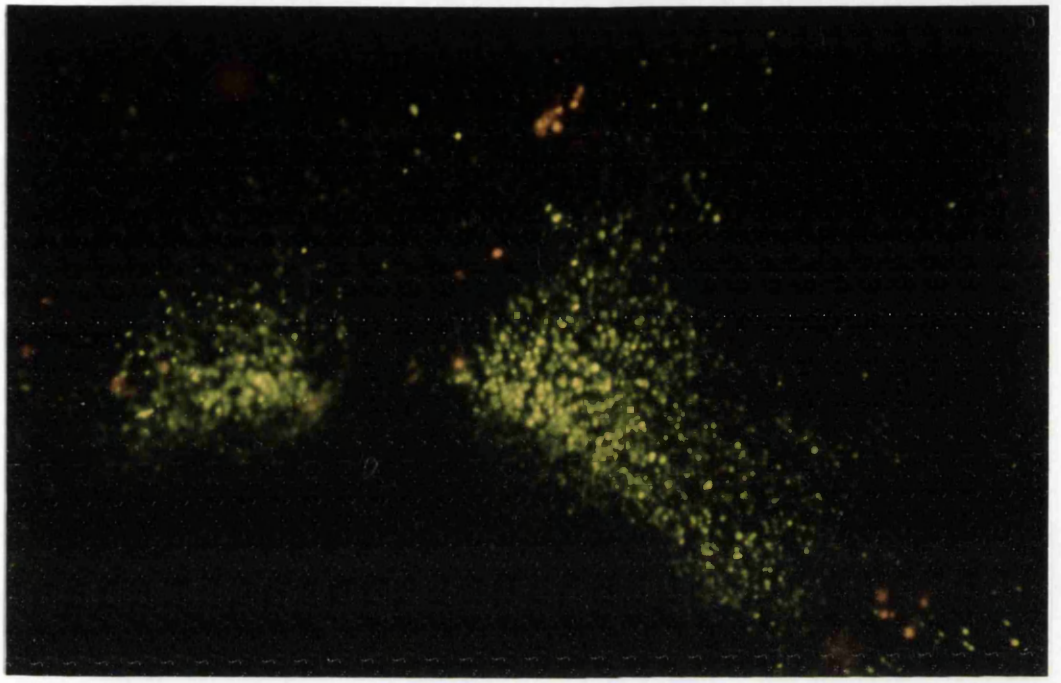
(c) Surface IF staining with Mab 5F6 of 5 day cultures of human TEC grown in the absence of IFN-γ. (d) Phase contrast of the same field.



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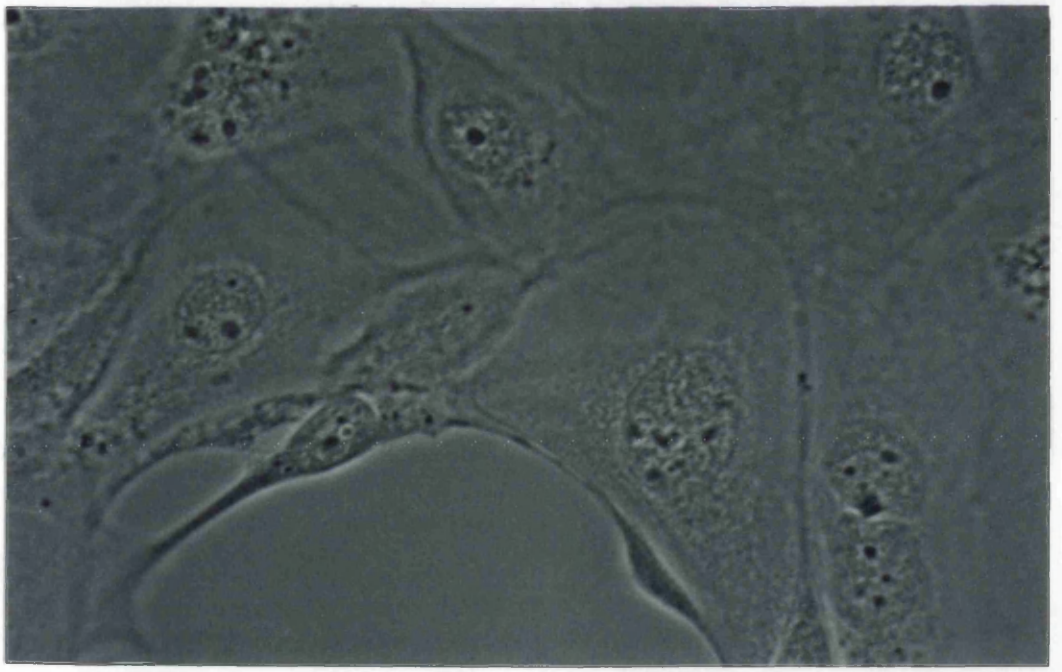


b

FIGURE 6.9A: TG epitopes expressed on the surface of human thyroid follicular cells. Surface expression of TG epitopes detected on human TEC with Mab 5F6. (a) IF of 5 day cultures of human TEC stimulated with IFN-γ. (b) Phase contrast of the same cells.

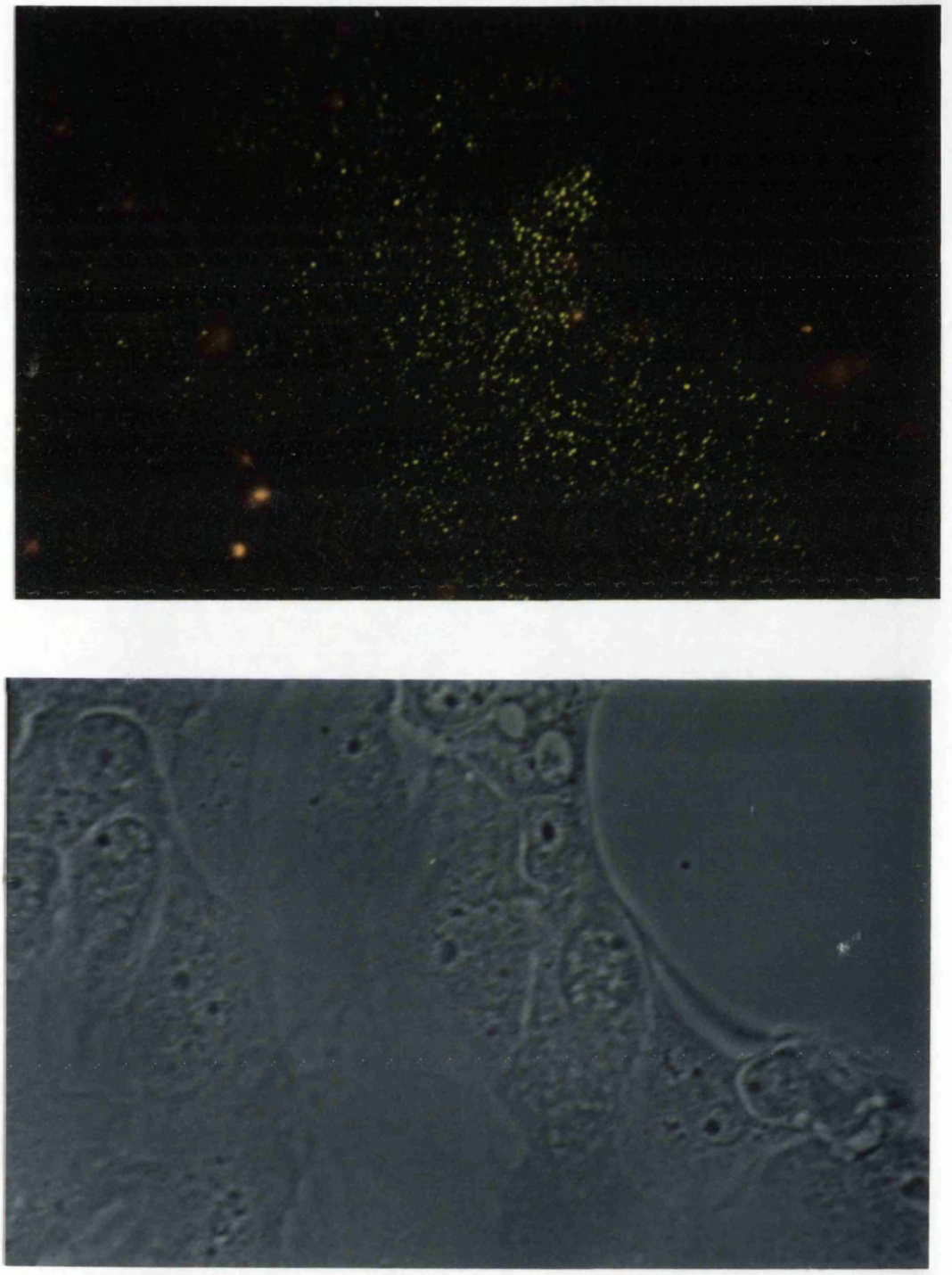
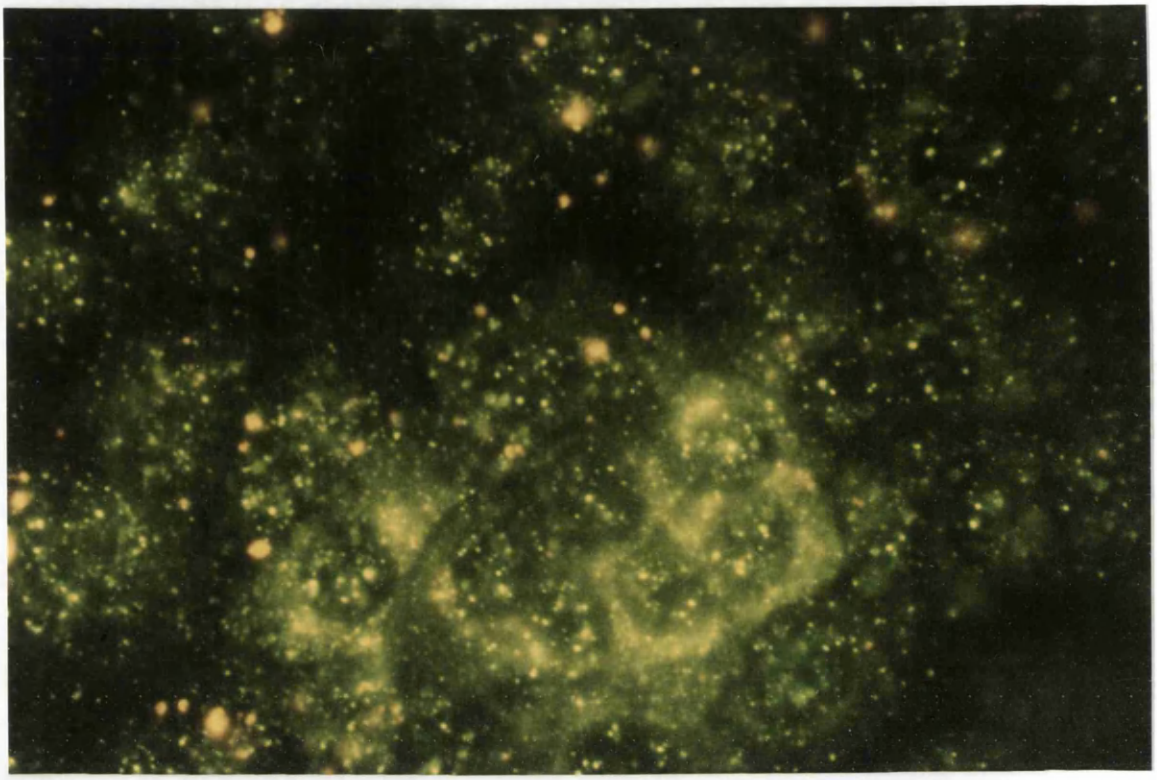


FIGURE 6.9B: TG epitopes expressed on the surface of human thyroid follicular cells. 5 day cultures of human TEC stimulated with IFN-γ. A granular surface stain was observed when mab 3B3 was used.

c



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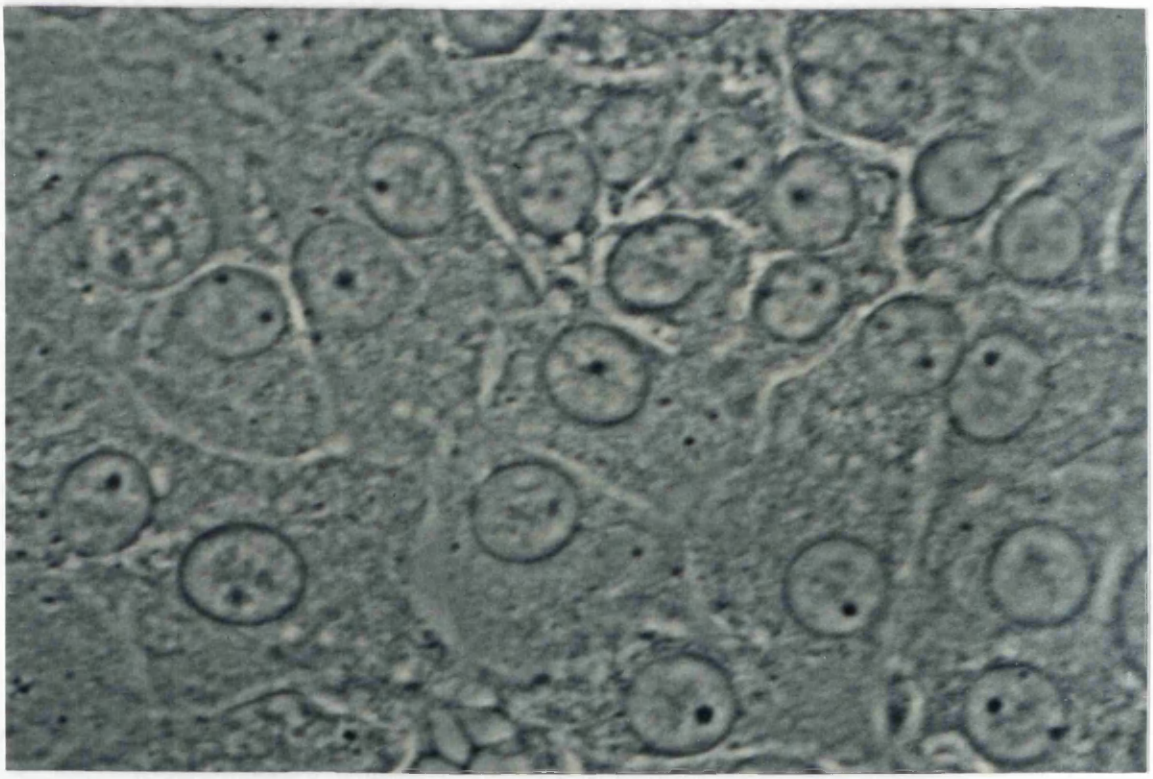


FIGURE 6.10: Mab 3B3 was used to stain 5 day human TEC cultures stimulated with IFN-γ.

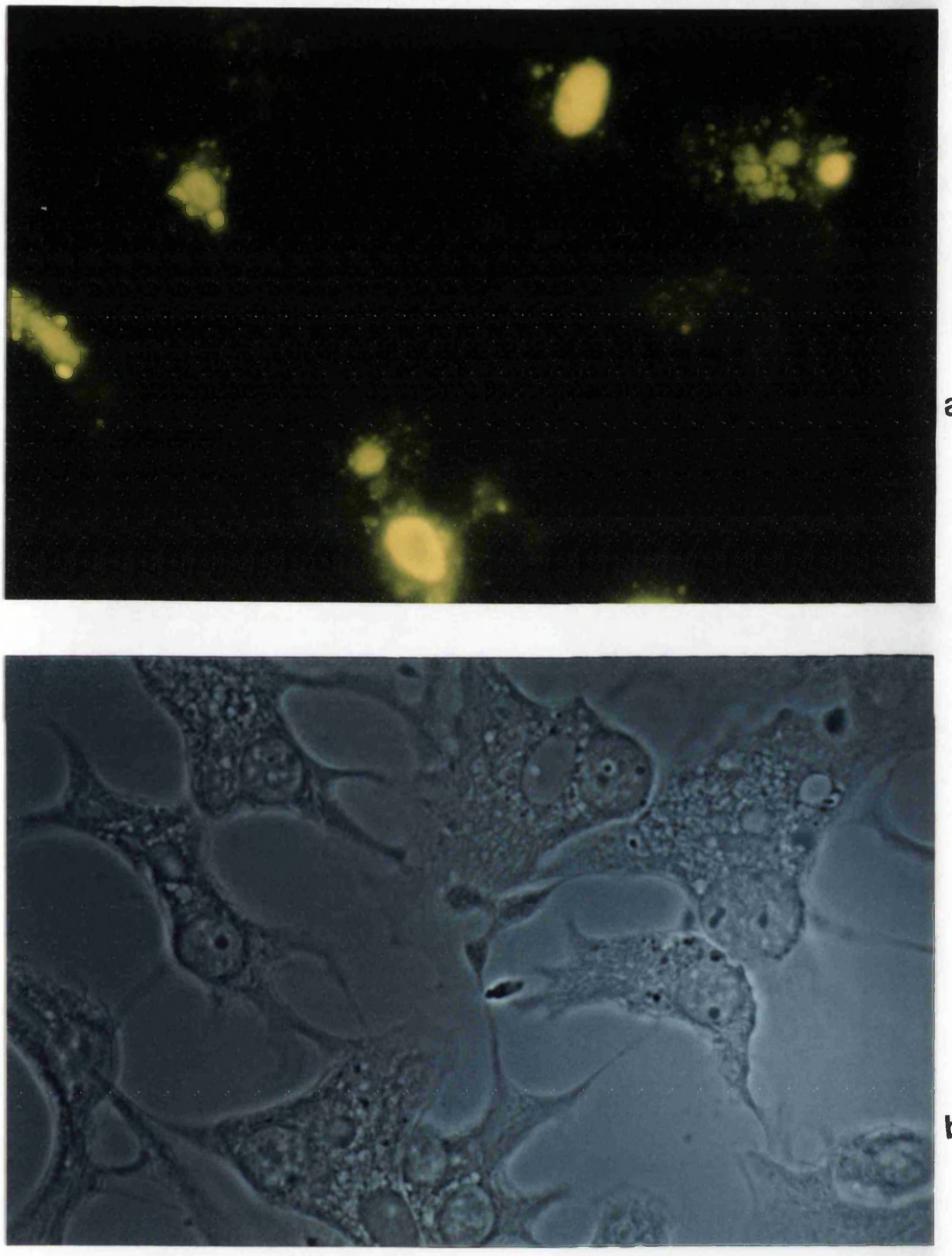
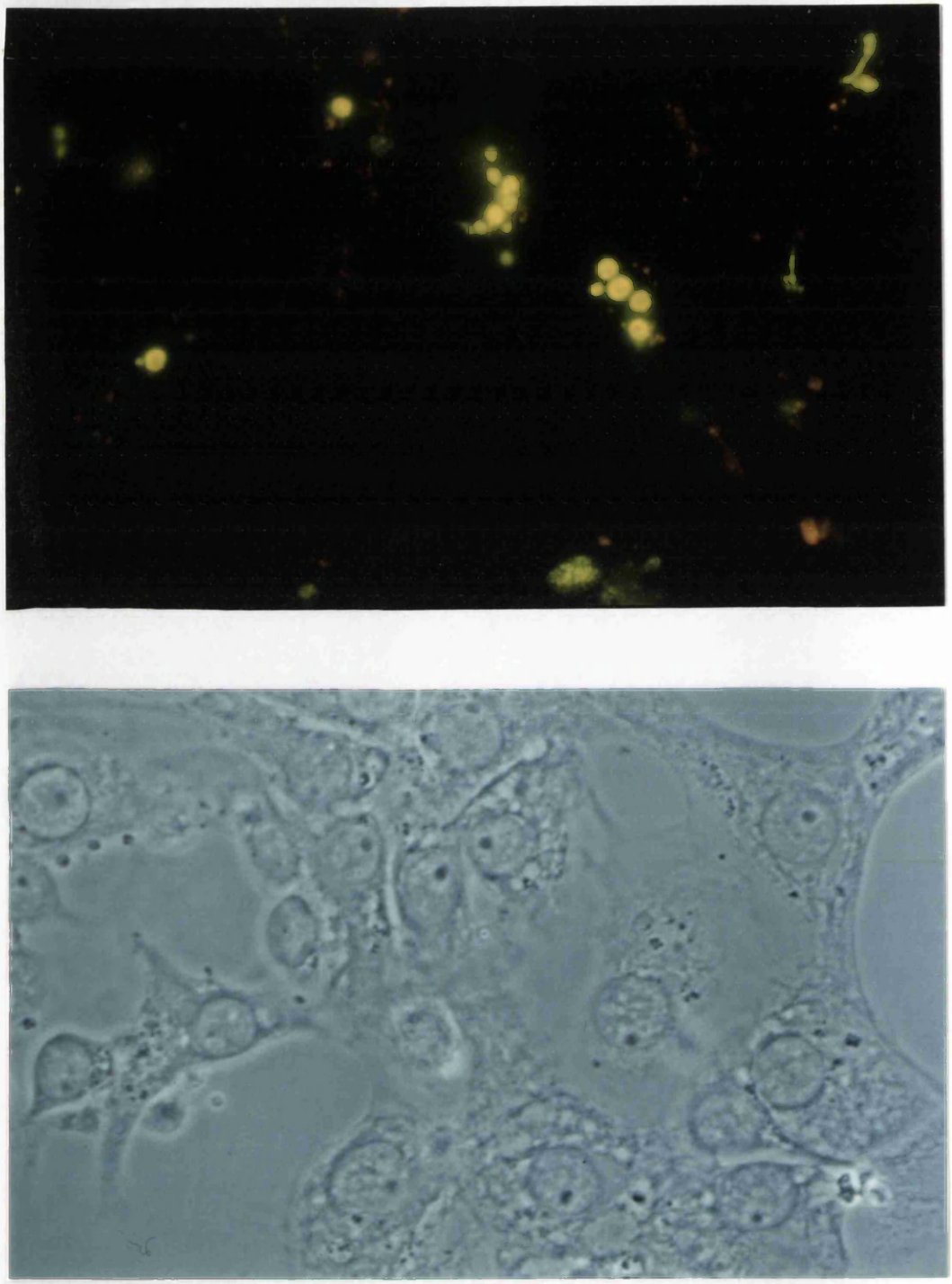
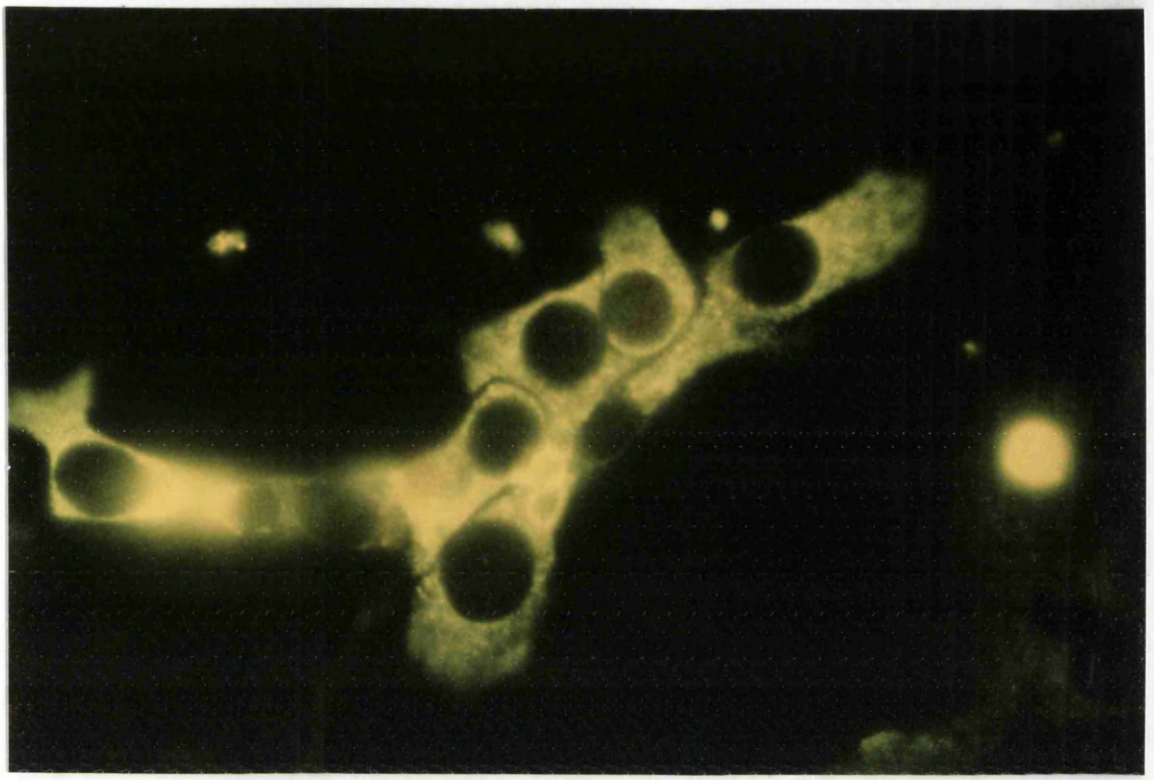


FIGURE 6.10: Variable distribution of TG in the cytoplasm of human TEC. Mab 3B3 was used to stain 5 day human TEC cultures stimulated with TSH (a). This mab was unusual in that the nuclei of cells grown in the absence of TSH were consistently seen to have a granular fluoresence (c). By contrast, intense fluorescence was observed in large vesicular structures within cells stimulated with TSH. In these cultures there was no evidence of granular staining over the nuclei (compare (a) with the phase contrast (b) of the same field. X 630.

b



Distribution of TG epitopes recognised by Mab 6B6 in human TEC stimulated with both TSH and IFN-γ.



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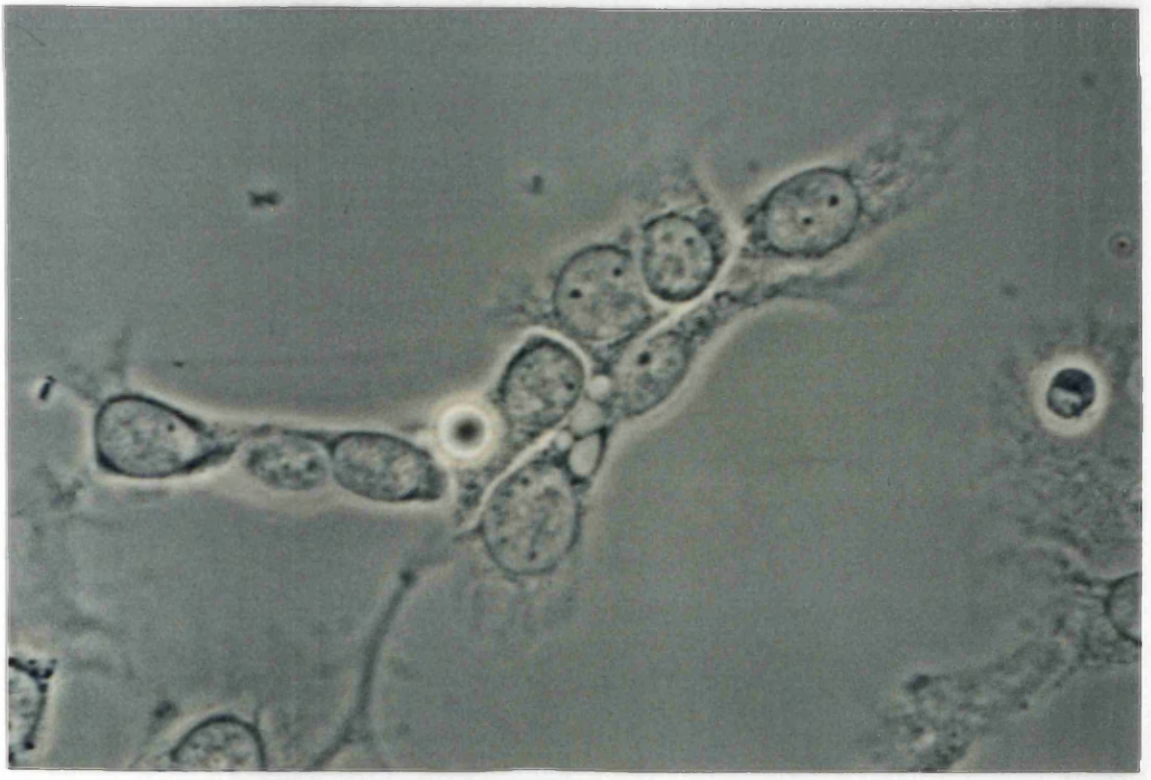


FIGURE 6.11: Distribution of TG epitopes recognized by mab 6B6. In 24 hour cultures of human TEC this mab uniformly stained the cytoplasm of many of the cells (a). However, by 5 days cells grown in the absence of TSH did not express this mab specific determinant. In cells cultured with TSH large positively stained vesicles were observed (data not shown). IFN-γ appeared to counteract this effect of TSH (b).

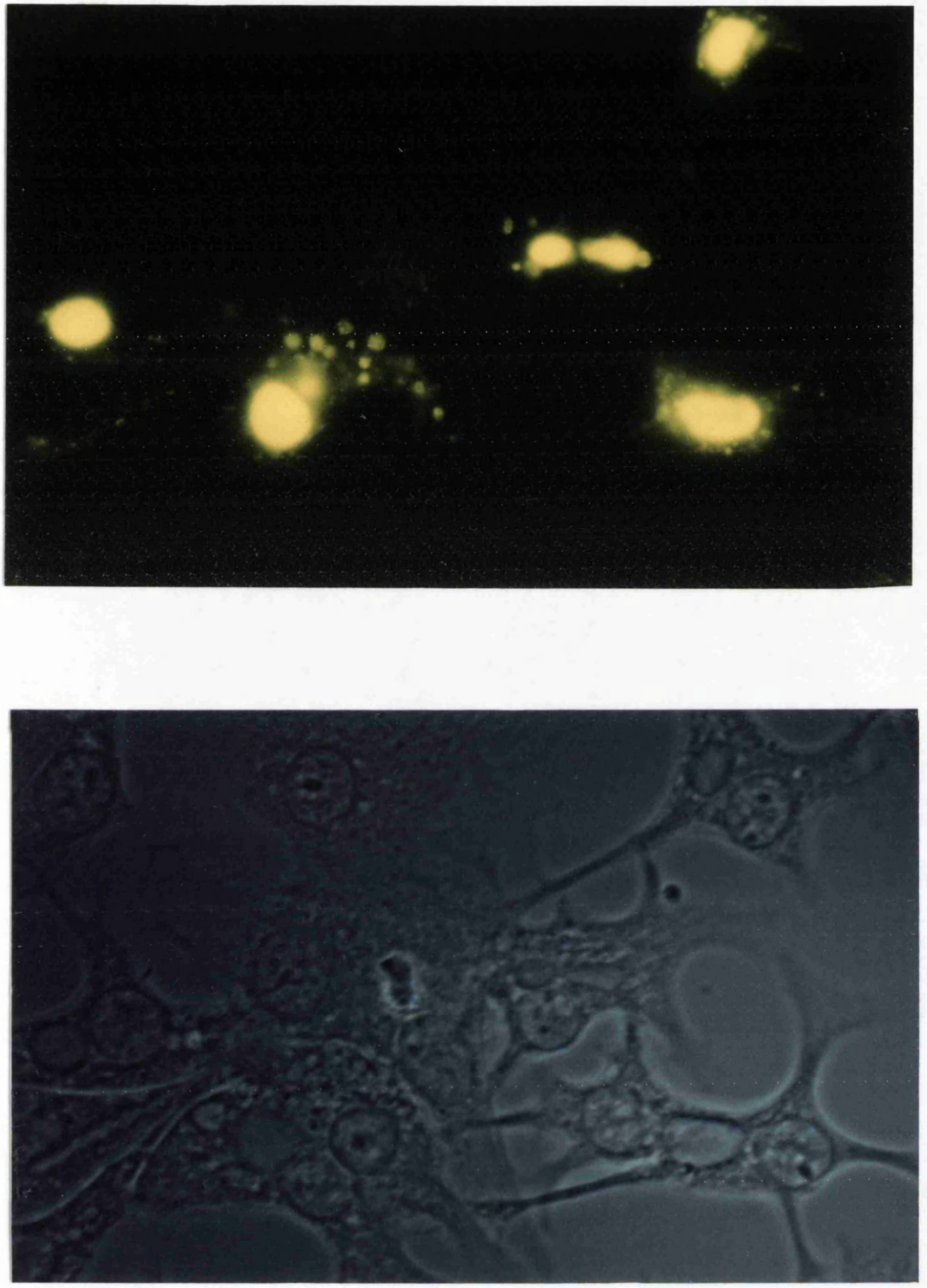
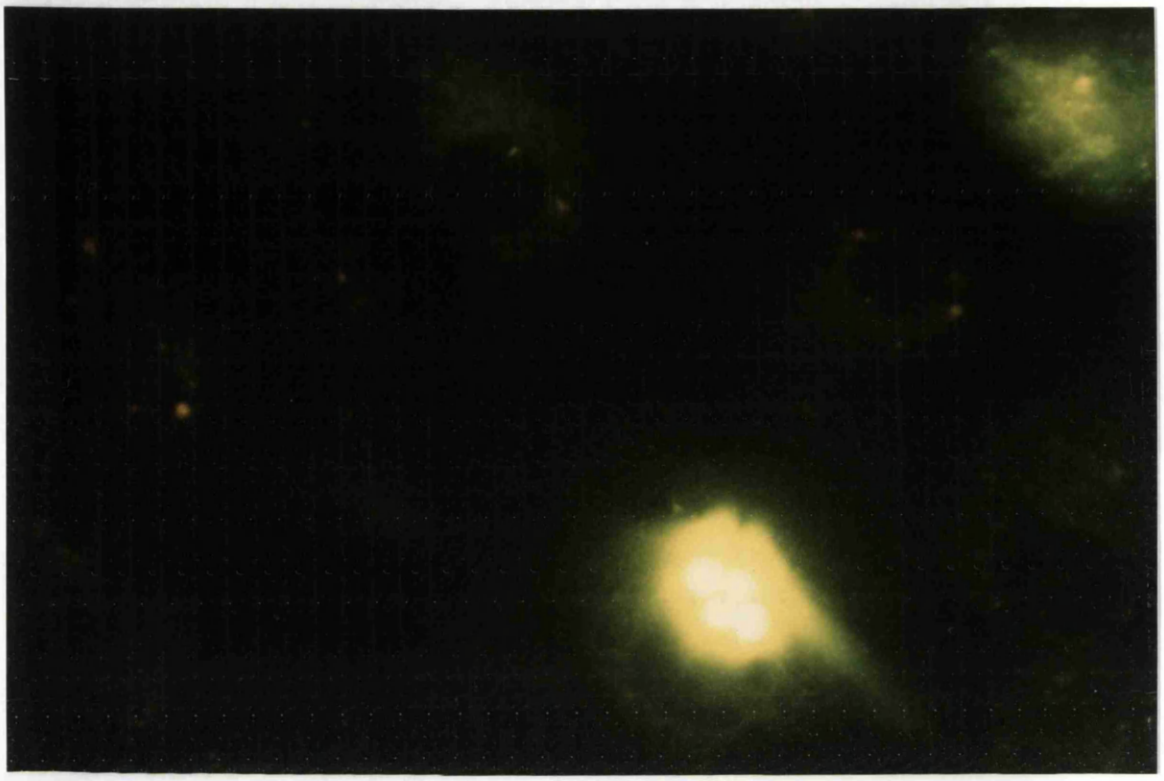


FIGURE 6.12: TG molecules recognized by mab G4F6 are restricted to vesicular structures. 5 day cultures of human TEC stimulated with TSH. Expression of this epitope was not observed under any other condition, or in cells grown for only 24hr.

c



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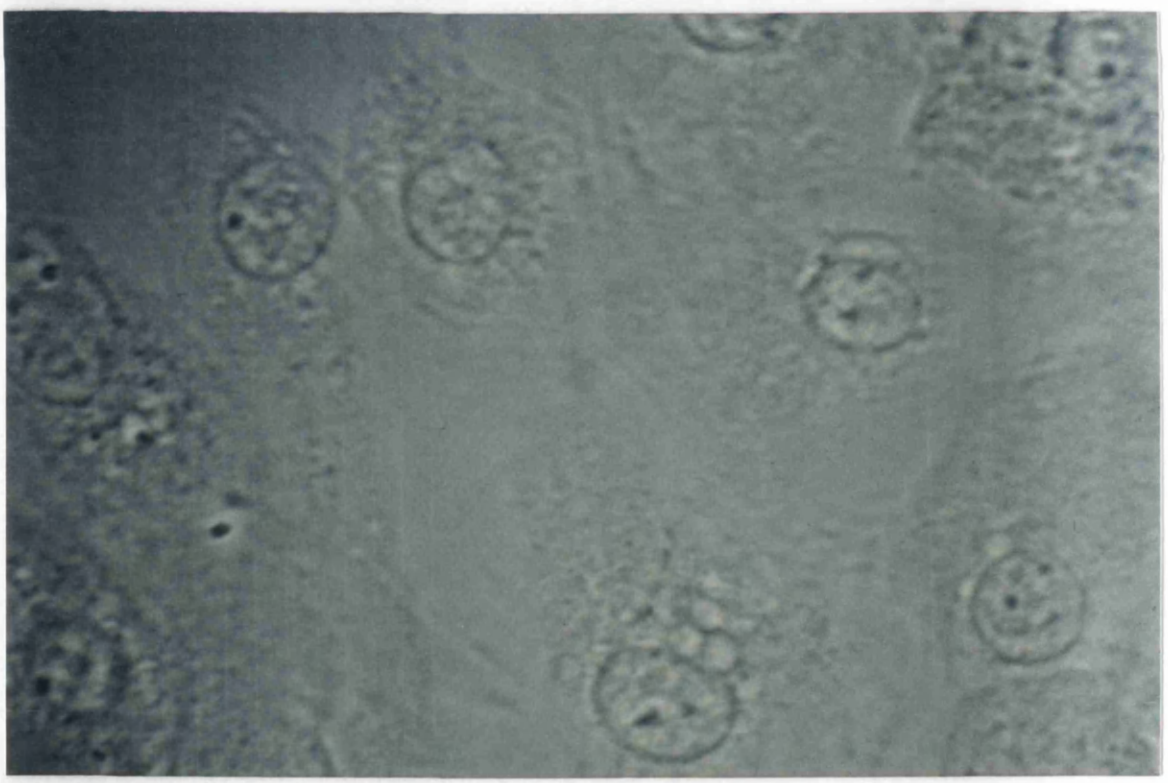
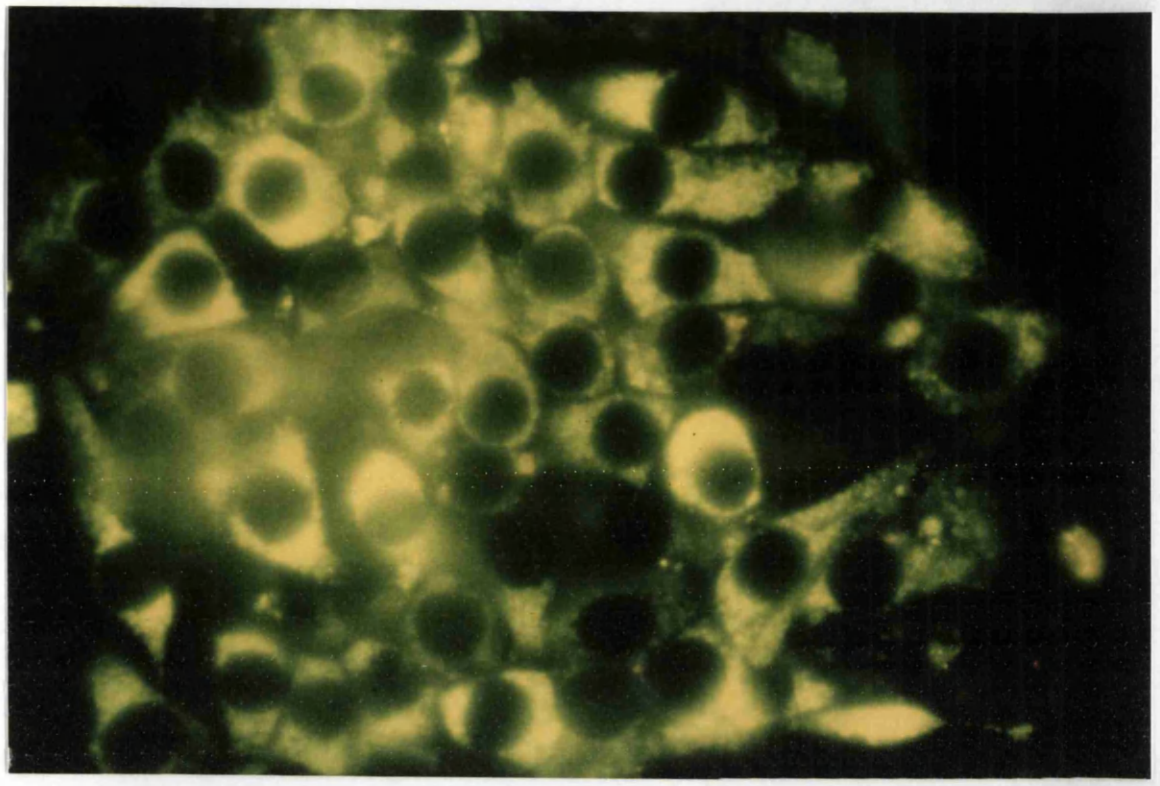


Distribution of TG molecules recognised by Mab 6D2 in 5 day cultures of human TEC grown in the absence of TSH.



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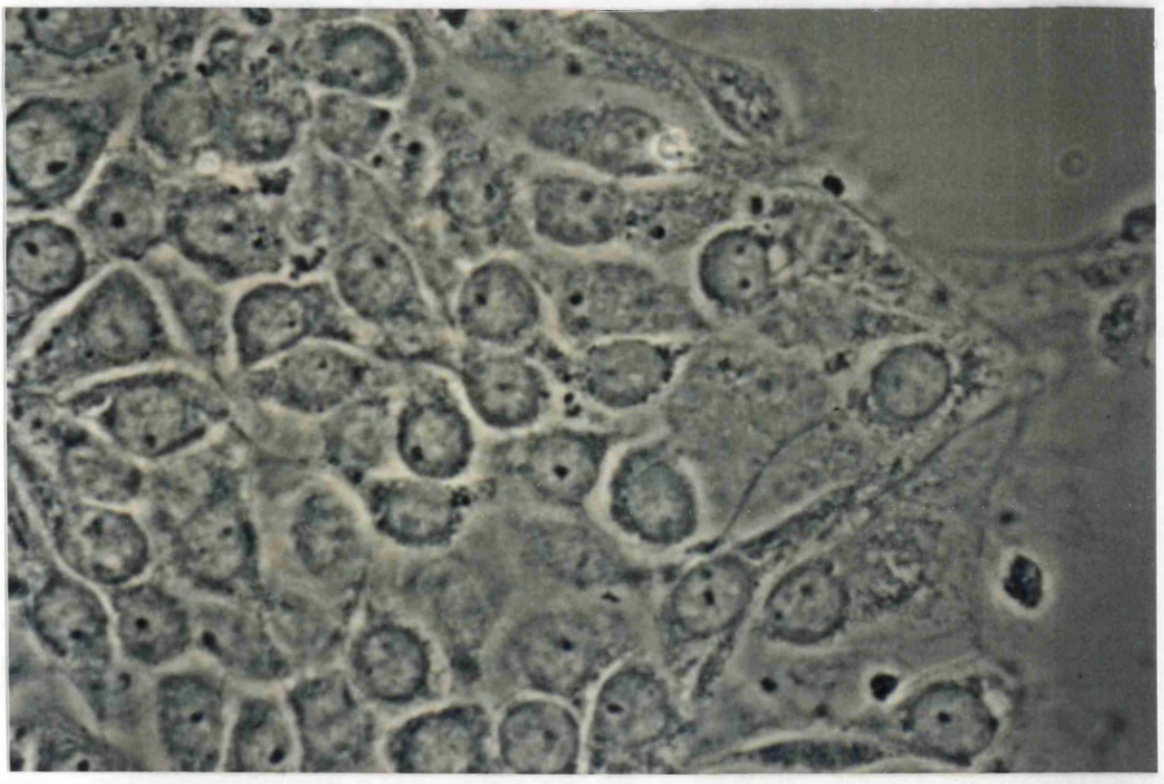


b

FIGURE 6.13A: Distribution of TG molecules recognized by mab 6D2. In 24 hr cultures of human TEC this mab uniformly stained the cytoplasm of the majority of cells (a). However, after 5 days in culture there was virtually no expression of these TG epitopes except in a few isolated vesicular structures (c). The continued expression of this epitope was dependent on TSH stimulation (see figure 6.13B). (No fluorescence could be detected on cells stained with second antibody only - photographs not shown).

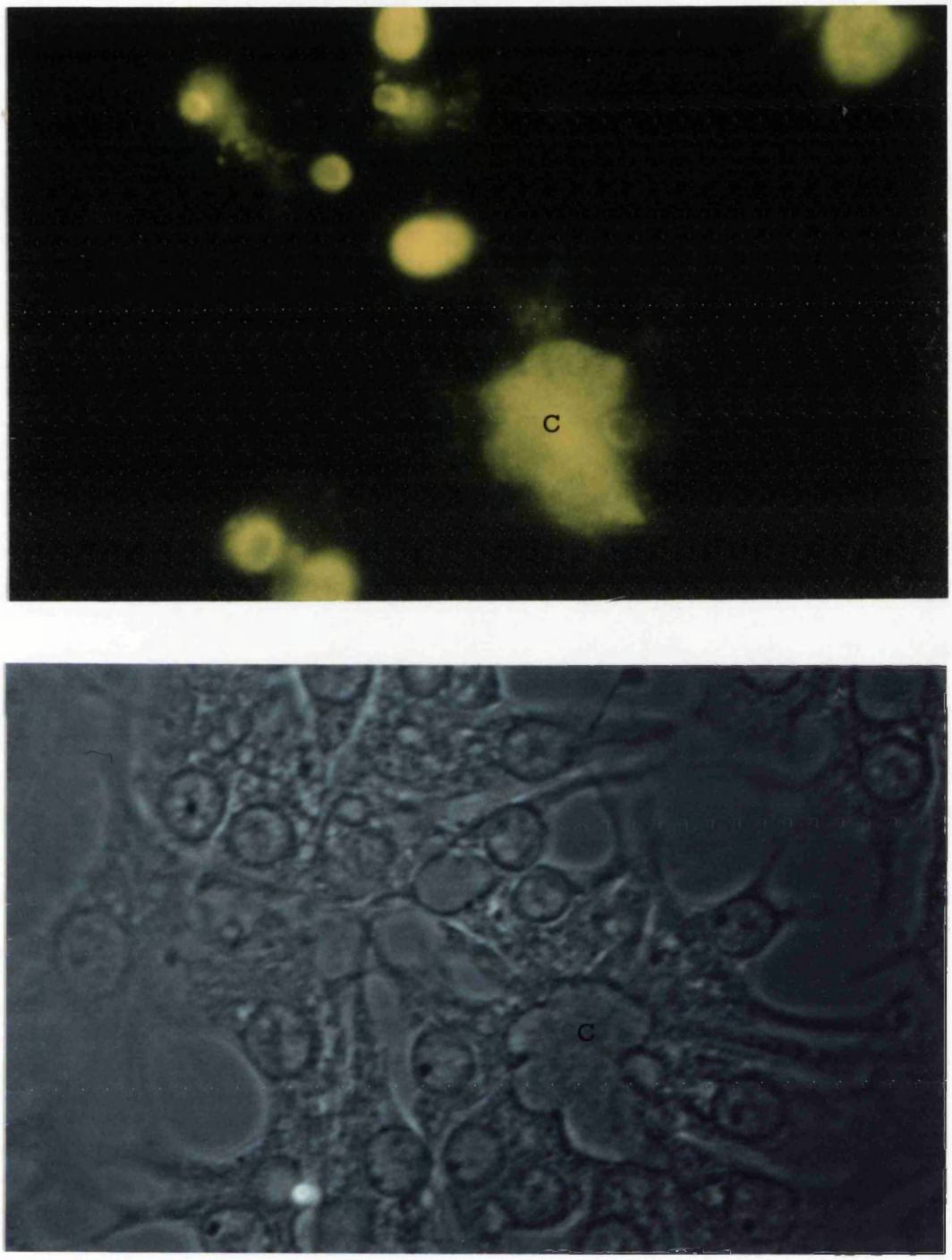
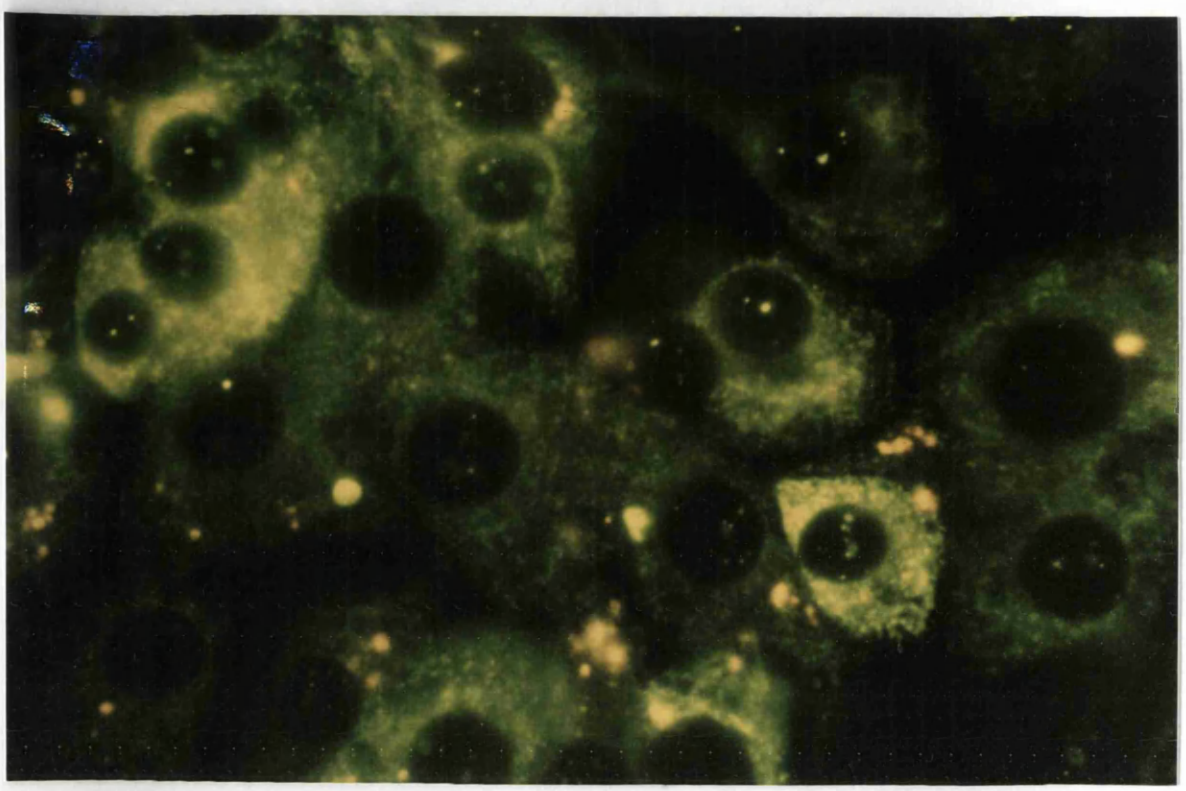


FIGURE 6.13B: Activation of "follicular" structures by TSH. 5 day cultures of Human TEC stimulated with TSH. A number of cells could be seen to form what appeared to be an open follicle enclosing a colloidal space (C), which positively stained for TG recognized by mab 6D2.

c



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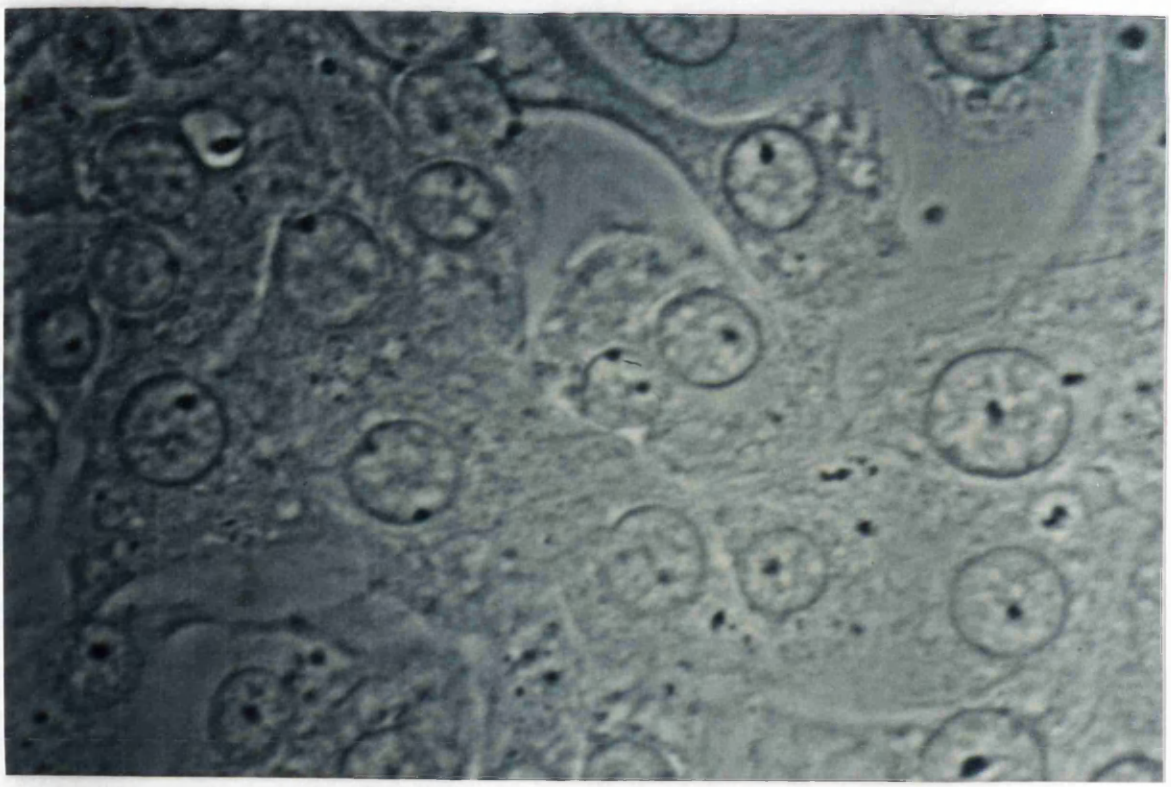
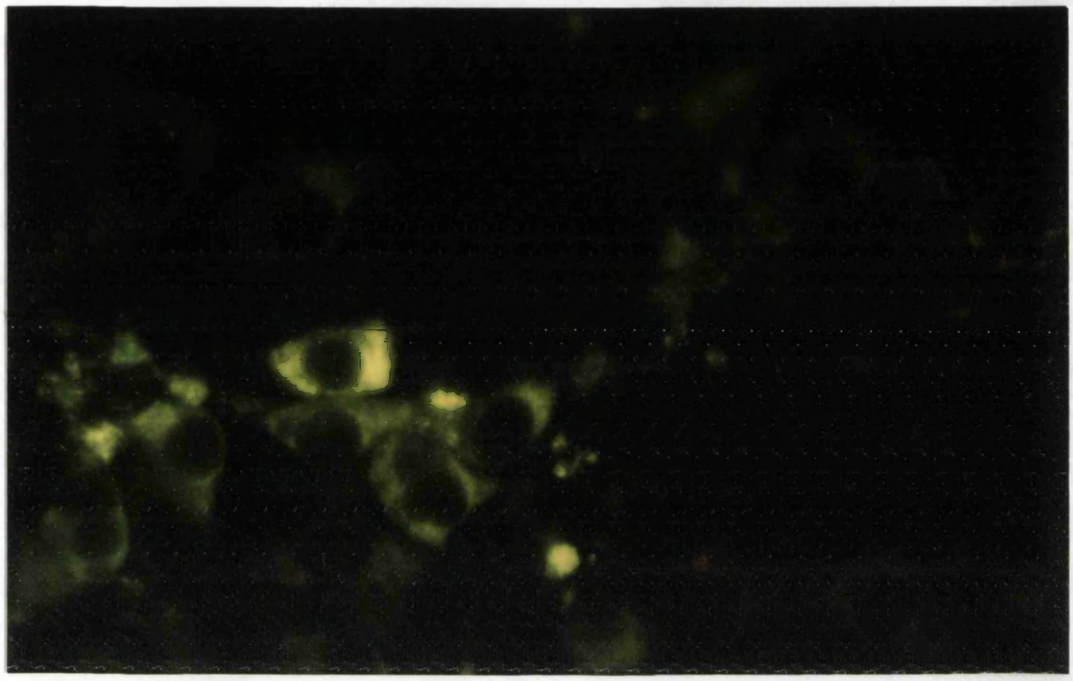
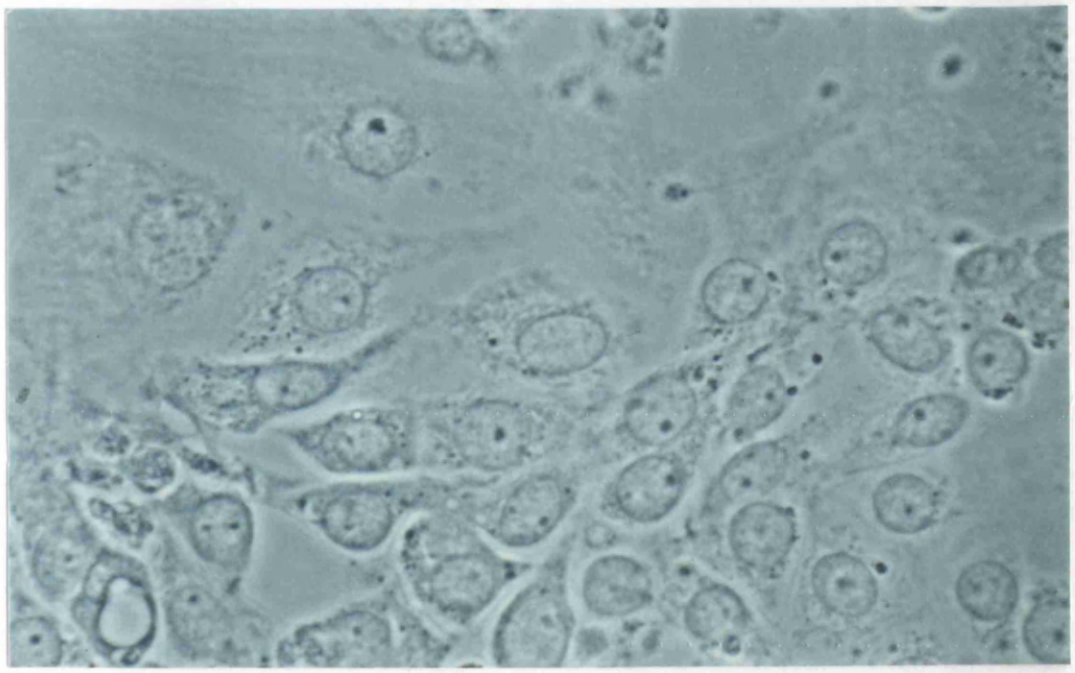


IF detected by Mab 2C6 in 5 day cultures of human TEC stimulated with IFN-γ.

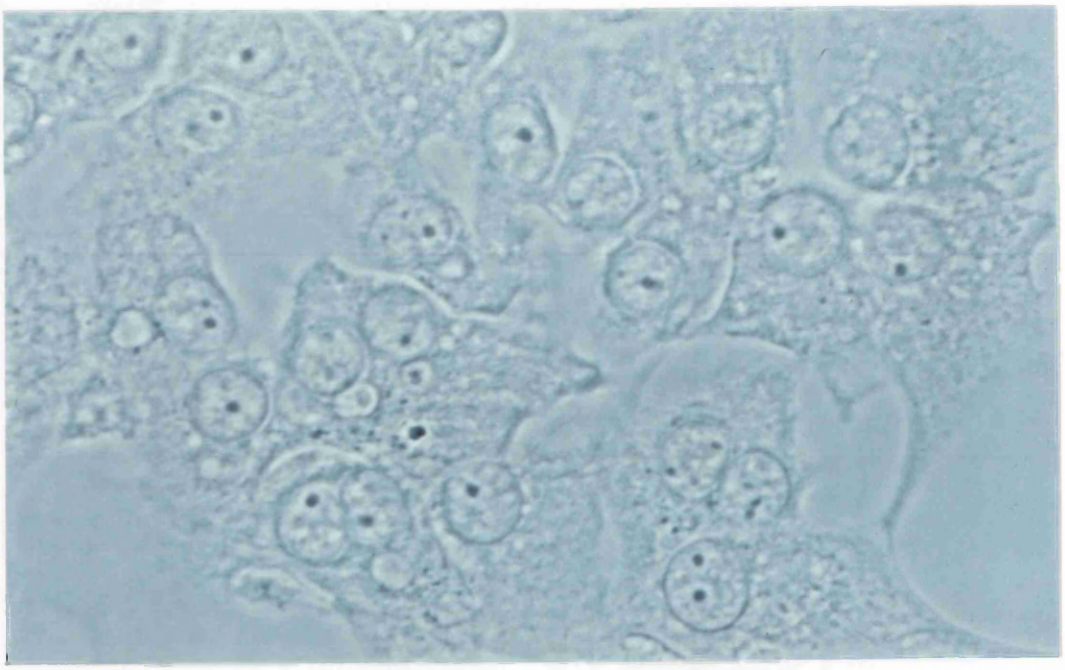


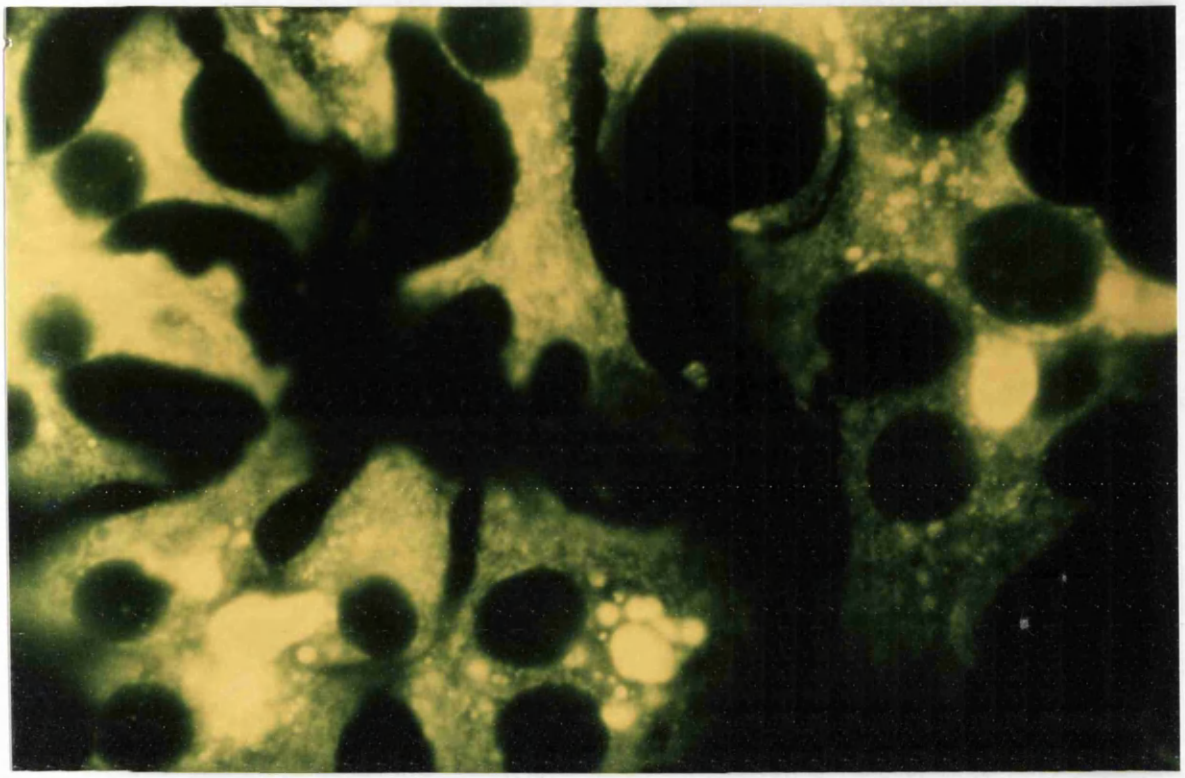
a



b

FIGURE 6.14A: Some TG epitopes are constitutively expressed. Cytoplasmic staining was consistently observed in human TEC when mab 2C6 was used. Cells stained after 24hr in culture (a). 5 day cultures stimulated with IFN-γ also expressed a high concentration of these TG determinants (c). (Original magnification X 630)





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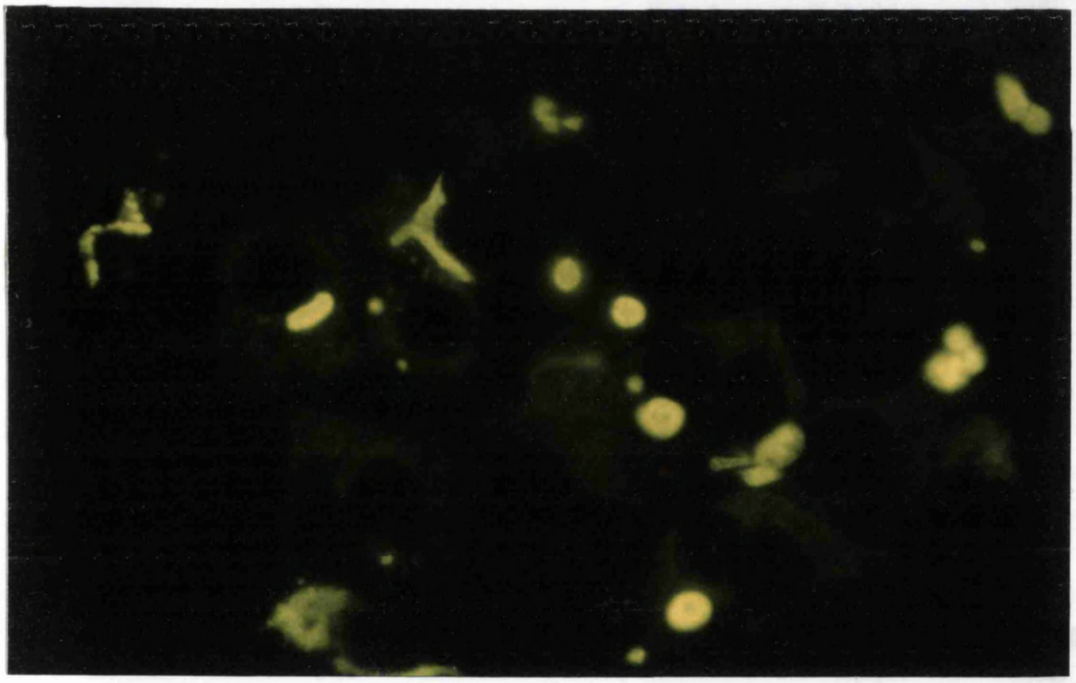


b

FIGURE 6.14B: TSH increases the concentration of TG throughout the cell cytoplasm. Using mab 2C6 it was clear that as well as increasing the concentration of TG in vesicular structures, TSH upregulates the TG content in the rest of the cytoplasm (a). IFN-γ may counteract the TSH stimulated response; (b) 5 day cultures stimulated with both IFN-γ and TSH. (Note that the phase contrast of this field is shown as a mirror image.)

TABLE 6.2

SUMMARY OF OBSERVATIONS FROM IF STUDIES ON MONOLAYER CULTURES OF HUMAN TEC

Monoclonal Antibody (Mab)	Distribution of Mab Defined TG Epitopes Within Human TEC After 5 Days in Culture			
	Membrane - Influenced by IFN-γ	Uniform Distribution Within Cytoplasm	Vesicular Structures	Dependency on TSH for Expression of Epitopes in Cytoplasmic Areas
2A4	NO	HIGH LEVEL	YES	NO
2C6	NO	HIGH LEVEL	YES	NO
2B1	NO	LOW LEVEL	YES	PARTIAL
6B6	NO	LOW LEVEL	YES	YES
6D2	NO	LOW LEVEL	YES	YES
5B1	NO	LOW LEVEL	YES	YES
3B3	YES	NO	YES	YES
G4F6	NO	NO	YES	YES
F5B3	NO	NO	YES	YES
5F6	YES	NO	NO	YES

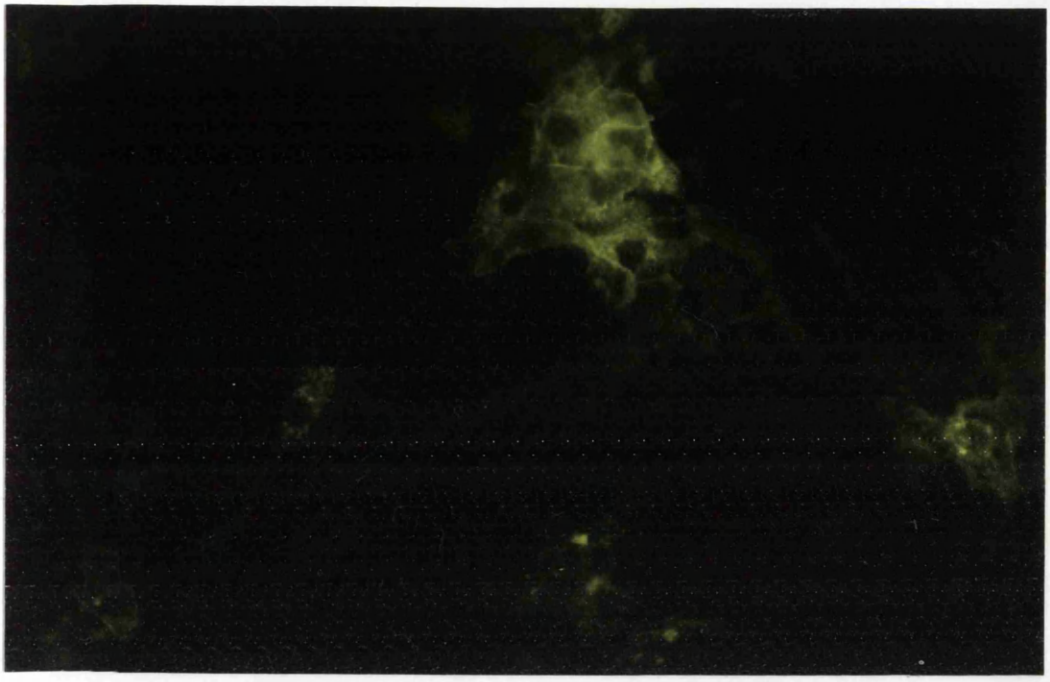
TEC were from a patient presenting with colloid goitre. The data shows that TG molecules within the thyroid cell are heterogeneous. Some epitopes are expressed only if cells are stimulated by TSH. Furthermore, some of these are specifically localised within the vesicluar structures.

6.2.5 Modulation of MHC class II antigens in mouse thyrocytes

The cytoplasmic and membrane expression of the MHC class II antigens, I-A and I-E was assessed. The cytoplasmic expression of these antigens was observed only in IFN-γ stimulated cultures (FIG. 6.15). The expression of I-E antigens was apparently greater than I-A antigens, but this difference may have been due to the different affinities of the monoclonal antibodies used. In these studies the anti-I-A monoclonal antibody did not show any surface staining of cells cultured in the absence of IFN-γ. However, the surface expression of I-E antigens was observed in cultures stimulated by TSH only, although this expression was far more prominent in IFN-γ stimulated cells (FIG. 6.16).

6.2.6 Antigen-presenting function of murine thyrocytes

As the mouse TEC cultures could be activated to express surface class II antigens, and the surface expression of some TG epitopes could be demonstrated by IF studies, the antigen presenting function of these cells was analysed. Specific stimulation of the I-A^k restricted TG-dependent T-cell hybridoma CH9 (Rayner et al., 1987) was observed if the TEC cultures were pretreated with IFN-γ ($P = 0.001$) (FIG. 6.17). Cultures pretreated with TSH alone did not have TG-specific antigen-presenting function, but TSH did not inhibit IFN-γ activated stimulation of CH9 (FIG 6.17c). By comparison, mouse TG specific T cell lines MTG12B & MTG9B3 (Champion et al., 1985), could not be activated by TEC cultures (data not shown). IFN-γ pretreated TEC could also stimulate the TG-independent T-cell hybridomas U3 and U10, which are restricted to the I-A/E region of k haplotypes. U10 was also stimulated by thyrocytes cutured in 10% FCS supplemented with TSH (FIG. 6.17a). A factor other than TSH was responsible for this activation, because U10 did not respond when the TEC were grown in medium containing 1% FCS and TSH (FIG. 6.17c). TSH and IFN-γ did not directly affect the CTLL assay.



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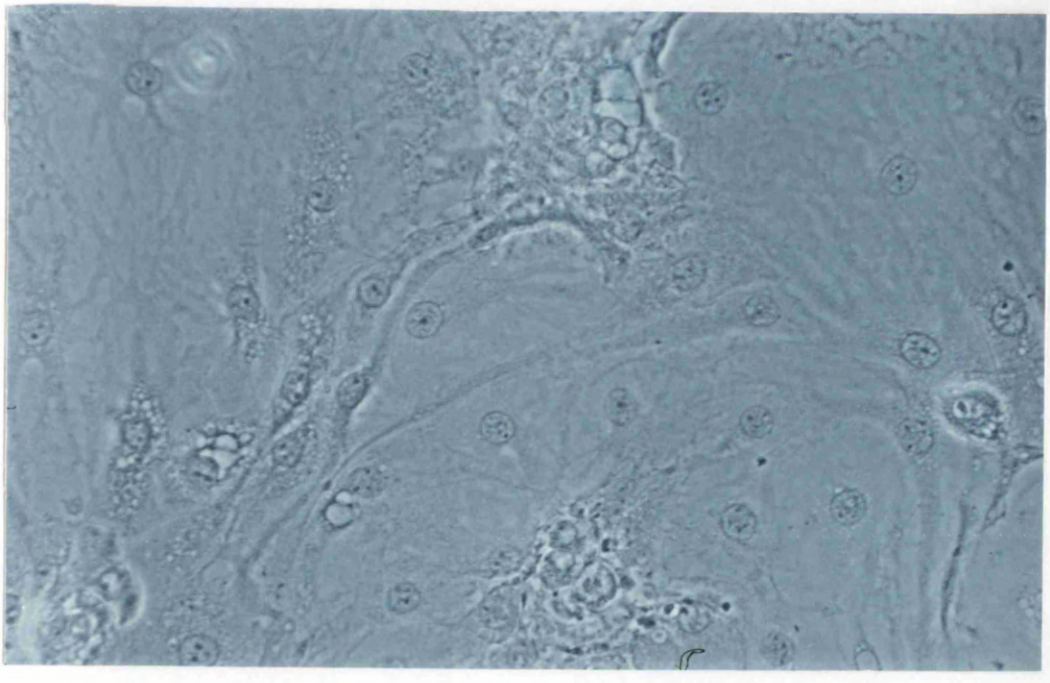
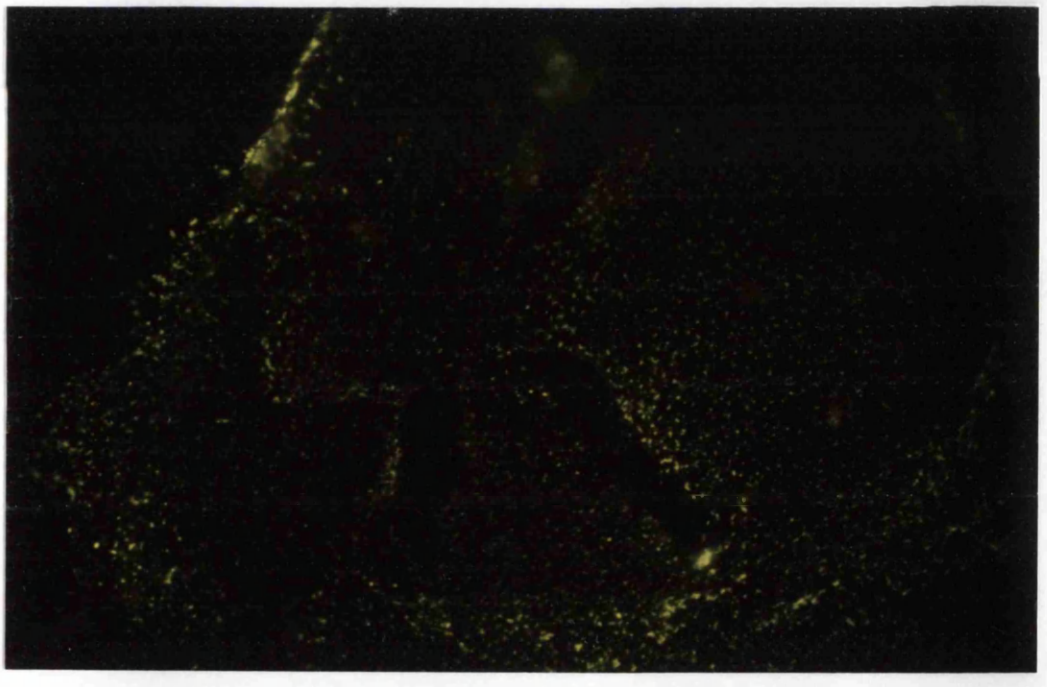


b

FIGURE 6.15: Cytoplasmic expression of class II antigens in mouse TEC. (a) 5 day cultures of mouse TEC stimulated with IFN-γ for 72hr. Mab to class II I-E^k antigen was used. (b) Shows the phase contrast of the same field.

a



b

FIGURE 6.16: Class II antigen expression on the surface of mouse TEC. Surface IF detected by Mab 1a.7, which is specific for class II I-E antigens. 5 day mouse TEC cultures stimulated with TSH (a) or IFN-γ (b).

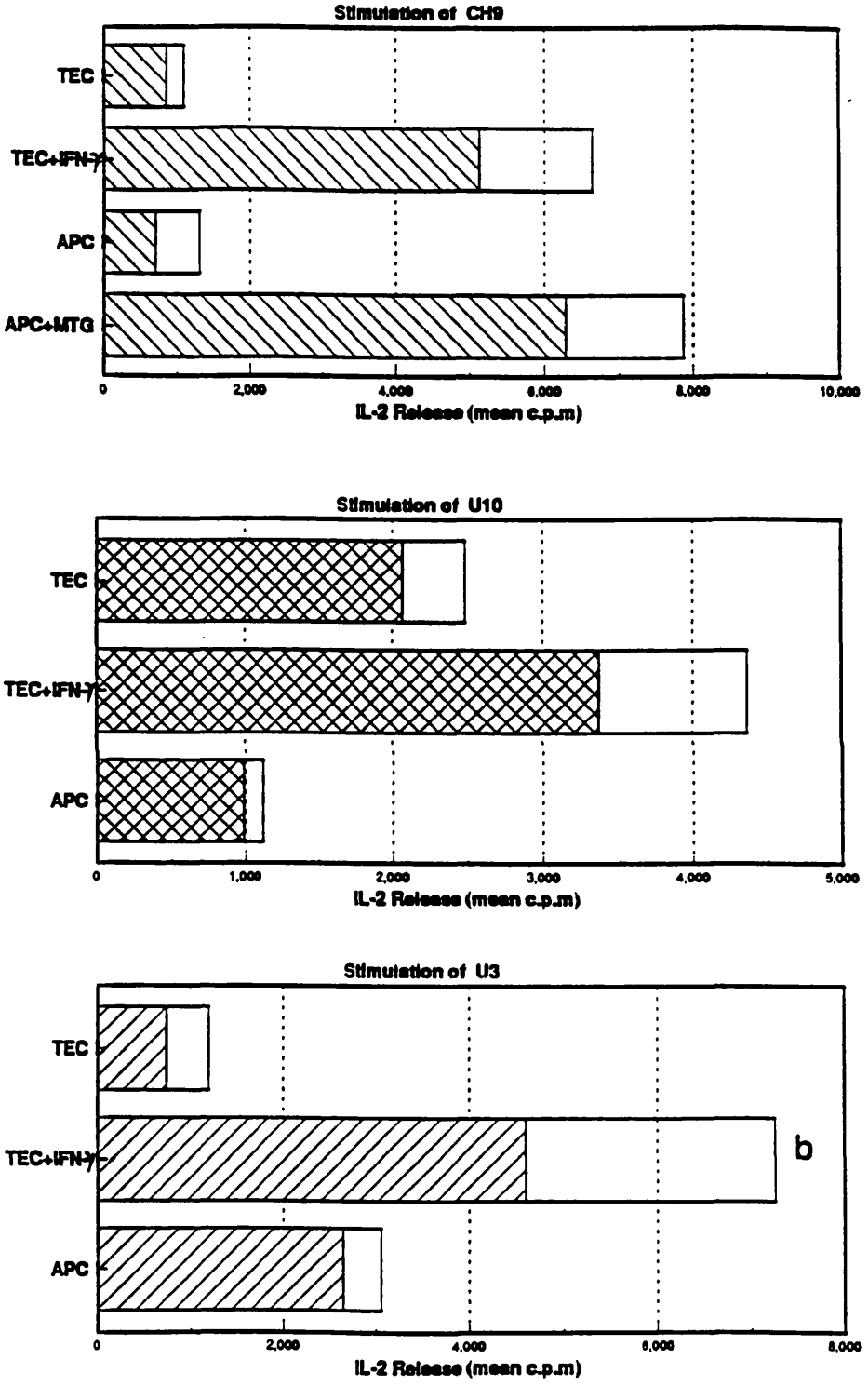


FIGURE 6.17A: *In vitro* stimulation of T cell hybridomas was determined by IL-2 release assay (see Materials & Methods). 4 day cultures of CBA/J TEC grown in medium supplemented with 10% FCS and 60μU/ml TSH were used. All three hybridomas were activated by TEC pretreated (72 hrs) with IFN-γ (100 U/ml). Irradiated spleen cells ($4x10^4$) pulsed with or without MTG (50μg/ml) were used as control antigen presenting cells (APC). Each control value is the mean of triplicate cultures. Other values are the mean of five separate samples, except for (b), where 4 samples were used. Unshaded areas of each bar represents the S.D. (Background counts were subtracted in each case).

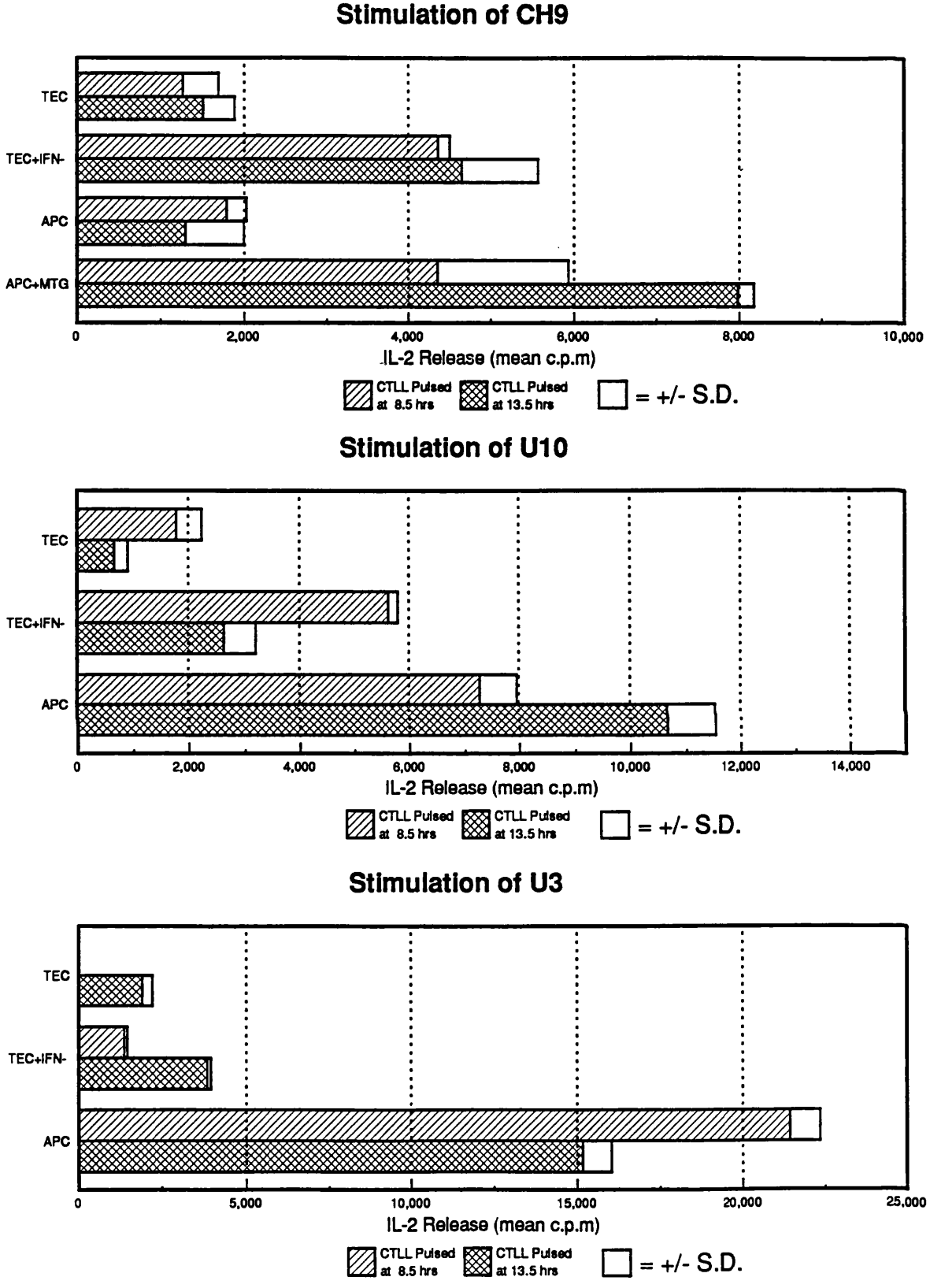
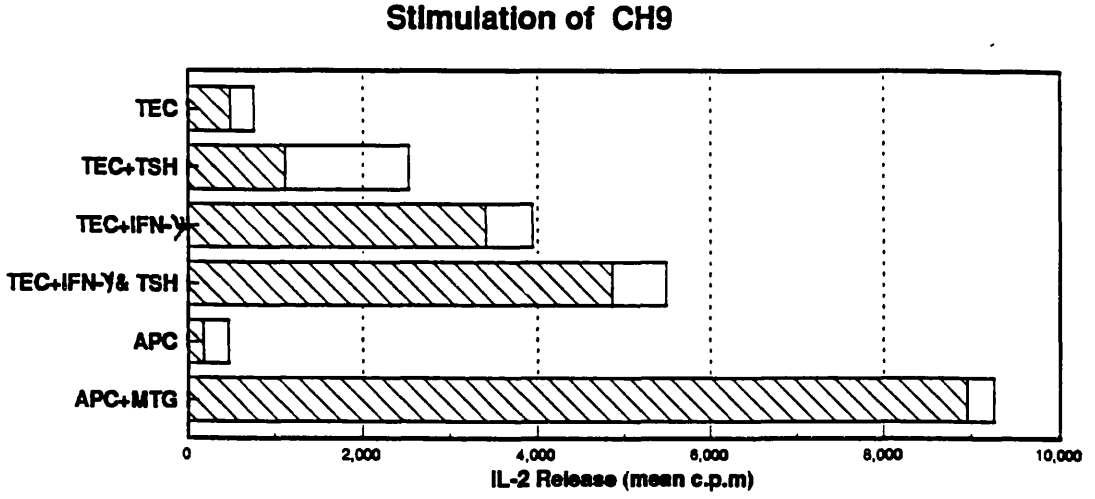


FIGURE 6.17B: *In vitro* activation of T cell hybridomas by 5 day cultures of CBA/J TEC (10% FCS & TSH supplemented medium) stimulated with IFN-γ (100 U/ml) for 72 hrs. For control APC $5x10^5$ irradiated spleen cells were used. For some samples the maximum incorporation of ^{125}IUdR by CTLL cells was dependent on the time at which the cells were pulsed. All values are the means of triplicate samples.



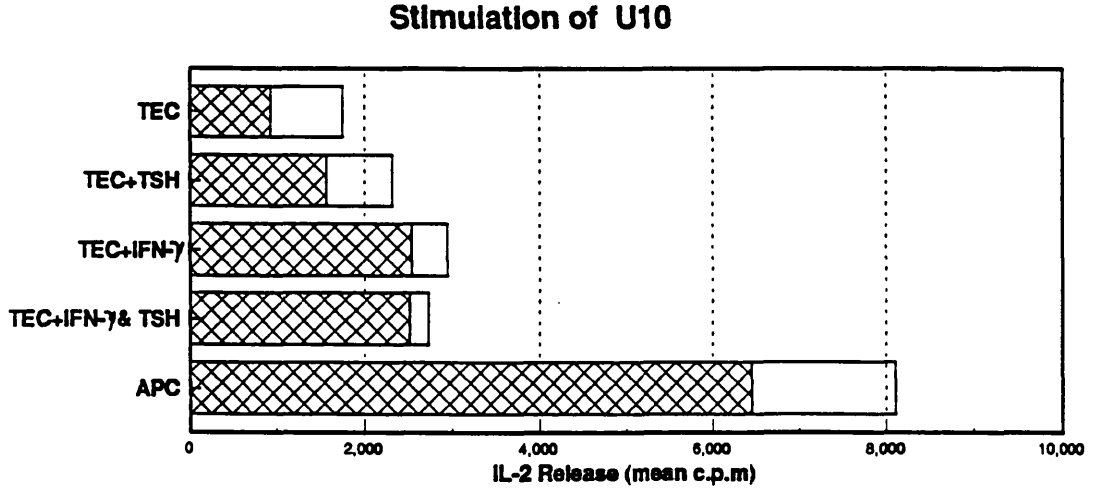


FIGURE 6.17C: TEC were grown in medium supplement with 1% FCS alone, or with TSH (60 μU/ml) and IFN-γ (100 U/ml) as indicated. $5x10^5$ irradiated spleen cells were used as control APC. Each value is the mean (shaded areas) of triplicate samples. Unshaded areas of each bar represents the S.D.

6.3 DISCUSSION

6.3.1 Modulation of TG pathways by TSH and IFN-γ

The post-translational modulation of TG by TSH and IFN-γ was studied in primary cultures of mouse thyroid cells. The main advantage of using a primary culture system, is that their metabolic pathways are likely to remain unaltered: In the rat thyroid cell line FRTL-5 there is a dramatically reduced synthesis of TG, which may be due to a stable alteration in a post-transcriptional step of TG biosynthesis (Avvedimento et al., 1985).

In these studies there was no detectable TSH induced increase of secreted TG (FIG. 6.4), in contrast to observations made by Creemers et al. (1983). The reason for this discrepency is not clear, but there are a number of plausible explanations. One explanation may be that the effect of TSH was masked by the inclusion of insulin in my culture medium. There is evidence to support the view that the control of TG gene expression does not involve TSH alone. Insulin also appears to play a role in basal expression of TG gene transcription (Aouani et al., 1987, Santisteban et al., 1987). Alternatively, the explanation may lie in the differences in the assays used. In these studies polyclonal rabbit anti-MTG sera (which did not recognize trypsinized TG (FIG. 2.2, see materials & methods)) was used, while the Creemers group used two monoclonal antibodies to different mouse-specific determinants of MTG. Therefore, one possible explanation for this discrepency is that TSH stimulates the release of processed TG, which may be recognized in the assay used the Creemers group. The IF studies clearly show that the specificity of the antibody determines whether an influence of the culture condition on TG expression is observed.

In the majority of cells, cytoplasmic TG was expressed under all culture conditions (FIG. 6.6). This was also observed in human thyrocytes (FIG. 6.14), but only if mab 2C6 was used. Other monoconal antibodies such as 3B6 and 6D2, showed that the expression of some TG epitopes can be highly

dependent on culture conditions (FIG. 6.13, 6.11). For example, between day 1 and day 6 of culture in the absence of TSH, TG expressing the epitope recognized by mab 6D2 was virtually lost (FIG. 6.13). Interestingly, under TSH stimulation this fraction of TG preferentially accumulated in large vesicular structures. It is difficult to know whether such structures are endocytosed colloid droplets, or exocytotic vesicles.

It is well known that in addition to subapical vesicles, thyroid follicular cells contain numerous cytoplasmic granules; less dense granules (colloid droplets), and both heterogeneously and homogeneously electron-dense granules, which are thought to be associated with production of thyroid hormones (Uchiyama et al., 1986). Furthermore, it seems that the colloid droplets migrate from the apical through the intermediate to the basal parts of follicular cells, changing their appearance and properties as they go. It is tempting to suggest that mab G4F6 recognizes a TG epitope which is retained (or exposed) in this catabolic pathway (FIG.6.12). These studies show that in human thyroid cells the vesicular structures (which are considerably increased by TSH) contain a heterogeneous population of TG molecules. Some of these epitopes appear not to be expressed in the rest of the cytoplasm. This was observed with mab 3B3 (FIG. 6.10). Interestingly, this mab, which is cross-reactive with many different species of TG (Appendix 2), did recognize TG in the cytoplasm of mouse thyroid cells, but expression of this epitope was restricted to the smaller cells (FIG. 6.7).

The same level of antigenic variance was not observed in the mouse cultures, but it seems likely that they do exist and may be revealed by using a more extensive range of antibodies. Importantly, although vesicles were induced in the presence of TSH, they were not as prominant and not as uniformly expressed as in the human thyroid cells. This may be related in part to the possibility that the two TSH induced responses; TG secretion (Ekholm et al., 1975; Ofverholm et al., 1985) and endocytosis of follicular colloid (Neilsen et al., 1985; Westermark & Porter, 1982), occurs concurrently in the different cells of a heterogeneous population. (Even cultures of the FRTL-5 cells immunostained for TG show variability between the cells (Huber et al., 1990).) Importantly, if the two functional activities do occur concurrently in

in vitro TEC cultures, then the overall concentration of TG in the medium should remain constant.

Vesicular structures were not preferentially stimulated by IFN-γ, and the size and number of these structures was down-regulated in cells stimulated with both TSH and IFN-γ (FIG. 6.10). The effect of IFN-γ on TSH induced processes is not clear and is an area which deserves more attention. Interestingly, cells stimulated with IFN-γ alone expressed surface TG epitopes recognized by mab 5F6 and mab 3B3 (FIG. 6.9). In mouse thyroid cultures, mab 3B3 did not show surface binding, but surface expression of TG epitopes recognized by mab 5F6 TG was induced by IFN-γ (Fig. 6.8). A similar pattern of expression was also observed with mab H3D7. Interestingly, these epitopes were not expressed in the cytoplasm.

The implications from these observations are far reaching, assuming that the use of staining intensities as a measure of quantitation for antigen expression (Bontrop et al., 1986) is justified. If, for instance, the surface TG induced by IFN-γ are epitopes on TG peptides which are associated with MHC class I molecules, then even this level of induced expression may be sufficient for cytotoxic T-cell mediated lysis. Recently it was calculated that as few as 200 class-I-peptide complexes can sensitize EL4-thymoma cells for lysis by cytotoxic T-lymphocytes (Christinck et al., 1991). Alternatively, these may be epitopes on TG in association with class II molecules; again between 60 and 300 peptides are sufficient to stimulate IL-2 production in class II- restricted T cell hybridomas (Harding & Unanue, 1990; Demotz et al., 1990).

Supporting the IF study of the stimulatory effect of IFN-γ on TG metabolism in murine thyrocytes, is the observation that in two separate experiments, IFN-γ induced an increased level of TG in the medium as detected by solid phase RIA, whereas TSH consistently failed to induce an increased response. Interestingly, in two other studies, the combined use of TSH and IFN-γ decreased the basal level of TG measured (TABLE 6.1). Either this effect of TSH is regulated by the past history of the cell; as was shown to be the case for the TSH effect on cell growth and thymidine incorporation (Valente et al., 1983), or the effect of IFN-γ on thyrocytes is multifactorial and may be dependent on the expression of the IFN-γ receptors. As on other cell types, human thyrocytes express

both high and low affinity receptors for IFN-γ and treatment with recombinant tumour necrosis factor-alpha (rTNF-α) can increase the binding of rIFN-γ, probably due to increased numbers of receptors per cell (Buscema et al., 1989). Conversely, on tumour cell lines, IFN-γ increases the expression of TNF-α receptors (Ruggiero et al., 1986). Furthermore, like TSH (Todd et al., 1987), TNF-α can enhance the effects of IFN-γ on thyrocytes (Taverne et al., 1987; Weetman & Rees, 1988). From these observations it is only a short step to the suggestion that IFN-γ and TSH can also modulate (either increasing or decreasing) the opposing receptors.

Nagayama et al. (1987) have reported that human thyrocytes which have been pretreated with IFN-γ and express DR antigens loose their sensitivity to TSH induced TG secretion. The relationship between these two pathways is not clear. One possibility is that prolonged pretreatment with IFN-γ switches off TG synthesis, or the synthesis of a protein essential to exocytosis. In this context it is interesting to note that in a recent study (Ashizawa et al., 1989) it was demonstrated that IFN-γ inhibits TSH induced TPO gene expression in cultured human thyrocytes. The coupling between exocytosis and TPO synthesis seems reasonable considering that peroxidase is present in the membrane of typical exocytotic vesicles, the content of which is mainly made up of newly synthesized thyroglobulin (Bjorkman et al., 1976).

In the intact organ, the thyroid epithelial cells are organized into follicular structures in which the cells are polarized with their apical part facing the follicle lumen and their basal part the basement membrane (Mauchamp et al., 1979). Free communication is prevented by intercellular junctional complexes (tight junctions) (Dragsten et al., 1981). In suspension cultures, thyroid follicles will initially maintain their functional polarity, but depending on culture conditions they will gradually invert so that the apical part faces the culture medium (Nitsch & Wollman, 1980).

It has been suggested (Mauchamp et al., 1989) that when thyroid cells are cultured on impermeable surfaces, they form polarised monolayers, but under these conditions they lose some of their specific functions: iodide concentration and organification, sensitivity to acute TSH stimulation.

These authors suggest that this may be related to the basal location of the TSH receptor (Chambard et al., 1987), which makes them inaccessible to TSH stimulation. Both the mouse and human thyroid cell cultures described in this study were clearly responsive to TSH, since surface expression of microsomal/TPO antigen was induced. A number of cells expressed the typical (Khoury et al., 1984) granular pattern of surface staining, with each cell prominantly outlined, although the number of responsive cells was far less than in the human thyroid cultures (FIG. 6.3). These results extend and confirm the findings of Chiovato et al. (1985; 1989). The specificity of this response to TSH was demonstrated by the fact that IFN-γ could not induce a similar level of M/TPO-antigen expression. It remains to be determined whether the polarization of the thyroid cells in these systems are incomplete, or if limited transport of metabolites does occur via the basal receptors.

6.3.2 Antigen presentation to T-cell hybridomas

These experiments look at the presentation of endogenously synthesized TG by thyroid epithelial cells. The T-cell hybridoma CH9, specifically responsive to TG epitopes in association with I-A^k molecules, was used. The stimulation of IL-2 release by this hybridoma provides an assay for the presence of TG peptide-Ia complexes on the surface of thyroid epithelial cells. The epitopes recognized by CH9 has recently been characterized. Through the use of synthetic T4 containing peptides, representing the four major hormonogenic sites in TG, it has been demonstrated that CH9 recognizes an epitope containing the T4 position 2553 in human TG (Champion et al., 1991). The IF studies showed that mouse thyrocytes cultured in 1% FCS in the absence of TSH and IFN-γ did not express Ia molecules, but de novo synthesis was observed when these factors were added to the culture medium (FIG. 6.16)

Although the induction of Ia antigens on mouse TEC by IFN-γ has previously been reported (Salamero et al., 1985) the IFN used was prepared from PHA stimulated spleen cells, therefore contamination by other factors could not be ruled out. That factors other than IFN-γ are able to stimulate Ia antigens on mouse TEC, was demonstrated in these studies: the expression of I-E antigens was induced by TSH. The class II

molecules induced by IFN-γ on primary mouse thyroid cultures were shown to be functional (FIG. 6.17). The ability of human class II positive thyroid cells to stimulate autologous T cells has previously been demonstrated (Londei et al., 1985; Davies, 1985) and Charreire & Salamero (1984) have shown that mouse T cells can be stimulated by syngeneic thyrocytes, but in these studies the nature of the autoantigen presented by the class II molecules was not determined. The hybridomas U3 and U10, which do not recognize MTG (Rayner et al., 1987), were also stimulated by class II positive TEC. Thus some class II reactive T cells may be stimulated by class II positive antigen presenting cells, but the antigen to which they respond may not necessarily be endogenously synthesized. The data presented here suggest that TEC are able to present endogenously synthesized TG to the T cell hybridoma CH9. It was not established whether the epitopes recognized by CH9 correspond to the epitopes recognized by mab 5F6 or mab H3D7.

These assays were not consistently repeatable most probably because of the heterogeneity of the primary cell cultures. Stein & Stadecker (1987) have used a murine TEC line to look at their capacity for antigen presentation. After class II antigen induction by IFN-γ this cell line was able to present antigen to T cell hybridomas, but importantly this antigen presenting function was provided by the addition of the drug phorbol myristate acetate. In a previous report (Ebner et al., 1987) the same group was unable to show the antigen presenting function of primary cultures of murine TEC, but TG-specific clones were not used. This suggests that the specificity of the responding T cells has to be considered, when assessing the antigen-presenting capacity of murine thyrocytes. This also appears to be the case for human thyrocytes: Grubeck-Loebenstein et al. (1987) have demonstrated that IFN-γ pre-treated autologous thyrocytes stimulate uncloned T-cell lines from Graves' disease thyroids but fail to stimulate T-cell lines from non-toxic goitre.

In addition to presenting the antigen-MHC molecular complex, APC provide additional non-specific signals to T cells that promote antigen-induced responses. Indeed, the recognition of MHC plus antigen in the absence of accessory signals, may result in an abortive program of T-cell activation that leads to T-cell unresponsiveness, and could be critical to the

maintenance of self-tolerance. This is therefore an important parameter to consider in a primary culture assay. Antigen presentation can be restricted by adhesion molecules that bridge APC and T cells and antibodies against these structures block antigen recognition by proliferating T cells (Davignon et al., 1980; Dougherty et al., 1988; Bierer et al., 1988). The possible influence of an increased expression of the adhesion molecule ICAM-1 in perpetuating thyroid pathology was recently addressed by Weetman et al. (1988; 1990). These authors demonstrated that ICAM-1 may be spontaneously expressed on human thyroid cells, obtained from patients with autoimmune thyroid disease and cultured *in vitro*. Furthermore, ICAM-1 expression by thyroid cells may enhance immune recognition and play some role in cytotoxicity. The second type of non-specific signals which may be essential to appropriate T cell stimulation by APC are generally referred to as co-stimulators, exemplified by interleukin-1 (reviewed in Weaver & Unanue, 1990). The inventory of accessory molecules that form the basis of the APC-T cell interactions is, no doubt, still incomplete. Therefore, the system presented in this study may provide a useful foundation for future work in this area.

CHAPTER 7

DISCUSSION

7.1 INTRODUCTION

In the last thirty-five years since Hashimoto's thyroiditis was unequivocally defined as an autoimmune disease, animal models of autoimmune thyroiditis have provided insights into the possible aetiology and pathogenesis of the disease. In particular, the murine models of EAT (Vladutiu & Rose, 1971;Tomazic et al., 1974; Esquivel et al., 1977; Kong et al., 1978) proved to be valuable in establishing the influence of the MHC antigens on the susceptibility to thyroiditis. The rapid developments in technology in the last decade have precipitated research on autoimmunity at the molecular and cellular level and *in vivo* studies of the murine EAT model have largely been abandoned. However, animal models are invaluable for assessing plausible therapeutic methods for prevention or amelioration of disease, therefore we need to have an in depth understanding of the immune response in these models. For example, it is necessary to know the relative importance of the cellular and humoral immune response in pathogenesis. This is still an area of unresolved controversy.

The concept of autoimmune thyroiditis as an antibody mediated disease has fallen into disfavour in the last decade. However, the evidence for the involvement of B cells and autoantibodies cannot be ignored: A proportion of thyroid infiltrates from patients with Hashimoto's thyroiditis consists of activated and well differentiated B lymphocytes specific for thyroid antigens and actively synthesize autoantibodies *in vitro* (Del Prete et al., 1987; McLachlan et al., 1986). Bursal reconstitution experiments support the concept that antibody is an essential ingredient in the pathologic changes of spontaneous thyroiditis in the OS chicken (Polley et al., 1981).

Protagonists of a "cellular mediated disease" consider the observation that autoantibodies may be present in the absence of disease (Guilbert et al., 1982; Ruf et al., 1985) and that autoreactive T cells to MTG can mediate and transfer disease with little involvement of autoantibodies (Maron et al., 1983; Charreire & Michel Bechet, 1982; Simon et al., 1986) as proof that the humoral response is not important in the disease process. In view of recent developments in our understanding of T helper cell populations (reviewed in Mossman & Coffman, 1989), these observations may merely reflect an imbalance of TH1 and TH2 cells in favour of the former cell type, which can induce delayed type hypersensitivity, but may not be able to provide help for B cells. Although TH1 clones can help antigen-specific secondary responses in primed B-cell populations, immunoglobulin production from dense, resting B cells is not observed. Indeed, the clear dissociation between anti-TG autoantibodies and lymphocytic infiltration has recently been put into doubt by two sets of experiments: in female August rats conventionally immunized with TG in CFA (Hassman et al., 1988) and in T cell-depleted B mice reconstituted with normal L3T4 (Lyt-1 ^{dull}) T cells (Sugihara et al., 1988) a significant relationship was found between anti-TG autoantibodies and the severity of thyroiditis assessed by lymphocytic infiltration of the thyroid glands.

7.2 THE ROLE OF B CELLS IN EAT

In this project I have studied the long-term response to TG immunization in high-responder mice. Both thyroid pathology and antibody titres were assessed at various timed intervals (section 3.2). These studies show that the mode of antigen challenge may determine the severity of disease. Unlike MTG/CFA induced EAT, activation of autoreactive cells with MTG/LPS produced a prolonged chronic disease with greater similarity to the human disease. The evidence from these experiments point to a role for the MTG specific autoantibodies in perpetuating the disease process. In a wider context, as already discussed (section 3.3), B cells may contribute to the perpetuation of thyroid disease by acting as antigen-presenting cells (Rock et al., 1984; Abbas et al., 1985; Lanzavecchia, 1985).

The present study suggests that autoantibodies which recognize

self-restricted TG epitopes may be important in pathogenesis. This conclusion is supported by the finding that autoantibodies to TG in OS chickens are directed in the main against determinants unique to the species (Chan et al., 1989). The importance of autoantibody recognition domains on TG as markers of thyroid disorder is now being recognized. Recently, Bouanani et al. (1989), have confirmed that natural autoantibodies show wide heterogeneity with respect to their recognition of antigenic domains on the human TG molecule. Furthermore, these authors report that the domain recognized by autoantibodies from patients is very rarely recognized by autoantibodies from healthy subjects. Future work has to be directed at defining the epitopes recognized by MTG/CFA and MTG/LPS induced autoantibodies.

7.3 THE ANAMNESTIC RESPONSE TO TG CHALLENGE

Primary encounter with antigen stimulates specific B cells to differentiate into plasma cells which produce antibody at a high rate. Exposure also results in the T cell-dependent induction of B-cell memory (MacLennan et al., 1990; Gray & Leanderson, 1990). When re-exposed to antigen, memory cells generate secondary IgG responses that are enhanced in rate, titre and affinity. A large body of evidence now supports the idea that this affinity maturation is largely due to somatic hypermutation in antibody V genes and selection of B cells expressing high-affinity variants of this antigen receptor (Weigert et al., 1970; Rajewsky et al., 1987; Berek & Milstein, 1988). It appears that the developing memory B cells first undergo mutation as early as the fifth day of the primary response and that the frequency of mutation does not increase significantly after a few days (Levy et al., 1989; Tao & Bothwell, 1990). After an initial phase of extensive proliferation following primary immunization, memory cells can persist in the organism for extended periods of time in the absence of cell division (Schittek & Rajewsky, 1990). Furthermore, proliferation of isolated IgG_1+ memory B cells *in vitro* occurs in the absence of continued hypermutation (McHeyzer-Williams et al., 1991), which suggests that *in vivo* expansion of memory B cells after secondary challenge does not drive hypermutation events. Consistent with this result is earlier work on the stability of the adoptive secondary response and of V region genes in myeloma and

hybridoma cells (Askonas et al., 1970; Bruggemann et al., 1986).

Subsequent to antigenic challenge a number of B cell differentiation pathways can be envisaged (DIAGRAM. 7.1). In the primary response, antigen-specific B cells available in the preimmune repertoire are triggered into expansion and terminal differentiation. The sub-set of B cells activated clearly depends on the nature of the primary antigenic stimulus (section 3.3). One of the fundamental questions regarding the generation of B cell memory, is whether some B cells from the same sub-set are selected into the memory cell pathway. The difference in the cross-reactivity profile of MTG/LPS induced antibodies after primary and secondary challenge (FIG. 3.6), cannot be attributed to somatic mutation alone. Given our present understanding of B cell populations, MTG/LPS immunization may activate TG-specific CD5+ B cells to contribute to the primary antibody response. However, it is likely that the cells activated into the memory pathway would be selected from the repertoire of bone-marrow derived, recently generated B cells.

The suggestion that memory B cells have a distinct clonal origin from those dominating the primary response (Cumano & Rajewsky, 1986) is an argument in favour of this mechansim. Recruitment of other B cell specificities is likely to be mediated by activating specific T cells, either by direct presentation of antigen by the CD5+ B cells, or indirectly by antigen-presenting cells taking up antigen-antibody complexes (Rajewsky et al., 1987). The implication is that the immune system has a built-in system to prevent naturally autoreactive B cells from entering the long-lived memory cell pool. Strains of mice which have a propensity towards autoimmune disease are known to be associated with predominantly (such as the "moth-eaten" strain (Painter et al., 1988)) or, expanded (as in NZB mice (Dighiero et al., 1988)) Ly-1 B cell populations. There are also data suggesting that increased levels of CD5+ B cells are associated with autoimmune thyroid disease (Iwatani et al., 1989) and in rheumatoid arthritis these cells have been shown to be responsible for rheumatoid factor production in humans (Plater- Zyberk & Maini, 1988; Hardy et al., 1988).

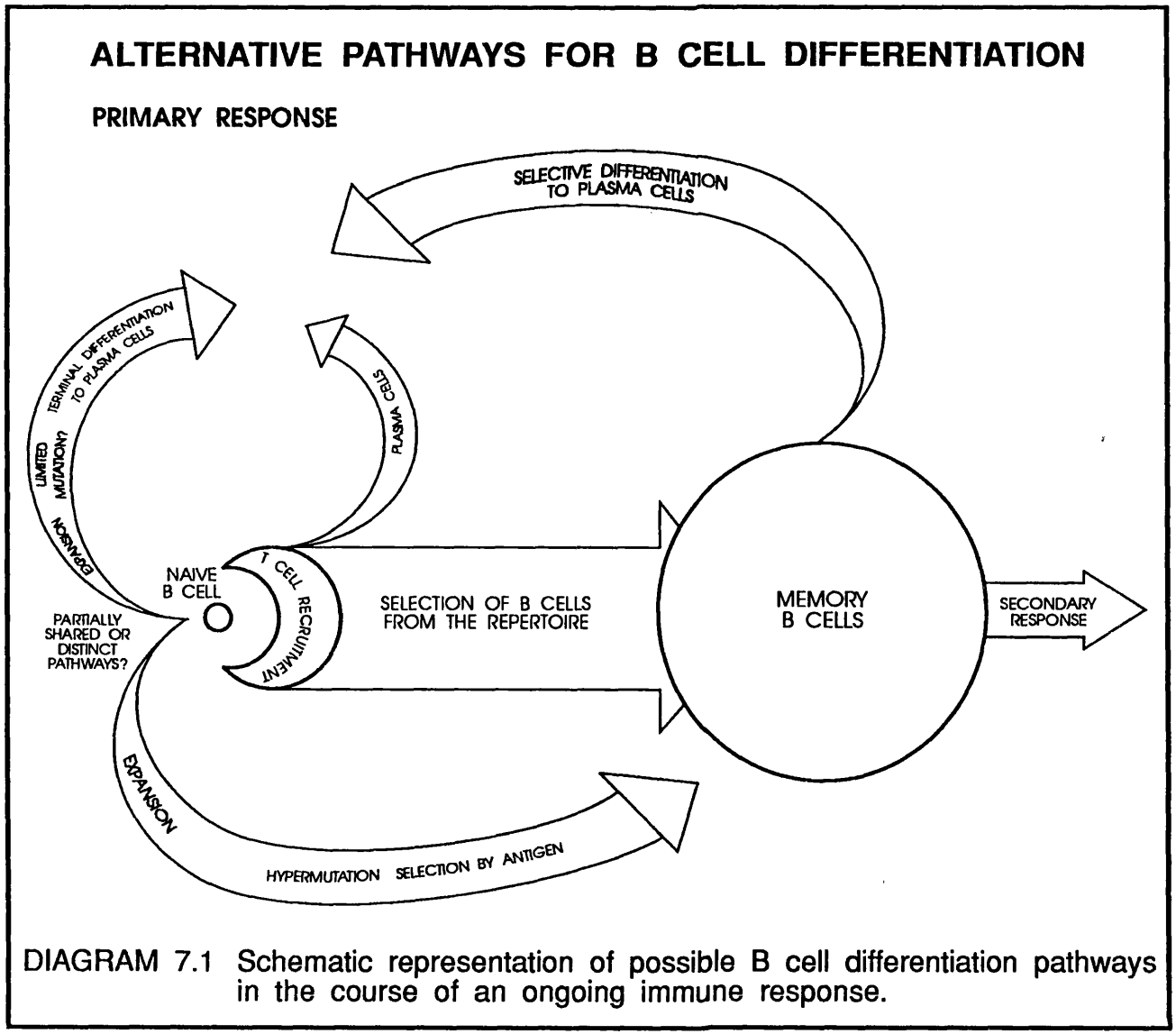


DIAGRAM 7.1 Schematic representation of possible B cell differentiation pathways in the course of an ongoing immune response.

An often overlooked fact is that an individual may continue to make an antibody response for many months following a single challenge with antigen (Tew et al., 1980; Gray et al., 1986). CBA/J mice also have a prolonged humoral response to challenge with both MTG/CFA and MTG/LPS (FIG. 3.1). This continued antibody production raises the question of whether some of the memory B-cell clones are activated to terminal differentiation during an ongoing immune response. Lettenberg et al. (1988) have presented data which suggests that virgin and memory B cells differ in the cytokines they require for differentiation into Ig-secreting cells. If, in the course of a prolonged primary response the ratios of TH1 and TH2 cells change (Mosmann & Coffman, 1989) a variation in cytokine synthesis may be sufficient to allow the differentiation of some memory B cells. There was no increase in cross-reactivity of the MTG/LPS induced antibodies with time, therefore this fraction of B memory cells does not contribute to the ongoing immune response. The question of simultaneous activation of both naive and memory T cells has recently been addressed (Akbar et al., 1991). These authors suggest that synergy between these two populations will occur when activated in close proximity and in response to alloantigens. This presents the interesting possibility that if cross-reactive anti-TG antibodies mediate the down-regulation of MTG/CFA induced EAT, then the specific recruitment of cross-reactive memory B cells may provide two ways of down-regulating the response in the MTG/LPS model; by antigenic competition (Adorini & Nagy, 1990) and through the anti-idiotypic network (Zanetti et al., 1986; De Kozak et al., 1987; Carteron et al., 1989). A final point to consider is the encounter with antigen which does not lead to antibody synthesis, but a specific recollection of the encounter (section 4.2). Does an early (in the neonatal period) encounter with antigen lead to an altered preimmune repertoire, or can the memory cell pathway be activated in the total absence of a primary antibody response?

7.4 NETWORK INTERACTIONS IN THE PREIMMUNE REPERTOIRE

The detection of self-reactive T and B cells in secondary lymphoid tissue (Nossal, 1983; Ternynck & Avrameas, 1986 ; Wood et al., 1987), has generated continual controversy surrounding the mechanism of

self-tolerance. It has been postulated that self-reactive lymphocytes remain in a functionally silent form as a result of early, or repeated, exposure to antigen (clonal anergy) (reviewed in Goodnow, 1989), contact with antigen-specific suppressor T cells (reviewed in Germain & Benacerraf, 1981) or regulatory elements of an idiotypic network (Paul & Bona,1982). In other cases it has been suggested that autoimmunity is prevented by a lack of specific T-cell help, rather than by B-cell tolerance (Weigle, 1981). Most of these mechanisms have been advanced to explain the tolerance of lymphocytes with specificity for TG (Kong et al., 1982; Zanetti et al., 1985; Zanetti & Rogers, 1987; Weigle, 1981). Considerably less attention has been paid to the functional relevance of anti-self lymphocytes in the preimmune repertoire. This is despite the fact that in the preimmune repertoire anti-self lymphocytes may be generated at similar frequencies as lymphocytes specific for foreign antigens (Goodnow, 1989; Portnoi et al., 1986).

An intriguing aspect of thyroid autoimmunity is that some patients have autoantibodies reacting with the iodothyronines, T4 and T3 (Pearce et al., 1981). Thyroxine-binding antibodies are also present in all sera from OS chickens (Chan et al., 1989). Iodination-related epitopes may also be important at the T-cell level, as suggested by the failure of autoreactive T cell hybridomas, CH9 and ADA2 and the T cell clone MTg9B3 to recognize noniodinated mouse TG (Champion et al., 1987b). These observations have promoted the view that most TG-reactive lymphocytes are silenced by continuous exposure to circulating TG, but cells which have specificity for iodine related epitopes may escape tolerance mechanisms and so are activated by exposure to highly iodinated TG. By inference the total absence of iodinated epitopes should allow greater numbers of specific cells to escape clonal anergy and an antigen driven response to normally iodinated TG should be increased. The validity of this argument was tested in an *in vivo* model of mouse EAT by using the thyroid-peroxidase inhibitor, aminotriazole (ATA) (Chapter 5).

Although the available literature suggests that this drug specifically inhibits thyroid hormone formation, it cannot be ruled out that in this experiment other TG epitopes were depleted (or altered). ATA treatment effectively down-regulated the humoral response to MTG. Analysis of the cross-reactivity pattern of the immune sera further suggested that

alterations in the form of circulating TG may influence the preimmune repertoire sufficiently to change the outcome of a subsequent antigen-driven response (FIG. 5.3,5.4). The inference from these observations is that MTG-specific lymphocytes do exist in the pre-immune repertoire and that a particular sub-set is highly susceptible to the concentration of circulating TG. Furthermore, this set may be important in regulating the activity, or recruitment, of lymphocytes which respond to more cross-reactive epitopes on the TG molecule. The scheme proposed in chapter 4 suggests that a tolerogenic dose of antigen may have a similar effect: Clonal anergy may be induced in a regulatory set of lymphocytes (which recognize self specific epitopes) and concurrently lymphocytes with more cross-reactive receptor specificities are activated or recruited.

As the outcome of the two treatments is similar, an intriguing possibility is that in both cases the same population of cells is affected. This apparent paradox might be explained if we consider that lymphocyte responses are a function of receptor occupancy and can be described by a bell-shaped dose-response curve of ligand binding (Perelson & Weigel, 1981). This implies that too high a concentration of antigen may lead to clonal anergy and insufficient antigen would eventually result in cell death, or if there is not an absolute requirement for persistence of antigen, then the cells may remain arrested in the cell cycle. Speculations regarding the phenotype of the critical regulatory cells naturally have to be based on our present knowledge of T and B cell physiology. In rodents the life span of B and T cells has been estimated in weeks and months respectively (Sprent & Basten, 1973), therefore the time-frame of these experiments suggests that the cells which are affected by a depletion of (or alteration) ATA susceptible epitopes are more likely to be B cells.

The mechanism of *in situ* tolerance induced by increased levels of antigen may be sufficiently explained by clonal anergy in a population of activated B cells. However, it has been established that induced tolerance to erythrocyte antigens (Cooke et al., 1978) and to TG (Kong et al., 1982; Parish et al., 1988) can be transferred to naive recipients, and is mediated by T cells. Although it is not clear whether the transferred cells themselves are the final effectors, or whether they

merely induce suppressor cells in the recipients (Huthchings et al., 1989). These authors acknowledge that B cells may also play a part and Howles & Cox (1983) have reported that suppressor populations depleted of B cells and cells that adhered to rat RBC lost the ability to suppress autoantibody production in normal mice injected with rat RBC.

An alternative to the suppressor cell hypothesis would be that suppression is mediated through the activation of T cells (and possibly memory B cells) specific for the cross-reactive epitopes on the self-antigen. The activation of these cells would in turn be dependent on the existence of regulatory networks. It is generally accepted that helper T cells are critical for the proliferation and maturation of antigen-specific B lymphocytes, but B cells do influence the development of the T-cell repertoire. For example, B cell idiotypes are required in some systems to establish the repertoire of idiotype specific T cells (Bottomly & Mosier, 1979). Furthermore, the suppression of B-cell development with antibodies to IgM also results in deficiencies in T-cell reactivity (Gray, 1984).

The proposed critical regulatory cells in the experimental systems discussed here may be peritoneal Ly-1+ (CD5+) B cells (possibly together with the "sister" peritoneal Ly-1$^-$ B cells) (Herzenberg et al.,1986). This would be in line with the observation that naive spleen cells do not abrogate the effect of a tolerizing regime with MTG (Parish et al., 1988). Further evidence implicating the role of these cells comes from the observation that SJL mice may be tolerised *in situ* with TG, but this tolerance cannot be transferred (Hutchings et al., 1989). The percentage of Ly-1 B cells in the peritoneal cavity of SJL mice is significantly lower (Hayakawa et al., 1984; Poncet et al., 1985) and these mice do not respond normally to LPS (Hutchings et al., 1986) suggesting that MTG/LPS induced responses may be derived from this population. Importantly, CD5+ B cells have been shown to be committed to the production of antibodies reactive with self-antigens, including autologous thyroglobulin and erythrocytes (Portnoi et al., 1986; Casali & Notkins, 1989). These autoantibodies can be of both IgM and IgG class and are present in healthy subjects and in normal mouse serum (Avrameas, 1991).

By utilising CD5+ B cells in an immunoregulatory network, inadvertant

central tolerance of these cells may be avoided in early ontogeny, because unlike other immature B cells (which are easily modulated by anti-immunoglobulin (Nossal & Pike, 1985) and specific antigen (Bruyns et al., 1976)) human CD5+ B cells in the foetal spleen fail to cap their surface Ig (Antin et al., 1986). By contrast, if susceptibility to tolerance is a consequence of engaging the surface Ig receptor of a B cell in cycle (Scott & Klinman, 1987) then these autoreactive cells which are blast, cycling cells (Portnoi et al., 1986) would be more likely to be tolerized by a high level of antigen than the majority of adult B cells which are non-cycling (de Franco et al., 1982).

If autoregulation is maintained by a particular cell lineage, then such cells should have the capacity to regulate both B and T cell activities. Since the antigen presenting capacity of B cells has been unequivocally demonstrated, CD5+ B cells have the potential to specifically activate T cells. In addition, it has been postulated that CD5+ B cells help in the activation and development of conventional CD5 negative B cells (Hardin et al.,1990). The recent observation that the B-cell surface protein CD72/Lyb-2 is the ligand for CD5 (Van de Velde et al., 1991) lends credence to this view.

7.4.1 The structure and control of immune networks

Despite the lack of molecular evidence in support of the existence of a separate lineage of suppressor T cells, the idea resists change, because the defence of this idea is persuasive (Sercarz & Krzych, 1991). In the absence of further experimentation, the credibility of the above proposal as an alternative to the idea of regulation mediated by suppressor T cells remains open to question. Nevertheless, a discussion of this idea in the context of the structure and function of the immune system as a whole throws up less anomalies than the concept of T suppressor cells. For example, we cannot readily explain why T-cell repertoires generated in a given thymus should be both deleted of "autoreactivities" and enriched for complementarities towards the deleted specificities (Coutinho & Bandeira, 1989).

The main body of *in vivo* experimental evidence in defence of suppressor T cells is indirect and has been based on depletion of T lymphocytes by

thymectomy (reviewed by Cooke & Lydyard, 1981). The interpretation of these results requires that we view the Ts cell component of the immune system as a disconnected unit or collection of independent clones. This exhibits some conflict with other notions (factual or preconceived) of the structure of the immune system, which promotes the idea of connectivity (Jerne, 1974; Varela & Coutinho, 1991; Cohen & Young, 1991). Furthermore, experimental evidence suggests that the spatial organization of the connected components is fluid; they can expand or contract to fill the available space. For example, there has been limited success of idiotypic mainpulation of autoimmunity, because even if a dominant idiotype (Id) of an autoantibody response can be suppressed, silent Ids arise to replace the dominant suppressed Id (Male, 1986). From this conceptual stance, Ts cells can only play a minor role in preventing autoimmune disease. Thus it seems that the regulation of autoimmunity must be a property of the overall structure of the immune system. How is this structure organized?

7.5 A MODEL OF REGULATED AUTOIMMUNITY

If the regulation of autoimmunity is a property of the structure of the immune system, then any model of this structure must also provide an adequate explanation for protective immunity. The first question to consider is the extent of the network. At one extreme, strict adherence to clonal selection predicts that the 10^6 T cells exported daily from the thymus remain inert in peripheral lymphoid tissues pending the appearence of specific peptide-MHC complexes on antigen-presenting cells. The other extreme is that the immune system consists of a network of idiotypes (Ids) (the antigenic determinant present on the antibody molecule which is common to antibodies of the same composition and fine specificity) and anti-idiotypes (anti-Ids). These Ids and anti-Ids create an endless web of interactions, and constitute the sole means of regulating the immune system (Jerne, 1984). One of the modifications to this network theory was that the immune system is not a single, highly interconnected network, but rather a collection of many "mini-networks" each based on a single family of related regulatory idiotopes (Paul & Bona, 1982). These authors considered that the mini-networks would be interconnected with one another through rather fragile links. The demonstration that natural

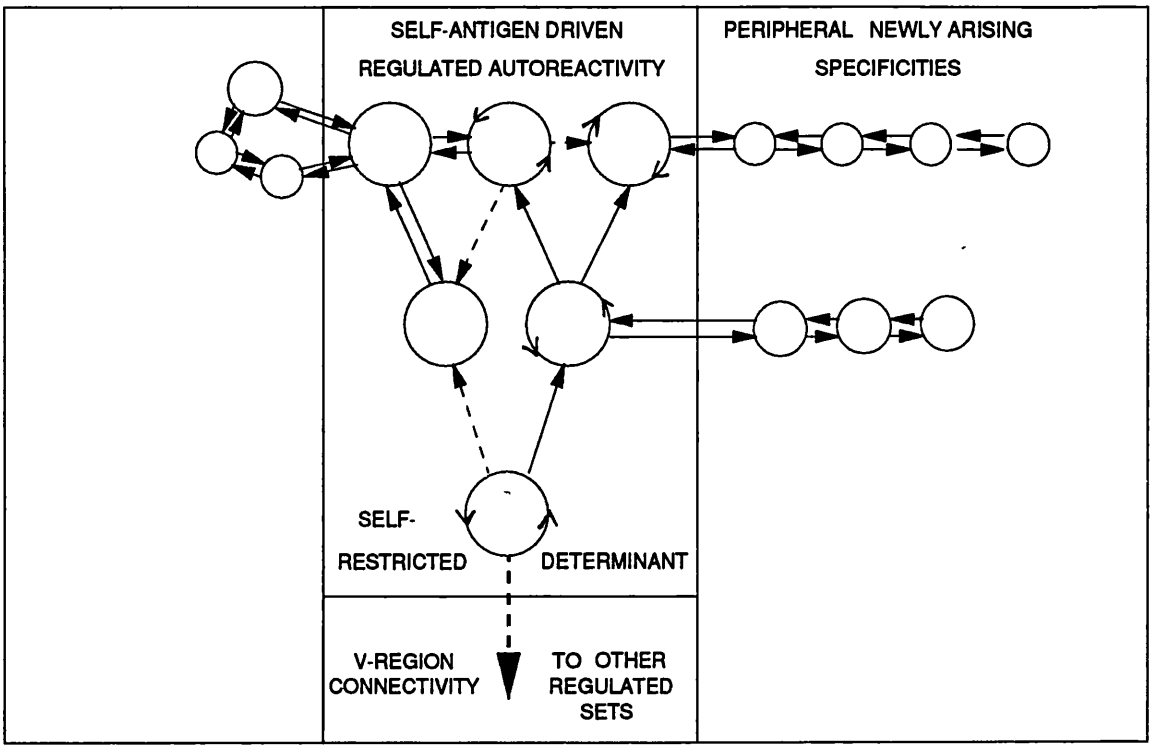
autoantibodies exist in measurable quantities (Prabhakar et al., 1984; Avrameas, 1991), has shifted the emphasis from an idiotypic network corresponding to all specificities, to one which is concerned with self. Holmberg and Coutinho (1985) suggested that the normal immune systems can be described as consisting of two generally distinguishable compartments: The immune response set comprising of 85-90% of all lymphocytes which make specific immune responses by large clonal expansions and production of effector molecules when stimulated by foreign antigen. By contrast, the internal activity set contains all self-reactive lymphocytes which are "naturally" activated and connected by an idiotypic network of high connectivity antibodies. A two tier immune system has also been proposed by Kocks and Rajewsky (1989).

By maintaining a tightly regulated network for all autoreactive cells, overt autoimmunity can normally be avoided, but it seems unlikely that this is the only reason for maintaining specific self-reactive cells. Cohen and Young (1991) believe that the immune system, by priming-for-self, is able to mount an effective reponse to many infections. They suggest that this is why immunity to conserved, self-like microbial antigens (such as the heat shock proteins) are dominant over more microbial specific antigens. These authors view the lymphocyte network as one in which regulated autoimmunity is connected with effective microbial immunity. Both regulated autoimmunity and effective immunity is maintained in this network, because for any given conserved antigen, the antigen presenting cell (a natural autoimmune B cell, or macrophage that has bound natural antibody) will present self and microbe-specific epitopes. Any autoreactive cell which may become activated would be tightly regulated by network connections, while T cells bearing receptors for the microbe-specific epitopes will not be under regulatory constraints. This idea is appealing, because the activation of immunity to a pathogen is not left to the fortuitous presence of lymphocytes bearing the specific receptors. However, there are insufficient variables in this model to allow autoimmune diseases to occur spontaneously or to be induced by pathogens.

It has been speculated that viruses, bacteria or parasites may induce autoimmunity by molecular mimicry (Oldstone, 1987), increased expression of MHC class II antigens (Bottazzo et al., 1983), polyclonal B-cell

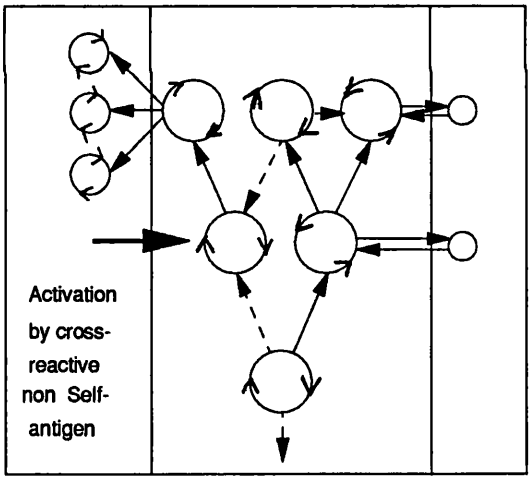
activation (Izui et al., 1978; Klinman & Steinberg, 1987), or idiotypic cross-reaction (Plotz, 1983; Cooke et al., 1983). It is possible to accept these mechanisms in a immune system of total connectivity as suggested by Jerne or in a system such as that suggested by Paul and Bona, but not in a two tier system or one which argues for the existence of both efficient antigen presentation and control of autoreactive cells. Furthermore, none of these models adequately explain some of the immune responses to parasite infections. For example, resistance to reinfection in already infected hosts, which is a feature of the immunology of schistomiasis and referred to as concomitant immunity. Therefore an alternative model of the immune network is proposed here (DIAGRAM 7.2).

DIAGRAM 7.2 A) Representation of the connectivity of lymphocytes with specificity for a set of epitopes on a conserved self-antigen. Each circle represents a set of lymphocytes with specificity for a single epitope on the antigen, but the determinants recognized are increasingly less self-specific. Circles with arrows indicate an activated state. Appropriate activation is suggested to be mediated by circulating self-antigen being taken up and presented by B cells (or macrophages). Positive or negative (broken arrows) control is exerted by the connected lymphocytes either by cytokine release or through antigen-presenting functions. Some sets may remain in a resting state unless activated by foreign antigen (B). This would allow an effective immune response towards the foreign antigen. Although limited autoimmunity may also occur, elimination of the antigen would limit the activity of these cells. In the situation where high levels of cross-reactive antigen are able to induce clonal anergy in a given set, effective immunity towards that antigenic determinant would be prevented (C). Some parasites may escape immune surveillance in this way. In addition, they may secrete low levels of the antigen (with different exposed epitopes) which would activate other lymphocyte sets and so allow an effective immunity towards related pathogens or early infective forms. If a pathogen expresses an epitope which mimicks self, then clonal anergy of the self-restricted lymphocytes, would lead to deregulation of the immune network and autoimmunity (D). Tandem infection with a related pathogen may enhance the autoimmune response (E).



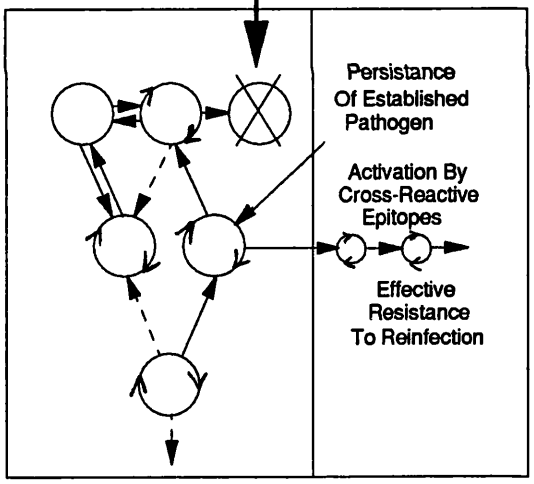
A

AGGRESSIVE
IMMUNE
RESPONSE



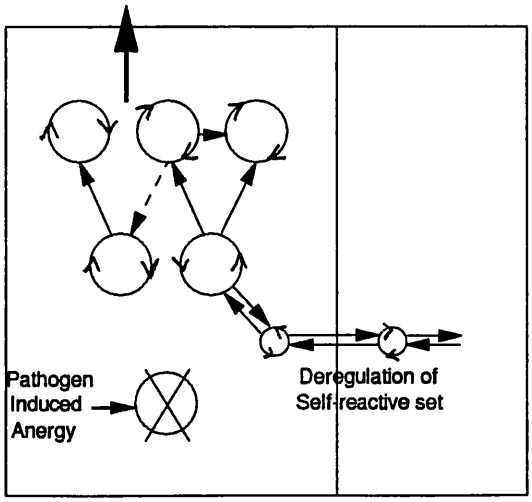
B

CLONAL ANERGY
INDUCED BY INFECTING PATHOGEN



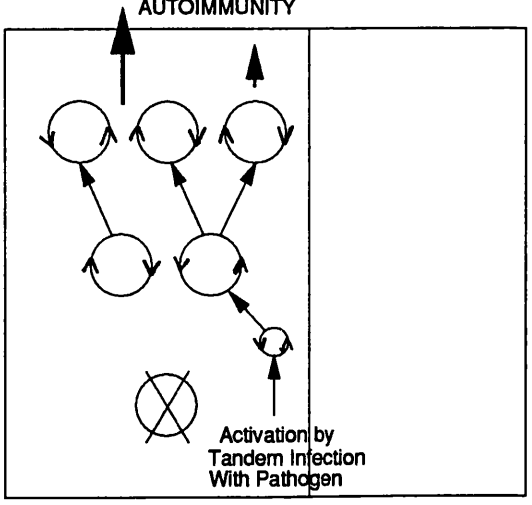
C

AUTOIMMUNE RESPONSE



D

ENHANCED
AUTOIMMUNITY



E

The model presented is based on the assumption that for every functionally important self-antigen there exists one or more preformed sets of highly connected naturally activated lymphocytes. The functional activity of these cells requires the persistence of a defined level of self-antigen, as suggested by the data in chapter 5. Moreover, there is connectivity with parallel sets of lymphocytes which have receptors with increasingly less specificity for self. These lymphocytes would include the newly emerging B and T cells - perhaps the short-lived T1 cells, which are rapidly lost after adult thymectomy (Kappler et al., 1974; Araneo et al., 1977; Simpson and Cantor, 1975). These cells would not normally be activated by self- antigen, because of competition for the available antigen. However, non-specific connectivity could be mediated by cytokines produced from the naturally activated set. An obvious advantage of this mechanism would be to regulate the level of cytokines and so prevent excess proliferation of autoreactive cells by paracrine or autocrine mechanisms. Although it is not known whether increased levels of cytokines initiate autoimmune diseases, the association between elevated levels of cytokines and autoimmune disease has frequently been observed. For example, elevated levels of IL-6 can be detected in the synovial fluid from affected joints and sera of patients with active rheumatoid arthritis (Hirano et al.,1988; Houssiau et al.,1988). IL-2 hypersecretion may be one of the putative accelerating factors modulating spontaneous autoimmune thyroiditis (Kroemer and Wick, 1989). *In vivo* studies have revealed that de-novo induction of autoimmune disease as well as aggravation of pre-existing autoaggression may be side effects of IL-2. If administered with rat erythrocytes, IL-2 induces autoimmune haemolytic anaemia in nude mice (Reimann & Diamanantstein, 1981) and *in vivo* application of recombinant human IL-2 accelerates and aggravates autoimmune diabetes in BB rats (Kolb et al., 1986).

Direct, non-specific cellular interactions may also occur between the two sets. Analysis of T-cell clones raised and selected according to their reactivity to a variety of antigens associated with different class II MHC products revealed that a significant portion responded to Class II MHC products on activated B cells in the absence of antigen (De Kruyff et al., 1985). CD4+ clones selected for efficient recognition of foreign peptides and class II products can also display low levels of reactivity

to syngeneic APC in the absence of foreign antigen (Patarca et al., 1990). It is tempting to suggest that this interaction with the internal set is required prior to T cells seeding or replacing peripheral lymphocyte pools - a post thymic education event perhaps? In the absence of this continuous supply of newly migrating T cells, the internal set of autoreactive cells may increase. This may be why in mice, neonatal thymectomy 2-4 days after birth induces organ-specific autoimmune diseases affecting a variety of endocrine organs and the stomach (Kojima & Prehn, 1981; Fukuma et al., 1988). The observation that removal of the source of self antigen prior to thymectomy prevents the development of the autoimmune response (Taguchi & Nishizuka, 1980) is also compatible with this model.

Importantly, this model suggests that there is a progressive change in fine specificity leading to the acquisition of cross-reactivities with homologous antigens. This dictates that preformed networks are directed to house-keeping enzymes and proteins which are ubiquitous (eg. basic structural proteins such as myosin and tubulin, for enzymes such as glutathione S-transferase (GST) and protein kinases and for circulating antigens such as insulin and thyroglobulin). The functional importance of these proteins implies that they are likely to be phylogenetically conserved, but necessarily have interspecies heterogeneity. For example, actin is a major component of the tegument of the blood fluke *Schistosoma* and, as in vertebrates, a number of actin isotypes can be identified (Abbas & Cain, 1989) and molecular analysis shows that there is considerable homology between rat GST isoenzymes and the 26kDa isoenzyme expressed by *Schistosoma japonicum* (Smith et al., 1986).

This strategy maximizes the probability that any member of the antigenic universe can be recognized and expeditiously dealt with by a limited number of clones. In addition the system does not have to rely on random antigen driven somatic mutation and clonal selection. Since the internal set cannot be driven in the absence of specific antigen (note the absence of high affinity anti-TG antibodies in mice challenged with adjuvant alone), the chances of activating those clones with highly restricted specificity for self are minimised. By extrapolation, low responder strains such as BALB/c mice which are unable to respond to LPS challenge, either have an extremely tightly regulated autoreactive network, or lack

specific LPS responsive populations. If the first explanation is true, we should expect these mice to be highly resistent to infections as well as autoimmune diseases, but these mice were seen to be highly susceptible to infections. Interestingly, while CBA strains are resistant to *L. major* infection, BALB/c mice are exceptionally susceptible and develop fatal disseminating disease, even with a minimal infecting dose (Liew. 1989).

There is likely to be some sort of fragile link (possibly mediated by Id/anti-Id networks) between unrelated antigenic networks. Moreover, this would account for the observation that the steady state of the immune system can be perturbed by idiotype (Forni et al., 1980) and anti-idiotype (Wikler et al.,1980; Reth et al.,1981). Networks which are related to specifically secreted antigens, such as insulin, thyroglobulin and intrinsic factor (a secretory product of gastric parietal cells) stand a greater chance of being dysregulated because the basal concentrations of these antigens may well fluctuate according to the active state of the relevant secretory cell. Thus a combination of a previous history of network perturbations together with an acute reponse to a related epitope on a foreign antigen may eventually lead to a chronic autoimmune disease.

7.5.1 The relationship between infection and autoimmunity

The immune system is frequently unable to effectively eliminate invading organisms. One reason for this is that pathogens often exploit the immune system to enhance dissemination. A number of protozoa inhabit and multiply in the cells of the immune system: *Theileria* species inhabit lymphocytes (Morrison et al., 1986), macrophages provide a safe habitat for organisms such as *Toxoplasma gondii*, which have evolved the ability to circumvent attack. Infectious agents have been suspected to be involved in triggering autoantibodies and autoimmune disease. Behcet's syndrome (a disease affecting a spectrum of tissues with a number of immunological manifestations) is one such example. Although the etiology of this disease is unknown, a number of antigens from streptococcal species have been implicated. Interestingly, some of these antigens may be associated with heat shock or stress proteins (Lehner et al., 1991).

In other cases, pathogen induced immunity later expresses itself as an autoimmune disease, displaying both humoral and cell-mediated immunity to self antigens. This is exemplified by Coxackie virus B3 (CB3)-induced myocarditis in the mouse (Rose et al., 1991). Early myocarditis is due to infectious myocarditogenic virus, while the late phase, which occurs in only a few genetically susceptible strains of mice, is associated with heart-specific autoantibodies. Importantly, only myocarditogenic variants of CB3 share an epitope with myosin (Chow et al., 1991)

The mechanism by which pathogens may induce autoantibodies is poorly understood. Activation of autoreactive cells by cross-reactive antigens on pathogens (molecular mimicry) has been suggested as a possible initiating event for autoimmune diseases. This appears as a complete paradox if we consider that complex parasites seem to produce host-like molecules as a strategy for evading host surveillance (Bloom, 1979). However, if autoreactive cells are regulated as suggested above, molecular mimicry - by inducing clonal anergy at the B-cell level, rather than clonal activation - may represent a common mechanism through which natural self-tolerance can be circumvented. Such a mechanism also provides a suitable explanation for concomitant immunity (DIAGRAM 7.2C). A candidate antigen in the case of schistosome infections may well be glutathione-S-transferase, which is located at the surface of the adult schistosome and shows both quantitative and immunogenic variation between different species (Henkle et al., 1990). Interestingly, trypanosomes are thought to escape the immune response by varying their surface glycoprotein coat and by inducing generalized suppression of immunological function (Pentreath, 1989).

Other pathogens may induce functional anergy in a specific clone of cells which is central to the normal autoimmune network. Thus over a period of time down-regulation of regulated autoreactivity may occur and so lead to autoimmunity (DIAGRAM 7.2D). This view of the autoimmune response is strengthened by the observation that tandem infection by myocarditogenic CB3 and non-myocarditogenic CB2 produces a more severe myocarditis than the myocarditogenic CB3 variant alone (Beck et al., 1990). A number of non-mutually exclusive ways in which deregulation might occur can be envisaged: One possibility which is suggested by the recent demonstration that clonal anergy in B cells is a reversible process

(Goodnow et al., 1991), is that in the absence of continuous exposure to the mimicking antigen there may be partial recovery of the relevant autoreactive B cells. Such partial recovery, in which clonal proliferation may initially be disassociated from antibody secretion, would disrupt an integrated network where ratios of cells are likely to be important. Additionally, we have to consider the possibility that if differentiation to a memory cell requires a large number of rounds of cell division (perhaps accompanied by somatic mutation), high affinity clonotypes would preferentially enter the memory pool. Activation of such cells at a later time by a second episode of infection would obviously have severe consequences. Alternatively, homing of memory cells to sites of specific antigen secretion may lead to their activation.

7.6 QUESTIONS ON ANTIGEN PRESENTATION

This data (chapter 6) unequivocally demonstrates that endogenously synthesized TG can be expressed on the surface of thyroid cells and is recognized by TG specific MHC class II restricted T cell hybridomas. However, this observation is paradoxical to current views on antigen processing and presentation. Whereas MHC class II-restricted T cells usually recognize peptides derived from exogenous antigens entering antigen presentation cells, class I-restricted T cells generally recognize peptides derived from processing that bind to fragments from endogenously synthesized cellular antigens (Townsend & Bodmer, 1989). Several mechanisms, including differences in antigen processing in different intracellular compartments, have been proposed to account for these pathway differences (Morrison et al., 1986; Germaine, 1986). The growing conviction is that MHC class I molecules interact with specific peptide in the endoplasmic reticulum (ER) prior to translocation to the cell surface (Townsend et al., 1989; Nuchtern et al., 1989; Yewdell & Bennick, 1989). It appears that interaction with peptide is also necessary for the stability of the assembled class I molecules (Ljunggren et al., 1990). These endogenously derived peptides are shorter and more potent than the T-cell epitopes deduced from experiments using synthetic peptides (Rotzschke et al., 1990). Endogenous peptides directly isolated from class I molecules in cells infected with vesicular stomatitis virus

also show homogeneity and are composed of only eight residues (Van Bleek & Nathenson, 1990). This homogeneity has been suggested to be imposed by the peptide-generating or translocating machinery. However, selection may also be an intrinsic property of the class I molecule (Schumacher et al., 1991). These authors found that empty class I molecules of the mutant cell line RMA-S preferentially bound short peptides even when they were a minor component in a mixture of longer peptides.

By contrast, class II MHC glycoproteins associate with peptides derived from material endocytosed by antigen-presenting cells and processed along the endocytotic pathway (Allen, 1987; Long, 1989). At present there is some discrepency regarding the intracellular site where class II meet antigen. Guagliardi et al. (1990), have defined an intracellular site (early endosomes) where peptides from endocytosed antigen could bind class II molecules en route to the cell surface. Other authors (Peters et al., 1991) disagree with this conclusion and show that class II molecules are routed to compartments related to lysosomes. A characteristic of intracellular class II molecules is their association with an additional glycoprotein, the invariant chain. This glycoprotein associates with the class II molecules in the ER and remains associated through the Golgi apparatus (Blum & Cresswell, 1988). The presence of the invariant chain appears to be important for the presentation of some exogenous antigens by the endosomal pathway (Stockinger et al., 1989) and may also play some role in preventing the association of antigen with newly synthesized class II molecules in the ER (Elliot et al., 1987; Roche & Cresswell, 1990). However, the distinction between available antigen presentation pathways for class I and class II MHC molecules may not hold for all antigens (Nuchtern et al., 1990). These authors suggest that class II molecules are able to bind some endogenous antigens in the ER.

Given this current understanding of antigen processing and presentation, we have to question how TG, an endogenously synthesized molecule, can become associated with class II molecules. Thyroglobulin is rather an unusual protein; not only does it have a regulated pathway of synthesis and secretion into the lumen via the apical membrane (Van Herle et al., 1979; Bjorkman et al., 1974; Spiro & Gorski, 1986), it is also subject to endocytosis and proteolytic degradation within the same cell (Unger et

al., 1985; Fouchier et al., 1987; Rousset et al., 1989). Thus the observed *in vitro* stimulation of MHC class II restricted cells may be due to presentation of TG peptides which have associated with class II molecules in the endocytic pathway. The TG in this case could be considered as an "exogenous" antigen. The alternative would be to suggest that TG is another example of an "endogenously synthesized" protein which is able to associate with class II molecules in the ER of thyroid epithelial cells, when these cells are appropriately stimulated. Significantly, the ten type 1 repetitive domains of the N terminal part of the TG monomer present an important homology with an alternatively expressed domain of the invariant chain (Koch et al., 1987) and one interpretation would be that newly synthesized TG monomers may inadvertently substitute the association of invariant chains with class II molecules.

7.7 IMMUNE SURVEILLANCE OF THYROID CELLS

The above considerations lead to the obvious question of whether TG peptides associate with MHC class I molecules in the ER of normal thyrocytes. Given that appropriate expression of MHC I molecules requires peptide binding, and TG synthesis represents 70% of the total protein synthesis of thyroid cells (Van Herle et al., 1979) it is reasonable to postulate that under normal conditions a percentage of the class I molecules expressed on the thyroid cell will be associated with TG peptides. Indeed, the constitutive expression of class I-TG complex may be important for immunological surveillance of thyroid cells. TG is a large molecule which undergoes a number of post-translational modifications in the Golgi apparatus (Godelaine et al., 1981). The tertiary structure of the molecule also appears to be important for coupling of iodotyrosine residues (Malthiery et al., 1989). In order to maintain the integrity of thyroid function, mechanisms must exist to prevent the transfer of incomplete molecules from the ER to the Golgi for export to the lumen. If abnormal peptides become associated with class I molecules then in most cases they would be recognized as foreign and these cells would be eliminated by circulating class I restricted cytotoxic cells. (Such a mechanism would also suffice to eliminate those cells producing excess TG.)

In vitro cytotoxicity studies on mouse thyroid cells (Creemers et al., 1983) have shown that thyrocytes are susceptible to lysis by class I restricted cells and so lend support to the above hypothesis. The T-cell receptor specificity of these cells have to be defined. As some class II antigens can be induced with TSH (section 6.2; Todd et al.,1987) class II restricted T cells may also be important for immune surveillance. If class II cells are involved in immune surveillance, then we need to determine whether the same TG peptides associate with class I and class II molecules. Recognition of MHC-peptide complexes would probably be insufficient to activate the T cells in the absence of accessory signals such as co-expression of adhesion molecules and costimulators (reviewed in Weaver & Unanue, 1990). Perhaps this would be a secondary level of regulation for immune surveillance cells. These questions clearly have to be investigated, because the answers may shed some light on how a putative surveillance system might be triggered to cause "mayhem".

7.8 SOME THOUGHTS ON THE KINEMATICS OF "REVERSED POLARITY"

One of the puzzles of thyroid pathogenicity is to understand how the normally secluded "microvillar" antigens may become transposed to the vascular pole of thyroid follicles where they could come into direct contact with cytotoxic antibodies or sensitized immunocytes. A similar problem is encountered in pernicious anaemia. The evidence to date suggests that the major molecular targets recognized by the parietal cell autoantibodies in this disease are the alpha and beta subunits of the gastric H+,K+ ATPase (gastric proton pump) (Gleeson & Toh, 1991). Although sera containing parietal cell autoantibodies are cytotoxic to gastric parietal cells *in vitro* (De Aizpurua et al., 1983), the role of these antibodies in the pathogenesis of the disease remains unresolved, since *in vivo* the gastric proton pump is located on the apical surface of parietal cells.

To explain the conundrum of the availability of secluded microvillar antigens to cytotoxic antibodies *in vivo*, Hanafusa et al., (1984) postulated that "spontaneous reversal" of thyroid cell polarity occurs. This is not improbable; the data in chapter 6 suggests that atleast partial redistribution of surface antigens on thyrocytes in culture does

occur and this distribution may be further influenced by cytokines such as IFN-γ. If we accept that surface antigen expression may be easily altered in thyrocytes, then it becomes necessary to consider that reversal of polarity is likely to be associated with the re-routing of the TG metabolic pathways. Atleast three separate states may be envisaged (DIAGRAM 7.3). In view of the relationship of the traffic of MHC class II molecules and antigen destined for processing and presentation, this is an issue that remains to be precisely defined. The results presented in chapter 6 suggests that the use of epitope-defined monoclonal antibodies may provide a means of identifying the steps involved in the processing and presentation of TG molecules. Although Den Hartog et al. (1990) have attempted to study the mechanism of thyroid hormone formation (and mutations in this process), by using three different groups of monoclonal antibodies raised against iodinated and non-iodinated human TG, these studies were done using cell-free systems.

Although "spontaneous reversal" would allow the recognition of "apical surface" antigens by cytotoxic antibodies, antigen redistribution *per se* would not activate cytotoxic T cells. A feasible suggestion would be that peptide transport from the ER in association with MHC antigens (as discussed in section 7.6) represents a "limited reversed polarity". A second more dramatic process by which such "inside-out" follicles could occur is suggested by two recent reports (Lippincott-Schwartz et al., 1989; Bonifacino et al., 1990) on the regulation of protein transport within the ER. The well-organized stack of Golgi cisternae is fed at one end (*cis* or immature face) by secretory (and membrane) proteins from the ER, whereas the opposite (*trans*, mature) face releases these proteins in membrane containers for a variety of destinations within the cell (Rothman & Orci, 1990). Lippincott-Schwartz et al. have shown that the antibiotic, brefeldin A, induces rapid redistribution of the marker enzymes of the early and late stages of the Golgi complex. From these observations they have postulated that cells normally have transport pathways not only from the ER to the Golgi, but back again. By analogy with vesicle traffic in the nerve axon, they referred to these routes as "anterograde" and "retrograde" transport respectively.

The paper by Bonifacino et al. provides strong evidence that the level of expression of membrane proteins can be regulated, post-translationally, within the ER.

These investigators studied the expression of surface T cell receptor (TCR) expression on immature CD4+CD8+ thymocytes and the influence of anti-CD4 antibody on this expression. They found that immature CD4+CD8+ thymocytes normally retain and degrade in the endoplasmic reticulum more than 90% of some endogenously synthesized TCR chains, however, anti-CD4 induced increased surface TCR expression. They could not ascribe this to increases in RNA levels, translation, or assembly, and concluded that it was due to the escape of newly synthesized receptor chains from the ER. One might surmise that the uderlying mechanism causing the retention and degradation of these receptors in the ER involves "retrograde" transport.

Extending this hypothesis to thyroid cells, it is intriguing to suggest that under normal conditions the majority of TG-MHC complexes (or indeed other thyroid antigen-MHC complexes) are retained and degraded within the ER, however, signalling through surface molecules may inhibit this process and so induce a dramatic increase in their surface expression, thereby inducing an apparent "reversed polarity". Therefore we have to consider the possibility that the IFN-gamma induced surface expression of TG epitopes (FIG.6.8, 6.9) are neodeterminants resulting from the interaction of MHC antigens with TG peptides (Kourilsky et al., 1987). If the above described pathways occur in thyrocytes (and future research should be aimed at delineating these events), there is a possibility that non-specific activation of the peripheral immune repertoire could also lead to thyroiditis in the susceptible individual. In addition, if the ER mechanism of retention and degradation is employed by other endocrine organs, the observed association of thyroiditis with other endocrinopathies (Khoury et al., 1982) may also be explained.

THE POLARITY OF THYROID CELLS *IN VITRO*

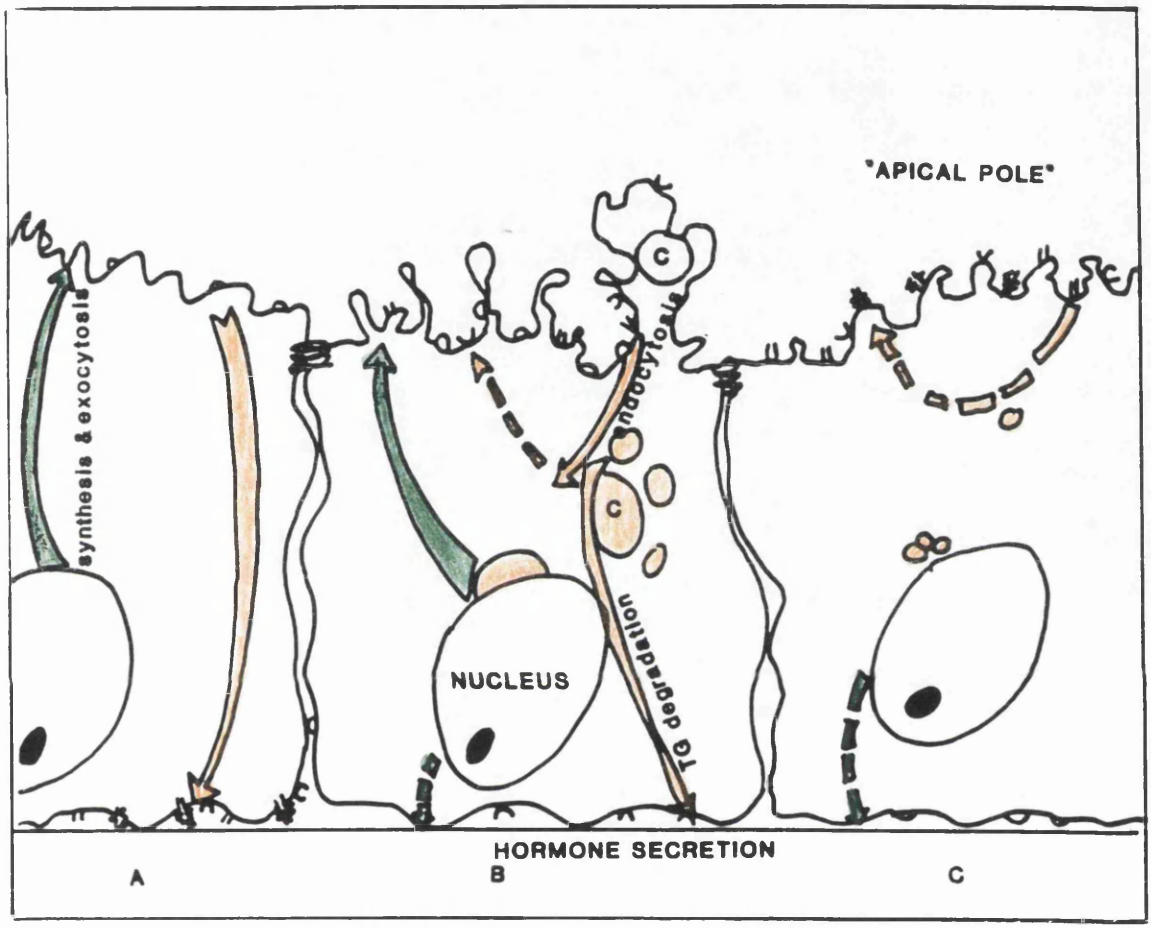


DIAGRAM 7.3: Diagrammatic representation of the possible distribution of surface antigens and the route of the TG metabolic pathways in cultured thyrocytes. Three alternative states are suggested: A) Normal direction of TG exocytosis + hormone secretion. B) Partial redistribution of directed pathways. C) Complete deregulation of TG metabolism (Broken arrows). TSH receptors (⌒); M/TPO (⌓); MHC class II (⫯); Surface TG (||); Colloid droplets (c).

A final note

To paraphrase de Bono (1972), "The ideas you find may or may not be better than presently held concepts. If they are not better you can always return to the current pattern of thinking. Even if you have not gained a new idea, you have gained a better appreciation of the value of the old idea, which is no longer held simply because you had never considered any alternative, but because it is better than the alternatives...At worst, you (and I) have lost a little thinking time."

APPENDIX 1

VALUES FOR IODOTHYRONINE CONTENT OF THYROGLOBULINS USED

SPECIES	T4	T3 Residues Per Mole	rT3	PROTEIN (mg/ml)
Chicken	2.23	0.02	0.03	0.33
Dog	5.36	0.32	0.06	0.33
Guinea Pig	5.08	0.29	0.08	0.90
Reindeer	4.59	0.14	0.03	0.64
Markhor	4.37	0.21	0.05	0.94
Human 1	0.56	0.07	0.04	0.79
Human 2	4.32	0.23	0.05	0.59
Pig	6.03	1.06	0.08	0.96
Monkey (1957)	7.04	0.61	0.14	1.53
Rat	2.50	0.18	0.04	0.46
Rabbit	5.32	0.71	0.10	0.94
Mouse (CBA)	2.97	0.38	0.05	0.43
Flying Squirrel	4.64	0.32	0.05	0.76
Pig-tailed Monkey	6.33	0.54	0.11	0.88
Sheep	2.10	0.31	0.08	0.66
Braz. tree porcupine	4.36	0.11	0.03	0.69
Cat	4.00	0.18	0.12	0.69
Hedgehog	1.85	0.09	0.05	N.D.
Wombat	5.42	0.14	0.11	1.01
Bovine	6.27	0.41	0.07	0.88
Zebra	3.99	0.53	0.04	1.09
Wallaby	2.06	0.06	0.07	0.73

The thyroxine content of TG was analysed by C.T. Chan by previously described methods (Chan et al 1987).

APPENDIX 2

CROSS-REACTIVITY PROFILE OF MONOCLONAL ANTIBODIES TO HUMAN TG

SPECIES OF TG

GROUP	mAb	HUMAN	BABOON	SHEEP	PIG	BOVINE	MOUSE	CHICKEN	RABBIT
1	6C1								
	6D2	+++	-	-	-	-	-	-	-
	1D4	+++	+++	-	-	-	-	-	-
	2B4	+++	+++	-	-	-	-	-	-
	2C4	+++	+++	-	-	-	-	-	-
	2C6	+++	+++	-	-	-	-	-	-
	6B2	+++	+++	-	-	-	-	-	-
	6B6	+++	+++	-	-	-	-	-	-
2	1D6	+++	-	-	++	+++	-	-	-
	2B1	+++	+++	++	++	+++	-	-	-
	5D2	+++	+++	+	+++	-	+++	-	-
3	3B3	+++	+++	+++	++	+++	++	++	-
	2A4	+++	+++	+++	+++	+++	+++	-	++
	3C4	+++	+++	+++	+++	+++	+++	+	++

This data was kindly provided by Phil shepherd

+++ indicates 100 to 75 %
++ 75 to 25 %
+ 25 to 10 % of C.D. reached by HuTG sensitised wells
Binding activities to TG species was assessed by ELISA.
5 µg/ml of TG was used for sensitising the microtitre plates

APPENDIX 3

EFFECT OF ADJUVANT ON THE ANTI-TG CROSS-REACTIVITY PROFILE OF MURINE EAT SERA

Classification of TG used in binding assays					Adjuvant used in immunization		
INFRA-CLASS	ORDER	SUB-ORDER	FAMILY	SPECIES	LPS/ MTG	CFA/ MTGx2	CFA/ MTGx1
EUTHERIA		MYOMORPHA	Muridae	Mouse	100%	100%	100%
				Rat	5	5	5
	RODEN-TIA		Criceti-dae	Chinese-hamster	5	5	4
		SCIUROMORPHA	Sciuri-dae	Flying squirrel	1	3	3
		HYSTRICO-MORPHA	Cavidae	Guineapig	0	3	1
			Erithi-zontidae	B.Tree-porcupine	5	4	4
	LAGO-MORPHA		Lepori-dae	Rabbit	0	3	1
	CARNI-VORA	AELUROIDEA	Felidae	Cat	0	3	1
		ARCTOIDEA	Canidae	Dog	0	3	0
	PERISSO-DACTYLA	HIPPO-MORPHA	Equidae	Zebra	1	3	2
	ARTIO-DACTYLA	SUIFORMES	Suidae	Pig	2	4	3
		RUMINANTIA	Cervidae	Reindeer	0	3	4
			Bovidae	Bovine	2	4	4
				Sheep	1	4	2
	PRI-MATES	ANTHROPOIDAE	Cercopi-thecidae	Pig-tail monkey	5	4	4
			Humini-dae	Human (1)	3	4	N.D
				Human (2)	4	4	4
	INSECTI-VORA		Erina-ceidae	Hedgehog	0	1	0
META-THERIA	MARSU-PIALIA		Vombati-dae	Wombat	0	3	4
			Macropo-didae	Wallaby	0	2	3

The cross-reactivity to different TG species was calculated as a % of the binding to MTG for individual sera. (see Chapter 5, section 5.2.1, for details). Percentage binding was scored on a 0 to 5 scale representing 0-10%, 10-20%, 20-30%, 30-50%, 50-70%, and > 70%. The model score for 5 or more samples is given.

* B.Tree Porcupine = Brazillian tree porcupine

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