

**T CELL EFFECTOR MECHANISMS IN  
EXPERIMENTAL AUTOIMMUNE UVEITIS (EAU)**

by

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### ***Abstract***

Experimental autoimmune uveitis (EAU) is a T cell-mediated autoimmune model for posterior uveoretinitis in man. There is increasing evidence to suggest an important role for CD4<sup>+</sup> T cells and their lymphokines in EAU induction, although the mechanisms involved are still not clear. Adoptive transfer of EAU in Lewis rats can be achieved using activated CD4<sup>+</sup> T cell lines specific for retinal soluble antigen (S-Ag). However there is little information on the regulation of lymphokine expression by these uveitogenic T cell lines. In order to further understand the pathogenic mechanisms involved in EAU, S-Ag specific CD4<sup>+</sup> T cell lines were established from Lewis rats immunized with S-Ag in CFA and were used to examine the regulation of lymphokine expression. Lymphokines present in the supernatant were assessed by bioassays and their mRNA was detected by polymerase chain reaction (PCR). All S-Ag specific cell lines established were found to be CD4<sup>+</sup>CD8<sup>-</sup>OX22<sup>low</sup> T cells.

The mRNA samples were taken at 6 h and supernatant samples were taken at 24 h after stimulation. Results demonstrated that S-Ag specific T cell lines when stimulated with antigen express IL-2, IFN- $\gamma$  and IL-4 mRNA. Furthermore, IL-2 and IFN- $\gamma$  bioactivities correlated with gene expression of respective lymphokines except in one cell line which had no detectable IL-2. These findings suggest that the production of IL-2 and IFN- $\gamma$  may play important role in EAU induction. Furthermore, the time course data (3, 6, 24, 48 and 72h) with one of S-Ag specific T cell lines showed that the proliferation of murine HT-2 cells induced by the supernatant following ConA stimulation was only partially inhibited by rat IL-2R mAb, while proliferative response induced by supernatant after S-Ag activation could be totally blocked by rat IL-2R mAb, suggesting that there are HT-2 cell growth factors other than IL-2 in the supernatants following ConA, but not S-Ag stimulation. All these suggest that the way in which the T cell is activated has an effect on its resultant lymphokine secretion.

Mouse CD4<sup>+</sup> T cell clones have been divided into Th1 and Th2 based on the patterns of lymphokines expressed, but in the rat there is still no evidence to support this. Rat CD4<sup>+</sup> T cell clones specific for S-Ag or PPD established from antigen specific CD4<sup>+</sup> T cell lines were therefore examined for lymphokine expression. The results indicated that S-Ag

specific CD4<sup>+</sup> T cell clones exhibit mainly a Th1 pattern but also a mixed (Th0) cytokine pattern on the basis of lymphokine mRNA expression, whereas the PPD specific CD4<sup>+</sup> T cell clones exhibit mostly a mixed (Th0) pattern but also Th1 and Th2 cytokine patterns.

In the rat, CD4<sup>+</sup> T cells could be functionally divided into OX22<sup>high</sup> and OX22<sup>low</sup> subsets based on phenotypic expression of CD45 isoforms. It has been found that lymphokine expression by OX22<sup>high</sup> and OX22<sup>low</sup> CD4<sup>+</sup> T cells seems to correlate with that of murine CD4<sup>+</sup> T cell clones. However, our results suggested that there is no simple correlation between rat CD4<sup>+</sup> cell subsets (based on phenotypic expression of CD45 isoforms) and their lymphokine gene expression.

Apart from inflammatory cytokines, immunosuppressive factors such as transforming growth factor- $\beta$  (TGF- $\beta$ ) could play an important role in the down-regulation of EAU. To examine this further, the *in vivo* role of TGF- $\beta$ 1 on the course of EAU was also examined. The results showed that intraocular TGF- $\beta$ 1 had the effect of delaying the onset of EAU.



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### *List of abbreviations*

Ab	-	antibody
Ag	-	antigen
APC	-	antigen-presenting cells
BT	-	biotinylated
CD	-	cluster designation
CFA	-	complete Freund's adjuvant
ConA	-	concanavalin A
cpm	-	counts per minute
DMSO	-	dimethylsulfoxide
EAU	-	experimental autoimmune uveitis
FACS	-	fluorescence Activated Cell Sorter
FCS	-	foetal calf serum
FITC	-	fluorescein isothiocyanate
H37RA	-	non-viable M. tuberculosis H37RA strain
<sup>3</sup> H	-	tritiated
hr	-	human recombinant
IFN- $\gamma$	-	interferon gamma
IL-2	-	interleukin 2
IL-4	-	interleukin 4
i.p.	-	intraperitoneal
i.v.	-	intravenous
i.o.	-	intraocular
mAb	-	monoclonal antibody
2-ME	-	2-mercaptoethanol
$\alpha$ -MM	-	methyl- $\alpha$ -D-mannoside
MHC	-	Major Histocompatibility Complex
NRS	-	normal rat serum
OVA	-	ovalbumin
PCR	-	polymerase chain reaction

PKC	- protein kinase C
PMA	- phorbol 12-myristate 13-acetate
PPD	- purified protein derivative of tuberculin
r	- recombinant
R	- receptor
RT	- reverse transcription
S-Ag	- retinal soluble antigen
SD	- standard deviation
SN	- supernatant
TcR	- T cell receptor
Th	- T helper (cell)
Thy	- thymidine
Ts	- T suppressor
T cell	- T lymphocyte
U	- units

## **Chapter 1 Introduction**

### **1.1 Brief introduction to uveitis**

#### **1.1.1 Uveitis**

"Uveitis" is a general term used to describe the myriad of intraocular inflammatory conditions which can lead to visual impairment and blindness in man. It is a relatively common clinical disorder occurring at a yearly rate of around 20/100,000 of the population [Forrester et al., 1990].

Uveitis can be categorized on the anatomical location of the inflammation, i.e. anterior uveitis (iris and ciliary body) or posterior uveitis (choroid and retina), each of which is pathogenetically and clinically a discrete entity. Certain types of anterior uveitis are an acute self-limiting disease, closely linked with Major Histocompatibility (MHC) Class I antigens, particularly HLA-B27 [Brewerton, 1989], while posterior uveitis is more frequently associated with MHC Class II antigens (HLA-DR) and is often chronic in nature [Lightman & Chan, 1990]. In anterior uveitis, the disorder is more closely linked with infectious agents such as viral infection and *Yersinia enterocolitis* [Byrom et al., 1979; Wakefield et al., 1990]. Much of the posterior uveitis is of unknown aetiology. Posterior uveitis has been shown to be associated with CD4<sup>+</sup> T cell infiltration in the retina [Lightman & Towler, 1992] and this thesis is concerned solely with posterior uveitis.

#### **1.1.2 Posterior uveitis and autoimmune disease**

##### **1.1.2.1 The retina and the immune system**

The retina is part of the central nervous system (CNS). It has a specific relationship with the immune system. Due to its developmental origin and the presence of physiological factors, most parts of the eye are avascular and absence of lymphatic drainage. Furthermore there are strong barriers between the blood and the eye (blood-retinal barrier, BRB). Under physiologic conditions, these factors modify the induction

(afferent limb) of immunity directed at ocular antigens. Thus, the eye is offered as a privileged site in which immune responses are probably not initiated [Cserr & Knopf, 1992; Niederkorn, 1990; Rocha & Baines 1992]. However, it has been recently shown that under normal conditions activated T cells can across the BRB at a low level so that there is no strict "immunologic privilege" for the nervous system [Greenwood, 1992].

#### **1.1.2.2 Autoimmunity and autoimmune disease**

Autoimmunity and autoimmune disease are not the same. The former is often benign whereas the latter is potentially fatal. Autoimmunity is often reflected only by the presence of serum autoantibodies; it is a normal consequence of aging, is readily inducible by drugs or infectious agents and is potentially reversible, in that it disappears when the offending drug or agent is removed or eradicated. Autoimmunity might even be a normal physiological state, such that we are all probably autoimmune, but relatively few of us develop autoimmune disease. Autoimmune disease results from activation of self-reactive T and B cells, following stimulation by genetic or environmental factors, which result in actual tissue damage (reviewed in [Mountz & Talal, 1993]).

Immunological tolerance to self antigens is of paramount important in the prevention of autoimmune diseases. Tolerance can be established both in the B lymphocytes (reviewed in [Goodnow, 1992] and T lymphocytes (reviewed in [von Boehmer & Kisielow, 1990; Miller & Morahan, 1992]. A well established mechanism to achieve T cell tolerance is clonal deletion of autoreactive thymocytes (negative selection) [Fowlkes et al., 1988; Kappler et al., 1987 and 1988; Kisielow et al., 1988; MacDonald et al., 1988a and 1988b; Pircher et al., 1989; Sha et al., 1988]. However, many self antigens are not produced in the thymus and T cell tolerance has also be established to such antigens. This could be achieved if extrathymic antigens, soluble or bound to cells, could gain access to the thymus and be presented as processed short peptides bound to a MHC molecule on antigen-presenting cells [Bogen et al., 1993]. In the periphery, several mechanisms of tolerance induction in T cells have been identified. Clonal deletion of peripheral T cells has been demonstrated for antigen- and MIs-specific T cells [Jone et al., 1990; Kawabe & Ochi, 1991]. Peripheral tolerance can be mediated by CD8<sup>+</sup> T cells [Schwartz, 1990]. It can also be manifested by T cell anergy [Jenkins et al., 1987; Miller et al., 1991]. The nonresponsiveness of anergic T cells can be overcome in some

systems by stimulation with antigen *in vitro* in the presence of interleukin-2, while in others it cannot [Lo et al., 1989; Morahan et al., 1989; Rammensee et al., 1989]. CD28-mediated signalling could block anergy induction *in vitro* [Harding et al., 1992]. Results reported by Yule et al. have shown that Hen Egg-White Lysozyme (HEL)-reactive T cells were anergic *in vivo*, but could be partially activated with a strong stimulus to the immune system, suggesting T cell indifference on the effects of breaking anergy for T cells rendered anergic *in vivo* [Yule et al., 1993]. Down-regulation of TCRs and associated coreceptors on self-reactive T cells has been observed as another mechanism of tolerance induction in peripheral T cells [Schonrich et al., 1991].

### **1.1.2.3 Evidence for autoimmune disease in posterior uveitis**

The theory of an autoimmune mechanism to explain the origin of ocular disease was first developed in order to explain the pathogenesis of sympathetic ophthalmia. Elshnig suggested that the resorption of antigen in the damaged eye led to a hypersensitivity that involved the second eye. Thus, the mildest disturbance in the sensitized second eye could lead to inflammation and blindness (reviewed by [Silverstein, 1990]). Elshnig's hypothesis was one of the first formulations of the autoimmune theory in human disease, and he focussed attention on the uveal pigment as the source of sensitization (reviewed in [Faure, 1980]). Over the years, the notion that immune perturbation leads to an autoimmune response has become an accepted explanation for several intraocular inflammatory entities [Gery et al., 1986].

Like other autoimmune diseases such as rheumatoid arthritis (RA), multiple sclerosis (MS) and insulin-dependent diabetes mellitus (IDDM) [Feldmann & Londei, 1989], posterior uveitis is now commonly presumed to be autoimmune in origin since most cases occur in the absence of an identifiable infectious agent, have large numbers of infiltrating CD4<sup>+</sup> T cells and enhance HLA-DR expression in the target organ. Its acceptance as an autoimmune disease is partly due to the existence of good experimental models resembling aspects of the human diseases in which the antigens (e.g. S-Ag and IRBP) are well-defined. The experimental disease induced by these retinal antigens is termed experimental autoimmune uveitis.

## **1.2 Experimental autoimmune uveitis (EAU)**

### **1.2.1 History of EAU**

The first successful induction of eye disease by systemic injections of uveal preparations was performed by Collins [Collins, 1949]. He chose a sensitive animal species, the guinea pig; used homologous uveal homogenate as antigen; and injected it emulsified with complete Freund's adjuvant (CFA). Histologically, 3-6 months later, among the 25 animals treated, 12 (24 eyes) were positive, showing choroiditis and retinal lesions. None of the control animals, injected with liver + CFA or CFA alone, had ocular pathology. This disease was reproduced by Aronson et al. and Wacker et al., and extensive studies have subsequently been carried out (reviewed by [Faure, 1980]).

The term experimental allergic uveoretinitis (EAU) was originally described by Wacker and Lipton [Wacker & Lipton, 1965] when they used crude extracts of retinal tissue to induce an organ-specific retinal and choroidal inflammation after a single inoculation. They have shown that the potent uveitogenic antigens localize in the retina, a very fruitful finding leading in a few years to great developments in the field. Since then, several retinal antigens have been described, including S-antigen [Wacker et al., 1977], interphotoreceptor retinoid binding protein (IRBP) [Gery et al., 1986], (rhod)opsin [Broekhuysen et al., 1984] and phosducin [Dua et al., 1992], all with the ability to induce EAU. Considerable information is now available concerning the amino acid sequence and secondary structure of these proteins [Borst et al., 1989; Kuhn, 1984; Shinohara et al., 1988] and more recently immunodominant epitopes on these antigens have been described at least for retinal S-antigen [Donoso et al., 1987] and IRBP [Sanui et al., 1989].

### **1.2.2 Variation of EAU among species**

EAU can be reproducibly induced in a variety of animals, including primates, rabbits, guinea pigs, rats and mice (reviewed by [Caspi, 1989]). Features of EAU may differ among the tested species, due to anatomical or other factors (e.g. absence of retinal blood vessels in guinea-pigs).

EAU in the Lewis rat is a good model for posterior uveitis in man due to the similarities

both in the pathology of the disease processes (an infiltration of CD4<sup>+</sup> T cells and enhanced expression of MHC class II molecules within the retina) [Forrester et al., 1990] and in the functions of the blood-retinal barrier in both rat and man [Faure, 1980].

### **1.2.3 Induction of EAU**

EAU is most often induced by sub- or intra-cutaneous inoculation of a single dose of uveitogenic emulsion containing either whole retinae or retinal proteins in an equal volume with CFA, a mineral oil containing *Mycobacterium tuberculosis*. The addition of a bacterial component to the adjuvant has a marked augmenting effect on the subsequent disease and decreases the time between injection and the first sign of disease. The mechanism of action of CFA is probably that when CFA material is mixed with antigen, it forms an insoluble complex with the antigen. This complex slows the escape of antigen from a subcutaneous depot, by increasing the physical size of the antigen. They also provide microdroplets of antigen in oil to phagocytic cells. Phagocytes can imbibe these antigen droplets more easily than antigen in solution [Barrett, 1988].

Recent studies have shown that the induction of EAU is further enhanced by a simultaneous injection of the animals with *Bordetella pertussis* (*B. pertussis*) bacteria [de Kozak et al., 1981]. Of particular interest was the finding that *B. pertussis* converted rats of strains with low responsiveness to EAU induction to become highly responsive (see [section 1.2.4](#)). The mechanism of this enhancing effect of *B. pertussis* is not known, but may be explained in part by its histamine-sensitizing property [Duijvestijn & Hamann, 1989; Munoz & Bergman, 1968; Stanford et al., 1988]. Indeed, it has been reported that *B. pertussis* increased the vascular permeability in the central nervous system of mice by the histamine-sensitizing effect [Linthicum & Frelinger, 1982]. Thus, it may be possible that the histamine-sensitizing effect leads to increased vascular permeability and enhancement of the emigration of recirculating lymphocytes into the pathological lesions. Furthermore, it was found that the whole *B. pertussis* bacteria could be replaced with a purified preparation of a single component of these bacteria, termed pertussis toxin [McAllister et al., 1986].

### **1.2.4 Genetic basis for susceptibility to EAU**



Models of EAU have been developed in guinea pigs, rats, rabbits and mice although the uveitogenic responses within species are varied. McMaster et al. [McMaster et al., 1976] studied EAU induction with retinal and uveal antigens in several strains of guinea pigs. Their results showed that the Hartley and NIH strains could be classified as high responders, strain 13 animals, intermediate responders, and strain 2 guinea pigs, non-responders.

In mice, EAU has only been recently achieved, and its induction requires the use of IRBP as opposed to S-Ag which appears to be poorly uveitogenic in this species [Caspi et al., 1988].

Differences in susceptibility to EAU among rats of various strains have been reported [Gery et al., 1985]. The findings showed that high responder strains were Lewis and CAR, with virtually all animals developing EAU. BN, MAXX and AVN were defined as low responders, with approximately one quarter of the rats developing the disease. Intermediate responsiveness was exhibited by rats of LBNF strains, which are hybrids of Lewis and BN, while no EAU was found among RCS and the LeR rats.

Recent immunogenetic studies by Hirose et al. [Hirose et al., 1991] showed that both MHC and non-MHC genes were involved in the development of EAU in rats. The effect of non-MHC gene(s) was demonstrated by the finding that none of the WKAH.1L congenic rats developed S-Ag-induced EAU, although this strain has the same MHC as the Lewis rat which is highly susceptible to EAU. However the significant influence of non-MHC gene(s) on the development of EAU could not be observed when an additional injection of *B. pertussis* was given.

The relationship between susceptibility to EAU and MHC haplotype was tested in rats [Hirose et al., 1991] and strains which have haplotype *I* at the MHC locus (Lewis and WKAH.1L *RTI* congenic rats) developed EAU. The other two strains which developed EAU, F344 (*RTI*<sup>lv1</sup>) and NIG-III (*RTI*<sup>q</sup>), have the specificity *I* at B and D loci respectively of the class II MHC. On the other hand, none of the other strains which do not have allele *I* at the class II loci developed peptide M-induced EAU (given *B. pertussis*). However, these refractory strains could develop EAU when immunized with S-Ag in CFA concurrently with *B. pertussis* adjuvant. The MHC restriction of

susceptibility to peptide M-induced EAU may possibly be explained by differences in the capacity of polymorphic MHC class II molecules to bind the uveitogenic antigen represented by this peptide since T cell recognition of certain epitopes on antigen depends on the binding capacity of class II molecules with the antigen [Guillet et al., 1987; Ogasawara et al., 1990].

### **1.2.5 Histopathology of EAU**

Early histologic changes in the retina of Lewis rats immunized with S-Ag in CFA are accumulation of inflammatory cells in the photoreceptor cell layer. Other components of the eye rapidly become involved as well, with inflammatory cells infiltrating the vitreous, iris, ciliary body and anterior and posterior chambers. The choroid becomes usually affected only at the peak of the pathologic process, which is reached approximately 2-4 days after the disease onset. The infiltration in the rat eye consists of a mixture of polymorphonuclear (PMN) and mononuclear cells (MNL, i.e., lymphocytes). However, the proportions of PMN and MNL differ in the various eye compartments, with PMN always being the majority in the anterior segment, whereas in the posterior segment, MNL were predominately found [de Kozak et al., 1981; Gery et al., 1986].

An analysis of the T cell subsets by immunohistochemistry in retinal lesions at different stages was studied by Chan et al. [Chan et al., 1985]. After 11-14 days' immunization with S-Ag emulsified in CFA, increased numbers of inflammatory cells (predominantly CD4<sup>+</sup> T cells) can be found within the retinal layers and the ratios between CD4<sup>+</sup> and CD8<sup>+</sup> T cells were found to be from 5:1 to 5:2. At the later postinflammatory stage of disease (> day 17 onwards), the number of CD8<sup>+</sup> T cells were increased and the ratio found to be from 1:1 to 1:2. The inflammatory process leads to partial or complete loss of the photoreceptor cell layer.

## **1.3 Retinal specific antigens**

### **1.3.1 General review of retinal specific antigens**

A substantial number of tissue-specific antigens have been identified in vertebrate retinas. The retinal homogenate has been separated into two fractions, designated

"soluble" (S) and "particulate" (P). The major component of S fraction is a highly immunogenic protein, designated "S-Ag", while the active antigenic component of P fraction is thought to be rhodopsin. In addition to these antigens, other molecules with apparent tissue specificity have been identified in the retina. These include the interphotoreceptor retinoid binding protein (IRBP), phosducin, the 'A' antigen, transducin, cGMP phosphodiesterase (PDE) the 'large intrinsic membrane protein', and membrane-associated proteins which bind antibodies to the retina [Gery et al., 1986] .

### **1.3.2 Retinal soluble antigen (S-Ag)**

#### **1.3.2.1 Role of S-Ag in the pathogenesis of EAU**

The role of retinal antigens, including S-Ag, in the pathogenesis of EAU has been reviewed previously [Faure, 1980; Gery et al., 1986]. Summary of most studies indicate that S-Ag is highly efficient for the induction of EAU in many different animal species including rats [de Kozak et al., 1981], rabbits [Wacker et al., 1981], guinea pigs [Rao et al., 1979] and primates [Nussenblatt et al., 1981]. The immunological and histological features of the disease are dependent, in part, on the susceptibility of the animal strain, the dose of immunizing antigen, the type of adjuvant used and on certain anatomical features of the experimental animal such as the presence or absence of a retinal circulation.

#### **1.3.2.2 Distribution of S-Ag**

S-Ag has been considered a specific protein of photoreactive cells [Shinohara et al., 1988]. The high levels of cross reactivity were observed between preparations of S-Ag purified from retinas of a variety of animals [Wacker, 1981]. Studies at the electron microscope level showed that S-Ag localizes mainly in the rod outer segments [Yajima et al., 1983]. Furthermore, the unique relationship between S-Ag and the visual system was proposed by the finding of this antigen in the pineal gland [Kalsow & Wacker, 1977; Mirshahi et al., 1984], which has a photoreceptor function in lower vertebrates [Deguchi, 1981; Oksche, 1965]. It is of note that in accord with its content of S-Ag, the pineal gland is often affected by the inflammatory process which develops in animals immunized with S-Ag [Kalsow & Wacker, 1978]. However, recent experiments

demonstrated that S-Ag is present in low amounts in many other cells in the body [Faure & Mirshahi, 1990]. Whether any systemic pathology is associated with uveitis and pinealitis after S-Ag immunization is unclear.

#### 1.3.2.3 Structure of S-Ag

The entire amino acid sequence of human [Yamaki et al., 1988], bovine [Shinohara et al., 1987; Yamaki et al., 1987], and mouse [Tsuda et al., 1988] retinal S-Ag as well as rat pineal gland [Abe et al., 1989] S-Ag has been determined, and there is a high degree of sequence homology among these species. The molecular weight of S-Ag is approximately 45 kilodaltons and the number of residues are 403, 404 or 405 respectively (for more detailed description see [Shinohara et al., 1991]). Some regions of internal similarity were detected, perhaps reflecting a repeating structure of S-Ag. The data bank revealed no extensive sequence homology between S-Ag and other proteins, but there were local regions of sequence similarity with  $\alpha$ -transducin [Shinohara et al., 1988; Wistow et al., 1986].

Analysis of nucleotide sequences of S-Ag cDNA also showed a high degree of sequence homology among the mouse, rat, bovine and human [Shinohara et al., 1991]. These results suggest that the S-Ag in the retina and pineal gland are virtually identical at the transcript and polypeptide level.

#### 1.3.2.4 Immunogenic sites in S-Ag

Initially, a series of 23 oligopeptides were synthesized corresponding to the entire amino acid sequence of the bovine S-Ag, and tested for their ability to induce EAU in Lewis rats and guinea pigs (reviewed by [Shinohara et al., 1991]). In these studies, four synthetic peptides, designated peptide M (18 amino acid residues) and peptide N (22 amino acid residues) corresponding to amino acid position 282-320 in bovine S-Ag consistently induced EAU when immunized at a 50-100  $\mu$ g dose (major sites). In addition, other synthetic peptides, peptide K (20 amino acid residues) and peptide 3 (20 amino acid residues), corresponding to amino acid position 221-260 also induced mild EAU with high doses (100-500  $\mu$ g) of immunization (minor sites).

In an effort to define more precisely the peptide M pathogenic site, smaller peptides corresponding to the amino and carboxy terminus were tested [Shinohara et al., 1988]. These studies indicated that EAU could be induced with a peptide as small as 12 amino acids and the amino-terminal portion of peptide M was essential for uveitopathogenicity.

## **1.4 Pathogenetic mechanisms of EAU**

### **1.4.1 Immunological background**

#### **1.4.1.1 Components of the immune system**

Cells of the immune system originate from pluripotent hematopoietic stem cells in the bone marrow which give rise to two different cell lineages: lymphoid cells, which are the precursors of B and T cells, and myeloid cells, which give rise to monocytes (macrophages), neutrophils, eosinophils and mast cells.

The two classes of lymphocytes, T cells and B cells, differ in their functional properties. T cells are responsible for cell-mediated immunity, as well as coordinating the functions of other cell types, including B cells. T cells can be phenotypically divided into two subsets by cell surface markers: one expressing the CD4 molecule and the other expressing the CD8 molecule. The expression of CD4 or CD8 molecules was initially believed to correlate with lymphocytes exhibiting distinct effector functions, i.e. helper and killer T lymphocytes [Cantor & Boyse, 1975]. A few years later, it became apparent that the effector functions of CD4 and CD8 lymphocytes were not as distinct as initially believed, i.e. CD4 cells could become cytotoxic [Bell & Stastny, 1982; Krensky et al., 1982] and CD8 cells could produce at least some lymphokine [Von Boehmer & Hass, 1981; Widmer & Bach, 1981]. It was suggested that the CD4 and CD8 accessory molecules may correlate with T cell specificity, i.e. MHC restriction rather than lymphocyte function (reviewed in [Swain, 1983]). Recently, it has been shown that the TCR recognizes an MHC-peptide complex, with the CD8 and CD4 molecules functioning as co-receptors binding to the non-polymorphic regions of MHC class I and class II molecules, respectively (reviewed in [Julius et al., 1993]). The CD4 and CD8 molecules also play major roles in the thymic T cell differentiation process that leads to

the mature T cell repertoire and the expression of CD4 and CD8 on mutually exclusive T cell subsets (reviewed in [Janeway, 1992; Miceli, & Parnes, 1993]).

B cells are primarily effector cells and B cell activation often requires the successful interaction with helper T cells. They secrete antigen-specific immunoglobulin molecules that mediate humoral immunity. B cells also express cell surface immunoglobulins that function as specific antigen receptors.

Macrophages are an additional class of cells (accessory cells) and critical for the function of the immune system. These cells do not possess any specific antigen-recognition capacity, but play an essential role in the process of antigen presentation to T cells by allowing T cell recognition of foreign antigens, and also provide to both T cells and B cells the extracellular signals (cytokines) required for functional activation. Macrophages are considered to be professional antigen presenting cells (APC), although a number of other cell types, including dendritic cells, Langerhans cells, B cells and monocytes, have this property.

#### 1.4.1.2 Activation of T cells

T cell activation requires two signaling events. The first, an Ag-specific signal delivered by the MHC/Ag complex interacting with TCR, results in early biochemical and molecular changes in the activated T cells. However, complete cellular activation depends on a second signal, defined as costimulation [Schwartz, 1990]. One of the most well characterized of these T cell costimulatory molecules is CD28 [June et al., 1990]. Unlike other T cell activation/adhesion molecules, CD28-mediated signaling is TCR independent [Ledbetter et al., 1990] and cyclosporin A resistant [June et al., 1987]. The natural ligand for CD28 is B7. It has been shown that the CD28-B7 pathway is crucial in T-cell activation and that stimulatory CD28 antibodies may block anergy induction *in vitro* [Harding et al., 1992]. Another protein, CTLA-4, which has a similar sequence and maps closely to CD28 [Harper et al., 1991; Howard et al., 1991] also binds to B7, in fact with higher affinity than does CD28 [Linsley et al., 1991]. Unlike CD28 which is expressed by both resting and activated T cells, the expression of CTLA-4 is apparently restricted to activated T cells [Freeman et al., 1992]. Furthermore, studies have demonstrated the existence of an additional CTLA-4 ligand, termed B7-2, which also

play a critical role in T cell costimulation [Hathcock et al., 1993; Lenschow et al., 1993]. Recent discovery that CD28 and B7 are each members of large gene families suggests that the regulation of co-stimulation is more complex than imagined (reviewed by [June et al., 1994]).

#### 1.4.1.3 Antigen presentation

A striking feature of immune recognition is that it operates at two quite different levels. The B cell humoral response, via antibody, recognises with exquisite sensitivity the three-dimensional specific conformation of antigen molecules, and is sensitive to very small changes in the outer "accessible" surface, even when primary structure or chemical composition remains unchanged. In contrast, T cells, which make up an important component of the cellular immune system, have evolved a separate mechanism which recognises the primary structure of proteins (reviewed in [Levine & Chain, 1991; Brodsky & Gualgliardi, 1991]). T cells generally recognize antigen only if associated with cell surface glycoproteins encoded by the MHC, a phenomenon known as MHC restriction [Zinkernagel & Doherty, 1979]. For T cells to respond, antigens must be processed and presented as peptide-MHC complexes. Such processing is a function of APC in which foreign antigen must compete with self components for binding sites on class I or class II molecules, if they are to be successfully displayed for T cell recognition [Buus et al., 1987]. The CD4<sup>+</sup> T cells recognize peptides complexed with class II MHC molecules and the CD8<sup>+</sup> T cells recognize peptides in association with class I molecules. MHC class I molecules, which are expressed on most cell types, normally present peptides derived from proteins that are produced endogenously in the cell. MHC class II molecules, which are expressed on only a few cell types, such as B cells and macrophages, can present peptides derived from exogenous antigens to MHC-restricted T cells (reviewed in [Braciale et al., 1987]). The APC are present in large numbers in lymph nodes, the spleen and the thymus. They are also present in peripheral tissues, particularly in the skin. A typical APC of the skin and mucosae is the Langerhans dendritic cell. After acquiring the antigenic information, this cell migrates from the skin or mucosa to the lymph node to present the antigen to the T lymphocyte. Class II-positive cells are normally present in all organs but are rare in the eye and the brain. However, ocular tissue cells such as RPE cells, Muller cells and retinal endothelial cells can be induced to express MHC class II antigens aberrantly during inflammation

[Lightman & Chan, 1990].

It has long been believed that macrophages are the major APC of the immune system [Unanue, 1984]. However, it is becoming clear that the major route for primary stimulation is via bone-marrow derived dendritic cells [Steinman, 1991].

#### 1.4.1.4 The classic immune hypersensitivity reactions

Inflammatory reactions were originally classified into four types or "hypersensitivity reactions" by [Coombs & Gell, 1975]. The first, or type I hypersensitivity reaction, is thought to be mediated by immunoglobulin E. The type II hypersensitivity involves cytotoxic antibodies affecting cells directly or with the help of cytotoxic cells. The type III mechanism is immune complex mediated, with a deposition of an antigen-antibody complex onto susceptible cell surfaces that initiate a series of reactions triggered by complement activation. The final classic mechanism is of the type IV hypersensitivity reaction, which is mediated by T-cells, not by antibodies, as in the other three reactions.

Type III hypersensitivity reactions were once thought to be the predominant mechanism of ocular inflammation. Immune complexes can be demonstrated in the aqueous humour of patients with uveitis [Char et al., 1979; Dernouchamps et al., 1977]. Circulating immune complexes have been reported in patients with Behcet's disease [Lehner et al., 1978]. However, recent observations would not support the notion that immune complexes are playing a pivotal role in posterior uveitis but rather demonstrated that T cells (CD4<sup>+</sup> T cells) play the major role in these process especially due to lack of neutrophils and PMN in the inflamed tissue (see [section 1.4.4 and 1.4.5](#)).

#### **1.4.2 Infection and autoimmunity**

There are many ways such as superantigenic stimulation [Marrack & Kappler, 1990] and the involvement of the 65-kDa heat-shock proteins [Cohen & Young, 1991; Kaufmann, 1990] in which microbial agents could influence autoimmunity, but the mechanism which has attracted most interest, is that of molecular mimicry between micro-organisms and autoantigens including, of course, idiotypes. Many bacterial, viral and fungal proteins share antigenic sites with normal host cell proteins, a phenomenon known as



molecular mimicry [Fujinami & Oldstone, 1985]. These homologous but not identical determinants, differing in one or more amino acids, may be foreign enough to elicit an immune response. The immune response initiated against the foreign epitope may also react with a closely homologous 'self' host protein. In this way, an antibody or cytotoxic lymphocyte generated against a microbial agent may cross-react with a self-protein, resulting in cellular injury and disease.

Singh et al. have presented evidence that molecular mimicry may occur in the ocular microenvironment. These studies have identified sequence homology between the uveitopathogenic site of S-Ag and selected viral proteins [Singh et al., 1990], *Escherichia coli* protein [Singh et al., 1989] and yeast histone H-3 protein [Singh et al., 1989]. Sequence homology with S-Ag was identified in the following viruses: hepatitis B virus DNA polymerase, *gag*polyprotein of baboon endogenous virus and *gag*polyprotein of Akv murine leukemia virus. Inoculation of Lewis rats with these different synthetic peptides triggered the process of EAU. Moreover, native histone H-3 alone was demonstrated to induce EAU [Singh et al., 1989]. Infection with the appropriate pathogenic strain of the organism might initiate an autoimmune response to retinal antigen. Clearly there would be a time-lag between the initial infection and the development of the autoimmune ocular inflammation and this, in fact, is often the pattern of development of endogenous uveitis.

Recently, the term superantigen has been proposed to describe novel immunostimulatory molecules that cause polyclonal T cell activation [White et al., 1989]. To date, superantigens are known to be produced by bacteria and viruses and they have common ways of stimulating T cells. Unlike conventional antigens, superantigens can activate up to 20% of T cells and interact mostly with the V $\beta$  element of the TCR [Mourad et al., 1993]. Superantigen binding to MHC class II molecules is a prerequisite for T cell stimulation. The ability of superantigens to induce polyclonal activation of MHC class II-positive cells may confer to the superantigen its capacity to trigger autoimmune disease.

### **1.4.3 General immune mechanisms in EAU**

When an antigen comes in contact with the immune system, two types of responses,

classically termed humoral and cellular, can occur. The former is produced when an antigen induces B-cell transformation into a plasma cell, with subsequent production of antibodies. The latter involves helper T (Th) cells and cytolytic effector T cells, both of which in turn produce cytokines. Both humoral and cellular responses require the processing and presentation of antigen by APC and the assistance of Th cells. The distinction between humoral and T cell-mediated responses may reside at the level of the Th cell. A subpopulation of Th cells, called Th1 cells, primarily induces cell-mediated immunity and DTH, while Th2 cells induce humoral immunity. Th1 and Th2 cells differ in the cytokines they produce [Mosmann & Coffman, 1989a and 1989b] as described later in this thesis.

#### **1.4.4 Possible roles of antibodies in EAU**

There is some evidence pointing to the role of antibodies in the perpetuation of EAU. In guinea pigs, injection of hyperimmune sera to rod outer segments directly into the eye can elicit EAU-like pathology [De Kozak et al., 1976]. Antibodies of the IgE subclass are detectable in rats immunized with S-Ag before the onset of EAU, and might mediate the mast cell degranulation that occurs shortly before disease onset [De Kozak et al., 1981]. Release of vasoactive amines contained in the mast cell granules may contribute to EAU induction by increasing the permeability of ocular blood vessels. In support of this theory is the observation that sensitivity to EAU of different rat strains appears to be correlated with the number of choroidal mast cells [Mochizuki et al., 1984], and that local treatment with pharmacological agents that block mast cell function can delay or suppress EAU [De Kozak et al., 1983]. A monoclonal antibody (mAb) specific for S-Ag or a polyclonal anti-S-Ag rat antiserum can suppress the ocular disease and reduce the antibody response of rats to S-Ag [De Kozak et al., 1985]. Furthermore, the results presented by De Kozak et al. showed that EAU can be blocked by antiidiotypic antibodies generated against certain murine monoclonal anti-S-antigen idiotypes [De Kozak et al., 1987]. The mechanism of resistance to EAU by idotype immunization is unknown. It has been suggested that antiidiotypic antibodies may block the antigen receptors of uveitogenic T cells [Caspi, 1989]. Alternatively, it is conceivable that idotype immunization might be initiating a regulatory circuit, resulting in the induction of antiidiotypic T suppressor cells [Lider et al., 1988].

It seems that antibody-mediated mechanisms are involved in the pathogenesis of EAU. However, EAU can be induced by adoptive transfer with T lymphocytes, but not serum, of genetically identical donors immunized with S-Ag [Quinby & Wacker, 1967; Aronson & McMaster, 1971; Mochizuki et al., 1985], and suppressed (by cyclosporin A treatment) without apparent decrease in the titre of serum antibodies to S-Ag [Nussenblatt et al., 1981], suggesting that humoral immunity is not mandatory to the disease.

#### **1.4.5 Role of T cells in EAU**

##### **1.4.5.1 Evidence for cell-mediated immunity in EAU**

Indirect evidence in support of the role of cell-mediated immunity in the pathogenesis of EAU has been obtained in two systems: (1) Nude rats, which congenitally lack a thymus and have no endogenous T cells, were found incapable of developing EAU when immunized with S-Ag [Gery et al., 1986]. Yet, EAU was induced in these rats by adoptive transfer of lymphocytes from heterozygous donors immunized with S-Ag [Salinas-Carmona et al., 1982]. (2) Development of EAU was inhibited in rats by treatment with cyclosporin, an immunosuppressive drug which selectively affects T lymphocytes [Nussenblatt et al., 1981; Nussenblatt et al., 1983]. Although the role of T lymphocytes could hypothetically be to 'help' B lymphocytes to produce antibodies, other data have demonstrated directly the capacity of T lymphocytes to induce EAU using the system of adoptive transfer.

The usefulness of adoptive transfer experiments for analysis of the pathogenic mechanisms of various immune-mediated disease is well established. Experiments attempting to transfer EAU with antibodies were described above (see Section 1.4.4). The capacity of lymphocytes to adoptively transfer EAU was shown by several authors [Aronson & McMaster, 1971; Faure & De Kozak, 1981; Meyers, 1976], using splenic cells or lymph node cells collected directly from the immunized donors. The immunological capacities of lymphocytes are enhanced profoundly by activation with the specific antigen or a polyclonal mitogen such as concanavalin A (ConA) [Mochizuki et al., 1985]. Uncultured lymph node or spleen cells from donor rats immunized with S-Ag were unable to induce EAU in naive recipients when injected at numbers as high as  $10^8$

or  $10^9$ , respectively. On the other hand, these cells became highly uveitogenic when injected following incubation with S-Ag. Incubation with ConA was also found to enhance the uveitogenicity of spleen cells but not that of lymph node cells.

#### 1.4.5.2 The role of T cell subsets in the pathogenesis of EAU

The adoptive transfer system made it possible to identify the subset of lymphocytes responsible for EAU induction. Using purified subsets of lymphocytes, it has been shown that only  $CD4^+$  T lymphocytes were capable of transferring EAU; no such activity was found among  $CD8^+$  cells [Mochizuki et al., 1985]. The uveitogenic efficacy of  $CD4^+$  lymphocytes has been further demonstrated by studies in which long-term lines of T lymphocytes sensitized against S-Ag were employed. Only lines of  $CD4^+$  T cells were found to induce EAU in naive recipient rats [Caspi et al., 1986; Gregerson et al., 1986; Rozenszajn et al., 1986]. The importance of  $CD4^+$  T cells in the initiation of EAU can be seen in inhibition of EAU by anti- $CD4$  mAb [Atalla et al., 1990].

The negative regulation of EAU by  $CD8^+$  T cells has also been reported, mostly from studies in the rat model. Chan et al. [Chan et al., 1985; Chan et al., 1985] have shown that at later stages of disease an increase in proportion of  $CD8^+$  T cells can be seen within the retina and it has been proposed that these cells could down-regulate the disease process. Furthermore, the results showing *in vivo* suppression of the disease by a long-term  $CD8^+$  T cell line derived from S-Ag-primed donors indicated that suppressor T cells may play a role in the downregulation of EAU [Caspi et al., 1988]. However, more recently our results showed that no change was found in the course of disease when Lewis rats were depleted of their  $CD8^+$  T cells before induction of and during EAU [Calder et al., 1993], suggesting that  $CD8^+$  T cells do not play an important role in initiating or down-regulating EAU.

#### 1.4.5.3 $CD4^+$ T cell mechanisms in EAU

Exactly how these  $CD4^+$  T cells, when injected peripherally, induce EAU remains unclear. Studies using radioisotope labelling to follow the migration of the  $CD4^+$  T cells after adoptive transfer of EAU found only very few of the labelled cells reached the eye [Lightman et al., 1987; Palestine et al., 1986]. Thus a clonal expansion of the inoculated

autoreactive T cells within the tissue has been suggested as the amplification mechanism whereby very few cells can induce EAU in syngeneic hosts [Caspi, 1989].

In EAU, successful adoptive transfer of disease requires that T cells are activated. Furthermore, it is likely that T cells need to be activated to cross the BRB similar to findings in blood brain barrier [Hickey et al., 1991]. The reason for this requirement is not well understood, but it may involve the effector cytokines secreted by these CD4<sup>+</sup> T cells. It seems that at the early stages of the disease the first lymphocytes through will release cytokines which will induce a highly localized increase in expression of endothelial cell adhesion molecules. This will, in turn, bring about further recruitment of inflammatory cells and a further increase in the local concentration of cytokines. A more widespread stimulation of the endothelium will ensue, propagating an increase in cellular infiltration and perpetuation of the cycle [Greenwood, 1992].

Cytokines secreted by CD4<sup>+</sup> T cells are believed to play a very important role in regulating immune responses. For example, induction or augmentation of expression of MHC class II antigen expression and adhesion molecules requires local cytokine production. In addition, chemo-attraction of cytotoxic cells, such as macrophages, natural killer cells and cytotoxic T cells, all of which cause tissue damage, is dependent on cytokine production, predominantly by CD4<sup>+</sup> T cells.

## **1.5 Possible roles of cytokines in the pathogenesis of EAU**

### **1.5.1 Introduction**

Cell-free soluble factors, which are generated during interaction of sensitized lymphocytes with specific antigen, were discovered in 1969 and termed lymphokines [Dumonde et al., 1969]. Lymphokines were originally thought to be produced only by lymphocytes and to communicate with other cells of the immune system. It is now clear that lymphokines are also produced by non-lymphoid cells, affecting the growth or functions of many types of cells. For these reasons, the more general term "cytokines" is often used now to refer to all cell-derived soluble factors. These factors modify the activity of the same cells that produce them in an autocrine fashion, as well as modulate responses of other cells in a paracrine fashion. It has been suggested that cytokines may

be involved in both the generation and the maintenance of autoimmune responses, as well as in the final effector mechanisms acting on target cells (reviewed in [Cavallo et al., 1994]). Cope et al. reported that anti-TNF treatment in patients with active rheumatoid arthritis (RA) restored the diminished proliferative responses of peripheral blood mononuclear cell to mitogens and recall antigens towards normal, suggesting that TNF plays very important role in the pathogenesis of RA [Cope et al., 1994]. The recent advance of embryonic stem cell technology has made it possible to study the *in vivo* functions of cytokines through mouse mutants specifically deficient for a given cytokine. Mice deficient for IL-2, IL-10 and TGF- $\beta$ 1 are born without any obvious abnormalities, but later develop autoimmune disease. TGF- $\beta$ 1-deficient mice suffer from inflammation in multiple organs, mainly heart, liver, lungs and muscle [Shull et al., 1992]. In IL-10-deficient mice, most animals are growth retarded, anemic and suffer from chronic enterocolitis [Kuhn et al., 1993]. Similar to IL-10-deficient mice, mutants deficient for IL-2 also develop a chronic bowel inflammation, probably by a different pathogenetic mechanism [Sadlack et al., 1993]. All these provided insights into the essential functions of these cytokines for the immune system.

### **1.5.2 Biological properties of some important cytokines**

Many cytokines have been identified so far. The following is a description of the cytokines related to work carried out in this thesis.

#### **1.5.2.1 IL-2**

IL-2, originally, called T cell growth factor, is a lymphokine synthesized and secreted by T cells following activation with antigen or mitogen [Morgan et al., 1976]. IL-2 allows the long-term growth *in vitro* of T cells [Gillis et al., 1978]. In order to exert its biological effects, IL-2 must interact with its specific membrane receptor which is synthesized during T cell activation [Robb et al., 1981]. This interaction is critical to the development of a normal immune response. Binding of IL-2 by T cells results in proliferation of these cells and enhanced secretion of other lymphokines such as IFN- $\gamma$  and lymphotoxin. IL-2 can also induce T lymphocyte cytotoxicity [Zarling & Bach, 1979] and stimulate natural killer cell activity [Henney et al., 1981; Ortaldo et al., 1984]

and activate B cells [Coffman et al., 1988]. IL-2 has anti-tumour effects, derived from its ability to stimulate the cytotoxic activity of lymphokine-activated killer (LAK) cells [Malkovsky et al., 1987], but no direct cytotoxic or cytostatic effects. IL-2 may play a major role in the immune response to viral and mycobacterial infections [Jeevan & Asherson, 1988; Nabavi & Murphy, 1986]. It has been noted that in addition to IL-2 as T cell growth factor, there are several cytokines such as IL-4 [Kupper et al., 1987], IL-12 [Trinchieri, 1993], IL-7 [Londei et al., 1990] and IL-6 [Tosato and Pike, 1988; Lotz, 1988] which promote the growth of T cells.

#### 1.5.2.2 IL-4

IL-4 plays a pivotal role in the functions of B cells in the immune system. It causes activation, proliferation and differentiation of B cells [Hamaoka & Ono, 1986; Jelinek & Lipsky, 1987], and leads to the production and secretion of IgG1 and IgE antibodies [Lebman & Coffman, 1988; Vitetta et al., 1985]. However IL-4 also has the property of inhibiting some B cell responses such as inhibiting the production of IgG2a, IgG2b, IgG3 and IgM [Snapper & Paul, 1987]. In addition to its capacity to support B cells, IL-4 increases the viability, and stimulates the growth of T lymphocytes [Paul & Ohara, 1987; Yokota et al., 1988] and some T cell lines [Kupper et al., 1987]. IL-4 can induce the expression of Ia (MHC class II molecule) on B cells [Roehm et al., 1984; Rousset et al., 1988] and macrophages [Zlotnik et al., 1987] to enhance their ability to present antigens to T cells. It has been shown that IL-4 is active in the development of cytotoxic T cells [Pfeifer et al., 1987; Widmer & Grabstein, 1987]. IL-4 also inhibits the synthesis of IFN- $\gamma$  by human peripheral blood mononuclear cells and mouse CD4<sup>+</sup> T cells, and stimulates the degranulation of mast cells [Powrie & Coffman, 1993].

#### 1.5.2.3 Interferon- $\gamma$

Interferons were initially identified for their ability to "interfere" with viral replication in infected cells. There are three kinds of interferons: IFN- $\alpha$ , IFN- $\beta$  and IFN- $\gamma$ . Although the antiviral activity of IFN- $\gamma$  is important, its other functions in the immune system are considered as being of equal or greater importance. One of the immunological activities of IFN- $\gamma$  is its ability to induce and augment MHC class II antigen expression on a variety of cells [Basham & Merigan, 1983; Detrick et al., 1985; Pober et al., 1983]. In

addition to its effects on MHC class II expression, IFN- $\gamma$  stimulates macrophage activity [Nathan et al., 1983] and cytolytic T lymphocyte differentiation [Chen et al., 1986; Simon et al., 1986].

IFN- $\gamma$  has a strong inhibitory effect on B cells, in particular inhibiting most or all of the effects of IL-4 on B cells [Coffman & Carty, 1986; Mond et al., 1986; Rabin et al., 1986]. In addition to the inhibition of IL-4 activities, IFN- $\gamma$  also has positive effects on B cells, such as the stimulation of antibody secretion and the specific enhancement of IgG2a production [Snapper & Paul, 1987].

#### 1.5.2.4 IL-10

Mouse IL-10 is produced by Th2 cells, B cells, macrophages/monocytes and keratinocytes [De Waal Malefyt et al., 1992; Zlotnik & Moore, 1991], and was originally called cytokine synthesis inhibitory factor (CSIF). It can inhibit the production of IL-2, IL-3, lymphotoxin (LT), IFN- $\gamma$  and granulocyte-macrophage CSF (GM-CSF) by Th1 cells responding to antigen and APC, but Th2 cytokine synthesis is not significantly affected [Fiorentino et al., 1989]. IL-10 inhibits the antigen-presenting capacity of monocytes through downregulation of class II MHC expression and in this way prevents antigen-specific T cell proliferation and cytokine production by these cells [De Waal Malefyt et al., 1992; Fiorentino et al., 1991]. IL-10 also directly affects the function and growth of T cells, B cells and mast cells [Spits & de Waal Malefyt, 1992]. In humans, IL-10 is not restricted to Th2 cells, since most Th0 and Th1 clones are also able to produce considerable levels of IL-10 following antigen or mitogen stimulation [De Waal Malefyt et al., 1992].

#### 1.5.2.5 TGF- $\beta$

TGF- $\beta$ , originally described as a factor that induced phenotypic transformation of fibroblast-like cells, appears to play a central role in embryonic development, tumorigenesis, wound healing and fibrosis [Nilsen-Hamilton, 1990]. It is a pleiotropic factor that can stimulate or inhibit the proliferation and differentiation of various cells. Furthermore, it appears to play an important role in immunoregulation. TGF- $\beta$  has several immunosuppressive properties, such as inhibition of proliferation of T and B



lymphocytes and inhibition of macrophage activation [Miller et al., 1992; Wahl et al., 1989]. Recently it was also shown that TGF- $\beta$  mediates T cell suppressor activity [Miller et al., 1992]. In addition to its immunosuppressive effects, TGF- $\beta$  also has inflammatory properties, such as chemotaxis and activation of monocytes [Wahl et al., 1989].

There are five isoforms of TGF- $\beta$ , however only three (TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3) have been described in man and other mammals. TGF- $\beta$  is mainly secreted in a latent form and can be activated by plasmin and cathepsin proteases or transient exposure to acid conditions [Wahl, 1992].

#### 1.5.2.6 Other cytokines

A few other cytokines, including tumor necrosis factor (TNF), IL-1 and IL-6 will be briefly summarized here.

TNF- $\alpha$  (TNF) and TNF- $\beta$  (lymphotoxin, LT) are mainly produced by T cells and macrophages. These two cytokines can cause direct killing of certain transformed cell lines *in vitro* and destruction of solid tumor tissue *in vivo*. TNF may also be involved in the activation of PMN and induction of expression MHC class I and II and ICAM-1 [Mosmann, 1989].

IL-1, originally called lymphocyte activating factor (LAF), is produced by a variety of different cell types, including macrophages, lymphocytes, fibroblasts, neutrophils and endothelial cells. IL-1 can be regarded as a basic mediator of intercellular communication, both within the immune system as well as between the immune system and almost all other organ systems [Di Giovine & Duff, 1990]. Its inflammatory effects may be activation of granulocytes and lymphocytes, chemotaxis of lymphocytes and monocytes and induction of endothelial cells to synthesize prostaglandins and express ICAM-1 on their surface.

IL-6 can be produced by a variety of cells, both immune cells, such as macrophages, monocytes, lymphocytes and PMN, as well as by other cells, like fibroblasts, epithelial cells, endothelial cells and smooth muscle cells. It can activate B cells, induce T cell activation and differentiation and macrophage differentiation [Mosmann, 1989].

### **1.5.3 Interactions between cytokines**

It has been shown that most of the cytokines have pleiotropic biological effects that overlap with each other and act on many different cell types. Likewise, one type of cell may express various cytokine receptors and can respond to multiple cytokines. In addition, some cytokines are produced by a number of different cell types. All these studies suggest that cytokines interact in a complex network, in which they can induce the production of other cytokines, modulate the expression of cytokine receptors or have synergistic or antagonistic effects on other cytokines [Balkwill & Burke, 1989].

Synergistic interactions are more likely to occur between cytokines that exert related but not identical actions than between cytokines that are closely related functionally. For example, IL-2 and IFN- $\gamma$  synergize in the generation of cytotoxic effectors CTL [Takai et al., 1986]. In the presence of lymphotoxin (LT), IFN- $\gamma$  has more potent growth inhibitory and cytolytic activities [Stone-Wolff et al., 1984]. The interaction between cytokines may also induce a response not seen with either signal alone, allowing such a response to be more tightly regulated. For example, although normal human pancreatic islet cells do not express class II when stimulated by IFN- $\gamma$  alone, in combination with tumor necrosis factor (TNF) the class II MHC gene is upregulated [Pujol-Borrell et al., 1987].

Lymphokines can also act antagonistically to each other. IFN- $\gamma$  suppresses many of the B cell activation and differentiation activities of IL-4 [Rabin et al., 1986; Snapper & Paul, 1987] and, conversely, IL-4 suppresses the isotypes of Ig that IFN- $\gamma$  enhances [Snapper & Paul, 1987] and interferes with IFN- $\gamma$  action. TGF- $\beta$  inhibits the generation of cytotoxic T cells and its inhibition can be reversed by the addition of exogenous TNF- $\alpha$  [Ranges et al., 1987]. IL-10 inhibits lymphokine secretion by Ag/APC-stimulated Th1 cells [Fiorentino et al., 1989; Fiorentino et al., 1991].

Cytokines therefore play a complex role in the regulation of immune and inflammatory responses [Mosmann, 1989; Scott & Kaufmann, 1991]. Thus as for the regulation of cell growth, the effect which a particular mediator exerts depends on its target cell, its

state of activation and the presence of other cytokines [Sporn & Roberts, 1988]. Therefore meaningful results cannot be obtained from studying the regulation of a single cytokine in isolation since the results are unlikely to apply *in vivo*. Cells *in vivo* are exposed to a variety of cytokines which may have synergistic or antagonistic effects, although relatively little is known concerning the precise nature of how these signals modulate the response to injury or infection.

#### **1.5.4 Cytokines and autoimmunity**

A striking feature of many autoimmune diseases including rheumatoid arthritis, multiple sclerosis, diabetes mellitus and posterior uveitis [Feldmann & Londei, 1989; Lightman & Chan, 1990] is the enhanced expression of MHC class II antigen on non-lymphoid cells such as endothelial and epithelial cells which normally do not express it. This led to the proposal that the inappropriate expression of MHC class II allowed the target tissue cells to act as antigen presenting cells in autoimmunity, presenting previously sequestered autoantigens to autoreactive T cells [Bottazzo et al., 1983]. It has been shown that CD4<sup>+</sup> T cells can recognise class II molecules on non-traditional accessory cells and this recognition results in activation of the T cell [Wagner et al., 1984]. One of the principal modulating influences on MHC expression *in vitro* and *in vivo* is probably IFN- $\gamma$  [Basham & Merigan, 1983; Pober et al., 1983]. In the eye, there is evidence that intravitreal injection of IFN- $\gamma$  can induce MHC class II expression on rat ocular tissues [Hamel et al., 1990; Lee & Pepose, 1990], and retinal pigmental epithelial (RPE) cells in human [Liversidge et al., 1988a] and rat and guinea pig [Liversidge et al., 1988b] can be induced to express class II antigens *in vitro* when stimulated by IFN- $\gamma$ . The importance of expression of MHC-class II has been demonstrated by *in vivo* treatment with mAbs where anti-Ia (MHC class II) antibodies can effectively inhibit EAU by blocking class II-mediated processes [Rao et al., 1989; Wetzig et al., 1988]

#### **1.5.5 Cytokines and adhesion molecules**

Cytokines such as TNF- $\alpha$ , IL-1 and IFN- $\gamma$  can act as communication signals between leukocytes and endothelial cells [Mantovani & Dejana, 1989]. They can increase expression of adhesion molecules such as intercellular cell adhesion molecule (ICAM)-1, endothelial adhesion molecule (ELAM)-1 and vascular adhesion molecule (VCAM)-1 on

endothelial cells, which could facilitate the homing of lymphocytes to sites of inflammation (reviewed in [Issekutz, 1992]). In the retina, T cells have to traverse the BRB in order to arrive at the target cell i.e. the photoreceptor cell. Human RPE cells constitutively express ICAM-1, and retinal endothelial cells can be induced by IFN- $\gamma$  to express ICAM-1 antigen on their cell surfaces [Forrester, 1992]. Blockade of this molecular response by anti-ICAM-1 specific mAbs inhibits adhesion of activated T lymphocytes [Liversidge et al., 1990]. Interaction between ICAM-1 and LFA-1 are therefore probably important in directing lymphocyte traffic during ocular immune responses [Forrester, 1992].

### **1.5.6 Role of cytokines in inflammation of the eye**

#### **1.5.6.1 Detection of cytokines during posterior uveitis and EAU**

Immunohistochemical analysis have shown that elevated expression of IL-2 and IFN- $\gamma$  was detected in inflamed ocular material obtained from patients with uveitis [Hooks et al., 1988; Mondino et al., 1990]. Increased expression of IL-2R on peripheral blood lymphocytes of patients with posterior uveitis [Feron et al., 1992] and elevated levels of soluble IL-2R (sIL-2R) in serum of patients with uveitis [Arocker-Mettinger et al., 1990] have been reported. In aqueous humor and vitreous, obtained from patients with uveitis, IL-6 could be detected [Murray et al., 1990; Van Der Lelij et al., 1991]. In EAU, it has been shown that IFN- $\gamma$ , IL-2, LT and IL-4 mRNA could be detected using *in situ* hybridization within the retina during disease [Charteris & Lightman, 1992 and 1993].

#### **1.5.6.2 Inflammatory properties of intraocular injected cytokines**

Several cytokines, including IL-1, IL-6, IFN- $\gamma$  and TNF, have been shown to have inflammatory properties. Brosnan et al. [Brosnan et al., 1989] and Martiney et al. [Martiney et al., 1990] injected recombinant human (rh) IL-1 $\beta$  into the vitreous of rabbits and observed uveitis. Intravitreal injection of rhIL-6 in rats [Hoekzema et al., 1990; Hoekzema et al., 1991; Hoekzema et al., 1992] and rabbits [Hoekzema et al., 1990; Malecaze et al., 1991] resulted in uveitis. Intravitreal injection of recombinant rat IFN- $\gamma$  in rats caused an infiltrate of cells in the inner retinal layers [Hamel et al., 1990].

Analysis of intravitreal injection of TNF in rats showed an infiltrate of PMN and MNL cells in the retina [De Vos et al., 1992].

#### **1.5.6.3 Prevention of EAU by inhibition of cytokine activities**

As mentioned above, cytokines are involved in the pathogenesis of inflammatory eye diseases. Evidence that inhibition of cytokine activity by specific inhibitors, such as antibodies, receptor antagonists and soluble receptors, may reveal the exact contribution of cytokines to uveitis. Martiney et al. showed a considerable reduction of the cellular inflammation in rabbit retina when IL-1 was injected together with anti-IL-1 antibodies [Martiney et al., 1990]. The results reported by another group showed that intravitreal injection of IL-1R antagonist almost completely reduced the inflammatory response induced by intravitreally injected IL-1 in rabbits [Rosenbaum & Boney, 1992]. Rosenbaum et al. also investigated the effect of soluble IL-1 receptor (sIL-1R) on IL-1-induced uveitis in rabbits [Rosenbaum & Boney, 1991]. Intravitreal injection of soluble human IL-1R at the same time as recombinant human IL-1 reduced markedly both cellular infiltration and protein extravasation. Intravitreal injection of IL-2-PE40, which is a fusion protein of IL-2 with the toxic part of *Pseudomonas* exotoxin, inhibited protein synthesis in cells after binding to the IL-2R on cells, and caused a marked reduction of the incidence and severity of EAU [Roberge et al., 1989]. The successful prevention of EAU in Lewis rats with anti-IFN- $\gamma$  or IL-2R mAbs demonstrate the importance of these cytokines in the pathogenesis of EAU [Atalla et al., 1990; Higuchi et al., 1991].

Finally, the dramatic suppressive effect of cyclosporin A in controlling uveitis has been repeatedly demonstrated in both EAU [Nussenblatt et al., 1982] and human disease [Nussenblatt et al., 1983]. This immunosuppressive drug is known to inhibit the synthesis of several cytokines, particularly IL-2 and IFN- $\gamma$  [Shevach, 1989] thus interfering with T cell activation.

### **1.6 Current knowledge of cytokine secretion patterns**

#### **1.6.1 Heterogeneity of CD4<sup>+</sup> T cells**

Mouse CD4<sup>+</sup> T cell clones can be distinguished by their lymphokine production

patterns. Two distinct, mutually exclusive lymphokine production profiles were defined. Type 1 helper (Th1) cells produce IL-2 and IFN- $\gamma$  and LT, whereas type 2 helper (Th2) cells secrete IL-4, IL-5, IL-6 and IL-10 [Cherwinski et al., 1987; Mosmann et al., 1986]. The existence of Th1 and Th2 cells was originally demonstrated *in vitro* [Mosmann et al., 1986]. More recently, Th1 and Th2 immune responses were also shown to occur *in vivo* [Mosmann & Coffman, 1989a; Street et al., 1990]. Generally, Th1 responses are preferentially associated with delayed-type hypersensitivity (DTH), whereas Th2 responses are associated with high antibody levels [Mosmann & Coffman, 1989b]. In addition to T cell clones that fit the Th1 or Th2 classification, a Th0 cell subset has also been described whose cells produce almost all cytokines and are probably precursors of the Th1 and Th2 cells [Firestein et al., 1989].

In humans, there is now considerable evidence for CD4<sup>+</sup> T cell clones with cytokine patterns and functions that are comparable to murine Th0, Th1 and Th2 cells, although the expression of a few cytokines, such as IL-2 and IL-10, may be less restricted [Yssel et al., 1992]. Based upon recent *in vivo* reports, it is likely that Th1 and Th2 type cells exist likewise in rats [Papp et al., 1992; Saoudi et al., 1993], although Th1 and Th2 subtypes have not yet been cloned.

In the rat, CD4<sup>+</sup> T cells can be functionally divided into naive (OX22<sup>high</sup>) and memory (OX22<sup>low</sup>) subsets based on reaction with MRC OX22 mAb [Spickett et al., 1983]. The OX-22<sup>high</sup>CD4<sup>+</sup> subset has been described as Th1-like cells (high IL-2 and IFN- $\gamma$  production but low IL-4) whereas the OX-22<sup>low</sup>CD4<sup>+</sup> subset as Th2-like cells (the provision of B-cell help but low IL-2 and IFN- $\gamma$  production) [Fowell et al., 1991]. However, this classification is not based on lymphokine expression. It has become increasingly clear that these subsets do not represent homogeneous populations [Powrie et al., 1991].

### **1.6.2 Reciprocal regulation of Th subsets**

Evidence from a number of recent studies suggests that the differentiation and/or growth of Th1 cells might be inhibited during a strong Th2 response, and *vice versa*. The separation of immune responses into DTH or antibody responses has been thoroughly documented [Crowle & Hu, 1966; Heinzel et al., 1989; Katsura, 1977; Parish, 1972].

These two types of reactions generally are mutually exclusive, which has led to the hypothesis that they are reciprocally regulated *in vivo*. Recently, it has become clear that these two arms of the immune response are regulated by Th1 and Th2 CD4<sup>+</sup> T cell subsets [Powrie & Coffman, 1993]. As described above, Th1 cells effectively mediate DTH reactions whereas Th2 cells are the major regulators of antibody production. The distinct array of cytokines produced by each of these subsets dictates their effector functions and also plays a major role in inhibiting the induction and effector functions of the reciprocal subset. For example, IFN- $\gamma$ , a Th1 product, inhibits the proliferation of Th2 but not Th1 cells [Gajewski & Fitch, 1988] and IL-10 inhibits the synthesis of IFN- $\gamma$  and other cytokines by Th1 clones [Fiorentino et al., 1991]. IL-4 also inhibits the synthesis of IFN- $\gamma$  by human peripheral blood mononuclear cells and mouse CD4<sup>+</sup> T cells [Powrie & Coffman, 1993]. It is apparent that cytokine synthesis by the Th subsets is tightly regulated to maintain the critical balances necessary for immune cells to function.

### **1.6.3 Selective activation and differentiation of Th cell subsets**

Several factors can influence the development of Th subsets, including the kind of antigen, antigen concentration, the signalling pathway, the route of immunization and/or the nature of APC, the presence of exogenous lymphokines, and the genetic factors.

#### **1.6.3.1 The role of structure of antigen and antigen concentration**

It is becoming clear from a number of recent studies that *in vivo* somehow the nature of the stimulating antigen is important for the selection of the responding T cell subset. Most T cell clones specific for *Dermatophagoides pteronyssinus* or for *Toxocara canis* excretory-secretory antigen generated from atopic patients or healthy individuals secrete high levels of IL-4 and IL-5, whereas T cell clones specific for tetanus toxoid or *Mycobacterium* secrete IFN- $\gamma$  and IL-2 but not IL-4 [Del Prete et al., 1991; Romagnani, 1991; Wierenga et al., 1990]. It is intriguing to speculate that the physicochemical properties of the stimulating antigen might be responsible for the selection of T cell phenotype versus the other [Baum et al., 1990; Liew et al., 1990]. Furthermore, it has been found that human CD4<sup>+</sup> T cell clones specific to bee venom phospholipase A2 from allergic and hypersensitized individuals could produce both IL-4 and IFN- $\gamma$  at a higher

concentration of antigen, whereas at low antigen dose, only IL-4 was produced [Carballido et al., 1992], suggesting that different CD4<sup>+</sup> T cell subsets (on the basis of lymphokine production) may occur for the same antigen responses.

#### 1.6.3.2 The signalling pathway

The notion that CD4<sup>+</sup> T cells can independently regulate the profile of lymphokines produced has been reported as dependent upon the type and intensity of the activation signal. Data from Yokoyama et al. showed that some Th2 clones in the mouse could produce IFN- $\gamma$  and IL-2 after stimulation with immobilized anti-CD3 mAb [Yokoyama et al., 1989]. It has also been found that there is altered lymphokine mRNA expression in human HBs-antigen specific CD4<sup>+</sup> T cell clones after specific antigen (HBs) and non-specific mitogen stimulation [Tsutsui et al., 1991]. Similarly results reported by another group suggested that CD4<sup>+</sup> T cells producing IL-2 and IFN- $\gamma$  (Th1-like) or IL-4 (Th2-like) may have different requirements for activation [Monteyne et al., 1992]. They analysed the expression of IL-4 and IFN- $\gamma$  mRNA in anti-CD3 stimulated mouse spleen cells and observed a shift in the lymphokine pattern induced in the presence of PMA. Therefore, it is possible that mechanisms exist for the selective production of appropriate cytokines in the response by the same cells to the different stimuli.

#### 1.6.3.3 The route of immunization and/or the nature of APC

Differences in antigen processing have also been used to explain why some antigens induce Th1 cytokines while other antigens cause activation of Th1 cells. Studies demonstrated that mice immunized intravenously with killed whole *Leishmaniasis major* promastigotes (or their soluble antigen extract) developed substantial resistance to a challenge infection [Howard et al., 1982]. The protection is mediated by the equivalent of Th1 cells. In contrast, the same antigen preparation injected subcutaneously was not only ineffective but induced a Th2-like population which inhibited the induction of effective protection by the i.v. route of immunization, or the transfer of immunity by protective T cells [Liew et al., 1985]. It has also been shown that clonal expansion of ovalbumin-specific Th1 and Th2 cells was preferentially stimulated by two distinct APC; Th1 cells stimulated by macrophages and dendritic cells and Th2 cells stimulated by B



cells [Gajewski et al., 1991].

#### 1.6.3.4 The cytokine environment.

The cytokine environment present during differentiation may have an important influence on the type of Th cell that will be generated. Gajewski et al. [Gajewski et al., 1989] have shown that Th1 cells are preferentially obtained when CD4<sup>+</sup> cells are cloned in the presence of IFN- $\gamma$ . Conversely, the presence of IL-4 during Th effector generation *in vitro* leads to a strong polarization of the T cells towards the Th2 subset [Swain et al., 1991].

#### 1.6.3.5 Genetic factors

Genetic factors appear to play an important role at least in some experimental models. Following infection with *Leishmania major*, BALB/c mice develop a Th2-type immune response with IL-4 mRNA containing cells in the draining lymph node and in the spleen and suffer from progressive disease, whereas C57BL/6 mice, which are able to control the infection, develop a Th1-type response with strong expression of IFN- $\gamma$  mRNA but no expression of IL-4 mRNA in both organs [Heinzel et al., 1991]. In another model, mice differing in their MHC class II haplotype respond differently to the same antigen with regards to IL-4 and IFN- $\gamma$  production [Murray et al., 1989].

### 1.6.4 Molecular differences between Th1 and Th2 cells

Several lines of evidence exist which show differences in the signal transduction apparatus between Th1 and Th2 clones. Elevation of cytosolic free Ca<sup>2+</sup> and the generation of inositol phosphates in response to ConA or to a T cell receptor antibody was demonstrated in Th1 clones, but not in Th2 clones [Gajewski et al., 1990]. A CD3 antibody induced unresponsiveness to IL-2 in Th1 clones but not in Th2 clones [Williams et al., 1990]. Whereas induction of anergy in Th1 cells led to abrogation of a subsequent proliferative response to antigen plus APC, cell proliferation of Th2 cells remained unimpaired under such conditions [Gilbert et al., 1990].

### 1.6.5 Inflammatory autoimmune diseases

Several animal models of human inflammatory autoimmune diseases suggest that preferential activation of Th1 responses is central to the pathogenesis of these diseases. Experimental allergic encephalomyelitis (EAE), an animal model of multiple sclerosis in humans, is a CD4<sup>+</sup> T cell mediated autoimmune disease of the CNS. T cell lines and clones that transfer EAE in the rat and the mouse produce the Th1 cytokines IFN- $\gamma$ , IL-2, TNF- $\alpha$  and TNF- $\beta$  [Ando et al., 1989; Sedgwick et al., 1989] and these cytokines are present in the CNS of animals with active disease [Khoury et al., 1992]. There is also evidence of Th1 cell involvement in insulin-dependent diabetes mellitus (IDDM) in humans and in animal models of the disease. IFN- $\gamma$  production correlates with diabetes in non-obese diabetic mice whereas anti-IFN- $\gamma$  prevents disease induction [Campbell et al., 1991]. A high frequency of IFN- $\gamma$ -containing lymphocytes (40%) infiltrating the pancreatic islet cells were detected in autopsy pancreases from patients with IDDM [Foulis et al., 1991]. However, there is little information regarding regulation of cytokine expression by uveitogenic S-Ag specific CD4<sup>+</sup> T cells in the rat except for the Charteris work previously discussed (see [section 1.5.6.1](#)) and Savion et al. work which showed Th1-like cytokines in their uveitogenic cell lines (see [section 3.3](#)).

### **1.7 Aims of this study**

As discussed in the previous sections of this chapter, there are several sets of data that implicate CD4<sup>+</sup> T cells in the pathogenesis of EAU. Firstly, active EAU (i.e. that induced by the injection of S-Ag/CFA into the test animal) was shown to be completely preventable by intravenous administration of anti-CD4 mAb or anti-Ia (MHC class II) mAbs. Secondly, passive EAU was shown to be inducible in syngeneic Lewis strain recipients by the intraperitoneal injection of lymph node cells or spleen cells from S-Ag immunized animals after culturing the donor cells in the presence of S-Ag or mitogen (ConA). The identity of the lymphocytes that transferred disease was determined to be of the CD4<sup>+</sup> T cell subclass and not of the CD8<sup>+</sup> T cell subclass. Serum could not transfer disease. Furthermore, adoptive transfer of EAU in Lewis rats can be achieved using activated CD4<sup>+</sup> T cell lines specific for S-Ag. Thirdly, depletion of CD8<sup>+</sup> T cells by repeated injections of anti-CD8 mAb had no effect on the induction of EAU. All these results taken together suggest that CD4<sup>+</sup> T cells are important in both inducing disease and perpetuating the disease process. However the exact mechanisms by which these

CD4<sup>+</sup> T cells cause damage within the eye are still unclear. It is likely that the process involves the production of effector lymphokines by CD4<sup>+</sup> T cells since *in vitro* activation of CD4<sup>+</sup> T cells is required for successful adoptive transfer of disease. In order to further understand the pathogenic mechanisms involved in EAU, S-Ag specific CD4<sup>+</sup> T cell lines and clones were established from Lewis rats immunized with S-Ag in CFA and were used to examine the regulation of lymphokine expression with antigen or mitogens.

In the rat, CD4<sup>+</sup> T cells could be functionally divided into OX22<sup>high</sup> and OX22<sup>low</sup> subsets based on phenotypic expression of CD45 isoforms. It has been shown that the OX-22<sup>high</sup>CD4<sup>+</sup> subset has a Th1-like lymphokine repertoire (high IL-2 and IFN- $\gamma$  production but low IL-4) whereas the OX-22<sup>low</sup>CD4<sup>+</sup> population has characteristics of Th2-like cells (the provision of B-cell help but low IL-2 and IFN- $\gamma$  production). Yet it is unclear if their patterns of lymphokine expression depend on the mode of stimuli. For this reason, lymphokine expression by OX22<sup>high</sup> and OX22<sup>low</sup> CD4<sup>+</sup> T cells by different stimuli was investigated.

Apart from inflammatory cytokines, immunosuppressive factors such as transforming growth factor- $\beta$  (TGF- $\beta$ ) could play an important role in the down-regulation of EAU. To examine this further, the *in vivo* role of TGF- $\beta$ 1 on the course of EAU was also examined.

The purposes of this study:

1.7.1 Investigate the lymphokine expression by rat S-Ag T-cell lines and T-cell clones, to see if there is restricted lymphokine expression in these S-Ag specific T cells and attempt to assess the classification of CD4<sup>+</sup> T-cells based on cytokine expression patterns in the rat.

1.7.2 Analyze the kinetics of the lymphokine expression in S-Ag specific T cells in response to specific antigen (S-Ag) or non-specific mitogens (ConA and PMA).

1.7.3 Determine lymphokine expression by OX22<sup>high</sup> and OX22<sup>low</sup> CD4<sup>+</sup> T cells in response to different stimuli.

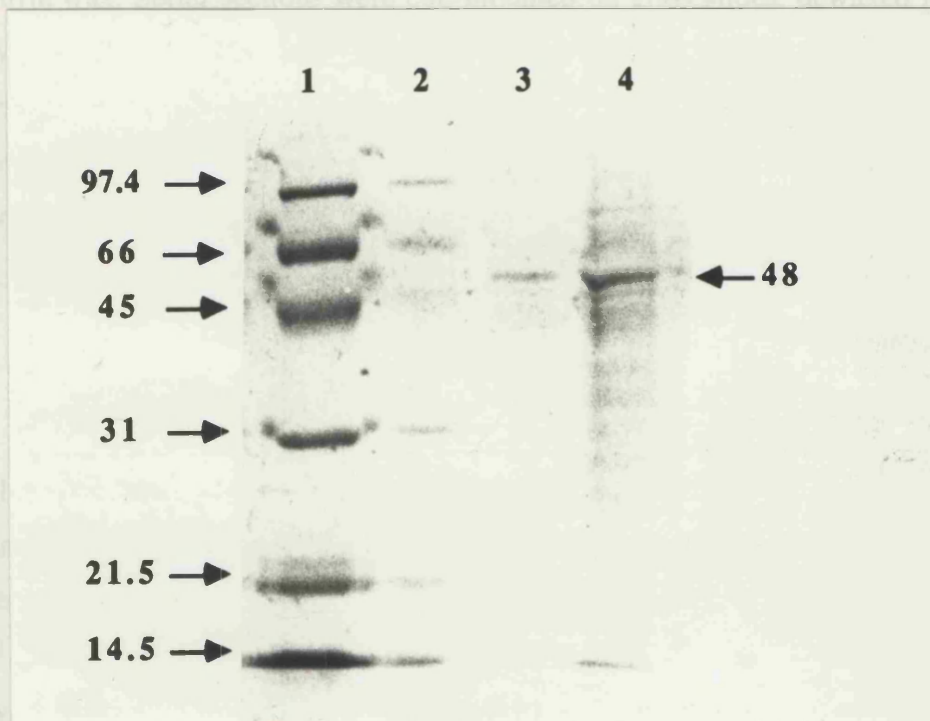
1.7.4 Study the effect of TGF- $\beta$  administration on EAU development.

## Chapter 2 Materials and methods

### 2.1 S-Ag preparation and induction of EAU

#### 2.1.1 S-Ag preparation

Retinal tissue was obtained from fresh bovine eyes and retinal S-Ag was purified using a modified method described elsewhere [Calder et al., 1993]. Briefly, 50-100 retinae were homogenized, protein precipitated by ammonium sulphate at 4°C and large insoluble proteins removed by ultracentrifugation. Following separation by gel chromatography, the positive fractions were screened for the presence of S-Ag by ELISA and by immunodiffusion on Micro-Ouchterlony plates using a monoclonal anti-S-Ag antibody (a gift from J. Liversidge, Aberdeen, U.K.) and those which were positive were pooled and fractionated by ion-exchange chromatography. Protein was concentrated by dialysis in 35% polyethylene glycol, aliquoted and stored at -70°C. Fig.2-1 shows SDS gel electrophoresis of S-Ag. A predominant band (MW 48,000) was observed.



**Figure 2-1** SDS gel electrophoresis of purified bovine retinal soluble antigen. Lanes 1 and 2: molecular weight; Lanes 3 and 4: samples.

### **2.1.2 Induction of EAU**

Purified bovine S-Ag after preparation as above was tested *in vivo* by inoculating Lewis rats with 50 µg of this antigen in each hind foot pad and the base of tail in a 1:1 (v/v) emulsion in complete Freund's adjuvant (CFA) containing 2.5 mg/ml of heat-killed *Mycobacterium tuberculosis* (strain H37Ra). Animals were also given 5x10<sup>9</sup> killed *Bordetella pertussis* organisms intraperitoneally. Control rats included naive animals and animals treated with CFA emulsified in phosphate-buffered saline (PBS) and immunized with and without *B. pertussis*. Disease onset usually occurred at days 12-14 with peak inflammation at days 14-17 and by days 21-25 no evidence of disease.

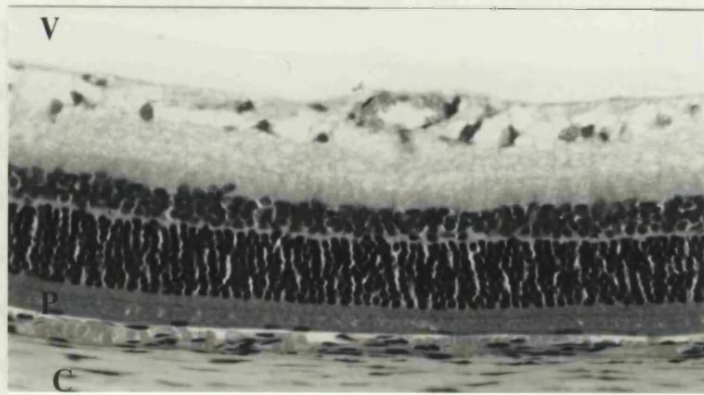
### **2.1.3 Histology of rat retina by light microscopy**

Rats were killed by carbon dioxide asphyxiation after day 12-15 post-immunization. Eyes were enucleated for histology, fixed in 4% glutaraldehyde for 15 min followed by 10% formaldehyde at least 48 h, dehydrated through graded alcohols and embedded in paraffin wax. Serial sections were cut, mounted on glass slides, dewaxed in xylene, rehydrated through graded alcohols to water, stained with haematoxylin and eosin for assessing the cellular infiltration/ inflammation, dehydrated through graded alcohols, cleared in xylene and mounted in resin under cover slips. All rats immunized with S-Ag developed EAU (Fig.2-2 c and d). There was no disease in control groups (Fig.2-2 a and b).

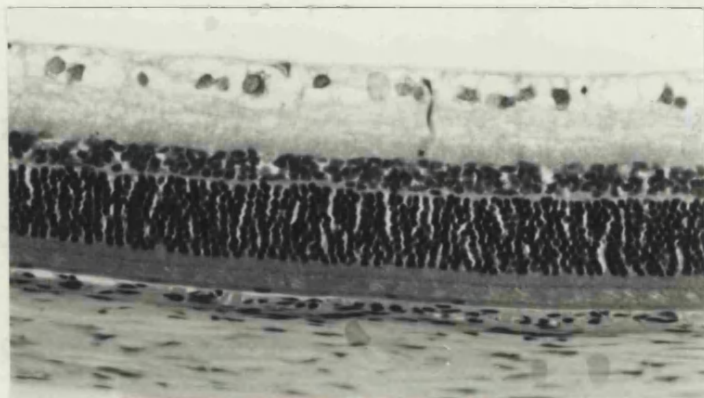
## **2.2 Cell counting**

Cell counts were performed using a Neubauer haemocytometer. Cell viability was estimated by diluting the cells 1:1 in 0.4% trypan blue. Dead cells stained blue while viable cells remained unstained, thus allowing a viability count as: Number viable cells ml<sup>-1</sup> = (number cells counted/ number triple-ruled squares) x 25 x10<sup>4</sup> [see Hudson & Hay, 1989a].

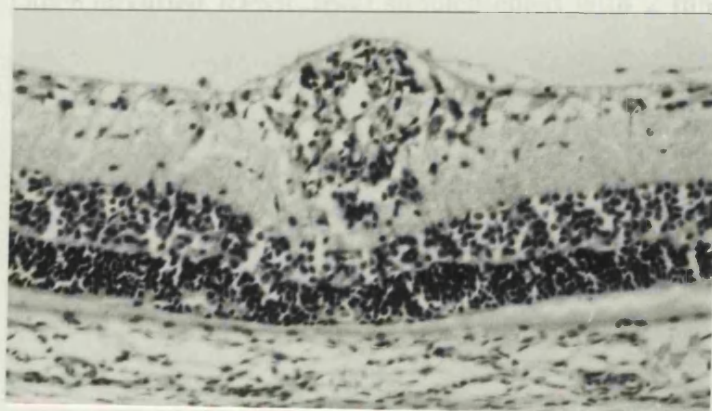
## **2.3 Freezing and thawing of T cells**



**Figure 2-2A** Histology of enucleated eyes from normal Lewis rats. A retina with its intact layers from the outer choroid layer (C) through the photoreceptor layer (P) to the vitreous (V). No inflammatory cells are present in the retina. Magnification x250.



**Figure 2-2B** Histology of enucleated eyes from control CFA- and pertussis-treated Lewis rats. No inflammatory cells are present in the retina.



**Figure 2-2C** Histology of enucleated eyes from Lewis rats after 11-14 days' immunization with S-Ag. Inflammatory cells were found in the vitreous and within the retinal layers.



**Figure 2-2D** Histological examination of enucleated eyes from Lewis rats at the late post-inflammatory stage of disease (>21 days). The extensive photoreceptor cell layer was disrupted.

T cells were resuspended up to  $1 \times 10^7$  cells/ml in 10% FCS-RPMI complete medium and 1 ml of the cell suspension was added to sterile freezing vials. Dimethyl sulfoxide (DMSO, 10% V/V) was added dropwise into each freezing vial on ice. The vials were immediately transferred to an insulated box at  $-70^{\circ}\text{C}$  overnight before being transferred to a liquid nitrogen tank for longterm storage. To recover the frozen cells, vials were quickly thawed in a  $37^{\circ}\text{C}$  water bath and added to warm 15 ml of 10% FCS-RPMI complete medium. After washing twice, the cells were cultured in IL-2 containing 10% FCS-RPMI complete medium or stimulated with antigen and irradiated thymocytes in 1% NRS-RPMI complete medium.

## **2.4 Antigen specific $\text{CD4}^+$ T cell lines**

### **2.4.1 Reagents**

#### **Cell culture medium and conditions**

Dutch modified RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, 50  $\mu\text{g/ml}$  gentamycin,  $5 \times 10^{-5}\text{M}$  2-mercaptoethanol (2-ME), and either 1% syngeneic rat serum or 10% heat-inactivated fetal calf serum was used as a complete medium in all experiments. Cells were incubated at  $37^{\circ}\text{C}$  in a 5% carbon dioxide atmosphere.

#### **PPD**

PPD was diluted in RPMI-1640 at a concentration of 10 mg/ml and stored at  $4^{\circ}\text{C}$ . For stimulating lymphocytes it was diluted to 10  $\mu\text{g/ml}$ .

#### **ConA**

ConA was dissolved in RPMI-1640 at a concentration of 1 mg/ml and stored at  $4^{\circ}\text{C}$ . For stimulating lymphocytes it was diluted to 5  $\mu\text{g/ml}$ .

#### **PMA**



PMA was dissolved in PBS at a concentration of 1 mg/ml and stored at 4<sup>0</sup>C. For stimulating T cells it was diluted to 10 ng/ml.

### OVA

OVA was dissolved in RPMI-1640 at a concentration of 1 mg/ml and stored at 4<sup>0</sup>C. For stimulating lymphocytes it was diluted to 10 µg/ml.

### Geneticin (G418)

G418 was dissolved in RPMI-1640 at a concentration of 1 g/ml and stored at 4<sup>0</sup>C. For growing hybridoma cells transformed with mouse IL-2 cDNA it was diluted to 1 mg/ml.

### Sources of IL-2

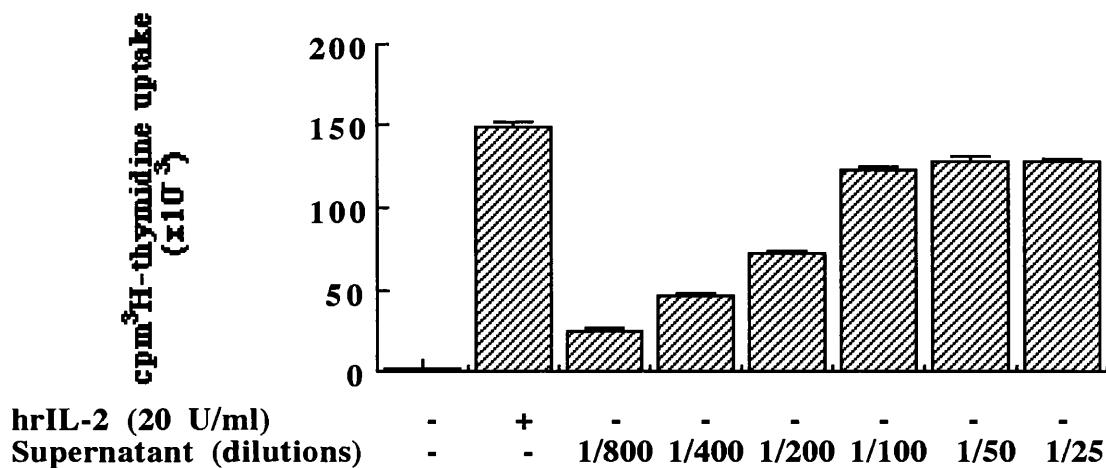
#### *Supernatant of hybridoma cells transformed with mouse IL-2 cDNA*

Hybridoma cells transfected with mouse IL-2 cDNA (a kind gift from Prof. F. Melchers) [Karasuyama & Melchers, 1988] at a concentration of  $2 \times 10^5$  cells/ml were cultured with G418 (1 mg/ml) in 5% FCS-RPMI complete medium without 2-ME. Cells were split every two days.

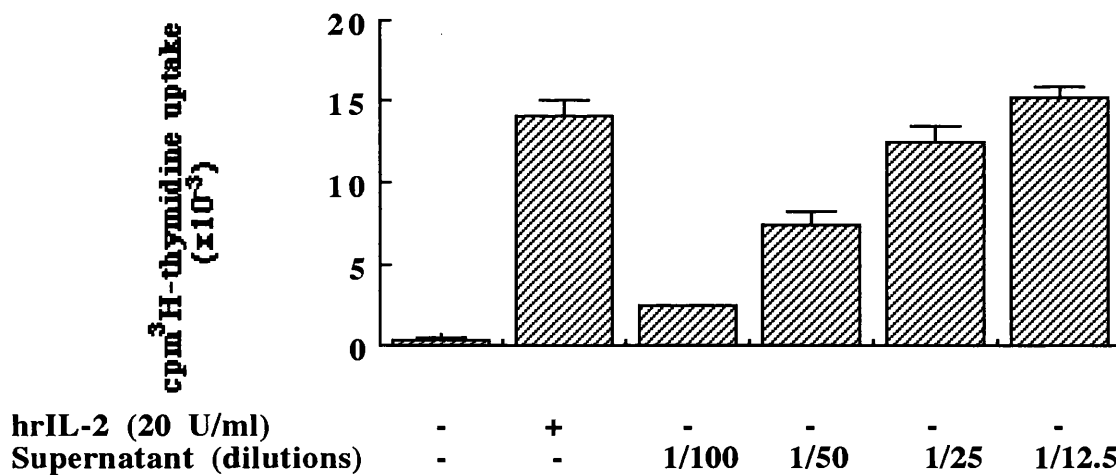
For collecting supernatant, the cells were washed twice to remove G418, cultured for 48 h and conditioned media collected. All supernatant was filter-sterilized and stored at -20<sup>0</sup>C. Fig.2-3 shows the proliferative response of IL-2-dependent cells (rat spleen ConA blasts) to different dilutions of supernatant from the hybridoma (for description of the assay see section 2.8.2). The different batches of the supernatant were assayed each time in comparison with 20 U/ml human recombinant IL-2 (hrIL-2, Boehringer-Mannheim).

#### *Supernatant of ConA-activated rat spleen cells*

Preparation of IL-2 from rat spleen cells is based on the method described by Eichmann [Eichmann, 1982]. Spleens were removed from Lewis rats. Cell suspensions were



**Figure 2-3** Proliferation of IL-2-dependent cells (rat spleen ConA blasts) to supernatant from hybridoma cells transformed with mouse IL-2 cDNA. Rat spleen ConA blasts were cultured in 96-well, flat-bottomed plates at a concentration of  $2 \times 10^5$  cells/well (0.1 ml). Different dilutions of supernatants were added to the wells in a final volume of 0.2 ml. After incubation at 37°C for 72 h, the plates were pulsed with 1  $\mu$ Ci of [<sup>3</sup>H] thymidine for 8 h and harvested. Each value is the mean  $\pm$  SD of three samples. hrIL-2 (20 U/ml) standard was included in each assay.



**Figure 2-4** Proliferation of IL-2-dependent cells (rat spleen conA blasts) in response to supernatant from ConA-activated rat spleen cells. IL-2-dependent cells were cultured in 96-well, flat-bottomed plates at a concentration of  $2 \times 10^5$  cells/well (0.1 ml). Different dilutions of supernatants were added to triplicate wells in a final volume of 0.2 ml. After incubation at 37°C for 72 h, the plates were pulsed with 1  $\mu$ Ci of [<sup>3</sup>H] thymidine for 8 h and harvested. Each value is the mean  $\pm$  SD of three samples. hrIL-2 (20 U/ml) standard was included in each assay.

prepared by pressing the spleen through a sterile nylon mesh using the rubber plunger of a 5 ml disposable plastic syringe. Splenocytes at  $4 \times 10^6$  cells/ml medium were stimulated with  $5 \mu\text{g/ml}$  ConA in 5% FCS-RPMI complete medium for 3 days. Cells were then pelleted and the conditioned media collected. For some experiments,  $\alpha$ -methyl-D-mannopyranoside (AMDM) was added to the supernatant at a final concentration of 20 mg/ml to remove any residual ConA present in the lymphokine preparations [Andersson et al., 1979]. All ConA supernatant was filter-sterilized and stored at  $-20^\circ\text{C}$ . Fig.2-4 shows the proliferative responses of IL-2-dependent cells to different dilutions of supernatant from ConA blasts (for detailed procedure see section 2.8.2). The different batches of the supernatant were assayed each time in comparison with hrIL-2.

#### Normal rat serum (NRS)

NRS was obtained from Lewis rats, and on the same day was heat-inactivated ( $56^\circ\text{C}$ , 45 min), filter-sterilized and stored in aliquots at  $-20^\circ\text{C}$ .

#### Preparation of antigen-presenting cells (APC)

Thymuses were harvested from Lewis rats and made into a single cell suspension in 10% FCS-RPMI complete medium by grinding the tissues through a sterile nylon mesh. The cells were washed twice and finally resuspended in 5 ml 1% NRS-RPMI complete medium and exposed to 2000 rads gamma radiation ( $^{137}\text{Cs}$ , 88 rads/min, Gamma Cell 40).

#### Metrizoate-Ficoll (M-F) separation of cells

To separate live T cells from irradiated accessory cells, M-F at a specific density of around 1.088 was used [Sedgevick et al., 1989]. Ficoll 400 (Pharmacia) was dissolved in distilled water at 14 g/100ml. This was mixed with 32.9% (W/V) sodium metrizoate (Sigma) at a ratio of 10 vols Metrizoate to 24 vols Ficoll then filter sterilized. M-F was stored at  $4^\circ\text{C}$  in the dark.

#### 2.4.2 Establishment of antigen-specific T cell lines

Th cell lines specific for soluble proteins can only be initiated with lymphoblasts, previously activated *in vivo*, since resting Th cells do not proliferate *in vitro* [Taylor et al., 1987].

S-Ag specific T cell lines were derived from draining lymph node cells of Lewis rats immunized with S-Ag/CFA plus *B. pertussis*. Two methods were used to set up S-Ag specific T cell lines: (1) the cells were not purified at the beginning of the culture [Sedgwick et al., 1989], and (2) separation of CD4<sup>+</sup> T cells was carried out at the beginning of the culture [Caspi et al., 1986].

#### Lines established without selecting CD4<sup>+</sup> T cells prior to Ag stimulation

Three S-Ag-specific T cell lines (called SAG1, SAG2 and SAG3) were selected *in vitro* as previously described by [Sedgwick et al., 1989]. Briefly, draining lymph nodes were removed from Lewis rats 7-10 days after immunization with 50 µg S-Ag in CFA (containing 2.5 mg/ml *M. tuberculosis*) and *B. pertussis*. Single cell suspensions were cultured with 10 µg/ml S-Ag at a cell number of  $2 \times 10^6$  cells/ml in 1% NRS-RPMI complete medium with irradiated (2000 rads) thymocytes ( $10^7$  cells/ml) for 3 days, after which the blasts were isolated by density centrifugation over M-F at 2500 rpm for 30 min. Cells from the interface were collected, washed twice and recultured in 20 U/ml of IL-2-containing 10% FCS-RPMI complete medium for 6-9 days. After that, cells ( $2 \times 10^5$ ) were passaged weekly by restimulation with S-Ag (10 µg/ml) and irradiated (2000 rads) thymocytes ( $10^7$  cells/ml). The T cells were returned to the growth medium and alternately stimulated and expanded; the specificity of the lines was improved at each step. Growth curve for one of these cell lines was shown in Fig.2-5. The cells were used for the present studies after the third or fourth cycle of antigen restimulation.

Following the same process, two control PPD-specific T cell lines (PPD1 and PPD2) were selected using PPD (10 µg/ml) prepared from rats immunized with CFA alone (containing extra *M. tuberculosis*).

#### Lines established by purifying CD4<sup>+</sup> T cells prior to stimulation with Ag

Two S-Ag-specific T cell lines (SAG4 and SAG5) were selected *in vitro* as previously

described by Caspi et al. [Caspi et al., 1986]. Draining lymph nodes were removed from antigen-primed rats as described above. Cell suspensions were prepared. Cells were then washed twice in 10% FCS-RPMI medium using centrifuge at 600 g for 10 min. The protocol for purifying the CD4<sup>+</sup> T cells was as follows:

### *Purification of T cells*

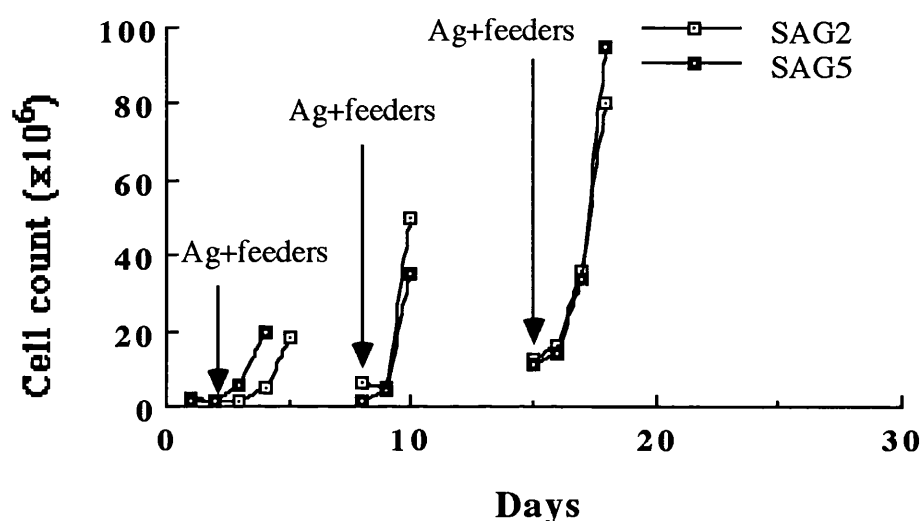
Preparation of Nylon wool columns: Nylon wool columns were prepared using the method of Julius et al. [Julius et al., 1973] with minor modifications. Nylon wool was boiled twice in distilled water for 30 min each time and then dried. To prepare the column, 0.6 g of nylon wool was loosely packed into the barrel of a 10 ml sterilizing plastic syringe, sealed and autoclaved. Before use, each column was washed with 40 ml of 10% FCS-RPMI warm complete medium.

For purification of T cells, draining lymph node cells were layered over M-F. After density centrifugation at 2500 rpm for 30 min, the cells at the interface were collected and washed twice in 10% FCS-RPMI complete medium. After equilibration of the column with warm 10% FCS-RPMI complete medium, up to  $5 \times 10^7$  cells suspended in warm 20% FCS were added dropwise in one ml volume. The column was then incubated at 37°C for 40 min. After this period, T cells (non-adherent cells) were eluted with 40 ml warm 10% FCS-RPMI complete medium. The cells passed nylon wool were stained with FITC-conjugated OX19 mAb (see [section 2.4.3](#)). Purity of T cells was > 90% (data not shown).

### *Panning for CD4<sup>+</sup> T cells*

T cells obtained from nylon wool column were coated with a monoclonal W3/25<sup>+</sup> antibody (50 µg/ml) and panned on rabbit anti-mouse Ig (1/100 dilution) coated dishes (for more details see [section 2.6.2](#)). The strongly adherent cells (W3/25<sup>+</sup>) were stimulated *in situ* with 10 µg/ml S-Ag plus irradiated 2000 R thymocytes, at  $5 \times 10^6$  cells/ml in 1% NRS-RPMI complete medium. After 3 days, cells were collected over M-F and resuspended for expansion in IL-2-containing (20 U/ml, see [section 2.4.1](#)) 10% FCS-RPMI complete medium at  $10^5$  cells/ml. Four days later, two more rounds of panning were performed: a negative selection for OX8<sup>-</sup> cells and a positive selection for

W3/25<sup>+</sup> cells. The final population was restimulated at  $2 \times 10^5$  cells/ml with  $10 \mu\text{g/ml}$  S-Ag+APC for 3 days, and was expanded in 20 U/ml of IL-2-containing 10% FCS-RPMI medium, after which the T cell lines were passaged weekly by restimulation with S-Ag. A growth curve for one of these two cell lines was shown in Fig.2-5. The cells were used for the present studies after the third or fourth cycle of antigen restimulation.



**Figure 2-5** Growth curves of S-Ag specific T cell lines. The cells ( $2 \times 10^5$  cells/ml) were restimulated weekly with S-Ag ( $10 \mu\text{g/ml}$ ) and irradiated thymocytes ( $10^7$  cells/ml) for 3-4 days. After that, the cells were grown in IL-2-containing medium.

#### 2.4.3 Phenotyping of T cell lines

Cell surface immunofluorescence was performed using FITC- or PE- conjugated mAbs and quantitated on a flow cytometer. Isotype-matched mAbs were used as controls. Cells were incubated with either W3/25 (anti-CD4) [Mason et al., 1983], MRC OX-8 (anti-CD8) [Mason et al., 1983], MRC OX19 (anti-CD5) [Dallman et al., 1982] or MRC OX22 (anti-CD45R) [Spickett et al., 1983] mAbs (1:10 dilution, all from Serotec) at  $4^{\circ}\text{C}$  for 30 min and then washed twice with PBS before for flow cytometry. Only cells with high forward and side-scatter were examined and gates were set so as to exclude non-viable cells, counting at least 5000 events within the gate. Results are expressed as percentage of positive cells following subtraction of background staining with the

fluorescent conjugate alone (IgG1 FITC and PE). The pattern of cell surface phenotype of each cell lines was shown in Table 2-1.

***Table 2-1 Phenotyping of T cell lines***

Cell lines	Percentage (positive cells)			Passage No
	W3/25/OX19	OX8/OX19	OX22/OX19	
SAG1	88.0±00	<8	<5	4
SAG2	96.0±00	<5	<7	3
SAG3	95.7±1.5	<4	<3	3
SAG4	89.5±6.5	<6	<5	4
SAG5	95.5±3.5	<2	<4	3
PPD1	89.3±3.0	<5	<2	4
PPD2	94.9±2.3	<4	<1	4

#### 2.4.4 Proliferation assay

The antigen specificity of these T cell lines was examined in a proliferation assay as described elsewhere [Sedgwick et al., 1989].  $2 \times 10^4$  cells were added to wells containing  $10^6$  irradiated thymocytes (APC, 2000 rads) and either S-Ag (0.3 to 10  $\mu\text{g/ml}$ ), ConA (5  $\mu\text{g/ml}$ ), PMA (10 ng/ml), PPD (10  $\mu\text{g/ml}$ ) or ovalbumin (OVA, 10  $\mu\text{g/ml}$ ), as an irrelevant antigen. After 64 h, each well was pulsed-labeled with 1  $\mu\text{Ci}$  of [ $^3\text{H}$ ] thymidine and incubated for an additional 8 h. Cultures were harvested with a cell harvester and thymidine incorporation was measured by  $\beta$ -scintillation counting. The results were express as the means±SD of triplicate cultures. A stimulation index (SI) of  $> 2$  was regarded as a significant proliferative response.

#### 2.5 Establishment of T-cell clones (This work was mainly done by Dr. V.

Calder, and both Dr. Calder and the author of this thesis did the preparative work and the maintenance of the cell clones.)

The methodology for cloning rat T cells was based on Sedgwick [Sedgwick et al., 1989]. The T cell lines (SAG5 and PPD2) were cloned after four cycles of antigen stimulation. Resting cells (i.e., after 10 days in IL-2-containing media) were separated over M-F to isolate viable lymphocytes and resuspended at 10 cells/ml in 10% FCS-RPMI complete medium. 0.1 ml of each of these suspensions was added to each well of a 96-well flat-bottom plate to give one cell/well respectively. Irradiated thymocytes were resuspended at  $10^7$  cells/ml in 10% FCS-RPMI medium containing 20% ConA supernatant (with or without AMDM) and 20  $\mu\text{g/ml}$  S-Ag, and 0.1 ml of this mixture added to each well to give  $10^6$  thymocytes per well with a final concentration of 10% ConA supernatant and 10  $\mu\text{g/ml}$  S-Ag. Plates were incubated for 3 days, and then 80  $\mu\text{l}$  of medium were carefully removed from each well and replaced with 80  $\mu\text{l}$  of the same media-i.e., complete media-10% FCS, 10% ConA supernatant and 10  $\mu\text{g/ml}$  S-Ag. Starting at about day 7-10, clones began to appear although they were best seen after about 10-14 days. At this time, 100  $\mu\text{l}$  medium was carefully removed from positive wells and replaced with 100  $\mu\text{l}$  same medium (no antigen). After a further 3-4 days, 100  $\mu\text{l}$  of medium were removed from each well and replaced with 100  $\mu\text{l}$  of the same medium (with 10  $\mu\text{g/ml}$  S-Ag). Wells exhibiting clonal growth were expanded into 24-well plates with 200  $\mu\text{l}$  complete medium containing 10% FCS, 10% ConA supernatant and 10  $\mu\text{g/ml}$  S-Ag. After 3-4 days, 200  $\mu\text{l}$  of medium was carefully removed from each well and replaced with 200  $\mu\text{l}$  same medium (no antigen). Unfortunately, we were unable to expand these clones successfully from 24-wells. After about a month culture in 24-wells, the cloned cells were used for the experiment to look for lymphokine gene expression.

## **2.6 Enrichment for $\text{CD4}^+\text{OX22}^{\text{high}}$ and $\text{CD4}^+\text{OX22}^{\text{low}}$ T cells**

### **2.6.1 Enrichment of $\text{OX22}^{\text{high}}$ and $\text{OX22}^{\text{low}}$ cells by magnetic activated cell sorter (MACS)**

#### **Biotinylated OX22 monoclonal antibody (BT-OX22 mAb)**



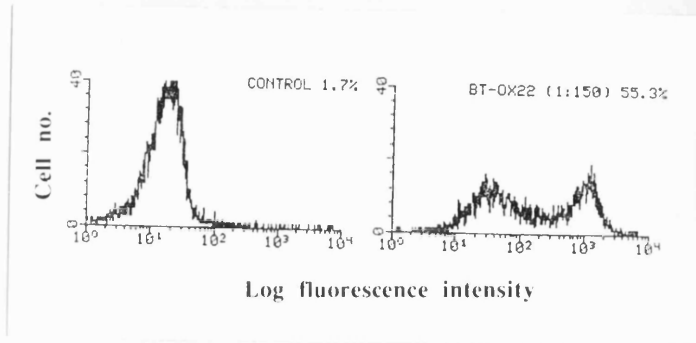
BT-OX22 mAb was prepared as described [Bayer et al., 1979; Miltenyi et al., 1990]. Briefly, 1mg/ml of pure OX22 mAb was dialyzed overnight in bicarbonate buffer (0.1M, pH.8) at 4°C and then stirred with 1mg/ml biotinamidocaproate N-Hydroxysuccinimide Ester at 40/3(V/V) at room temperature for 4 h. The mixture was dialyzed in 0.1M Tris-HCl pH8 for at least 1 h.

### Titration of BT-OX22 mAb

Lymph node cells obtained from normal Lewis rats were stained with BT-OX22 mAb at a range of concentrations (1:5 to 1:200) at 4°C for 30 min and then washed once with PBS. The cells were then incubated with avidin-FITC (1:50 dilution) for an additional 30 min. After that, the cells were washed twice and analysed by FACS. Controls included cells alone and cells with avidin-FITC as negative controls and cells stained with OX22-FITC as positive controls. Table 2-2 shows that the optimal concentration of BT-OX22 was 1:150 dilution. Fig.2-6 shows the results of another experiment in which the cells were stained with BT-OX22 mAb (1:150 dilution) in comparison with OX-22-FITC and W3/25-PE.

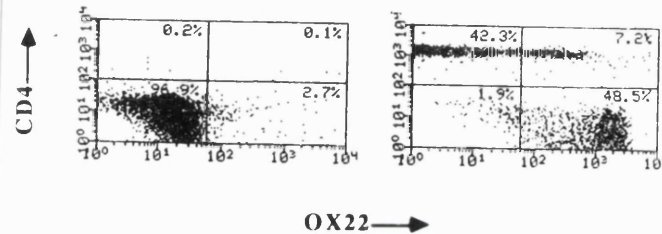
***Table 2-2 Titration of BT-OX22 mAb***

		Percentage (positive cells)
Negative control:	None	2
	Avidin-FITC (1:50 dilution)	2
Positive control:	OX22-FITC	62
BT-OX22 mAb (dilution):		
	1/5	89
	1/10	86
	1/50	78
	1/100	69
	1/150	60
	1/200	49



**Figure 2-6A** Lymph node cells were stained with BT-OX22 mAb (1:50 dilution). Percent positive OX22 cells: 55.3%.

**Figure 2-6B** The cells were stained with OX22-FITC and W3/25-PE (1:10 dilution). Percent positive OX22 cells: 55.7%.



#### Enrichment of OX22<sup>high</sup> and OX22<sup>low</sup> with MACS

Lymph node cells were obtained from normal Lewis rats and separated over M-F to obtain viable mononuclear cells and to remove red blood cells and debris. Isolated cells ( $2 \times 10^7$  cells/ml) were incubated with BT-OX22 mAb at 1:150 dilution (described above) for 30 min at 4°C. After the incubation, 5 ml of PBS were added. The mixture was centrifuged, and then the supernatant was removed. The cell pellet was resuspended in 90  $\mu$ l PBS per  $10^7$  cells and 10  $\mu$ l streptavidin microbeads per  $10^7$  cells added. This was gently mixed and incubated on ice for 15 min. Fluorochrome-conjugated (strept)avidin (1:50 dilution) was added and incubated for 5 min, washed with PBS/1%BSA and the cell pellet resuspended in 500  $\mu$ l PBS/1%BSA. This mixture was applied to the top of the separation column, equilibrated with PBS/1%BSA in the MACS separator with the flow rate defined by a 24 G needle. Unbound cells were washed out with 3 ml PBS/1%BSA. The eluted fraction gave the population of OX22<sup>low</sup> cells. The purity of OX22<sup>low</sup> cells was  $\geq 90\%$  (see [chapter 6](#)). To remove any non-specific cells retained within the column, the column was taken out from the magnetic field and flushed to the

top of column with PBS/1%BSA using a side syringe. The column was then replaced into the magnetic field. Unbound and untight-bound cells were passed through the matrix with the flow rate defined by a 25 G needle and rinsed with 3 ml PBS/BSA.

To obtain OX22<sup>high</sup> cells the column was removed from magnetic field and rinsed with 10 ml PBS/BSA without needle. The eluted fraction gave the population of OX22<sup>high</sup> cells. The purity of OX22<sup>high</sup> cells was  $\geq 90\%$  (see chapter 6).

### 2.6.2 Separation of CD4<sup>+</sup> T cells from OX22<sup>high</sup> and OX22<sup>low</sup> populations by panning

#### Preparation of anti-Ig-coated plastic dishes

100 mm polystyrene petri plates suitable for cell culture (Nunc) were each coated overnight at 4°C with 5 ml of rabbit anti-mouse immunoglobulin (100 µg/ml). The plates were then washed 3 times with PBS as described by [Mage et al., 1977].

#### Panning for CD4<sup>+</sup> T cells

CD4<sup>+</sup> T cells were enriched using mAb according to the method described by Mochizuki et al. [Mochizuki et al., 1985]. Briefly, OX22<sup>high</sup> or OX22<sup>low</sup> cells at a concentration of 10<sup>7</sup> cells/ml in 5% FCS-RPMI complete medium were incubated with mouse IgG mAb against rat-helper T cells, clone W3/25 (50 µg/ml). Following incubation (4°C, 45 min), the cell suspensions were washed twice to remove excess antibody and resuspended to a concentration of 10<sup>7</sup> cells/ml in 5% FCS-RPMI complete medium. The cell suspensions (3 ml/dish) were incubated in the 100-mm tissue culture dishes coated with rabbit anti-mouse mAbs. Following incubation at 4°C for 60 min, nonadherent cells were harvested and the plates washed three times carefully to avoid losing adherent cells. The cells adhering to the antibody-coated surface were collected by forcefully pipetting the medium with the pipette tip and incubated in 10% FCS-RPMI complete medium at 37°C overnight. 10<sup>5</sup> cells were analyzed for their purity and the resultant cells were found to be >90% CD4<sup>+</sup> T cells, as determined by FACS analysis.

## **2.7 Activation of T cells**

Antigen-specific T cell lines and clones were "rested" in IL-2-containing medium for at least 10 days. At that time point, the cells were resuspended at a concentration of  $2 \times 10^5$  cells/ml plus APC in 1% syngeneic rat serum (for cell lines) and in 10% FCS (for cloned T cells). Cells ( $2 \times 10^5$  to  $10^6$ ) were cultured in the absence or presence of S-Ag (10  $\mu\text{g/ml}$ ) or PPD (10  $\mu\text{g/ml}$ ). For some experiments the cells were also stimulated with ConA (5  $\mu\text{g/ml}$ ) or PMA (10 ng/ml). After the incubation periods, the supernatants were harvested and frozen at  $-20^\circ\text{C}$  prior to bioassays, and RNA was extracted from the cells for mRNA detection as described below. Unstimulated cells were used as controls for each timepoint.

## **2.8 Bioassays**

### **2.8.1 Introduction**

Bioassays and immunoassays (radioimmunoassay and ELISA) can be used to detect the secretion of cytokines. The difference between bioassays and immunoassays is that the bioassay is the only way to measure biologically active protein; immunoassays are more sensitive but also can detect denatured biologically inactive cytokine and are more specific in their ability to measure the presence of a particular cytokine.

Bioassays rely on the biological effects of the cytokine on specific cells. A number of considerations need to be born in mind when using bioassays in order to ensure that the data generated is specific for the cytokine of interest. Biological assays are rarely entirely specific for a particular cytokine and can respond to a number of cytokines and other molecules. In these assays, specificity for a particular lymphokine can only be established by using a monospecific neutralizing antibody. In some cases bioassays will underestimate the cytokine content due to inhibitors present in the same samples.

Bioassays will be particularly subject to day-to-day variations in assay performance . It is therefore essential that assays of cytokine activity are standardized to minimize intra- and inter-assay variation. All assays include a standard cytokine preparation, to assess the responsiveness of the cells within an assay.

### **2.8.2 Bioassay for IL-2**

IL-2 assays are based on the principle that IL-2 is required for the proliferation of IL-2-dependent T-cell lines or mitogen-activated T cell blasts [Hudson & Hay, 1989b]. It is usual to measure increased DNA synthesis by increased incorporation of [<sup>3</sup>H] thymidine into DNA. It has been reported that interleukin-2 receptor (IL-2R) monoclonal antibodies can block IL-2-induced proliferation *in vitro* [Leonard et al., 1982], making it possible to test the specificity of this assay.

Mouse HT-2 cells respond to mouse IL-2- and IL-4 [Ho et al., 1987; Watson, 1979]. These cells have been shown to respond to human [Ho et al., 1987] and rat [Shipley et al., 1985], as well as mouse IL-2 [Watson, 1979]. In this study, HT-2 cells were used as target cells for rat IL-2 bioassay.

HT-2 cells at concentration of  $2 \times 10^5$  cell/ml were grown in 5% FCS-RPMI complete medium containing IL-2 from supernatant of hybridoma cells transformed with mouse IL-2 cDNA and split every two days.

#### Procedure of the assay

HT-2 cells were cultured in 96-well, flat-bottomed plates at a concentration of  $2 \times 10^4$  cells/well (0.1 ml). Two-fold serial dilutions of supernatants (SN) were added to triplicate wells in a final volume of 0.2 ml. After the plates were incubated at 37°C for 48 h, the plates were pulsed with 1  $\mu$ Ci of [<sup>3</sup>H] thymidine for 8 h and harvested. [<sup>3</sup>H] thymidine incorporation is determined by scintillation spectroscopy. A hrIL-2 standard was included in each assay.

A similar procedure was adopted for the IL-2 assay using rat spleen ConA-blasts. ConA-blasts were prepared using the method described by Gearing et al. [Gearing et al., 1985]. Briefly rat spleen cells ( $2 \times 10^6$ /ml) were cultured for 3 days in the presence of the mitogen ConA (5  $\mu$ g/ml) to induce blast formation. After 3 days, the cells were re-cultured with ConA (5  $\mu$ g/ml) for further 3 days. At this stage the cells were found to be dependent on exogenous IL-2 for further proliferation, and did not to respond further to ConA and thus were utilized for IL-2-indicator assays.

The effect of anti IL-2R monoclonal antibody (mAb) on HT-2 cells or rat spleen ConA-

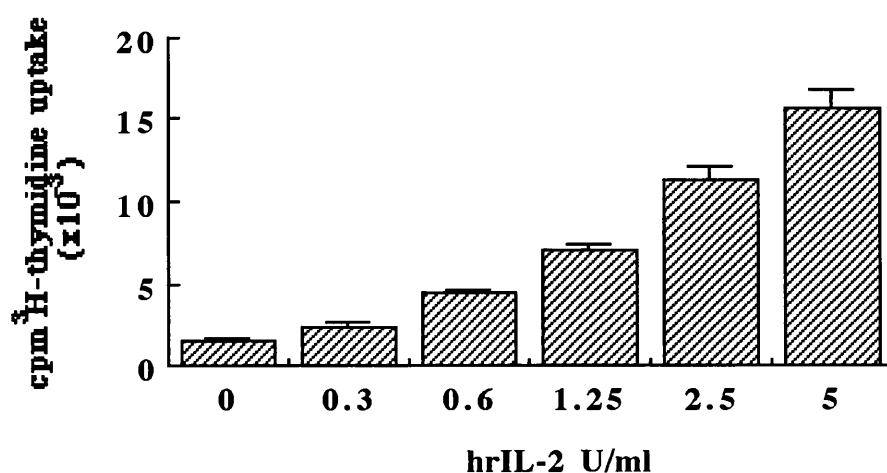
blasts were tested without or with different dilutions of anti-IL-2 receptor mAb.

### Effect of hrIL-2 on the proliferation of HT-2 cells and rat ConA-blasts

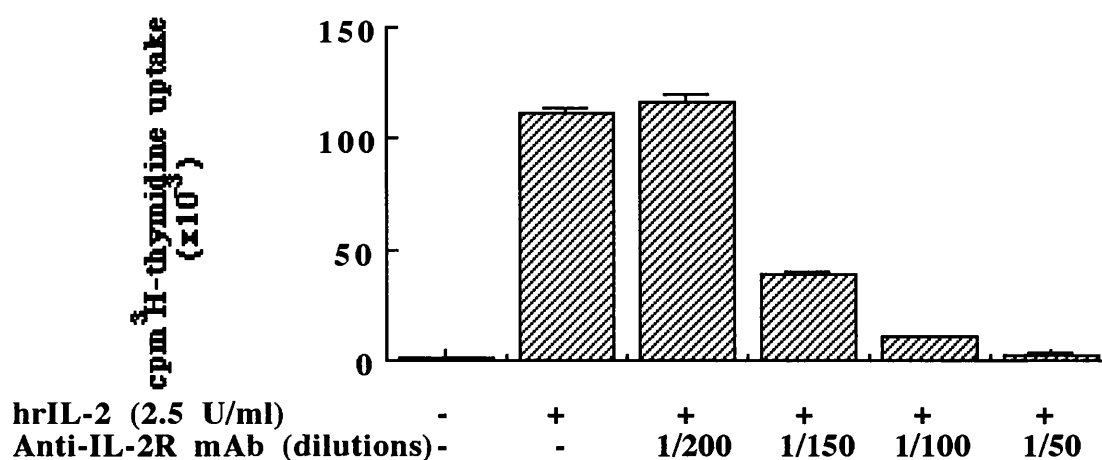
Work was done to validate the bioassay for detection of IL-2. Different concentrations of hrIL-2 were tested. The proliferative response of HT-2 cells (Fig.2-7) was in a dose-dependent ( $R^2=0.99$ ) fashion at concentration of 0.3 to 5 U/ml of hrIL-2. Likewise, Fig.2-9 shows that the proliferative response of rat spleen conA-blasts was in a dose-dependent fashion ( $R^2=0.95$ ) between the concentrations of 0.1 to 10 U/ml of hrIL-2.

### Inhibitory effect of anti-rat IL-2R mAb on mouse HT-2 cell and rat ConA-blasts proliferation

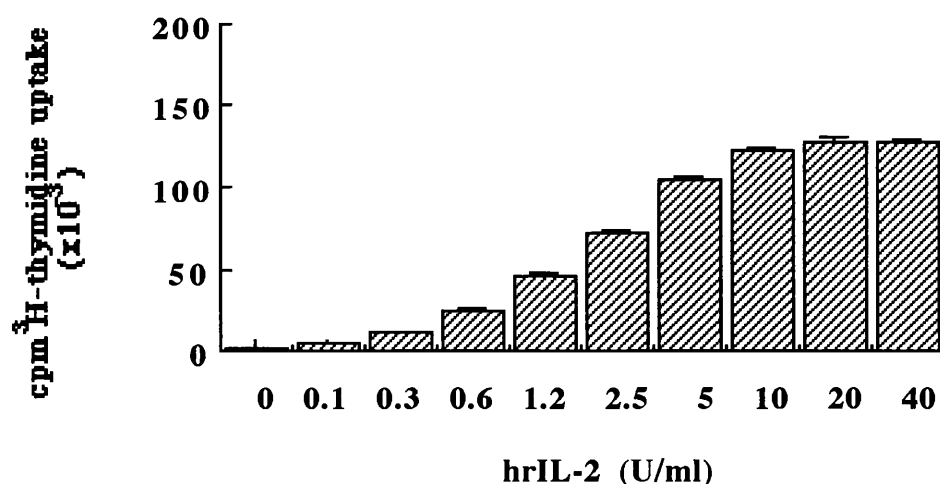
It has been shown that anti-IL-2R mAbs do not cross-react with receptors of different species [Diamantstein & Osawa, 1986]. We set out to search for IL-2R antibodies to block IL-2-dependent T-cell proliferation. As expected, the proliferative responses of rat spleen conA blasts were inhibited by the addition of anti-rat IL-2R mAb (NDS 64, Serotech) [Tellides et al., 1989] dose dependently. The use of the mAb diluted to 1:100 totally blocked the stimulation of hrIL-2 (Fig.2-10). By testing the effect of this antibody



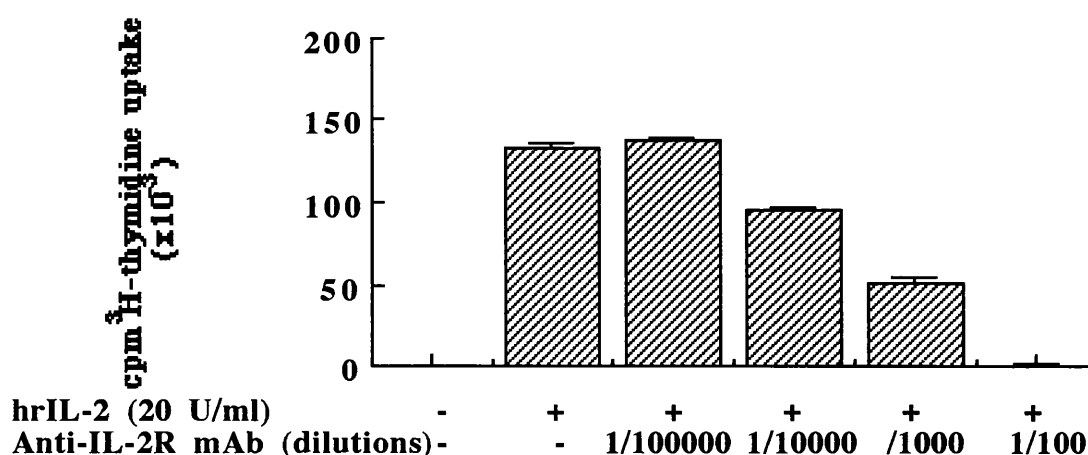
**Figure 2-7** Proliferation of HT-2 cells in response to hrIL-2. HT-2 cells were cultured in 96-well, flat-bottomed plates at a concentration of  $2 \times 10^4$  cells/well (0.1 ml). Different concentrations of hrIL-2 were added to triplicate wells in a final volume of 0.2 ml. After incubation at 37°C for 48 h, the plates were pulsed with 1  $\mu$ Ci of [<sup>3</sup>H] thymidine for 8 h and harvested. Each value is the mean  $\pm$  SD of three samples.



**Figure 2-8** Inhibitory response of anti-IL-2R mAb on proliferation of HT-2 cells induced by hrIL-2. HT-2 cells were cultured in 96-well, flat-bottomed plates at a concentration of  $2 \times 10^4$  cells/well (0.1 ml). Different concentrations of anti-IL-2R mAb with 2.5 U/ml hrIL-2 were added to triplicate wells in a final volume of 0.2 ml. After incubation at 37°C for 48 h, the plates were pulsed with 1  $\mu$ Ci of [<sup>3</sup>H] thymidine for 8 h and harvested. Each value is the mean $\pm$ SD of three samples.



**Figure 2-9** Proliferative response of IL-2-dependent cells (rat spleen ConA blasts) to hrIL-2. IL-2-dependent cells were cultured in 96-well, flat-bottomed plates at a concentration of  $2 \times 10^5$  cells/well (0.1 ml). Different concentrations of hrIL-2 were added to triplicate wells in a final volume of 0.2 ml. After incubation at 37°C for 72 h, the plates were pulsed with 1  $\mu$ Ci of [<sup>3</sup>H] thymidine for 8 h and harvested. Each value is the mean $\pm$ SD of three samples.



**Figure 2-10** Inhibitory response of anti-IL-2R mAb on proliferation of IL-2-dependent cells (rat spleen ConA blasts) induced by hrIL-2. IL-2-dependent cells were cultured in 96-well, flat-bottomed plates at a concentration of  $2 \times 10^5$  cells/well (0.1 ml). Different concentrations of IL-2R mAb with 20 U/ml hrIL-2 were added to triplicate wells in a final volume of 0.2 ml. After incubation at 37°C for 72 h, the plates were pulsed with 1  $\mu$ Ci of [ $^3$ H] thymidine for 8 h and harvested. Each value is the mean  $\pm$  SD of three samples.

on the proliferation of mouse HT-2 cells, it was found to inhibit HT-2 cell proliferation dose dependently, although less potent compared with rat spleen ConA-blasts. The mAb at 1:50 totally blocked the stimulation of 2.5 U/ml (Fig.2-8), but not 5 U/ml (data not shown) hrIL-2 on HT-2 cells. Subsequently, this antibody was used for to block HT-2 cell proliferation in response to rat T-cell supernatant.

### 2.8.3 Bioassay for IFN- $\gamma$

The IFN- $\gamma$  sensitive cell line WEHI-279 (ECACC) was used to assess production of IFN- $\gamma$  by a T cell supernatant. Inhibition of growth demonstrated the presence of IFN- $\gamma$  lymphokine [Katz & Michalek, 1991; Reynolds et al., 1987; Wang et al., 1992].

WEHI-279 cells were grown in 5% FCS-RPMI complete medium at a concentration of  $2 \times 10^5$  cell/ml and split every two days.

### Procedure of the assay

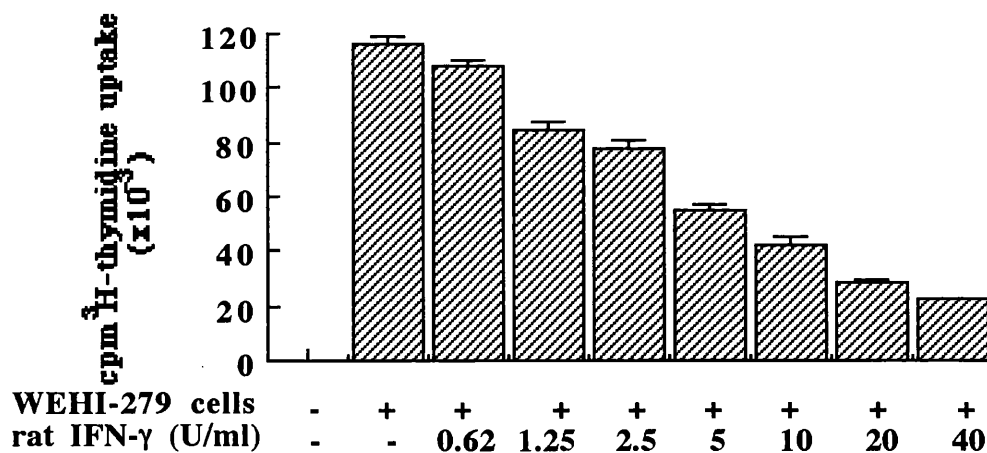


WEHI-279 cells were cultured in 96-well flat-bottom plates at a concentration of  $2 \times 10^4$  cells/well (100  $\mu$ l) in 5% FCS-RPMI complete medium. Ten-fold dilutions of SN were added with and without anti-IFN- $\gamma$  antibody in a final volume of 200  $\mu$ l. In order to confirm the specificity of this assay, control wells containing cells alone, different concentrations of rat IFN- $\gamma$  (Holland Biotechnology b.v., The Netherlands) and anti-rat IFN- $\gamma$  mAb (TechGen, London, UK) were included. Cultures were incubated for 72 h, and then assessed for proliferative activity, measuring the incorporation of [ $^3$ H] thymidine as above. A recombinant rat IFN- $\gamma$  standard was included in each assay.

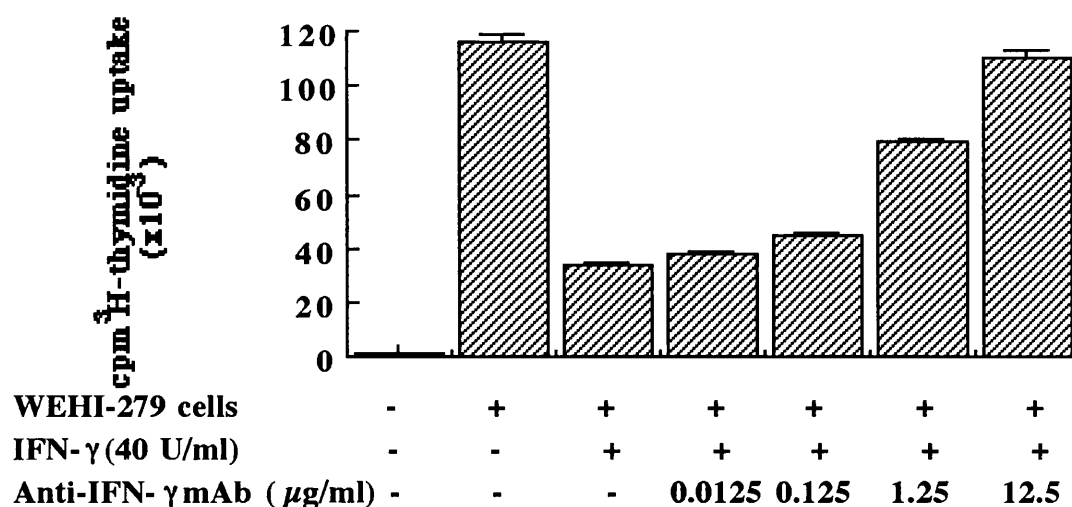
#### The inhibitory effect of rat IFN- $\gamma$ on WEHI-279 cell proliferation

The effect of different concentrations of rat IFN- $\gamma$  on WEHI-279 cells were tested. The inhibitory effect of rat INF- $\gamma$  was shown in [Fig.2-11](#). This response was dose-dependent ( $R^2=0.98$ ) between concentrations of 0.62 and 40 U/ml.

The inhibition of WEHI-279 cells proliferation by IFN- $\gamma$  could be reduced by anti-rat IFN- $\gamma$  mAb at concentrations of 0.0125 to 12.5  $\mu$ g/ml, and at the highest concentration used, the anti-inhibition effect of rat IFN- $\gamma$  (40 U/ml) on WEHI-279 cells was reversed ([Fig.2-12](#)).



**Figure 2-11** Inhibition of WEHI-279 proliferation by rat IFN- $\gamma$ . WEHI-279 cells were cultured in 96-well flat-bottom plates at a concentration of  $2 \times 10^4$  cells/well (0.1 ml) in 5% FCS-RPMI complete medium. Different concentrations of IFN- $\gamma$  were added in a final volume of 0.2 ml. Control wells containing cells alone. After incubation at 37°C for 48 h, the plates were pulsed with 1  $\mu$ Ci of [ $^3$ H] thymidine for 8 h and harvested. Each value is the mean $\pm$ SD of three samples.

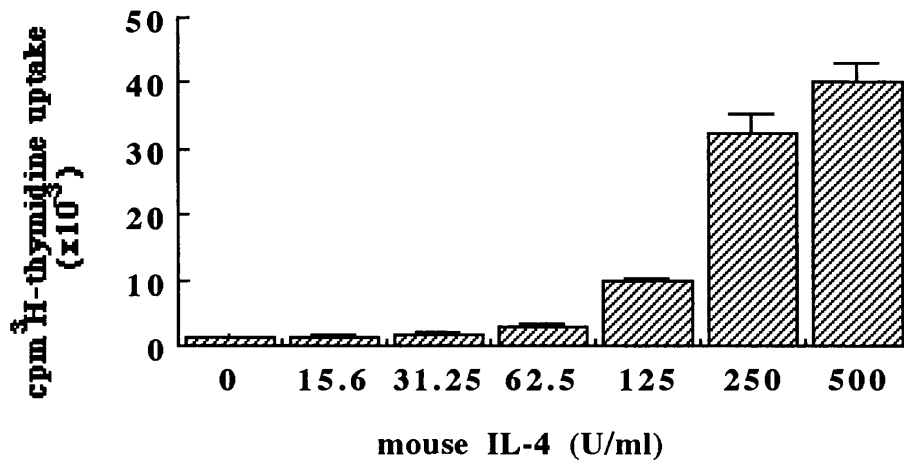


**Figure 2-12** The inhibitory effect of anti-IFN- $\gamma$  mAb on the anti-proliferative effect of IFN- $\gamma$  of WEHI-279. WEHI-279 cells were cultured in 96-well flat-bottom plates at a concentration of  $2 \times 10^4$  cells/well (0.1 ml). Different concentrations of anti-IFN- $\gamma$  mAb were added in a final volume of 0.2 ml with or without IFN- $\gamma$  (40 U/ml). After incubation at 37°C for 48 h, the plates were pulsed with 1  $\mu$ Ci of [ $^3$ H] thymidine for 8 h and harvested. Each value is the mean  $\pm$  SD of three samples.

#### 2.8.4 Bioassay for IL-4

##### Effect of mouse IL-4 on the proliferation of HT-2 cells

The proliferative response of HT-2 cells to mouse IL-4 were tested. HT-2 cells ( $2 \times 10^4$ ) were incubated with different concentrations of mouse IL-4 for 48 h, before pulsing and harvesting as described above. The proliferative response of HT-2 cells was in a dose-dependent ( $R^2=0.91$ ) fashion at concentrations of 15.6 to 500 U/ml (Fig.2-13). As there was no commercial rat IL-4 available, the IL-4 secreted by rat antigen specific T cells were not evaluated due to that IL-4 is a highly species-specific lymphokine [Leitenber & Feldbush, 1988].



**Figure 2-13** The proliferative response of HT-2 cells to mouse recombinant IL-4. HT-2 cells ( $2 \times 10^4$ ) were incubated with different dilutions of IL-4 in 96 well plates for 48 h. After incubation at 37°C for 48 h, the plates were pulsed with 1  $\mu$ Ci of [ $^3$ H] thymidine for 8 h and harvested. Each value is the mean  $\pm$  SD of three samples.

## 2.9 Molecular biological techniques

### 2.9.1 Analysis of cytokine gene expression -- general points

There are different techniques to detect mRNA expression such as Northern blotting, *in situ* hybridization, ribonuclease protection analysis and polymerase chain reaction (PCR). Choosing the method for analysis of lymphokine mRNA depends on the number of cells available, the level of gene expression and the source of cells or tissues.

Northern blotting is widely used for the analysis of gene expression and is generally thought to provide semi-quantitative data, but suffers from a lack of sensitivity and requires a huge amount of RNA. Ribonuclease protection analysis is a more sensitive technique for mRNA detection. It has been used for analyzing certain cytokine mRNAs [Lowry et al., 1989] in models of transplantation. The rather large amounts of mRNA often required for this approach make its use prohibitive in many situations. Analysis of cytokine gene expression may be approached by *in situ* hybridization. The most important aspects of *in situ* hybridization is that it allows identification of the precise

morphological distribution of nucleic acid sequences directly in cells or tissue sections.

Since its introduction in 1985, PCR [Mullis & Faloona, 1987; Saiki et al., 1985] has transformed the DNA analysis carried out both research and clinical laboratories. The PCR, which was developed by scientists at Cetus, involves the *in vitro* enzymatic synthesis of millions of copies of a specific DNA segment. The reaction is based on the annealing and extension of two oligonucleotide primers that flank the target region in duplex DNA; after denaturation of the DNA, each primer hybridizes to one of the two separated strands such that extension from each 3' hydroxyl end is directed toward the other. The annealed primers are then extended on the template strand with a DNA polymerase. These three steps (denaturation, primer binding and DNA synthesis) represent a single PCR cycle. Consequently, repeated cycles of denaturation, primer annealing and primer extension result in the exponential accumulation of a discrete fragment whose terminal are defined by the 5' ends of the primers. PCR is particularly useful in studying cytokine gene expression since most cytokine genes are expressed transiently at low levels. The high sensitivity and specificity of PCR make it an extremely useful method for the analysis of cytokine gene expression. The housekeeping gene  $\beta$ -actin, was successfully used as an internal control for both reverse transcription and PCR. Using this method, we were able to study the kinetics of cytokine gene expression in T cell subsets with satisfactory reproducibility.

The main problem with interpreting data based on analysis of mRNA is that, although some gene expression has been reported at the translational level, the presence of message does not necessarily indicate the presence of protein [Dallman & Clark, 1991]. However, the precision of this method does offer certain advantages in the assessment of the ability of cells to synthesize a particular lymphokine. These methods, in conjunction with the monospecific bioassays, have been very important in recent efforts to define the patterns of lymphokine synthesis in different T cell clones [Cherwinski et al., 1987].

## 2.9.2 Amplification of cDNA

### Extraction of RNA

Total RNA was isolated by a guanidinium thiocyanate/phenol/chloroform/isoamyl

alcohol procedure [Chomczynski & Sacchi, 1987]. Briefly, T cells (up to  $10^6$ ) were resuspended in 500  $\mu$ l of solution D [4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% sarcosyl and 0.1 M 2-ME] in a sterile eppendorf tube. Then, 50  $\mu$ l of 2 M sodium acetate (pH4.3), 500  $\mu$ l of phenol/ $H_2O$ , and 100  $\mu$ l of chloroform/isoamyl alcohol (49/1, V/V) were added. The tubes were vigorously shaken, incubated on ice for 15 min, and microfuged (13,000 g) at 4°C for 15 min. The aqueous layer was removed to another tube and RNA precipitated with an equal volume of cold isopropanol at -20°C over night. RNA was centrifuged (13,000 g) in the Eppendorf, resuspended in 300  $\mu$ l solution D, and reprecipitated with an equal volume of isopropanol. The RNA pellet was then washed twice with 75% ethanol/25% diethyl pyrocarbonate (DEPC) water, vacuum dried for 15-30 min and dissolved in DEPC- $H_2O$ . The amount of RNA was determined by OD<sub>260</sub> using the spectrophotometer as 1 unit of OD at 260 equals 40  $\mu$ g of RNA. The ratios of OD<sub>260/280</sub> were around 1.8. Isolated RNA was stored at -70°C or reverse transcribed into cDNA immediately.

### Reverse transcription (RT)

cDNA synthesis was performed as described by Dallman et al. [Dallman et al., 1991]. For each sample, 5 or 2  $\mu$ g of total RNA and 4  $\mu$ g oligo dT<sub>12-18</sub> were incubated for 5 min at 60°C and chilled on ice for 3 min. The reaction was carried out in a final volume of 40  $\mu$ l containing 400 U MMLV reverse transcriptase, 40 U RNAsin, 1 mM deoxynucleoside triphosphates and 8  $\mu$ l RT-buffer. The reagents were incubated at 37°C for 40 min. After that, another 400 U of reverse transcriptase was added to the reaction and incubated for a further 40 min at 37°C. The re-action mixture was heat-inactivated at 70°C for 10 min to denature the MMLV reverse transcriptase. The cDNA was diluted to a total volume of 100  $\mu$ l by adding 60  $\mu$ l of water per sample and frozen at -20°C.

### Polymerase chain reaction (PCR)

The standard amplification reaction consisted of 5  $\mu$ l of the resulting cDNA, 1  $\mu$ M of each primer, 0.2 mM deoxynucleoside triphosphates, 10  $\mu$ l 10x polymerization buffer [500 mM KCl, 100 mM Tris-HCL, pH 8.3, 15 mM MgCl<sub>2</sub>, 0.1% (W/V) gelatin] and 2.5 U of Taq polymerase and water to a total volume of 100  $\mu$ l in the eppendorf. The

sequences of the primers used are shown in table 2-3. The reaction mixture was covered with 50  $\mu$ l of mineral oil to prevent evaporation and then amplified by a repeated 3-temperature cycle on the thermocycler programmable heating block. The temperature used in the annealing cycle was varied because groups of primers had different melting temperatures. Each cycle consisted of 1 min at 93°C, 1-2 min at 50°C-60°C and 1 min at 72°C. For the first round of the amplification, the denaturing step was performed for 5 min and subsequently 1 min was used. The last extension was 10 min. The amplification

**Table 2-3** *Primer sequences for amplification of rat IL-2, IL-4, IFN- $\gamma$ , IL-10 and  $\beta$ -actin*

Molecule	Primer/probe	Sequences (5'-3')	References
IL-2	A	AACAGCGCACCCACTTCAA	McKnight et al. (1989)
	B	TTGAGATGATGCTTTGACA	
	Internal probe	GTTCATCTTCTAGGCACTG	
IL-4	A	TCTCACGTCACTGACTGTA	McKnight et al. (1991)
	B	CTTTCAGTGTTGTGAGCGT	
	Internal probe	GGGGTTCTCGGTGAACTGAGGAACTC	
IFN- $\gamma$	A	ATGAGTGCTACACGCCGCGTCTTGG	Dijkema et al. (1985)
	B	GAGTTCATTGACAGCTTTGTGCTGG	
	Internal probe	CAGATTATCTCTTTCTACCTCAGAC	
IL-10	A	GTGAAGACTTTCTTTCAA	Dallman, M.J. (personal communication)
	B	TGATGAAGATGTCAAACCTC	
	Internal probe	GTTTTACCTGGTAGAAG	
$\beta$ -Actin	A	ATGGATGACGATATCGCTG	Nudel et al. (1983)
	B	ATGAGGTAGTCTGTCAGGT	
	Internal probe	AGCAAGAGAGGTATCCT	

reaction was carried out for 25 to 50 cycles to obtain appropriate amount of products.

Table 2-4 shows the programmes used for each lymphokine.

***Table 2-4 PCR reaction conditions.***

Molecule	Concentration (Mg <sup>++</sup> )	Reaction Conditions			
		Denature	Annealing	Extension	Fragment
IL-2	1.5 mM	93 <sup>0</sup> C, 1 min	60 <sup>0</sup> C, 2 min	72 <sup>0</sup> C 1 min	400 bp
IL-4	1.5 mM	93 <sup>0</sup> C, 1 min	55 <sup>0</sup> C, 2 min	72 <sup>0</sup> C 1 min	406 bp
IFN-γ	1.5 mM	93 <sup>0</sup> C, 1 min	60 <sup>0</sup> C, 2.5 min	72 <sup>0</sup> C 1 min	405 bp
IL-10	1.5 mM	93 <sup>0</sup> C, 1 min	55 <sup>0</sup> C, 2 min	72 <sup>0</sup> C 1 min	372 bp
β-Actin	2.5 mM	93 <sup>0</sup> C, 1 min	55 <sup>0</sup> C, 2 min	72 <sup>0</sup> C 1 min	568 bp

#### Agarose gel electrophoresis of PCR products

To determine whether the amplification worked, one tenth of the final volume was taken, mixed with 1 µl 10x loading buffer [0.25% Bromophenol blue, 0.25% Xylene cyanol, 15% Ficoll], and electrophoresed on 1.5% agarose checking gel (containing 0.5 µg/ml ethidium bromide ) together with a size marker (ØX174 RF DNA/Hae III Fragment) in 1x TBE-buffer [0.05M Tris, 0.05M Boric acid, 1mM EDTA]. A clear band with the appropriate size on the gel could be viewed under ultraviolet transilluminator.

#### 2.9.3 Southern blot analysis

The product of PCR was probed with a third oligonucleotide whose position in the

cDNA sequence lay between that of the original two primers.

### Denaturation of DNA

The DNA was denatured by soaking the gel for 25 min in a solution of 1.5 M NaCl, 0.5 N NaOH with constant, gentle agitation. This step was repeated with fresh solution for another 25 min. The gel was rinsed briefly in deionized water, and then neutralized by soaking for 30 min in a solution of 1 M Tris (pH 7.4), 1.5 M NaCl at room temperature with constant, gentle agitation. The soaking procedure was continued with fresh neutralization solution for a further 15 min. At that stage, the gel was ready for blotting.

### Blotting

The blotting apparatus comprised two wicks 3 MM filter paper soaked in 10x SSC [20x SSC: 3 M NaCl, 0.3 M Na<sub>3</sub>Citrate] laid at right angles to each other across a raised platform, with each end reaching a bath of 10x SSC. The gel was laid onto the wicks on the platform, and any air bubbles smoothed out. The blotting membrane (Hybond-N) and two sheets of 3 MM paper previously cut to the size of the gel were soaked in 10x SSC and 2x SSC respectively. The blotting membrane was laid onto the gel, and any air bubbles smoothed out. The edges of the membrane were then covered with cling film, which was extended to the sides of the blotting tray, to reduce evaporation of 10x SSC in the base of the tray, and to prevent it from passing the gel into the blotting materials above the membrane. Two sheets of pre-wet 3 MM paper were laid on the membrane and on top of which a pack of dry paper towels were laid. A small weight was placed on the top of the paper towels. Blotting was carried out overnight at room temperature. The towels and filter papers were then removed. The membrane was labelled with the position of the wells, dried between two layers of 3 MM, and DNA was fixed onto the membrane by baking for 2 h at 80°C.

### Hybridization

#### *5'-end labelling*

The labelling reaction was carried out in a total volume of 50 µl containing internal probe



(maxim 300 ng), 3  $\mu$ l 10x kinase buffer [0.5 M Tris-HCl (pH 7.5), 0.1 M  $\text{MgCl}_2$ ], 5  $\mu$ l ( $\gamma$ - $^{32}\text{P}$ )dATP, 1  $\mu$ l polynucleotide kinase and water. The reaction was incubated at 37°C for 45 min, and then 450  $\mu$ l 10mM sodium phosphate buffer (pH 6.8) was added, and the mixture were passed through a NAP<sup>TM</sup>-5 column (sephadex G-25).

#### *Purification of labelling probe by NAP<sup>TM</sup>-5 column*

The gel column was equilibrated with approximately 10 ml of 10 mM phosphate buffer (pH 6.8). The labelling mixture (500  $\mu$ l) was added to the top of the column. The eluted fraction (500  $\mu$ l) was collected. Then another 1 ml of phosphate buffer was added to the top of the column. The labelled probe was eluted with 1 ml of buffer.

#### *Hybridization with radioactively labelled probes*

The baked filter was soaked in 2x SSC, rolled up and fitted into the hybridization tube. The membrane was prehybridized in a rotary oven at 42°C in 10 ml prehybridization solution [10% PEG, 40% deionized formamide (100 ml formamide, 5 g AG501X8), 20% 20x SSC, 1% 100x denhardt's solution (2% PVP, 2% BSA and 2% Ficoll), 2 M Tris pH 7.4 and 0.02 mg/ml] for 1 h. The prehybridization solution was replaced by 10 ml hybridization solution [prehybridization solution with prepared radioactively labelled probe]. The hybridization tube was then transferred back to the 42°C rotary oven, and incubated overnight.

#### Washing and autoradiography

After hybridization, the radioactive hybridization solution was poured away and the membrane were washed in 2x SSC at room temperature for 20 min. They were then washed in 2x SSC, 0.1% SDS solution at room temperature for 20 min followed by washing in 0.5x SSC, 0.1% SDS at 60°C for 15 min. At this stage they were monitored with a hand-held Geiger counter, and washed with higher stringency if it was still excessively radioactive (by reducing the SSC concentration to a minimum of 0.1% if necessary). The blots were then reassembled in their original orientation on cards covered in plastic food-wrap, and exposed to X-ray film with intensifying screens overnight at -70°C. The films were then developed and they would re-exposed for

longer period if the image was too faint.

#### 2.9.4 Semi-quantitative PCR

There are different methods of quantitative PCR. Competitive PCR technique amplifies the target DNA sequences simultaneously with either endogenous or exogenous internal standard. As the input of the internal standard is known, the target DNA sequences can be measured. However, compared to competitive PCR, dot blot is simpler, and it is easier to assay larger number of samples [O'Garra and Vieira, 1992].

##### The procedure of semi-quantitative PCR by dot-blot

The cDNA pooled from the reverse transcription reaction was diluted 1/10, 1/20 and 1/40 in sterile water. 10  $\mu$ l of the separated dilutions was each subjected to PCR and 15  $\mu$ l of the amplification mix was removed every 5 cycles from 15-45 cycles in the PCR.

One piece each of nitrocellulose membrane and Whatman 3MM paper was wetted in 10x SSC. Whatman 3MM paper was first placed in apparatus and then nitrocellulose membrane placed on top of the paper. The apparatus was covered and subjected to a vacuum for 20 min. During this time the wells were first rinsed with 100  $\mu$ l of 20x SSC. The PCR products (15  $\mu$ l) taken from different cycles were heated at 90°C for 10 min and then 85  $\mu$ l of 20x SSC were added. The mixture was dot-blotted to nitrocellulose membrane. Each well was rinsed with 100  $\mu$ l of 20x SSC. After 20 min, the vacuum was switched off. The nitrocellulose membrane was carefully removed, baked for 2 h at 80°C and probed using  $\gamma$ -<sup>32</sup>P-end-labeled internal oligo-probe as described above. The variable size of the spots could be examined for semi-quantity. For semi-quantitative PCR an internal control ( $\beta$ -actin) was included.

#### 2.9.5 Validation of the detection of lymphokine mRNAs by PCR

##### Production of positive control cDNA

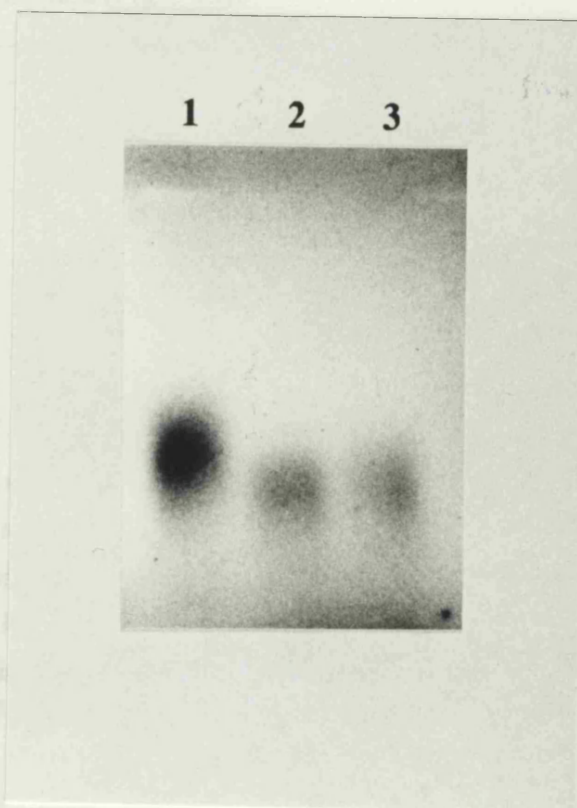
For the positive control, spleen cells (10<sup>6</sup> cells/ml) from Lewis rats were stimulated with 5  $\mu$ g/ml of ConA for 6 or 24 hrs. RNA was isolated, reverse transcribed and used for

PCR analysis as described above.

### Monitor of RT by including $\alpha$ - $^{32}\text{P}$ -dCTP in the reaction mixture

In order to check the effect of the first and second strands of cDNA synthesized by reverse transcriptase. 5  $\mu\text{Ci}$  [ $\alpha$ - $^{32}\text{P}$ ]dCTP (0.5  $\mu\text{l}$ ) were added to the reaction together with 5  $\mu\text{l}$  mixed reverse transcription reagents. The mixture was incubated for 40 min at 37°C and then 5  $\mu\text{l}$  running buffer [50 mM NaOH and 1 mM EDTA] and 1  $\mu\text{l}$  alkaline loading buffer [300 mM NaOH, 6 mM EDTA, 18% Ficoll, 0.15% bromocresol green and 0.25% xylene cyanol] were added. cDNA synthesis was analyzed by alkaline

**Figure 2-14** Incorporation of  $\alpha$ - $^{32}\text{P}$ -dCTP in to cDNA synthesis. 5  $\mu\text{Ci}$  [ $\alpha$ - $^{32}\text{P}$ ]dCTP (0.5  $\mu\text{l}$ ) were added to the reaction together with 5  $\mu\text{l}$  mixed reverse transcription reagents. After incubation for 40 min at 37°C, 5  $\mu\text{l}$  running buffer were added, and fractionated on the agarose gel. The gel was soaked in 7% trichloroacetic acid (TCA) for 30 min at room temperature. After soaking, the gel was dried under vacuum and exposed to the film. Lane 1: Hybridoma cells transformed with mouse IL-2 cDNA. Lanes 2 and 3: Rat spleen ConA blasts.

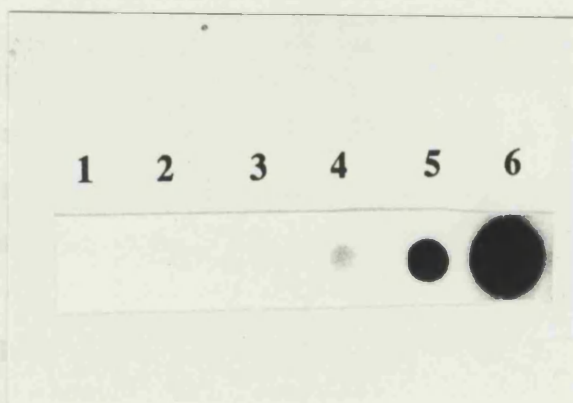


agarose gel (1.5% agarose, 50mM NaOH and 1mM EDTA) electrophoresis. Electrophoresis was carried out at voltages of < 0.25 V/cm. A glass plate was placed directly on the top of the gel after the dye had migrated out of the loading slot. At the end of the electrophoresis, the gel was removed from the tank and soaked in 7% trichloroacetic acid (TCA) for 30 min at room temperature. After soaking, the gel was dried under vacuum on a gel dryer. The gel was exposed at -70°C. The positive signal could be detected after electrophoresis on the agarose gel and exposed to the film (Fig.2-

14).

*Figure 2-16 Semi-quantification of*PCR for the detection of mRNA coding for  $\beta$ -actin from different cell numbers

RNA from  $5 \times 10^3$ ,  $10^4$ ,  $5 \times 10^4$ ,  $10^5$ ,  $5 \times 10^5$  and  $10^6$  rat spleen ConA blasts was reverse transcribed, and the PCR analysis was performed with  $\beta$ -actin oligonucleotide primers. PCR products were dot-blotted to a nitrocellulose membrane after 30 cycles and hybridized with  $\gamma$ - $^{32}\text{P}$ -dATP-end-labelled internal probe as described above. With increasing RNA input (RNA extracted from  $5 \times 10^3$  to  $10^6$  rat spleen ConA blasts), the signal appeared stronger. We have found that  $\beta$ -actin mRNA can be detected using as few as  $10^5$  rat spleen ConA blasts by this method (Fig.2-15).



**Figure 2-15** PCR detection of  $\beta$ -actin mRNA with different numbers of cells. RNA from  $5 \times 10^3$ ,  $10^4$ ,  $5 \times 10^4$ ,  $10^5$ ,  $5 \times 10^5$  and  $10^6$  rat spleen ConA blasts was extracted, reverse transcribed, and the PCR analysis was performed with  $\beta$ -actin oligonucleotide primers. PCR products were dot-blotted to nitrocellulose membrane after 30 cycles and probed using  $\gamma$ - $^{32}\text{P}$ -dATP-end-labelled internal probe. Lane 1:  $5 \times 10^3$  cells, lane 2:  $10^4$ , lane 3:  $5 \times 10^4$ , lane 4:  $10^5$ , lane 5:  $5 \times 10^5$  and lane 6:  $10^6$ .

Bio-Rad Laboratories, Richmond, CA, USA

Boehringer-Mannheim House (BCL), Bell Lane, Lewes, East Sussex, U.K.

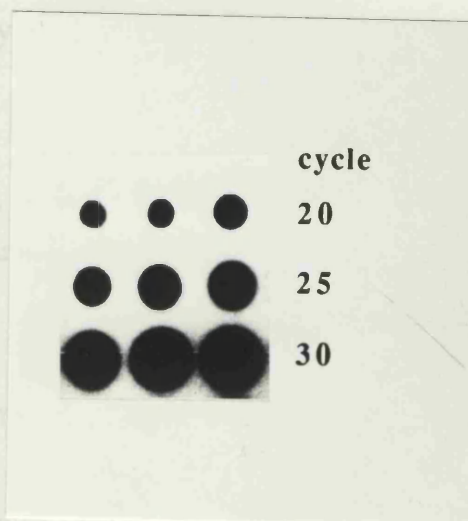
Semi-quantification of  $\beta$ -actin mRNA by sampling from various cycles

RNA from rat spleen ConA blasts was reverse transcribed, and the PCR analysis was performed at different cycles (20, 25 and 30) with  $\beta$ -actin primers. The PCR products were dot-blotted onto a nitrocellulose membrane and hybridized with  $\gamma$ - $^{32}\text{P}$ -dATP-end-labelled internal probe as described above. Fig.2-16 shows that with increasing numbers of PCR cycles, the signal appeared stronger.

DENLEY Instruments Ltd., England, U.K.



**Figure 2-16** Semi-quantification of  $\beta$ -actin mRNA by sampling at various cycles. RNA from rat spleen ConA blasts was extracted, reverse transcribed, and PCR amplified at different cycles of  $\beta$ -actin primers. The PCR products were dot-blotted to a nitrocellulose membrane and hybridized with  $\gamma$ - $^{32}$ P-dATP-end-labeled internal probe.



## 2.10 Statistical analysis

Results of triplicate readings were expressed as Means $\pm$ SD throughout the study. Significance was calculated using the student t-test.  $P < 0.05$  was accepted to be a significant difference. All samples to be compared were analysed in the same assays to eliminate interassay variations.

## 2.11 Materials

### 2.11.1 Suppliers' addresses

**Amersham** International plc, White Lion Road, Amersham, Bucks, U.K.

**BDH** Chemical Ltd., Poole, Dorset, U.K.

**Becton Dickinson** Ltd., Between Towns Road, Cowley, Oxford, U.K.

**Bio-Rad** Laboratories, Richmond, CA, USA

**Boehringer-Mannheim** House, (BCL), Bell Lane, Lewes, East Sussex, U.K.

**British Bio-technology** Products Ltd., 4-10 The Quadrant, Barton Lane, Abingdon, Oxon, U.K.

**Cadish** Ltd., London, U.K.

**Canberra-Packard** Instrument Company, Pangbourne, U.K.

**Carl Zeiss** (Oberkochen) Ltd., PO Box 78, Woodfield Road, Welwyn Garden City, Herts, U.K.

**Damon/Iec** Ltd., Lawrence Way, Brewers Hill RD, Dunstable, Beds, England, U.K.

**DENLEY** Instruments Ltd., England, U.K.

**Difco** Laboratories, East Molesey, Surrey, U.K.

**Dynatech** Billingshurst, Sussex, U.K.

**ECACC** Porton Down, Salisbury, Wilts, U.K.

**Edward** Manor Royal, Crawley, Sussex, U.K.

**EVANS** medical ltd., Langhurst, Horsham, England, U.K.

**Flow** Laboratories Inc, U.K.

**Gibco (BRL)**, Paisley, Scotland, U.K.

**Grand** Instruments Ltd., Barrington, Cambridge, England, U.K.

**Fuji** photo film Co, Japan

**Holland** Biotechnology b.v., Niels Bohrweg 13, The Netherlands

**Hybaid** Ltd., Waldegrave Road, Teddington, Middlesex, U.K.

**Jencons** Starbridge Road, Leighton Buzzard, Beds, England, U.K.

**Kelvinator** Commercial Products Inc., Manitowoc, Wis., USA

**Leec** Ltd., Private Road No.7 Colwick Industrial Estate, Nottingham, U.K.

**Medical** International Ltd., 239 Liverpool Road, London, U.K.

**Miltenyi Biotec** Laboratory Impex Limited, Teddington, Middlesex, U.K.

**Nen** Research Products, 549 Albany Street, Boston, Massachusetts, USA

**OSWELL** DNA Service, Department of Chemistry, University of Edinburgh, U.K.

**Oxoid** Ltd., England, U.K.

**PARKE-DAVIS** Pontypoll, Gwent, U.K.

**Peteric** Ltd., Shepperton studios, Studios Road, Shepperton, Middlesex, England, U.K.

**Pharmacia** Ltd., Pharmacia House, Midsummer Boulevard, Central Milton Keynes, Bucks, U.K.

**Polysciences** Ltd., Handelsstr. 3, Postfach 1130, D-6904 Eppelheim, Germany

**Quatro** Biosystems Ltd., Broadoak Business Centre, Ashburton Road, Trafford Park, Manchester, U.K.

**Raymond** A Lamb, 6 Sunbean Road, London, U.K.

**Serotec** Station Approach, Kidlington, Oxford, U.K.

**Sigma** Chemical Company Ltd., Poole, Dorset, U.K.

**Shandon** Scientific Ltd., Chadwick Road, Astmoor, Runcorn, Cheshire, England, U.K.

**Stratagene** Northumberland, U.K.

**TechGen** International Ltd., Sullivan Road, London, U.K.

**Wellcome** Ltd., Beckenham, London, U.K.

**Whatman** International Ltd., Mainstone, U.K.

#### 2.11.2 Equipment and suppliers

**Becton Dickinson** Ltd

Fluorescence-activated cell sorter (FACS), Magnetic cell sorter (MACS)

**Bio-Rad** Laboratories

Electrophoresis power supply, Gel dryer, Dot blot apparatus

**Cadish** Ltd

125 mesh

**Canberra-Packard** Instrument Company

TRI-CARB 1900 CA Liquid Scintillation Analyzer

**Carl Zeiss** (Oberkochen) Ltd

ID 03 microscopy

**Damon/Iec** Ltd

Microcentrifuge, Centrifuge

**DENLEY** Instruments Ltd

R100/TW rotatest shaker

**Dynatech**

Minimash 2000 cell harvestor

**Edward**

High vacuum

**Grant** Instruments Ltd

Waterbath

**Hybaid** Ltd

Electrophoresis plates, spacer, combs, gel tanks, Hybridization oven, Ultraviolet transilluminator

**Jencons**

Liquid nitrogen tank

**Kelvinator** Commercial Products Inc.

-70<sup>0</sup>C Freezer

**Leec** Ltd

CO<sub>2</sub> incubator

**Medical** International Ltd

Dialysis membrane (size 3-20/32")

**Peteric** Ltd

Hood

**Pharmacia** Ltd

LKB ultrospec plus Spectrophotometer

**Quatro**

TC-40 PCR cyclers

**Shandon** Scientific Ltd,

Citadel 2000 tissue processing machine

### 2.11.3 Reagents and chemicals and suppliers

AG501X8(Resin), Bio-Rad

Agarose, A-9539, Sigma

Albumin, A-7641, Sigma

AMDM,  $\alpha$ -methy-D-mannoside, M-6882, Sigma

Ammonium sulphate, A-5132, Sigma

Anti-rat IFN- $\gamma$  antibody, TechGen

Aquasol, Nen

Biotinamidocaproate N-hydroxysuccinimide ester, B-2643, Sigma

Bordetella pertussis, Wellcome

Boric acid, B-6768, Sigma

Bromocresol green, B-6771, Sigma

BPB, Bromophenol blue, B-6896, Sigma

BSA, Bovine serum albumin, B-2518, Sigma

CFA, complete Freund's adjuvant, Gibco

Chloroforme, 10077, BDH

Citric Acid trissodium dihydrate, C-8532, Sigma

ConA, concanavalin A, C-7275, Sigma

DEPC, Diethyl Pyrocarbonate, D-5758, Sigma

DMSO, Dimethyl Sulfoxide, D-5879, Sigma

dNTP's 100mM, 27-2035-01, Pharmacia



DTT, Y00147, Gibco  
EDTA, Ethylene Diamine Tetra-acetic Acid, E-5134, Sigma  
Eosin Y (1% aqueous), A/S025-D, Raymond  
Ethidium bromide, E-8751, Sigma  
F(ab')<sub>2</sub> Rabbit anti-mouse IgG-FITC conjugate, ST-AR 41, Serotec  
FCS, Fetal calf serum, Gibco  
Ficoll 400, F-2637, Sigma  
Formamide, F-7503, Sigma  
Geneticin (G418), Gibco  
Gentamycin, Gibco  
Glass filter paper, 1827842, Whatman  
L-Glutamin, Gibco  
Guanidine thiocyanate salt, G-6639, Sigma  
Haematoxylin (Ehrlin), A/S047-C, Raymond  
HCl, 10125, BDH  
HT-2 clone A5E, ECACC  
Human recombinant IL-2, Boehringer-Mannheim  
Hybond™-N, Amersham  
8-Hydroxyquinoline, H-6878 Sigma  
Isoamyl alcohol, I-885, Sigma  
Isopropanol anhydrous 405-7, Sigma  
Ketamine, PARKE-DAVIS  
Ladder, ØX174 RF DNA/Hae III Fragment, 5611SA, Gibco  
N-Lauroylsarcosine, L-5125, Sigma  
Magnesium Chloride, M-1028, Sigma  
2-Mercaptoethanol, M-6250, Sigma  
MACS microbeads, 481-01, Miltenyi Biotec Lab Impex  
Metrizoic acid, M-4762, Sigma  
Mineral oil, M-5904, Sigma  
Mouse IgG1 FITC, anti-chicken negative control for FITC, MCA 689F, Serotec  
Mouse IgG1 PE, anti-chicken negative control for PE, MCA 689PE, Serotec  
M-MLV (Moloney murine leukemia virus) reverse transcriptase, 8025SA, Gibco  
Mouse recombinant IL-4, I-1390, Sigma  
Mycobacterium tuberculosis strain H37Ra, Difco

Na<sub>2</sub>HPO<sub>4</sub> disodium hydrogen phosphate, S-3264, Sigma  
NaH<sub>2</sub>PO<sub>4</sub> sodium dihydrogen phosphate, S-3139, Sigma  
NAP<sup>TM</sup>-5 Columns, 17085301, Pharmacia  
NDS 64, MCA 495, Serotec  
Nonessential amino acid, Gibco  
Nylon mesh, Hybaid  
Nylon wool fiber, Polysciences  
OX8, MCA 48, Serotec  
OX8 FITC, MCA 48F, Serotec  
OX19 FITC, MCA 52F, Serotec  
OX22 MCA53, Serotec  
OX22 FITC, MCA 53F, Serotec  
[γ-<sup>32</sup>P] dATP 10mCi ml<sup>-1</sup>, PB10168, Amersham  
[α-<sup>32</sup>P] dCTP 10mCi ml<sup>-1</sup>, PB10385, Amersham  
Parafilm, P-7543, Sigma  
Pd(T)<sub>12-18</sub>, 27-785801, Pharmacia  
PBS, Phosphate buffered saline, Oxoid  
Pertussis vaccine B.P., Wellcome  
Phenol, P-1037, Sigma  
PEG, Polyethylene glycol, P-2139, Sigma  
PMA, Phorbol 12-myristate 13-acetate, P-8139, Sigma  
Polymerization buffer, 600131, Stratagene  
Polynucleotide kinase, 270736, Pharmacia  
PPD, Tuberculin purified protein derivative, EVANS  
Primers for IL-2, IL-4, IFN-γ, IL-10 and β-actin), OSWEL  
PVP, Polyvinylpyrrolidone, P-5288, Sigma  
R7.3, MCA 453, Serotec  
Rabbit anti-mouse Ig(A,G,M), SERT 100, Serotec  
Rat recombinant gamma interferon, Holland Biotechnology b.v.  
RT (reverse transcription) Buffer, Y00146, Gibco  
RNase inhibitor, 799025 Boehringer-Mannheim  
RPMI-1640, Gibco  
Salmon sperm DNA = DNA type III sodium salt from Salmon testes, D-1676, Sigma  
SDS, Sodium Dodecyl Sulfate, Lauryl Sulfate, L-4390, Sigma

Sephadex, G-25, 17-0043-01, Pharmacia  
Serum-free nutridoma, Boehringer-Mannheim  
Sodium Acetate, S-2889, Sigma  
Sodium Chloride, S-3014, Sigma  
Sodium Citrate, S9625, Sigma  
Sodium Hydroxide pellets, S-5881, Sigma  
Sodium pyruvate, Gibco  
Taq Polymerase, 600131, Stratagene  
TCA, Trichloroacetic acid, T-4885, Sigma  
TGF- $\beta$ 1 (human natural), BDA5, British Bio-technology  
Toluene, 30454 6R, BDH  
Tris, Trizma Base, T-8524, Sigma  
Trypan blue, Flow  
W3/25, MCA 55, Serotec  
W3/25 PE, MCA 55P, Serotec  
WEHI-279, ECACC  
Whatman 3MM, Whatman  
X-rayfilm, Fuji  
Xylene cyanol, X-2751, Sigma

#### 2.11.4 Animals

Specific pathogen-free female Lewis rats aged between 4 and 6 weeks were used in all experiments (Bantin & Kingman, Hull, U.K.).

## **Chapter 3 Lymphokine expression by S-Ag specific rat CD4<sup>+</sup> T cell lines**

### **3.1 Introduction**

With the discovery [Morgan et al., 1976; Ruscetti et al., 1977] and characterization [Smith, 1980] of T cell growth factor (IL-2), it became possible to maintain T cells in rapid division after each stimulation, resulting in a large expansion of the number of antigen specific cells. Long-term antigen specific T cell lines or clones appear to retain the features of their normal growth *in vivo* [Kimoto & Fathman, 1982] and have proven extremely useful in studies of the pathogenesis of autoimmune diseases [Ben-Nun et al., 1981; Caspi, 1989; Sakai et al., 1986].

T cells play a central role in immune responses, carrying out a number of effector and regulatory functions. Although some T cells have direct cytotoxic effector functions, T cells mainly exert their influence on other leukocytes via lymphokines or cytokines. Lymphokines secreted by CD4<sup>+</sup> T cells play a key role in the immune response and it is important that their pattern of synthesis is carefully regulated. Therefore, it has been suggested that mechanisms would exist for the selective production of appropriate lymphokines in the response to a given antigen. CD4<sup>+</sup> T cells in the mouse can be divided into two major groups based on lymphokine secretion: Th1 CD4<sup>+</sup> T cells, which mediate DTH and release IL-2 and IFN- $\gamma$ , and Th2 CD4<sup>+</sup> T cells, which provide help for antibody production and release IL-4 [Cher & Mosmann, 1987; Cherwinski et al., 1987]. It is possible that autoimmune diseases with characteristic features such as excessive autoantibody production in the model of systemic lupus erythematosus (SLE), or abnormal DTH responses to retinal antigen in EAU, may be due to differing profiles of CD4<sup>+</sup> T cell lymphokine release in addition to antigenic reactivity. Understanding the specific characteristics of pathogenic CD4<sup>+</sup> T cells in autoimmune disease may provide further insight into disease pathogenesis and may ultimately lead to novel therapeutic strategies.

As outlined in chapter 1, EAU is a CD4<sup>+</sup> T cell-mediated autoimmune disease. It became possible to induce EAU by adoptive transfer of uveitogenic CD4<sup>+</sup> T cell lines specific for S-Ag [Caspi et al., 1986; Gregerson et al., 1986; Rozenszajn et al., 1986]. There is

increasing evidence to suggest that the uveitogenic role of S-Ag specific T cell lines may be ascribed to the production of certain effector lymphokines since one of the requirements for successful adoptive transfer of EAU is that CD4<sup>+</sup> T cells must be activated by antigen or mitogen before injection into syngeneic recipients [Caspi, 1989]. However, there is limited information regarding lymphokine expression of S-Ag specific CD4<sup>+</sup> T cell lines.

In order to further understand the role of S-Ag specific CD4<sup>+</sup> T cell lines in immune regulation of EAU, it is very important to investigate the S-Ag specific CD4<sup>+</sup> T cell lymphokine expression regulation *in vitro*. The purpose of this chapter was to establish the S-Ag specific rat T cell lines and investigate lymphokine expression by these cell lines, to see if restricted lymphokine expression exists. The investigation was carried out at two levels: (1) lymphokine gene expression was detected by PCR combined with Southern analysis, and (2) lymphokines in the supernatants were analyzed by bioassays. Two PPD cell lines were also established as control cell lines and their lymphokine expression compared in parallel with those of the S-Ag T cell lines.

## **3.2 Results**

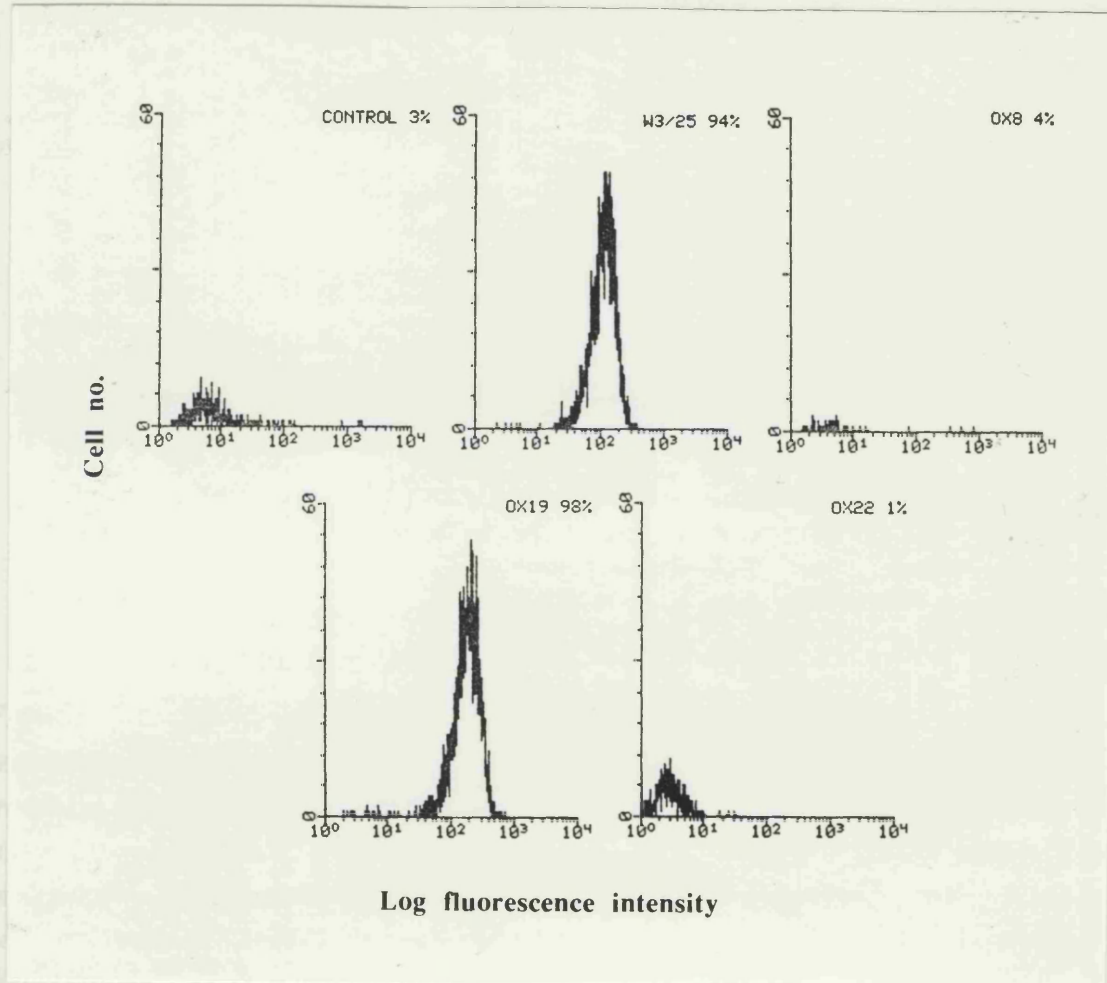
### **3.2.1 T cell lines**

S-Ag specific T cell lines were derived from draining lymph node cells of Lewis rats immunized with S-Ag/CFA plus *B. pertussis*. Three S-Ag specific T cell lines (SAG1, SAG2 and SAG3) were selected *in vitro* as previously described by Sedgwick et al. [Sedgwick et al., 1989] and two S-Ag specific T cell lines (SAG4 and SAG5) were also established by Caspi et al. [Caspi et al., 1986] (for more detail see [section 2.4.2](#)). Two PPD specific cell lines (PPD1 and PPD2) were also established as control cell lines using PPD lymph node cells from rats immunized with CFA alone (containing *M. tuberculosis*) following the Sedgwick method.

It has been shown that the pattern of lymphokine production by PPD-specific T cell lines derived from LN cells of BALB/c mice with either interleukin-2 or interleukin-4 as growth factors does not change significantly after the third restimulation with antigen [Schmitt et al., 1990]. The pattern of lymphokine gene expression in two of S-Ag

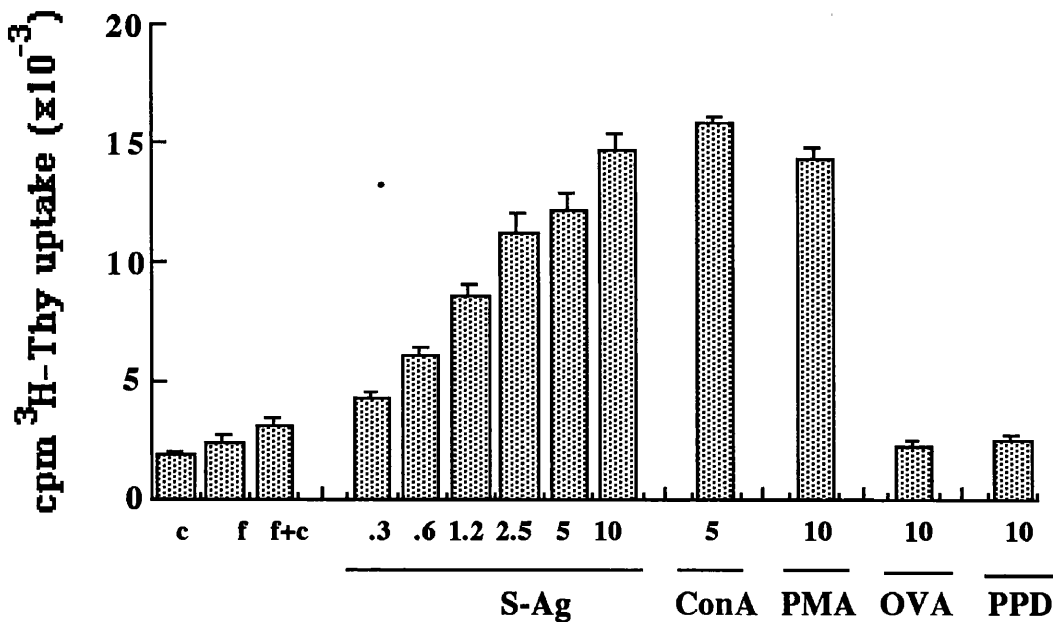
specific T-cell lines was shown to be stable from 2- to 5-passages of antigen restimulation [McLauchlan et al., 1993]. These experiments presented in this thesis were performed on cells 9-11 days after the third or fourth cycle of antigen restimulation (S-Ag specific T cell lines: SAG1. passage IV, SAG2. III, SAG3. III, SAG4. IV, SAG5. III; PPD specific T cell lines: PPD1. IV, PPD2. IV).

The S-Ag and PPD specific T cell lines were analysed for T cell subset markers CD4 (mAb W3/25), CD8 (OX8), CD5 (OX19) and CD45RC (OX22). The pattern of cell surface phenotype was similar in all S-Ag cell lines, although the absolute percentage of positive cells varied. All cell lines were found to be  $CD4^+CD8^-OX22^{low}$  as described in [section 2.4.3](#). A typical phenotype of staining of one of these S-Ag T cell lines is shown in [Fig.3-1](#).



**Figure 3-1** Phenotypic analysis one of S-Ag specific T cell line. Expression of CD4, CD8, OX19 and OX22 on S-Ag specific T cells by immunofluorescence as described in [section 2.4.3](#). Negative control: 3%; W3/25: 94%; OX-8: 4%; OX-19: 98% and OX-22: 1%.

The antigen specificity of the T cells was determined by significant proliferation to S-Ag in the presence of autologous APC. The results showed that all cell lines were highly specific for reactivity to S-Ag, to which they responded in a dose-dependent manner, giving maximal responses at 10  $\mu\text{g/ml}$  (SI>4). This concentration of S-Ag was subsequently used throughout the study. ConA and PMA (mitogens) were also effective stimuli. On the other hand, the nonselecting antigens, PPD and OVA, failed to elicit any significant response. Reciprocal responses to S-Ag and PPD were obtained with two PPD T cell lines. Fig.3-2 shows a typical proliferation assay of one of these S-Ag T cell lines.



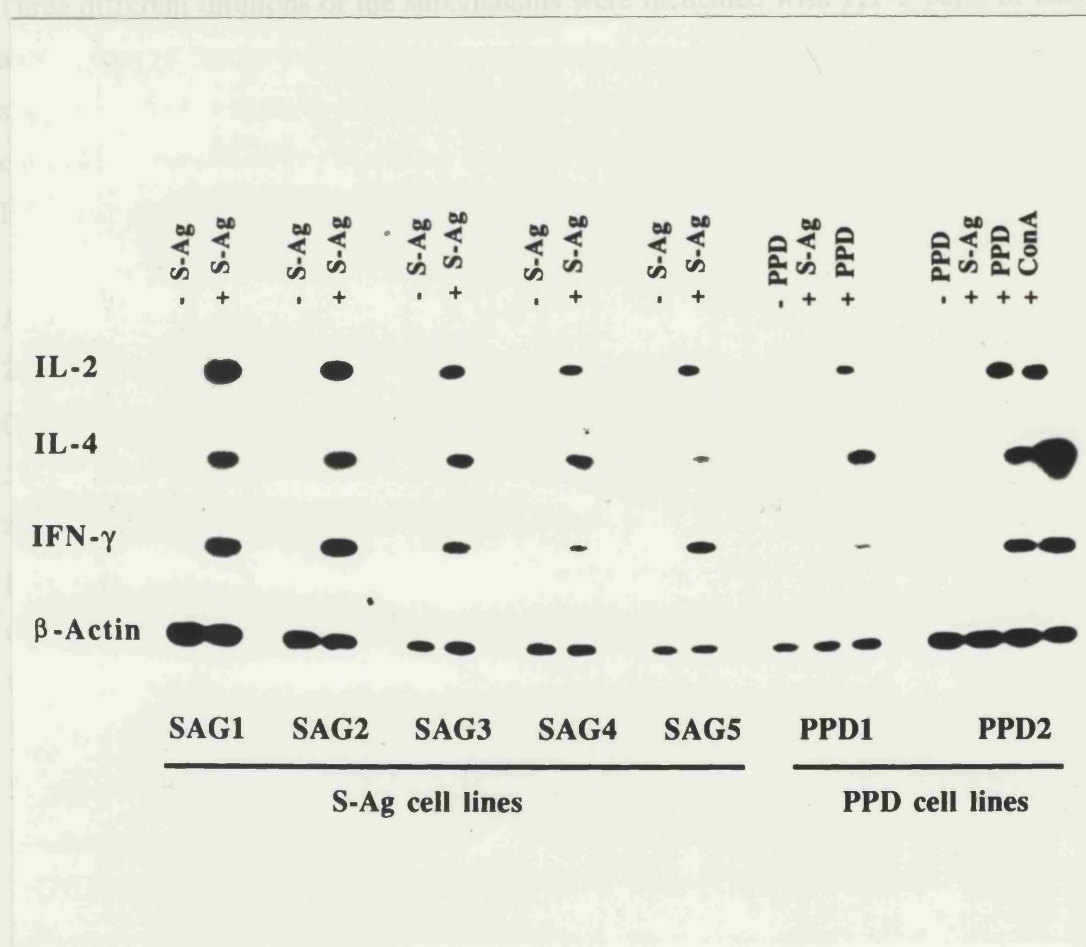
**Figure 3-2** Effects of S-Ag, ConA, PMA, OVA and PPD on the proliferative response of one S-Ag specific T cell line. Cells ( $2 \times 10^4$  cells/well) were cultured with irradiated thymocytes ( $10^6$  cells/well) and either different dilutions of S-Ag (0.3 to 10  $\mu\text{g/ml}$ ), ConA (5  $\mu\text{g/ml}$ ), PMA (10  $\text{ng/ml}$ ), OVA (10  $\mu\text{g/ml}$ ) and PPD (10  $\mu\text{g/ml}$ ). The proliferative response was determined by [ $^3\text{H}$ ]-thymidine incorporation as described in section 2.4.4. Each value represents the mean  $\pm$  SD of triplicate wells. c: cells alone; f: feeders alone; c+f: cells and feeders.

### 3.2.2 Lymphokine gene expression and production by S-Ag specific $\text{CD4}^+$ T cell lines

The mRNA samples were taken at 6 h and culture supernatants were taken 24 h after incubation with antigen and APC. These time periods had been found to be optimal for IL-2, IL-4 and IFN- $\gamma$  lymphokines (see chapter 4).

### 3.2.2.1 Lymphokine gene expression by S-Ag specific CD4<sup>+</sup> T cell lines

To evaluate the lymphokine gene expression of the five S-Ag specific T cell lines, the cells were incubated with S-Ag and APC for 6 h. The results demonstrate that all cell lines examined were able to express IL-2, IFN- $\gamma$  and IL-4 (Fig.3-3). No signals were detected in unstimulated cells.



**Figure 3-3** Lymphokine gene expression by S-Ag-specific and PPD-specific rat CD4<sup>+</sup> T cell lines after 6 h activation. cDNAs were reverse transcribed and amplified by PCR, as described in section 2.9.2. 10  $\mu$ l of PCR amplification products was electrophoresed through 1.5% agarose gel, Southern blotted to nitrocellular membranes and hybridized with internal probes.  $\beta$ -actin mRNA was a positive control.



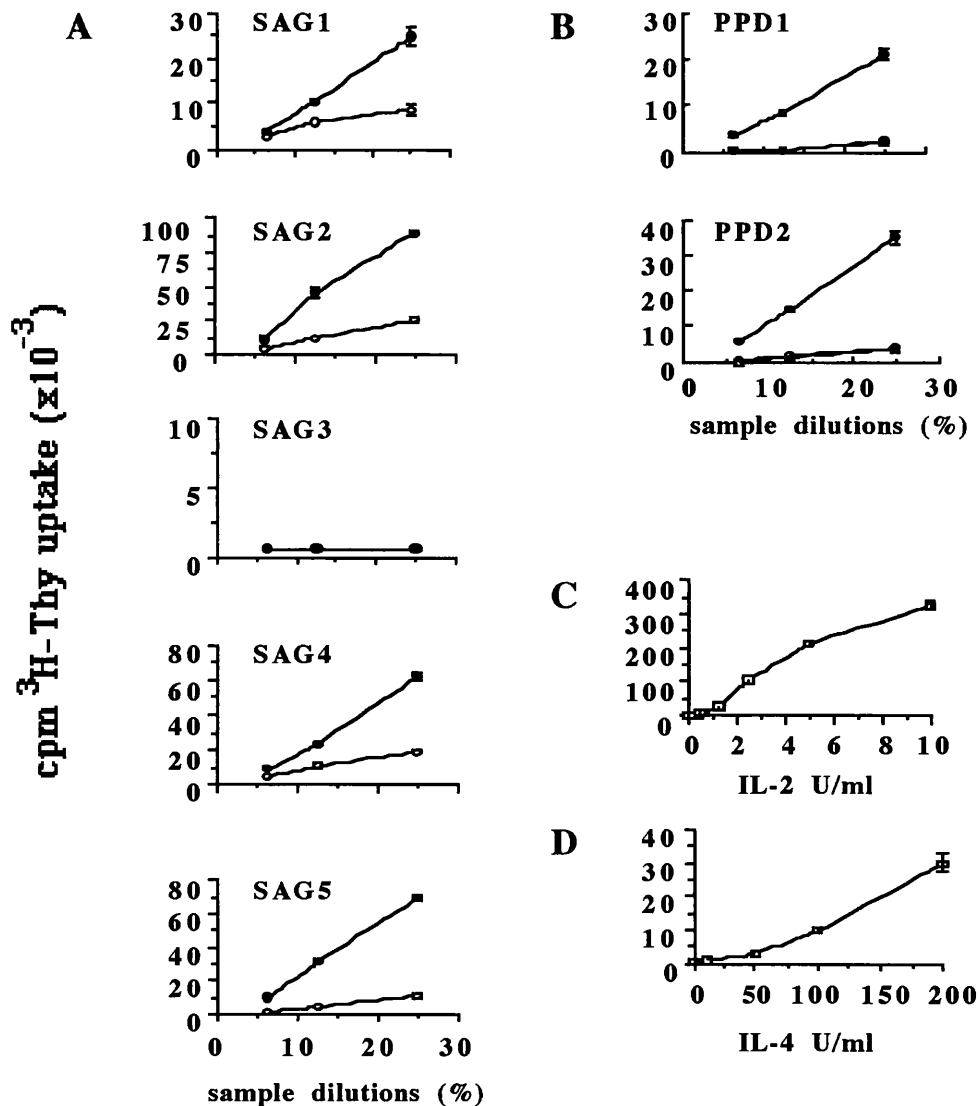
Two PPD cell lines were assessed for their lymphokine gene expression upon stimulation with S-Ag or PPD (one PPD cell line was also stimulated with ConA) and APC (Fig.3-3). It was found that PPD cell lines could not be induced to express any lymphokine gene by S-Ag, but were able to express lymphokine mRNA when activated by their specific antigen (PPD).

#### 3.2.2.2 Lymphokine production by S-Ag specific CD4<sup>+</sup> T cell lines

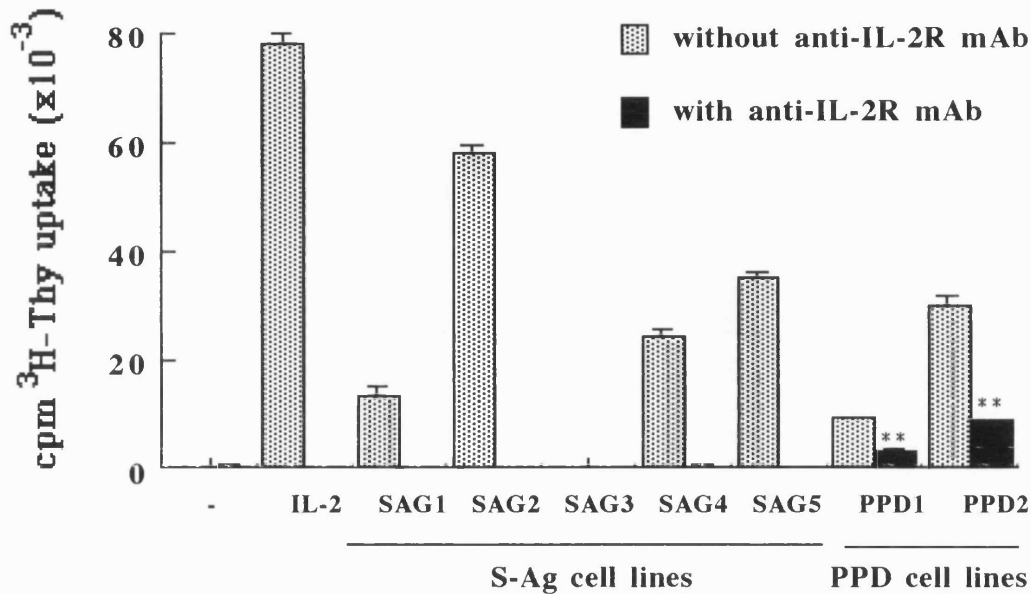
Supernatants were collected from cultured cells in the same experiments after 24 h stimulation. IL-2 bioactivities measured using HT-2 indicator cells are shown in Fig.3-4. Three different dilutions of the supernatants were incubated with HT-2 cells in each assay. One of the five S-Ag T cell lines failed to induce HT-2 cell proliferation, suggesting there was no IL-2 in the sample, although IL-2 mRNA was detected in this T cell line. It was also found that some unstimulated cell supernatants were able to induce HT-2 cell proliferation, suggesting a background level of IL-2.

As shown in Chapter 2 anti-rat IL-2R mAb (NDS, 64), known to prevent binding of IL-2 to its receptor [Tellides et al., 1989], was able to inhibit IL-2 dependent mouse T-cell (HT-2) proliferation. The inhibition by this antibody was tested at dilutions of  $10^{-5}$  to  $5 \times 10^{-1}$ , and the proliferative response to hrIL-2 (2.5 U/ml) was found to be inhibited in a dose-dependent fashion, and totally blocked at 1:50 dilution. Similarly, the proliferative response of HT-2 cells to the supernatants which were diluted (1/4) was completely blocked in the presence of this mAb (Fig.3-5), verifying that IL-2 existed in the supernatants.

In contrast, the supernatants from two PPD cell lines after stimulation with S-Ag could not induce proliferation of HT-2 cells and the proliferative responses of HT-2 cells to supernatants from PPD cell lines after stimulation with PPD were only partially blocked by anti-IL-2R mAb, suggesting that HT-2 cell stimulating factors other than IL-2 is likely to exist in the culture supernatants (Fig.3-5).



**Figure 3-4** Proliferative responses of HT-2 cells to supernatants from S-Ag and PPD specific T cell lines. Supernatants were collected after 24 h activation. The proliferative response was determined by [ $^3\text{H}$ ]-thymidine incorporation as described in section 2.8.2. Each value represents the mean $\pm$ SD of 3 individual wells. a: Dilutions of the supernatants from S-Ag T cell lines.  $\circ$ : supernatants from unstimulated cells;  $\bullet$ : supernatants from cells stimulated with S-Ag. b: Dilutions of the supernatants from PPD T cell lines.  $\circ$ : supernatants from unstimulated cells;  $\blacktriangle$ : supernatants from cells stimulated with S-Ag;  $\bullet$ : supernatants from cells stimulated with PPD. c: Human recombinant IL-2 standard curve. d: Mouse recombinant IL-4 standard curve.

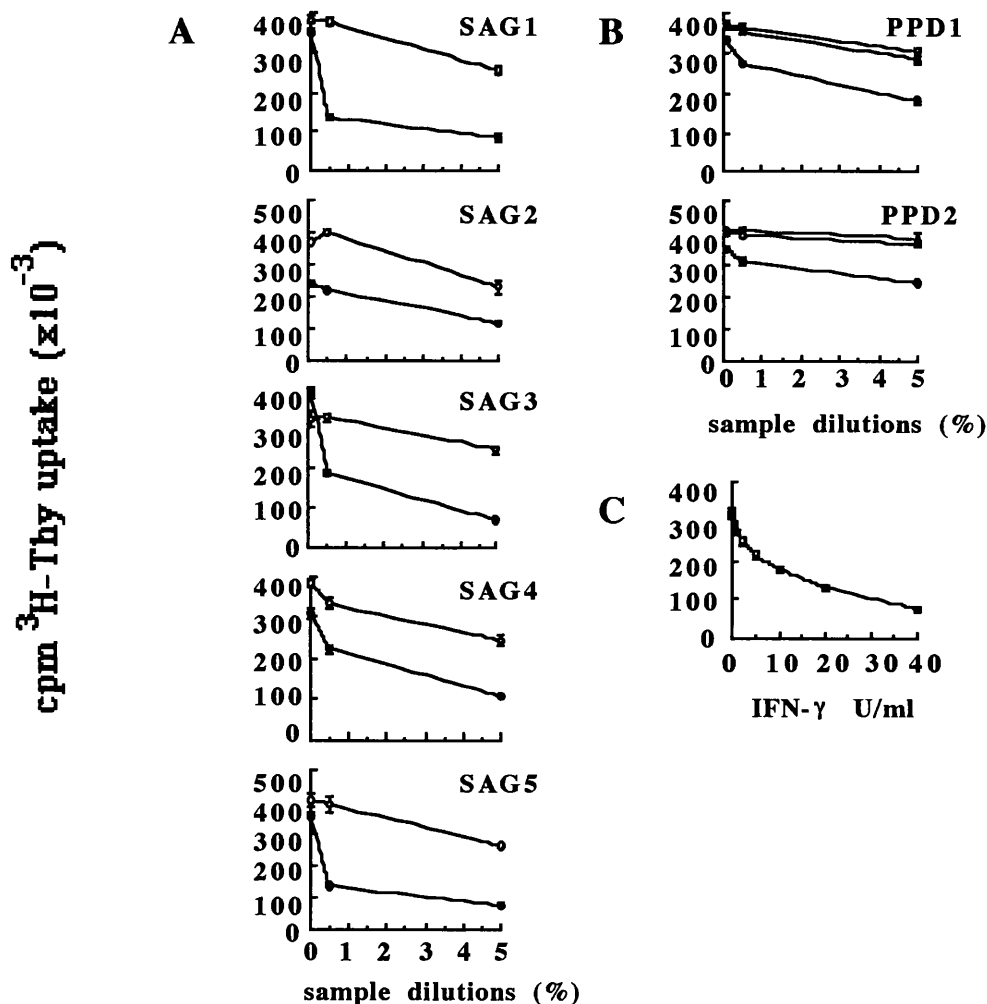


**Figure 3-5** Effect of anti-IL-2R mAb (1/50 dilution) on proliferative responses of HT-2 cells induced by supernatants (1/4 dilution) from S-Ag and PPD specific T cell lines 24 h after activation with antigen. Each value represents the mean $\pm$ SD of 3 individual wells. hrIL-2 (2.5 U/ml) in the absence and presence of anti-IL-2R mAb was as positive controls. \*\*  $p < 0.01$ .

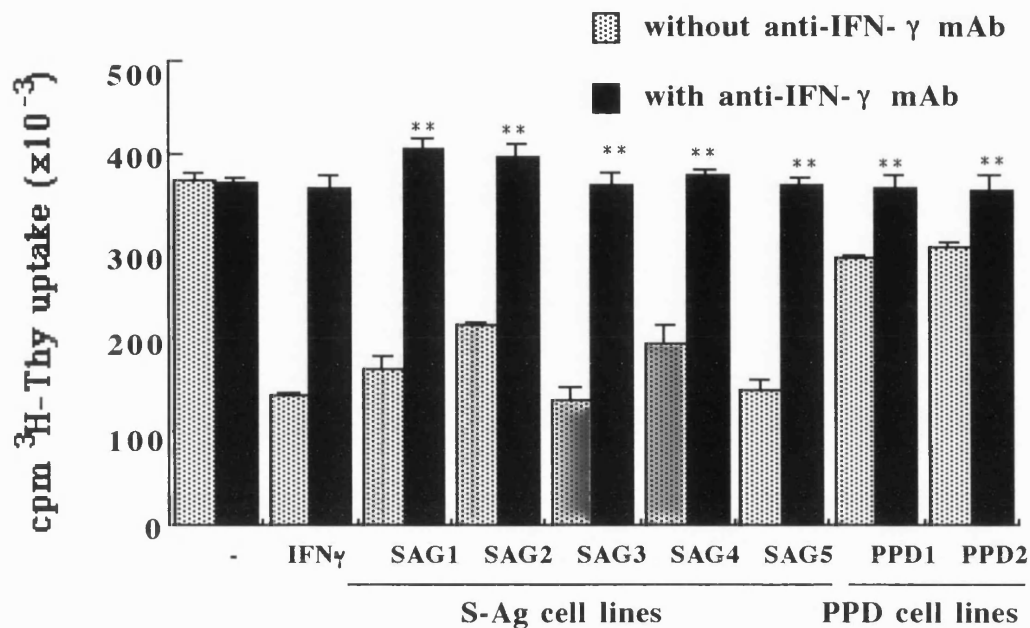
Fig.3-6 shows IFN- $\gamma$  production by S-Ag specific T line cells after stimulation with S-Ag. Three different dilutions of the supernatants were incubated with the IFN- $\gamma$ -sensitive cell line WEHI-279. The results show that all five S-Ag specific cell lines produced high levels of IFN- $\gamma$ . Similarly S-Ag failed to induce PPD line cells to produce IFN- $\gamma$ , but PPD could induce these cells to secrete IFN- $\gamma$ .

Anti-rat-IFN- $\gamma$  mAb was used to confirm that the IFN- $\gamma$ -induced inhibitory effect on WEHI-279 cells was indeed due to IFN- $\gamma$  present in the supernatants. It has been shown in chapter 2 that the inhibition of WEHI-279 cell proliferation by IFN- $\gamma$  (40 U/ml) could be reduced by anti-rat-IFN- $\gamma$  antibody at concentrations of 0.0125 to 12.5  $\mu$ g/ml dose-dependently, and at the highest concentration used, the anti-inhibitory effect of IFN- $\gamma$  on WEHI-279 cell could be reversed. Similarly, the inhibitory response of WEHI-279 cells

to supernatants which were diluted (1/20) was blocked in the presence of this antibody 12.5  $\mu\text{g/ml}$  (Fig.3-7).



**Figure 3-6** Inhibitory responses of dilutions of supernatants from different cell lines on WEHI-279 cells. Supernatants were collected after 24 h activation. The proliferative response was determined by [ $^3\text{H}$ ]-thymidine incorporation as described in section 2.8.3. Each value represents the mean  $\pm$  SD of 3 individual wells. A: Dilutions of the supernatants from S-Ag T cell lines. ○: supernatants from unstimulated cells; ●: supernatants from cells stimulated with S-Ag. B: Dilutions of the supernatants from PPD T cell lines. ○: supernatants from unstimulated cells; ▲: supernatants from cells stimulated with S-Ag; ●: supernatants from cells stimulated with PPD. C: Dose dependent response of WEHI-279 cells to rat recombinant IFN- $\gamma$ .



**Figure 3-7** Effects of anti-IFN- $\gamma$  mAb (12.5  $\mu$ g/ml) on inhibitory effects of WEHI-279 by supernatants (1/200 dilution) from S-Ag-specific and PPD-specific CD4<sup>+</sup> T cell lines. Each value represents the mean  $\pm$  SD of 3 individual wells. Rat recombinant IFN- $\gamma$  (40 U/ml) in the absence and presence of anti-IFN- $\gamma$  mAb was as positive controls. \*\*  $p < 0.01$ .

### 3.3 Discussion

CD4<sup>+</sup> T cells stimulated with antigen or mitogen do not normally secrete only a single lymphokine but secrete several lymphokines in a defined pattern. In order to investigate lymphokine profile by S-Ag specific CD4<sup>+</sup> T cells in the rat, we measured lymphokines produced by these lines, and compare their pattern with those of PPD T cell lines.

An unexpected, but repeatable, finding is the production of IL-2 and IFN- $\gamma$  by unstimulated resting cells. Furthermore, the IL-2 and INF- $\gamma$  bioactivities in unstimulated cell supernatants were blocked, or inhibited with respective antibodies, verifying that the lymphokines existed in the medium released by the cells (Table 3-1). Our results are in agreement with other investigators [Fernandez-Botran et al., 1988; Kurt-Jones et al., 1987] who observed that rested antigen-specific murine T cell lines and clones expressed

lymphokine receptors such as IL-2 and IL-4 which were not expressed on resting T lymphocytes. This phenomenon could be due to irradiated feeder cells in the unstimulated population which might be nonspecifically stimulating a low level of lymphokine expression or may reflect a continual state of activation of the T cell lines.

**Table 3-1** *Production of IFN- $\gamma$  and IL-2 by unstimulated cells (9-11 days after last stimulation) of antigen specific CD4<sup>+</sup> T cell lines.*

	IFN- $\gamma$ (cpm $\times 10^{-3}$ )		IL-2 (cpm $\times 10^{-3}$ )	
	-mAb	+mAb	-mAb	+mAb
Cells alone	321.4 $\pm$ 12.2	318.5 $\pm$ 10.8	-	-
IFN- $\gamma$ (10 U/ml)	177.3 $\pm$ 8.5	324.2 $\pm$ 9.5	-	-
Cells alone	-	-	0.51 $\pm$ 0.35	0.50 $\pm$ 0.09
IL-2 (2.5 U/ml)	-	-	108.40 $\pm$ 1.62	0.51 $\pm$ 0.08
SAG1	232.4 $\pm$ 18.4	307.3 $\pm$ 10.5	25.60 $\pm$ 2.02	0.45 $\pm$ 0.01
SAG2	256.3 $\pm$ 9.1	324.1 $\pm$ 15.6	9.40 $\pm$ 1.10	0.50 $\pm$ 0.13
SAG3	247.1 $\pm$ 10.2	331.6 $\pm$ 15.2	0.50 $\pm$ 0.17	0.52 $\pm$ 0.14
SAG4	244.8 $\pm$ 11.4	319.2 $\pm$ 14.1	19.90 $\pm$ 1.56	0.48 $\pm$ 0.12
SAG5	262.2 $\pm$ 3.8	325.5 $\pm$ 12.9	10.40 $\pm$ 1.32	0.50 $\pm$ 0.10
PPD1	305.5 $\pm$ 5.7	340.3 $\pm$ 16.1	2.60 $\pm$ 0.50	0.50 $\pm$ 0.11
PPD2	316.4 $\pm$ 4.9	351.4 $\pm$ 15.4	4.40 $\pm$ 0.12	0.65 $\pm$ 0.10

Upon stimulation of five S-Ag specific T cell lines with S-Ag, an increased production of IL-2 and IFN- $\gamma$  was observed, except in one cell line which had no detectable IL-2. The gene expression of the lymphokines was also studied and results showed that the patterns of expression correlated with the observed lymphokine bioactivities. The reason for the failure to detect IL-2 in this T cell line is not clear, although IL-2 mRNA was detected. It could be explained by the fact that we have inadvertently selected low IL-2-producing cells due to the continued presence of IL-2 in the medium [Tokuchi et al., 1990] or that IL-2 produced in culture is immediately consumed by activated cells and is

therefore no longer available in the supernatant for detection [Mizochi et al., 1986]. The increase in IL-2 and IFN- $\gamma$  production by S-Ag specific T cells after S-Ag activation which we have observed is in agreement with another study of rat CD4<sup>+</sup> T cells using ELISA techniques [Savion et al., 1992]. These results are also in agreement with other animal models of human inflammatory autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) where T cell lines and clones that transfer EAE in the rat and the mouse produce IFN- $\gamma$ , IL-2, TNF- $\alpha$  and TGF- $\beta$  [Ando et al., 1989; Sedgwick et al., 1989].

Mouse HT-2 cells respond to both IL-2 or IL-4 [Watson, 1979]. With this HT-2 system, we could estimate IL-2 bioactivity, but were unable to assure if there was IL-4 bioactivity in the supernatant due to the species specific of IL-4 in its activities [Mosmann et al., 1987; Leitenbergand & Feldbush, 1988]. When in the presence of rat IL-2R mAb, the proliferation of HT-2 cells induced by supernatants from S-Ag specific T cell lines was totally blocked, while the proliferative response induced by supernatants from PPD T cell lines was only partially inhibited, suggesting that there are HT-2 cell growth factors other than IL-2 in the supernatant of PPD T-cell lines, but not S-Ag T-cell lines. Savion et al. [Savion et al., 1992] failed to detect IL-4 production in rat uveitogenic CD4<sup>+</sup> T cells using ELISA. As we detected IL-4 mRNA expression in the S-Ag T-cells, IL-4 secretion would also be expected in these cells. But it must be born in mind that there may be discrepancy between mRNA expression and protein secretion, and that the presence of message does not necessarily indicate the production of bioactive protein [Beutler & Cerami, 1989; Cherwinski et al., 1987; Sariban et al., 1988].

IL-4 has a major role in B lymphocyte activation, proliferation and differentiation [Jelinek & Lipsky, 1987]. It is also involved in T lymphocyte activation and growth [Paul & Ohara, 1987]. However, in our study we were not sure if IL-4 was secreted from any of the S-Ag specific CD4<sup>+</sup> T cell lines, although IL-4 mRNA was detected. It is difficult to interpret the importance of IL-4 in inducing EAU. However it has been shown that few B cells are present in the intraocular pathology in EAU [Chan et al., 1985] and posterior uveitis in man [Lightman & Chan, 1990].

IL-2 plays a key role in the initiation of immune responses [Smith, 1988] and could be detected in ocular material obtained from patients with posterior uveitis [Hooks et al.,

1988]. An increase in IL-2 receptor positive T cells in peripheral blood have been demonstrated in patients with various uveitis including posterior uveitis [Deschenes et al., 1988]. In EAU, it has been shown that IL-2 mRNA was expressed *in vivo* by cells infiltrating the eye [Charteris & Lightman, 1993]. EAU induced by adoptive transfer of S-Ag specific T cell lines can be prevented by *in vivo* treatment with monoclonal antibody against IL-2 receptor [Higuchi et al., 1991]. The demonstration of production of IL-2 by S-Ag specific CD4<sup>+</sup> T cell lines is further evidence that this mediator is central to the immunopathology in intraocular inflammatory disease.

All S-Ag specific T cell lines reported here produced high levels of IFN- $\gamma$ . This lymphokine is known to play an essential role in DTH responses [Paul & Ohara, 1987] and to induce or up-regulate MHC class II antigen expression on a variety of cell types [Trinchieri & Perussia, 1985]. The fact that S-Ag specific T cell lines produce IFN- $\gamma$  may explain why *in vivo* S-Ag usually results in the development of a significant DTH response towards this antigen. Production of IFN- $\gamma$  is indeed essential for the expression of DTH reactions [Fong & Mosmann, 1989]. It has been shown that IFN- $\gamma$  can induce MHC class II expression *in vitro* in rat, guinea-pig [Liversidge et al., 1988] and human [Liversidge et al., 1988] retinal pigment epithelial (RPE) cells, a layer of cells lying between retina and choroid forming part of the blood-retinal barrier. RPE cells are known to express MHC class II antigens *in vivo* in human posterior uveitis [Chan et al., 1986] and in EAU [Chan et al., 1986]. It has been proposed that such aberrant expression of class II may be important in the development of autoimmunity [Bottazzo et al., 1983]. Recently it has been shown that IFN- $\gamma$  can be produced *in vivo* by cells infiltrating the eye in the Lewis rat of EAU [Charteris & Lightman, 1992]. EAU induced by active immunization can be prevented by *in vivo* treatment with monoclonal antibody against IFN- $\gamma$  [Atalla et al., 1990]. The production of high level of IFN- $\gamma$  by S-Ag specific cell lines provides a possible source for inducing aberrant expression of class II antigens on organ resident cells during EAU.

We have taken advantages of previously reported phenomenon that after 3 rounds of antigen stimulation, the pattern of lymphokine expression of antigen specific T-cell lines maintains a stable pattern [Schmitt et al., 1990]. Moreover, the gene expression of IL-2, IL-4 and IFN- $\gamma$  in two of S-Ag T-cell lines has been shown to be in a relatively stable



pattern from 2 to 5 rounds of antigen restimulation [McLauchlan et al., 1993]. However, it is clear that all the cells in the lines do not have the same cytokine repertoire. The technique of *in-situ* hybridization that allows identification of the precise morphological distribution of nucleic acid sequences directly in cells should be very useful in identifying a stable pattern of cytokine expression. Furthermore, caution should be taken to interpret the results obtained from gene expression studies, especially to apply this observation to bioactivity of cytokine. As has been reported, cytokine bioactivity may not always be well related to gene expression [Dallman & Clark, 1991].

Taken together, in the present studies, we found that S-Ag specific CD4<sup>+</sup> T cell lines produced IFN- $\gamma$  and IL-2, suggesting that IFN- $\gamma$  and IL-2 might play a key role in EAU induction. It should be noted that the ability of these cell lines to induce EAU is still unknown. Further investigation of the relationship of the secreted lymphokines with the ability to induce EAU will provide an insight into disease pathogenesis.

## **Chapter 4 Comparison of antigen and mitogens for inducing lymphokines in a rat S-Ag specific CD4<sup>+</sup> T cell line**

### **4.1 Introduction**

The specificity for antigen is determined by the T-cell receptor (TcR) expressed on the surface of T lymphocytes. The TcR, rearranged during thymic ontogeny, is polymorphic. This clonally unique TcR is associated on the surface of the cell with CD3 (T3), a multichain complex which is thought to be involved in signal transduction from the TcR to the cytoplasm of the cell. Antigen specific activation of the T cell is dependent upon the TcR-CD3 complex binding the appropriate antigen in association with MHC molecules [Weiss et al., 1986]. However, T cell triggering can also be modulated by a number of other cell surface molecules such as CD4 and CD2 [Bierer & Burakoff, 1989]. Mitogens (ConA and PMA) activate T cells in a non-antigen specific manner. For example, PMA, an activator of protein kinase C, can bypass signalling via TcR and activate directly the intracellular pathway leading to gene activation [Nishizuka, 1984]. ConA mediates T cells by interacting with the T3 antigen [Palacios, 1982].

Activation of CD4<sup>+</sup> T cells with different stimuli may directly or indirectly result in different signals being delivered to the cells, which in turn initiate a cascade of biochemical events leading to the eventual transcription of lymphokine genes [Carding et al., 1989]. There are two major biochemical pathways which inactivate or activate the programmed immune effector functions of T lymphocytes: adenylate cyclase-cAMP-protein kinase A and Ca<sup>2+</sup>/calmodulin-phosphoinositide-protein kinase C [Hadden, 1988]. The adenylate cyclase-cAMP-protein kinase A pathway conveys the inhibitory signal resulting in a downregulation of T cell effector functions [Kammer, 1988]. The positive signal is associated with Ca<sup>2+</sup>/calmodulin-phosphoinositide-protein kinase C pathway [Hadden, 1988]. The activation of T cells leads to the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdInsP<sub>2</sub>) which generates inositol 1,4,5-trisphosphate (InsP<sub>3</sub>) and diacylglycerol (DAG). DAG stimulates PKC, while InsP<sub>3</sub> appears to be an extremely potent and selective agent for the release of calcium from the endoplasmic reticulum.

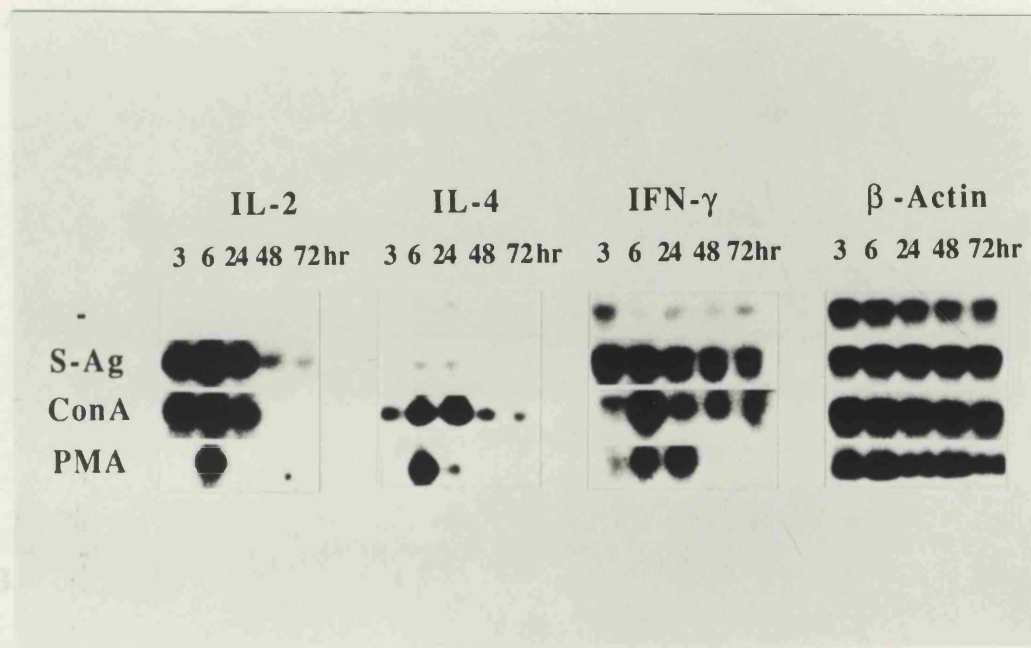
Recently, the notion that CD4<sup>+</sup> T cells can independently regulate the profile of lymphokines produced has been reported to depend upon the type and intensity of the activation signal. Data reported by Yokoyama et al. showed that some Th2 clones in the mouse could produce IFN- $\gamma$  and IL-2 after stimulation with immobilized anti-CD3 mAb [Yokoyama et al., 1989]. Patarca et al. [Patarca et al., 1991] showed differential induction of IFN- $\gamma$  gene expression after activation of Th1 cells either by conventional antigen or by Mls superantigens. Results reported by Torbett et al. [Torbett et al., 1989/1990] showed that PMA and ionomycin induced secretion of both IL-2 and IL-4 whereas antigen and lectin led to secretion of only IL-4. Similarly results reported by Monteyne et al. [Monteyne et al., 1992] suggested that CD4<sup>+</sup> T cells producing IL-2 and IFN- $\gamma$  (Th1-like) or IL-4 (Th2-like) may have different requirements for activation. They analysed the expression of IL-4 and IFN- $\gamma$  mRNA in anti-CD3 stimulated mouse spleen cells and observed a shift in the lymphokine pattern induced in the presence of PMA. Therefore, it is possible that mechanisms would exist for the selective production of appropriate cytokines in the response by the same cells to different stimuli.

It has been shown in chapter 3 that almost all the S-Ag specific rat CD4<sup>+</sup> T cell lines express IFN- $\gamma$ , IL-2 and IL-4 mRNA and secrete IFN- $\gamma$  and IL-2. It could be very interesting to know whether IL-2, IL-4 and IFN- $\gamma$  expression by these S-Ag specific rat T cell lines depends on the mode of activation. In this chapter, one of the S-Ag-specific CD4<sup>+</sup> T cell lines (SAG5) was used to investigate lymphokine expression in response to either specific antigen (S-Ag) or to non-specific mitogens (ConA and PMA).

## **4.2 Results**

### **4.2.1 Time course of lymphokine gene expression by S-Ag specific CD4<sup>+</sup> T cells**

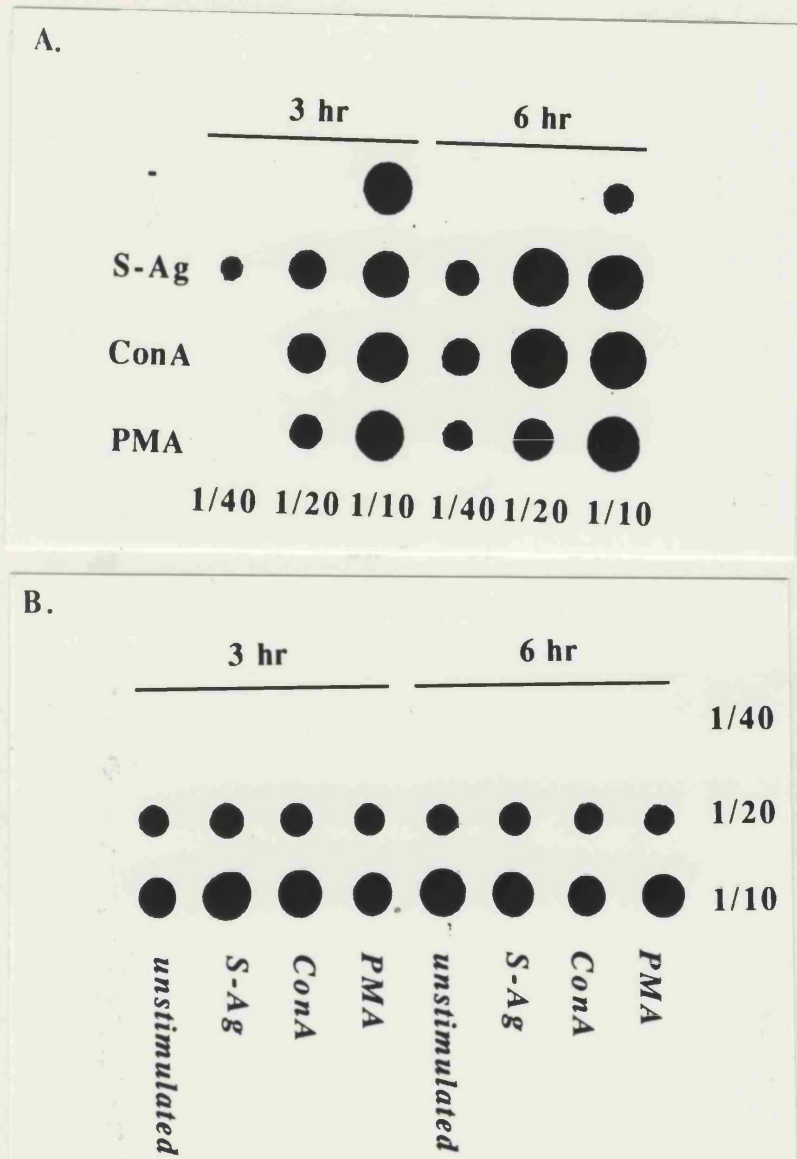
The S-Ag specific cells were stimulated with S-Ag, ConA or PMA for 3, 6, 24, 48 and 72 h. There was no detectable IL-2 mRNA in the unstimulated cells. IL-2 mRNA was detected at all time points after stimulation with S-Ag (Fig.4-1). IL-2 mRNA could be detected 6-24 h following PMA treatment, but was not detectable at other time points. The expression of IL-2 mRNA following activation by ConA was detected at 3, 6 and 24 h and no positive signals were detected after 48 h.



**Figure 4-1** Time course of IL-2, IL-4 and IFN- $\gamma$  gene expression in the S-Ag specific T cells after stimulation with either S-Ag (10  $\mu\text{g/ml}$ ), ConA (5  $\mu\text{g/ml}$ ) or PMA (10 ng/ml). cDNAs were reverse transcribed and amplified by PCR as described in section 2.9.2. 10  $\mu\text{l}$  samples were fractionated on 1.5% agarose gel, Southern blotted to nitrocellular membranes, hybridized to the internal probes labelled with  $\gamma\text{-}^{32}\text{P}\text{-dCTP}$ , and exposed to X-ray film.  $\beta$ -actin mRNA was a positive control.

In contrast to IL-2 mRNA, some blots showed a low level of mRNA for IFN- $\gamma$  in the unstimulated cells (Fig.4-1). Semi-quantitation of PCR by the dot blot technique was performed to assess IFN- $\gamma$  gene expression at 3 and 6 h in response to S-Ag and mitogen stimulation. IFN- $\gamma$  mRNA in the unstimulated cells is much less than that in the S-Ag, ConA and PMA activated cells (Fig.4-2A). There was no significant difference in the level of  $\beta$ -actin mRNA expression (Fig.4-2B).

IL-4 mRNA could be weakly detected in the unstimulated cells at 6 and 24 h (Fig.4-1), and could also be detected in the S-Ag stimulated cells at the same time point, but little or no visible induction was observed compared with the unstimulated cells. However, IL-4 mRNA was clearly induced after stimulation with PMA or ConA compared with the unstimulated and S-Ag-stimulated cells, suggesting that IL-4 gene expression is differentially regulated by signals transmitted through the TcR and other surface molecules.

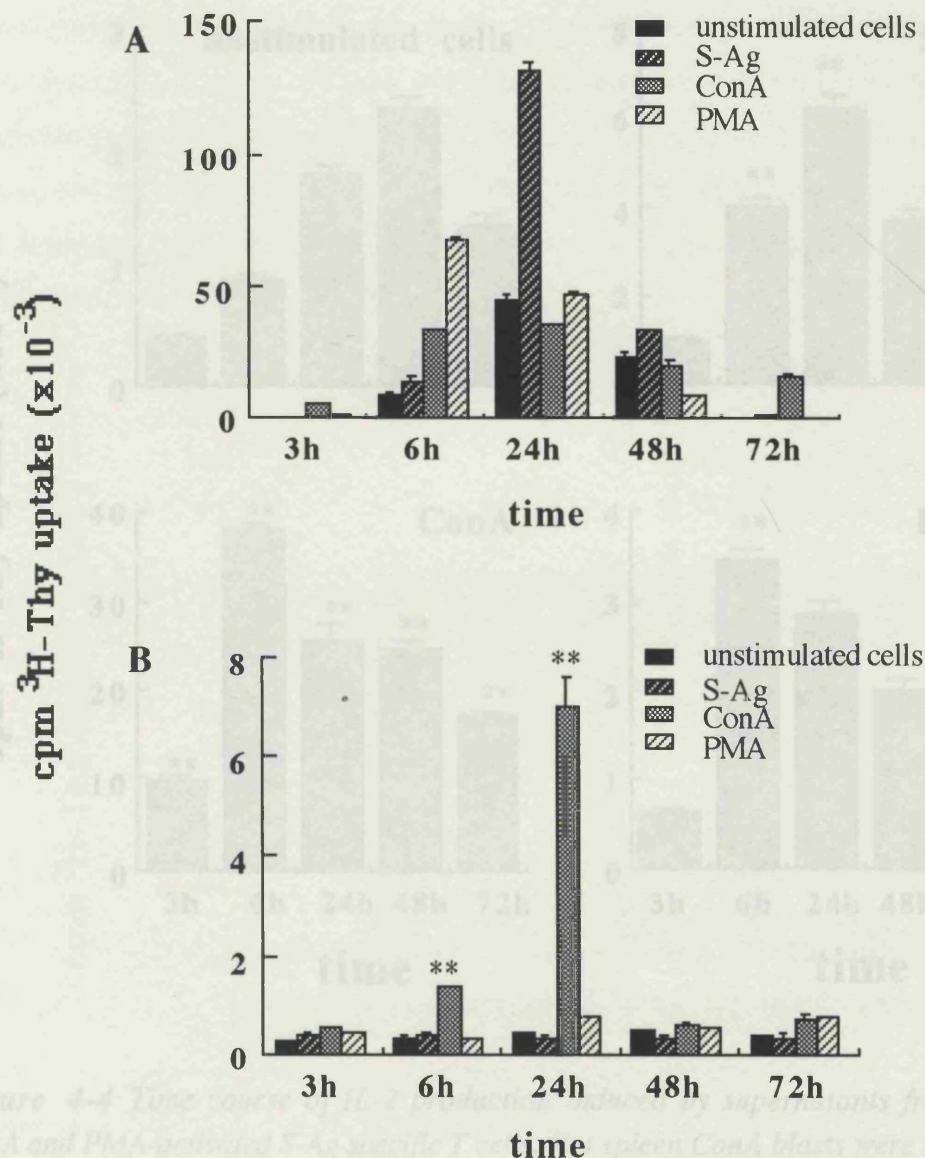


**Figure 4-2** A: Semi-quantitation of IFN- $\gamma$  mRNA by dot blot. The cDNA pooled from the reverse transcription reaction was diluted 1/10, 1/20 and 1/40 in sterile water. 10  $\mu$ l of the separated dilutions was each subjected to PCR. After 25 cycles, 10  $\mu$ l samples were dot blotted to a nitrocellular membrane, hybridized to the internal probe labelled with  $\gamma$ - $^{32}$ P-dCTP, and exposed to X-ray film. B: Dot blot analysis of  $\beta$ -actin mRNA from S-Ag, ConA and PMA groups as described above.

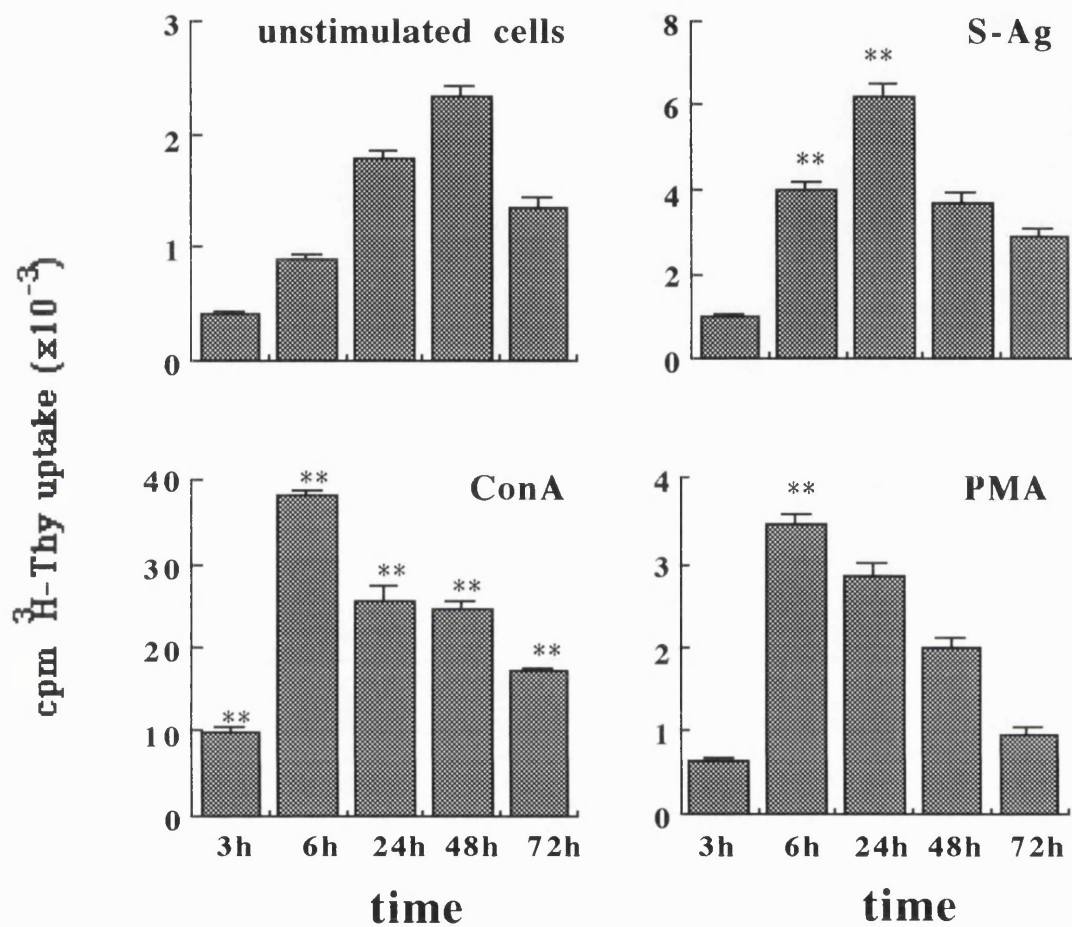
#### 4.2.2 Time course of lymphokine production by S-Ag-specific CD4<sup>+</sup> T cells

The proliferative responses of HT-2 cells were increased by incubation with supernatants from different groups shown in Fig.4-3. After the simultaneous treatment of HT2 cells with supernatants and anti-IL-2R mAb, the proliferative responses of the cells to the diluted supernatants (1/4) from unstimulated, S-Ag- and PMA-treated groups were completely inhibited. However, IL-2R mAb only partially blocked the proliferative responses to the diluted supernatants (1/40) from the ConA-treated group after 6 and 24 h ( $p < 0.01$ ), suggesting that ConA may induce other HT-2 growth factor(s) besides IL-2.





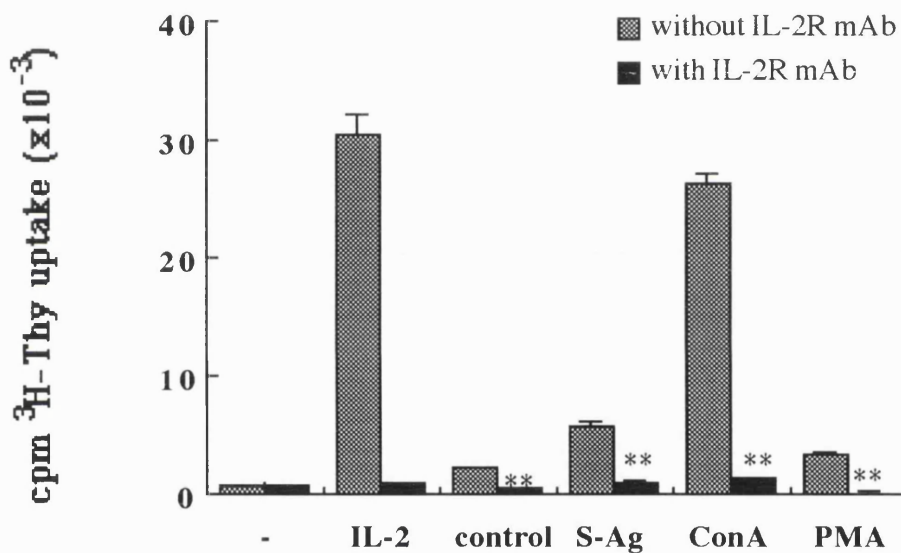
**Figure 4-3** Time course of proliferative responses of HT-2 cells to supernatants from unstimulated cells, S-Ag, PMA and ConA groups. After simultaneous treatment of the cells with supernatants in the absence or in combination with anti-IL-2R mAb (1/50 dilution), the proliferative responses of the cells to supernatants from unstimulated cells, S-Ag-, ConA- and PMA-treated groups were examined. Each value represents the mean $\pm$ SD of 3 individual wells. A: Proliferative response of HT-2 cells to supernatants (unstimulated cells, S-Ag- and PMA-groups: 1/4 dilution; ConA-group: 1/40 dilution). B: Proliferative responses of HT-2 cells to supernatants, diluted as above, in combination with anti-IL-2R mAb. \*\*  $p < 0.01$ .



**Figure 4-4** Time course of IL-2 production induced by supernatants from S-Ag, ConA and PMA-activated S-Ag specific T cells. Rat spleen ConA blasts were incubated with 1/4 diluted supernatants from different groups for 64 h. The plates were pulsed with 1  $\mu\text{Ci}$  of [ $^3\text{H}$ ] thymidine for 8 h, and harvested. Each value represents the Mean $\pm$ SD of 3 individual wells. \*\*  $p < 0.01$ .

The production of IL-2 was also measured with IL-2-dependent cells (rat spleen ConA blasts). Fig.4-4 shows that the production of IL-2 after S-Ag treatment was significantly increased ( $p < 0.01$ ) above the level of the unstimulated cells at 6 and 24 h. IL-2 bioactivity was significantly increased ( $p < 0.01$ ) by the treatment of ConA at all the time points tested. The release of IL-2 bioactivity was significantly increased ( $p < 0.01$ ) at 6 h after treatment with PMA as compared with the unstimulated cells. There was no

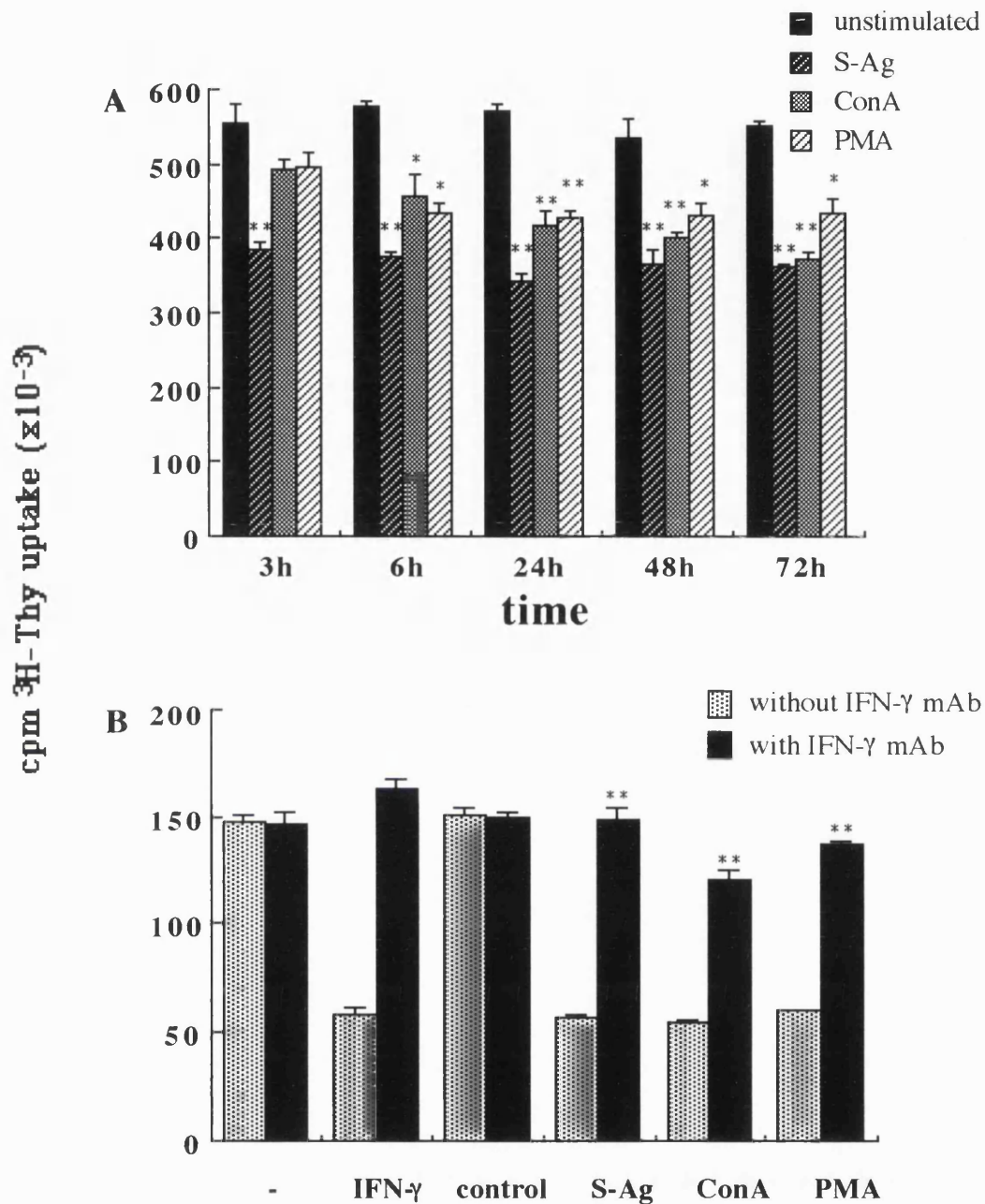
significant difference from the unstimulated group at other time points. An anti-rat IL-2R mAb (NDS 64) was used to demonstrate specific IL-2 activity in the supernatants from various groups. It has been shown in [chapter 2](#) that inhibitory proliferation of rat spleen ConA blasts by anti-rat IL-2R mAb was tested at dilutions of 0.00005 to 0.5, and the proliferative response to hrIL-2 (20 U/ml) was found to be inhibited in a dose-dependent fashion, and totally blocked at a 1:100 dilution. Similarly, the proliferative response of IL-2-dependent cells (rat spleen ConA blasts) to supernatants which were diluted (1/4) was blocked in the presence of this antibody ([Fig.4-5](#)).



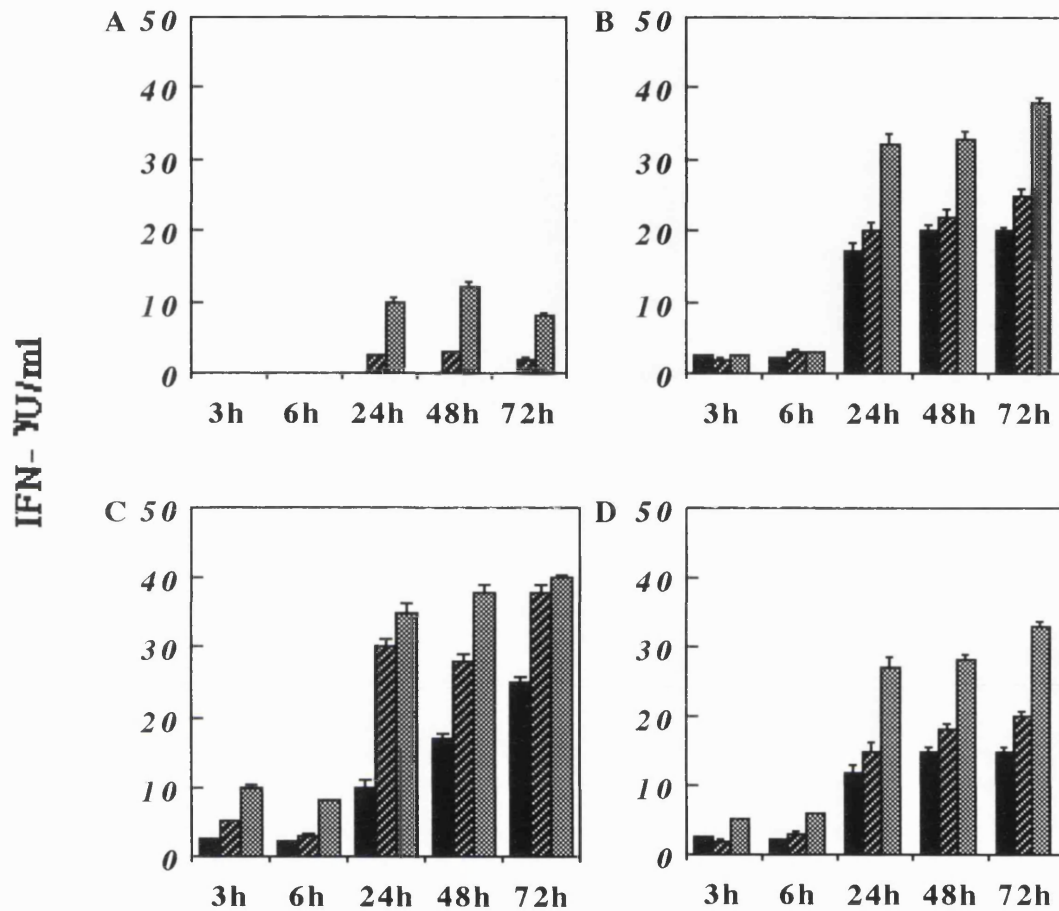
**Figure 4-5** Effects of anti-IL-2R monoclonal antibody (1/100) on the proliferative responses of IL-2 dependent cells (rat spleen ConA blasts) to factors induced by S-Ag, ConA and PMA. Cells were incubated with 1/4 diluted supernatants from S-Ag, ConA and PMA groups in absence or presence of anti-IL-2R monoclonal antibody. Each value represents the Mean $\pm$ SD of 3 individual wells. hrIL-2 (5 U/ml) in the absence and presence of anti-IL-2R mAb was as positive controls. \*\*  $p < 0.01$ .

The time-dependent IFN- $\gamma$  release in response to S-Ag stimulation is shown in [Fig.4-6A](#). The production of IFN- $\gamma$  was significantly increased ( $p < 0.01$ ) at all time points above the level of the unstimulated cells. PMA and ConA were also effective in enhancing IFN- $\gamma$  production ([Fig.4-6A](#)). The release of IFN- $\gamma$  bioactivity was





**Figure 4-6** A: Timecourse of inhibitory responses of WEHI-279 cells to supernatants from S-Ag, ConA and PMA-groups. WEHI-279 cells were incubated with supernatants diluted (1/200) from different groups for 64 h. The plates were pulsed with 1  $\mu$ Ci of [ $^3$ H] thymidine for 8 h and harvested. Each value represents the mean $\pm$ SD of triplicate wells. B: WEHI-279 cells were incubated with supernatant 1/200 dilution collected at 72 h from different groups in absence or presence of anti-rat IFN- $\gamma$  mAb (12.5  $\mu$ g/ml). IFN- $\gamma$  (40 U/ml) was a positive control. \*  $p < 0.05$ , \*\*  $p < 0.01$ .



**Figure 4-7** Time course of IFN- $\gamma$  production by diluted supernatants from S-Ag specific T cells after stimulation with S-Ag, ConA and PMA. Three different dilutions of the supernatants were incubated with the IFN- $\gamma$ -sensitive cell line WEHI-279. A: unstimulated cells. B: S-Ag. C: ConA. D: PMA. ■ 0.25% dilution. ▨ 0.5% dilution. ▩ 1% dilution.

significantly increased at 6 h ( $p < 0.05$ ), 24 h ( $p < 0.01$ ), and 48 and 72 h ( $p < 0.05$ ) after treatment with PMA as compared with the unstimulated cells at corresponding time points. The effect of ConA on the release of IFN- $\gamma$  bioactivity from the cells was significantly increased at 6 ( $p < 0.05$ ), 24, 48 and 72 h (all  $p < 0.01$ ).

Fig.4-7 shows time course of IFN- $\gamma$  production by diluted supernatants from S-Ag specific T cells after stimulation with S-Ag, ConA and PMA. Three different dilutions of the supernatants were incubated with the IFN- $\gamma$ -sensitive cell line WEHI-279. The

proliferation of WEHI-279 could be inhibited by the supernatants from S-Ag, ConA and PMA activated cells. The level of IFN- $\gamma$  was calculated from the standard curve.

Anti-IFN- $\gamma$  mAb blocked the inhibiting effect of supernatants (1/200 dilution) collected after 72 h activation from S-Ag-, ConA- and PMA-group on the proliferation of WEHI-279 cells (Fig.4-6B), confirming that IFN- $\gamma$  existed in the supernatants.

### **4.3 Discussion**

In this study, we found that unstimulated control cells expressed a low level of INF- $\gamma$  and IL-4 mRNAs. These findings are consistent with observations reported by others [Dallman et al., 1991; Iizawa et al., 1992], who used a PCR amplification technique to demonstrate IFN- $\gamma$  mRNA expression in unstimulated normal cardiac tissue and spleen cells. Some investigators however did not detect lymphokine gene expression by Northern blot analysis in unstimulated cells [Cherwinski et al., 1987; Pallard et al., 1988].

It has been shown in chapter 3 that almost all S-Ag T cell lines released IL-2 and IFN- $\gamma$  after 24 h activation with S-Ag. In this chapter, we investigated the lymphokine expression of S-Ag specific T cells in response to specific antigen and non-specific mitogens. The proliferative response of HT-2 cells induced by the supernatants after S-Ag stimulation could be totally blocked by rat IL-2R mAb. However, the time course data showed that the proliferative response of HT-2 cells induced by supernatants following ConA stimulation was only partially inhibited by rat IL-2R mAb, suggesting that these cells have the capacity to produce certain factors other than IL-2 under certain conditions. This alteration in the pattern of lymphokine production depending on the mode of stimulation has been recently reported by several groups in other species. Tsutsui et al. have used hepatitis B surface antigen (HBsAg) specific human CD4<sup>+</sup> T cell clones to investigate whether expression of lymphokines by these cells varies after antigen and mitogen stimulation [Tsutsui et al., 1991]. They found that different stimuli resulted in different lymphokine expression in cloned human CD4<sup>+</sup> T cells. Using *in situ* hybridization, it has been demonstrated that in normal mouse CD4<sup>+</sup> T cells (mixed populations), the production of IL-2 and IL-4 appears to be stimulus-dependent [Carding

et al., 1989]. Others have analysed the expression of IL-4 and IFN- $\gamma$  mRNA in anti-CD3 stimulated mouse spleen cells and observed that a shift in the lymphokine pattern was induced in the presence of PMA [Monteyne et al., 1992]. Our results provide evidence for a differential activation and production of lymphokines by S-Ag specific rat CD4<sup>+</sup> T cells after stimulation with antigen and mitogen *in vitro*. If the *in vitro* findings are reflecting an *in vivo* situation, it is of interest to determine what regulates lymphokine production after these cells are adoptively transferred into naive animals during the induction of EAU. We were not able to claim that the unidentified factors other than IL-2 in the supernatant is IL-4. Furthermore, it should be noted that in addition to IL-2 and IL-4 as T cell growth factors, there are several cytokines such as IL-12 [Trinchieri, 1993] and IL-6 [Tosato and Pike, 1988; Lotz, 1988] which promote the growth of T cells. As the secretion of IL-4, together with other cytokines, i.e. IL-2 and IFN- $\gamma$  is important in defining the functional classification of CD4<sup>+</sup> T-cells, it will be of importance for us to develop specific IL-4 assay, and study its secretion pattern.

Antigen-specific activation may be mediated through the antigen-MHC class II TcR/CD3-CD4 complex [Bierer & Burakoff, 1989; Finkel et al., 1991], while ConA or PMA operate through the TCR/CD3 complex [Crabtree, 1989] and the PKC pathway [Nishizuka, 1984] respectively. In this study, we have treated the cells with PMA, and found that PMA induced IL-4 as well as IL-2 and IFN- $\gamma$  mRNA expression, but the effect was transient. The reason for this might be due to the physiological diglycerides being short-lived, and, hence PKC activation is also transient and reversible [Manger et al., 1987; Meuer & Resch, 1989]. The production of IL-2 and IFN- $\gamma$  could be detected after stimulation with PMA, and IL-4 mRNA was detected in the PMA-treated group. The results presented here clearly imply that PKC is partly involved as an intracellular second messenger for IL-2, IFN- $\gamma$  and IL-4 expression in this T cell line. ConA, a plant lectin, can induce IL-2, IL-4 and IFN- $\gamma$  mRNA expression and protein release from the S-Ag-specific T cells and the level of IL-2 secreted by the cells stimulated with ConA is much higher than with S-Ag. The reason for this effect is not clear. It is of note that a similar situation was described by others [Flamand et al., 1990], who showed that ConA as a polyclonal activator can induce higher levels of lymphokine secretion than specific antigen in mouse OVA- and keyhole limpet hemocyanin-specific T cells. The expression of IL-4 mRNA after treatment with ConA by S-Ag-specific T cells agrees with a previous study [Lightman et al., 1989]. S-Ag activates T-lymphocytes through the T cell

antigen receptor, although there is still no direct evidence to indicate which intracellular messengers are involved in S-Ag-specific T-cell lines stimulated by S-Ag. Further studies with specific inhibitors of different intracellular messengers should help clarify this issue.

## **Chapter 5 Lymphokine gene expression by rat CD4<sup>+</sup> T cell clones specific for S-Ag and PPD**

### **5.1 Introduction**

T lymphocytes play an important role in the regulation of host responses to a variety of antigens. During the past several years, much information has been obtained on the mechanisms involved in antigen recognition by T cells and on the phenotypic and functional characteristics of T cell subsets involved in various immune processes. These studies have been greatly facilitated by the development of cloned T cells.

The concept of functional heterogeneity of CD4<sup>+</sup> T cells was proposed more than 10 years ago, based on data using *in vivo* primed T cells [Imperiale et al., 1982; Keller et al., 1980; Swierkosz et al., 1979; Tada et al., 1978], but the definition of subsets has only become possible by investigating CD4<sup>+</sup> T cell clones. Kim et al. [Kim et al., 1985] defined four types of CD4<sup>+</sup> clones which were functionally distinct in terms of B cell activation. In 1986, Mosmann et al. have shown that allogeneic and antigen-specific CD4<sup>+</sup> T cell clones in the murine system can be subdivided into two subpopulations, termed Th1 and Th2, based on their patterns of lymphokine secretion [Coffman et al., 1988; Mosmann et al., 1986]. Th1 produce IL-2 and IFN- $\gamma$ , as well as several other lymphokines, but not IL-4 and IL-10, while Th2 secrete IL-4 and IL-10, as well as other lymphokines, but neither IL-2 nor IFN- $\gamma$ .

The existence of Th1 and Th2 cells was originally demonstrated *in vitro* [Mosmann et al., 1986]. More recently, Th1 and Th2 immune responses were also shown to occur *in vivo* [Mosmann & Coffman, 1989a and 1989b]. The difference in the pattern of lymphokine secretion appears to result in differences in function of the T cell. Th1 clones are involved in cell-mediated immunity like delayed-type hypersensitivity (DTH), and Th2 subset participates in humoral immunity, providing help for specific antibody production [Mosmann & Coffman, 1989]. Furthermore, production of Th1 and Th2 cells may negatively regulate each other. IFN- $\gamma$  produced by Th1 cells inhibits Th2 proliferation and IL-10 produced by Th2 cells inhibits the synthesis of Th1 cytokines [Fernandez-Botran et al., 1988; Fiorentino et al., 1989; Gajewski & Fitch, 1988].

In the human system, the majority of CD4<sup>+</sup> T cell clones obtained from healthy donors produce IFN- $\gamma$ , IL-2, IL-4 and IL-5 upon stimulation with various mitogens, thus resembling the murine Th0 cells that produce almost every cytokine [Paliard et al., 1988]. There is increasing evidence that human CD4<sup>+</sup> T cells involved in certain inflammatory or allergic disease show restricted cytokine synthesis patterns comparable to the murine Th1 and Th2 subsets. A Th2 profile seems to dominate in human T cell clones specific for allergens [Wierenga et al., 1991] or the parasite *Toxocara canis* [Del Prete et al., 1991], and a Th1 pattern has been shown for human T cell clones specific for mycobacteria [Del Prete et al., 1991; Haanen et al., 1991], *Borrelia burgdorferi* [Yssel et al., 1991] and Yersinia [Schlaak et al., 1992].

Evidence for subsets of Th cells in rats is limited due to the difficulty in generating cloned T cells in this system. However based upon recent *in vivo* experiments, several groups have suggested similar CD4<sup>+</sup> T cell subsets exist in rats [Papp et al., 1992; Saoudi et al., 1993]. However only with cloned T-cells is it possible to clarify this issue.

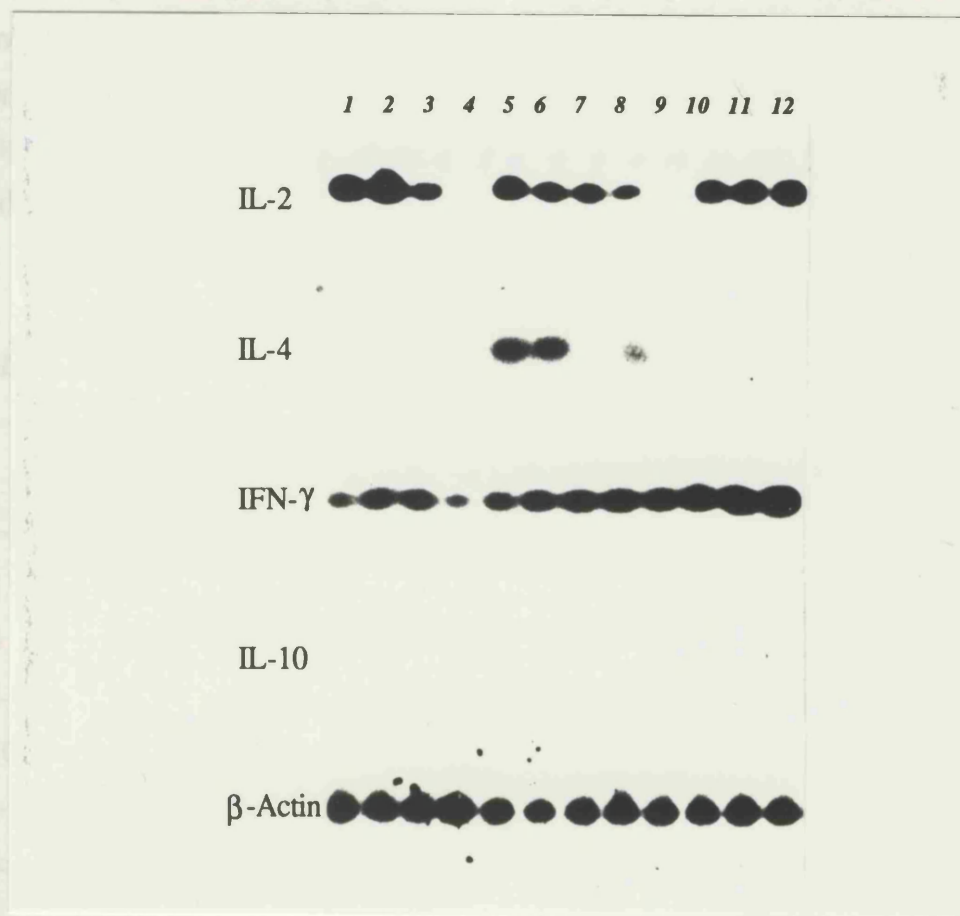
In the rat, it has been shown that CD4<sup>+</sup> T cells can be functionally divided into naive (OX22<sup>high</sup>) and memory (OX22<sup>low</sup>) subsets based on reactivity with MRC OX22 mAb (for more detail see [chapter 6](#)). OX22<sup>high</sup> CD4<sup>+</sup> T cells produce high IL-2 and IFN- $\gamma$  but low IL-4, whereas OX22<sup>low</sup> CD4<sup>+</sup> T cells produce high IL-4 but low IL-2 and IFN- $\gamma$ . Thus, it appears that the OX-22<sup>high</sup>CD4<sup>+</sup> subset has a Th1-like lymphokine repertoire whereas the OX-22<sup>low</sup>CD4<sup>+</sup> population has characteristics of Th2-like cells [Fowell et al., 1991]. However, this classification is not based on the patterns of lymphokine expression.

In this study, we have used S-Ag and PPD specific rat CD4<sup>+</sup> T cell clones derived from SAG5 and PPD2 CD4<sup>+</sup> T cell lines to investigate lymphokine expression. Our results show that S-Ag T cell clones express a mostly Th1-like pattern but also a mixed (Th0-like) cytokine pattern on the basis of lymphokine mRNA expression, whereas most PPD rat CD4<sup>+</sup> T cell clones express predominately a mixed (Th0-like) pattern but also Th1 and Th2 cytokine patterns.

## **5.2 Results**

### 5.2.1 Lymphokine gene expression by S-Ag specific rat CD4<sup>+</sup> T cell clones

Twelve S-Ag specific CD4<sup>+</sup> T cell clones were obtained from *in vitro* stimulated long-term CD4<sup>+</sup> T cell lines, and were stimulated with S-Ag and APC for 6 h to assess their ability to express IL-2, IFN- $\gamma$ , IL-4 and IL-10 mRNA. Individual mRNA lymphokine profiles from these clones are shown in Fig.5-1. Of these, all T cell clones expressed



**Figure 5-1** Lymphokine gene expression by S-Ag specific rat CD4<sup>+</sup> T cell clones. RNA was extracted from these clones 6 h after culture with S-Ag and APC, reverse transcribed in cDNA and amplified by PCR. 10  $\mu$ l of PCR amplification products was electrophoresed through 1.5% agarose gel, Southern blotted to nitrocellular membranes and hybridized with internal probes.  $\beta$ -actin mRNA was a positive control.

IFN- $\gamma$  mRNA, and all but two produced IL-2 mRNA. IL-4 mRNA was expressed by three T cell clones which also expressed IFN- $\gamma$  and IL-2. None of these clones



expressed IL-10.

According to the criteria of Mosmann and Coffman [Mosmann & Coffman, 1989], lymphokine expression by S-Ag T cell clones is summarized in Table-5-1. Seven of the 12 S-Ag specific CD4<sup>+</sup> T cell clones expressed IFN- $\gamma$  and IL-2 without expressing any IL-4 or IL-10. This is typical for a Th1-like pattern according to the criteria of Mosmann and Coffman [Mosmann & Coffman, 1989]. Two of the 12 CD4<sup>+</sup> clones expressed IFN- $\gamma$  alone, presenting a Th1 pattern. Three of twelve expressed IFN- $\gamma$ , IL-2 and IL-4 but not IL-10, presenting a mixed (Th0-like) cytokine pattern according to the same criteria. None of these clones exhibited a Th2-like pattern. It should be noted that cytokine mRNA expression does not correlate with secretion of protein so some of Th0 could be Th1 or Th2 since the Th classification is based on cytokine secretion.

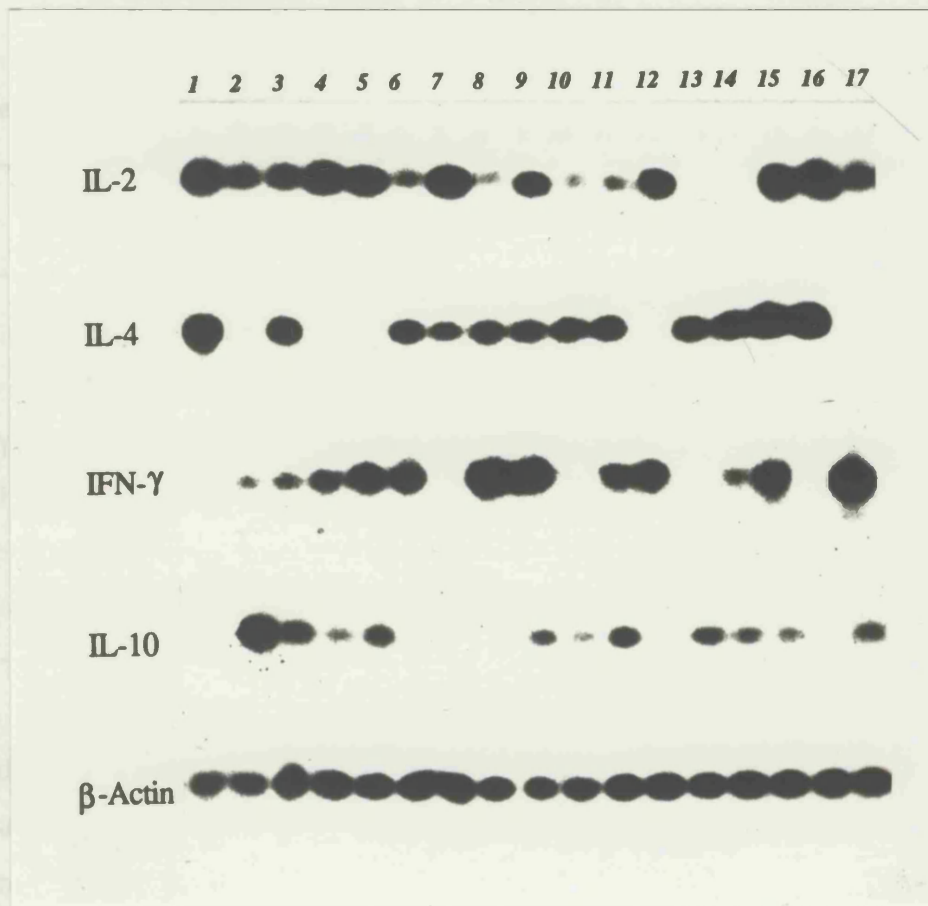
**Table 5-1** *The patterns of lymphokine gene expression by S-Ag T cell clones.*

No.	1	2	3	4	5	6	7	8	9	10	11	12
IL-2	+	+	+	-	+	+	+	+	-	+	+	+
IL-4	-	-	-	-	+	+	-	+	-	-	-	-
IFN- $\gamma$	+	+	+	+	+	+	+	+	+	+	+	+
IL-10	-	-	-	-	-	-	-	-	-	-	-	-
Type	Th1	Th1	Th1	Th1	Th0	Th0	Th1	Th0	Th1	Th1	Th1	Th1

### 5.2.2 Lymphokine gene expression by PPD specific rat CD4<sup>+</sup> T cell clones

Seventeen PPD specific CD4<sup>+</sup> T cell clones were studied and results are shown in Fig.5-2. IL-2 was produced by 15, IL-4 by 12, IFN- $\gamma$  by 12 and IL-10 by 11 out of 17 T cell clones, respectively. According to the cytokine production pattern, typical "Th1- and Th2-type" clones can be identified, but also clones which do not fit into the Th1 or Th2 scheme. In summary there are different patterns among the PPD CD4<sup>+</sup> T cell clones

(see Table 5-2). (1) Six clones had a mixed pattern with expression of IL-2, IFN- $\gamma$ , IL-4 and/or IL-10. Four had a mixed pattern with expression of IL-2, IL-4 and/or IL-10 but not IFN- $\gamma$ . One had a mixed pattern with expression of IL-4, IFN- $\gamma$  and IL-10 but not IL-2. (2) Five had a Th1 pattern with the expression of IL-2, IFN- $\gamma$  and/or IL-10 but not IL-4. (3) One had a Th2 pattern with the expression of IL-4 and IL-10 but not IL-2 and IFN- $\gamma$ .



**Figure 5-2** Lymphokine gene expression by PPD specific rat CD4<sup>+</sup> T cell clones. RNA was extracted from these clones 6 h after culture with S-Ag and APC, reverse transcribed in cDNA and amplified by PCR. 10  $\mu$ l of PCR amplification products was electrophoresed through 1.5% agarose gel, Southern blotted to nitrocellular membranes and hybridized with internal probes.  $\beta$ -actin mRNA was a positive control.

### 5.3 Discussion

It has been shown that mouse CD4<sup>+</sup> T cell clones could be subdivided into two subsets (Th1 and Th2) based on their cytokine production [Mosmann & Coffman, 1989a and

1989b]. For human, CD4<sup>+</sup> T cell clones with similar properties have been detected [Romagnani et al., 1992; Umetsu et al., 1988]. In the present study, we have investigated IL-2, IL-4, IFN- $\gamma$  and IL-10 mRNA expression of rat S-Ag and PPD specific CD4<sup>+</sup> T cell clones. The clones described here were generated using the same method so as to allow the comparison of these two different clones. Additionally, mRNA were analyzed at approximately the same time for all these clones.

**Table 5-2** *The patterns of lymphokine gene expression by PPD T cell clones.*

No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
IL-2	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+
IL-4	+	-	+	-	-	+	+	+	+	+	+	-	+	+	+	+	-
IFN- $\gamma$	-	+	+	+	+	+	-	+	+	-	+	+	-	+	+	-	+
IL-10	-	+	+	+	+	-	-	-	+	+	+	-	+	+	+	-	+
Type	Th0	Th1	Th0	Th1	Th1	Th0	Th0	Th0	Th0	Th0	Th0	Th1	Th2	Th0	Th0	Th0	Th1

Based on the production of IL-2/IFN- $\gamma$  as indicators of Th1 and that of IL-4/IL-10 as indicators of Th2 respectively, Nine out of 12 S-Ag CD4<sup>+</sup> T cell clones had a cytokine pattern characterized by expression IFN- $\gamma$  and/or IL-2 but not IL-4 and IL-10, consistent with a Th1 pattern. Three had a pattern with expression of IFN- $\gamma$ , IL-2 and IL-4 but not IL-10 and were regarded as mixed Th0-like pattern. The results clearly indicate that Th1 is a predominant pattern among T cell clones specific for S-Ag, although clearly this is only 12 clones and many more need to be examined to confirm such a pattern. This was also suggested in our study with S-Ag specific long-term CD4<sup>+</sup> T cell lines (see [chapter 3](#)).

S-Ag, a potent uveitopathogenic antigen from retina, induces EAU [Shinohara et al., 1991]. Recent *in vivo* data reported by Saoudi et al. showed T cells from rats immunized with S-Ag exhibited a Th1 pattern of cytokine production [Saoudi et al., 1993]. All S-Ag specific T cell clones did not express IL-10, suggesting that it is

possible that the absence of IL-10 may be due to the present of IFN- $\gamma$  since it has been shown that IFN- $\gamma$  can inhibit IL-10 production by monocytes [Chomarat et al., 1993]. It has also been reported that IL-10 inhibits IFN- $\gamma$  expression by Ag/APC-stimulated Th1 cells [Fiorentino et al., 1989; Fiorentino et al., 1991]. Three S-Ag specific CD4<sup>+</sup> T cell clones which expressed IL-2, IFN- $\gamma$  and IL-4 were also found. The interpretation of these results is consistent with a model in which Th1 and Th2 subsets represent final stages in Th differentiation, while their precursors express different lymphokine secretion pattern [Bottomly et al., 1989; Firestein et al., 1989; Gajewski et al., 1989; Street et al., 1990].

In our studies, PPD specific rat CD4<sup>+</sup> T cell clones exhibit mostly a mixed pattern with expression IL-2, IFN- $\gamma$  and IL-4, or IL-2 and IL-4, or IFN- $\gamma$  and IL-4. The expression of IFN- $\gamma$  associated with IL-4 in PPD T cell clones suggests that PPD stimulates Th1 as well as Th2 type of cells or that it alternatively activates Th0 cells capable of producing both cytokines. The patterns with the expression of IL-2 and IL-4, or IFN- $\gamma$  and IL-4 found here were also shown in murine CD4<sup>+</sup> T cell clones whose lymphokine secretion patterns are intermediate between Th1 and Th2 [Bottomly et al., 1989; Kelso & Gough, 1988; Street et al., 1990; Swain et al., 1990; Weinberg et al., 1990].

Typically, mycobacteria preferentially induced a cell-mediated type of immunity and human CD4<sup>+</sup> T cell clones responding to PPD raised *in vitro* correspond to the Th1 type [Del Prete et al., 1991]. Our results showed that PPD clones exhibit mostly a mixed pattern but also Th1 and Th2 cytokine patterns. The reason for this difference is not clear at the moment. The different culture conditions used to generate clones may be one of the reasons since it has been found that the pattern of lymphokine expression by PPD specific CD4<sup>+</sup> T cells could be significantly influenced by the cytokine environment during the cloning process. It has been reported that a 5-day culture of PPD stimulated human T cells in the presence of IL-4 prior to cloning induced Th0 and Th2 rather than Th1 cells [Maggi et al., 1992]. Similar results were also found in the mouse where PPD-primed lymph node T cells could be reproducible into distinct T cell lines with Th1 and Th2 under identical conditions with the exception that either IL-2 or IL-4 was employed as growth factors. However, under the same conditions neither OVA- nor insulin-specific T cell lines show such a clear separation into different T cell subsets [Schmitt et al., 1990].

Murine IL-10 is a Th2 type cytokine credited with major inhibitory activity on Th1 cytokine synthesis [Mosmann & Moore, 1991]. However, production of IL-10 in human CD4<sup>+</sup> T cell clones is not restricted to Th2 cells since most Th0 and Th1 clones are also able to produce this cytokine [De Waal Malefyt et al., 1992]. Similar results were obtained in rat T cells in the present study. Our results indicate from the expression of IL-10 that rat PPD T cell clones are not typical Th1 or Th0 cells, or that IL-10 can be produced by Th1 or Th0.

We intended to investigate both cytokine production and gene expression using T-cell clones. The criteria for Th1 and Th2 classification is based on cytokine production [Mosmann et al., 1986]. Although limiting dilution procedure described in section 2.5, are probably sufficient to obtain single T cell clones, it is nevertheless apparent that statistical estimates which may indicate a high probability of monoclonality may not always be valid even when a lower initial cell concentration is used. Thus it is important to reclone the initial T cell clones. Unfortunately it was very difficult to clone these T cells and we were unable to expand them successfully from 24-wells. This prevented us from recloning the initial T cell clones as suggested by Sedgwick et al., [Sedgwick et al., 1989]. Therefore, we only extracted RNA from cloned cells in 24-wells which provided sufficient RNA to look for lymphokine gene expression but did not result in enough tissue culture supernatant in order to look for lymphokine production by these clones.

In conclusion, the present results indicate that in a limited number of T cell clones rat S-Ag specific CD4<sup>+</sup> T cell clones exhibit mainly a Th1 pattern but also a mixed (Th0) cytokine pattern on the basis of lymphokine mRNA expression, whereas the PPD specific CD4<sup>+</sup> T cell clones exhibit mostly a mixed (Th0) pattern but also Th1 and Th2 cytokine patterns. It should be noted here that mRNA expression does not necessarily correlate with lymphokine production and thus further investigation of lymphokine production by more clones will be carried out in future studies.

## **Chapter 6 Lymphokine gene expression by rat OX22<sup>high</sup> and OX22<sup>low</sup> CD4<sup>+</sup> T cells**

### **6.1 Introduction**

It is well-known that CD4<sup>+</sup> T cells in human [Ralph et al., 1987; Streuli et al., 1987], rat [Barclay et al., 1987] and mouse [Saga et al., 1987; Thomas et al., 1987] can be subdivided into subpopulations based on expression of different isoforms of CD45 (leukocyte common antigen). The CD45 is expressed on lymphocytes and all other hematopoietic cells, with the exception of erythrocytes and platelets [Thomas & Lefrancois, 1988]. The molecule consists of three distinct regions: a large cytoplasmic carboxy-terminal that possesses two tandem domains with a protein-tyrosine phosphatase (PTPase) activity; a single membrane-spanning region; and an amino-terminal extracellular glycosylated region that is likely to function as a ligand-binding domain. Six different CD45 isoforms have been described. All are identical in their cytoplasmic and transmembrane regions but differ in their extracellular sequence near the NH<sub>2</sub> terminus. The various isoforms are generated by differential usage of three exons which code for peptide sequences A, B and C (reviewed in [Thomas, 1989]).

In the rat the mAb MRC OX22 [Spickett et al., 1983], which recognizes a restricted epitope on CD45 encoded by the C exons of the leukocyte common antigen (LCA) gene [Fowell et al., 1991], separates CD4<sup>+</sup> T cells into two subpopulations: OX22<sup>high</sup> and OX22<sup>low</sup>. The phenotypic heterogeneity of CD4<sup>+</sup> T cells revealed by mAb OX22 correlates with functional differences. Previous studies have demonstrated that OX22<sup>high</sup> CD4<sup>+</sup> T cells are able to mediate graft-versus-host reactions and provide B cell help in primary immune responses [Arthur & Mason, 1986; Powrie & Mason, 1989; Powrie & Mason, 1990]. CD4<sup>+</sup> T cells that express the low-molecular mass LCA (OX22<sup>low</sup>) provide B cell help in secondary immune responses [Powrie & Mason, 1989] and passively transfer EAE [Sedgwick et al., 1989] and EAU [Caspi et al., 1986].

Previously in this thesis the CD4<sup>+</sup> T cell lines and clones examined for lymphokine expression were all OX22<sup>low</sup>. Among these lines and clones a heterogeneous pattern of lymphokine expression was observed. The aim of this study was to examine a

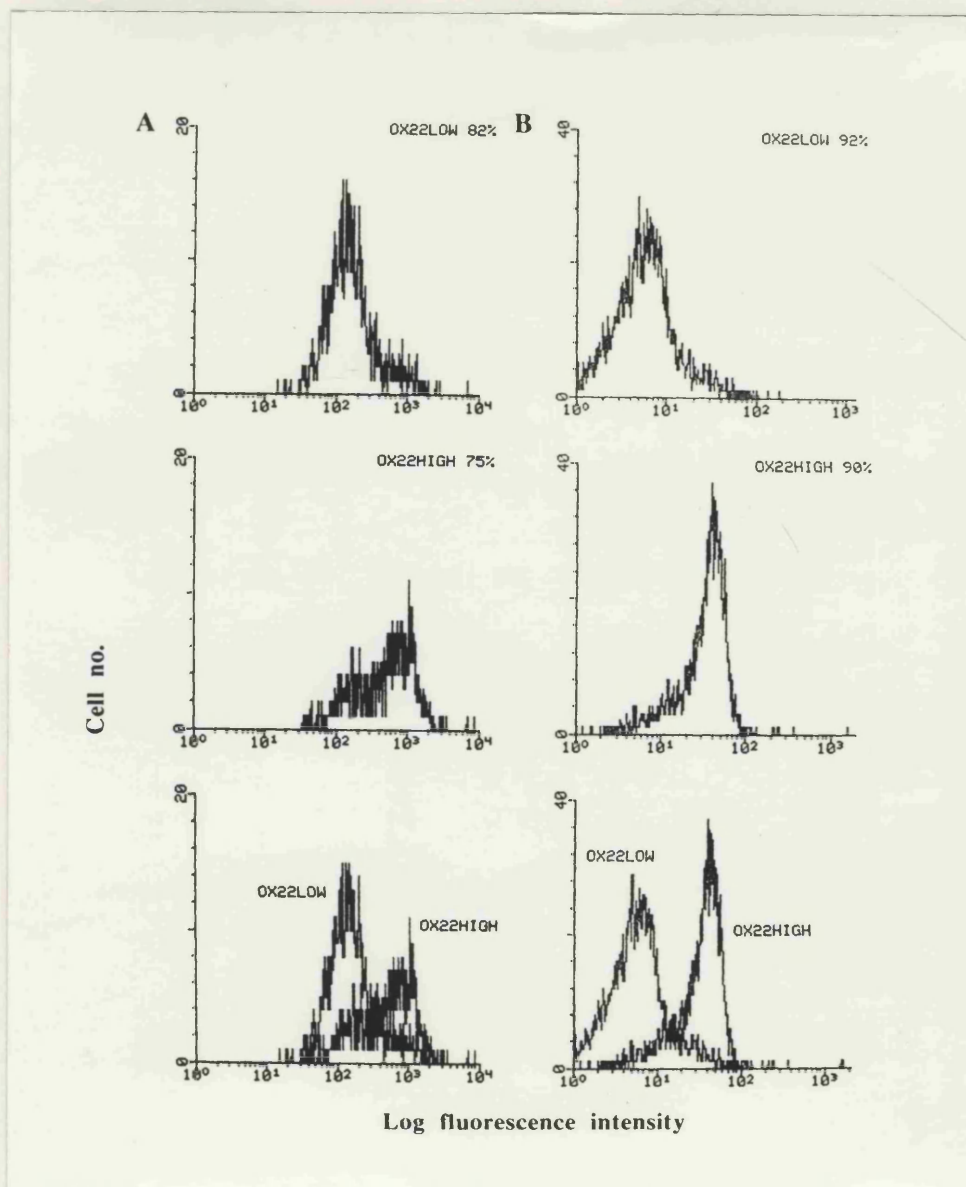
population of OX22<sup>high</sup> T cells to find out if they also exhibit such varied profiles. In order to obtain these cells, MACS sorting was carried out and the OX22<sup>low</sup> cells from the same rats were used as a comparison, since OX22<sup>low</sup> cells freshly isolated differ from the OX22<sup>low</sup> cells in longterm culture [Powrie & Mason, 1989]. There were some reports to show lymphokine gene expression and production by freshly isolated OX22<sup>high</sup> and OX22<sup>low</sup> CD4<sup>+</sup> T cells, but they were from the cells after 24 h stimulation *in vitro*. It is of interest to investigate lymphokine gene expression by cells following a shorter stimulation period (6 h), especially since mRNA expression occurs at early stage after activation based on our previous findings in T cell lines (see [chapter 4](#)). Furthermore, from the observation that lymphokine expression changes in response to different stimuli [Patarca et al., 1991; Torbett et al., 1989; Yokoyama et al., 1989], it is also of interest to know whether lymphokine expression by OX22<sup>high</sup> and OX22<sup>low</sup> CD4<sup>+</sup> T cells is changeable after activation with different stimuli. In the present study, we investigated lymphokine gene expression by OX22<sup>high</sup> and OX22<sup>low</sup> CD4<sup>+</sup> T cell subsets from unprimed rats after 6 h stimulation with S-Ag, ConA and PMA.

## **6.2 Results**

### **6.2.1 Separation of OX22<sup>high</sup>CD4<sup>+</sup> and OX22<sup>low</sup>CD4<sup>+</sup> T cells from normal Lewis rats**

OX22<sup>high</sup> and OX22<sup>low</sup> cells from normal lymph nodes were enriched by MACS as described in [section 2.6.1](#). We found that in addition to the speed of elution, the antibody concentration used in the process could affect staining and separation efficiency. [Fig.6-1A](#) show that when a higher concentration of the antibody (1/100 dilution) was used, the purity of OX22<sup>high</sup> and OX22<sup>low</sup> population obtained was less than 85%, as determined by FACS analysis. However, the purity of both separated populations could be up to 90% ([Fig.6-1B](#)) when the optimum concentration (1/150 dilution, 50 µg/ml) was used. CD4<sup>+</sup>-enriched OX22<sup>high</sup> and OX22<sup>low</sup> cells were prepared by panning CD4<sup>+</sup> T cells as described in [section 2.6.2](#). Panned cells were >90% positive for W3/25, as determined by FACS analysis ([Fig.6-2](#)).

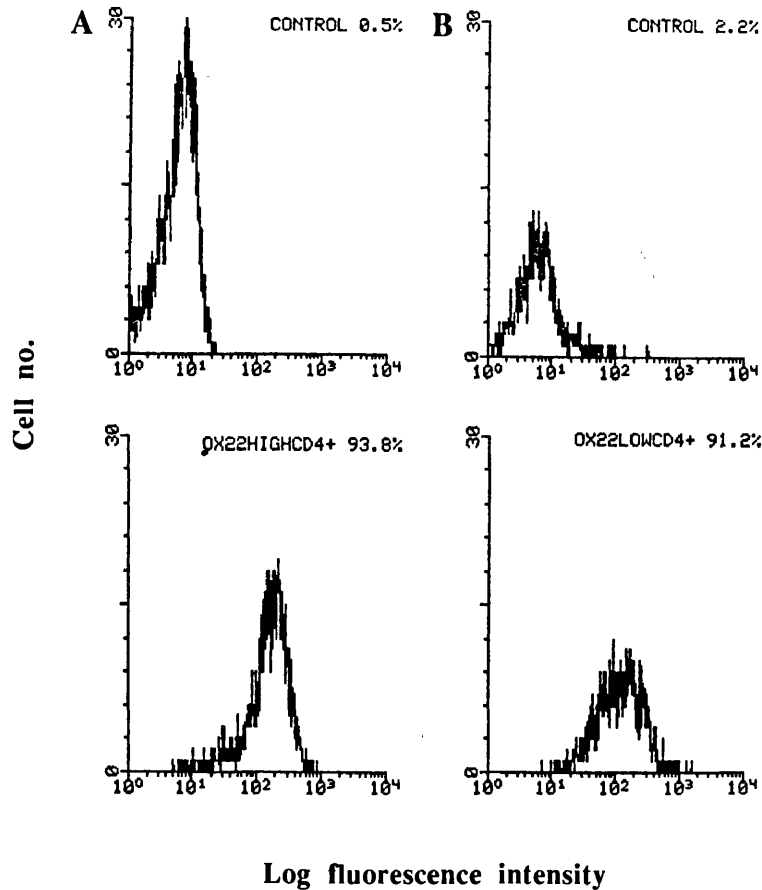
### **6.2.2 Lymphokine gene expression by OX22<sup>high</sup> and OX22<sup>low</sup> CD4<sup>+</sup> T cells with different stimuli.**



**Figure 6-1** Analysis of OX22<sup>high</sup> and OX22<sup>low</sup> cells after MACS. A: A high concentration of BT-OX22 mAb (1:100) was used for MACS process. The purity of OX22<sup>high</sup> was about 75% and OX22<sup>low</sup> 82%. B: The optimum concentration of BT-OX22 mAb (1:150) was used for MACS process. The purity of OX22<sup>high</sup> was 90% and OX22<sup>low</sup> 92%.

Freshly isolated OX22<sup>high</sup> and OX22<sup>low</sup> CD4<sup>+</sup> T cells ( $4 \times 10^5$ ) from healthy Lewis rats were stimulated with S-Ag (10  $\mu$ g/ml), ConA (5  $\mu$ g/ml) or PMA (10 ng/ml) plus irradiated thymocytes (APC). After 6 h, RNA was extracted. 2  $\mu$ g of total RNA were reverse transcribed and amplified in the PCR. All combinations were carried out in parallel in the same experiment at least twice.

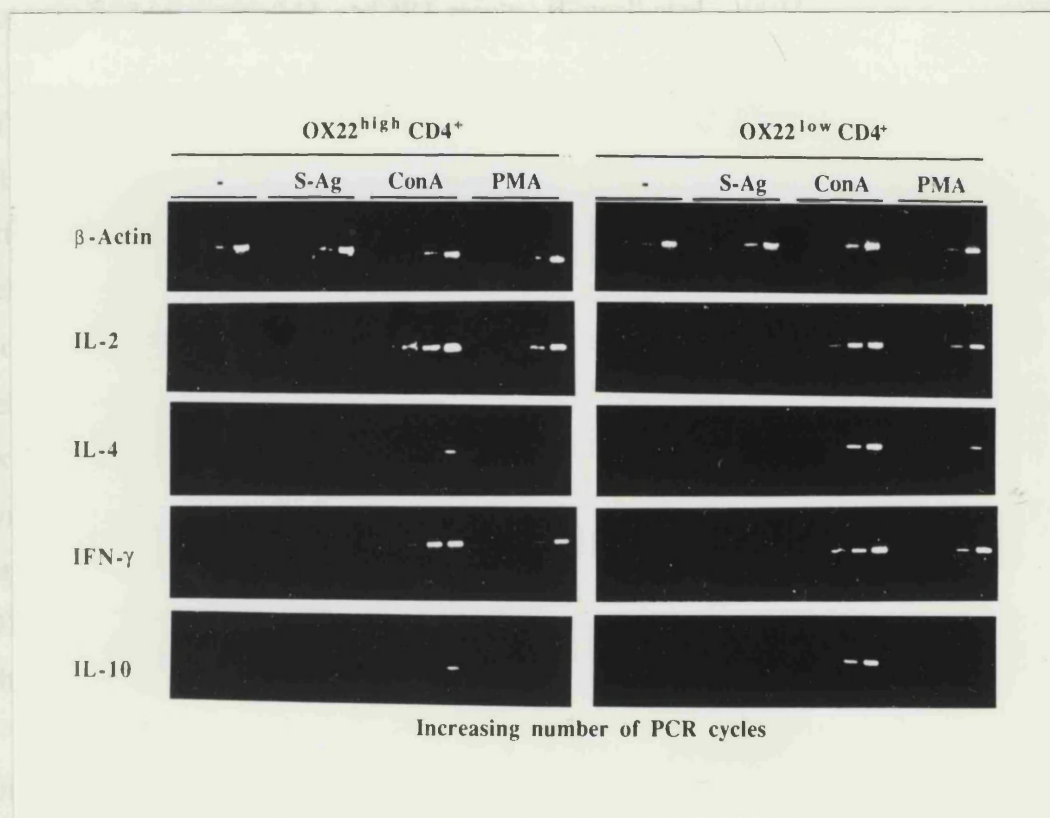




**Figure 6-2** Analysis of CD4<sup>+</sup> T cells after panning. A: OX22<sup>high</sup> cells after panning for CD4<sup>+</sup> T cells cells. The purity of CD4<sup>+</sup> T cells was 93.8%. B: OX22<sup>low</sup> cells after panning for CD4<sup>+</sup> T cells cells. The purity of CD4<sup>+</sup> T cells was 91.2%.

Using semiquantitative PCR we compared IL-2, IL-4, IFN- $\gamma$  and IL-10 gene expression in rat OX22<sup>high</sup>CD4<sup>+</sup> and OX22<sup>low</sup> CD4<sup>+</sup> T cells following different stimuli (Fig.6-3). There was no significant difference in the level of  $\beta$ -actin mRNA expression. No signals for IL-2, IL-4, IFN- $\gamma$  or IL-10 were detected in unstimulated and S-Ag-treated OX22<sup>high</sup>CD4<sup>+</sup> and OX22<sup>low</sup> CD4<sup>+</sup> T cells. In contrast, ConA could induce expression of all four lymphokines in both OX22<sup>high</sup>CD4<sup>+</sup> and OX22<sup>low</sup> CD4<sup>+</sup> T cell subsets. PMA

induced IL-2 and IFN- $\gamma$  but not IL-4 and IL-10 in the OX22<sup>high</sup>CD4<sup>+</sup> T cell subset; IL-2, IFN- $\gamma$  and IL-4 but not IL-10 in the OX22<sup>low</sup>CD4<sup>+</sup> T cell subset. There was no significant difference in IL-2 and IFN- $\gamma$  gene expression between two subsets after stimulation with ConA and PMA, although the signals from the cells stimulated by ConA were much stronger than those of the cells stimulated by PMA. OX22<sup>low</sup> CD4<sup>+</sup> T cells expressed higher levels of IL-4 and IL-10 mRNAs than OX22<sup>high</sup> CD4<sup>+</sup> T cells after ConA stimulation. The same phenomena could be observed after stimulation with PMA except that PMA could not induce IL-10 mRNA expression in OX22<sup>high</sup> CD4<sup>+</sup> T cell subset.



**Figure 6-3** IL-2, IL-4, IFN- $\gamma$  and IL-10 gene expression in rat OX22<sup>high</sup> and OX22<sup>low</sup> CD4<sup>+</sup> T cells after 6 h stimulation. RNA was extracted, reverse transcribed and subjected to PCR as described in section 2.9.2 and 2.9.3. PCR were analyzed at cycles 21, 24, 27 and 30 for  $\beta$ -actin, cycles 27, 30, 33 and 36 for IL-2, IL-4, IFN- $\gamma$  and IL-10. A negative control, containing all reagents except cDNA, was included in every batch of PCR.

### 6.3 Discussion

It has been noted that one of the critical differences between OX22<sup>high</sup> and OX22<sup>low</sup>

CD4<sup>+</sup> T cells from unprimed healthy rats is the profile of cytokines that they express. OX22<sup>high</sup> CD4<sup>+</sup> T cells from PVG and DA strain rats produced high IL-2 and IFN- $\gamma$  but low IL-4 after activation with ConA, whereas OX22<sup>low</sup> CD4<sup>+</sup> T cells produced high IL-4 but low IL-2 and IFN- $\gamma$  (reviewed in [Fowell et al., 1991]). It seems that normal CD4<sup>+</sup> T cells in the rat belong to functionally distinct subpopulations similar to murine T cell clones. Furthermore, the results of lymphokine gene expression by these OX22<sup>high</sup> and OX22<sup>low</sup> CD4<sup>+</sup> T cell subsets correlated well with lymphokine bioactivities with the exception of IFN- $\gamma$ . It has been shown that a higher frequency of cells expressing IFN- $\gamma$  mRNA was found within OX22<sup>low</sup> subset, although this high level of expression was not sustained (compare 24 h and 48 h results) [Fowell et al., 1991].

In the present study we investigated lymphokine gene expression by freshly isolated rat CD4<sup>+</sup> T cell subsets (based on CD45 isoform expression) after 6 h stimulation with different stimuli. No signals could be detected in both OX22<sup>high</sup> and OX22<sup>low</sup> CD4<sup>+</sup> T cell subsets after stimulation with S-Ag, which they had not previously encountered. ConA activated IL-2, IFN- $\gamma$ , IL-4 and IL-10 gene expression in the OX22<sup>high</sup>CD4<sup>+</sup> T cell subset whereas PMA only induced IL-2, IFN- $\gamma$ , but not IL-4 and IL-10 gene expression. In the OX22<sup>low</sup>CD4<sup>+</sup> T cell subset, ConA also induced expression of all four lymphokine genes, and PMA induced IL-2, IFN- $\gamma$ , IL-4 but not IL-10. These results suggest that the patterns of lymphokines expressed by freshly isolated OX22<sup>high</sup> and OX22<sup>low</sup> CD4<sup>+</sup> T cells are not dependent upon expression of a particular CD45R phenotype, but upon the mode of stimuli.

Our results that OX22<sup>low</sup> CD4<sup>+</sup> T cell subset expressed higher IL-4 mRNA than OX22<sup>high</sup> CD4<sup>+</sup> T cell subset is in accord with another study [Powrie et al., 1991]. Our data also suggested that there was no significant difference in expression of IFN- $\gamma$  mRNA between these two subsets at 6 h post-stimulation. Yet the finding that there was no significant difference of expression of IL-2 mRNA between these two subsets is not in agreement with others who found that the OX22<sup>high</sup> CD4<sup>+</sup> T cell subset expressed higher IL-2 mRNA than the OX22<sup>low</sup> CD4<sup>+</sup> T cell subset [McKnight et al., 1991; Powrie et al., 1991]. The discrepancy between our results and their findings may reflect the different time points examined. A similar finding was reported in human cells that CD45RA<sup>low</sup> T helper cells are early producers of IL-2 (24 h) [Dohlsten et al., 1988]. In the present study, we also observed that IL-10 could be expressed by OX22<sup>high</sup> and

OX22<sup>low</sup> CD4<sup>+</sup> T subsets after ConA stimulation and by OX22<sup>low</sup> CD4<sup>+</sup> T subsets after PMA stimulation. However, the OX22<sup>low</sup> CD4<sup>+</sup> T cell subset expressed higher IL-10 mRNA than the OX22<sup>high</sup> CD4<sup>+</sup> T cell subset. To our knowledge, this is the first report of expression of IL-10 *in vitro* by freshly isolated rat CD4<sup>+</sup> T cell subsets.

It has been proposed that OX22<sup>low</sup> CD4<sup>+</sup> T cells respond to antigens to which the donor animals had been primed *in vivo*, but OX22<sup>high</sup> CD4<sup>+</sup> T cells do not, suggesting that the expression of the OX22 antigen is a marker for naive T cells and this marker is lost when these cells encounter specific antigen [Arthur & Mason, 1986]. However, recent data has suggested that this change from OX22 high to low is not a stable long-term event and may only reflect the physiological status of T cells since OX22<sup>low</sup> CD4<sup>+</sup> T cells adoptively transferred into nude recipients can re-express CD45RC protein [Bell & Sparshott, 1990; Sparshott et al., 1991].

The relationship between CD45 subsets of CD4<sup>+</sup> T cells and functionally heterogeneous T cell clones is still not clear. In the mouse Th1 and Th2 cells have been shown to differ in the cytokines they release [Mosmann et al., 1986], in the functional capabilities [Cher & Mosmann, 1987; Stout & Bottomly, 1989] and in their activation requirements [Greenbaum et al., 1988; Lichtman et al., 1988], but there are few reports as to whether Th1 and Th2 cells are phenotypically distinct. Recently, one study reported a differential expression of CD45 isoforms in murine Th1 and Th2 clones [Luqman et al., 1991] but the significance of this is unclear. The OX-22<sup>high</sup>CD4<sup>+</sup> and the OX-22<sup>low</sup>CD4<sup>+</sup> subsets have been described as Th1-like and Th2-like cells respectively [Fowell et al., 1991]. Our results reported in this study suggest that OX-22<sup>high</sup>CD4<sup>+</sup> and the OX-22<sup>low</sup>CD4<sup>+</sup> subsets do not have distinct lymphokine expression patterns with respect to lymphokine gene expression .

The results presented here suggest that there is no simple correlation between rat CD4<sup>+</sup> cell subsets (based on phenotypic expression of CD45 isoforms) and their lymphokine expression.

## **Chapter 7 The role of transforming growth factor- $\beta$ (TGF- $\beta$ ) on S-Ag-induced EAU**

### **7.1 Introduction**

The treatment of uveitis is still a clinical challenge. Immunosuppressive drugs have many side effects [Lightman, 1991; Palestine et al., 1986] and are not always successful in controlling the inflammation and restoring sight. Biological agents that can suppress the inflammatory responses deserve attention as alternative approaches. So far EAU has been down-regulated by anti-class II MHC mAb [Rao et al., 1989; Wetzig et al., 1988], anti-CD4 mAb [Atalla et al., 1990], anti-IL-2R mAb [Higuchi et al., 1991], anti-IFN- $\gamma$  mAb [Atalla et al., 1990], cyclosporin A [Caspi et al., 1988; Nussenblatt et al., 1982] and FK506 [Kawashima et al., 1988]. Another cytokine which was shown to have profound immunosuppressive effects and might have therapeutic role in EAU is transforming growth factor  $\beta$  (TGF- $\beta$ ).

TGF- $\beta$  is a family of proteins (TGF- $\beta$ 1, - $\beta$ 2 and - $\beta$ 3 in mammals) and has been shown to control many different aspects of the immune system (reviewed in [Wahl, 1992]). The role of TGF- $\beta$  is likely to be complex and appears to play a role from the onset of inflammatory response to its resolution [Wahl et al., 1989]. TGF- $\beta$  released by platelets at the early stage has a very important role in mononuclear cell recruitment and activation, and induces its own production by monocytes. Following recruitment of monocytes, TGF- $\beta$  activates monocytes to release inflammatory mediators such as IL-1, platelet-derived growth factor, fibroblast growth factor and tumor necrosis factor which have an impact on inflammatory events. TGF- $\beta$  inhibits T cell proliferation which may reduce the inflammatory response while promoting healing. Although TGF- $\beta$  can promote cell recruitment and inflammation at the site of tissue damage, it can also suppress immune function. It has been shown that TGF- $\beta$  suppresses the development, activity and/or differentiation of T cells [Kehrl et al., 1986; Ranges et al., 1987], LAK cells [Espevik et al., 1988; Mule et al., 1988] and natural killer cells [Rook et al., 1986; Su et al., 1991]. TGF- $\beta$  also can influence the development of Th subsets. It has been shown that the presence of TGF- $\beta$  in the early stages of CD4<sup>+</sup> T cell stimulation (polyclonal and antigen specific) *in vitro*, leads to strong polarization of the T cells

towards the Th1 subset [Swain et al., 1991; Swain et al., 1991].

Based on its powerful anti-inflammatory effects, which in some respects mimics the beneficial effects of immunosuppressive drugs, TGF- $\beta$ 1 has been used in treatment of experimental autoimmune diseases such as collagen-induced arthritis (CIA, a model for rheumatoid arthritis) and experimental allergic encephalomyelitis (EAE, a model for multiple sclerosis), without discernable adverse effects [Johns et al., 1991; Kuruvilla et al., 1991; Racke et al., 1991]. Furthermore, neutralizing antibodies to TGF- $\beta$ 1 increase the severity of EAE, suggesting that endogenous TGF- $\beta$ 1 normally plays a role in modulating autoimmune disease [Miller et al., 1992; Racke et al., 1992]. These results support the hypothesis that the absence of TGF- $\beta$ 1 (the primary isoform in activated immune cells) could release immune cells from this negative regulation, potentially contributing to autoimmune activation.

In order to investigate the anti-inflammatory and immunosuppressive properties of the TGF- $\beta$ 1 in the *in vivo* regulation of EAU, we administer this cytokine locally in the eye to see whether TGF- $\beta$  can reverse EAU once it has been induced.

## **7.2 Results**

### **7.2.1 Intraocular treatment with TGF- $\beta$**

Human natural TGF- $\beta$ 1 (BDA5, British Bio-technology) which is apparent crossreactivity between species [Derynck et al., 1986; Derynck et al., 1985] was reconstituted at 2  $\mu$ g/ml in 4 mM HCl-normal saline supplemented with 0.1% BSA and stored at -70°C. Further dilutions were carried out in endotoxin-free saline.

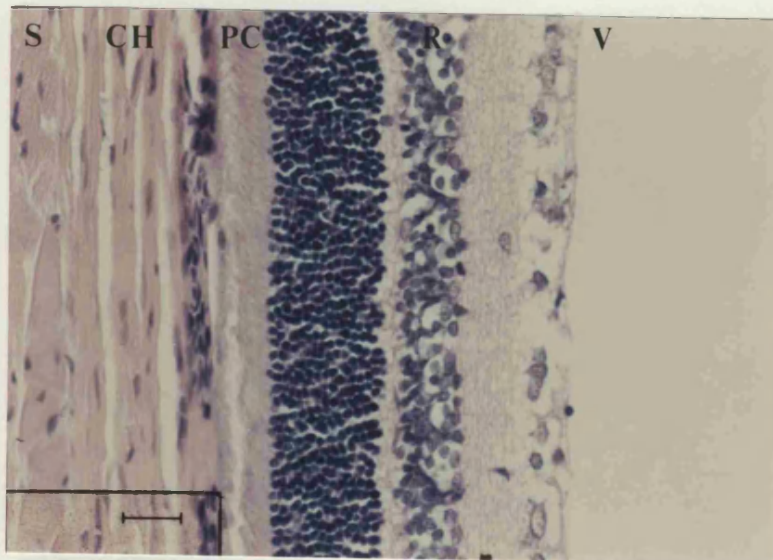
5-10 Lewis rats per group were injected intraocularly with 10  $\mu$ l volumes of 10, 20 or 50 ng per eye of human natural TGF- $\beta$ 1 on days 7 and 10 after immunization with S-Ag in CFA plus *B. pertussis* as described in [section 2.1.2](#). All intraocular injections were carried out under the microscope after rats were anaesthetized with 0.15 ml Ketamine (100 mg/ml). Controls included rats without intraocular injection and rats injected with 10  $\mu$ l endotoxin-free saline or solubilising buffer (4 mM HCl containing 0.1% BSA) at days 7 and 10 postimmunization. Rats with active EAU (day 13-15) and



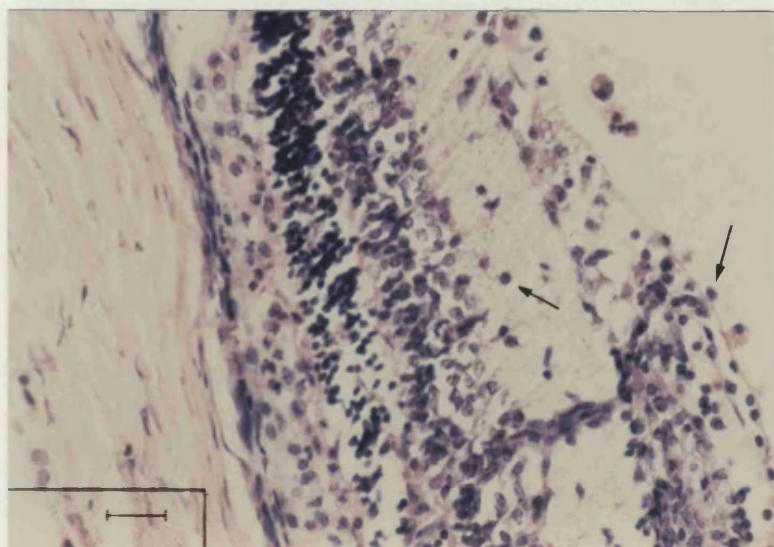
postinflammatory disease (day 17-20) were sacrificed and enucleated eyes examined histologically as described in section 2.1.3.

### 7.2.2 The role of TGF- $\beta$ on the course of EAU

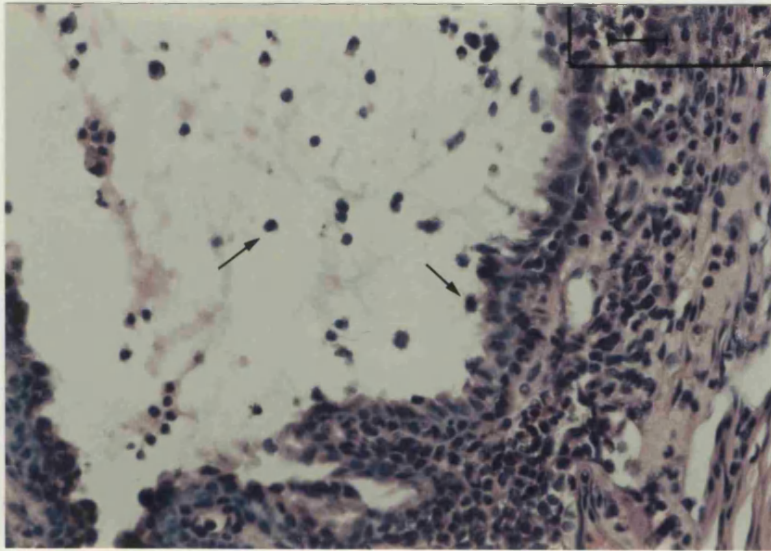
The severity of EAU was reduced at day 13 of disease (active stage) by 10-50 ng of TGF- $\beta$ 1 given intraocularly (Fig.7-2 C and D) in comparison with controls (Fig.7-2 A and B). However by day 17, no reduction of disease was observed (Fig.7-3 C) in comparison with controls (Fig.7-3 A and B). Fig.7-1 shows normal Lewis rat retina.



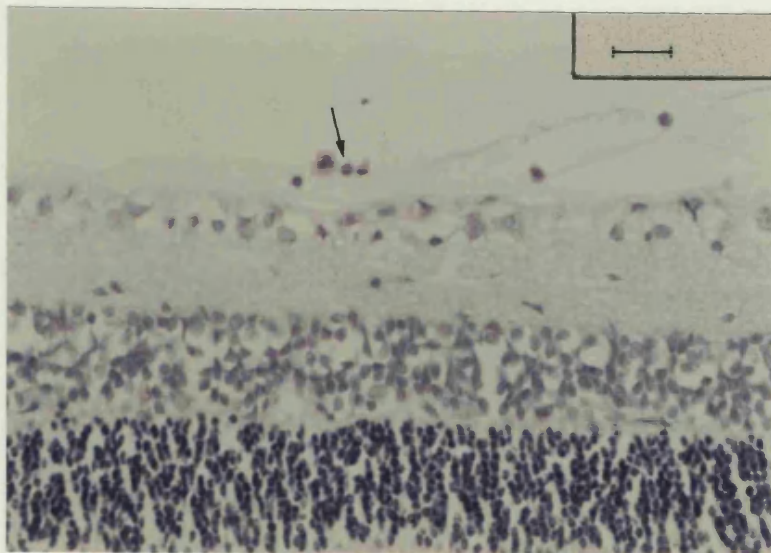
**Figure 7-1** Normal Lewis rat retina (R), showing the vitreous (V), photoreceptor cell layer (PC), choroid (CH) and sclera (S). Magnification x250



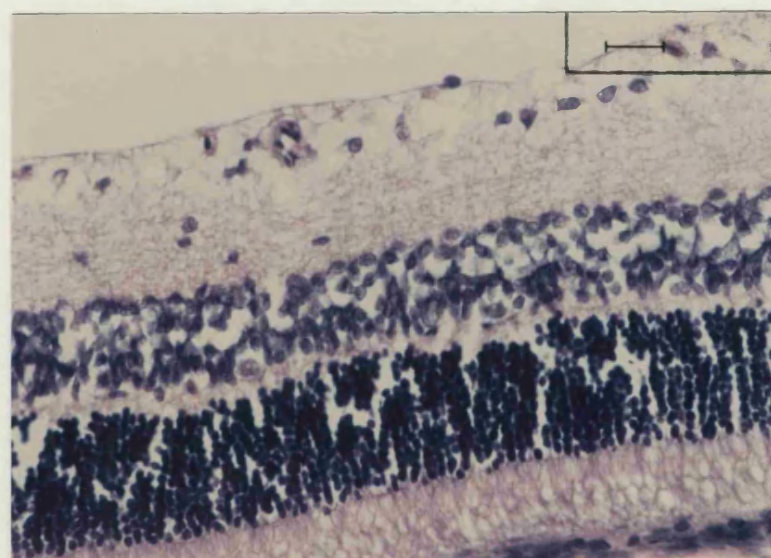
**Figure 7-2A** Day 13 EAU (active). Inflammatory cells are observed within the vitreous ( $\rightarrow$ ) and through the retina.



**Figure 7-2B** Day 13 EAU. Ciliary body is heavily infiltrated with densely stained mononuclear cells.

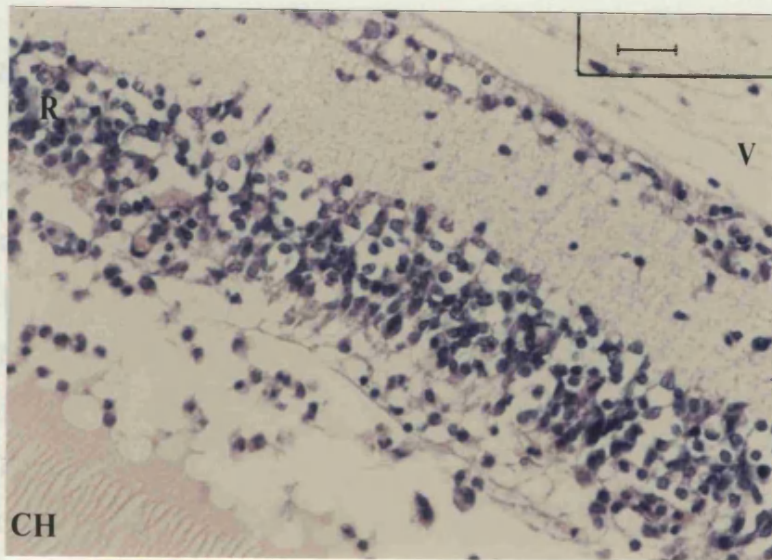


**Figure 7-2C** Day 13 EAU with TGF- $\beta$  (10 ng). Note the few cells in the vitreous but little infiltration of the retinal layer.

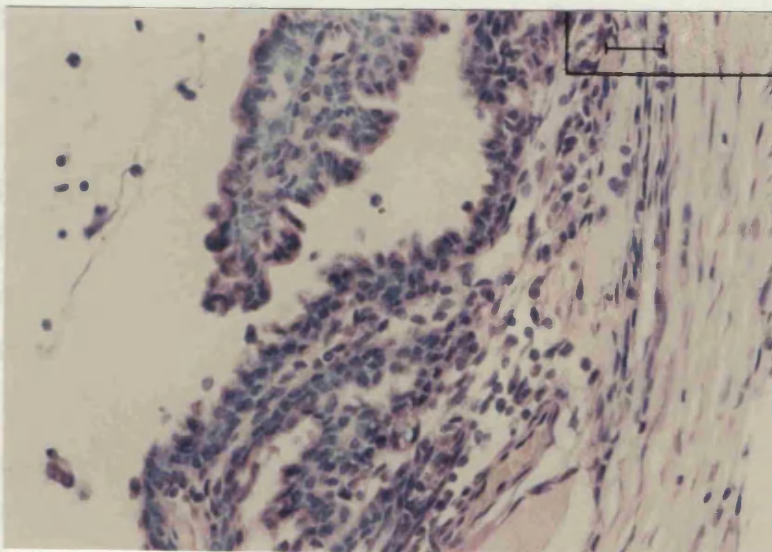


**Figure 7-2D** Day 13 EAU with TGF- $\beta$  (50 ng). There is little evidence of disease at this higher dose of TGF- $\beta$ .

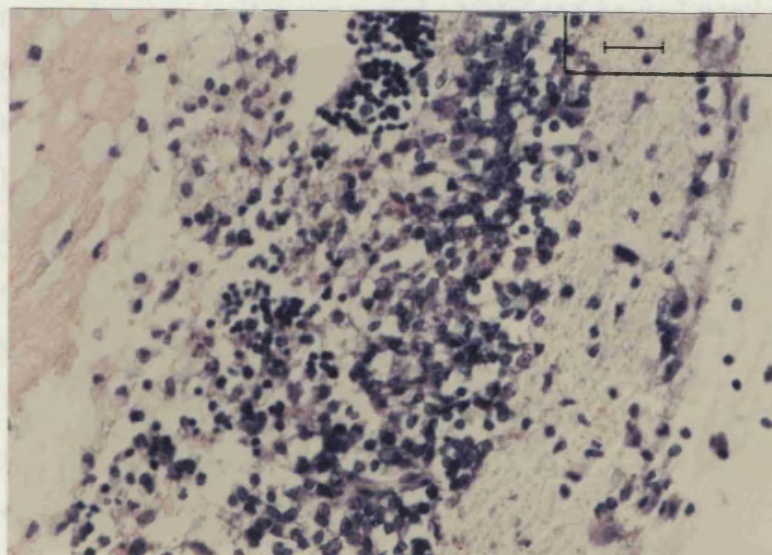




**Figure 7-3A** Day 17 EAU (late). There is a disorganization of the retinal layers at this later stage of EAU and a loss of photoreceptor cells.



**Figure 7-3B** Day 17 EAU (late). The degree of cellular infiltration in the ciliary body has declined by this stage of disease.



**Figure 7-3C** Day 17 EAU with TGF- $\beta$  (50 ng). Note the intense cellular infiltration throughout the retina with a loss of photoreceptors as seen in Figure 7-3A.

**7.3 Discussion**

It has been shown that administration of TGF- $\beta$  can prevent the occurrence of some autoimmune diseases [Johns et al., 1991; Kuruville et al., 1991; Racke et al., 1991]. As the clearance of circulating active TGF- $\beta$  is very rapid ( $<3$  min) [Coffey et al., 1987], TGF- $\beta$  is normally administered repeatedly in short intervals. In this experiment we injected TGF- $\beta$  locally in the eye on days 7 and 10 days after immunization with S-Ag. The results suggest that TGF- $\beta$ 1 delayed the onset of EAU in Lewis rats with our treatment protocol. This provides direct evidence that this cytokine can play an important role in immune responses within the eye. Considering the rapid clearance of circulating TGF- $\beta$ , the effect of TGF- $\beta$  on delaying the EAU induction with relatively long intervals of TGF- $\beta$  injection may be explained, at least in part, by the fact that it is bound to the proteoglycan and betaglycan [Andres et al., 1989], and this binding might protect TGF- $\beta$  from degradation, or might function as a long-term reservoir. As a possible immunoregulatory factor, TGF- $\beta$  appears to be one of the most interesting cytokines in relation to inflammatory eye disease. Normal iris, ciliary body and corneal tissue in tissue culture have been shown to produce TGF- $\beta$  [Helbig et al., 1991; Knisely et al., 1991; Runyan et al., 1983; Streilein & Bradley, 1991]. TGF- $\beta$  has been found by immunohistochemically in various parts of the eye. It has been detected in aqueous humor of normal non-inflamed eyes [Cousins et al., 1991; Granstein et al., 1990; Jampel et al., 1990], in vitreous aspirates from human eyes with intraocular fibrosis or retinal detachments [Connor et al., 1989] and normal porcine vitreous [Yoshitoshi & Shichi, 1991]. Immunohistochemical location of TGF- $\beta$  revealed that it was present in the human photoreceptor layer, associated with both rods and cones [Lutty et al., 1991]. It seems that it is secreted in a latent form that, by mechanisms that are not yet clear (but might include an acidic microenvironment and protease cleavage), is converted to its active form. A reduced pH often occurs at inflammatory sites and this local acidic condition is beneficial for activation of TGF- $\beta$ , resulting in its participation in the down-regulation of inflammation. The presence of TGF- $\beta$  in aqueous humor appears to be important for the maintenance of the immunosuppressive environment of the anterior chamber [De Vos et al., 1992].

The mechanism whereby TGF- $\beta$  had the effect of delaying the onset of EAU *in vivo* is

unknown. It has been shown that inappropriate expression of MHC class II antigens may allow cells to present autoantigens and initiate a circle of self-perpetuating inflammation leading to organ damage [Bottazzo et al., 1983; McCarron et al., 1985]. Recent results reported by Geiser et al. [Geiser et al., 1993] suggest potential applications for TGF- $\beta$  in the management of autoimmune disease, allograft rejection and other problems associated with altered MHC expression. TGF- $\beta$  prevents the increased expression of MHC class II antigens on epidermal Langerhans cells induced by IL-1, TNF- $\alpha$ , IFN- $\gamma$  and GM-CSF *in vivo* and *in vitro*; however, it does not down-regulate the normal MHC class II antigen expression on these cells [Epstein et al., 1991]. A similar antagonistic effect of TGF- $\beta$  on other cytokines has been noted for MHC class II expression on human melanoma cells [Czarniecki et al., 1988]. Thus regulation of MHC class II expression on target cells may be an important aspect of the effect of TGF- $\beta$  in EAU, although there is no direct evidence to support this.

There is evidence to show that TGF- $\beta$  plays an important role in the inhibition of leukocyte adherence to endothelium [Gamble & Vadas, 1988; Lefer et al., 1993] and has also been shown to regulate integrin synthesis [Ignatz et al., 1989; Ignatz & Massague, 1987], which is needed for cell-cell and cell-matrix attachment. Thus, the presence of TGF- $\beta$ 1 may also influence the access of infiltrating cells to tissues expressing elevated MHC antigens. The results demonstrated by Mahalak et al. showed that during the course of EAU development, TGF- $\beta$  can be synthesized by retinal vascular endothelial cells and pericytes and stimulates the synthesis and composition of extracellular matrix components in the vascular basal lamina [Mahalak et al., 1991]. Our results which show that TGF- $\beta$ 1 only delayed, but did not totally prevent EAU disease, might be due to both inhibition of adhesion of inflammatory cells to endothelial cells, and enhancement of cell surface integrin expression.

The inflammatory response of autoimmune disease is thought to be mediated by release of many cytokines. *In vitro*, exogenous TGF- $\beta$  has been shown to inhibit IL-1-dependent lymphocyte proliferation [Wahl et al., 1988] and prevent the expansion of IL-2- and IL-4-dependent T cells [Rueger et al., 1990]. The data reported by Espevik et al. showed that TGF- $\beta$  blocks IFN- $\gamma$  production by peripheral blood mononuclear cells (PBM) in response to LPS [Espevik et al., 1987], suggesting that TGF- $\beta$  may suppress immune responses by inhibiting cytokine production. Although TGF- $\beta$ 1 does not appear

to inhibit the ligand binding to its receptor, it appears to alter the early events of T cell activation after binding of lymphokines to their receptors, such as inhibition of c-myc expression, protein synthesis, and transcriptional initiation of genes and protein phosphorylation [Massague et al., 1990; Ortaldo et al., 1991].

It has also been shown that TGF- $\beta$  inhibited Ag-specific proliferation of MBP-specific T cell lines and the capacity of these cell lines to transfer EAE in Lewis rats [Schluesener & Lider, 1989]. We have observed that TGF- $\beta$ 1 has significant effects on inhibition of proliferative responses of S-Ag-primed spleen cells but not S-Ag specific CD4<sup>+</sup> T cell lines (Hu et al., unpublished data). However intraocular TGF- $\beta$ 1 (50 ng/rat) were unable to prevent S-Ag-induced EAU. The reasons for this could also be that there are some factors which are resistant to the effects of TGF- $\beta$ 1 or that the highest dose was insufficient for preventing the development of disease.

In summary, our results suggested that TGF- $\beta$  can delay the inflammatory responses of S-Ag-induced EAU *in vivo*. The mechanism(s) of this effect remains to be investigated.

## **Chapter 8 Concluding Remarks**

Most of the mechanisms which have been implicated in the pathogenesis of autoimmune diseases seem to be influenced by cytokines. These include the loss of self-tolerance and activation of autoreactive T cells, hyperexpression of MHC class I and aberrant expression of class II molecules by target cells, hyperexpression of target antigens and T cell-mediated cytotoxicity [Kroemer et al., 1991; Cavallo et al., 1994]. Cytokines may be involved in both the generation and the maintenance of autoimmune responses, as well as in the final effector mechanisms acting on target cells.

EAU is a T cell-mediated autoimmune model for human posterior uveitis. There have been some studies investigating the immunopathological mechanisms of this disease. Adoptive transfer of EAU in Lewis rats using activated CD4<sup>+</sup> T cell lines specific for S-Ag shows that CD4<sup>+</sup> T-cells play a key role in EAU induction [Caspi et al., 1986]. Although CD4<sup>+</sup> T cells have been demonstrated to have a cytotoxic effect in some circumstances [Ozdemirli et al., 1992; Strack et al., 1990], the majority of their effects appear to be mediated via the production of different cytokines [Mosmann & Coffman, 1989a and 1989b]. However there is little information on the regulation of lymphokine production by uveitogenic T cell lines. S-Ag specific CD4<sup>+</sup> T cell lines and clones were established in this laboratory to study the immunopathological mechanisms of EAU. As a part of this project, my thesis addressed the question of which lymphokines were expressed and how they were regulated in S-Ag-specific T cell lines and clones. Bioassays and PCR techniques were validated and employed for the study of lymphokine secretion and mRNA expression.

It was reported previously [Charteris & Lightman, 1992 and 1993] in our laboratory that IL-2, IFN- $\gamma$ , IL-4 and LT mRNA were expressed *in vivo* by cells infiltrating the eye during EAU. The importance of IL-2 and IFN- $\gamma$  in pathogenesis of EAU were also demonstrated by Atalla et al. [Atalla et al., 1990] and Higuchi et al. [Higuchi et al., 1991] who showed that anti-IFN- $\gamma$  and IL-2R mAbs could prevent EAU in Lewis rats. In the present studies, S-Ag specific CD4<sup>+</sup> T cell lines when stimulated with antigen expressed IL-2, IFN- $\gamma$  and IL-4 mRNA, and IL-2 and IFN- $\gamma$  could be detected in the supernatants. Our results from twelve S-Ag specific CD4<sup>+</sup> T cell clones showed that all

clones expressed IFN- $\gamma$  mRNA, and all but two expressed IL-2 mRNA. IL-4 mRNA was expressed by three T cell clones which also expressed IL-2 and IFN- $\gamma$ . All these results suggest that the expression of IL-2, IFN- $\gamma$  and IL-4 may be relevant to the biological features of S-Ag responses and could be important in EAU induction. However, it must be borne in mind that the detection of a particular cytokine does not necessarily point to a causal role for that mediator in *in vivo* pathology. The presence of IFN- $\gamma$  in the supernatants might act as an antagonist for the biological activity of IL-4 in the same supernatants [Paliard et al., 1988] and preferentially favour the development of Th1-like cells [Gajewski et al., 1989]. Furthermore the presence of some cytokines at the site of the autoimmune lesions may be related to inflammatory reactions which are often associated with the autoimmune process, and may be secondary to the induction of the disease. The production of a particular cytokine *in vivo* arises through a complex interaction of both stimulatory and inhibitory factors.

It is of interest to notice the different patterns of cytokine expression with individual clones as described in chapter 5. It is possible that the differences in lymphokine expression by S-Ag specific T cell clones may influence their abilities to induce EAU. However, the ability of these clones to induce EAU is still unknown. More information needs to be obtained to examine the uveitogenicity of these S-Ag specific clones, and correlate the pathogenesis with the lymphokine expression of individual clones. Sedgwick et al. (1989) showed that CD4<sup>+</sup> T cell clones that were able or not able to transfer EAE in the rat produced similar amounts of IFN- $\gamma$ , and concluded that IFN- $\gamma$  alone is unlikely important in EAE induction [Sedgwick et al., 1989].

In the present thesis, IL-2, IFN- $\gamma$ , IL-4 and IL-10 lymphokine gene expression by S-Ag-specific CD4<sup>+</sup> T cell lines and clones was investigated with PCR. Only IL-2 and IFN- $\gamma$  production by S-Ag-specific CD4<sup>+</sup> T cell lines was studied due to the lack of commercial cytokines for rats. Clearly, there are several other cytokines which might be involved in the disease processes. Cytokines such as TNF- $\alpha$  and IL-6 might be responsible for the inflammatory processes [De Vos et al., 1992]. Further studies are needed to clarify these cytokines in the pathogenesis of the disease, and to investigate the possible role of individual cytokines or the combination of these cytokines in EAU mechanism.

The lymphokine production by antigen specific T cells might be responsible for the selection of a particular T cell subset over others. For example, most T cell clones specific for *Dermatophagoides pteronyssinus* or for *Toxocara canis* excretory-secretory antigen generated from atopic patients or healthy individuals secrete high levels of IL-4 and IL-5, whereas T cell clones specific for tetanus toxoid or *Mycobacterium* secrete IFN- $\gamma$  and IL-2 but not IL-4 [Del Prete et al., 1991; Liew et al., 1990; Romagnani, 1991; Wierenga et al., 1990]. Our results with rat S-Ag and PPD specific T cell lines add more evidence that lymphokine production by antigen-specific T cells depends on the nature of the stimulating antigen [see [chapter 3](#)]. Furthermore, our data that HT-2 cells proliferated differently to supernatants of S-Ag specific T cells treated with S-Ag and Con-A suggested that different factors may be produced upon S-Ag and Con A stimulation. These data indicated a particular pattern of lymphokine expression is not a stable function of rat CD4<sup>+</sup> T cells and may be influenced by the ligand responsible for receptor-mediated activation. The phenomenon of stimuli-dependent lymphokine production has also been observed in human CD4<sup>+</sup> T cell clones specific for hepatitis B surface antigen (HBsAg) [Tsutsui et al., 1991], mouse CD4<sup>+</sup> clone specific for OVA [Patarca et al., 1991], and anti-CD3 stimulated mouse spleen cells in the presence of PMA [Monteyne et al., 1992]. If the *in vitro* findings in our studies are reflecting an *in vivo* situation, it is of interest to determine the factor(s) which regulates lymphokine production after these cells are adoptively transferred into naive animals during the induction of EAU.

Mouse CD4<sup>+</sup> T cell clones have been divided into Th1 and Th2 on the patterns of lymphokines expressed. Evidence for subsets of Th cells in rats is limited. Based upon recent *in vivo* experiments, it has been suggested that similar CD4<sup>+</sup> T cell subsets exist in rats [Papp et al., 1992; Saoudi et al., 1993]. However only with cloned T-cells is it possible to clarify this issue. S-Ag and PPD specific T cell clones were therefore examined for lymphokine expression and results indicated that most S-Ag specific CD4<sup>+</sup> T cell clones expressed Th1-like pattern but some clones expressed a mixed pattern (Th0) on the basis of lymphokine mRNA expression, whereas the PPD specific CD4<sup>+</sup> T cell clones exhibit mostly a mixed (Th0) pattern but also Th1 and Th2 patterns. It should be noted here that mRNA expression does not necessarily correlate lymphokine production and thus further investigation of lymphokine production by these and other clones will be carried out in future studies.



Although much interest has been generated by the Th1/Th2 paradigm, a number of observations indicate that it must be interpreted with some caution [see [section 1.6.3](#)]. For example, the cytokine environment that is present during differentiation may be an important influence on the type of Th cells that will be generated. Th1 cells are preferentially obtained when CD4<sup>+</sup> cells are cloned in the presence of IFN- $\gamma$  [Gajewski et al., 1989]. Conversely, the presence of IL-4 during Th generation *in vitro* leads to polarization of cells towards to Th2 subset [Swain et al., 1991]. Furthermore, results by many investigators [Erb et al., 1991; Firestein et al., 1989; Gajewski et al., 1989; Hom et al., 1989; Kelso & Gough, 1988] and our results indicate that a classification based on lymphokine pattern alone is not sufficient. It has been suggested that definitions of subsets should be made on the basis of lymphokine expression in combination of effector function such as B cell help, cell surface markers, APC killing, and others.

In the rat the mAb MRC OX22 separates CD4<sup>+</sup> T cells into two subpopulations: OX22<sup>high</sup> and OX22<sup>low</sup> [Spickett et al., 1983]. One of the critical differences between OX22<sup>high</sup> and OX22<sup>low</sup> CD4<sup>+</sup> T cells is the profile of cytokines that they express. OX22<sup>high</sup> CD4<sup>+</sup> T cells produced high IL-2 and IFN- $\gamma$  but low IL-4 after activation with ConA, whereas OX22<sup>low</sup> CD4<sup>+</sup> T cells produced high IL-4 but low IL-2 and IFN- $\gamma$  [Fowell et al., 1991]. In present thesis, we investigated lymphokine gene expression by freshly isolated OX22<sup>high</sup> and OX22<sup>low</sup> CD4<sup>+</sup> T cell subsets after 6 h stimulation with S-Ag, ConA and PMA. Our results suggest that there is no simple correlation between rat CD4<sup>+</sup> subsets (based on expression of CD45 isoforms) and their lymphokine gene expression under our experimental conditions. The patterns of lymphokines expressed by OX22<sup>high</sup> and OX22<sup>low</sup> CD4<sup>+</sup> T cells are dependent upon the mode of stimuli. Similar results have also been reported in mouse T cells [Lee & Vitetta, 1992] and human T cells [Butch et al., 1991] where the patterns of lymphokine production are not dependent upon expression of a particular CD45R phenotype.

The treatment of autoimmune diseases with cytokine agonists and antagonists has been attempted in order to establish their clinical usefulness for the immunotherapy. Studies on the effects of TGF- $\beta$  in the modulation of experimental autoimmunity *in*



*vivo* have provided evidence for the potential involvement of this cytokine in autoimmune processes [Johns, et al., 1991; Kuruvilla et al., 1991; Racke et al., 1991]. TGF- $\beta$  causes profound immunosuppressive effects, including inhibition of T cell proliferation, activation, cytotoxicity and cytokine production [Wahl, 1992]. In our studies (chapter 7) TGF- $\beta$ 1 was found to have the effect of delaying the onset of EAU. However, intraocular TGF- $\beta$ 1 (50 ng/rat) were unable to prevent S-Ag-induced EAU. This may be due to that there are some factors which are resistant to the effects of TGF- $\beta$ 1 or the dose was insufficient for preventing the development of disease. Further studies are needed to identify the mechanisms of this effect.

Future direction:

The present thesis has attempted to address the question of lymphokine expression regulation of S-Ag specific T-cells *in vitro* and investigate T-cell effector mechanism of EAU. Based on the lymphokine expression patterns of these T cells we also intended to assess the classification of CD4<sup>+</sup> T-cells. However there are still some fundamental questions remain unanswered. Further studies could be performed in the following areas.

#### 1. Rat CD4<sup>+</sup> T cell subsets

Establish specific IL-4 and IL-10 bioassays to facilitate the investigation of their production by S-Ag specific T cell clones. Based on bioactivities of IL-2, IFN- $\gamma$ , IL-4 and IL-10, the correlation between the detection of these cytokine mRNA expressed by S-Ag specific clones and their secretion can be determined.

Cytokine gene expression by rat S-Ag- and PPD-specific T-cell clones have been studied in the present studies. Further investigation on cytokine production by S-Ag T-cell clones will be carried out to obtain firm evidence whether a similar classification of CD4<sup>+</sup> T cell subsets (Th1 and Th2) exists in the rat as in the mouse.

#### 2. Adoptive transfer and effector mechanism of EAU

S-Ag specific T-cell lines and clones used in this thesis have not yet tested for

adoptive transfer. In the future, it would be of importance to investigate the uveitogenicity of these T-cell lines and clones. Furthermore, studies would be carried out to examine the relationship between the pathogenesis of the T cell clones and their lymphokine expression to find out the roles of individual lymphokines in induction of EAU.

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