

Analysis of candidate retinal autoantigens in autoimmune uveitis

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I would like to dedicate this thesis to my parents.

Abstract

Autoimmune uveitis is a non-infective ocular inflammation of humans that potentially causes blindness. It is mediated primarily by autoreactive CD4⁺ T-lymphocytes that target antigens within the retina. Autoantibody responses play a secondary role. This thesis describes various investigations into humoral and cellular immune responses in autoimmune uveitis and assesses several methodologies for their suitability to applied human research.

Recombinant human retinal S antigen (RSAg), an important candidate autoantigen in uveitis, was cloned and expressed in bacterial and human cells. Purified RSAg was tested by ELISA against sera from uveitis patients and controls. The recombinant antigens performed well in ELISA. No significant differences in antibody titres were detected between the groups.

B-cell epitope preferences of anti-RSAg polyclonal antibodies were investigated by screening several random phage display libraries. One library produced results, but no defining epitope was identified for either uveitis or control sera. In uveitis research this technique might be better suited to delineating minimal epitope requirements of monoclonal antibodies.

As yet undiscovered uveitis autoantigens may exist. A human retinal complementary DNA library was constructed and screened with uveitis and control sera. Seven potentially autoantigenic peptides were identified and expressed as fusion proteins. At least one peptide displayed significantly higher ELISA readings for uveitis over control sera. The full potential of this technique is still to be realised.

Responses of peripheral CD4⁺ T-lymphocytes to antigen-specific stimulation were studied at the single-cell level using cytokine flow cytometry. A definite response to RSAg was detected in human uveitis and control lymphocytes using this method. This technique has great potential for identifying autoantigenic proteins/epitopes, and analysing resultant cytokine profiles in uveitogenic T-cells.

Several of the new strategies and techniques described here have already produced exciting findings. It is envisaged that they will make further significant contributions to applied human uveitis research in the near future.

Chapter 1: General introduction

1.1 Introduction

This thesis describes investigations carried out into autoimmune posterior uveitis, an ocular inflammatory condition that is a leading cause of visual disability. The emphasis in this work is placed on investigative techniques that could be applied directly to human research without having to rely on animal models of disease. The subjects of autoimmune posterior uveitis and organ-specific autoimmune disease - the disease category to which it belongs - are discussed in detail in this chapter. The immune system's response to antigen under normal circumstances and the mechanisms by which self-tolerance to the body's own tissues is established, are also discussed.

1.2 The nature of autoimmune posterior uveitis

1.2.1 Definitions

Strictly speaking the term "uveitis" means an inflammation of the uveal tract of the eye i.e. the choroid, ciliary body or the iris. However, it is usually used to describe any form of intraocular inflammation. The uveal tract provides most of the blood supply to the intraocular structures and serves as a conduit for immune cells entering the eye – therefore it usually becomes inflamed irrespective of the primary target of the immune response. The target antigens themselves are rarely located within the uvea and therefore some prefer the term intraocular inflammation (IOI) (Forrester and McMenamin 1999).

The term uveitis alone does not imply aetiology, and it can variously be categorised as autoimmune, infectious, traumatic or even neoplastic in origin (Opremcak and Kachelein 1994). It can also be categorised anatomically as anterior, intermediate, posterior or pan-uveitis (involving all ocular layers)(Bloch-Michel and Nussenblatt 1987).

Traumatic and neoplastic causes of intraocular inflammation are relatively easily recognised on clinical grounds. The differentiation of infectious from non-infectious uveitis is determined clinically and by laboratory testing. Typical organisms that cause infectious uveitis include *Toxoplasma gondii*,

Toxocaris canis, cytomegalovirus, *Borrelia burgdorferi* and *Mycobacterium tuberculosis*. Infectious uveitis is different from “endophthalmitis” – the former is inflammation mediated by the immune system in response to a specific microbial pathogen, while the latter is the inflammation and destruction mediated directly by such pathogens (e.g. the release of lytic enzymes) during frank intraocular infection.

When no other cause of uveitis is found, it is described as “endogenous”, which is usually taken to mean of autoimmune origin, or at the very least immune-mediated. This is particularly so for many types of posterior, intermediate and pan-uveitis as opposed to anterior uveitis, which seems to be a distinct entity (Forrester 1992) (see below). Under recent guidelines posterior, intermediate and pan-uveitis have been designated as posterior segment intraocular inflammation (PSII), whereas anterior uveitis is now called anterior segment intraocular inflammation (ASII) (Forrester et al. 1998a). In theory, either category can be sub-divided further into infective or non-infective types.

The focus of this thesis is the investigation of PSII of presumed autoimmune origin. The terms "autoimmune uveitis" or “autoimmune PSII” are usually used to denote this specific disease category, but other expressions including "endogenous PSII" and even "uveitis" are also used where appropriate. Anterior uveitis (including that associated with juvenile rheumatoid arthritis) and infectious uveitis are specifically excluded from investigation in this study, but are discussed at various points. Where uveitis types other than autoimmune PSII are being discussed, this is explicitly stated.

1.2.2 Features of autoimmune PSII

Autoimmune PSII consists of a heterogeneous group of clinical conditions, each of which has its own characteristic features, natural history and even prognosis (see Table 1.1). However, these conditions share certain core clinical and immunopathological features that allow them to be considered as one group – in fact, some authorities regard the various autoimmune posterior uveitides as essentially different manifestations of a common pathogenetic process (Forrester 1990). The key clinical features are macular/retinal oedema, vitritis, chorioretinal infiltrates and retinal vasculitis, any one of which may predominate in a given uveitis subtype but all of which are recognised features of the

condition. The archetypical autoimmune posterior uveitic condition is sympathetic ophthalmia.

Around 50% of cases of autoimmune uveitis are confined to the eye, while the remainder form part of more generalised diseases such as multisystem connective tissue and granulomatous disorders (Dick 2000). Autoimmune PSII subtypes that form part of a multisystem disorder include sarcoid uveitis, Behcet's uveitis and Vogt-Koyanagi-Harada (VKH) syndrome.

Autoimmune PSII appears to be distinct from anterior uveitis in several ways. Anterior uveitis is usually an acute, self-limiting condition that rarely causes significant visual impairment. It is associated with Major Histocompatibility Complex (MHC) class I antigens and although never proven, an association with gram-negative bacteria has long been suspected (Wakefield et al. 1990; Careless et al. 1997). Autoimmune PSII on the other hand, is more associated with MHC class II antigens (the MHC and its disease associations are discussed later). It tends to be recurrent or chronic and because it causes destruction of delicate ocular tissues, in particular the macula, is a leading cause of blindness. There is no association with active infection.

Immunopathological studies in human autoimmune PSII are limited in number because of obvious difficulties in obtaining biopsy samples from affected posterior ocular structures. Also, most pathological material has been obtained from eyes with end-stage uveitis, which may not be particularly relevant to the initiation or perpetuation of early forms of the disease. Existing studies, however, show similar features for the various subtypes of autoimmune PSII. A predominance of CD4⁺ T-cells is found in the target tissues early in the disease, often with greater CD8⁺ and B-cell involvement later on (Boyd et al. 2001).

It is noticeable that virtually every clinical feature of autoimmune PSII can be reproduced using the same animal model of the disease, Experimental Autoimmune Uveoretinitis (EAU). This further strengthens the argument that the various clinical subtypes are closely related, or at least share a final common pathway in the manifestation of inflammation.

Table 1.1 Clinical subtypes of autoimmune PSII.

Sympathetic ophthalmia	Intermediate uveitis	Multifocal choroidopathy
Birdshot chorioretinopathy	Pars planitis	Sarcoid uveitis
Serpiginous choroidopathy	Panuveitis (non-infective)	Behcet's uveitis
Retinal vasculitis	Punctate inner choriopathy	Vogt-Koyanagi-Harada uveitis (VKH)
Multiple evanescent white dot syndrome (MEWDS)	Acute multifocal placoid pigment epitheliopathy (AMPPE)	

1.2.3 Epidemiology

Uveitis, mainly posterior, is an important cause of blindness, and is *estimated* to cause around 10% of visual handicap in the Western world (Darrell et al. 1962; Nussenblatt 1990). However, accurate figures for the overall prevalence and incidence of uveitis and its various sub-categories are lacking. Proper population based studies on the extent of uveitis as a cause of blindness and visual impairment do not exist (Suttorp-Schulten and Rothova 1996). In most epidemiological studies dealing with blindness, uveitis is not considered as a distinct entity, and many secondary ocular problems caused primarily by uveitis (e.g. cataract, maculopathy) are not recognised as such. Differing study designs make comparison of data difficult. No aetiology in terms of a causal systemic disease is found in over 25% of uveitis cases. This figure rises to around 50% if established uveitis entities without known cause (such as Fuch's cyclitis or idiopathic vasculitis) are included (Rothova et al. 1992). In most uveitis studies, "autoimmune PSII" is not considered as a distinct entity.

In 1995, it was estimated that 38 million people were blind worldwide, the majority in Africa and Asia, with a further 110 million being visually impaired (Thylefors et al. 1995). Cataract is the leading cause, followed by trachoma and glaucoma, accounting together for 71% of the total. The exact percentage of worldwide blindness due to uveitis is unknown.

In the developing world, the commonest cause of blindness is again cataract, followed in order by trachoma, glaucoma and onchocerciasis (Thylefors et al. 1995). Onchocerciasis causes blindness as a result of inflammation, and may therefore be categorised as a uveitis of infectious origin. If other causes of uveitis were included, such as leprosy, uveitis might rise to third or higher in the list of leading causes of blindness. Separate data for non-infective or autoimmune posterior uveitis are not available.

In the Western world, the commonest causes of blindness overall are age-related macular degeneration and diabetic retinopathy. It is estimated that uveitis causes 10 to 15% of all cases of total blindness in the USA, but again there are relatively few specific data in this area. European studies mention figures of 3-6% (ten Doesschate 1982). When over-65's and children are excluded, the remaining section of the population (20-65 years) comprise 26 to 32% of registered blindness. The major causes of blindness in this group are tapetoretinal degeneration (20%), congenital anomalies (20%), diabetic retinopathy (20%), accidents (5%) and uveitis (10%). In economic terms this is the most important part of the population, as it represents the working population. The two largest *treatable* disease categories are diabetes (20%) and uveitis (10%).

The prevalence of uveitis in Western countries has been reported as 38 per 100,000 and the incidence as 14-17 per 100,000 (Suttorp-Schulten and Rothova 1996). Several reports on the frequency of different types of uveitis use the IUSG classification and find anterior uveitis to be the most common category (> 50%), followed by posterior, and then pan-uveitis or intermediate uveitis (Smit et al. 1993; Tran et al. 1995). There are no studies on the incidence of vision-threatening complications of uveitis, so the impact of prevention and therapy (including surgery) remains unclear. Approximately 70-90% of uveitis patients present between 20 and 60 years of age (working age), particularly in the 3rd and 4th decades. Between 25 and 50% of uveitis patients have an underlying systemic disease. It is not known exactly what percentage of each anatomical category of uveitis patients suffers from blindness or visual impairment, but posterior or pan-uveitis are thought to be the worst types. In a retrospective study of 582 patients with both infective and non-infective uveitis, 35% exhibited blindness or visual impairment in at least one eye (Rothova et al.

1996). Visual impairment was mainly due to posterior uveitis, with CMO as a leading cause.

Prognosis varies with the aetiological subtype of uveitis. Acute anterior uveitis, accounting for at least 30% of all uveitis, leads to visual impairment in only 1% of cases. Toxoplasmosis is the aetiological factor in about 10% of all uveitis patients, and leads to severe visual impairment or blindness in at least 9% of affected eyes. Ocular sarcoidosis accounts for around 7% of all uveitis patients (Rothova et al. 1992) and causes blindness in at least one eye in 10% of cases. Several forms of posterior uveitis, such as ocular Behcet's and serpiginous choroidopathy (Weiss et al. 1979), are rare in the West but have a poor visual prognosis.

The socio-economic impact of uveitis is considerable. It is estimated that in the USA alone 2.3 million suffer from uveitis (Suttorp-Schulten and Rothova 1996). The economic costs are even comparable to those of diabetic eye disease.

1.2.4 PSII as an autoimmune disease

The clinical conditions listed in Table 1.1 are accepted by most authorities as having an autoimmune aetiology. This is based on several complementary arguments. Perhaps the most compelling is that despite extensive testing, no clinical or laboratory evidence of microbial infection can be found during active inflammatory episodes. This coincides with the probable decrease in the proportion of uveitis cases directly caused by infection. Overall, an infectious agent can be identified in less than 20-25% of uveitis cases in recent studies (Tran et al. 1995; PivettiPezzi et al. 1996; Rodriguez et al. 1996; Merrill et al. 1997), except in areas where there is high exposure to pathogens (Biswas et al. 1996; Kaimbo Wa Kimbo et al. 1998). This contrasts with the situation in the 1940's – Woods diagnosed tuberculosis in 80% of cases of granulomatous uveitis in 1941 (Guyton and Woods 1941) but in only 20% in 1960 (Woods 1960).

Evidence that endogenous PSII is an autoimmune disease is found in its very close resemblance to EAU, an experimental disease model directly caused by immunisation with retinal autoantigens (detailed discussion in a later section). This model, which can induce intraocular inflammation using any of

several candidate autoantigens, can be made to mimic practically all the features of clinical PSII by adjusting the immunisation protocol in terms of antigen dose, route of administration, timing of immunisation, use of adjuvant, etc. EAU and endogenous PSII resemble each other not only clinically, but also in terms of histopathological features and immunobiological findings (though studies on human tissue are relatively few) (Forrester et al. 1990).

The MHC in humans is called the Human Leucocyte Antigen (HLA) complex. The HLA, through its influence on antigen presentation, is intimately involved in the immune response, and several established autoimmune diseases have HLA associations (see later). Several subtypes of autoimmune PSII are also associated with HLA haplotypes, as outlined below. This suggests a role for autoimmunity in the aetiology of these diseases.

Clinically, many types of endogenous PSII respond favourably to specific immunosuppressive treatments such as cyclosporin and tacrolimus. These anti T-cell therapies are also used successfully in established autoimmune diseases such as rheumatoid arthritis, thereby strengthening the case for an immunological/autoimmune aetiology in endogenous PSII. In addition, such immunosuppression does not unmask infection in such cases.

Laboratory evidence of autoimmunity in human endogenous PSII does exist – both T-cell and B-cell responses have been demonstrated to human retinal antigens. However, immunoreactivity in healthy controls has also been demonstrated, and this confuses matters. Therefore, to date, there is no direct proof that autoimmunity to retinal or other ocular antigen(s) actually causes endogenous PSII in humans. Nevertheless, when the various strands of circumstantial evidence are presented together, the case seems compelling.

1.2.5 Aetiology of autoimmune PSII

1.2.5.1 Genetic studies

There appears to be a genetic predisposition to autoimmune PSII and it is considered to be a multifactorial, polygenic disease. It is clinically heterogeneous and this is reflected in its genetic heterogeneity. The MHC is the main target for scrutiny regarding the genetic basis of PSII and other autoimmune diseases. However, there are also some non-MHC genes linked to

immune disease. Genetic studies in polygenic disorders such as autoimmune PSII are made more difficult by low penetrance of individual genes, interaction between individual alleles (epistasis) or additivity between alleles. This is further complicated in PSII by genetic heterogeneity sometimes resulting in the same phenotype, and by the relatively low incidence of some PSII subtypes. Genetic aspects of human uveitis have been studied using twin studies, familial aggregation and segregation studies, cytogenetic studies and association studies (Pennesi and Caspi 2002). Genetic analysis of animal strains in EAU has also contributed greatly.

1.2.5.1.1 Twin studies

In autoimmune PSII, only a few twin pairs have been studied so far, due to the relative rarity of some of the conditions and their clinical heterogeneity. Therefore, a clear estimate of concordance is not possible. Concordant twin pairs for Behcet's disease (Hamuryudan et al. 1991), birdshot chorioretinopathy (Fich and Rosenberg 1992), VKH (Itho et al. 1992; Ishikawa et al. 1994; Rutzen et al. 1995) and intermediate uveitis (Biswas et al. 1998) have been reported. Of less relevance to autoimmune PSII, but interesting nonetheless, is a large Finnish twin cohort study of ocular diseases in which 58 twin pairs with anterior uveitis were included. No concordant pairs were detected among these (Teikari et al. 1987).

1.2.5.1.2 Family studies

Family studies are useful in detecting the clustering of diseases in families ("familial aggregation") or the identification of a recurrent pattern ("segregation") down through generations. Aggregation has been noted in uveitic disease but no pattern of inheritance has been recognised. Uveitis in Behcet's disease may be associated with the presence of HLA-B51. A number of affected individuals within the same family ("co-segregation"), have been found to share the same non-HLA B51 phenotype in non-ocular Behcet's (Sant et al. 1998). Also in families segregating for Behcet's disease, the sibling recurrence rate, a measure of the increased risk of disease for the sibling of a patient, has been found to be 4.2% in Turkey (with a "lambda S value" of between 11.4 and 52.5) (Gul et al. 2000). Again in Behcet's disease, a phenomenon known as genetic

anticipation has been observed in families (Fresko et al. 1998). This results in earlier onset or increased severity of disease between successive generations. There is also some evidence for relatives of a patient with Behcet's disease being at increased risk of developing another autoimmune disease (Chamberlain 1978).

1.2.5.1.3 Cytogenetic studies

There is no strong evidence for karyotype modifications being a predisposing factor for uveitis (Pennesi and Caspi 2002).

1.2.5.1.4 Association studies

Association studies, in which allele frequencies between affected and unaffected individuals are compared, are the primary method of studying the role of a potential disease-susceptibility gene. Most autoimmune PSII susceptibility genes are unsurprisingly located within the MHC complex on chromosome 6p. Interestingly, there are associations with both class I and II MHC in autoimmune PSII.

The strongest HLA association for any known disease is between birdshot chorioretinopathy and HLA-A29, especially HLA-A29.2 (Tabary et al. 1990). The relative risk in some populations can reach 157, and this HLA type therefore assumes diagnostic significance. One report associates HLA-A28 with intermediate uveitis accompanied by arthralgia (Martin et al. 1995). Behcet's disease is mainly associated with HLA-B51 (Chajek-Shaul et al. 1987; Mizuki et al. 1992). It carries a relative risk of between 6.3 to 11.5, being higher in Mediterranean populations and lower in Northern Europeans.

MHC class II alleles associated with autoimmune PSII include HLA-DRB1*0405 (Shindo et al. 1994; Kim et al. 2000). This codes for a variant of the HLA-DR4 antigen and is significantly increased in VKH patients, with a relative risk of 46.7. Sympathetic ophthalmia is also associated with HLA-DRB1*04, as well as with DQA1*03 (Shindo et al. 1997). However, European patients tend to be associated more with the allelic variant DRB1*0404, and Japanese patients with DRB1*0405 (Kilmartin et al. 2001). DR2 is associated both with pars planitis and multiple sclerosis (MS), and may provide a link between these conditions (Malinowski et al. 1993). Intermediate uveitis without MS is

associated with HLA-DR3, while pan-uveitis has been associated with HLA-DR4 in an Italian population (Cuccia Belvedere et al. 1986). Sarcoidosis is associated with HLA-DRB1.

Non-MHC genes seem to have a major role in the expression of autoimmune PSII (Caspi et al. 1992). A polymorphism within the ICAM-1 gene may contribute to a susceptibility to Behcet's disease (Verity et al. 2000). An additional risk of Behcet's can be conferred by mutations of the MEFV gene on chromosome 16p (Touitou et al. 2000).

1.2.5.2 Environmental factors

Although endogenous PSII is regarded primarily as an autoimmune disease under significant genetic influence, there are probably multiple initiating stimuli for disease onset. What exactly these triggers are, however, remains obscure in the majority of cases (Whitcup 1997). Those that are known are outlined below.

1.2.5.2.1 Infection

In common with many other autoimmune diseases, infection is suspected of being a trigger in certain cases of autoimmune PSII. This is generally thought to be mediated by molecular mimicry (see later), though release of sequestered autoantigens through tissue destruction is also possible. Homology has been noted between a yeast histone and RSAg (Singh et al. 1989b). Reiter's syndrome may occur after Gram-negative dysentery, or urethritis caused by *Chlamydia trachomatis* or *Ureaplasma urealyticum* (Keat 1983). Although Reiter's syndrome relates to anterior rather than posterior uveitis, this also suggests that intraocular inflammation can be triggered following infection.

1.2.5.2.2 Trauma

Sympathetic ophthalmia is a case where autoimmune PSII appears to be triggered by trauma. The autoimmune response is thought to be caused by the release of previously sequestered autoantigens. Infectious agents or toxic substances introduced at the time of injury may also have some role (Whitcup 1997).

1.2.5.2.3 Diet

There is little evidence of diet having a role in human autoimmune PSII. However, in one report experimental uveitis seemed to be suppressed by dietary calorie restriction in rats (Abe et al. 2001).

1.3 Autoimmunity

Autoimmunity occurs when a specific adaptive immune response is mounted against self-antigens. In this section general features of autoimmune disease are discussed, such as disease susceptibility, tolerance to self-antigens and its breakdown. The emphasis is on mechanisms that are of most relevance to autoimmune PSII and other cell-mediated, organ-specific autoimmune diseases.

1.3.1 Innate and adaptive immune responses

The immune system has evolved primarily with the function of protecting us from microorganisms, which are an ever-present feature of our environment. Traditionally, its components have been described separately in terms of innate and adaptive immune systems, although there are many areas of interaction between the two. Innate immunity refers to immune defence mechanisms that are pre-existing, and do not rely on the recognition of previously encountered agents to mount a response. Typical elements of the innate system are epithelial barriers, the complement system, many phagocytic white cell types, natural killer cells and many other cells involved in inflammatory and allergic type effector responses. The key features of innate immune responses are that they are non-specific but rapid – this defends the host during the first 7 days of infection, while the adaptive immune response is developing.

Adaptive responses on the other hand are mediated by lymphocytes, and are specific for particular immunogenic molecules (antigens) that are recognised as “foreign” (Abbas and Lichtman 2003), or alternatively antigens that represent “danger” to the host (Matzinger 1994). Specifically, lymphocytes recognise and bind to one or more well-defined sites called epitopes on the antigen. These responses take longer to develop to novel antigens, but display “immunological memory” where an enhanced response is rapidly produced to previously encountered antigens. The adaptive system is normally able to differentiate “self” from “non-self” antigens. Adaptive immunity is divided into cell-mediated immunity, whose principal functions are orchestrated by T-lymphocytes (T-cells) and humoral immunity, whose main functions are carried

out by antibodies, the immunoglobulin (Ig) molecules secreted by B-lymphocytes (B-cells).

1.3.2 Humoral and cellular adaptive immunity

The function of the humoral immune system is to eliminate pathogens from the extracellular compartment. B-cells recognise epitopes on intact antigen molecules through the immunoglobulin receptors on their surfaces. Subsequent B-cell activation results in the production of antigen-specific antibody. Pathogens that are bound by antibody are eliminated, usually with the help of mononuclear phagocytes, complement and other effector systems. Complexes of soluble antigen and antibody (immune complexes) may be deposited in organs and blood vessels and precipitate inflammation. Although B-cells can generate antibody independently, this function is greatly enhanced and prolonged with the help of certain types of T-cell (below).

The cellular immune system has probably developed to eliminate intracellular microorganisms, but also carries out additional roles. T-cells detect peptide antigens that are processed and presented on the surfaces of the body's own cells. T-cells carrying the surface marker CD8 recognise antigens that are synthesised in the target cell's cytoplasm and presented in association with MHC class I molecules. CD8⁺ T-cells differentiate into cytotoxic T-cells (Tc), and target virus-infected and tumour cells, as well as being involved in transplant rejection. T-cells bearing the marker CD4 generally recognise antigen presented by specialised antigen presenting cells (APC) in the context of MHC class II molecules. The CD4⁺ T-cells, also known as T-helper (Th) cells, occupy a central role in co-ordinating the whole adaptive immune response. Through a variety of mechanisms, including the release of cytokines and cell-cell activation, CD4⁺ cells can stimulate activation of macrophages, CD8⁺ cells and B-cells. Subcategories of T-helper cells often seem to be polarised towards the secretion of different patterns of cytokines, and this can influence whether a predominantly cell-mediated or antibody response ensues. These so-called Th1 and Th2 type responses have relevance in autoimmunity, and are discussed later.

1.3.3 Autoimmune responses vs. autoimmune disease

The adaptive immune response is essential for the effective recognition of pathogens and their removal. However, antigens from the body's own tissues are sometimes mistakenly recognised as “foreign” or “danger”, and an inappropriate immune response occurs. This autoimmune response can be mediated by either the cellular or humoral systems.

Most potentially autoreactive T- and B-cells are deleted during development (discussed below), but some low-affinity autoreactive cells persist, even in healthy subjects. These autoreactive lymphocytes are kept in check by mechanisms of peripheral tolerance. Also, transient immune responses may be noted secondary to tissue damage, but rarely go on to cause sustained autoimmune disease. It is therefore obvious that some degree of autoimmune response can exist without causing overt disease. The conversion of autoimmune response to overt disease may depend on the recruitment of a certain number of effector cells and the level of tissue destruction, which can lead to a self-sustaining reaction. For a disease to be categorised as definitively autoimmune, autoreactive T-cells or autoantibodies must be shown to cause the tissue damage, rather than be the result of it (Rose and Bona 1993).

1.3.4 Antibody mediated vs. cellular autoimmunity

Autoimmune diseases are often categorised as being either cell- or antibody-mediated, depending on their primary mode of tissue damage. Both mechanisms, however, often work together in the same disease. Autoimmune diseases can also be classified as types, in a similar manner to hypersensitivity reactions, with the notable absence of Type I allergic responses which are not known to cause autoimmune disease. T-cells possibly fulfil a more central role than B-cells in autoimmune disease, even where the tissue damage is primarily antibody-mediated. CD8⁺ T-cells can cause extensive tissue damage via direct cytotoxic responses, whereas CD4⁺ T-cells can mediate Th1 driven macrophage activation, while also providing T-cell help to autoreactive B-cells. Cell mediated autoimmune diseases, corresponding to Type IV delayed type hypersensitivity (DTH) responses, are listed in Table 1.2. In some conditions

such as rheumatoid arthritis, more than one disease mechanism appears to operate.

Table 1.2 Examples of T-cell mediated (Type IV) autoimmune diseases.

Disease	Autoantigen	Pathological Effect
Type I diabetes (insulin dependent)	Pancreatic β -cell antigens, insulin, GAD	Destruction of pancreatic islet β -cells
Rheumatoid arthritis	Unknown synovial joint antigen	Inflammatory joint destruction
Multiple sclerosis/ Experimental autoimmune encephalomyelitis (EAE)	Myelin basic protein, myelin oligodendrocyte glycoprotein, proteolipid protein	Infiltration of CNS with $CD4^+$ T-cells, paralysis
Autoimmune uveitis	Unknown	Ocular inflammation, visual impairment

Antibody mediated diseases can resemble Type II hypersensitivity responses, with the damage resulting from direct antibody binding to target antigens, or Type III responses, with immune complex deposition provoking the inflammation. Many of these conditions require $CD4^+$ T-cell help to the autoreactive B-cells, to provide sustained autoantibody responses. Conversely, B-cells may be important for antigen presentation in sustaining specific T-cell responses. Antibody-mediated autoimmune diseases are listed in Table 1.3.

Table 1.3 Examples of direct antibody-mediated (Type II) and immune-complex mediated (Type III) autoimmune diseases.

Disease	Response Type	Autoantigen	Pathological Effect
Goodpasture's Syndrome	Type II	Fragment of basement membrane collagen type IV	Glomerulonephritis Pulmonary bleeding
Acute rheumatic fever	Type II	Cardiac muscle crossreacting with streptococcal cell wall antigens	Myocarditis and valvular damage Arthritis
Pemphigus vulgaris	Type II	Cadherin	Blistering of skin
Autoimmune haemolytic anaemia	Type II	Rhesus blood group antigens	Destruction of red blood cells and anaemia
Idiopathic thrombocytopenic purpura	Type II	Platelet integrin	Destruction of platelets and bleeding
Systemic lupus erythematosus (SLE)	Type III	DNA, histones, ribosomes, snRNP, scRNP	Glomerulonephritis Vasculitis Rash
Rheumatoid arthritis	Type III	Rheumatoid factor IgG complexes	Destructive arthritis
Mixed essential cryoglobulinaemia	Type III	Rheumatoid factor IgG complexes +/- others	Systemic vasculitis

Similarly, some antibody-mediated autoimmune diseases exert their effects by blocking or stimulating particular cell-surface receptors (Table 1.4).

Table 1.4 Diseases caused by effects of autoantibodies on cell surface receptors.

Disease	Target	Pathological Effect
Grave`s disease	Thyroid stimulating hormone receptor	Hyperthyroidism
Myasthenia gravis	Acetylcholine receptor	Progressive muscle weakness
Insulin-resistant diabetes	Insulin receptor (effect depends on specificity of antibody)	Hyperglycaemia, ketoacidosis (agonist antibody) Hypoglycaemia (antagonist antibody)

1.3.5 Organ-specific vs. systemic autoimmune diseases

Autoimmune diseases can also be categorised by whether they are confined to particular organs (organ-specific autoimmunity), or involve many tissues of the body (systemic autoimmunity). The distribution of autoimmune disease reflects whether the autoantigens are expressed in specific organs or are ubiquitous in all the body`s cells. For example, in Hashimoto`s thyroiditis, autoantibodies are specifically directed against thyroid stimulating hormone receptors which are confined to the thyroid gland. By contrast, antibodies are found against ubiquitous cellular components such as chromatin and mRNA splicing proteins in SLE, a systemic autoimmune disease. Examples of organ specific and systemic autoimmune diseases are contained in Tables 1.5 and 1.6 respectively.

Table 1.5 Organ-specific autoimmune diseases

Autoimmune uveitis	Graves' disease	Myasthenia gravis
Type I diabetes	Hashimoto's thyroiditis	Autoimmune pernicious anaemia
Multiple sclerosis	Vitiligo	
Goodpasture's syndrome	Autoimmune Addison's disease	

Table 1.6 Systemic autoimmune diseases

Rheumatoid arthritis	Sarcoidosis	Primary Sjogren's syndrome
SLE	Scleroderma	
Behcet's disease	Polymyositis	

It is thought that organ-specific and systemic autoimmune diseases may have different aetiologies. This is supported by the finding that more than one organ-specific autoimmune disease is sometimes found within the same individual, or within a family. This phenomenon also applies to clusters of systemic autoimmune diseases.

1.3.6 Susceptibility to autoimmune disease

Twin and family studies in humans have shown that both inherited and environmental factors are important in susceptibility to autoimmune disease. Of the inherited factors, association with certain MHC genes seems to be the most important.

1.3.6.1 Genetic factors

1.3.6.1.1 The MHC and its association with autoimmune diseases

The MHC is a cluster of genes located on Chromosome 6. It codes for a number of important molecules in antigen processing, presentation and recognition. Foremost among these are the MHC class I and II molecules, glycoproteins that are expressed on cell surfaces and which present antigenic peptides to compatible T-cell receptors (TCR) of T-cells. The MHC locus is polygenic, and the genes within it the most polymorphic in the body. This

provides a very wide range of MHC molecules with which T-cells can interact. An individual T-cell will only recognise antigenic peptide if it is presented on a particular MHC allelic variant and not others – this is known as MHC restriction of T-cells.

MHC class I molecules are expressed on all nucleated cells in the body. The molecule comprises of an α -chain, together with a smaller subunit, B_2 microglobulin. MHC class I molecules bind proteins fragments that have been degraded in the cytosol, and the MHC:peptide is presented to the TCR at the cell surface. MHC class I molecules present antigen to $CD8^+$ T-cells, which upon becoming activated can kill the presenting cell. In humans, the 3 main class I genes are called HLA-A, -B and -C.

MHC class II molecules are expressed on immune cells capable of presenting antigen to T-cells, mainly dendritic cells, macrophages and B-cells. The molecule is a dimer composed of an α and β chain. It binds to degraded antigenic peptides derived from endocytosed protein, which are contained in endosomes. The MHC:peptide complex is displayed on the cell surface and presented to $CD4^+$ T-cells. The 3 main class II genes in humans are HLA- DR, -DP and -DQ.

The high levels of polymorphism in MHC molecules can mean that binding and presentation of antigen, including self-antigen, to T-cells can vary markedly between individuals. Therefore it is apparent that particular MHC haplotypes can have a crucial bearing on an individual's susceptibility to immune-mediated disease (Wicker 1997; McDevitt 2000). Indeed, statistical associations have been found between MHC alleles and particular diseases, including established autoimmune diseases. Alternatively the association between autoimmunity and HLA may derive from the role of MHC alleles in the shaping of the T-cell repertoire during T-cell development. Examples of autoimmune diseases and their associated MHC alleles are contained in Table 1.7.

Table 1.7 Autoimmune diseases and their HLA associations.

Disease	HLA allele	Relative risk
Ankylosing spondylitis	B27	87.4
Goodpasture`s syndrome	DR2	15.9
Multiple sclerosis	DR2	4.8
Grave`s disease	DR3	3.7
Myasthenia gravis	DR3	2.5
SLE	DR3	5.8
Type-1 diabetes	DR3/DR4	25
Rheumatoid arthritis	DR4	4.2
Pemphigus vulgaris	DR4	14.4
Hashimoto`s thyroiditis	DR5	3.2

1.3.6.1.2 Non-MHC genetic factors

Genetic factors other than those associated with MHC are known to determine susceptibility to autoimmune disease. This is demonstrated by identical twins (who share all genes) being much more likely to share the same autoimmune disease than siblings who merely share the same MHC haplotype. Several non-MHC disease susceptibility loci have been shown for autoimmune diabetes. There is also evidence that the levels of expression of a potential autoantigen within the thymus can influence the development of autoimmunity. Higher levels of transcription of the human insulin gene in the thymus, which shows genetic variation between individuals, tends to protect against diabetes.

There has been some interest in the possible association of autoimmune disease with the preferential expression of genes coding for particular TCR chain subtypes, in particular the V β 8.2 gene. There is evidence of suppression of EAE by immunisation with fragments from V β 8.2. This effect could not be reproduced in EAU, although preferential - but not exclusive - V β 8.2 expression has been found here also.

1.3.6.2 Environmental factors

Twin studies indicate that although genetic factors are the major factor in some autoimmune disorders, environmental influences also contribute, and indeed may dominate in some disorders.

Infection is thought to trigger autoimmunity in certain cases, the most clear-cut association probably being that of infection by group A streptococci with rheumatic fever. Other possible associations exist such as HLA-B27 associated reactive arthritis with infection by Chlamydia, Yersinia or Salmonella. Other associations are also being investigated. Non-specific infection is also believed to precipitate autoimmune disease in susceptible individuals. In Wegener's granulomatosis, a relapse of vasculitis is frequently observed in asymptomatic patients with high levels of anti-neutrophil cytoplasmic antibodies (ANCA) who develop an infection (Pinching et al. 1980).

Other possible environmental co-factors have been noted. The pulmonary haemorrhage component of Goodpasture's disease is almost exclusively found in patients who smoke (Donaghy and Rees 1983). Goodpasture's can also be precipitated by exposure to organic solvents. Several autoimmune diseases including SLE, myasthenia gravis and haemolytic anaemia can be induced by drugs. Sunshine is a trigger of skin lesions in SLE. Diet, however, may have a protective role in autoimmunity. There is currently some interest in a possible role for omega-3 fatty acids (found in fish oil) in the prevention of human autoimmune diseases.

1.3.7 Immunological tolerance

The immune system must recognise and remove foreign or "danger" antigens, while remaining unresponsive to antigens from the body's own tissues. This state of immunological non-responsiveness to self-antigens is called tolerance. Tolerance is antigen-specific and is acquired during development. The maintenance of tolerance requires the persistence of the antigen throughout life. Tolerance can be acquired in the primary lymphoid tissues during development (central tolerance), or later in the periphery during adult life (peripheral tolerance). A number of mechanisms exist to maintain self-tolerance, both for T-cells (Anderton and Wraith 2002; Shevach 2002) and B-cells (Goodnow et al. 1995).

1.3.7.1 T-cell tolerance

1.3.7.1.1 Central tolerance

Both T- and B-cell precursors arise from the bone marrow and undergo maturation and differentiation during foetal life. Whereas this process takes place in the bone marrow for B-cells, T-cells migrate to the thymus for "T-cell education". Here they acquire their surface markers in a step-wise manner, and eventually differentiate into mature CD4⁺ or CD8⁺ T-cells. The highly variable, unedited T-cell repertoire is put through two stages of selection, via interactions with MHC molecules and self-antigens encountered in the thymus. In the first stage, "positive selection", only those T-cells capable of recognising self-MHC antigens receive the necessary signals to survive. In the second stage, "negative selection", T-cells that interact with high or very low affinity to self-antigens presented on APC are destroyed. This mechanism is called clonal deletion, and is similar to activation induced cell death, a mechanism also found in peripheral tolerance. Central tolerance is probably more effective in deleting autoreactive T-cells to ubiquitous self-antigens, rather than those that are organ-specific. However, organ-specific antigens have been shown to take part in central tolerance mechanisms e.g. RSAg and interphotoreceptor retinoid binding protein (IRBP) from the eye and myelin basic protein (MBP) from brain. It has been noted that resistance of some animal strains to EAU after immunisation with particular retinal autoantigens, correlated with expression of the same autoantigens in the thymus (Egwuagu et al. 1997). After the completion of central tolerance, a vastly reduced repertoire of T-cell clones emerges, that is restricted to self-MHC and purged of self-reactive T-cells.

1.3.7.1.2 Peripheral tolerance

Central tolerance is incomplete, especially for organ-specific self-antigens, and peripheral mechanisms are required to maintain non-responsiveness during adulthood (Walker and Abbas 2002). Some of these mechanisms are listed below.

1.3.7.1.2.1 Ignorance

This term has been used to describe two slightly different ideas. One relates to self-antigen that is sequestered behind anatomical barriers (e.g. blood ocular

barrier), and which maintains non-responsiveness by excluding autoreactive cells. Tolerance via this mechanism would, however, be incomplete, as migrating APC are capable of presenting such sequestered antigens in lymph nodes or the spleen. The second meaning relates to the maintenance of self-tolerance, despite interaction between potentially autoreactive lymphocytes and autoantigen. This may happen because antigen binds to the T-cell either with low affinity or in very low levels (Janeway et al. 2001).

1.3.7.1.2.2 *Anergy*

Anergy is induced when self-peptide:MHC is recognised by a T-cell in the absence of co-stimulatory signals, or with inhibitory signals as in B7/CTLA-4 rather than B7/CD28 interactions. The T-cell is not deleted, but persists in a state of non-responsiveness. Anergic cells may eventually be removed by apoptosis (programmed cell death). Anergy is thought to be the main mechanism of peripheral tolerance. It is closely related to clonal deletion and the same mechanisms may be involved. It may be involved in central tolerance, where lymphocytes that fail to receive the appropriate rescue signals at each stage of development eventually undergo apoptosis.

1.3.7.1.2.3 *Apoptosis*

Peripheral T-cells may undergo apoptosis in the periphery, in a manner similar to deletion during central tolerance induction. This occurs particularly where the cells are exposed to persistent or high levels of self antigen.

1.3.7.1.2.4 *Suppression*

There is strong evidence for the existence of T-cell subsets that actively suppress autoreactivity in the periphery. These cells are now referred to as T regulatory (Tr) cells (Roncarolo et al. 2001) and exert their actions by cell-to-cell contact or the expression of the immunosuppressive cytokines interleukin 10 (IL-10) or transforming growth factor β (TGF- β). Although CD8⁺ T-cells were previously thought to be the "suppressor" population, this is now considered less likely. One probable regulatory population is the CD4⁺ CD25⁺ subset, but the mechanism of action is still unclear.

Other CD4⁺ subsets are probably involved in cellular and humoral tolerance. These include the so-called Th3 population, which secrete TGF-β. Other inhibitory populations may include Th2 cells, particularly through their secretion of IL-10.

1.3.7.1.2.5 *Idiotypic networks*

Idiotypic and anti-idiotypic networks, as originally proposed by Jerne (Jerne 1984), may contribute to B-cell and T-cell peripheral tolerance. Antibodies or TCR (both being proteins) can themselves induce an "idiotypic" antibody or T-cell response. These responses can further induce tertiary responses, the eventual result being a self-regulatory network that dissipates the original immune disturbance. However, despite the plausibility of this theory, there is little hard evidence for it in practice.

1.3.7.2 **B-cell tolerance**

Tolerogenic mechanisms that apply to CD4⁺ T-cells, through the abolition of T-cell help, will also have a major inhibitory effect on the production of autoantibodies. However, specific B-cell tolerance mechanisms are also important, particularly for non-protein self-antigens such as polysaccharides and lipids (T-independent antigens).

B-cells develop central tolerance in the bone marrow. Cells that recognise self-antigen with high affinity and in high concentration will undergo apoptosis in a similar manner to T-cells. Multivalent antigens are most likely to cause deletion by cross-linking several immunoglobulin receptors. Alternatively, the B-cell may respond to self-antigen by changing the specificity of its immunoglobulins, a process known as "receptor editing".

In the periphery, B-cells that recognise self-antigen in the absence of specific CD4⁺ T-cells are rendered functionally unresponsive. These anergic cells are more likely to undergo apoptosis by the Fas/Fas ligand (FasL) pathway if encountered by a specific Th cell. Similarly, B-cells that encounter self-antigen in the periphery lose the ability to enter lymphoid follicles, and therefore cannot be activated to produce antibody against the antigen. The exact mechanisms of B-cell tolerance are not yet fully known, and the phenomenon is not a complete

one, as shown by the frequent occurrence of autoantibodies, even in healthy subjects.

1.3.8 Initiation of autoimmune disease

Although some degree of autoimmunity is often detected in healthy individuals, it is usually kept in check by the tolerogenic mechanisms described above, and does not cause autoimmune disease i.e. tissue damage or dysfunction. Therefore autoimmune disease can be regarded as a breakdown of self-tolerance. The exact cause of this is still unclear, but some of the more likely mechanisms are outlined below.

1.3.8.1 Molecular mimicry

As mentioned above, infections are a major susceptibility factor for the onset of autoimmune disease (Wucherpfennig 2001). One possible mechanism is molecular mimicry, where there is immunological cross-reactivity between antigens present on foreign pathogens and self-tissues. This is plausible, given the wide range of antigenic peptides in infectious microbes and there are several examples of sequence homology between microbial and self-antigens in autoimmune disease. These include homology between myocardial antigens and short sequences in streptococci, *Chlamydia* and *Trypanosoma cruzi* (see Environmental factors). However, molecular mimicry has not yet been proven to actually cause a spontaneous autoimmune disease.

1.3.8.2 Bystander activation

Another side-effect of infection is the upregulation of co-stimulatory molecules on APC. This could potentially "rescue" anergic, autoreactive lymphocytes in the lymphoid tissues, and thereby cause autoimmune disease.

1.3.8.3 Trauma/tissue destruction

The traumatic release of previously sequestered autoantigen may be the trigger for autoimmune disease. This is probably the case in sympathetic ophthalmia following eye trauma, and autoimmune orchitis following vasectomy. Tissue destruction or inflammation may modify previously tolerated antigens, or reveal

previously cryptic epitopes, and provide new targets for potentially autoreactive lymphocytes.

1.3.8.4 Superantigen stimulation

Superantigens are proteins derived from staphylococcal enterotoxins that are capable of activating all T-cells expressing a particular set of V β TCR genes, in a non-antigen-specific manner. The antigen achieves this by directly binding to non-polymorphic regions on class II MHC and conserved regions on the TCR. This may cause simultaneous activation of several subsets of T-cells, potentially leading to autoimmune disease.

1.3.8.5 Polyclonal B-cell activation

Certain products such as bacterial endotoxin or glycolipids can activate B-cells directly. These can act as APC and activate autoreactive T-cells, or can cause disease by expressing autoantibody.

1.3.8.6 Idiotypic network disturbance

In response to a foreign antigen, an idiotypic antibody or T-cell response may develop. This may reveal an idiootype with homology to an autoreactive lymphocyte and lead to activation.

1.3.8.7 Failure of deletion

Deletion of autoreactive lymphocytes by Fas/FasL mediated apoptosis is important for the induction of central and peripheral tolerance. Failure of this mechanism can cause autoimmune disease.

1.3.8.8 Failure of regulatory T-cell activity

Regulatory T-cells are thought to play an essential role in the maintenance of peripheral tolerance. Failure of this mechanism could have obvious consequences for the induction of autoimmune disease.

1.3.9 Cytokines and the Th1/Th2 paradigm

Cytokines are small proteins, involved in short-range intercellular signalling. They are essential in co-ordinating the various elements of the adaptive

immune response, and are secreted by many tissue cells and leucocytes, especially CD4⁺ (Th) T-cells. The pro- or anti-inflammatory actions of individual cytokines can determine the outcome of an immune response, and they are particularly important in autoimmune disease. Th subsets can be functionally categorised in terms of their predominant cytokine profiles, the so-called Th1/Th2 paradigm.

Polarised cytokine secretion patterns in Th cells were first demonstrated in mice (Mosmann et al. 1986), and later confirmed in humans (Romagnani 1991). In the traditional Th1/Th2 model, Th1 cells typically secrete the pro-inflammatory cytokine interferon- γ (IFN- γ), and may also secrete IL-2 and tumour necrosis factor (TNF) (Liew 2002). These cells promote cell-mediated (DTH type) immunity and defence against intracellular organisms. They also promote the secretion of IgG_{2a}. Th1 cells differentiate from Th precursor cells, under the influence of IL-12. Th2 cells on the other hand, typically secrete IL-4, and also IL-5, IL-6, IL-10 and IL-13. They promote allergic and anti-helminthic type responses through the production of IgE, IgG₁ and recruitment of eosinophils. Th2 cells develop from the same precursors as Th1, but under the influence of IL-4. The powerful anti-inflammatory cytokine TGF- β is sometimes included in the Th2 category, but others designate TGF- β -secreting cells as Th3.

The factors that cause polarisation towards a Th1 or Th2 phenotype, IL-12 and IL-4, are derived from cells in the microenvironment during development. Particularly influential are two subtypes of dendritic cells (DC), DC1 and DC2, which promote respective Th1 and Th2 lineages. Cells developing under neutral conditions are termed Th0, and may secrete cytokines from either subset. The archetypical cytokines from each subset, IFN- γ and IL-4, promote the expansion of their own subset, while inhibiting the other. The two subsets therefore regulate each other's activity.

Autoimmune diseases are traditionally thought of as being due to the unopposed/dysregulated actions of pro-inflammatory cytokines like IL-2, IFN- γ , TNF and IL-12, and are presumably driven by a Th1 type response. In recent years however, it has emerged that these same cytokines can also have immunoregulatory effects, under certain conditions (Falcone and Sarvetnick

1999; O'Shea et al. 2002). This is particularly so when large amounts of cytokine are secreted late in the inflammatory process. Conversely, Th2 type cytokines can sometimes be found to mediate pro-inflammatory effects. This emphasises the complicated nature of cytokine effects and their interactions, and may have relevance for the development of future cytokine/anti-cytokine therapies. While the paradigm of Th1 and Th2 subsets remains valid, their exact role in autoimmunity is still unclear, with many diseases attributable to neither subset.

1.3.10 Treatment of autoimmune disease and induction of tolerance

Treatments applicable to autoimmune diseases in general are described later, in the context of treatment of autoimmune PSII. The mainstay of current treatment is non-specific anti-inflammatory drugs, especially corticosteroids, and is aimed at reducing tissue injury and dampening down the effector mechanisms of the disease. More specific therapies are being introduced. These include antagonists to pro-inflammatory cytokines such as TNF- α , and may take the form of monoclonal antibodies or solubilised receptors. Other new treatments specifically target co-stimulatory molecules such as B7, and other T-cell molecules such as CTLA-4 or CD40 ligand. Treatments such as anti-adhesion molecule antibodies, which may prevent the migration of leucocytes into tissues, are also being tested (Kalden et al. 1998).

Few current treatments aim to prevent induction of disease or restore tolerance in an antigen-specific manner. Experimentally, it is known that the manner in which a protein autoantigen is administered can result in either autoimmunity or tolerance. Factors favouring an immune response are subcutaneous/intradermal administration and the presence of adjuvant, while persistent, high doses of protein given systemically in the absence of adjuvant favours tolerance. In this situation, tolerance may result from the absence of co-stimulation by APC because of lack of adjuvant, or induction of regulatory or Th2 type T-cell subpopulations.

One potentially attractive therapy for autoimmunity is the induction of autoantigen-specific "mucosal tolerance" (Weiner 1997). In experimental

studies, protein antigens administered orally (or nasally) have been found to produce suppression of humoral and cellular immune responses on subsequent immunisation. This mechanism may have evolved to prevent immune reactions to antigens in food. Mucosal tolerance may involve the induction of anergy in autoreactive T-cell clones, or the secretion of TGF- β (Miller et al. 1993), which is involved in IgA production in mucosal tissues and the inhibition of lymphocyte proliferation. The treatment requires the accurate identification of the autoantigen/peptide, and optimisation of the dose. It has been successfully demonstrated in experimental models of autoimmune disease (Maassen et al. 2003). Mucosal tolerance therapy has been tested in trials in human autoimmune diseases, including multiple sclerosis, rheumatoid arthritis and autoimmune PSII, but its clinical efficacy has not yet been clearly established.

Peptides are used as signalling molecules for an important subgroup of human cellular receptors. Modification of these signalling peptides is an approach for highly selective and specific modulation of receptor function. So-called altered peptide ligands (APLs) can be synthesised by amino acid substitutions at crucial receptor contact sites. APLs have a potential therapeutic role in infectious diseases or cancer immunotherapy (Slansky et al. 2000), by enhancing specific T-cell stimulation. They may also selectively suppress immune responses in autoimmune diseases such as MS (Steinman and Conlon 2001).

1.4 Autoimmune PSII in animal models

Much of what we know today about autoimmune PSII derives from animal models of the disease. These are particularly useful in uveitis research, where it is very difficult to obtain biopsy material from intraocular structures, especially during disease initiation. Also, the availability of inbred strains allows more sophisticated analysis of individual features of the disease in isolation, while research using animal uveitis models is unhindered by the lack of numbers encountered in some of the human counterparts. Animal models have also been useful in evaluating the mechanisms of immunosuppressant drugs in uveitis such as cyclosporin A, and in studying mucosal tolerance. The central animal model for autoimmune PSII for many years has been experimental autoimmune uveoretinitis, EAU. However, other models of PSII exist, some of which are induced with ocular antigens and others with non-ocular antigens, and these are also described below.

1.4.1 Experimental autoimmune uveoretinitis

In 1906 specific antigenicity to retinal rods was reported by Hess and Romer. In 1910 Elschnig suggested a link between sympathetic ophthalmia and specific ocular tissue antigens. For many years an autoantigen from the uveal tract was sought without success, till in 1965 a paradigm shift occurred when Wacker and Lipton demonstrated specific ocular autoimmunity in animals to homologous retinal tissue (Wacker and Lipton 1965). EAU (then called experimental *allergic* uveitis) was first induced by a purified antigen in 1977 (Wacker et al. 1977) when what is now known as RSAg, mixed in complete Freund's adjuvant (CFA), was injected into the footpads of guinea pigs, causing bilateral intraocular inflammation.

EAU manifests itself as a destructive inflammatory response of the choroid, photoreceptors and retina, and anterior segment. The features closely resemble those of PSII in humans. T-cells are essential in the induction of EAU, and the disease cannot be transferred with hyperimmune sera alone (Salinas-Carmona et al. 1982). Since then EAU has been induced with different antigens

in a variety of animals including rats, mice and primates. The autoantigens identified so far are highly conserved retinal proteins usually involved in phototransduction and as well as RSAg include IRBP, rhodopsin, phosducin and recoverin (Adamus and Chan 2002). The features of EAU vary somewhat depending on the species and strain of animal, type and dose of antigen, method of administration and use of adjuvant. However, in all cases CD4⁺ T-cells play the central role in disease initiation.

In RSAg-induced EAU, CD4⁺ T-cells invade the eye initially, but later on CD8⁺ T-cells predominate (Chan et al. 1985b). This pattern has also been noted in studies on human eyes with sympathetic ophthalmia (Chan et al. 1985a). Mast cells may play a role in the development of EAU. Disease susceptibility in rats has been shown to be associated with the number of mast cells in the choroid (Mochizuki et al. 1984). It has also been demonstrated that choroidal mast cells degranulate just before the entry of T-cells into the eye in EAU (de Kozak et al. 1981a).

EAU differs from human autoimmune PSII in that the disease is mostly self-limiting and requires adjuvant for induction. Nevertheless, it is valuable in studying general mechanisms of uveitis, identifying disease-specific antigens and epitopes and evaluating disease-modifying strategies. The features of EAU induced by the main retinal autoantigens will be described in turn.

1.4.1.1 Retinal S antigen

RSAg (also known as retinal soluble antigen or visual arrestin) is a major component of rod outer segments (Beneski et al. 1984; Gery et al. 1986a), where it is involved in the quenching of the phototransduction cascade (Wilden et al. 1986). It is one of 6 related and highly homologous arrestin proteins, but unlike the others is mainly confined to the rods and cones of the retina (Craft et al. 1994). It is also found in the pineal gland (Abe and Shinohara 1990). The human RSAg gene is located at chromosome 2q24-37 and contains 16 exons. Human RSAg is 48 kilodaltons (kDa) in weight. Amino acid (a.a.) sequences for various species have been deduced by complementary DNA (cDNA) sequencing. These include human (Yamaki et al. 1988) and bovine RSAg (Shinohara et al. 1987), which share 81% sequence homology. The a.a.

sequences for mouse (Tsuda et al. 1988) and rat RSAg (Abe and Shinohara 1990) have also been deduced – these share 97% homology.

RSAg can induce EAU in many strains of rodents (Wacker et al. 1977; de Kozak et al. 1981b; Gery et al. 1986a) and also in primates (Nussenblatt et al. 1981). The predominant features of RSAg-induced EAU in the guinea pig, which has an avascular retina, are choroiditis with some anterior uveitis. In the monkey, mouse and rat, retinal vasculitis is more prominent. EAU onset varies from 10 days to 2 months, and in duration from days to 12 months or more, depending on animal species, antigen dose and use of adjuvant (Gery et al. 1986a). In the Lewis rat the disease is acute and self-limited, but leads to considerable damage of the retina and surrounding tissues in a few days. In less susceptible strains of rat, guinea pigs and primates the disease is more subacute and may continue for a number of months. In mice, induction of EAU is very difficult with RSAg and requires the use of CFA, pre-treatment with cyclophosphamide, and pertussis toxin to increase vascular permeability (Caspi et al. 1988).

1.4.1.1.1 Cellular immunity to RSAg in EAU

Based on experiments on the Lewis rat, several fragments of RSAg in adjuvant are capable of eliciting EAU (pathogenic sites), or in-vitro T-cell proliferation (proliferative sites). In human and bovine RSAg, three sites were originally identified as being immunopathogenic – peptide M (human a.a. sequence 303-320) (Donoso et al. 1987) and peptide N (bovine 281-302) (Singh et al. 1988) which are non-dominant, and peptide G (human 343-362) (Gregerson et al. 1990) which is immunodominant. The sites may be spatially dissociated (Gregerson et al. 1989; Gregerson et al. 1990), but are often close to each other (Merryman et al. 1991). Using overlapping synthetic peptides, the most pathogenic site seems to be within the a.a. sequence 340-360 of human RSAg (huRSAg) (de Smet et al. 1993). The minimal uveitogenic epitope possibly lies between 352-364 (Gregerson et al. 1990). Other pathogenic sequences are listed in Table 1.8.

Table 1.8 Uveopathogenic sequences of retinal S antigen (human RSAg except where stated).

Sequence Number	Amino Acid Sequence	References
181-200	VQHAPLEMGPQPRAEATWQF	1
301-320	GKIKHEDTNLASSTIIKEGI	1
341-360	GFLGELTSSEVATEVPFRLM	1
351-370	VATEVPFRLMHPQPEDPAKE	1
273-289 (bovine)	SLTKTLTLVPLLANNRE	2
286-305	PLLANNRERRGIALDGKIKH	3
306-325	EDTNLASSTIIKEGIDRTVL	3
339-352 (bovine)	LGELTSSEVATEVP	4
352-364 (bovine)	PFRLMHPQPEDPD	5

References: (1) de Smet 1993 (2) (Fling et al. 1991) (3) (Donoso et al. 1988) (4) Merryman 1991 (5) Gregerson 1990.

huRSAg epitopes that are immunopathogenic for the Lewis rat can induce EAU in other rat strains including Wistar Furth rats. Fischer (F344) rats respond poorly to the same peptides, but immunopathogenicity can be restored with the addition of pertussis toxin. EAU susceptibility does not seem to be solely related to strain MHC class II type. Rat experiments have also demonstrated that activated B-cells are required for T-cell activation in response to autologous rat RSAg peptide 270-289. The B-cells appear to provide an essential stimulatory co-ligand (Prasad and Gregerson 1997). Similar effects are observed in mice, where blocking B7-1 can reduce IRBP-induced disease expression (Fukai et al. 1999).

A classic mechanism for the induction of autoimmune diseases is molecular mimicry. The pathogenic peptide 303-320 (peptide M) of bovine RSAg (bovRSAg) is known to share sequence homology with several bacterial, viral and fungal peptides. Synthetic peptides from several of these non-self proteins have been shown to elicit proliferative responses in cells from peptide M-immunised rats, and can even induce EAU (Shinohara et al. 1990). A synthetic peptide (DTNLA) derived from yeast histone H3, containing 5 residues identical to peptide M of RSAg, can induce EAU in Lewis rats (Singh et al. 1989a). Native *S. Cerevisiae* histone is also capable of inducing the disease.

Viral peptides of 3 and 4 a.a. length, with homology to RSAg, have also been found to be pathogenic (Singh et al. 1990). Of particular interest is the partial sequence homology between RSAg 342-355 and HLA-B27 125-138 ("B27PD"). Immunisation of Lewis rats with B27PD causes EAU, while it can suppress RSAg-induced disease when administered orally (Wildner and Thureau 1994). Molecular mimicry may therefore provide an explanation for the apparently paradoxical statistical association between MHC class I antigens and organ-specific autoimmune disease, including autoimmune PSII.

1.4.1.1.2 Humoral immunity to RSAg in EAU

T-cell mediated immunity is the main feature of EAU but humoral responses are also present. Serum antibodies to RSAg are detectable by enzyme-linked immunosorbent assay (ELISA) within two weeks of a single immunization, which induces high titres of circulating antibodies directed towards different epitopes on the RSAg molecule (de Kozak et al. 1992). Immunoglobulin deposited on retinal outer segments can also be demonstrated. However, antibody production is unnecessary for the induction of EAU as demonstrated by studies in which the disease can be induced solely by adoptive transfer of RSAg-specific, autoreactive T-cells (Mochizuki et al. 1985; Caspi et al. 1986).

Anti-RSAg antibodies may, however, have a role in disease downregulation. Monoclonal antibodies (mAbs) against RSAg epitopes can abrogate the onset of EAU when injected simultaneously with the antigen (de Kozak et al. 1985; Dua et al. 1989a). Also, pre-immunisation with the anti-RSAg monoclonal antibody S2D2 leads to suppression of EAU (de Kozak et al. 1987). This antibody binds to an epitope (S2) away from known immunogenic sites in the rat or human antigen, and which also shares sequence homology with an epitope of TNF- α . After co-immunisation with RSAg and S2D2, rats that are disease free are found to have antibodies not only to S2, but also to sites of homology on RSAg and to TNF- α (de Kozak et al. 1992). This is taken as evidence of idiotypic and anti-idiotypic antibody immune regulation (de Kozak 1997).

1.4.1.2 Interphotoreceptor retinoid binding protein (IRBP)

IRBP is a 140 kDa highly conserved glycoprotein, which is produced by the photoreceptors (Fong et al. 1984; Borst et al. 1989). It is the major component of the interphotoreceptor matrix (Chader 1989) and serves to transport retinoids between the neural retina and the retinal pigment epithelium (RPE). It is also found in the pineal gland. Bovine (Borst et al. 1989) and human (Fong and Bridges 1988; Fong et al. 1990) IRBP genes have been sequenced - they encode proteins of 1,264 and 1,262 a.a. respectively, and share 84% sequence homology. The molecule consists of 4 repeating subunits of approximately equal size and with 30-40% sequence homology between any 2 subunits.

IRBP is highly uveitogenic in Lewis rats (Gery et al. 1986b), rabbits (Eisenfeld et al. 1987), monkeys (Hirose et al. 1986) and mice (Caspi et al. 1988), but evokes a poor response in guinea pigs (Vistica et al. 1987). As in RSAg-induced EAU there are different patterns of response between species. In the Lewis rat, EAU is acute and severe, with the inflammation starting in the anterior segment and spreading to the retina and choroid. The EAU starts around 8 days post-immunisation and subsides within another 7 days (Gery et al. 1986b). In the monkey, however, the onset is delayed for 3-4 weeks and the process continues for over 5 months. Here the main focus of inflammation is the choroid, with lesser amounts in the retina and virtually none in the anterior chamber (Hirose et al. 1986). The cellular infiltration is characterised mainly by granulomas in the monkey but by polymorphs in the rat. Autoantibody responses to IRBP have also been noted in EAU induced by the protein or its fragments (Waldrep and Donoso 1990).

The features of EAU in the mouse can vary depending on the mouse strain, the dose of IRBP and the use of pertussis toxin. Induction of EAU in some strains of mice but not others requires the use of pertussis toxin (Silver et al. 1999). A high dose of both IRBP and pertussis toxin will induce an acute inflammation of early onset and short duration, whereas a lower dose of either may lead to a later onset and extended course. High doses typically induce diffuse retinal damage, while low doses cause more focal damage and choroidal thickening. The chronic, relapsing nature of IRBP-induced EAU in the mouse, may more closely resemble the course of autoimmune PSII in humans

than more acute models and provide better opportunities of testing therapies (Adamus and Chan 2002).

1.4.1.2.1 T-cell epitope recognition in IRBP

Experimental studies have demonstrated several uveopathogenic epitopes on IRBP. Using 120 synthetic overlapping peptides encompassing the entire sequence of human IRBP, 9 uveitogenic peptides were identified (Donoso et al. 1989). The most pathogenic peptide was "HIRBP 715", between amino-acid positions 521-540 (Table 1.9).

In a different approach, synthetic peptides of bovine IRBP, predicted to bind simultaneously to both TCR and MHC, were tested for ability to induce EAU in Lewis rats. These experiments revealed 3 uveitogenic sequences: 1091-1115 (Kotake et al. 1991b), 1158-1180 (Sanui et al. 1988) and 1169-1191 (Sanui et al. 1989). The latter two were also pathogenic in monkeys (Sanui et al. 1990). Of particular interest is peptide 1169-1191, which has been shown to be immunodominant for IRBP in the Lewis rat (Sanui et al. 1989). This epitope was further localised to sequence 1179-1191 (Kotake et al. 1990), the minimum active peptide being found at 1182-1190 (WEGVGVVPD) (Kotake et al. 1991a). The importance of epitope binding to MHC was demonstrated when EAU was induced by a markedly lower dose of uveitogenic peptide 273-283, after amino acid substitutions to optimise the peptide-MHC binding (Kozhich et al. 1997). An additional human IRBP peptide (161-180) has been reported to be immunopathogenic in B10.RIII mice (Silver et al. 1995).

Table 1.9 Uveopathogenic sequences for IRBP.

Sequence No.	Name	Species	Amino acid sequence	Ref.
521-540	HIRBP 715	Human	YLLTSHRTATAAEEFAFLMQ	1
531-550	HIRBP 778	Human	AAEEFAFLMQSLGWATLVGE	1
821-840	HIRBP 730	Human	KDLYILMSHTSGSAAEFAH	1
1121-1140	HIRBP 745	Human	SKKSMVILTSTVTAGTAEF	1
1131-1150	HIRBP 808	Human	TVTAGTAEETYIMKRLGRA	1
621-640	HIRBP 720	Human	ALVEGTGHLLRAHYARPEVV	1
661-680	HIRBP 722	Human	DLESLASQLTADLQEVSGDH	1
701-720	HIRBP 724	Human	PAVPSPEELTYLIEALFKTE	1
1051-1070	HIRBP 804	Human	EHIWKKIMHTDAMIIDMRFN	1
271-283		Bovine	SQTWEGSGVLPCV	2
880-892		Bovine	GEAWDLAGVEPDI	2
1179-91		Bovine	GSSWEGVGVVPDV	2
518-529	TA12	Bovine	ALDRAQEVLEFH	3
1158-1180	R4	Bovine	HVDDTDLYLTIPTARSVGAADGS	4
1169-1191	R14	Bovine	PTARSVGAADGSSWEGVGVVPDV	5
1177-1191	R16	Bovine	ADGSSWEGVGVVPDV	5
1091-1115	R23	Bovine	PNDVSELWTLSQLEGERYGSKKSM	6

References: (1) Donoso 1989 (2) Kotake 1990 (3) (Tanaka et al. 1993) (4) Sanui 1988 (5) Sanui 1989 (6) Kotake 1991b

1.4.1.2.2 Factors influencing susceptibility to IRBP-induced EAU

As with RSAg-induced disease, susceptibility to IRBP EAU varies with the strain of animal. Lewis rats develop EAU at very low doses of IRBP, whereas Fischer (F344) rats are resistant, despite both species sharing the same class II MHC. Development of EAU appears to be related to the strong expression of a Th type1 (pro-inflammatory) cytokine profile in the Lewis rat (Caspi et al. 1997b). Interestingly, Fischer rats can develop severe EAU after adoptive transfer of T-cells treated with pertussis toxin, which has been found to cause a switch to a strong Th1 cytokine profile in primed lymph node cells. Reversal of the EAU phenotype in Lewis rats can be achieved by antigen immunization in incomplete Freund's adjuvant, which induces a Th2 type cytokine profile. Further evidence of a Th1 cytokine profile being associated with susceptibility to EAU has been found in mice (Caspi et al. 1997b; Sun et al. 1997), though resistant strains are not necessarily associated with a Th2 profile. Other factors such as MHC receptor affinity or the presence of appropriate co-factors are also known to influence the direction of T-cell differentiation. Other factors unrelated to Th1 vs. Th2 differentiation also have a role in the induction of EAU with IRBP (Silver et al. 1999). Possible influences include hormonal levels in the adrenal-pituitary axis, neural immunoregulation and the effect of regulatory cell populations.

1.4.1.3 Rhodopsin

Rhodopsin is the visual pigment in rod photoreceptors and in response to the detection of photons, triggers the phototransduction cascade. It is a membrane protein of two parts; a polypeptide chain called opsin and a covalently bound chromophore, 11-cis-retinal. It is part of the G protein-coupled receptor family.

Rhodopsin is capable of eliciting EAU in guinea pigs, rabbits, rats and monkeys. In mice, it appears to be immunogenic but not pathogenic in most strains (Adamus et al. 1991). In rats, high dose rhodopsin immunisation with CFA and pertussis toxin causes acute inflammation of the anterior segment at day 10-12, followed by chorioretinitis (predominantly retinitis) which results in complete elimination of the photoreceptor cells (Schalken et al. 1988).

There is controversy concerning the uveitogenicity of rhodopsin versus opsin. Broekhuysse reported that the uveitogenicity of opsin was much lower

than that of RSAg or IRBP (Broekhuysse et al. 1986). Rhodopsin (with CFA and toxin) seemed to cause earlier onset, more severe and more frequent EAU than opsin under similar conditions (Schalken et al. 1988; Schalken et al. 1989). The difference was thought to be due to conformational changes in the protein as a result of dark/light adaptation. However, later studies did not confirm these differences (Adamus and Chan 2002). Using synthetic peptides, three distinct uveitogenic epitopes on the polypeptide chain have been identified. These include sequence 230-250 on extracellular loop V-VI, which induces EAU of comparable severity to that of RSAg or IRBP (Adamus et al. 1992).

Rhodopsin has not been studied as an autoantigen in human uveitis. However, it has been implicated in the pathogenicity of retinitis pigmentosa.

1.4.1.4 Phosducin

Phosducin is a 33 kDa photoreceptor phosphoprotein that is a cytosolic regulator of G-protein mediated signalling. It is found in the retina and pineal gland, as well as in liver, heart, lung and brain. It was first reported as inducing a mild to moderate EAU and pinealitis in Lewis rats (Dua et al. 1992). The disease was late in onset, low grade in severity and predominantly affected the posterior segment. Later studies, using synthetic peptides corresponding to rat phosducin to map pathogenic epitopes, reported severe EAU in Lewis rats within 12 days of inoculation (Abe et al. 1997). A potent uveitogenic site located between residues 65-96 was identified, whose potency was comparable to that of RSAg. A further study reported 5 uveitogenic epitopes on phosducin, one of which was dominant (Sato et al. 1998). Phosducin's role in human uveitis is, however, uncertain.

1.4.1.5 Recoverin

Recoverin is a 23 kDa protein found mainly in photoreceptor cells, but also retinal bipolar cells and the pineal gland. It regulates phosphorylation of rhodopsin in a calcium-dependent manner. Recoverin has also been detected in cancer cells. It causes a severe EAU in Lewis rats at low doses (Adamus et al. 1994; Gery et al. 1994). Histopathologically, recoverin-induced EAU resembles

that of RSAG but may require a higher antigen dose. The sequence 61-82 is a key region for anti-recoverin antibody binding, contains a major T-cell epitope and is highly uveitogenic in Lewis rats (Adamus and Amundson 1996). More recently, an additional uveitogenic epitope (sequence 149-167) has been identified using recoverin-derived peptides (Ohkoshi et al. 2001). The presence of anti-recoverin antibodies is associated with cancer-associated retinopathy (CAR) (Thirkill et al. 1992). This phenomenon, in which non-inflammatory retinal degeneration occurs leading to decreased visual acuity and night-blindness, may develop because of cross-reactivity between B-cell epitopes on recoverin and antigens expressed on tumour cells.

1.4.1.6 RPE 65

RPE 65 is a 61 kDa protein found specifically in the RPE (Hamel et al. 1993). It appears to play a role in vitamin A metabolism. Immunization of several inbred rat species resulted in an acute widespread uveitis (Ham et al. 2002). Interestingly this included a strain (Brown Norway) normally resistant to RSAG-induced EAU. Unlike EAU induced by several other retinal proteins, no pinealitis was noted.

1.4.1.7 Cytokine profiles in EAU

The predominant pattern of cytokine secretion in EAU is generally believed to be a Th1 type (Xu et al. 1997; Foxman et al. 2002). Susceptibility to the disease seems to occur with a conversion from a Th0 or Th2 to a Th1-type response, whereas a Th2-type response appears to confer resistance (Saoudi et al. 1993b). Pathogenic T-cell populations in EAU produce large amounts of IFN- γ (Caspi et al. 1996). Endogenous IL-10 may limit the expression of EAU and help in its resolution. Whereas IL-10 is capable of suppressing a Th1 response, the addition of IL-4 can skew it towards a Th2 pattern (Rizzo et al. 1998). Similarly endogenous IL-12 is required for the development of EAU (Tarrant et al. 1998). IL-12 "knockout" mice fail to develop EAU and instead develop a Th2-type response, but are capable of developing the disease when infused with cells pre-primed in IL-12.

The above findings seem to imply a clear causative role for Th1-type cytokine patterns in EAU, but as is seen in other experimental diseases, contradictory roles for these apparently pro-inflammatory, Th1 cytokines have been noted. For example, IFN- γ deficient knockout mice have been found to be susceptible to EAU despite the development of a Th2-type profile (Jones et al. 1997). Endogenous systemic IFN- γ seems to play a protective role in EAU in mice (Caspi et al. 1994). It has also been found that the systemic administration of IL-12 protects mice from the development of EAU, in response to immunisation with IRBP (Caspi et al. 1997a). It also appears that more than just a predisposition towards a Th2 response is required to confer genetic resistance to EAU (Sun et al. 1997). Perhaps other factors, such as the stage of disease at which particular cytokines are administered/secreted, could account for these apparently contradictory effects.

1.4.2 Non-retinal ocular antigen models of uveitis

1.4.2.1 Experimental melanin-protein induced uveitis (EMIU)

A non-soluble, melanin-associated protein derived from the RPE, choroid, ciliary body and iris, has been found capable of inducing experimental uveitis. The protein can be solubilised by proteases but has not yet been fully characterised (Simpson et al. 1997). Pathological changes were initially thought to be confined to the anterior chamber, hence the term experimental autoimmune anterior uveitis (EAAU) (Broekhuysse et al. 1991). However, choroidal involvement was later found to be a more constant feature (Chan et al. 1994), and the consensus term experimental melanin-protein induced uveitis (EMIU) was coined. The prime mediators of the inflammation are CD4⁺ cells (Smith et al. 1999), but accumulations of polymorphs are also observed. Disease onset occurs approximately 14 days after inoculation and regresses around 1 month afterwards. Spontaneous recurrence is seen clinically in a quarter of rats around 37 days after immunization, but after the addition of low dose endotoxin all eyes showed some histopathological evidence of choroiditis (Chan et al. 1994). Small subretinal inflammatory foci may be seen in recurrences, in addition to the other features described. In contrast to EAU, the Th1 response seen in EMIU is less

intense and expression of Fas and FasL is weaker, and this may account for the more limited inflammatory response and increased rate of recurrences (Li et al. 1999). The onset of recurrences may also be facilitated by relatively higher expression of Bcl-2, which may be responsible for uveitogenic cells avoiding apoptosis and surviving in the choroid. EMIU recurrences can be prevented by intraperitoneal injection of TGF- β , probably by upregulating expression of ocular IL-10 (Li et al. 1996). Therefore the EMIU model of uveitis is particularly useful for studying recurrences.

1.4.2.2 Lens induced uveitis

Uveitis can be induced in animals by disrupting the lens capsule, leading to the exposure of lens proteins (crystallins) (Marak et al. 1976). The reaction induced is an acute granulomatous uveitis with features of an immune complex-mediated inflammation. The experimental disease is histopathologically identical to a human condition known as phacoanaphylactic endophthalmitis or lens-induced uveitis, which can be cured by lens extraction. Posterior subcapsular cataract development has been found as a result of an immune response induced by bovine lens membrane protein (Tanemoto et al. 2000). A recent hypothesis proposes that posterior subcapsular cataracts may be induced by low-grade inflammation, initially raised against bacterial proteins and then directed against β -crystallins (Shinohara et al. 2000).

1.4.3 Non-ocular antigen models of uveitis

Direct injection of non-ocular antigen into the eye induces a progressive immunisation reaction. An inflammatory reaction of lymphocytes and macrophages is detectable in the first week following injection of endotoxin-free human serum albumin into the vitreous of rabbits (Sher et al. 1976), and a humoral immune response is active within 10 days. A severe “reinduction uveitis” can be initiated by the intravenous injection of human serum albumin after 30 days, presumably due to the persistence of memory cells. The initial immunological response seems to take place in the spleen and regional lymph nodes, with primed cells then migrating back to the eye. Immune-complex

mediated inflammation has also been implicated after injection of intraocular antigen in hyperimmunised animals. Furthermore, uveitis can be induced by systemic immunization of non-ocular antigens and these models are outlined below.

1.4.3.1 Endotoxin-induced uveitis (EIU)

Uveitis can be induced by intravenous injection of specific "Shwarzman" bacterial toxins in previously injected (Sanders 1939) or non-prepared (Ayo 1943) eyes. Anterior uveitis can be induced by intravenous, intraperitoneal or footpad injection of low doses of bacterial endotoxin without causing inflammation in other organs (Rosenbaum et al. 1980; Kogiso et al. 1992). In rats, inflammation begins a few hours after lipopolysaccharide (LPS) injection and disappears within a few days. After repeat injections, a state of tolerance to the LPS develops (Howes et al. 1985). In the C3H/HeN mouse, a first wave of inflammation characterised by polymorphs is induced on Day 1 post-immunisation, followed by a second wave on Day 5 with macrophages predominating (Shen et al. 2000).

Most of the effects of LPS in EIU are thought to be related to the lipid part of the molecule. In Lewis rats, the effect is probably initiated by macrophages within the stroma of the iris and ciliary body (McMenamin and Crewe 1995; Pouvreau et al. 1998). This leads to the recruitment of T-cells and polymorphs (Kogiso et al. 1992; Guex-Crosier et al. 1996; Brito et al. 1999). Apoptosis of mononuclear cells occurs early in EIU, but polymorphs survive, producing inducible nitric oxide synthase (iNOS) that may contribute to disease pathogenesis (Smith et al. 2000).

There is variation in disease susceptibility in different animal strains. This seems to be related to animals' abilities to induce expression of pro-inflammatory cytokines (de Vos et al. 1994a; de Vos et al. 1994b) and adhesion molecules (Suzuma et al. 1997). Decreased levels of inflammation can be caused by modulation of cytokine expression, or systemic use of TGF β (Peng et al. 1997) or IL-10 (Hayashi et al. 1996). Oestrogen can also reduce the severity of inflammation in EIU (Miyamoto et al. 1999), as may pregnancy in other uveitis animal models (Agarwal et al. 1999) and possibly also in humans (Steahly 1990).

1.4.3.2 Adjuvant induced uveitis

Intravesical BCG (Bacille Camette Guerin) infusion therapy has been reported to cause a reactive arthritis in some patients, and in some of these a transient uveitis develops (Missioux et al. 1995; Chevrel et al. 1999; Clavel et al. 1999). In rats, immunisation with CFA induces uveitis in 22% of cases (Petty et al. 1994). The inflammation is localised in the iris and ciliary body and may be recurrent. Addition of *Mycobacterium butyricum* to the adjuvant increases the rate of uveitis. In adjuvant-induced arthritis, mycobacterial heat shock protein (HSP) 65 is known to play an important role. Peptide sequences derived from HSP 65 of *Mycobacterium bovis* are able to provoke inflammation in the iris and ciliary body. Extensive photoreceptor loss can be seen at times on histology, but little posterior segment inflammation (Uchio et al. 1998). Antibodies against HSP 70 have been found to be elevated in certain human uveitides, including pars planitis, sarcoid uveitis and Behcet's uveitis (de Smet and Ramadan 2001).

1.4.3.3 Uveitis associated with experimental autoimmune encephalomyelitis (EAE)

Intraocular inflammation has also been noted in experimental autoimmune encephalomyelitis (EAE), a disease model for MS. EAE is a classic experimental model for an organ-specific, T-cell mediated, autoimmune disease and is induced in susceptible animals by immunisation with MBP or its pathogenic epitopes (Wraith et al. 1989). Intraocular inflammation, including pars planitis and retinal periphlebitis (Tola et al. 1993), is known to occur in human MS patients.

Intraocular inflammation in EAE may affect the anterior or posterior segment. Posterior segment inflammation involves the optic nerve and retina (Bullington and Waksman 1958). This is characterised by a mononuclear cell infiltration, periphlebitis and demyelination (Rao 1981). The severity of inflammation depends on the amount of myelin in the target tissue, animal species and strain, and the dose of MBP used.

In Lewis rats, anterior segment inflammation often develops at the same time as EAE (Verhagen et al. 1994), which develops around 11 days post-

immunization with MBP. However, the peak of ocular inflammation occurs as the neurological disease is subsiding, demonstrating that at least the kinetics of the diseases are different. Myelinated nerve fibres in the iris may be the primary target of inflammation and there is some evidence that the same subset of autoreactive T-cells is responsible for both the anterior segment uveitis and EAE (Buenafe et al. 1998).

1.4.3.4 Other experimentally induced models of uveitis

Recently, an experimental model for autoimmune uveitis using the tyrosinase-related proteins TRP-1 and TRP-2 has emerged. These proteins are tissue differentiation antigens found in melanocytes, and immunization of Lewis rats causes anterior and posterior segment uveitis, as well as some extraocular manifestations (Yamaki et al. 2000). TRP1 is also capable of causing CD4⁺, T-cell mediated vitiligo (Overwijk et al. 1999). This model therefore seems particularly well suited to the study of VKH.

1.4.3.5 Genetic control of uveitis in animals

Animal models, where the inbreeding and manipulation of various strains is possible, allow more precise and sophisticated genetic studies in uveitis. They have shown that susceptibility to EAU is genetically controlled and appears to follow a polygenic, multifactorial pattern of inheritance. Inter-species and inter-strain differences are apparent. In mice, the expression of EAU is found to be controlled by MHC and non-MHC genes. The MHC-controlled component appears to be located in the mice MHC class II locus (Caspi 1992), possibly implicating antigen presentation as a factor. In rats, two non-MHC chromosomal regions have been found to be associated with EAU (Sun et al. 1999). Many immunologically relevant genes are located in these regions. The EAU susceptibility regions overlap with genetic regions associated with other human and animal autoimmune diseases. This suggests that autoimmune disease markers may be shared between autoimmune diseases, and in different species (Becker et al. 1998). An exciting recent development is the generation of a transgenic (TG) mouse model of EAU that expresses human HLA-class II but is

deficient in mouse class II (Pennesi et al. 2003). EAU was induced in DR3, DR4, DQ6 and DQ8 mice with IRBP. Importantly, HLA-DR3 TG mice also developed severe EAU with RSAg, to which wild-type mice are highly resistant. This humanized TG mouse model should therefore represent a better model of human uveitic disease in future studies.

1.5 Regulation of ocular immunity

Animal models of autoimmune PSII have shown a central role for CD4⁺ T-cells in the generation of the immune response, with B-cells - and possibly others such as mast cells – playing an accessory role. The regulatory mechanisms active within the eye, including those involved in immune privilege, antigen presentation and ocular T-cell priming are described below. Again, most of what we know has been derived from animal models.

1.5.1 Ocular immunity and immune privilege

The eye responds to immune stimuli with a modified, generally less destructive, response than most other tissues. This is thought to be an evolutionary adaptation that has developed because the degree of inflammation required to eliminate pathogens in other tissues, would cause deleterious effects in the eye. For example, even moderate inflammation of the cornea can result in sight-threatening scarring, while inflammation of the posterior pole can very easily destroy the delicate tissues of the macula and retina.

Specialized anatomical and physiological features mediate the modified inflammatory response seen in the eye. The cornea, anterior chamber, vitreous cavity and sub-retinal space are termed “immune privileged” sites, meaning that foreign tissue grafts can survive at these sites (but not conventional sites) for prolonged or indefinite periods (Streilein 2003). Other immune privileged sites include the brain, pregnant uterus, ovary, testis, adrenal cortex and hair follicles. Immune privileged *tissues* are those that experience extended survival when transplanted to conventional sites that normally reject foreign tissue transplants. Immune privileged ocular tissues include cornea, lens, retinal pigment epithelium and neuroretina. Other immune privileged tissues include brain, placenta, ovary, testis, liver and certain tumours. Ocular immune privilege is in part related to the eye’s lack of lymphatics and the presence of effective blood tissue barriers. It is also related to the reduced expression of MHC (particularly class II) in the anterior chamber, vitreous cavity and sub-retinal space. The expression of FasL on ocular cells can cause apoptosis of activated T-cells (Griffith et al. 1995; Dick et al. 1999). Ocular immune privilege is an active

process, with ocular antigens being recognised by the immune system, but producing a deviant effector response – anterior chamber associated immune deviation (ACAID) (Streilein 2003). Ocular antigens are thought to be carried to the spleen in the bloodstream, where the deviant immune response is generated (Wilbanks and Streilein 1991; Wilbanks et al. 1992). The eye-derived APC are themselves primed in a microenvironment that contains immunosuppressive mediators such as TGF- β (Cousins et al. 1991), vasoactive intestinal peptide (VIP) and somatostatin (Biros and Taylor 2001). The deviant response results in suppression of the expected cellular, DTH responses and the production of non-complement-fixating antibodies. There is also some evidence of suppression of EAU by idiotype/anti-idiotype antibody networks (de Kozak and Mirshahi 1990).

1.5.2 T-cell priming in the retina and uvea

Resident RPE, Muller and microglial cells within the retina were initially thought to fulfil the role of antigen presentation, through the expression of class II MHC. However, RPE and Muller cells have subsequently been found to be poor antigen presenters and probably down-regulate immune responses (Caspi and Roberge 1989; Liversidge et al. 1993). Retinal microglial cells probably function similarly. It is now thought that antigen presentation in the eye occurs through classic mechanisms involving macrophages and dendritic cells (DC). Dense networks of these cells have been found in the iris, ciliary body and choroid (Forrester et al. 1994; McMenamin 1997). DC reside in the eye for only a few days, before migrating to the secondary lymphoid organs, mainly the spleen. In the early stage of EAU, inflammation (and presumably antigen presentation) occurs around either the RPE or surrounding retinal vessels. Later it spreads to deeper retinal structures, where antigen presentation is probably carried out by MHC class II-positive macrophages. Marrow-derived activated macrophages are required for the full development of inflammation in EAU and EMIU. Depletion of these cells or absence of the appropriate class II MHC reduces the severity of EAU (Forrester et al. 1998b). These cells are recruited early in the effector phase of EAU. In the early stages, resident DC numbers remain

relatively low but then increase during the active inflammatory phase, remaining high for over 6 weeks (Butler and McMenamin 1996).

1.5.3 Immunoregulatory role of dendritic cells

As well as their role in antigen presentation, DC are also thought to influence the differentiation of CD4⁺ T-cells to a Th1 or Th2 cytokine profile. This may happen through co-stimulatory signals conveyed during antigenic stimulation of the TCR (Kalinski et al. 1999; Banchereau et al. 2000). The nature of these signals is determined during DC development. After leaving the bone marrow, immature DC temporarily migrate to the tissues where they take up local antigen, while being exposed to the local cytokine environment. The local environment determines whether immature DC will adopt a pro-inflammatory (DC1) or suppressive (DC2) profile. DC1 cells are polarised towards expression of IL-12 and TNF- α , whereas DC2 preferentially express IL-6 (Kalinski et al. 1999). The cytokine environment of the ocular anterior chamber seems to promote the development of DC2-type cells. Once established, the DC profile is relatively fixed and upon maturation uptake of further antigen ceases. Mature, antigen-bearing DC migrate back to the lymph nodes, where they may activate and polarise T-cells with compatible TCRs.

1.6 Autoimmune PSII in humans

"Endogenous" PSII in humans is generally accepted to be an autoimmune disease in the majority of cases, as discussed above. When searching for the cause of this condition, particularly when using in vitro assays, it is important to distinguish between an autoimmune response and autoimmune disease. Given that ocular immune privilege is due to an active downregulation of a pro-inflammatory response rather than antigen sequestration, some autoimmune responses to antigen in vitro are to be expected in normal individuals. In addition, an autoimmune response can be the result rather than the cause of the disease process. Generally speaking, it is the degree of autoimmunity that differentiates patients from healthy individuals. It is thought that autoimmune disease will follow from an autoimmune response if a sufficient number of effector cells are recruited, after which an amplification of non-specific inflammatory events will occur. After the initial disease process, chronicity or recurrences may occur if sufficient numbers of autoreactive cells remain.

1.6.1 Human autoreactivity to retinal antigens

Because retinal antigens can induce EAU in many animal models including primates, it is tempting to conclude that retinal autoimmunity is the cause of autoimmune PSII. This is especially so when considering how EAU can be made to closely mimic many of the individual features of clinical autoimmune PSII. However, caution is advised as a direct causal link between retinal antigens and autoimmune PSII has not yet been established in humans. Overall, retinal autoimmunity probably has some role in the generation of clinical PSII, but exactly how and at which level has yet to be determined.

1.6.1.1 Cellular immunity to retinal proteins in human PSII

Positive lymphocyte proliferative responses have been noted in humans in response to retinal extract, RSAg and IRBP. T-cell responses to RSAg have been studied in greater detail than IRBP. Greater RSAg-specific (bovine and

human) in vitro lymphocyte proliferation has been noted in a variety of clinical, autoimmune uveitic conditions compared to controls, using peripheral blood samples (Nussenblatt et al. 1980). This is especially so for birdshot retinochoroidopathy, where over 90% of patients showed significant responses (Nussenblatt et al. 1982). Long-term T-cell lines have been established in response to RSAg stimulation using vitreous samples from uveitis patients (Nussenblatt et al. 1984). Doekes (Doekes et al. 1987) has noted much higher cellular immune responsiveness to human RSAg in posterior and pan-uveitis patients compared to controls, by measuring the production of migration inhibitory factor (MIF) during overnight culture of peripheral blood mononuclear cells (PBMC) with the antigen. In all the above studies, minimal or no responses to RSAg were reported in controls. However, Hirose, et al, found that PBMC from a large proportion of healthy donors reacted positively to bovine RSAg using a highly sensitive proliferation assay (Hirose et al. 1988b), and later reported the establishment of a RSAg-specific T-cell line from the blood of a healthy donor (Hirose et al. 1988a).

Several studies have been carried out to determine the response to peptide fragments of RSAg in a number of autoimmune PSII conditions (de Smet et al. 1990; Hirose et al. 1990; Nityanand et al. 1993; Yamamoto et al. 1993; de Smet et al. 2001). Some studies suggest that peptides M and N, previously found to be immunopathogenic in EAU, cause increased proliferation rates in patients with uveitis, including active Behcet`s disease (de Smet et al. 1990; Yamamoto et al. 1993). De Smet (de Smet et al. 2001), using 40 overlapping synthetic peptides from human RSAg, tested the cellular responses to PBMC from uveitis patients with Behcet`s, sarcoid, sympathetic ophthalmia and VKH. Immunodominant peptides were identified for Behcet`s and sarcoid disease categories. Above-baseline proliferative responses were found to multiple epitopes in several individuals, representing all disease categories except sympathetic ophthalmia, which did not show any response to RSAg. A changeable response pattern was noted in selected patients monitored for between 6 and 12 months, but this was not noted in the single control subject followed. "Epitope spreading", where immunoreactivity is directed against different epitopes on the same antigen over time, was also seen. Rai (Rai et al. 2001) also used synthetic peptides spanning the entire sequence of human

RSAg, to screen a group of uveitis patients (anterior and posterior) for proliferative responses. Eleven out of 26 patients responded to at least one peptide. It would appear that multiple epitopes of human RSAg are immunoreactive in different subtypes of autoimmune PSII, and this can vary between individuals and over time. Cellular responses to RSAg peptides have also been seen in conditions such as retinitis pigmentosa (Yamamoto et al. 1992) and Eale's disease (Saxena et al. 1999).

The frequency of autoreactive T-cells has been estimated in autoimmune PSII. Opremcak used limiting dilution analysis to determine the minimal frequency of (bovine) RSAg-reactive T-cells in PBMC (Opremcak et al. 1991). Significant frequencies of RSAg-specific CD4⁺ T-cells were detected in anterior and posterior uveitis, but none in healthy controls. Similarly, cell culture-based limiting dilution assays have detected an increased frequency of RSAg-specific T-cell lines in PBMC from patients with active ocular Behcet's disease (de Smet and Dayan 2000). The range of autoreactive RSAg-specific cells is estimated at 0 - 400 per 10⁷ PBMC for uveitis patients and 0 - 4 per 10⁷ PBMC for healthy controls. These figures are comparable to those found for autoreactive cell frequencies in MS. This paper also suggests the expansion of certain T-cell subpopulations in response to episodes of uveitis, with a return to normal levels within 3 months.

Cellular responses to IRBP have been found in PBMC from autoimmune PSII patients. Hirose (Hirose et al. 1988b) detected low but significant responses to whole bovine IRBP in autoimmune PSII patients. However, the responsiveness was lower than that detected for RSAg in the same experiment and similar levels of response were also detected in the control group. De Smet (de Smet et al. 1990) found greater proliferative responses to whole bovine IRBP in certain uveitis subtypes compared with controls. Again the responses were lower than those of RSAg in the same patients, but the response profiles of the 2 antigens paralleled each other. Responses were detected to the IRBP peptides R-4 and R-14 in a minority of cases. Yamamoto (Yamamoto et al. 1993) found that 35% of Behcet's patients without uveitis responded to whole bovine IRBP, compared to 14% of controls. This figure increased to 52% for Behcet's patients with active uveitis. Responses to the peptides R-4 and R-14 were not significantly different to controls.

Overall, although there is ample evidence of proliferative cellular responses to stimulation by RSAg, IRBP or some of their peptides, no unequivocally immunodominant epitope has emerged for either antigen in human disease. This may be because of immunoreactivity to multiple epitopes in the same antigen, heterogeneous responses of different disease subtypes or the effects of epitope spreading as the disease progresses. It may also reflect the limited ability of proliferation assays to detect subtle cellular responses to uveitogenic antigens or peptides.

1.6.1.2 Humoral immunity to retinal proteins in human PSII

The role of antibodies in autoimmune uveitis is controversial. Antibodies directed against specific antigens or organs could either participate in or suppress the disease process, or could be seen as epiphenomena resulting from other processes e.g. tissue destruction. Generally speaking, autoantibodies to retinal antigens are not considered to play an active part in the induction of autoimmune PSII. Much of what we know in this area is derived from animal models, especially EAU as discussed above.

In humans, autoantibodies to retinal antigens have been detected both in uveitis and normal populations. Several studies have detected the presence of anti-RSAg antibodies in autoimmune uveitis patients, and these are described more fully in Chapter 3. Hoekzema found anti-IRBP antibodies using human and bovine antigen in uveitis patients (Hoekzema et al. 1990). However, similar responder frequencies and antibody titres have been detected in uveitis and control groups in all these studies.

Despite considerable evidence to the contrary, there is some evidence that autoantibodies to retinal antigens may be involved in pathological manifestations. Using immunostaining techniques, autoimmune antibodies from the sera of patients with VKH and to a lesser degree Behcet's syndrome and sympathetic ophthalmia, were found against the outer segments of photoreceptors and Muller cells from normal human retina (Chan et al. 1985c). This was despite the absence of serum anti-retinal antibodies.

As briefly mentioned above, the serum of patients suffering from the paraneoplastic disease cancer-associated retinopathy (CAR) have been found to contain autoantibodies to recoverin. The immune response seems to be

directed against recoverin in both rods and cones (Polans et al. 1993). In a similar condition, cutaneous melanoma-associated retinopathy (MAR), onset of night-blindness is seen in patients with metastatic cutaneous melanoma. Specific autoantibodies have been detected against human retinal bipolar cells, which possibly represent cross-reactivity with a melanoma antigen (Milam et al. 1993). This induces specific abnormalities of the rod and cone systems.

Immune complexes have been found in the circulation of patients with active Behcet's disease (O'Connor 1983) and the aqueous humour of posterior uveitis patients (Dernouchamps 1977). Low levels of immune complexes in serum have been associated with cases of severe isolated retinal vasculitis (Dumonde et al. 1982), while some investigators suggest that high levels of immune complexes may have a protective effect in uveitis (Stanford et al. 1988). It is suggested that the immune complexes, possibly of an idiotype/anti-idiotype nature, may be a compensatory mechanism accompanying anti-retinal autoimmunity. Current evidence does not support a role for immune complexes as a *cause* of autoimmune uveitis.

Pooled human immunoglobulin from multiple donors (IVIG) has been found to modify inflammation in both EAU and EIU (Saoudi et al. 1993a; Obrador et al. 1999). This potential method of treatment has shown promising results (in an open trial) when applied to humans with birdshot chorioretinopathy (LeHoang et al. 2000). The mechanism of action is possibly by induction of the idiotype/anti-idiotype network.

It is well established that anti-retinal antibodies exist in human autoimmune PSII, but on balance the evidence seems to be against a role in the induction of disease. However, the potential role of autoantibodies as immunoregulatory molecules or disease markers has not been fully explored. It is possible that autoantibodies specifically directed against pathogenic or suppressive B-cell epitopes exist and this is an area worthy of further investigation.

1.6.1.3 Cytokines and adhesion molecules in autoimmune PSII

1.6.1.3.1 Cytokines

Ocular fluids have been analysed for cytokines and other immunologically relevant factors in human autoimmune PSII. The levels of IL-6, but not IL-2, were found to be significantly elevated in the aqueous humour of patients with VKH (Norose et al. 1994). Cytokine expression patterns of infiltrating CD4⁺ T-cells from the aqueous of patients have also been studied. Cloned CD4⁺ cells from the aqueous of patients with sarcoid and VKH were found to express greater levels of pro-inflammatory cytokines, especially IL-6 and IL-8, than cloned cells from a healthy control (Sakaguchi et al. 1998).

Serum samples have also been studied in autoimmune PSII. One study found increased IL-8 levels in 27 out of 61 patients with intermediate uveitis, 12 of 27 patients with sarcoid uveitis, and in five of 29 healthy controls (and also in 19 of 30 patients with HLA-B27 associated acute anterior uveitis) (Klok et al. 1998). Macrophage migration inhibitory factor (MIF) was found by ELISA to be significantly higher in the sera of patients with autoimmune PSII compared with controls (Kitaichi et al. 1999). This was particularly so for patients with active ocular Behcet's or sarcoid. It has also been reported that in Behcet's patients, uveitis was seen significantly less frequently in those with anti-CTLA-4 antibody in their sera (Matsui et al. 1999).

Unfortunately most other studies measuring intraocular cytokines in uveitis concern groups of mixed types of uveitis, including infectious and anterior uveitis, and the results are not necessarily applicable to autoimmune PSII. However, increased levels of the inflammatory cytokines IL-1, IL-8, IL-12, TNF- α and especially IL-6 have been reported in these studies (de Boer et al. 1992; Franks et al. 1992; Wakefield and Lloyd 1992; El-Shabrawi et al. 1998; Ongkosuwito et al. 1998; Petrinovic-Doresic et al. 1999). One particular study, comparing 2 forms of non-infective anterior uveitis, noted higher IFN- γ and IL-10 expression in aqueous from patients with Fuch's cyclitis (a relatively non-destructive form of anterior uveitis) and greater IL-12 expression in "idiopathic" anterior uveitis (Muhaya et al. 1998). Many intraocular inflammatory cytokines apparently raised in uveitis, are also elevated in other conditions such as

proliferative vitreoretinopathy (de Boer et al. 1992; Elner et al. 1995) and following cataract surgery (Nishi et al. 1992).

Overall, there are few comprehensive studies of cytokine expression in ocular fluids or sera, in well-defined groups of patients with autoimmune PSII. Once again, most of what we know in this area of uveitis research derives from animal models.

1.6.1.3.2 Cellular adhesion molecules

The expression of cellular adhesion molecules, and the successful use of blocking monoclonal antibodies to suppress inflammation, has been demonstrated in various animal models of uveitis (Whitcup 2000). In comparison there are relatively few studies on human PSII. Nevertheless, in human PSII, upregulation of ICAM-1 has been demonstrated in the retina, choroid and RPE and expression of LFA-1 demonstrated on infiltrating lymphocytes (Whitcup et al. 1992). Elevated levels of soluble ICAM-1 have been associated with active intermediate uveitis and a predisposition to systemic disease (Klok et al. 1999). The blockade of cellular adhesion molecules with monoclonal antibodies or immunoadhesins is one of the more promising approaches for the future treatment of human PSII.

1.6.2 Treatment of autoimmune PSII

The following section describes the medical treatment of autoimmune uveitis. The first part describes the more conventional treatments currently employed. The second discusses immunotherapies, many of which are still being developed, but some which are already being used.

1.6.2.1 Established uveitis treatments

1.6.2.1.1 Corticosteroids

Corticosteroids are still the mainstay of anti-inflammatory therapy in autoimmune PSII, and can be administered systemically or locally by injection e.g. orbital floor, sub-Tenon`s or even intraocular. The drug alters the number and functions of lymphocytes, polymorphonuclear leucocytes (PMN), and macrophages and increases vascular permeability (Forrester et al. 1996). It also

interferes with the effects of phospholipase A₂, prostaglandin, thromboxane, leukotrienes and histamine. While corticosteroid treatment is often effective in controlling inflammation, it does not effect a cure, and long-term use of steroids is associated with ocular and systemic side-effects (Dick et al. 1997). Side-effects may eventually force cessation of therapy. Some patients are refractory to treatment with steroids.

1.6.2.1.2 Immunosuppressive agents

A wide range of immunosuppressive agents has been used systemically in the treatment of uveitis. The alkylating agent chlorambucil is effective in the short term, but is potentially more toxic than other agents for long-term use. Azathioprine, an antimetabolite, is also used and is usually reasonably well tolerated. However, it is less effective than some of the newer drugs, and may cause myelosuppression. Recently more specific drugs have been developed. Cyclosporin A (CsA) is a biologically active fungal metabolite and specifically targets T-cell function by inhibiting intracellular signalling (and therefore activation) after antigen recognition, suppressing IL-2 gene transcription and downregulating IL-2 receptor expression on the cell surface (Dick et al. 1997). CsA is more suitable than alkylating agents for long-term treatment of chronic PSII, as it is less likely to cause long-term adverse effects such as neoplasia. However, it can cause nephrotoxicity and hypertension, as well as minor adverse effects. Tacrolimus (FK506) acts in a similar manner to CsA, but is more potent and can be used to “rescue” failed CsA treatment (Kilmartin et al. 1998). It has a similar side-effect profile to CsA, but these are less common and it is therefore often better tolerated. The newest and possibly most effective of these agents is mycophenolate mofetil, a purine analogue that inhibits the cell cycle by disrupting DNA synthesis (Larkin and Lightman 1999). It is particularly inhibitory for lymphocytes, because it inhibits de novo purine synthesis, on which these are particularly dependent. It is better tolerated than either CsA or tacrolimus, and does not cause nephrotoxicity or hypertension. It potentially causes lymphopaenia however, and is associated with a small increase in the number of viral infections.

In general, although current treatments represent an improvement on the past, there are still substantial limitations in terms of efficacy and side-effect

profiles. None of the above treatments is curative. It is hoped that specific immune-based therapies can overcome these limitations, and possibly even lead to abolition of the disease.

1.6.2.2 Immunotherapies, present and future

1.6.2.2.1 Oral and nasal tolerance induction

Oral and nasal tolerance (mucosal tolerance) induction to retinal self-antigens is currently an area of great interest in the development of treatments to autoimmune PSII. This approach has the potential advantages of selectively abrogating the immune response and inducing long-term tolerance to self-antigens, while causing minimal or no side-effects. Mucosal tolerance has been successful in the treatment of animal models of the disease using both retinal extract (Dick et al. 1994) and IRBP (Rizzo et al. 1994). There is a suggestion that induction of nasal rather than oral tolerance is more effective in reducing inflammation.

A number of small trials have been carried out to test the efficacy of oral tolerance induction to putative autoantigens in human autoimmune uveitis. A randomised controlled phase I/II trial tested a total of 45 patients with purified bovine RSAg alone, a mixture of soluble retinal antigens or a combination of the two (Nussenblatt et al. 1997). A trend towards reduction of immunosuppressive therapy in RSAg (alone) treated patients was reported, but was not statistically significant. No toxic effects were reported, but there may have been potential exacerbation of disease in the retinal mixture group. In another trial, low doses of chicken type-II collagen were fed to 13 patients with juvenile rheumatoid arthritis-associated uveitis (Thompson et al. 2002). While appearing safe, the treatment did not seem to have a significant effect on uveitis. In another uncontrolled trial of 9 patients with refractory uveitis, clinical improvement was demonstrated by the oral administration of peptide HLA-B27PD (Thureau et al. 1999). This synthetic peptide, derived from uveitis-associated MHC class I antigens, shares sequence homology with RSAg. Although all patients eventually relapsed after cessation of treatment, no adverse effects from the therapy were reported.

1.6.2.2.2 Monoclonal antibody and immunoadhesin therapy

Regardless of the initiating event, the chronicity of many autoimmune PSII conditions probably implies a chronic imbalance of cytokine production (Dick and Carter 2003). Under these conditions, continued secretion of pro-inflammatory cytokines maintains an infiltrate of activated T-cells and myeloid cells. Inflammatory cytokines or activated T-cells can now be specifically targeted using monoclonal antibodies or immunoadhesins. The latter refer to recombinant hybrid molecules consisting of a biologically active component e.g. a specific receptor, combined to a carrier component (e.g. the Fc part of an antibody molecule). Soluble TNF receptor/IgG fusion proteins have been shown to be effective in minimising rod outer segment damage in EAU (Dick et al. 1996). It has been suggested that anti-TNF- α treatments may have an immunomodulatory effect, as well an anti-inflammatory one, in the treatment of autoimmune diseases (Dick et al. 1998).

These new treatments are now being applied to human autoimmune diseases, including uveitis (Dick and Isaacs 1999). Success has been achieved in the treatment of rheumatoid arthritis using anti-TNF immunoadhesins and monoclonal antibodies (Elliott and Maini 1995). Anti-TNF mAb (Infliximab) therapy has been used successfully in a case of treatment-resistant, spondyloarthritis-associated uveitis (Kruithof et al. 2002), and in refractory posterior uveitis (Joseph et al. 2003). A recent report on the use of an anti-TNF alpha receptor fusion protein in 15 patients with PSII is encouraging (Greiner et al. 2004). Other molecules have also been targeted. Early successes have been noted in the treatment of autoimmune PSII, in single cases using anti-CD4 mAb (Thurau et al. 1994), and Campath-1 H (Isaacs et al. 1995). Encouraging results have also been reported for the use of anti-IL-2 receptor mAb (Daclizumab) in a small, non-randomised study (Nussenblatt et al. 1999). Other immune-related molecules have been targeted in experimental autoimmune disease, and co-stimulatory molecules such as CD28, B7.1 and CD40 are potential targets for future treatment of uveitis.

Despite early apparent successes, potential drawbacks of anti-cytokine treatment should be noted. It does not seem to restore immunological tolerance or immune regulation in autoimmunity. There is the potential risk of generating

other autoimmune diseases in the long term. There is also a potentially increased risk of infection, particularly with TNF blockade.

1.7 Aims of this study

In the past, the emphasis in basic science research in autoimmune PSII has been, of necessity, on animal models of the disease, in particular EAU. Basic research directly into human PSII has been limited by several practical problems. There is an overall scarcity of human ocular material for uveitis research. This reflects the impracticality of obtaining biopsy material from the retina/choroid without causing significant harm, the general lack of post-mortem or enucleation specimens from patients except those with end-stage disease, and the relative rarity of certain important subcategories of the disease. In recent years, however, immunological and molecular techniques have emerged that might circumvent some of the problems regarding access to and supply of ocular tissues. The cloning and expression of recombinant human antigens, the utilisation of serum antibodies from patients and the analysis of their binding preferences, and the analysis of T-cell responses in the peripheral blood by flow cytometry are some of the techniques that seem capable of lending themselves directly to the investigation of autoimmune PSII in patients.

In this thesis, four main areas of research are described. The emphasis, wherever possible, was on the application and evaluation of techniques that could be directly applied to human uveitis research. The specific aims were:

To clone and express the human form of retinal S antigen, one of the major candidate autoantigens in autoimmune uveitis.

To screen by ELISA, sera from autoimmune uveitis patients and controls, for the presence of antibodies against human recombinant RSAg. Also to use bacteriophage display technology to delineate the preferential binding sites of human anti-RSAg antibodies from the same groups of subjects.

To identify novel potential autoantigens for human uveitis, by constructing a human retinal cDNA expression library and screening it with sera from human uveitis patients or controls.

To demonstrate a specific response to RSAg stimulation of peripheral blood T-cells from uveitis patients, using the sensitive technique of cytokine flow cytometry.

Chapter 2: Materials and methods

2.1 General methods

In all molecular biology, bacterial culture, cell culture and transformation, and protein purification procedures described here (and subsequently), sterile reagents and consumables were used and aseptic technique practiced, unless stated otherwise. Similarly, all procedures were carried out at room temperature (RT°), except where stated.

2.1.1 Patient and control selection

The great majority of subjects were recruited from the Ophthalmology Outpatients` Department (OPD), Queen's Medical Centre, Nottingham at various times between 1998 and 2001. Some healthy laboratory workers were also recruited as controls from the Department of Immunology, University of Nottingham. Subjects were assessed clinically and their patient records reviewed. They were assigned to uveitis patient or control groups accordingly. Subjects were given an information sheet about the study and informed written consent obtained. The study adhered to the principles of the Treaty of Helsinki and formal local ethics committee approval was obtained.

Patient recruitment was carried out with a view to specifically selecting subjects with autoimmune PSII (these are usually referred to simply as "uveitis" patients). Specifically, patients with primarily anterior uveitis or those with uveitis of suspected infectious aetiology were excluded. Subjects with ocular or systemic inflammatory disease were excluded from the control group. None of the healthy control laboratory workers had been involved in the preparation or extensive handling of any of the putative ocular autoantigens subsequently used experimentally. Recruitment was consecutive in both groups and children (under-16s) were excluded. Various data were collected at the time of blood sampling from patients and controls, including age, sex and diagnosis. Uveitis subtype (both clinical diagnosis and IUSG classification), current level of uveitic activity, duration of current inflammatory episode, total disease duration and details of any systemic immunosuppressive therapy (including steroids) were recorded for uveitis patients.

2.1.2 Materials

2.1.2.1 Bovine retinal S-antigen

Bovine RSAg had previously been prepared in the Dept. of Ophthalmology, University of Nottingham from fresh retinas, as previously described (Dua et al. 1994). Antigen had been quantified and stored at -70°C at a concentration of 8.7 mg/ml. Prior to use the antigen was analysed by SDS-PAGE gel electrophoresis and Coomassie Brilliant Blue staining (see below). It was found to be pure and free of contaminants.

2.1.2.2 Random peptide (phage display) libraries

The f88-4 linear and f88-4/Cys4 filamentous bacteriophage peptide display libraries (described later) were the kind gift of Professor George Smith, University of Missouri, USA.

2.1.2.3 Monoclonal antibodies

Monoclonal anti-polyhistidine (Sigma, Poole, UK), an unconjugated antibody (mouse IgG_{2a} isotype) with specific reactivity to sequences of 6 contiguous histidine residues - a polyhistidine or "His" tag - in recombinant proteins, was derived from clone His-1 and supplied as mouse ascites fluid.

Anti-HisG (Invitrogen, Paisley, UK) was an affinity-purified mouse monoclonal antibody against the epitope HHHHHHG, found on recombinant proteins expressed from the pCR-T7-NT-Topo plasmid.

Anti-XPress (Invitrogen) was an affinity-purified monoclonal antibody against the XPress™ epitope found on recombinant proteins expressed from the same plasmid.

All other materials and reagents were purchased from commercial suppliers, as indicated.

2.1.3 Peripheral blood sampling and processing

2.1.3.1 Blood sampling

Uveitis patient and control peripheral venous blood samples were collected at the Ophthalmology OPD or in the Department of Immunology, University of Nottingham. Serum samples were collected in sterile plain vacuum tubes (Becton Dickson, U.K.), clotted and centrifuged within 3 hours of sampling. Serum fractions were aspirated and stored in 2ml, sterile, screw top tubes at -70°C. Samples for extraction of peripheral blood mononuclear cells (PBMCs) were collected in 10ml, sterile vacuum tubes containing heparin. All were processed within 3 hours of sampling.

Some of the serum samples used in this work had previously been collected under similar conditions by Dr. D. Lioumi for use in a preliminary study in 1997 which led to the current study. Samples had been stored at -70°C and were seen to be in good condition. Patient and control clinical details were verified using medical records for each sample subsequently used.

2.1.3.2 Separation of PBMCs from peripheral blood

All tissue culture work was carried out using sterile consumables and reagents, under sterile conditions in a Class 2 flow hood. Heparinised peripheral blood samples were transferred to universal polystyrene tubes and diluted 1:1 with sterile phosphate buffered saline (PBS) (Oxoid, Basingstoke, UK). These were underlaid with 10mls of "Histopaque 1077" (polysucrose and sodium diatrizoate, density 1.070) (Sigma) and centrifuged at 870 g for 20 minutes – "density gradient sedimentation". The "buffy" intermediate layers of PBMCs were aspirated by pastette into fresh tubes and washed by resuspending the cells in 10 volumes of RPMI 1640 tissue culture medium (Gibco BRL, Paisley, U.K.). After gentle mixing, PBMCs were pelleted by centrifugation at 440 g for 10 minutes and supernatant was poured off. Cells pellets were resuspended in all tissue culture procedures by gently tapping the side of the tube and then diluting. A total of 3 washings were carried out. Before the 3rd round of centrifugation, 20 µl of suspension were taken to calculate cell concentration. This was carried out using a standard haemocytometer (Weber Scientific

International, Teddington, U.K.) and trypan blue (Sigma) for vital staining as previously described (Shapiro 1988). Cells were resuspended in either "complete" RPMI medium or "freezing medium", depending on whether they were to be used immediately or frozen and stored in liquid nitrogen (see below). Complete medium consisted of 10% heat-inactivated fetal bovine serum (FBS) (Sigma) in RPMI 1640 containing penicillin 100 U/ml, streptomycin 100 µg/ml, 2 mM L-glutamine (all Gibco BRL) and 5mM Hepes buffer (Sigma). Cells were resuspended to a working concentration of 2×10^6 PBMCs/ml.

2.1.3.3 Freezing, storage and thawing of PBMCs

Freezing medium consisted of 10% dimethyl sulfoxide (DMSO)(Sigma), 25% heat-inactivated foetal bovine serum (hiFBS) and 65% complete RPMI as described above. In later procedures this was modified to 10% DMSO and 90% hiFCS, in an effort to better protect the cells during the freezing/thawing procedure. Freezing medium was kept $<4^{\circ}\text{C}$ to reduce cell toxicity to DMSO. Cell pellets were resuspended to a final concentration of $5\text{-}10 \times 10^6$ PBMCs/ml and gently mixed. Cells were transferred quickly to pre-labelled cryovials (Nunc, Roskilde, Denmark) in 1 ml aliquots. Vials were placed in a Mr Frosty (Nalgene, UK) cryofreezing container containing isopropanol and immediately placed in a -70°C freezer for a minimum of 18 hours. Vials were then stored long-term in the vapour phase of a liquid nitrogen storage tank.

Cells were thawed rapidly by transfer of the vials from the liquid nitrogen storage chamber to a 37°C waterbath for a few seconds, and after gentle agitation, transferral to a universal tube on ice. 20ml of RPMI were added dropwise, to avoid osmotically shocking the cells. Cells were washed twice more, by pelleting and resuspension in complete RPMI medium, as described above. They were either used in assays straight after the thawing procedure, or alternatively were left to "recover" overnight at 4°C . Cell viability counts were performed before proceeding with the assays in either case.

2.1.4 Maintenance of bacteria and phage

2.1.4.1 Bacterial strains

Bacterial strains used and their specific requirements are described in the relevant sections.

2.1.4.2 Antibiotics

Tetracycline:

Tetracycline hydrochloride powder (Sigma) was dissolved in 50% v/v ethanol solution at 10 mg/ml. This was filter sterilised and stored at -20°C till used.

Ampicillin:

Ampicillin (Sigma) sodium salt was dissolved in dH_2O at 50 mg/ml, filter sterilised and stored at -20°C till used.

Carbenicillin:

Prepared from powder (Sigma) as per ampicillin.

Kanamycin:

Kanamycin was prepared from powder (Sigma) as a 100 mg/ml stock solution in dH_2O . Solution was filter sterilised and stored at -20°C .

Chloramphenicol:

Prepared from powder (Sigma) in dH_2O at 34 mg/ml and filter sterilised. Stored at -20°C .

2.1.4.3 Bacterial media

2.1.4.3.1 Liquid media

2.1.4.3.1.1 LB broth

10 g bactotryptone, 5 g yeast extract (both Sigma), 5 g NaCl (Fischer Scientific), diluted to 1 litre in dH₂O. pH adjusted to 7.4 with 1M NaOH and autoclaved (121°C, 15 psi) for 20 minutes in Duran autoclavable bottles (Schott, Germany). Stored in sealed bottles at RT° till used.

2.1.4.3.1.2 M9LB

10 mls 20x M9 minimal salts (Sigma) (autoclaved)
4 mls 20% glucose (Fischer Scientific) (filter sterilised)
0.2 mls MgSO₄ (Fischer Scientific) (autoclaved)
Made up to 200 mls with LB broth (autoclaved)

2.1.4.3.1.3 Terrific broth

47 g Terrific broth base (Sigma), 4ml glycerol per litre dH₂O. Autoclaved and stored as above.

2.1.4.3.2 Bacteriological agar

Bacteriological agars were melted by microwave oven and mixed. Media were cooled to 50°C and antibiotic or other additives mixed in as appropriate. Molten agar was poured into sterile 90 mm single-vent Petri dishes (Sterilin, UK), approximately 20 mls per plate. Agar was left to solidify for 30 minutes. Plates were dried before use by inverting them and placing them in an incubator at 37°C for 2 hours or on the bench at RT° overnight. Below are recipes of agars used in the following experiments.

2.1.4.3.2.1 *LB agar*

14 g bacteriological agar (Sigma)
LB broth base 1 litre
Autoclaved, stored as above.

2.1.4.3.2.2 *Minimal agar*

(per 200 mls)

100 mls of 2x M9 minimal salts

95.2 mls of 3% agarose

0.4 mls 1M MgSO₄

0.02 mls 1M CaCl₂ (Fischer Scientific)

Autoclaved as described above. Molten agar let cool to 50°C and 4 mls 20% glucose and 0.2 mls 10 mg/ml thiamine (Sigma)(both filter sterilised) added and mixed. Poured into plates while still molten and set.

2.1.4.3.2.3 *Top agarose*

(per 100 mls)

1 g tryptone

0.5 g yeast extract (both Sigma)

0.5 g NaCl

0.6 g agarose

0.1 g MgCl₂·6H₂O (Fischer Scientific)

Diluted to 100 mls in dH₂O and autoclaved.

2.1.4.4 Growth of bacteria in liquid media

All incubations in liquid media of bacteria and phage were carried out in a shaking incubator at 225 revolutions per minute (RPM) at 37°C. Incubations on solid media were carried out at 37°C or at RT° where noted. Colonies were grown de novo from commercially supplied or previously frozen glycerol stocks. Stocks were melted and a flame-sterilised loop dipped into the stock tube. This was streaked several times on an agar plate, containing appropriate antibiotic

where necessary. The plate was incubated overnight at 37°C, and well-defined colonies identified next day. One colony was picked with a sterile loop, used to inoculate 5 mls of appropriate liquid medium and incubated overnight. Overnight culture was used to (1) generate reserve glycerol stocks by mixing 50% cells with sterile glycerol in a 1.5 ml Eppendorf tube and storing at -70°C (2) to streak out on an agar plate to grow a ready supply of colonies for medium term use and (3) to directly inoculate fresh medium/antibiotic to grow an immediate culture of mid-log cells. Subsequent cultures were inoculated from well-defined colonies on the stock agar plate.

2.1.4.5 Monitoring growth of cultures

Bacterial cells need to be in the logarithmic growth phase ("mid-log" cells) for several specific applications, particularly amplification of phage. This is because mid-log cultures contain substantial numbers of cells that are dividing rapidly, but which have not yet developed rigid cell walls, and are therefore easily infected by phage. Depending on how rapidly cells were needed, medium was inoculated with 1:20 to 1:200 of overnight cells. Cells were incubated as before and most cultures reached mid-log within 2-3 hours. 100 µl culture samples were taken at intervals and transferred to cuvettes containing 900 µl medium. Absorbances of 1:10 dilutions were measured at 600 nm using a CE272 spectrophotometer (Cecil Instruments, Cambridge, UK). Cells were considered to have reached mid-log phase when optical density (OD) of (undiluted) cultures reached 0.5 to 0.8.

2.1.4.6 Storage of bacterial strains

Primary supplies of bacterial strains or secondary amplified (see above) glycerol stocks were maintained at -70°C for long term storage. Frequent freeze-thaw cycles of original stocks were avoided where possible. For short-term maintenance of bacterial colonies, agar plates were sealed with Parafilm (Pechiney Plastic Packaging, USA), inverted and stored at 4°C for up to 1 month.

2.1.4.7 Amplification of phage

Bacterial host cells were grown to mid-log phase at 37°C. For cells containing an F-pilus the shaker was slowed down briefly to allow regeneration of sheared pili. Cells were infected by direct introduction of phage into culture and incubation continued. Lytic phage strains (e.g. T7) usually took 1-2 hours to lyse their host, which was detected by a dramatic reduction of culture density and the appearance of strands of cellular debris. Non-lytic strains (e.g. M13) were incubated for a further 3 hours after infection. Where necessary lysates could be clarified by centrifugation, with phage particles remaining in the supernatant. Phage titres were usually expressed in terms of plaque forming units (PFUs) rather than total number of particles, as the titre of *infective* phage in a given library was functionally more relevant.

Phage were grown on agar plates as plaques (clones). Phage were first mixed with 200 µl mid-log bacterial cells for 5 minutes in a sterile Bijou tube (Sterilin). 5 mls of top agarose, which had been melted and allowed to cool to below 50°C, was added and mixed. This was immediately poured onto a pre-warmed agar plate and distributed evenly by tilting the plate. Top agarose was let solidify for 10 minutes, the plate inverted and incubated at 37°C for several hours or else at RT° overnight. Phage growth was detected for both lytic and non-lytic strains by the appearance of clear plaques on the bacterial lawn. This method was also adapted to estimate phage titres in a solution (see later).

2.1.4.8 Maintenance of phage

Amplified phage were maintained long-term either by purification through PEG precipitation (see below) and storage in phage storage solution, or else maintenance in the original growth medium containing lysed bacterial cells with NaN₃ added to 0.02%. The latter method was found to be at least as effective and perhaps even better for preserving phage titres and number of clones. Long-term storage was at 4°C in both cases.

2.1.4.8.1 Bacteriophage storage buffer

(per 500 mls)

2.9 g NaCl

1.0 g MgSO₄·7H₂O

25 mls 1M Tris-HCl, pH 7.5

2.5 mls 2% gelatin

Diluted to 500 mls with dH₂O and sterilised.

2.1.4.9 PEG precipitation of phage

Phage were sometimes needed in purified form and were isolated from lysates by precipitation. 20 ml lysates were clarified by centrifugation and the supernatant mixed with 1/6 volume of 20% w/v polyethylene glycol (PEG) – 8000 (Sigma), 2.5 M NaCl. The mixture was incubated overnight at 4°C. Precipitated phage were pelleted by centrifugation at 10,000 RPM at 4°C for 15 minutes and the supernatant removed. The pellet was resuspended in 1 ml PBS, then centrifuged in a 1.5 ml tube for 5 minutes, to pellet residual bacterial cells. The supernatant was transferred to a new tube, and again mixed with 1/6 volumes PEG/NaCl. This was incubated on ice for 2 hours and centrifuged as before. Supernatant was removed and the purified phage pellet resuspended in 200 µl PBS/0.02% NaN₃ for short-term use, or phage storage buffer for long-term maintenance. The protocol was adjusted pro rata for larger volumes of phage lysates.

2.1.5 Miscellaneous general methods

2.1.5.1 Polymerase chain reaction (PCR)

PCR was used to amplify specific segments of DNA using small amounts of template from various sources (e.g. bacteriophage, plasmids, RNA, cells, etc). Reactions were carried out in sterile 0.2 ml wells, either in 96-well PCR plates (Elkay Lab Products, UK) or individual tubes (Alpha Lab Products, UK). A typical 25 μ l PCR mixture was set up as follows: 16.75 μ l PCR grade H₂O (Invitrogen), 5 μ l of 5x Buffer B (300 mM Tris-HCl pH 8.5, 75 mM (NH₄)₂SO₄, 10 mM MgCl₂) (GibcoBRL), 1 μ l of 5 mM deoxynucleotide triphosphates (dNTPs) (Invitrogen), 0.25 μ l Tween 1% (Sigma), 0.5 μ l each of 20 μ M forward and reverse primers (MWG-Biotech AG, UK, unless stated otherwise) and 1 μ l (0.5 U) "Platinum Taq" DNA polymerase (GibcoBRL). Template DNA was introduced directly into each well, in either cellular or liquid form. Larger or smaller reaction mixes were produced pro rata. Plates were covered with foil (Eurogentec, UK), heat-sealed and placed in a Hybaid Omnigene thermal cycler. PCR conditions varied for individual applications and between 30 and 40 cycles of amplification were carried out. Successful amplifications were confirmed by agarose gel electrophoresis (below).

2.1.5.2 Ethanol precipitation of DNA

A Micromax RF temperature-controlled, benchtop microcentrifuge (IEC, USA) was set to 4°C. 0.1 volumes of 3M sodium acetate (Fischer Scientific, UK) pH 5.2 and 2 volumes of ice-cold 100% ethanol (BDH Laboratory Supplies, UK) were added to a dilute solution of DNA in a 1.5 ml Eppendorf tube (Treff Lab, Switzerland). The sample was vortexed and DNA precipitated on ice for 1 hour. DNA was pelleted by centrifugation at 15,000 RPM for 15 minutes, after which supernatant was carefully aspirated by suction. The DNA pellet was rinsed with 500 μ l 70% ethanol (in sterile dH₂O), re-centrifuged and the ethanol once again aspirated off. The pellet was left to air dry for a few minutes, before being resuspended in an appropriate volume of nuclease free water. Where it was necessary to work with smaller volumes during precipitation, 1 volume of isopropanol (BDH) was used instead of the 2 volumes of ethanol. In addition,

4M ammonium acetate (Fischer Scientific) (1:1 volume with DNA solution) could be added with 5 volumes ethanol, instead of sodium acetate. This was used where purer DNA samples were required, even though overall DNA yield would be reduced.

2.1.5.3 Quantification of DNA by fluorimeter

Fluorimetry was used for quantification of DNA. A Hoefer TKO 100 DNA Fluorimeter (Hoefer Scientific Instruments, San Francisco, USA) was pre-heated 30 minutes prior to measurement and the "scale" reading set to maximum. 2 mls of x1TNE buffer with Hoechst 33258 dye (Hoechst) at 0.1 $\mu\text{g/ml}$ in a clean cuvette was used to "zero" the scale. 2 μl of calf thymus DNA (Sigma) 200 $\text{ng}/\mu\text{l}$ was added to the cuvette and mixed well. The scale was set to 200 $\text{ng}/\mu\text{l}$. 2 μl of the test DNA solution was then added into the same cuvette, mixed and the difference between readings noted. Concentration of the test DNA sample was recorded in $\text{ng}/\mu\text{l}$.

2.1.5.4 Extraction of plasmids from bacterial culture by "Mini-Prep"

Extractions of plasmids from bacterial cell cultures of up to 5 mls were done using the Concert™ Miniprep kit (GibcoBRL). An overnight culture of plasmid-containing cells was pelleted, and all growth medium aspirated off. 210 μl of cell suspension buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, RNase A 20 mg/ml) was added to the pellet and resuspended. 210 μl of cell lysis solution (200 mM NaOH, 1% SDS w/v) was added to the cells, mixed gently by inversion (to release plasmid DNA while keeping most genomic DNA attached to the cell membrane) and incubated at RT° for 5 minutes. The mixture was neutralised by adding 280 μl neutralization buffer (proprietary formulation containing acetate and guanidine hydrochloride) and again mixing gently by inversion. The mixture was centrifuged at 12,000 g for 10 minutes, to precipitate out insoluble matter including chromosomal DNA. The supernatant (containing plasmids) was then loaded into a spin cartridge, which contains a silica-based membrane that selectively absorbs plasmid DNA. This was centrifuged at 12,000 g for 1 minute, the flow through being collected in a tube and discarded. The membrane was

washed with 500 μ l wash buffer (proprietary formulation containing acetate, guanidine hydrochloride, ethylenediaminetetraacetate (EDTA) and ethanol) and centrifuged. It was then washed with 700 μ l of another wash buffer (proprietary formulation containing NaCl, Tris-HCl pH 8.0, EDTA and ethanol) and centrifuged once more as above. The membrane was centrifuged again for 1 minute to remove any residual wash buffer and 75 μ l of TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA), pre-heated to 70°C, directly applied to the centre of the membrane. After 1 minute incubation at RT°, the cartridge was centrifuged at 12,000 g for 2 minutes and the eluted plasmid solution collected in a sterile 1.5 ml recovery tube. Samples were stored at -70°C till used.

2.1.5.5 Agarose gel electrophoresis

Agarose gels were prepared by adding Tris-borate-EDTA (TBE) buffer (Sigma) to molecular biology grade agarose (Eurogentec, UK) in a 200 ml Erlenmeyer flask, to a final concentration of 1 or 2% agarose w/v. Agarose solution was boiled in a microwave oven and mixed till thoroughly dissolved and free of bubbles. The solution was let cool till "hand hot" (<60°C). Ethidium bromide (Sigma) was added to a final concentration of 0.5 μ g/ml and mixed. Liquid gel was poured into a horizontal plastic electrophoresis mould with its ends sealed with masking tape and distributed evenly to ensure a uniform depth of 5 mm. A comb containing the eventual number of wells was immediately inserted into the liquid gel, ensuring that there was at least 1 mm clearance between the tip of the comb's teeth and the bottom of the mould. The gel was let set at RT° till solid (usually 30 minutes), and the autoclave tape removed from the ends of the mould. The gel/mould was transferred to an electrophoresis tank (Bioscience Services, UK) and TBE buffer was added till the gel was submerged by 1mm. The comb was then removed.

DNA samples were mixed with 0.1 volumes of loading buffer (20% Ficoll 400 (Pharmacia), 0.25% bromophenol blue and 0.25% xylene cyanole FF (both Sigma) in H₂O) and then loaded into individual wells. DNA standards used were either 100 base pair (bp) DNA Ladder (Biorad, UK) for up to 1500 bp, or Smartladder (Eurogentec) for up to 10,000 bp. The lid (containing electrodes) was then fitted onto the gel tank, with the anode orientated so that the DNA will migrate towards it. Electric leads were checked for correct positioning and a

constant voltage of approximately 5 V/cm (distance between electrodes) applied across the gel. Migration of the DNA in the right direction was confirmed by observation of the bromophenol blue dye migrating from the wells into the main body of gel. Gels were run till the dye front was 80-90% towards the end. Prolonged electrophoresis was avoided to prevent excessive migration of ethidium bromide out of the gel (moves towards the cathode). The gel was let cool for several minutes and examined under an ultraviolet illuminator. The gel and its fluorescent DNA bands were photographed where appropriate.

2.1.5.6 Dot blot assays

Dot blots make use of specific antigen/antibody interactions and can be used to demonstrate the presence of either in a test solution. Depending on whether the composition of the test antigen or the antibody (or antiserum) solution is known, dot blots can be used to detect the presence of a particular antigen in blotted protein sample or cell lysate, or the presence of antibody specific for a particular antigen in a test solution. They can also be used to detect specific peptides displayed on the surface of bacteriophage e.g. in a phage display library.

Protein solutions (either purified antigen or test samples) were spotted onto pieces of nitrocellulose membrane in volumes of approximately 1 to 10 μ l and let air-dry for 20 minutes. Nitrocellulose, which binds non-specifically to proteins, was supplied as a roll of membrane with matrix support (Hybond C- Amersham Pharmacia Biotech, U.K.) or as discs, each the size of a standard agar plate (Millipore, U.K.). After blotting, non-bound sites on the membrane were blocked by incubating the membrane in a PBS /polyoxyethylenesorbitan monolaurate 0.1% [Tween 20 – Sigma] /5% Bovine Serum Albumin [BSA-Sigma] (PBST/BSA 5%) solution on a rocking platform at RT° for 1 hour. Once wet, membranes were not let dry out till after development was completed. Membranes were incubated with antibody solution (known or unknown) diluted in PBS, either in the form of purified antibody or serum dilutions, again for 1 hour at RT°. For direct probing of His-tagged recombinant proteins, horseradish peroxidase (HRP) conjugated anti-His monoclonal antibody (Sigma) was used, at a dilution of 1:1000 in PBS. Membranes were washed 4 times x 15 minutes in PBS/Tween 0.1% (PBST).

Primary antibody probed blots were incubated with secondary, enzyme-conjugated antibody in PBS, at a dilution of 1:5000, for 1 hour. Secondary antibodies were either alkaline phosphatase (AP)-conjugated, goat anti-human IgG or IgGAM (both Sigma) as appropriate. Membranes were washed again for 1 hour and finally incubated with substrate compatible for the antibody-conjugated enzyme. Substrates were 0.15 mg/ml 5-bromo-4-chloro-3-indolyl phosphate / 0.3 mg/ml nitro blue tetrazolium, 100 mM Tris buffer pH 9.5, 5 mM MgCl₂ (BCIP-NBT) (Sigma) for AP and liquid 3, 3'-diaminobenzidine tetrahydrochloride (DAB) (DAKO, U.K.) for HRP. After 5-10 minutes, membranes developed using the HRP-DAB system were washed in dH₂O and any spots containing specific antigen for the antibody solution were identified by the presence of insoluble dark brown precipitate. Development using the AP-BCIP/NBT system took up to 30 minutes and positive spots were revealed by the formation of a purple precipitate.

2.1.5.7 Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE), for the separation of protein fragments of various sizes into bands, was carried out under denaturing and reducing conditions using pre-cast Novex mini-gels and electrophoresis equipment (Invitrogen). Some samples were analysed using standard tris-glycine gels while others were studied using the newer NuPage™ bis-tris gel system.

Tris-glycine gels used were 8 cm x 8 cm, 1 mm thick, contained between 10 and 15 individual wells and contained stacking gel. The gel matrix consisted of acrylamide and bis-acrylamide, higher concentrations of the latter being used for analysis of smaller proteins. Wider ranges of protein sizes were analysed with gels with differential bis-acrylamide concentrations e.g. 10-20%. Protein samples for electrophoresis were prepared as a 50:50 mixture with 2x sodium dodecyl sulphate (SDS) sample buffer (25% v/v 0.5M Tris-HCl pH6.8, 20% v/v glycerol, 4% SDS w/v and 0.005% v/v bromophenol blue) (Invitrogen) with 5% v/v β-mercaptoethanol (Sigma) as reducing agent. 12 µl of each sample was prepared, heated to 95°C for 3 minutes, cooled on ice and centrifuged at maximum speed to pellet insoluble debris. Mark 12™ (Invitrogen) unmarked protein standards were prepared as per test protein samples, whereas

MultiMark™ (Novex, UK) multi-coloured standards were loaded directly into their wells (10 µl/well). Gel cassettes were removed from their storage pouches and packaging buffer rinsed off with dH₂O. The comb was removed from the top of the cassette (for contact with the upper, cathode buffer chamber) and tape peeled of the bottom slot (for lower chamber). Cassettes were inserted into the XCell SureLock™ (Invitrogen) electrophoresis tank with the exposed wells facing the inner (upper) chamber. Upper and lower chambers were filled with 1x SDS tris-glycine running buffer (SDS 1 g/L, Tris base 2.9 g/L pH 8.3 (both Sigma), glycine (BDH)14.4 g/L,) till wells were covered. Wells were rinsed out with buffer using a pastette, and 10 µl of each sample supernatant was carefully loaded using a 200 µl pipette. 1x sample buffer was loaded into unoccupied wells and the lid and electrodes fitted onto the electrophoresis tank. Gels were run at a constant voltage of 125 V for 90 minutes or until the bromophenol blue dye-front reached the bottom of the gel. Cassettes were split open and the gels carefully removed for subsequent analysis (see below)

NuPage bis-tris gels were also used because they may produce sharper bands under certain conditions. The 2 alternative denaturing running buffers, commercially supplied by Invitrogen, were MES-SDS (2-[N-morpholino] ethane sulfonic acid 50 mM, Tris base 50 mM pH 7.3, SDS 3.465 mM, EDTA 1.025 mM), and MOPS-SDS (3-[N-morpholino] propane sulfonic acid 50 mM, Tris base 50 mM pH 7.7, SDS 3.465 mM, EDTA 1.025 mM). Different band migration patterns are seen when running identical gels with one or other buffer; MES buffer provides greater resolution for lower molecular weight proteins, while the converse is true for MOPS buffer. Protein test sample or size standards were prepared by mixing 7.5 µl protein solution with 2.5 µl 4x NuPage LDS sample buffer (lithium dodecyl sulfate (LDS) 0.8 g, glycerol 4.0 g, Tris base 0.682 g, Tris-HCl 0.666 g, EDTA 0.006 g, 0.75 mls of Serva Blue G250 1%, 0.25 mls of Phenol Red 1%, total volume 10 mls at pH8.5) (Invitrogen). 1.1 µl of 10x NuPage Reducing Agent (0.5M liquid DTT) (Invitrogen) was mixed with the sample, which was then heated to 70°C for 10 minutes. Each 11.1 µl sample was centrifuged and cooled as described above. NuPage gel cassettes were prepared for the electrophoresis tank essentially as described above and 600 mls of running buffer poured into the lower

compartment. 500 μ l of NuPage Antioxidant (Invitrogen, proprietary formula) was added to another 200 mls of running buffer, and this was loaded into the upper buffer chamber. Antioxidant was used to prevent re-oxidation of reduced proteins during electrophoresis. 10 μ l of test sample or standard supernatants were loaded into each well and gels were run at a constant 200 v. Running time was approximately 35 minutes for MES buffer and 50 minutes for MOPS.

2.1.5.8 Coomassie Brilliant Blue gel staining

Coomassie Brilliant Blue dye binds with high affinity to protein bands separated by PAGE, and after de-staining of the gel reveals clear bands. After removal from cassettes, gels were transferred to fixative (45% v/v methanol, 10% v/v glacial acetic acid (both Fischer Scientific)) and incubated on a rocking platform for at least 1 hour. Coomassie solution was prepared by dissolving 0.25g of dye powder (Sigma) in 100 mls of fresh fixative. Gels were stained for at least 4 hours and then de-stained by removing the dye solution and replacing it with 50 mls of fixative (same volume used in subsequent steps). Fixative was replaced at least 4 times over 4 – 8 hours, till the gel backgrounds became clear again. More rapid de-staining could be achieved by using a solution of 30% methanol, 10% glacial acetic acid but could potentially result in band contrast deterioration. Gels were either photographed immediately or stored in a 20% glycerol (Courtin & Warner, UK) with 0.002% sodium azide (Sigma) solution.

2.1.5.9 Silver staining

Silver staining of protein bands after PAGE was also carried out on occasion, because of the greater sensitivity (but also possibly higher background) of this technique over Coomassie staining. Gels were fixed for at least 1 hour in a solution of methanol 500 ml/L, glacial acetic acid 120 ml/L and 37% formaldehyde (Fisons Scientific, UK) 0.5 ml/L. 50 mls of solution were used in this and subsequent steps. Gels were then washed in 50% ethanol for 1 hour, changing the solution every 20 minutes. Gels were incubated in "pretreatment" solution (sodium thiosulfate (Sigma) 0.2 g/L) for 1 minute and rinsed in ultrapure dH₂O 3 times for 20 seconds. They were then impregnated with silver by incubation for 20 minutes in a solution of silver nitrate (Sigma) 2g/L, 37% formaldehyde 0.75 ml/L. After rinsing twice more in dH₂O for 20 seconds, gels

were placed in a developing solution (sodium carbonate (Sigma) 60g/L, 37% formaldehyde 0.5 ml/L, sodium thiosulfate 4 mg/L). The rate of development was watched closely and when protein bands became distinct the reaction stopped by incubation in "stop" solution (50% methanol, 12% glacial acetic acid). Gels were rinsed in 50% methanol, and either photographed immediately or stored in 20% methanol solution.

2.1.5.10 Western blotting

Western blotting is used to transfer protein bands that have already been separated on the basis of size by PAGE, onto membranes where they can be probed with specific antibodies. For each polyacrylamide gel, 6 layers of QuickDraw Extra Thick™ blotting paper (Sigma), 2 layers of Whatman 3MM CHR chromatography paper (Whatman, Maidstone, UK) and 1 layer of Hybond-C nitrocellulose membrane were cut to the size of the body of the gel. Gels for Western blotting were run with Multimark coloured standards in their 2 outer lanes. 3 layers of Whatman blotting paper were soaked in 1/2 x Tris-Borate-EDTA (TBE)(Sigma) transfer buffer and aligned vertically on the anode surface of a semi-dry blotter (Sigma), making sure that no air bubbles were trapped between layers. One layer of wetted light blotting paper followed by nitrocellulose membrane were placed on top of the Whatman blotting paper. Cassettes containing polyacrylamide protein gels were split open and orientated facing upwards. Top and bottom edges of gels were cut off with a sharp knife, gels lifted and placed face-up on the nitrocellulose membranes. Positions of the coloured protein bands were marked onto membranes with pencil and gaps between membranes and gels filled in with insulating strips of Parafilm. Wetted layers of light blotting paper (x1) and Whatman paper (x3) were then placed over each gel. The lid (cathode) of the semi-dry blotter was fitted so that it was firmly in contact with each stack, while maintaining the stack's vertical alignment. Electrodes were fitted so that current flowed downwards, causing protein molecules to migrate onto the membranes. Constant current of 1 mA/cm² was applied across each gel and maintained for between 2 hours and overnight.

After transfer, stacks were disassembled and membranes briefly washed in PBS. Membranes were blocked for 1 hour at RT° in PBST/BSA 5%.

Membranes were washed briefly in PBST and then probed by incubation with specific antibody, as per dot blotting (above). Where His-tagged proteins were being analysed, anti-His monoclonal antibody conjugated to HRP, diluted to 1:1000 in PBS, was used. Development of membranes was as described for dot blotting.

2.1.5.11 Photography

DNA agarose gels were photographed using a Polaroid MP4 Land Camera with Polaroid 667 film (both Polaroid, USA). Camera settings were f4.5 – f11, for 1 second. DNA bands were visualised on an ultraviolet transilluminator (UVP, USA).

Coomassie and silver stained polyacrylamide gels were photographed on a white lightbox (Jensons Scientific, UK) using a Fujifilm FinePix SI Pro digital mounted camera (Fujifilm, UK) and processed using SI Pro Shooting Software (Fujifilm) software. Images were cropped and orientated using Adobe Photoshop 6.0 (Adobe).

2.1.5.12 Bicinchoninic acid (BCA) protein assay

This was used to quantify the total protein content of various crude and purified protein preparations. Proteins reduce acidic Cu^{2+} to Cu^+ in a concentration dependent manner. Cu^+ forms an intense purple complex with BCA and the OD of each sample can be measured using an ELISA plate reader.

BCA solution was prepared by mixing 160 μl 4% w/v $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ with 8 mls of bicinchoninic acid (both Sigma). Protein standards were prepared in 2-fold dilutions in sterile PBS, from 1mg/ml BSA (Sigma) down to 1.95 $\mu\text{g}/\text{ml}$. 190 μl of BCA solution were pipetted into duplicate wells of a 96-well, Maxisorp ELISA plate (Nunc), for each of the 10 standards and test protein solutions. 2 negative control wells were filled with 200 μl BCA. 10 μl of each standard or test solution were mixed into their respective wells without delay, the plate sealed with cling-film and incubated at 60°C for 60 minutes. Plates were let cool to RT° and ODs read at 550 nm on a Dynatech plate reader. A standard curve of mean standard readings was formed and best fit applied. Concentrations of test protein solutions were calculated from this.

2.2 Production of recombinant human retinal S antigen

Recombinant human RSAg was cloned and expressed in two forms: human antigen expressed in human cells and human antigen expressed in a bacterial host. Both methods are described below.

2.2.1 Human RSAg expressed in eukaryotic cells

2.2.1.1 Purification of human RNA

Special precautions (e.g. use of gloves, sterile polypropylene tubes, diethyl pyrocarbonate (DEPC) treated glassware, etc) were taken to avoid ribonuclease contamination at all stages of this and all subsequent procedures involving RNA. All samples containing RNA were disrupted in lysis buffer such as RLT buffer (proprietary formula, contains guanidine isothiocyanate)(Qiagen, Hilden, Germany) containing 10 μ l/ml β -mercaptoethanol, to minimise degradation by endogenous ribonucleases.

Human total RNA from a consented donor eye neuroretina preparation was used as template for the amplification of the human RSAg sequence. Total RNA was purified using the RNeasy extraction kit (Qiagen). RNeasy purification columns work through the selective binding and then elution of RNA to a (patented) silica gel based membrane. The sample was homogenised completely by 2 rounds of centrifugation through shredder spin columns. Homogenate was mixed with 5 mls of 70% ethanol in DEPC H₂O. It was then loaded into several RNeasy purification columns, 700 μ l at a time. Columns were centrifuged at >10,000 RPM for 15 seconds and flow-through discarded. Contaminants were washed away with 700 μ l and 500 μ l (twice) of 2 separate proprietary wash buffers. Total RNA was eluted by pipetting 50 μ l of nuclease-free H₂O onto the centre of the membrane, and centrifugation of the eluate into a 1.5 ml collection tube. The RNA was stored at -70°C till used.

2.2.1.2 Production of RSAg DNA by PCR

Total RNA was measured by fluorimetry and 5 µg used as template for a reverse transcription PCR reaction (RT-PCR) in an Amersham Ready-to-Go reverse transcription tube (Amersham BioSciences, UK) according to the manufacturers instructions. The 33 µl reaction was incubated for 1.5 hours at 42°C, to convert the RNA into single-stranded DNA (ssDNA), and then the enzyme inactivated by incubation at 70°C for 10 minutes. PCR primers to amplify human RSAg were designed to have ends compatible for ligation into EcoR1 and Not1 restriction sites, and the stop codon of RSAg was removed in the reverse primer sequence, which allowed translation through the affinity tag sequences within the vector. The forward primer (including EcoR1 site) was 5`-ATG GAA TTC TAG AGA CCC TCT CCT TGC CA-3`, the reverse primer (Not1) was 5`-TGC GAT CGC GGC CGC TCA TCA GCG TCA TTC TTG TC-3`.

PCR reactions were set up using 0.5 µl (of 20 µM stock) of each primer and 1 µl of the reverse-transcription reaction mix. A standard 25 µl PCR reaction was used as described previously, using the same forward and reverse primers as above with the single alteration of using Elongase DNA polymerase mix (Life Technologies Ltd, Paisley, Scotland) for improved fidelity of copying.

2.2.1.3 Ligation of DNA into a eukaryotic compatible vector.

The pcDNA4-TO-myc-HisA vector (Invitrogen), a 5151 bp plasmid, was used as the vector for cloning the RSAg gene sequence into mammalian cells. This plasmid contains multiple cloning sites, including EcoR1 and Not1 restriction sites, a resistance gene to the antibiotic Zeocin™ (phleomycin)(Invitrogen) and a carboxyl-terminal polyhistidine tag sequence. It contains a hybrid promoter consisting of the strong cytomegalovirus (CMV) immediate-early promoter and 2 x tetracycline operator (Tet O) sites that allows high levels of tetracycline-regulated protein expression. Control of the reading frame orientation is a feature of this plasmid.

PCR products were purified enzymatically before restriction digestion and ligation into the plasmid. 10 µl PCR product was mixed with 2 µl 10x One Phor All (OPA) buffer (Pharmacia, USA), 1 µl (1U) shrimp alkaline phosphatase (SAP) and 1 µl (5U) exonuclease 1 (both USB, Cleveland, USA), and 4 µl pure

H₂O. Excess oligonucleotides, primers and phosphates were enzymatically degraded by incubation at 37°C for 20 minutes, followed by inactivation at 85°C for 20 minutes. After brief centrifugation, 1 µl (20U) of EcoR1 and 1 µl (20U) of Not1 (both USB) were added to the mix and incubated at 37°C for 90 minutes and the reaction cleaned up in a Qiagen spin column as previously described. Cut PCR product was eluted in 30 µl of PCR-grade H₂O

Plasmid was restriction digested as follows: 10 µl (100 ng) pcDNA4-TO-myc-HisA plasmid was mixed with 2 µl 10x OPA buffer, 1 µl EcoR1, 1 µl Not1 and 6 µl H₂O. This was incubated at 37°C for 1 hour and the reaction cleaned up in a Qiagen spin column as previously described. Plasmid was eluted in 30 µl of PCR-grade H₂O.

A PCR/plasmid ligation reaction was then set up. 5 µl digested PCR product was mixed with 1 µl cut plasmid solution, 0.5 µl (5U) T4 DNA ligase, 1 µl 10 mM adenosine triphosphate (ATP) (both Novagen, UK) and 1.5 µl H₂O. This was incubated at 12°C for 12 hours. The ligase was inactivated by heating to 70°C for 10 minutes and the reaction allowed cool slowly to RT°. 1 µl of reaction was analysed by agarose gel electrophoresis, to ensure successful ligation. The correct sequence for RSAg was verified by carrying out a PCR using the specific primers (pcDNA4mha) 5'-CCTCCGGACTCTAGCGTTTA-3' and (pcDNA4mhb) 5'-TCTTCTGAGATGAGTTTTTGTTCG-3', followed by DNA sequencing (see later). The remainder was stored at -70°C till used.

2.2.1.4 Growth of TREx 293 human cells

TREx 293 is a human embryonic kidney cell line. It contains the pcDNA6/TR plasmid, which stably expresses a tetracycline repressor protein (TetR) and therefore allows controllable expression of recombinant protein when used together with the pcDNA4-TO-myc-HisA plasmid. pcDNA6/TR also confers resistance to the antibiotic blasticidin. They can be used for transient or stable transfections.

The TREx 293 cells for transfection were thawed and re-seeded 4 days before the procedure. Briefly, the supplied cryovial containing the 3x10⁶ supplied cells, was removed from liquid nitrogen storage and thawed quickly in a 37°C waterbath. Cells were transferred to a Falcon T-75 (75 cm²),

flat-bottomed cell culture flask (Becton Dickson) containing 12 mls of "complete medium" (without blasticidin). Complete medium consisted in this case of Dulbecco's Modified Eagles Medium (DMEM) High Glucose (Life Technologies), 10% pre-filtered FBS, 1% penicillin/streptomycin solution and 2mM L-glutamine (both Invitrogen). This was placed in a humidified incubator at 37°C and 5% CO₂ to allow the cells to adhere to the bottom of the flask. After 4 hours medium was aspirated and replaced with fresh complete medium, again without antibiotic. After overnight incubation, again under the same conditions, medium was aspirated and replaced with complete medium with blasticidin 5µg/ml (CMB). Incubation was continued and the cells checked daily till they reached 80-90% confluence – approximately 3 days.

Cells were then "passaged" once, one day before the transfection procedure. All medium was removed from the flask and cells were washed once with 10mls PBS (serum inhibits trypsin). 5 mls of trypsin-EDTA solution (Invitrogen) were added to the monolayer and incubated at room temperature (RT°) for 5 minutes. 5 mls CMB was added to inactivate the trypsin and cell clumps broken up by aspiration. Cell concentration was determined using a haemocytometer and trypan blue stain, as described above. 3x10⁵ cells were added to each 35mm well of a 6-well culture plate and made up to 1 ml with CMB. To maintain the cell line, 1 out of the 10 mls of cell suspension were transferred to a fresh T-75 flask and 15 mls of CMB added. Both culture plates and cells were incubated at 37°C as before – the first passage. After 24 hours the cells in the 6-well plate had reached 70-80% confluence and were ready to be transfected.

2.2.1.5 Transfection of plasmids into eukaryotic cells

Before transfection, the pcDNA4-TO-myc-HisA plasmid containing the sequence insert for human RSAg was precipitated and resuspended in nuclease free water, to rid the DNA of any contaminants. DNA concentration was determined by fluorimetry. Final plasmid DNA concentration was 400ng/µl. Transient transfections using uncut plasmid were carried out first. After they had been shown to be successful, stable cell lines were established by transfecting with cut plasmid - which was capable of integrating permanently into the host

cells genome. The basic procedure used is described below, with differences between transient and stable transfection methods pointed out where necessary.

100 μ l of DMEM at RT $^{\circ}$, serum and antibiotic free, was pipetted into each of 4 polystyrene tubes (12 x 75 mm, Elkay Lab Products, Basingstoke, UK). 6 μ l of GeneJammer Transfection Reagent (Stratagene) was pipetted directly into the medium and incubated at RT $^{\circ}$ for 5 minutes. 3 different amounts of either cut or uncut plasmid DNA – 1 μ g, 2 μ g or 3 μ g– were added directly to each of 3 tubes and mixed gently. The final tube received no DNA and acted as a negative control. The mixtures were incubated for a further 5 minutes at RT $^{\circ}$.

Growth medium was gently aspirated from the wells of the 6-well plate and replaced with 900 μ l CMB. Each of the 4 transfection mixtures was added dropwise into a pre-labelled well, while gently rocking the plate. The plate was incubated for 3 hours at 37 $^{\circ}$ C, then topped up with another 1 ml of CMB and incubated for a further 21 hours.

2.2.1.6 Maintenance and expansion of transfected cell cultures

At this stage cells undergoing transient transfection were induced with tetracycline (see below). Wells containing stably transfected cells were trypsinised and split 1:5 in CMB, as previously described. Each new 6-well plate contained 5 wells containing cells transfected with a particular concentration (1, 2 or 3 μ g) of plasmid DNA, while the last well contained the cells "transfected" with no plasmid (negative control). Wells were topped up with CMB to a final cell volume of 2.5 ml and incubated again for a further 24 hours.

Selection for cells stably transfected with the pcDNA4-TO-myc-HisA plasmid was then carried out by adding Zeocin to the culture medium i.e. 48 hours after transfection. The growth medium was partially replaced with Zeocin in CMB, giving a final concentration of 400 μ g/ml. Cells were then continuously incubated at 37 $^{\circ}$ C, replacing the medium/Zeocin every 3-4 days, while inspecting the wells for the appearance of colonies by direct visualisation and by microscope. Some colonies were noted 12 days post-transfection and by 18

days were large enough (though still separate) to be re-seeded in individual wells as individual clones.

12 candidate colonies with round edges and even growth patterns were identified for re-seeding by marking the underside of the culture plates. All were from the wells "positively" transfected with plasmid DNA, with all 3 plasmid concentrations yielding clones. Colonies together with medium were picked using a 200 μ l pipette and transferred to 2 new, labelled 6-well plates, each containing 2.5 mls CMB/Zeocin. Cell clumps were broken up by pipetting/re-pipetting and the plates re-incubated. Ten of the 12 colonies re-seeded successfully, the cell monolayers becoming confluent between 7 and 10 days. At this stage, individual cell clones were trypsinised and split 3 ways into wells – one to maintain the clone, one to be induced with tetracycline to express the recombinant protein and one to act as a negative control during expression (see below).

After induction of the different cell lines and analysis of their protein products by SDS-PAGE and Western blotting, those expressing significant amounts of RSAg were identified. One cell line was selected for immediate expansion and expression. These cells were trypsinised and re-seeded first into a T-75 flask and then into 2 Falcon T-175 (175 cm²) flasks. The cells were induced as described below. Frozen stocks were made of all RSAg cell lines and stored in liquid nitrogen (below).

2.2.1.7 Expression of recombinant RSAg from transfected cells

Both stable and transient transfections were induced using similar methods. In both, tetracycline at a final concentration of 1 μ g/ml in medium was added to each well, when the cells had reached 60% confluence. For trial expressions of both stable and transient transfections, incubation was for 24 hours at 37°C. Medium was CMB/Zeocin for stably transfected cells and plain CMB for transient transfections. Later, during substantive expression of RSAg from the chosen cell line, total induction time was increased to 72 hours.

2.2.1.8 Harvesting and lysis of induced cells

Harvesting and lysis of trial expression cells was done by aspirating the medium from the wells of the 6-well plates and replacing it with 1 ml of PBS. Cell monolayers were disrupted by scraping and pipetting, and each 1 ml of cell suspension transferred to a 1.5 ml Eppendorf tube. Cells were pelleted by centrifuging at 1500 g for 5 minutes and the pellet resuspended in 50 µl of cell lysis buffer (50 mM Tris pH7.8, 150 mM NaCl, 1% Nonidet P-40)(Invitrogen). The suspension was vortexed and incubated at 37°C for 10 minutes to allow complete cell lysis. Complete™ (Roche Diagnostics, Mannheim, Germany) protease inhibitor cocktail (25x) was added, and the mixture centrifuged at 10,000 g for 10 minutes to pellet insoluble cell debris. The supernatant fraction was aspirated and stored at –70°C till analysed.

2 flasks (T-175) of cells were induced for substantive expression of recombinant RSAg as described. After 72 hours, all medium was poured off and the monolayer washed with PBS. 20 mls of fresh PBS was added and the cell layer disrupted/resuspended as above. Cell concentration was determined by haemocytometer/trypan blue, and found to be 10×10^6 cells/ml. 1ml aliquots of cell suspension were placed in Universal (Sterilin) polystyrene tubes and centrifuged at 1,500 RPM for 5 minutes. Supernatant was poured off and pellets stored in a – 80°C freezer till ready for protein purification.

2.2.1.9 Lysis of mammalian cells in preparation for protein purification

Recombinant RSAg was ultimately extracted from cell lysates using the Probond Purification System (Invitrogen), which makes use of the polyhistidine tag incorporated onto the expressed protein (described below). As purification was found to be most effective under denaturing conditions, cell lysates were prepared with reagents compatible with this method.

Frozen cell pellets were thawed and 8 mls of Guanidinium Lysis Buffer (6M guanidine HCl, 20 mM NaPO₄ pH 7.8, 500 mM NaCl)(Invitrogen) used to resuspend the pellet. DNA was sheared by repeatedly passing the suspension through an 18-gauge needle till it became non-viscous. The lysate was centrifuged at 3,000 g for 15 minutes to pellet insoluble cellular debris.

Supernatant was separated from the pellet and either underwent immediate protein purification or else was stored at -20°C till needed. 10-20 µl was kept for analysis by SDS-PAGE and Western blotting analysis. Pellets of cellular debris were also kept for analysis in case the recombinant protein was unexpectedly insoluble and significant amounts were retained in inclusion bodies.

2.2.2 Human RSAg expressed in bacterial cells

2.2.2.1 Template DNA and design of RSAg primers

RSAg DNA for expression in bacterial cells was produced by PCR, using the previously cloned pcDNA4-TO-myc-HisA/RSAg plasmid as template. New primers were used, producing PCR products with ends compatible for ligation into the BamH1 and Hind III sites of the pCR-T7-NT-Topo plasmid (Invitrogen). Forward primer (BamH1) was 5`-ACT GGA TCC AAT GGC AGC CAG CGG GAA GAC C -3` and reverse primer (Hind III) 5`-TGG AAG CTT CAC TCA TCA GCG TCA TTC TTG TC-3`.

2.2.2.2 Ligation of DNA into plasmid suitable for prokaryotic expression

BamH1 and Hind III digested PCR products were ligated into the cut pCR-T7-NT-Topo plasmid, as carried out for the pcDNA4-TO-myc-HisA plasmid.

The pCR-T7-NT-Topo plasmid is 2870 bp in size, contains an ampicillin resistance gene, a sequence for an N-terminal polyhistidine tag, several other distinctive internal epitopes and an enterokinase cleavage site. Expression of the recombinant protein is under the control of a T7 promoter, which in turn is activated by T7 RNA polymerase. The T7 RNA polymerase gene is located on the DE3 lambda lysogen, which is found in the bacterial strain used for expression of the RSAg in these experiments - BL21(DE3)pLysS (Invitrogen). The T7 gene is under the control of a lacUV5 promoter, but this is usually rendered inactive by the action of a *lac* repressor. Expression of the recombinant protein is therefore achieved by either directly infecting the cell with helper phage that express the T7 RNA polymerase, or more commonly by activation of the lacUV5 promoter with isopropyl-β-D-thiogalactoside (IPTG) (Roche).

2.2.2.3 Transformation of plasmids into Top 10 F⁻ bacterial cells

The manufacturer (Invitrogen) recommended transforming the recombinant pCR-T7-NT-Topo/RSAg plasmid into Top 10 F⁻ chemically competent cells (Invitrogen) for characterisation, stable propagation and long-term maintenance of the construct. Top 10 F⁻ is a type of Escherichia coli (E. coli), but does not contain T7 polymerase, unlike strains used for expression of the recombinant protein. Basal levels of T7 polymerase would result in small amounts of recombinant protein expression – if the product was toxic this might result in cell death and possible loss of plasmid from the cell line.

For transformation, one 50 µl aliquot of chemically competent Top 10 F⁻ cells were thawed on ice. 2.5 µl of plasmid preparation (representing between 5 and 10 ng of DNA) was added directly to the cells and mixed by gently tapping. The mixture was maintained on ice for 30 minutes. Cells were heat-shocked by immersing the vial into a pre-heated waterbath at 42°C for 30 seconds exactly and then replacing the vial on ice. After 1 minute, 250 µl of sterile SOC growth medium (Invitrogen) was added to the vial. The vial was taped horizontally into an incubator/shaker and incubated at 37°C for 1 hour at 225 RPM. 30 and 90 µl aliquots of transformation reaction were plated out onto two LB agar plates containing 50 µg/ml ampicillin, under sterile conditions. Plates were inverted and incubated overnight at 37°C. Next day a number of well-spaced colonies were selected from either plate and re-amplified overnight as clones in LB broth/ampicillin. Aliquots (0.5 mls) were taken of each, placed in pre-labelled Eppendorf tubes and frozen in 50% glycerol. Plasmid mini-preparations were isolated for each clone (Concert Mini-prep Kit). A portion of this DNA underwent agarose gel electrophoresis and was sequenced (described later) to identify cell lines containing the correct RSAg construct in the right orientation. Recombinant plasmids were sequenced in both directions using sequencing primers pcDNA3.1 (5'-TAA TAC GAC TCA CTA T-3') and T7reverse (5'-CTA GTT ATT GCT CAG CGG TGG-3'). The remainder of the mini-preps were stored for subsequent transformation into cell lines more suitable for protein expression (see below).

2.2.2.4 BL21(DE3)pLysS bacterial cells

BL21(DE3)pLysS (Invitrogen) was chosen as the bacterial host cell for expression of RSAg from the pCR-T7-NT-Topo/RSAg recombinant plasmid. It is an E. coli B/r strain, does not contain the lon protease and is deficient in the outer membrane protease OmpT. The lack of these key proteases reduces the degradation of proteins expressed within the cells. Cells contain the λ DE3 lysogen that carries the gene for T7 RNA polymerase and is under control of the lacUV5 promoter. This promoter is inducible by IPTG. These cells also contain the pLysS plasmid, which produces T7 lysozyme, which in turn greatly reduces the basal levels of recombinant gene expression. This is particularly useful where the protein being expressed may be toxic and expression needs to be tightly regulated. pLysS also confers resistance to chloramphenicol and contains the p15A origin. This origin, in effect allows pLysS to be compatible with pUC- or pBR322-derived plasmids.

BL21(DE3)pLysS competent cells were supplied commercially in single-use aliquots of 50 μ l. While transformed cell clones could be frozen down in glycerol 50% and stored, possibly better levels of expression were found by transforming fresh competent cells for each individual expression experiment.

2.2.2.5 Transformation of plasmids into BL21(DE3)pLysS bacterial cells

BL21(DE3)pLysS chemically competent cells were transformed with the plasmid/RSAg constructs using essentially the same method as described for Top 10 F' cells. It was unnecessary, however, to plate the outgrown transformed cells onto LB agar/ampicillin plates and select specific colonies for amplification. Instead the whole outgrowth was used to inoculate a culture of LB broth/ ampicillin 50 μ g/ml / chloramphenicol 34 μ g/ml and amplified to mid-log phase. Pilot expressions were then carried out to identify the clones that best expressed the recombinant protein and also to determine the optimal incubation time post-induction.

2.2.2.6 Pilot and main expressions of recombinant RSAg from bacterial cells

It was necessary to carry out pilot expressions even when the cell line for expression had been selected. This is because peak rate of protein production may not be reached if the induction time is too short, but unnecessary degradation of expressed product may occur for prolonged inductions. Samples were therefore taken from cultures at different time-points, to determine the optimal incubation time for maximal yield of recombinant protein. The transformed cell cultures were split in 2 as soon as they reached mid-log growth phase (OD_{600} 0.5 - 0.8) as determined by spectrophotometer. One half of each was induced with IPTG, while the remaining non-induced culture acted as a measure of background expression. IPTG-induced and non-induced cultures were run in parallel, so as to aid identification of the correct size protein, when samples from both cultures were later run side-by-side on SDS-PAGE gels. For each time point, a 500 μ l sample was taken from both cultures and centrifuged at maximal speed for 30 seconds. Supernatants were aspirated and the pellets kept at -20°C till analysed on gels (by Coomassie Brilliant Blue staining and Western blotting). Time points were taken at 0, 1, 2, 3 and 4 hours and after overnight incubation. Cell lines (non-induced) were incubated overnight and used to re-seed fresh medium the next day. If time-point analyses of samples were available at this stage, the clone(s) for substantive expression could be identified and expression proceed without delay. Alternatively, glycerol stocks were made of each cell line and frozen, pending analysis of time-point samples.

A positive control plasmid pCR T7/NT-E3 (Invitrogen), encoding a 58 kDa protein kinase protein, was supplied with the expression kit. This plasmid, which codes for the distinctive epitopes and His tag found in the recombinant RSAg, was used to verify the success of the basic transformation and expression procedure. The 58 kDa protein would prove useful as a positive control in subsequent protein purification procedures and was also used as a His-positive control protein in ELISA experiments.

Once the particular pCR-T7-NT-Topo/RSAg – transformed cell-line and optimum induction times were determined, the main RSAg expression was carried out. 180 mls of LB broth/ ampicillin 50 $\mu\text{g/ml}$ / chloramphenicol 34 $\mu\text{g/ml}$

were inoculated with a 5 ml (1:36 dilution) of overnight growth of transformed cells in a 500 ml glass culture flask. After 3 ½ hours incubation in a 37°C/ 225 RPM incubator the OD₆₀₀ had reached 0.5. The culture was induced by adding IPTG to a final concentration of 1 mM. Incubation was continued for a further 4 hours, at which stage cells were harvested by centrifuging the culture in 50 ml Apex tubes (Alpha) at 5000 RPM for 30 minutes. Supernatants were discarded and the pellets stored at – 70°C till protein purification was carried out.

2.2.2.7 Lysis of bacterial cells in preparation for protein purification

Recombinant protein was purified from bacterial cell lysates under denaturing conditions. Bacterial cells have a tough outer cell wall and, in comparison with mammalian cells, require more vigorous disruption to ensure lysis. 8 mls of Guanidinium Lysis Buffer, pH 8.0, 37°C, was added to each pellet from 50 mls of bacterial cell culture. Pellets were lysed by rocking the tubes at RT° for 10-15 minutes. Lysates were sonicated (on ice) to complete lysis and to break up strands of DNA. 4 separate bursts of medium intensity were applied for 15 seconds each. Sonicates were centrifuged at 3,000 g for 15 minutes to pellet any insoluble cell debris, and supernatants aspirated off. Again samples of unprocessed supernatants and cellular debris were stored and analysed by SDS-PAGE /Western blotting, to ensure the recombinant protein was found in the supernatant as expected. Supernatants were either subjected to protein purification immediately or stored at – 20°C.

2.2.3 Purification of RSAg using Probond™ nickel-chelate resin

Once lysates of mammalian or bacterial cells had been prepared, protein purification under denaturing conditions was able to proceed. The Probond (Invitrogen) protein purification system makes use of the polyhistidine tag incorporated into the recombinant protein. The 6 tandem histidine residues bind with high affinity to the Probond nickel chelate resin, under appropriate conditions of pH and ionic charge. Purification can be carried out with the protein in native or denatured conformation. Both were tried, but denaturing

conditions were found to be most successful. The system works on the basis that His-tagged protein will bind preferentially to the resin at pH 6.0 – 8.0 (optimum 7.2 - 7.8), and in the presence of 500mM NaCl to prevent the positively charged Probond resin binding to non-specific negatively charged proteins. Residual non-specific proteins are removed by washing at pH 6.0 and then 5.3. The relatively pure His-tagged protein is finally eluted by washing the resin with elution buffer at pH 4.0.

The binding capacity of Probond resin is 1-5mg recombinant protein per ml of resin. Therefore 2mls of resin was used to extract the recombinant RSAg contained in 50 mls of induced bacterial culture. For RSAg expressed in human cells, 1 ml of resin was initially used to purify protein from no more than 20×10^6 cells (2 pellets). The ratio of cells to resin was increased later as it became apparent that the cell pellets contained low yields of recombinant protein. Volumes described below are for purification using 2 mls of settled resin, and were increased pro rata for larger purifications.

All solutions, buffers and the pre-prepared lysates in lysis buffer were equilibrated to RT° and their pHs measured before starting the purification process. pHs were adjusted with HCl or NaOH where necessary. This had to be rechecked every day, as pHs were found to fluctuate.

A small sample (10-20 μ l) was taken from each cell lysate (prior to purification) and stored at - 20°C. All binding supernatants, binding buffers, wash buffers and elutions were also stored and later analysed by SDS-PAGE and Western blotting. Comparisons of the various fractions allowed verification of the presence of the recombinant protein in lysates, verification that bound protein was not being eluted prematurely in the washes, and identification of the elution fractions containing significant amounts of recombinant protein.

2.2.3.1 Equilibration of resin

Resin is supplied as a 50% slurry in 20% ethanol. 2 mls of settled resin (4 mls of slurry) was put in a 15 ml Apex polypropylene tube, mixed and then centrifuged at 800 g for 1 minute. The supernatant was aspirated by pastette. 6 mls of sterile, distilled water (dH₂O) was added and the resin resuspended by tapping and gently inverting the tube. The mixture was again centrifuged and the dH₂O aspirated off. This was replaced with 6 mls of Denaturing Binding

Buffer (8M urea, 20 mM NaPO₄ pH 7.8, 500 mM NaCl) and the resin resuspended. Resin was re-pelleted by centrifugation and the supernatant aspirated off. The washing in denaturing binding buffer was repeated once more, and the resin was then ready for incubation with bacterial or mammalian cell lysates.

2.2.3.2 Binding of protein to resin

8 mls of cell lysate was added to 2 mls of equilibrated resin in the 15 ml tube. This was mixed gently and then left agitating slowly on a mechanical rocker, for 30 minutes at RT°. After centrifugation (as before), the unbound lysate supernatant was aspirated carefully and stored. Resin was washed with 4 mls of denaturing binding buffer by resuspending, agitating for 2 minutes, re-pelleting and aspirating (and storing) the supernatant. This step was repeated once more.

2.2.3.3 Washing at pH 6.0 and 5.3

The resin was washed with 4 mls of Denaturing Wash Buffers pHs 6.0 and 5.3 (8M urea, 20 mM NaPO₄, 500 mM NaCl), as described for binding buffer. The manufacturer recommended 2 washes at each pH, but up to 4 washes per step were carried out on occasion and was found to reduce the amount of non-specific protein found in subsequent elution fractions.

2.2.3.4 Elution of His-tagged protein

After the resin had been resuspended in wash buffer pH 5.3 for the last time, the whole 4 mls resin/wash buffer was gradually loaded into a 2.5 ml Mobicol (Mobicol, Germany) column, fitted with upper and lower 35 µm pore size filters, and held vertically in place by a benchtop clamp. A 2.5 ml syringe tube was fitted to the column's Luer lock to increase its capacity. Wash buffer was allowed to flow through and was saved. Elutions were then carried out by adding Denaturing Elution Buffer (8M urea, 20 mM NaPO₄ pH 4.0, 500 mM NaCl), 1 ml at a time to the column and catching the flow-through in a 1.5 ml Eppendorf tube. 10 elution fractions were usually collected and frozen till analysis. The resin was then washed by passing 10-20 mls of dH₂O through the

column, followed by 20% ethanol. Columns were sealed while the resin was still wet and stored at 4°C for future use.

2.2.3.5 Analysis of lysates, washes and elutions by SDS-PAGE and Western blotting

It was essential to analyse each fraction saved during the purification process, to ensure the process was working as planned and to make changes (e.g. slight changes in pH of wash buffers or number of washes) where necessary. Identification of elution fractions containing recombinant protein was found to be unreliable using a spectrophotometer, as it was not sensitive enough to detect the sometimes very low concentrations of RSAg eluted. Instead worthwhile elutions were identified by Coomassie Brilliant Blue or silver staining of samples run on SDS-PAGE gels, combined with Western blotting.

2.2.3.6 Concentration and measurement of recombinant proteins

Once elution fractions containing RSAg were identified, they were pooled and concentrated by Centriprep and Centricon (both Millipore, UK) centrifugal filter devices. This was done for 2 reasons: firstly to bring the protein to a concentration where it would be within the detection threshold of a quantification assay such as the BCA test, and secondly to remove the high levels of urea (i.e. 8M) contained in the denaturing elution buffer.

Both Centriprep and Centricon devices work on the same principle i.e. filtering the test solution through a cellulose filtration membrane, which has a designated molecular weight cut-off through which smaller molecules can pass, but the majority of larger molecules cannot. Centriprep devices were used for volumes up to 15 mls and filtration occurs in the opposite direction to sedimentation, which in theory reduces membrane clogging. Centricon devices were used to further concentrate volumes of 2 mls or less. Filtration occurs in the same direction as sedimentation and relies on the use of a fixed-angle rotor to minimise membrane clogging.

For both types of concentrators, membranes with molecular weight cut-offs of 30 kDa were selected (RSAg is approximately 48 kDa in size).

Membranes of both devices were pre-rinsed prior to use to wash out trace amounts of glycerine and sodium azide. A typical starting volume of protein/denaturing elution buffer was 10 mls (1ml x 10 elutions) but this had to be diluted in filtered sterilised PBS to 40 or 50 mls to prevent degradation of the membrane by the high urea content and possible loss of sample. The sample was concentrated step-by-step, by centrifugation in a Universal 30 RF bench-top centrifuge (Hettich, Germany) using a swinging-bucket rotor at 1500 g, till sample volume was less than 2 mls. This was rediluted to 20 mls and the procedure repeated till the volume was once again less than 2 mls, resulting in a 40-50 fold reduction in the urea concentration. At this stage, concentrates were transferred to pre-rinsed Centricon devices and spun at 5000 g till final volume was less than 1 ml. Concentrates were transferred to fresh 1.5 ml Eppendorf tubes, Complete protease inhibitor cocktail added and stored at -70°C.

Although removal of the leader sequence including the His tag was an option, through digestion at the enterokinase cleavage site, this was felt to be unnecessary for subsequent applications. Leader sequences were therefore left in situ and were later found to be useful as binding sites for capture antibodies and for verification of the presence of the recombinant protein at various stages.

Finally, the amounts of total protein in the concentrated samples were determined by BCA assay as described above.

2.3 ELISAs for measuring anti-RSAg activity

The extent and degree of humoral immunoreactivity in human subjects to RSAg was investigated by ELISA. This technique is well established, cheap, rapid and is capable of accurately detecting specific antibodies to a particular antigen, both qualitatively and quantitatively. ELISA screening of human autoimmune uveitis and control sera was carried out in 2 separate experiments: firstly bovine RSAg was used on its own and in a later experiment the technique was refined and immunoreactivity of individual patients/controls to bovine RSAg was compared to that of human recombinant RSAg expressed in a bacterial host.

2.3.1 Basic procedure for 3-step ELISA (anti-RSAg titres)

A 3-step, indirect ELISA, with antigen bound to solid phase (see Figure 2.1, p126), was used to determine the immunoreactivity of patient and control sera to RSAg. Optimal concentrations/dilutions of reagents are quoted here, and were determined prior to starting test ELISAs (see next section). In the initial experiments (sera versus bovine RSAg) individual sera were screened against duplicate antigen-coated wells, with duplicate wells coated overnight in coating buffer/ BSA 5% acting as negative controls for each 96-well plate. 50 µl of RSAg solution, at 10 µg/ml in carbonate coating buffer (Na₂CO₃ 1.59 g/L, NaHCO₃ 2.93 g/L, pH 9.6), was coated directly into duplicate wells of 96-well Maxisorp (Nunc) ELISA plates and incubated overnight at 4°C. Wells were washed 3 times with PBST from a wash bottle and blocked by filling them with PBST/BSA 1% for 2 hours at RT°. After repeat washing (x3), 50 µl of each serum was loaded into its respective well at a dilution of 1:100 in PBST/BSA 1% and incubated for 2 hours at RT°. After washing (x3), 50 µl goat anti-human IgG – alkaline phosphatase secondary antibody (Sigma) was added at a dilution of 1:5000 for 1 hour at RT°. After a final round of washing (x3), 75 µl of p-nitrophenyl-phosphate (pNPP) (Sigma) dissolved in diethanolamine buffer (97 ml/L diethanolamine (Sigma), 250 µl 1M MgCl₂, 0.1% NaN₃ at pH 9.8) at a final concentration of 1 mg/ml, was added to each well at RT°. After 1 hour, ODs were read on a Dynatech optical plate reader at a wavelength of 405 nm. It was

later realised that the layout of antigen/negative control wells was less than optimal, as each serum should have had its own negative control, and the experiments were repeated as below.

In repeat experiments (sera versus bovine and recombinant human RSAg), each serum sample was tested against a panel of duplicate wells; negative control, His-tagged protein control, bovine RSAg and recombinant human RSAg. The His-tagged protein was run as an additional control to detect inadvertent reactivity to His tags (still attached to recombinant human RSAg). All proteins were coated at a concentration of 5 µg/ml in carbonate coating buffer, overnight at 4°C. Negative control wells did not receive antigen (only coating buffer), but otherwise were treated at each stage as per test wells. All washes were carried out 5 times using PBST. PBST/5% fat-free dried milk (Sainsbury, UK) (PBSTM5%) was used to block wells at RT° for 2 hours. Sera and secondary antibody were diluted in PBSTM1%. Serum (50 µl of 1:25 dilution per well) was incubated at RT° for 2 hours. 50 µl of secondary antibody (goat anti-human IgGAM – alkaline phosphatase) was used at a dilution of 1:20,000 for 1 hour at RT°. Substrate was added as above at RT° and ODs read after 30 minutes.

2.3.2 Optimisation of ELISAs

Optimisation of the concentrations of antibodies, sera and antigens was performed before analysis of test samples began. In the initial experiments optimal reagent concentrations were determined on a trial-and-error basis. This approach was later changed to a more formal optimisation protocol, as outlined here.

For 3-step ELISAs, optimisation was in 2 stages. Firstly, a serum sample known to contain anti-RSAg antibodies was diluted to 1:25. Conjugated, secondary antibody was diluted to 1:20,000. Antigen was directly plated into duplicate wells in 2-fold dilutions, from 10 µg/ml to 0.635 µg/ml. ELISA was carried out as described above, a standard curve of ODs created and an optimal antigen concentration chosen, usually in the mid-range of the standard curve. In the second stage of optimisation, antigen concentration was kept constant at the previously determined optimal level. 2-fold dilutions of sera (1:10

to 1:200) and secondary antibodies (1:5000 to 1:40000) were used in a "criss-cross" grid assay. Blocked wells were used to determine background "noise" levels. A combination of the serum and secondary antibody dilutions that gave the highest signal:noise ratio combined with an acceptably low background were chosen for the substantive ELISAs that followed. Direct comparison of blocking buffers (PBST/BSA5% or PBST/M5%) was carried out with other reagents at their optimal concentrations.

2.3.3 Analysis of generated data

Mean values for test and control samples were calculated using a spreadsheet (Microsoft Excel 97). Test sample readings (ODs) were divided by their corresponding control values. The values for His-tagged protein wells were used as negative controls where they were higher than those of plain blocked wells. Positive values from sera of patients and controls were categorised as being "mildly", "moderately" or "highly" reactive to RSAg. Differences in immunoreactivity between bovine and human recombinant RSAg were analysed on an individual subject basis and also in terms of differences between patient and control groups (see Chapter 3).

2.4 Epitope mapping studies for RSAg

2.4.1 Overview of biopanning

Biopanning (affinity selection) is a technique used to screen phage display libraries with target ligand molecules, often antibodies or serum. Phage display libraries can be either random combinatorial (e.g. random peptide libraries) or non-random (e.g. cDNA libraries). Manipulation of the phage genome results in clones of particles expressing a particular peptide on their surfaces, as fusions with host capsid proteins. Only a tiny percentage of the original library of clones will bind specifically with the target ligand molecule(s), even in non-random cDNA libraries. However, by "panning" the library with immobilised target ligand and amplifying captured phage particles for use as input phage for subsequent rounds of panning, a steady enrichment of ligand-specific phage clones can be achieved. In fact, after 3 or 4 rounds of biopanning with a random peptide library, up to 99% of total clones should be specific for the target molecule. Clones can then be plated out as plaques and analysed individually, both in terms of their DNA sequences and in terms of specific binding properties as measured, for example, by ELISA. Much information about the antigen binding site(s) and epitope preferences of the target ligand molecule, and by inference its target antigen *in vivo*, can be gained in this way.

2.4.2 Random phage display libraries used in epitope mapping studies

2.4.2.1 T7 library

T7 is a robust, double-stranded DNA phage. It is a head-and tail unit with an icosahedral head (diameter 55 nm), from which random peptides are displayed as fusion proteins on pentamer/hexamer capsid subunits. Phage assembly takes place inside the *E. coli* cells and particles are released by lysis of the host. Time from infection of cells in culture to lysis (1-2 hours) is more rapid than for λ or filamentous phage. In this experiment a high-copy number, linear 9-mer library, that had originally been purchased commercially (T7Select™,

Novagen) and re-amplified, was used. The fusion is at the carboxyl terminus (C-terminal) of the recombinant protein. BL21 E. coli were used as host.

2.4.2.2 Ph.D.-12 library

This library was purchased commercially (New England Biolabs, Hitchin, U.K.). It is a combinatorial library of linear random peptide 12-mers, fused to the gene III minor coat protein (pIII) of the filamentous (single-stranded) M13 phage. The pIII protein is involved in phage infectivity and the 12-mer peptides are expressed on the N-terminus end of the protein. ER2537 E. coli was used as host.

2.4.2.3 f88-4 linear and f88-4/Cys4 (constrained) 15-mer libraries

These 2 libraries were the kind gift of Prof. G. Smith, University of Missouri, USA. The vector of both libraries is based on the filamentous fd-tet vector (9183 bp), which confers resistance to tetracycline and is inducible by the antibiotic. f88 vectors contain 1 recombinant and 1 wild-type gene VIII molecule, both of whose products contribute to the f88 virion capsid. Recombinant f88 molecules are 9273 bp in size. Up to 300 recombinant capsid proteins are displayed per virion, which is capable of displaying quite large recombinant peptides. In GVIII recombinant proteins, the fusion is at the amino terminus (N-terminal). Because f88 plaques are so small, phage are titred more easily by measuring the number of tetracycline transducing units (TU) i.e. the number of (infected) bacterial host colonies that grow on a tetracycline containing plate. Both libraries require amplification prior to use (see below). Because gene VIII is transcribed from a *tac* promoter, IPTG is added to a final concentration of 1mM to induce full expression of the protein during amplification.

The f88-4 linear library contains 2×10^9 primary clones and has an infectivity rate of 42%. The f88-4/Cys4 library displays the recombinant 15-mer peptides in constrained conformation, which may enhance binding to target ligand in certain cases. It has 1.7×10^8 primary clones with an infectivity of 9.4%.

2.4.3 Affinity purification of anti-RSAg polyclonal antibody

50 μ l aliquots of stored serum samples from autoimmune uveitis patients and healthy controls were taken and pooled in separate 15 ml Apex tubes designated "P" and "C" respectively. Serum samples were diluted to 4 mls in PBS. Protein G resin (Pharmacia Biotech, Sweden) in 20% ethanol was centrifuged at low speed (2000 RPM) in a Universal bench-top centrifuge. Supernatant was decanted and resin washed 3 times in 10 volumes of PBS. 1ml of settled resin was added to each serum-containing tube and allowed to mix on a rocking platform at RT° for 30 minutes. Resin was diluted to 10 mls with PBS and gradually loaded into 5 ml Mobicol affinity chromatography columns (Mobicol) with pre-fitted lower 35 μ m pore size filters. As resin was settling, upper filters were also fitted and all supernatant allowed flow through. Columns were washed through 5 times with 5 mls PBS. 1 ml of elution buffer (0.1M glycine-HCl, pH 2.2) was then added to each column, the outflow blocked and the mixture incubated for 2 minutes. Eluates were allowed flow through and were collected in 1.5 ml Eppendorf tubes. Each elution was neutralised to pH 7.0 with 250 μ l of 1M Tris base, pH 10. This process was repeated 4 times and columns finally washed through with 10 mls elution buffer. They were then washed 3 times with 5 mls PBS and stored in PBS/20% ethanol. Optical densities of eluted IgG fractions were determined by spectrophotometry at 280 nm using quartz cuvettes. The readings were translated to mg/ml by dividing by 1.43

Patient and control IgG fractions were pooled separately and underwent affinity purification for anti-RSAg antibody. 0.33 g of 6-aminohexanoic acid N-hydroxysuccinimide ester – Sepharose B4 resin (Sigma) was reconstituted with 1 ml of PBS. Resin was washed twice with PBS and 268 μ g purified bovine RSAg in 1 ml of PBS added to the dry resin. This was mixed at RT° for 1 hour and the mixture loaded into a 2.5 ml Mobicol as described above. The column was washed through 5 times with PBS and then patient sera IgG passed through. Flowthrough was collected and passed once more through the column.

The column was washed through 5 times with PBS and affinity purified anti-RSAg antibody finally eluted in 1 ml fractions of elution buffer (as above). Neutralised eluates were concentrated using Centricon centrifugal concentrators (30 kDa MW cut-off) and final IgG concentration determined by spectrophotometry. The affinity purification column was washed 5 times in PBS and stored in PBS/0.02% NaN₃ till re-used. Immunoglobulin from control subjects was affinity purified in the same way. The specificity of the anti-RSAg antibody was confirmed by screening it against dot blots of RSAg, using non-specific bacterial cell lysate as negative control.

2.4.4 Affinity selection of antibody reactive phage

2.4.4.1 T7 library biopanning

Patient and control affinity purified anti-RSAg antibody was diluted to 1 ml in PBS and placed in 2ml sterile, screw-top tubes (Alpha). 50 µl washed, paramagnetic, Dynabeads (Dyna, Norway), pre-coated with mouse anti-human IgG, was added to each tube, which was incubated on a rotating mixer for 2 hours. Antibody excess solution was separated from beads with a magnet, aspirated and saved. Beads were washed in PBS 4 times. 2 mls of T7 phage solution (clarified lysate) were negatively selected for non-specifically binding particles by incubation with 100 µl fresh Dynabeads. Supernatant was aspirated and 1 ml phage library (approximately 10¹⁰ PFUs representing 10⁸ combinations) added to each tube containing washed, anti-RSAg coated beads. This was incubated/rotated for 30 minutes. Supernatant phage solution was separated and decanted, and the beads washed 4 times with PBS. 1 ml of BL21 E. coli that had been grown to mid-log (OD 0.5) from an overnight culture was added to each tube of washed beads and incubated for 10 minutes. 10 µl of cells were taken from each tube for titration of pre-amplification numbers of captured phage (see below). The remaining 990 µl were used to inoculate separate flasks containing 20 mls of BL21 mid-log cells. These flasks were incubated till total lysis of cells was observed, usually 1-2 hours, or overnight. Phage titres were estimated as before, and lysates stored. Lysate which was clarified by centrifugation was used as input phage for the next round of biopanning.

Yields of pre- and post-amplification phage were determined by "plaque assay". 10 μ l of each phage solution was used to produce a series of 10-fold dilutions in LB. 10 μ l of each dilution was plated out on a bacterial lawn, as described in "amplification of phage". After overnight incubation at RT $^{\circ}$, a plate with 50 - 300 well-spaced plaques was selected and the exact number counted. Correcting for the dilution factor, the total number of infective particles in each original 10 μ l could be calculated and expressed as PFUs/ml. Knowledge of the percentage infectivity of each library could be used to calculate the total number of phage particles in each lysate if necessary.

Less stringent conditions (no detergent in PBS for washes, long incubation times) were used for the 1st round of biopanning to prevent premature loss of relevant phage clones from the experiment. In the 2nd and subsequent rounds, greater stringency was used to reduce the number of non-specific phage being selected and therefore amplified.

4 rounds of biopanning were carried out on both samples. A progressive increase in the number of amplified phage was confirmed for each round. The number and location of plaques expressing peptides specific for anti-RSAg was monitored after each amplification by performing "plaque lifts". Circular nitrocellulose membranes were laid on top of plates containing plaques and as they were lifted off, some of each plaque remained bound. Membranes were screened with anti-RSAg and developed as described in "dot blots". Levamisole (final concentration 5mM) was added to alkaline phosphatase substrate solution to neutralize the activity of bacterial alkaline phosphatase and better delineate plaques displaying high-affinity peptides. The exact positions on the master plates of specific plaques were determined by studying the membranes and marked. After the 4th round, positive plaques were identified and labelled on each master plate.

2.4.4.2 Ph.D.-12 library biopanning

Essentially the same method as above was used for biopanning with the Ph.D.-12 (M13) library, with the following adjustments. ER2537 grown on minimal medium was used as host bacterium. 4×10^{10} (10 μ l) of input phage were used for the first round of biopanning. Affinity-selected phage were eluted from

Dynabeads with 1 ml glycine-HCl 0.2 M, pH 2.2 and neutralised in 150µl 1M Tris-HCl, pH 9.1.

Problems were encountered amplifying the eluted captured phage after each round and other methods of amplification were tried. This included separation of phage DNA by phenol and electroporation into electrocompetent cells and the use of M13 K07 helper phage to assist with the Ph.D.-12 infection process. Neither of these approaches overcame the problem (discussed later) and the use of this library was abandoned.

2.4.4.3 f88-4 15-mer linear and f88-4/Cys4 15-mer library biopanning

2.4.4.3.1 Amplification of f88-4 linear and f88-4/Cys4 pentadecapeptide libraries

The procedure was identical for both libraries. K91Kan E. coli (which contain a kanamycin resistance gene) were grown overnight in a culture of LB containing 100 µg/ml kanamycin. Two 1-litre culture flasks containing 100 mls of terrific broth were each inoculated with 1 ml of overnight culture, and grown to late log phase (OD = 2.0). The shaker was slowed down for 5 minutes to allow sheared F-pili to regenerate and 10^{12} of the supplied phage particles (corresponding to 5×10^{10} TU) added to each flask. Slow shaking was continued for 15 minutes. Cultures were then poured into individual 3-litre flasks containing 1L of LB with 0.22 µg/ml tetracycline and 0.5mM IPTG. Incubation was continued for a further 35 minutes (shaker at full speed). Tetracycline was then added to an overall concentration of 18 µg/ml. 10 µl of each infected culture were taken for titration and the main culture incubations continued overnight. A broad range of serial dilutions were made from the 10 µl samples and 200 µl of each plated out on LB agar plates containing 100 µg/ml kanamycin and 40 µg/ml tetracycline. After overnight incubation the colonies were counted, and the number of phage-infected cells per original primary phage clone in both flasks was calculated (ideally >100 cells per clone). The phage titre of the overnight incubations was calculated as before, the library transferred to a 500 ml sterile Duran bottle and stored with 0.02% NaN₃ at 4°C till used.

2.4.4.3.2 Biopanning of f88-4 linear and f88-4/Cys4 pentadecapeptide libraries

The same general principles were applied as in the T7 biopanning experiments. Linear and constrained libraries were mixed in equal parts to produce a single solution containing 3×10^{11} TUs as input phage for the first round of biopanning. 1 ml of Protein G, loaded into a chromatography column, was used as the solid phase for patient and control affinity-purified anti-RSAg polyclonal antibody. Input phage in PBS were negatively selected with unbound Protein G before biopanning began. Phage were eluted from the antibody-coated resin using 500 μ l of 0.2M glycine-HCl, pH 2.2, and neutralised in 300 μ l of Tris-HCl, pH 9.1. After 3 rounds of biopanning, phage-transformed colonies were plated out on kanamycin/tetracycline agar plates and screened by PCR as described below.

2.4.5 Sequencing antibody reactive phage peptides

2.4.5.1 PCR amplification of phage DNA

After the final round of biopanning, plaques/transformed bacterial colonies were screened by PCR amplification of the DNA from individual phage clones. Each plaque was touched with a pipette tip and directly introduced into 25 μ l of PCR mix a 0.2 ml well.

T7 PCR primers were:

T7 forward primer: 5`-ACA ACG TTA TCG GCC TGT TC-3`

T7 reverse primer: 5`-TAC CGG AGG TTC ACC GAT AG-3`

PCR conditions for T7 amplification were:

94°C for 10 minutes – 1 cycle

94°C for 50 seconds, 50°C for 1 minute, 72°C for 2 minutes – 35 cycles

72°C for 10 minutes, 30°C for 5 minutes – 1 cycle

f88-4 linear /f88-4 Cys4 PCR primers were:

f88-4 (Gene VIII) forward primer: 5`-TCC CCC TGT TGA CAA TTA ATC-3`

f88-4 (Gene VIII) reverse primer: 5`-ATT AGG CGG GCT GGG TAT-3`

PCR conditions for f88-4 linear /f88-4 Cys4 amplification were:

94°C for 10 minutes – 1 cycle

94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds – 35 cycles

72°C for 20 minutes – 1 cycle.

Successful amplification was confirmed by agarose gel electrophoresis.

2.4.5.2 Enzymatic digestion of PCR products

PCR products were purified prior to sequencing by enzymatic digestion. The enzymes digest single-stranded DNA and remove unincorporated primers and dNTPs. 5 µl of PCR product was mixed with 0.5 µl (5U) exonuclease I and 1 µl (1U) SAP in a 0.2 ml PCR tube. The reaction was heated to 37°C for 15 minutes and then the enzymes inactivated by heating to 80°C for 15 minutes. The reaction was cooled to RT° and centrifuged briefly.

2.4.5.3 Cycle sequencing reaction of PCR products

Sanger`s enzymatic method, using fluorescent dideoxynucleotides (ddNTPs) as chain terminators during thermal cycling, was used for sequencing of the purified PCR products. 6µl of digested PCR products were mixed with 4 µl of Ready Reaction Mix™ from the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit v2.0 (Applied Biosystems, Warrington, UK). The Ready Reaction Mix contains a proprietary mixture of AmpliTaq™ DNA polymerase FS enzyme, a mixture of dideoxy- and deoxy- dye terminators, deoxynucleoside triphosphates, magnesium chloride and Tris-HCl buffer, pH 9.0. Appropriate sequencing primer (0.15 µl of 20 mM) was added to each reaction and sequencing carried out, as below:

T7 sequencing primer: 5`-TTA AGC TGC GTG ACT TGG C-3`

T7 sequencing reaction conditions:

96°C for 30 seconds, 50°C 15 seconds, 60°C for 4 minutes – 30 cycles

28°C for 1 minute – 1 cycle

This was later changed to a 2-step sequencing reaction, as follows:

96°C for 30 seconds, 50°C for 4 minutes and 15 seconds – 30 cycles

28°C for 1 minute – 1 cycle

f88-4 linear/Cys 4 libraries (GVIII) sequencing primer: 5`-TTC TTA ATG GAA
ACT TCC TC-3`.

Sequencing reaction conditions were identical to those for T7.

2.4.5.4 Clean-up of sequencing reaction

The DNA solution from the previous stage was centrifuged briefly, diluted to 20 μ l with ultrapure H₂O and transferred to a 0.5 ml Eppendorf tube. 2 μ l 3M sodium acetate, pH 5.0 and 50 μ l 95% ethanol were added, the mixture vortexed and placed on ice for 10 minutes. This was centrifuged at 15,000 RPM at 4°C for 15 minutes. DNA was pelleted and the supernatant aspirated off carefully. The pellet was rinsed with 250 μ l of 70% ethanol, the tube inverted several times and centrifuged again for 5 minutes. Supernatant was again aspirated off and the pellet left to air dry for 10 minutes. Pellets were stored at -20°C till sequenced.

2.4.5.5 Automatic sequencing

Automatic sequencing was carried out on an ABI Prism™ 310 Genetic Analyser. Each DNA pellet was resuspended in 15 μ l of Template Suppression™ reagent (contains 99.5% deionised formamide, 0.11% EDTA). After mixing, samples were denatured by heating to 95°C for 2 minutes. Samples were cooled immediately and placed into the machine for automated loading onto the sequencing capillary. Up to 500 bp were sequenced at a time.

2.4.6 Analysis of immunoreactivity of phage peptides

2.4.6.1 ELISA testing of phage selected by immunopanning

After analysis of their DNA sequences, plaques/colonies representing phage clones of interest were punched out of the master plate and amplified overnight in 5 mls of mid-log cells. Clarified lysates were PEG precipitated and the phage pellets resuspended in PBS/0.02% NaN₃. Panels of purified phage clones were used as solid phase antigen in indirect ELISA assays against individual uveitis patient and control sera. Purified phage preparations in carbonate buffer were coated in excess into duplicate wells of 96-well ELISA plates, and incubated overnight as previously described. Wells coated with wild-type phage were used as negative controls. 3-step ELISAs were carried out essentially as described for anti-RSAg screening. Wells were blocked in 5% PBSTB, sera diluted to 1:100 in 1% PBSTB and a 1:20,000 dilution of secondary antibody used. Mean test clone values were divided by mean negative control readings and the immunoreactivity of each phage clone designated as "low", "intermediate" or "high" on this basis.

2.5 Screening for new retinal autoantigens with a retinal cDNA library

2.5.1 Purification of mRNA from human retina

2.5.1.1 Harvesting, fixation and homogenisation of fresh human retina

Fresh human retinas were kindly provided by the Bristol Eye Bank. Whole human eyes were harvested from donors who were consented for research purposes, within 12 hours post-mortem. Each retina was immediately micro-dissected from its globe by Dr. V. Smith, University of Bristol, and the neuroretina separated from the retinal pigment epithelium (RPE)/choroid. Each was placed in a separate 15 ml Apex tube containing 5 mls of RLT buffer and 50 μ l β -mercaptoethanol. Retinas were vortexed in the fixative till homogenized, to prevent degradation of the RNA by tissue nucleases. Samples were stored at -70°C till needed.

2.5.1.2 Extraction and measurement of total RNA

Special precautions to avoid ribonuclease contamination were employed as before. Total RNA was extracted from 2 homogenised human retinas from a normal individual, using the RNeasy extraction kit, essentially as described for recombinant RSAg production (above). Total RNA concentration was determined by UV spectrophotometry at 260 nm absorbance and was stored at -70°C till used.

2.5.1.3 Purification and measurement of mRNA fraction

mRNA was purified from the total RNA sample using the Straight A's mRNA Isolation System (Novagen). This system depends on paramagnetic beads coated with deoxythymidine (dT) to selectively bind to the poly-adenine(A) tails found on all eukaryotic mRNA molecules. mRNA comprises approximately 5% of total RNA. 250 μ l total RNA solution was added to 250 μ l pre-washed Magnetight™ Oligo (dT) Particles and 750 μ l Lysis Buffer (proprietary). The

mixture was incubated at RT° for 5 minutes and the beads separated from the supernatant using a magnet. Separated beads were washed twice in 1 volume of Wash Buffer (proprietary) and all residual buffer removed. mRNA was eluted by mixing the beads with 0.5 mls of nuclease-free water and incubation at 60°C for 10 minutes. Magnetically separated supernatant was transferred to a fresh tube where it was precipitated (as described for DNA) using 0.1 volumes 3M sodium acetate, 0.6 volumes isopropanol and 2 µl 10 mg/ml glycogen as a carrier molecule. The pellet was resuspended in 50 µl nuclease-free water. The whole mRNA purification process was repeated once more, to enhance the purity of the sample. mRNA concentration was determined by UV spectrophotometry and was then concentrated further to a working volume of 5 µl.

2.5.2 Construction of a retinal cDNA expression library

Retinal mRNA was converted to a library of cDNA fragments of various sizes using the OrientExpress™ cDNA Synthesis kit (Novagen). All components belonged to this kit, except where noted. The Hind III Random Primer strategy was used, which produces more even sequence representation than other priming options.

2.5.2.1 First strand cDNA synthesis with random primer strategy

5 µl of purified retinal mRNA was mixed with 1 µl (0.5 µg) Hind III Random Primer (patented sequence) and 4 µl nuclease free water. This was heated to 70°C for 10 minutes, immediately placed on ice and centrifuged briefly. To this was added 5 µl 5x First Strand Buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl₂), 2.5 µl 100 mM dithiothreitol (DTT), 1.25 µl 10x Methylation dNTP mix and 4.25 µl nuclease free water. This was incubated at 37°C for 1 minute and then 2 µl of MMLV reverse transcriptase (400 U) added. The 25 µl reaction was incubated for 1 hour at 37°C, to convert the RNA into single-

stranded DNA, and then the enzyme inactivated by incubation at 70°C for 10 minutes. This was immediately chilled on ice and centrifuged briefly.

2.5.2.2 2nd strand synthesis

25 µl of first strand reaction was mixed with 25 µl 5x Second Strand Buffer (200 mM Tris-HCl pH 7.5, 22 mM MgCl₂, 425 mM KCl), 3 µl 100 mM DTT, 1µl 10x Methylation dNTP mix, 2.5 µl (25 U) DNA polymerase I, 0.8 µl (0.8 U) RNase H and 67.7 µl nuclease free water. To estimate (by radionucleotide incorporation) the total amount of double-stranded DNA (dsDNA) produced, 10 µl of the reaction was taken and mixed with 0.25 µl of (α- P32)dATP. Both this and the remaining 115 µl reaction were incubated at 15°C for 90 minutes.

After incubation, the 115 µl reaction was diluted to 250 µl with nuclease free water and mixed with an equal volume of Tris-EDTA (TE) buffered phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma). This was vortexed vigorously and centrifuged at 12,000 g for 1 minute. The aqueous phase was aspirated, transferred to a tube containing 1 µl glycogen, 250 µl 4M ammonium acetate and 300 µl isopropanol, and mixed well. After 5 minutes incubation (RT°) this was centrifuged at 12,000 g for 8 minutes. Supernatant was aspirated carefully and the pellet rinsed with first 70% and then 100% ethanol. It was air-dried and resuspended in 20 µl TE buffer.

The amount of double-stranded DNA produced was estimated in 2 ways. 5 µl of P32-labelled DNA reaction was spotted onto 2 pieces of membrane. One was washed (removing all excess P32-dNTPs, etc) and the radioactivity of the 2 membranes compared by measuring counts per minute on a DNA scintillometer. Once the proportion of incorporated versus unincorporated P32 was ascertained, the amount of total DNA from the second strand synthesis could be calculated. The presence of double-stranded DNA was also confirmed by PCR amplification of the HPRT housekeeping gene, and its detection by agarose gel electrophoresis. The PCR was carried out as before, using 1 µl DNA solution as template and HPRT forward (5'-GACCAGTCAA CAGGGGACAT-3') and reverse (5'-CGACCTTGACCATCTTTGGA-3') primers.

2.5.2.3 End modifications

Ends of the double-stranded DNA molecules were blunted (by T4 DNA polymerase) in preparation for ligation with directional linkers. The reaction mixture was as follows: 19 μ l cDNA, 3 μ l 10x Flush Buffer (proprietary), 1.5 μ l 100 mM DTT, 3 μ l 1 mM dNTPs, 0.6 μ l (1.5 U) T4 DNA polymerase and 2.9 μ l nuclease free water. This was incubated at 11°C for 20 minutes. 20 μ l of TE and then 50 μ l of TE-buffered phenol:chloroform:isoamyl (25:24:1) alcohol were added and vortexed. After centrifugation at 12,000 g for 1 minute the aqueous phase was transferred to a fresh tube. 50 μ l chloroform:isoamyl (24:1) alcohol was added, vortexed and centrifuged as before. The aqueous phase was aspirated and mixed with 50 μ l 4 M ammonium acetate, 1 μ l glycogen and 250 μ l ethanol. This was incubated at -20°C for 1 hour, centrifuged as above for 10 minutes and the supernatant discarded. The DNA pellet was rinsed successively in 70% and 100% ethanol, air dried, resuspended in 10 μ l TE and stored at -20°C till needed.

2.5.2.4 Ligation of EcoRI and Hind III directional linkers

These linkers were ligated to the blunt ended cDNA fragments to allow eventual ligation of the molecules into the T7 10-3 phage vector in the correct orientation. The EcoRI and HindIII linkers were phosphorylated immediately before the main ligation. The reaction was as follows:

10 μ l blunt ended cDNA, 2 μ l 10x Ligation Buffer (200 mM Tris-HCl pH 7.6, 50 mM MgCl₂), 2 μ l 1 mM adenosine triphosphate (ATP), 2 μ l 100 mM DTT, 2 μ l (100 pmol) directional EcoRI/HindIII linkers, 0.5 μ l (5U) T4 polynucleotide kinase (PNK). This was incubated at 37°C for 5 minutes. 1.5 μ l (6 Weiss units) of T4 DNA ligase was then added, mixed and the reaction incubated for 20 hours at 16°C. The ligase was inactivated by heating to 70°C for 10 minutes. The reaction was allowed cool slowly to RT°.

2.5.2.5 Digestion of linker EcoRI and Hind III restriction sites

The ligated linkers were prepared for ligation to phage vector arms by enzymatic digestion with the restriction endonucleases EcoRI and Hind III. The digestion reaction was set up as follows:

20 μ l ligated cDNA, 10 μ l 10x Hind III Buffer (proprietary), 65 μ l nuclease free water and 5 μ l (100 U) Hind III. This was incubated at 37°C for 2 hours. 10 μ l 10x EcoRI Adjustment Buffer (proprietary) and 5 μ l (100 U) EcoRI were then added and incubated at 37°C for 4 hours. After brief centrifugation, 115 μ l (1 volume) chloroform:isoamyl alcohol (24:1) was added, mixed and centrifuged, and the aqueous phase extracted as before. To this was added 1 μ l glycogen, 120 μ l 4M ammonium acetate and 500 μ l ethanol. The digested cDNA fragments were stored in this mixture at -20°C till size fractionated (next step).

2.5.2.6 Size fractionation

This was necessary to remove excess linkers and small cDNA fragments (less than 300 bp) from the cDNA mixture. The stored cDNA fragments were precipitated, rinsed with ethanol and air-dried as previously described. The cDNA pellet was resuspended in 100 μ l TE. 2 mls of Gel Filtration Resin (proprietary) slurry was loaded onto a filtration column and storage buffer let run through. The settled 1 ml of resin was washed through with 5 mls of Column Buffer (proprietary), till buffer had drained to the level of the resin bed. The 100 μ l of diluted cDNA was then loaded onto the gel bed. After this was settled, 200 μ l of Column Buffer was gently added to the column and let flow through (as void fraction). 250 μ l Column Buffer was loaded onto the resin and the flow-through (containing the largest cDNA fragments) collected. This was repeated once more. The cDNA in both eluates was pelleted as previously described and resuspended in 20 μ l TE. 2 μ l from each was analysed by agarose gel electrophoresis, to confirm the presence of fragments of the correct size. The cDNA was now ready for ligation into vector arms.

2.5.2.7 Ligation of retinal cDNA fragments to T7 10-3 vector arms

All T7Select Phage Display System kit components (Novagen) are designed to be compatible with cDNA products from the OrientExpress kit, and were used to express the retinal cDNA library polypeptides as a phage library. The T7Select 10-3b vector was used, which expresses 5 -15 copies per phage of polypeptides up to 1200 amino acids (aa) in size, on the 10 B capsid protein. This 36,249 bp size vector contains multiple cloning sites. It acts as a typical T7 vector (previously described) and uses *E. coli* BLT5615 as host. This host contains an ampicillin/carbenicillin resistance plasmid, which also serves as a source of essential exogenous capsid protein. The expression of the essential capsid protein 10A in BLT5615 is under the control of a lacUV5 promoter and therefore requires the presence of IPTG for induction. BLT5615 was grown in M9LB liquid medium containing 50 µg/ml carbenicillin.

Retinal cDNA was ligated with T7Select vector arms, whose multiple cloning sites had been pre-digested with EcoRI and Hind III. Maximal cloning efficiency required a molar insert:vector ratio of between 1:1 and 3:1. The remaining 18 µl of retinal cDNA was precipitated by the ammonium acetate method and resuspended in 5 µl of TE. The reaction was then set up as follows in a 0.5 ml tube:

1.5 µl insert cDNA, 1 µl (0.5 µg) T7Select vector arms, 0.5 µl 10x Ligation Buffer, 0.5 µl 10 mM ATP, 0.5 µl 100 mM DTT and 1 µl (containing 0.6 Weiss units) T4 DNA Ligase. The 5 µl reaction was mixed and then incubated at 16°C for 16 hours.

2.5.2.8 In vitro phage assembly

In vitro packaging of phage particles was carried out by incubation of the purified recombinant T7 10-3 vectors with a cell-free phage packaging extract. A vial containing 25 µl of T7 Packaging Extract (proprietary) was thawed on ice. The 5 µl of recombinant vector (ligation reaction) was mixed with this without vortexing, and incubated at RT° for 2 hours. The reaction was stopped by adding 270 µl LB medium. 10 µl of the mixture was taken for plaque assaying to determine the titre of primary recombinants. 10µl of each serial phage dilution

was mixed with 250 μ l of mid-log BLT5615, 100 μ l 1M IPTG and 2.5 mls molten top agarose and plated on LB plates containing 50 μ g/ml carbenicillin.

2.5.2.9 Amplification and titration of library

The remaining 290 μ l of packaged phage was amplified to produce a library containing numerous copies of each recombinant. 500 mls of M9LB/carbenicillin was inoculated with 5 mls of BLT5615 overnight culture. This was grown to mid-log and IPTG added to 1mM final concentration. After 30 minutes further incubation, the 290 μ l packaged phage solution was added to the culture and incubated till lysis was observed (3-4 hours). 10 μ l of lysate was taken for titration by plaque assay and the rest stored at 4°C in 0.02%NaN₃.

2.5.2.10 Estimation of percentage of retinal cDNA recombinant clones in library

One plate containing around 200 well spaced plaques was chosen for analysis. A PCR containing 10 μ l of PCR mix per well was set up as previously described for T7 phage. T7 Forward (5`-ACA ACG TTA TCG GCC TGT TC-3`) and T7 Reverse (5`-TAC CGG AGG TTC ACC GAT AG-3`) primers were used. 78 wells were directly inoculated with DNA template from individual clones by the plaque touch method. After thermal cycling, PCR products of individual phage clones were analysed by electrophoresis on a 2% agarose gel. Phage containing no retinal cDNA insert appeared as bands of approximately 400 bp in size. Recombinants containing retinal inserts produced larger PCR products and were easily identified. The percentage of recombinant versus non-recombinant phage in the amplified library was estimated.

The whole ligation/phage packaging process was repeated using a higher cDNA insert:vector molar ratio. The remaining 3.5 μ l of cDNA was precipitated and resuspended in 1.5 μ l TE. This was ligated to the T7 10-3b vector arms, packaged in vitro, amplified and analysed as already described. The resulting library was pooled with the first. Phage were stored as lysate with 0.02% NaN₃, at 4°C till required for biopanning. 100 mls of lysate was then taken, clarified and PEG precipitated. The phage pellet was resuspended in PBS at a concentration of 2.2 x 10¹¹ PFUs/ml.

2.5.3 Screening cDNA library against human sera

2.5.3.1 Micro-biopanning using capture antibody

The retinal cDNA library was biopanned against sera from 37 uveitis patients and 42 controls. A capture antibody "micro-biopanning" strategy, coating the wells of 96-well microtitre ELISA plates with sera from individual patients or controls, allowed enrichment for phage displaying immunoreactive retinal peptides. Before the main experiment, a number of optimisation procedures were carried out to determine the dilutions of capture antibody and sera to be used.

Each (labelled) well was coated with 100 μ l of goat anti-human IgGAM (Sigma) at 1:500 dilution (12.4 μ g/ml) in carbonate buffer, and incubated overnight at 4°C. 2 negative control wells were set up: one without capture antibody and another without human serum, both undergoing all other incubation steps. Wells were washed out x3 with PBST, blocked with PBST/BSA 5% and incubated for 2 hours at 37°C. Sera were diluted 1:10 in PBST/BSA 5%, 100 μ l loaded into each well and incubated for 2 hours at 37°C. Wells were washed 5 times and 100 μ l of cDNA library phage (containing 10^8 PFUs in 100 μ l blocker) added. This was incubated for 1 hour at 37°C. Excess phage were removed through 5 rounds of washing and 200 μ l of BLT5615 cells (mid-log, IPTG induced) added to each well. Affinity selected phage were allowed infect the host cells in-situ for 10 minutes. Each 200 μ l was then transferred to its respective well in a deep, 96-well plate, each well containing 500 μ l mid-log cells. Deep well plates were then incubated in a shaker at 37°C for 3 hours or until lysis was observed. Lysates were clarified by centrifuging the 96-well plate at 5000 RPM for 10 minutes. Average post-amplification titres were calculated by performing plaque assays on serial dilutions from 6 representative wells. Phage were stored in situ (with NaN₃ 0.02%).

3 rounds of biopanning were carried out in total. 10^8 PFUs (as clarified lysate) were used as input for the 2nd and 3rd rounds. After each round, individual sera capturing and enriching for specific phage were identified by PCR, essentially as described previously but using 0.5 μ l of each lysate as

template DNA. Titres of amplified lysates were calculated after each round and showed a round-on-round increase.

The biopanning experiments were later repeated with an additional blocking step during each of the 3 rounds, in order to reduce affinity-selection of non-specific T7 10-3 phage. A solution of PEG-precipitated wild-type T7 phage in PBS was rendered non-infective by irradiation with UV light. Wells were blocked for 1 hour with 100µl of this solution (originally containing 3×10^9 PFUs/ml), between the serum-coating and live phage-coating steps, and washed out as before.

2.5.3.2 Analysis and DNA sequencing of affinity-selected phage within pools.

Phage pools from the 2nd and 3rd rounds of biopanning from both experiments were analysed for the presence of phage clones displaying retinal-derived protein fragments. Various methods were employed as the process evolved. Once individual clones were isolated, their DNA was sequenced and sequences were subjected to analysis on established electronic nucleic acid and protein databases.

2.5.3.2.1 Gel extraction method

PCR amplification was carried out using 0.5 µl of each phage pool as input DNA and using standard T7 forward and reverse primers and conditions. PCR products from each phage pool were analysed by agarose gel electrophoresis and pools containing products greater than 370 bps (containing a retinal insert) identified. Products from these selected pools were re-run on extended length 1% agarose gels at 70V for several hours. Individual DNA bands (> 370 bps) were cut from the gel under UV light (allowing minimal exposure), placed in 1.5 ml Eppendorf tubes and weighed. DNA was purified from each gel slice using the QIAquick gel Extraction Kit (Qiagen). This uses a silica-based membrane, which selectively binds DNA under conditions of high salt concentration and pH < 7.5. The procedure was as follows: 3 gel volumes of Buffer QG (proprietary, contains guanidine thiocyanate and a pH indicator) were added to each tube. This was incubated in a 50°C waterbath till agarose was completely dissolved,

ensuring pH remained below 7.5. One volume of isopropanol was added, mixed, and the sample loaded into a QIAquick spin column. DNA was bound to the membrane by centrifugation at 10,000 g for 1 minute, and flow-through discarded. The membrane was washed with 0.75 mls of Buffer PE (proprietary, contains ethanol) and centrifuged through, and then centrifuged once more to remove residual ethanol. DNA was eluted by pipetting 50 μ l of Buffer EB (10 mM Tris-Cl, pH 8.5) directly onto the membrane, incubating for 1 minute and centrifuging as before into a fresh collection tube. 1 μ l of each sample was used as template for re-amplification, in a 15 μ l PCR reaction. Correct DNA fragment size was verified by agarose gel and 5 μ l of each reaction used for automated DNA sequencing as previously described. The T7 reverse primer (5`-TAC CGG AGG TTC ACC GAT AG-3`) was used for sequencing.

2.5.3.2.2 Random PCR screening of plaques derived from phage pools

Plaques from phage pools known to contain clones of interest were plated out and 10-20 plaques from each randomly chosen for initial screening. PCR amplification using T7 primers, followed by electrophoresis, identified any clones containing retinal inserts. Corresponding plaques were amplified and stored, while their PCR products were sequenced as above.

2.5.3.2.3 Amplification of phage pools using modified T7 primers coupled with enzymatic pre-digestion

This method was designed to eliminate/reduce the amplification of T7 10-3 recombinants without an insert ("baseline" PCR products) and therefore only amplify those recombinants that coded for a retinal protein fragment. PCR products from the original amplifications were digested for 1 hour with the EcoRV restriction endonuclease, as described for Sequencing. A modified T7 forward primer ("T7insertPCRa", 5`-ATG CTC GGG GAT CCG AAT TCA AGC-3`) was designed that would anneal to EcoRV digested vector/insert recombinant molecules, but which would not prime T7 vectors without an insert. The reverse primer used was 5`-GGT TAA CGT AGA TGG ATT GAC CGG A-3` ("T7b2"). Optimisation of the PCR reaction was carried out for the new primers,

and the standard T7 program combined with the reaction buffer "Buffer H" (5x = 300 mM Tris-HCl pH 9.0, 75 mM (NH₄)₂SO₄, 17.5 mM MgCl₂) [Invitrogen] was found to be optimal. Modified PCR products were further processed and sequenced as described for "gel extraction method". The sequencing primer used was 5`-TAC CGG AGG TTC ACC GAT AG-3`.

2.5.3.2.4 "Shotgun cloning" method of clone isolation and expression

This method is described in detail below. It enabled separation of individual clones from within affinity-selected phage pools and identification of their inserts by DNA sequencing, while at the same time facilitating the expression of affinity selected protein fragments.

2.5.4 Bioinformatic analysis of sequences

Sequencing readouts were analysed manually between the EcoR I and Hind III restriction sites. Inserts in the correct orientation and containing an open reading frame (ORF) were identified using GeneJockey Software (BioSoft Ltd). Sequences were translated to reveal their related amino acid sequence (if any). Both DNA and protein sequences were analysed on NCBI Genbank databases searched with the BLAST algorithm (www.ncbi.nlm.nih.gov/blast), for matches to previously identified sequences. cDNA library sequences corresponding to protein fragments likely to originate from human retinal or neural tissue were earmarked for further analysis at this stage. The remaining sequences were discarded.

2.5.5 Expression and purification of recombinant cDNA library polypeptides.

It would have been possible to test the immunoreactivity of displayed retinal protein fragments in-situ as amplified phage clones by, for example, ELISA. However, previous experience with T7 phage systems had shown high levels of non-specific cross-reactivity with T7 elements. Therefore, the affinity selected retina-derived protein fragments were produced in purified form by ligating the

DNA inserts into a new vector, expressing the recombinant protein fragment in a bacterial host and purifying the product.

2.5.5.1 Construction of recombinant expression vector

The pCR-T7-NT-Topo plasmid (previously described) was chosen for expression of the protein fragments. Both plasmids and inserts (PCR products) were restriction digested with BamH1 and Hind III.

PCR products corresponding to protein fragments for analysis were enzymatically purified by digestion with SAP and exonuclease 1 as previously described. 1 μ l (20U) of BamH I (USB) and 1 μ l (20U) of Hind III were then added to the mix and incubated at 37°C for 90 minutes.

Plasmid was restriction digested as follows: 10 μ l (100 ng) pCT-T7-NT-Topo plasmid was mixed with 2 μ l 10x OPA buffer, 1 μ l BamH I, 1 μ l Hind III and 6 μ l H₂O. This was incubated at 37°C for 1 hour and the reaction cleaned up in a Qiagen spin column. Plasmid was eluted in 30 μ l of PCR-grade H₂O.

A PCR/plasmid ligation reaction was then set up for each different PCR product. 5 μ l digested PCR product was mixed with 1 μ l cut plasmid solution, 0.5 μ l (5U) T4 DNA ligase, 1 μ l 10 mM ATP and 1.5 μ l H₂O. This was incubated at 12°C for 12 hours.

2.5.5.2 Propagation and maintenance of plasmids in Top 10 F⁻ host cells

Top 10 F⁻ cells were chosen for stable maintenance and propagation the recombinant plasmid. 2.5 μ l of each recombinant plasmid was used to transform an aliquot of chemically competent cells, as previously described for RSAg expression in bacteria. Well-spaced colonies from each transformation were grown on LB agar/ampicillin plates, and 20-40 colonies from each chosen for analysis. The presence or absence of insert-containing recombinant plasmids was determined by PCR amplification across the restriction sites, using forward primer "pcDNA3.1a" (5'-TAA TAC GAC TCA CTA T-3') and reverse primer "T7reverse" (5'-CTA GTT ATT GCT CAG CGG TGG-3'). PCR products from positive colonies were sequenced as previously described, using pcDNA3.1a as sequencing primer in a 2-step sequencing reaction. Positive colonies were

amplified by overnight incubation in liquid medium, and after mixing 50:50 with sterile glycerol, stored at -70°C till needed for expression.

2.5.5.3 Transformation of BL21(DE3)pLysS or pLysE strains and expression of cDNA library protein fragments

Expression of the retinal cDNA library-derived protein fragments was tried using both BL21(DE3)pLysS and BL21(DE3)pLysE cells as host. BL21(DE3)pLysE is practically identical to BL21(DE3)pLysS, but produces higher levels of T7 lysozyme, which provides even tighter control of basal level expression of genes. This may be useful if the gene product is toxic.

Transformed Top 10F` cultures were grown overnight from frozen stocks in 5 mls of LB/chloramphenicol/ampicillin. Plasmid "mini-preps" were carried out the next morning, as previously described. Aliquots of BL21(DE3)pLysS or BL21(DE3)pLysE cells were immediately transformed and pilot expressions carried out, inducing protein expression with IPTG as described for RSAg. Yields of the desired protein were analysed by SDS-PAGE/Coomassie staining and Western blotting, for each time point. Substantive expressions were carried out the next day, using optimal time-points for each individual cell line. Better yields were possibly found when transformed cell lines were not frozen/re-thawed between pilot and substantive expressions. Instead cultures were maintained overnight and used to re-seed fresh medium the next morning. Cells containing the positive control plasmid pCR-T7/NT-E3 were also induced in parallel with those expressing cDNA library fragments.

Plasmid "mini-preps" were repeated on all transformed Top 10 F` and BL21(DE3)pLysS cell lines. The plasmid DNA was then re-sequenced using the same primer, to verify that each cell line contained the correct plasmid/insert.

2.5.5.3.1 Induction by infection with T7 phage

Because of difficulties in expressing the recombinant proteins in BL21(DE3)pLysS (see Results), attempts were made to stimulate protein expression by directly infecting transformed cells with T7 phage. T7 DNA polymerase should in theory have provided a more robust induction signal to the lacUV5 promoter. Transformed BL21(DE3)pLysS and Top 10 F⁻ cells were grown to mid-late log phase and infected with wild-type T7 phage. Lysis was noted in 1-2 hours. Parallel non-infected cultures were also grown to check for basal recombinant protein expression. Lysates were pelleted and the supernatant separated. Pellets of non-infected cells and debris from lysates were resuspended in PAGE loading buffer. Supernatants were concentrated and de-salted using Centricon centrifugal concentrators (3,000 MW cut-off) and also resuspended in loading buffer. All samples were analysed by PAGE/Coomassie staining and Western blotting. No significant recombinant protein was produced in either phage-infected or non-infected samples and the method was abandoned.

2.5.5.4 Alternative "shotgun cloning" method for isolation and expression of affinity-selected cDNA library clones

This method enabled separation of individual clones from within affinity-selected phage pools and identification of their inserts by DNA sequencing. At the same time, it facilitated the expression of affinity-selected protein fragments. Lysates from the 3rd round of biopanning, each representing a pool of phage clones, were used as template for a PCR amplification. Each heterogeneous sample of PCR products was purified by enzymatic digestion and then restriction digested with BamH I and Hind III. The PCR products were ligated into similarly digested pCR-T7-NT-Topo plasmids, as previously described. Recombinant plasmids representing each pool were transformed into separate aliquots of Top 10 F⁻ cells and plated out as colonies. 20-40 colonies (clones) from each plate were labelled, their plasmid DNA amplified by PCR, and the plates stored. Agarose gel electrophoresis revealed which colonies contained cDNA inserts, and PCR products from these were sequenced. Important sequences were identified and the corresponding colonies amplified in liquid medium and stored as glycerol stocks. The end result was the separation, DNA

amplification, sequencing and transformation of individual clones within phage pools, without having to identify and excise (the sometimes indistinct) DNA bands from agarose gels.

2.5.5.5 Harvesting and protein purification

All steps were carried out essentially as previously described for RSAg. 100 mls of cells were induced for each recombinant protein fragment. Verification of expression of the correct protein was carried out by PAGE/Coomassie staining and with Western blotting using anti-His-HRP primary antibody. After expression, cells were harvested and lysed. Recombinant proteins were purified using the Probond system and concentrated using Centricon and Centriprep centrifugal concentrators. Protein concentration was determined by BCA assay. Protease inhibitor cocktail was added to purified samples and they were stored at -20°C .

2.5.6 ELISAs for measuring antibodies against cDNA library polypeptides.

Purified protein fragments derived from affinity-purified retinal cDNA library clones, were tested by ELISA for immunoreactivity against all 79 patient and control sera. Two indirect ELISA systems were tried: a 4-step capture system, in which a solid phase, antigen-specific monoclonal antibody was used to bind and orientate the polypeptide and a 3-step assay with recombinant antigen directly bound to the ELISA plate. Preliminary "checkerboard" assays were carried out for both types of ELISA (as previously described for other ELISA assays), to determine the optimal concentrations/dilutions of capture antibody, antigen, serum, secondary (conjugated) antibody and type of blocking buffer.

2.5.6.1 4-step indirect capture ELISA

Mouse anti-His1 monoclonal antibody was chosen as capture antibody for the (His-tagged) recombinant protein fragments. Mouse ascites anti-His (Sigma) was chosen over affinity-purified anti-His (Invitrogen), as it produced less non-

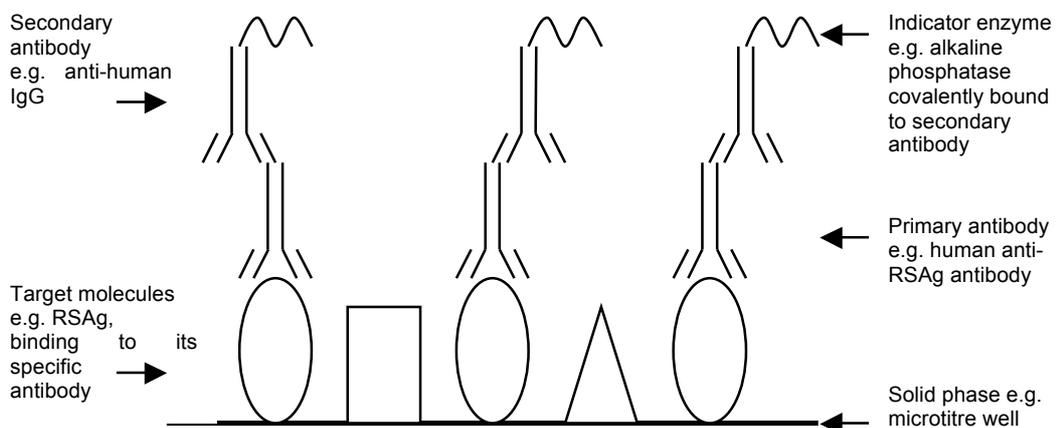
specific "background". PBST/ non-fat dried milk 5% w/v was found to block non-bound sites on ELISA plates more effectively than PBST/BSA 5%.

ELISA wells were coated with 50 μ l of capture antibody, at 10 μ g/ml (approx.1:3000) in carbonate buffer, and incubated overnight at 4°C. Wells were washed 3 times with PBST and blocked with 350 μ l of PBST/5% milk for 2 hours at RT°. After washing, recombinant antigens were coated in duplicate at 2.5 μ g/ml in PBST/1% milk and bound overnight at 4°C. Two negative control wells, one without capture antibody and the other without serum, were run in parallel with each panel of antigens. Another control, using an irrelevant His-tagged protein as antigen, was also run to detect inadvertent serum responses to the His tag epitope on recombinant antigens. ELISAs were then carried out essentially as previously described. Sera were coated at a concentration of 1:25 and the secondary antibody (anti-human IgGAM - alkaline phosphatase) at 1:20,000, both in PBST/1% milk.

2.5.6.2 3-step indirect ELISA

Recombinant antigens were bound directly in wells (in duplicate), at 2.5 μ g/ml in carbonate buffer, and incubated overnight. Sera were used at a dilution of 1:25 and secondary antibody as 1:20,000. Negative controls and His-tagged protein controls were carried out as above. PBST/5% milk was once again used as blocker. The procedure was carried out essentially as described before.

Figure 2.1 Schematic representation of a 3-step indirect ELISA



2.6 Studying T-cell responses to retinal autoantigen using cytokine flow cytometry

2.6.1 PBMC stimulation by RSAg

13 uveitis patients were selected consecutively from our outpatient clinic. Each had a clinical diagnosis of autoimmune posterior uveitis. Of the individual sub-diagnoses in this group 5 patients had intermediate uveitis, 4 had retinal vasculitis/periphlebitis, 2 had multifocal choroiditis and 2 had pan-uveitis. Ages ranged from 32 to 63 years (mean=42.6). 5 male patients and 8 females took part. 10 healthy laboratory workers were recruited as controls. These included 8 males and 2 females, ranging in age from 26 to 45 years (mean=34).

RSAg had previously been prepared from fresh bovine retinas, as described in Materials. After extraction, purified RSAg was made up to a final concentration of 4 $\mu\text{g}/\mu\text{l}$ in PBS. A PAGE-SDS gel was run prior to the experiment to check viability of the antigen. No contaminants were detected on the gel.

PBMCs were isolated from peripheral venous blood of uveitis patients and controls by density gradient sedimentation, and were resuspended in "complete" medium at a concentration of 2×10^6 /ml, as described previously. Fresh cells were used for each stimulation assay (frozen stocks were not used in these assays, but kept in reserve). Stimulation of cells commenced within 3 - 4 hours of harvesting.

Ex-vivo T-cell stimulation and cytokine staining were based on the method previously described by Waldrop (Waldrop et al. 1997), with the notable exception that an 18 hour incubation was used (see below). Briefly, 3 assays were set up for each test sample: a "positive control", "negative control" and "antigen specific" tube. In each, 1×10^6 cells in medium were placed in sterile 12x75 mm polystyrene tissue culture tubes (Elkay Lab Products). Positive control samples were stimulated with 20 ng/ml phorbol myristate acetate (PMA), and 1 μM ionomycin (both Sigma), providing robust non-specific T-cell activation. Negative control tubes contained cells, medium and 1 μg anti-CD28 monoclonal antibody (Beckman Coulter, High Wycombe, U.K.). Antigen specific

tubes contained 50 µg of RSAg and 1 µg of anti-CD28. All tubes were made up to 1 ml volume with medium prior to incubation.

Cells were incubated at 37°C and 5% CO₂ for 18 hours, with brefeldin A (Sigma), an exocytosis inhibiting agent, being added for the last 17 hours at a concentration of 10 µg/ml. Although previous studies have utilised a 6 hour incubation time, here a higher antigen-specific:negative control responder ratio was found when the incubation was extended to 18 hours. This was determined by repeating the stimulation assays over different incubation periods, on PBMCs saved from the first 2 patients found to elicit positive responses. The optimum dosage of antigen was also determined during these preliminary tests. It was felt that this incubation time was justified, as recent studies have indicated that peak expression of IFN-γ occurs at 8 hours with only slight reduction after 18 hours (Mascher et al. 1999) and levels of intracytoplasmic CD69 remain optimum up till at least 18 hours (Rostaing et al. 1999). Excessive T-cell loss due to activation-induced apoptosis was not thought likely after only 18 hours incubation.

2.6.2 Fixation and intracellular cytokine staining

After incubation, EDTA was added (final concentration 2 mM) and cells resuspended. After 10 minutes, PBMCs were placed in 0.5% formaldehyde/borate buffered saline and fixed overnight. Intracellular cytokine and surface marker staining was then carried out. Fluorochrome-conjugated anti-IFN-γ monoclonal antibody was used to detect any intra-cellular expression of this (typical Th1-type) cytokine. Similarly, expression of CD69 (a general marker of early activation in lymphocytes), CD4 and CD8 was detected using labelled monoclonal antibodies. Fluorochrome/monoclonal antibody combinations were as follows: interferon gamma-fluorescein isothiocyanate (FITC), CD69-phycoerythrin (PE), CD4-PCy5 (all Immunotech, Marseilles, France) and CD8-ECD (Coulter, Miami, USA). Positive control samples were split in 2 equal parts: one half was stained as above, and the other with isotype-matched FITC control antibody (Immunotech), to evaluate non-specific background staining. Cells to be stained were permeabilised with saponin 0.1% (Sigma) and directly labelled with 5µl of monoclonal antibody. After 2 ½ hours

staining, cells were washed 3 times in PBS/ 0.1% BSA / 0.1% saponin (Sigma) and finally resuspended in formalin 0.5%.

2.6.3 Flow cytometry and electronic analysis of data

Cells were analysed on a 4-colour flow cytometer (EpicsXL-MCL, Beckman Coulter) within 3 days of staining. For each subject, 50,000 PBMCs were analysed for each split positive control sample, while approximately 500,000 were analysed for negative control and antigen specific tubes. Data were analysed using WinMDI 2.8 software (<http://facs.scripps.edu/software.html>). Lymphocyte events were identified from within the total PBMC population using the parameters of forward versus side-scatter and these were electronically gated. This sub-population was analysed for CD4 versus CD8 expression and the CD4⁺, CD8⁻ population gated. These events were analysed for IFN- γ and CD69 co-expression and data presented as dot-plots. Negative control samples were analysed first and the quadrant markers set around the main body of baseline events for IFN- γ expression (horizontal axis) and at 10² Log fluorescence units for CD69. Using these quadrant settings, the positive control samples and then antigen-stimulated samples were analysed for levels of IFN- γ /CD69 co-expression. "Positive" events were those in the upper-right, "double-positive" quadrant. The numbers of "positive" events, CD4⁺ cells, lymphocytes and total PBMCs were automatically recorded, and expressed in absolute and percentage terms.

2.6.4 Analysis of individual patient and aggregate results

Results were produced both in terms of the responses of individual patients or control subjects *and* the aggregate responses of whole patient and control groups. For purposes of comparison, positive event numbers were converted to rates per 100,000 CD4⁺ cells for all analyses. The designation of a subject's response to RSAg stimulation as "positive" required co-expression of IFN- γ and CD69 in cells forming a distinct cluster of events in the double-positive quadrant of the dot-plot. It also required the negative control tube showing no or a negligible proportion of positive events.

Aggregate responses were determined by comparing the values for baseline-subtracted (i.e. antigen-specific minus negative-control) positive events, between patient and control groups. The 2 groups of values were compared using standard statistical tests. This approach was used because it was felt that analysis of absolute numbers of antigen-specific positive events was not valid without comparison to the number of positive events from the negative control tubes in the same group of subjects.

In the following four "results" chapters (Chapters 3, 4, 5 and 6), the format Introduction, Results and Discussion is used. Details of methods employed are found in the relevant sections of Chapter 2 (Materials and methods).

**Chapter 3: Production of recombinant
human RSAg and detection of serum
antibodies to RSAg by ELISA**

3.1 Introduction

RSAg is important because it is considered one of the foremost candidate autoantigens for the induction of human autoimmune uveitis. Its effects have been extensively studied in both animal models and humans, as discussed in Chapter 1. The majority of experiments to date have utilised the bovine form of the protein, presumably because supplies of bovine retina are more readily available. Although the human and bovine forms share similar physico- and immunochemical properties (Beneski et al. 1984), important differences have been noted (see also Figure 3.1). In particular, bovine RSAg carries epitopes not shared by other species (Wacker et al. 1977; Faure et al. 1984; Mirshahi et al. 1985). However, bovine RSAg is still used in experimental studies mainly because of the relative scarcity of human eyes. Even when available, yields from human eyes are low: in one report extraction of RSAg from 40 human eyes yielded only 2.5 mg of purified protein (Doekes et al. 1987).

Serum antibodies against bovine RSAg have previously been detected in autoimmune uveitis patients using passive haemagglutination (Dumonde et al. 1982). Gregerson found relatively elevated serum titres of anti-bovine RSAg in a mixed group containing anterior and posterior - including infectious - uveitis patients using ELISA (Gregerson et al. 1981). Abrahams (Abrahams and Gregerson 1982; Abrahams and Gregerson 1983) also found similar results by ELISA in patients with granulomatous uveitis, but antibody titres correlated only weakly with clinical activity in longitudinal studies. Forrester found no significant differences between patients and controls in terms of frequency of anti-bovine RSAg antibodies or range of titres (Forrester et al. 1989). One paper reported lower affinity anti-RSAg antibodies in patients with retinal vasculitis compared with controls (Kasp et al. 1992b). Another study reported no circulating anti-bovine RSAg antibodies in sera from 25 uveitis patients and 10 controls (Chan et al. 1985c).

Doekes (Doekes et al. 1987) found no quantitative or qualitative differences in the ELISA response to human RSAg in uveitis patients and controls, or in different types of uveitis patient. Anti-RSAg antibodies were found in around 25-30% of both groups. Anti-RSAg IgG, IgA and IgM levels were found to be equal in both groups. No correlation was found between humoral

and cellular reactivity to RSAg in individual subjects. Doekes (Doekes et al. 1992) also tested a large number of uveitis patients and controls for reactivity against both bovine and human forms of RSAg. Again no differences were demonstrated between patient and control groups. The numbers of positive sera and the levels of anti-RSAg antibodies correlated significantly between human and bovine RSAg groups, but there were many exceptions at an individual level. The same study compared the immunoreactivity of each form of purified RSAg to anti-human RSAg and anti-bovine RSAg antibodies raised in animals. This demonstrated a predominant role for species-specific epitopes on bovine RSAg but not human RSAg, and concluded that human RSAg should therefore be used for ELISA testing of individual human sera.

It is obvious from the above that the human form of RSAg should be used wherever possible in experiments on human uveitis samples. Given the known inter- and intra-species differences between different forms of antigens, it is not surprising that considerable variation in immunoreactivity has been found. The limited availability of human eyes for research, coupled with the low yields of RSAg from such eyes, would appear to be a considerable problem for those planning large-scale experiments with human RSAg. However, the possible solution of producing recombinant RSAg does not appear to have been fully exploited. The DNA and amino acid sequences of human RSAg are known and the production of human RSAg in either prokaryotic or eukaryotic cell hosts appears to be technically feasible. Recombinant RSAg has been produced before, but has mostly been either of the non-human form or has been expressed in bacterial hosts (Kasp et al. 1992a). The possible advantages of using a bacterial host would be rapid production of large amounts of antigen, whereas protein expressed in eukaryotic cells would include post-translational modifications and would therefore be more similar to the native protein confirmation in-vivo. The aim of these experiments was therefore to attempt to clone human RSAg and express it in both prokaryotic and eukaryotic cells. I also aimed to test any recombinant antigen for the detection of autoantibodies in uveitis patients and controls by ELISA, and compare its performance to that of bovine RSAg.

3.2 Results

3.2.1 Expression of recombinant human RSAg in eukaryotic cells

The human RSAg sequence was successfully cloned in the pcDNA4-TO-myc-HisA vector (Figure 3.2) and expressed in TReX 293 human embryonic kidney cells. A transient transfection was first carried out and when this was shown to be successful (by SDS-PAGE and Coomassie staining), was followed by a stable transfection. DNA sequencing of the recombinant vector confirmed the sequence was correct for human RSAg. Stably transfected clones SA3 and SA5 were found to contain RSAg, which was verified by SDS-PAGE/silver staining and Western blotting/probing with anti-His-HRP antibody. Cells from both cell lines were stored long-term in liquid nitrogen. Cells from clone SA5 were induced with tetracycline for 72 hours and harvested as pellets containing 1×10^7 cells each. The 20 pellets (total cells = 2×10^8) were stored at -70°C till purified using the Probond nickel-chelate resin system. RSAg/His tag fusion protein was successfully purified in small but definite amounts, as shown by SDS-PAGE/silver staining (Figure 3.6) and Western blotting (Figure 3.7). The His tags were not cleaved from the RSAg molecule. Total amount of recombinant RSAg, as determined by BCA assay, was approximately 29 μg .

3.2.2 Expression of recombinant human RSAg in bacterial cells

The human RSAg insert was successfully cloned into the pCR-T7-NT-Topo plasmid and recombinant antigen expressed as a fusion protein from BL21(DE3)pLysS bacterial cells. PCR products coding for RSAg (Figure 3.4) were first ligated into pCR-T7-NT-Topo plasmids (Figure 3.3) and used to transform Top 10 F['] cells. Several clones likely to contain the correct insert were identified by PCR amplification across the restriction site of recombinant plasmids (Figure 3.5). Plasmid preps and glycerol stocks were kept from each candidate cell line. 3 cell lines of BL21 (DE3)pLysS were established with plasmids from these candidate cell lines. Plasmid preps were again produced

from each of the 3 cell lines and DNA sequencing reactions carried out from both ends of the restriction site. Only one clone (RSAg2) was found to contain the correct sequence for RSAg. A pilot expression, using different time-points post induction, was carried out on this cell line. Time of maximal expression of RSAg was found to be 4 hours, as determined by SDS-PAGE/Coomassie staining and Western blotting (Figures 3.8 (a) and 3.9 (a)). A substantive expression (200 ml cell culture) was carried out. Fractions produced during protein purification with the Probond system (washes, elutions, etc) were kept and analysed by SDS-PAGE and Western blotting (Figures 3.8 (b) and 3.9 (b)). This confirmed the presence of the RSAg fusion protein. The pCR-T7-NT-Topo leader sequence, containing a His tag, enterokinase cleavage site and the XPress synthetic epitope, was again left in situ. Recombinant protein was concentrated to 129 $\mu\text{g/ml}$ in PBS. 290 μg of recombinant protein were produced in total.

3.2.3 Expression of positive control, 58 kDa protein

The pCR-T7-NT-E3 plasmid, which codes for a His-tagged, 58 kDa protein kinase protein, was successfully transformed into BL21 (DE3)pLysS cells and expressed (Figure 3.10). This protein too was purified using the Probond system. As well as confirming that the transformation/expression and purification procedures were carried out properly, the 58 kDa protein was used as a His tag positive control in ELISA experiments (see below).

Figure 3.2 Map of recombinant pCDNA4-TO-myc-HisA plasmid with human RSAG insert – for expression in human TREx 293 cells.

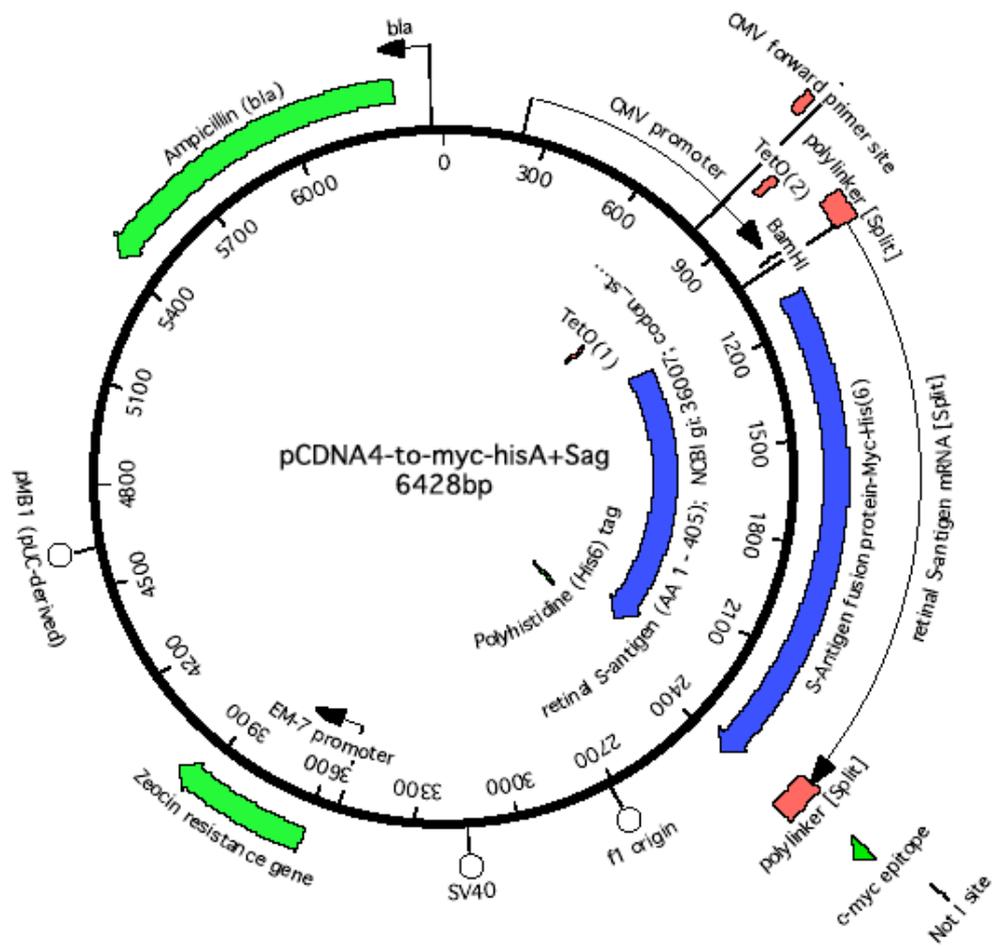


Figure 3.3 Map of recombinant pCR-T7-NT-Topo plasmid with human RSAG insert – for expression in BL21 bacterial hosts.

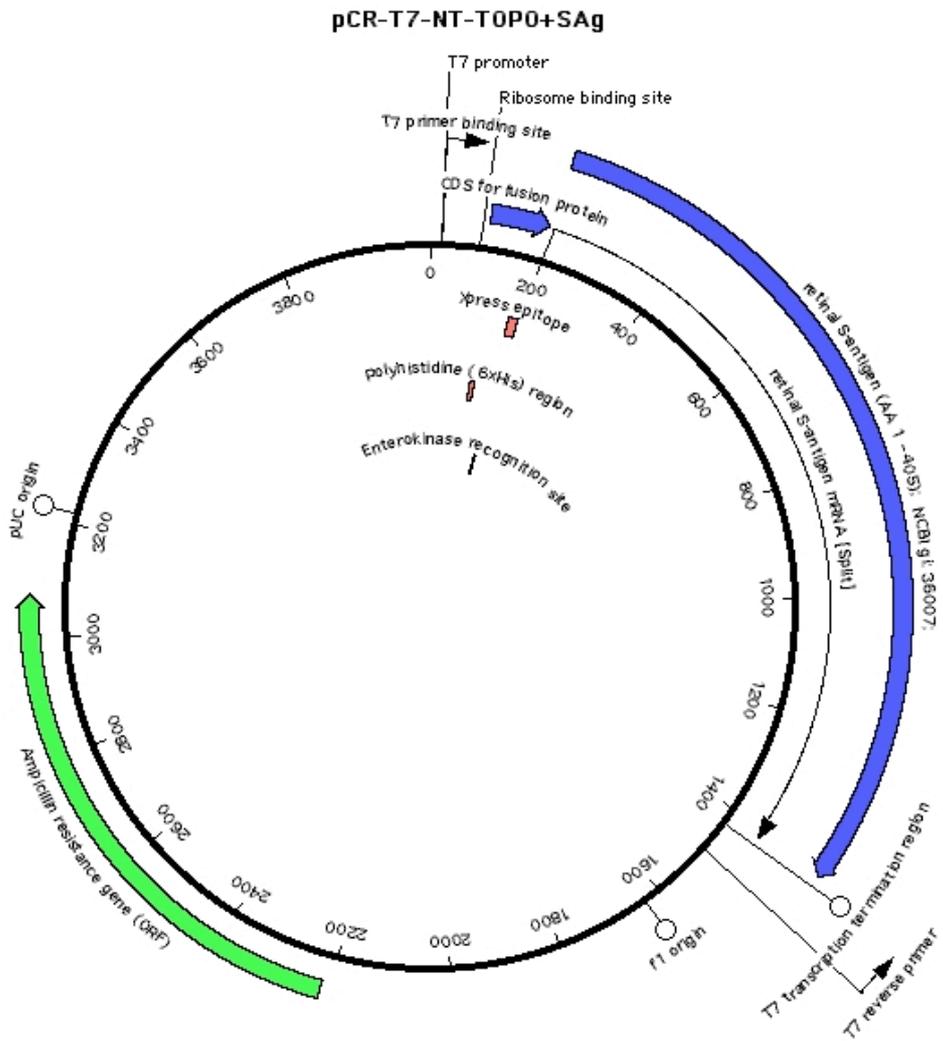


Figure 3.4 PCR products coding for human RSAg, produced by the enzyme ELONGASE™, on an agarose gel.

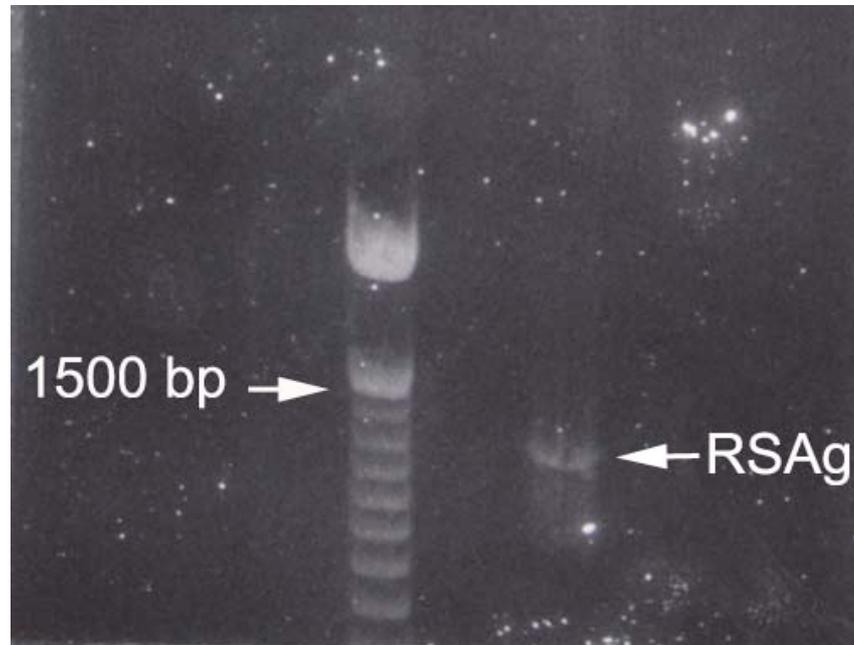
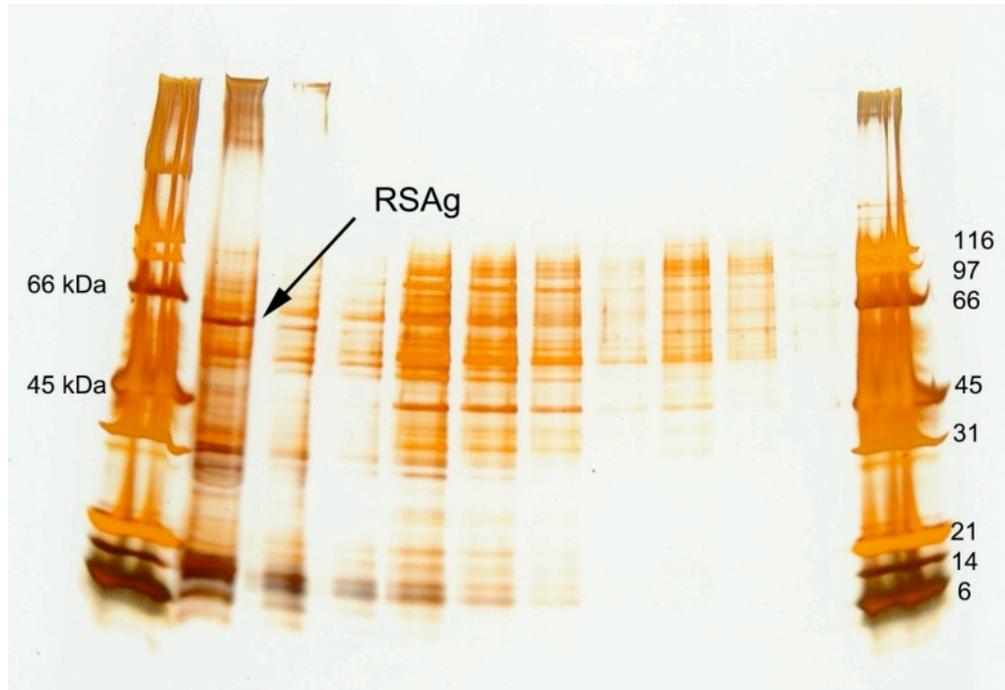


Figure 3.5 PCR amplifications of input DNA from Top10F cell clones containing human RSAg/ pCRT7-NT-Topo recombinant plasmids.



Figure 3.6 SDS-PAGE gels with silver staining of recombinant human RSAg: (A) expressed in eukaryotic cells and (B) purified using Probond™ resin.

(A)



(B)

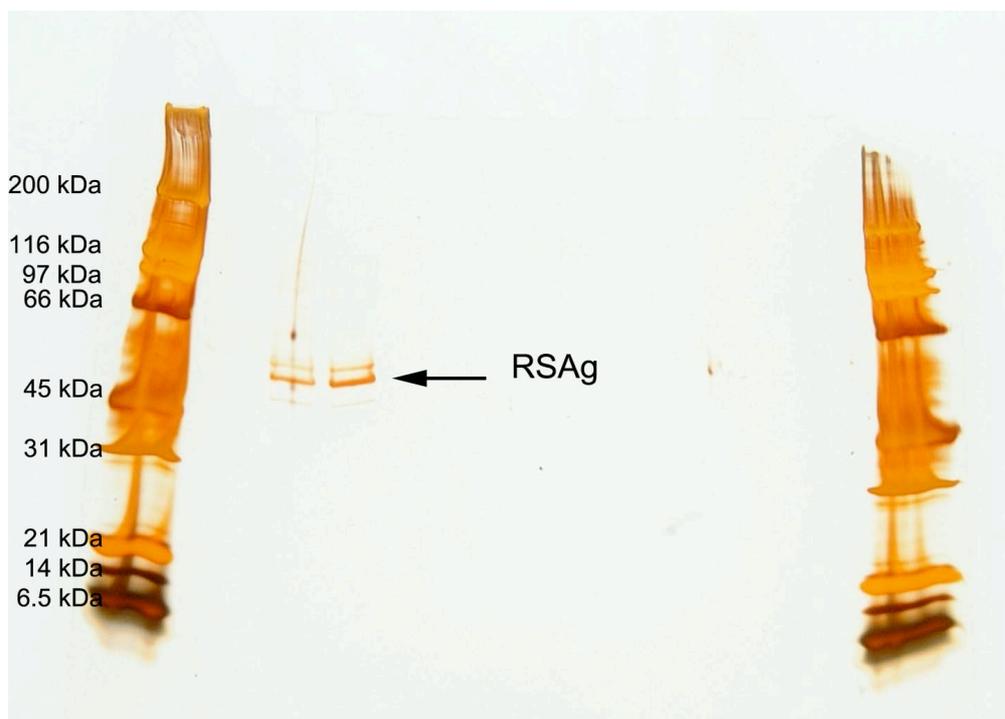
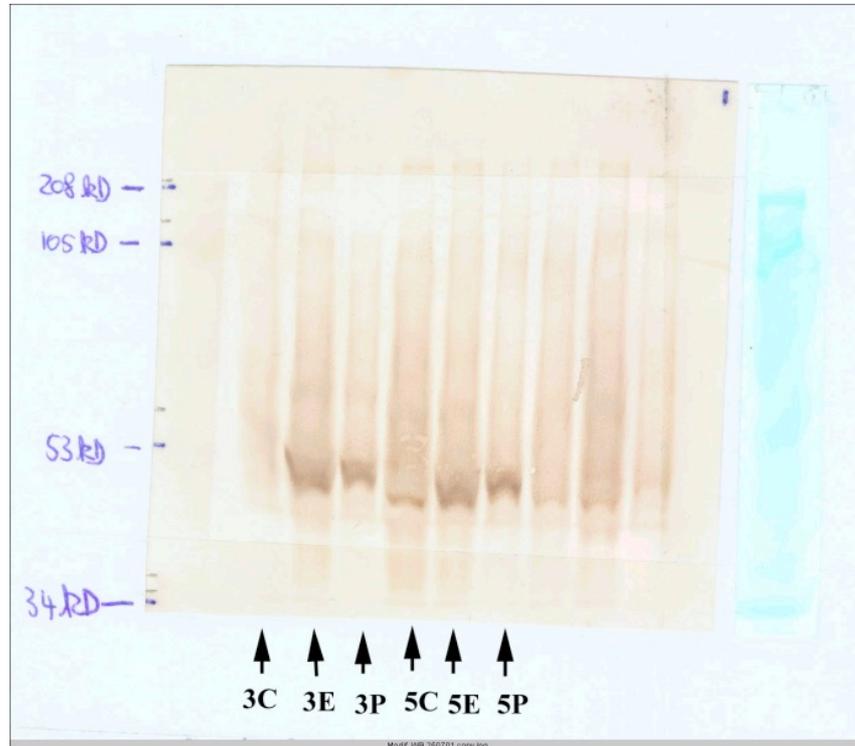


Figure 3.7 Western blots of recombinant human RSAg probed with anti-His monoclonal antibody: (A) expressed from eukaryotic cells clones and (B) elutions 1-7 after purification using Probond resin.

(A)



(B)

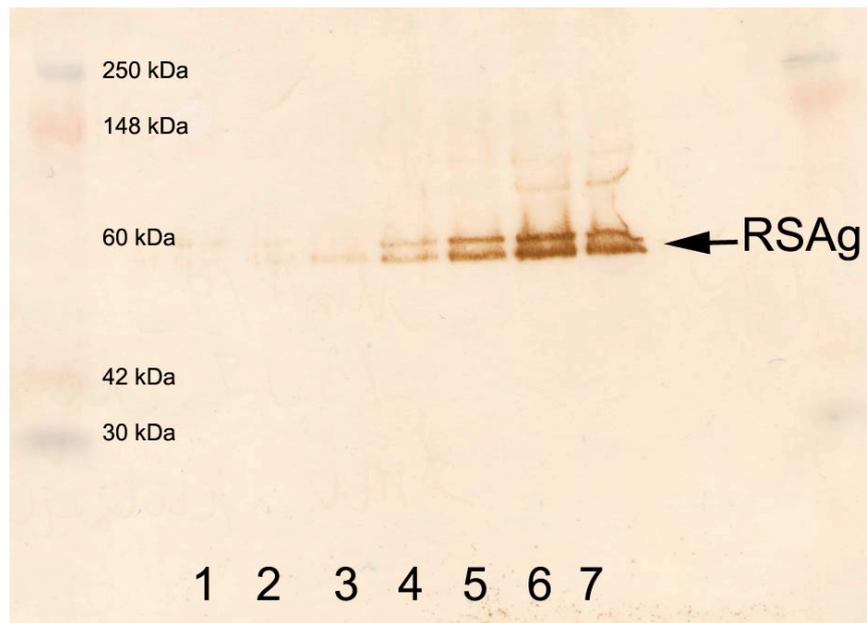
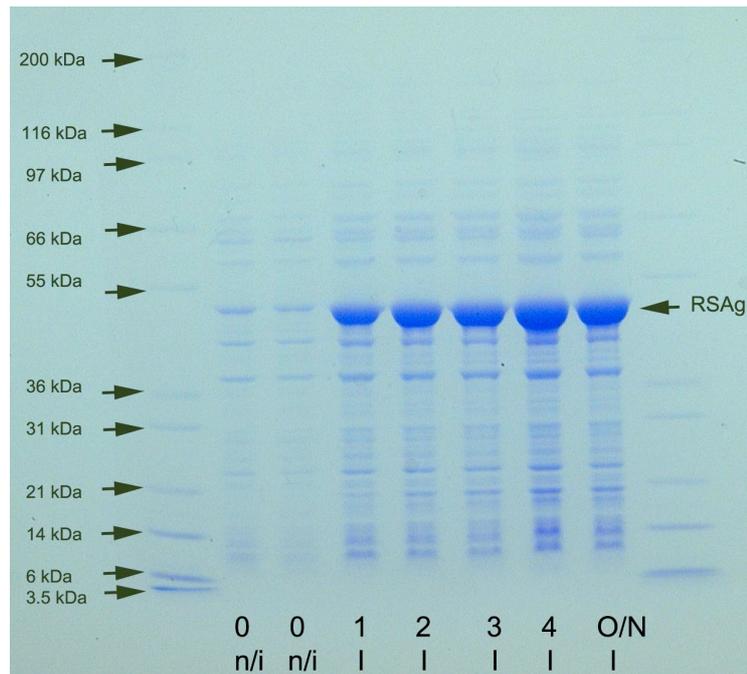


Figure 3.8 SDS-PAGE gels with Coomassie staining of recombinant human RSAg. (A) Expressed protein from bacterial cells at various time points in hours. n/i=non-induced, O/N=overnight. (B) Washes and elutions during Probond purification process using resin. R=raw lysate, B=binding buffer excess, w6.0=wash excess at pH 6.0, 1-7=elutions.

(A)



(B)

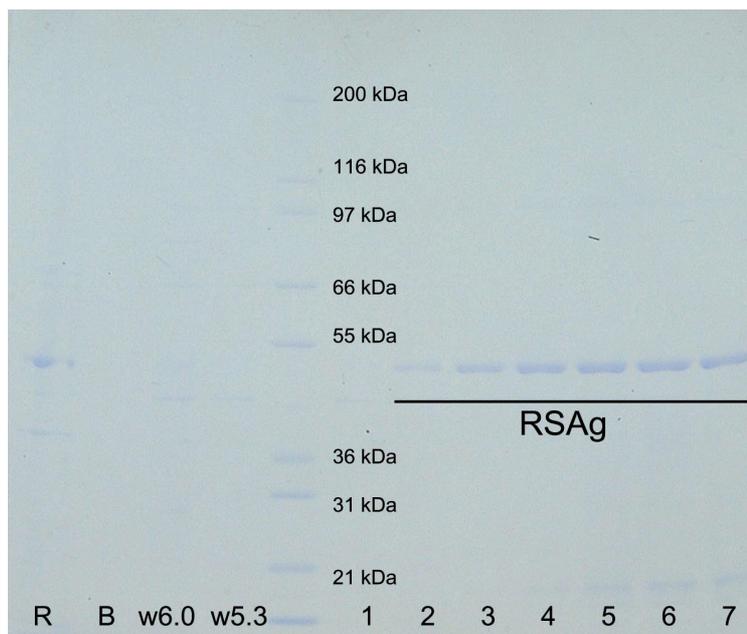
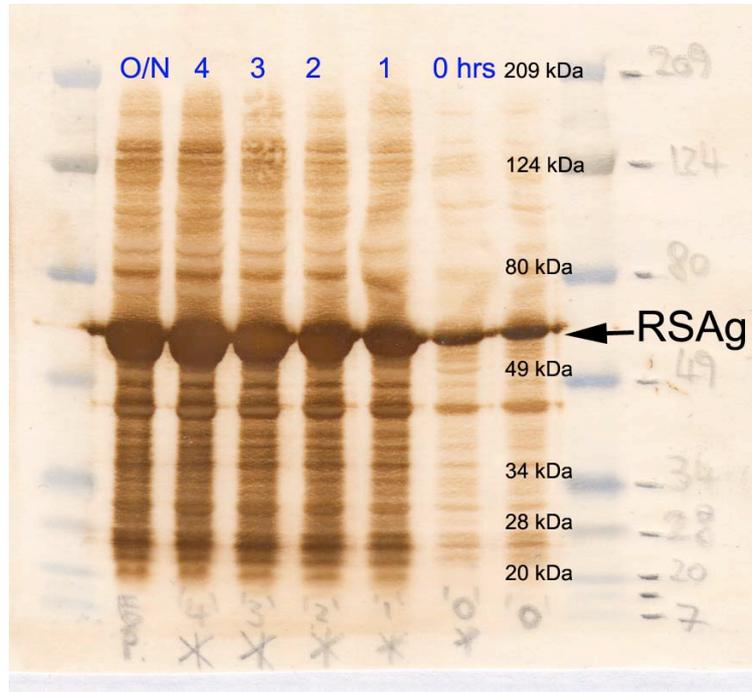


Figure 3.9 Western blots of gels from Figure 3.8, probed with anti-His monoclonal antibody.

(A)



(B)

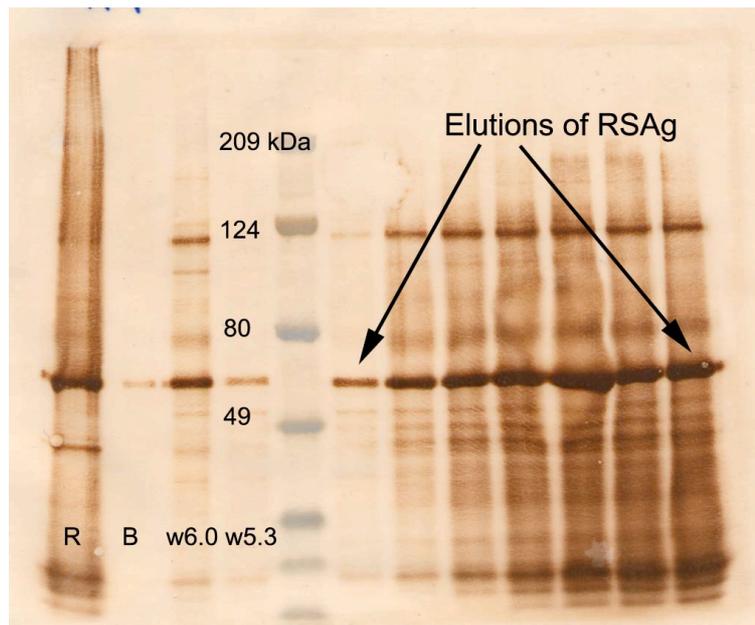
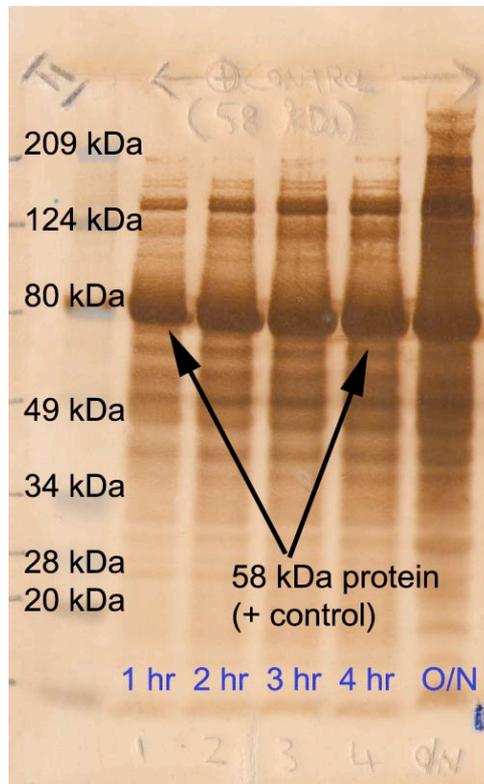


Figure 3.10 Western blot of 58 kDa protein kinase containing polyhistidine tag, expressed over 1-4 hours and overnight.

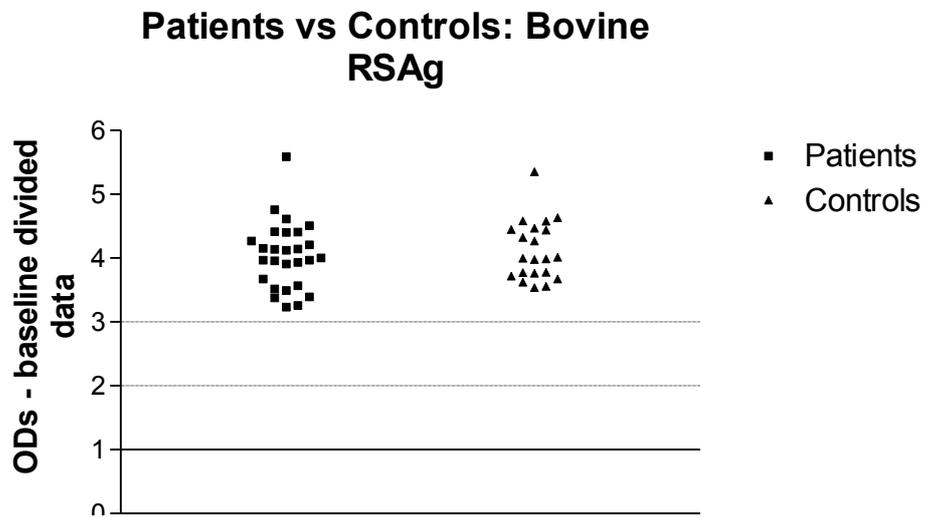


3.2.4 ELISA testing for measuring RSAg antibodies in serum

3.2.4.1 ELISA using bovine RSAg alone

In preliminary experiments using bovine RSAg, indirect, 3-step ELISAs were carried out in duplicate for each tube of uveitis or control serum, as described in Chapter 2. Twenty-seven individual patient samples, P1-15, 17, 19-28 and 30 (see Table 3.1) and 21 individual control sera C2, 4, 5, 7-10, 13, 15-17, 19, 22, 24, 26-31 and HS were analysed. 3 bovine RSAg-coated ELISA plates were used in total, one screening patient sera and the other 2 screening control sera. A pair of wells blocked with 5% BSA (not containing RSAg) acted as negative controls for each plate, and were screened with the sera of one patient/control chosen at random. The random serum sample was P30 (ii) for the Patient plate, C19 (ii) for Control Plate 1 (samples C2 to C19) and C31 (iii) for Control Plate 2 (samples C22 to HS). Average ODs for each serum test sample were divided by the average readings for their negative control wells. Where there were more than 1 tube of an individual serum, the negative control-divided readings were averaged. Patient versus control readings are presented as a scatter-graph (Fig. 3.11). Both groups of data followed a normal distribution. Comparison of the 2 groups showed no significant differences between the mean baseline-divided readings ($p=0.5632$), using a 2-tailed t-test with Welch's correction (GraphPad Prism, Version 2). However, given the lack of a negative control for individual serum samples, this result should be interpreted guardedly.

Figure 3.11 Scattergram of corrected ELISA OD readings from patient and control sera using bovine RSAg. Baseline-divided data are categorised as follows: <1.0 negative result, 1.0-2.0 “low” titre, 2.0-3.0 “medium” titre, >3.0 “high” titre.



3.2.4.2 Analysis of uveitis patient and control sera by ELISA using both recombinant human and bovine RSAg

The sera of 45 patients (P1-15, 17, 19-28, 30, 50-55, 58-69) and 45 controls (C2, 4, 5, 7-10, 13, 15-17, 19, 22, 24, 26-33, 35-43, HS, C50-62) were screened by ELISA against panels of duplicate wells as described in Chapter 2. Each panel contained duplicate wells of a negative control, a 58 kDa His-tagged protein, human recombinant RSAg (expressed in bacteria and containing a His tag), and bovine RSAg – this layout provided a robust internal control for each serum. Each pair of readings was averaged, and the test readings for bovine and human RSAg wells were divided by the negative control readings. Readings from wells containing the 58 kDa His-tagged protein were compared with those containing human recombinant RSAg on a case-by-case basis, to identify any cases displaying disproportionate immunoreactivity to the His tag. Results were expressed in terms of multiples of the negative control reading (i.e. "blocked" wells), values below 1.0 being regarded as negative. Immunoreactivity of each "positive" individual serum (to both types RSAg) was categorised as "low" (1.0 – 2.0), "medium" (2.0 – 3.0) or "high" (3.0 or above). Results were analysed on an individual subject basis, and patient and control sera were also compared with each other as groups. Human and bovine RSAg readings were correlated, as were human RSAg and 58-kDa protein readings (both contain a His tag). Cases where immunoreactivity to human RSAg but not bovine RSAg was demonstrated were highlighted, and a possible explanation sought.

Of a total of 45 uveitis patients, those with "positive" ELISA tests (displaying "medium" or "high" reactivity to either bovine or human antigen) were found to represent individual clinical conditions as follows: pars planitis, 8 out of 19 samples; sarcoid uveitis, 4 of 8; pan-uveitis, 2 of 4; multifocal choroiditis, 1 of 3; serpiginous choroiditis and MEWDS, each 1 of 1). None of the 5 samples from patients with ocular Behcet`s were positive, nor were the individual samples from patients with Wegener`s granulomatosis/white dot syndrome, neuro/chorioretinitis and AMPPE. Patients displaying "medium" reactivity to either form of antigen were P1, P3, P10, P15, P21, P23, P25, P28, P51, P53, P55 and P65. Those displaying "high" reactivity were P2, P11, P24,

P52 and P60. Patient samples displaying "medium" or "high" reactivity to *both* forms of antigen were P51, P60 and P65.

The immunoreactivities of patient and control groups were compared using Log-transformed data from both types of RSAg (Figures 3.12 and 3.13). No significant differences were detected – $p=0.169$ for bovine RSAg and $p=0.5298$ for human RSAg. The immunoreactivity of human sera was found to be significantly higher to human RSAg than to bovine RSAg, again using Log-transformed data (Figure 3.14). Data were analysed by t-test (with Welch's correction for unequal variances) and found to be significantly different for both uveitis patients ($p=0.0486$) and healthy controls ($p<0.0001$). No difference in immunoreactivity was detected between active and inactive uveitis patients (Figure 3.15), when tested using log-transformed data from human RSAg ($p=0.5119$). The readings for bovine and human RSAg correlated as groups (Figure 3.16), with a p value of 0.0003 using a 2-tailed Spearman correlation test ($r=0.3724$). Readings from human RSAg and the 58 kDa protein (both containing His tags) were correlated (Figure 3.17), using the Pearson correlation test on log-transformed data from the 2 groups. Statistically significant correlation was shown with a p value of <0.0001 . However, the "R squared" value was only 0.3145, indicating that 31% of the variance was shared between the 2 groups. Readings from bovine RSAg and His-tagged protein, showed no demonstrable correlation (Figure 3.18), using a 2-tailed Spearman test for non-normally distributed data ($p=0.3937$, $r=0.09099$).

Table 3.1 Individual patients: clinical diagnosis, activity and disease duration. IUSG, International Uveitis Study Group classification. Case numbers P2a, P2b, etc. denote repeat samples. Unavailable details are indicated by N/A.

Case/serum No.	Age	Clinical Diagnosis	IUSG Diagnosis	Activity	Duration Current Episode	Total Duration	Syst. Meds
P1	20	Pars planitis	Intermediate uveitis	1+	2 mths	8 years	No
P2a	51	Sarcoid uveitis	Pan-uveitis	Minimal	5 mths	54 mths	Yes
P2b	54	Sarcoid uveitis	Posterior	Inactive	N/A	90 mths	Yes
P3	46	Serpiginous choroiditis	Posterior	Inactive	N/A	15 mths	No
P4	34	Pars planitis + vitritis /Hodgkin's	Intermediate	2+	N/A	N/A	Yes
P5	77	Multifocal/birdshot choroiditis	Posterior	Inactive	N/A	2 years	Yes
P6	46	Behcet's disease	Pan-uveitis	N/A	N/A	N/A	N/A
P7	77	Sarcoid uveitis	Posterior	Inactive	N/A	17 mths	Yes
P8	34	Multifocal choroiditis	Posterior	Active	N/A	N/A	N/A
P9	50	Neuroretinitis + chorioretinitis	Posterior	Inactive	N/A	25 mths	Yes
P10	49	Multifocal choroiditis	Posterior	N/A	N/A	N/A	N/A
P11	29	Pars planitis + vitritis	Intermediate	1+	6 mths	39 mths	No
P12a	23	Pars planitis	Intermediate	N/A	N/A	N/A	N/A
P12b	26	Pars planitis	Intermediate	Inactive	N/A	5 years	Yes
P13	39	Behcet's disease	Pan-uveitis	N/A	N/A	N/A	N/A
P14	64	Wegener's/white dot choroiditis	Posterior	Inactive	N/A	2 weeks	Yes
P15	29	Pars planitis	Intermediate	Inactive	N/A	7 years	No
P17a	40	Pars planitis (MS)	Intermediate	N/A	N/A	N/A	N/A
P17b	44	Pars planitis (MS)	Intermediate	1+	2 mths	10 years	Yes
P19	22	Pars planitis /inter. uveitis	Intermediate	Inactive	N/A	11 mths	No
P20	28	AMPPE	Posterior	Active	1 week	23 mths	No
P21	49	Pars planitis	Intermediate	Inactive	N/A	16 mths	No
P22	29	Behcet's disease	Pan-uveitis	N/A	N/A	N/A	N/A
P23	41	M.E.W.D.S.	Posterior	N/A	N/A	N/A	N/A
P24	25	Anterior + inter. uveitis	Intermediate	Active	1 week	1 week	No

Case/serum No.	Age	Clinical Diagnosis	IUSG Diagnosis	Activity	Duration Current Episode	Total Duration	Syst. Meds
P25	47	Sarcoidosis	Posterior	N/A	N/A	N/A	N/A
P26	37	Uveitis+vasculitis	Posterior	N/A	N/A	N/A	N/A
P27	16	Pars planitis	Intermediate	1+	N/A	3 years	No
P28	38	Pars planitis	Intermediate	N/A	N/A	N/A	N/A
P30	40	Pars planitis	Intermediate	Active	N/A	N/A	N/A
P50	46	Post uveitis (? sarcoid)	Posterior	Active	N/A	N/A	Yes
P51	54	Pan-uveitis	Pan-uveitis	Minimal	23 mths	13 years	No
P52	32	Pars planitis +periphlebitis	Intermediate	Minimal	2 mths	2 years	No
P53	34	Intermediate/ pars planitis	Intermediate	1+	2 mths	2 mths	No
P54	40	Behcet's disease	Pan-uveitis	2+	3 weeks	13 years	Yes
P55	56	Pan-uveitis/vitritis	Pan-uveitis	1+	N/A	17 years	No
P58	30	Intermediate uveitis	Intermediate	Active	3 weeks	6 years	No
P59	29	Intermediate uveitis	Intermediate	2/3+	3 weeks	16 mths	No
P60	48	Sarcoid posterior uveitis	Posterior	Minimal	1 month	14 years	No
P61	36	Pars planitis (B/L)	Intermediate	Inactive	N/A	5 1/2 years	No
P62	40	Sarcoid uveitis	Posterior	Active	6 weeks	15 years	No
P63	56	Pan-uveitis	Pan-uveitis	Active	8 mths	10 years	Yes
P64	30	Behcet's disease	Pan-uveitis	Active	1 month	9 years	Yes
P65		Sarcoid kerato-uveitis	Posterior	1+	N/A	7 years	No
P66	69	Pan-uveitis	Pan-uveitis	Active	6 mths	4 years	Yes
P67	61	Intermediate uveitis	Intermediate	Inactive	N/A	32 mths	N/A
P68	42	Posterior/inter uveitis	Posterior	Active	N/A	N/A	N/A
P69	37	Ant. + posterior uveitis (MS)	Posterior	2+	3 mths	3 years	No

Figure 3.12 Patient and control sera screened against bovine RSAg by ELISA (2nd experiment).

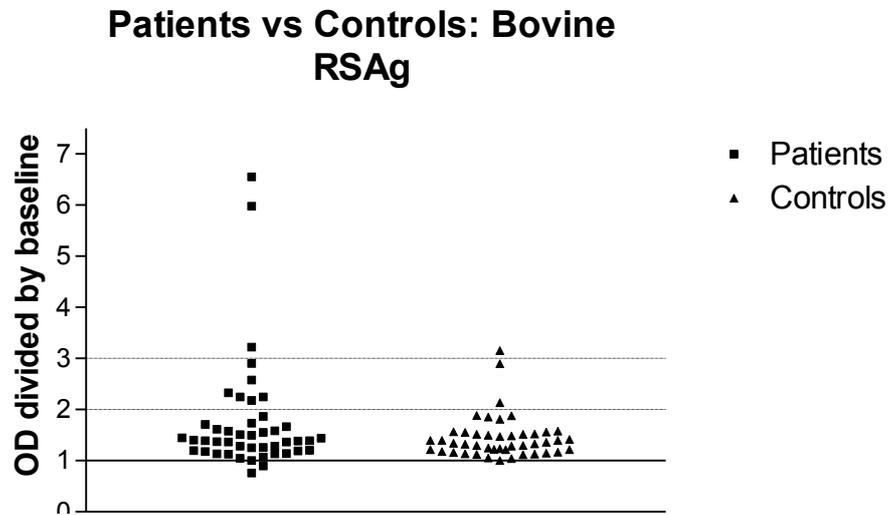


Figure 3.13 Patient and control sera screened against human recombinant RSAg by ELISA.

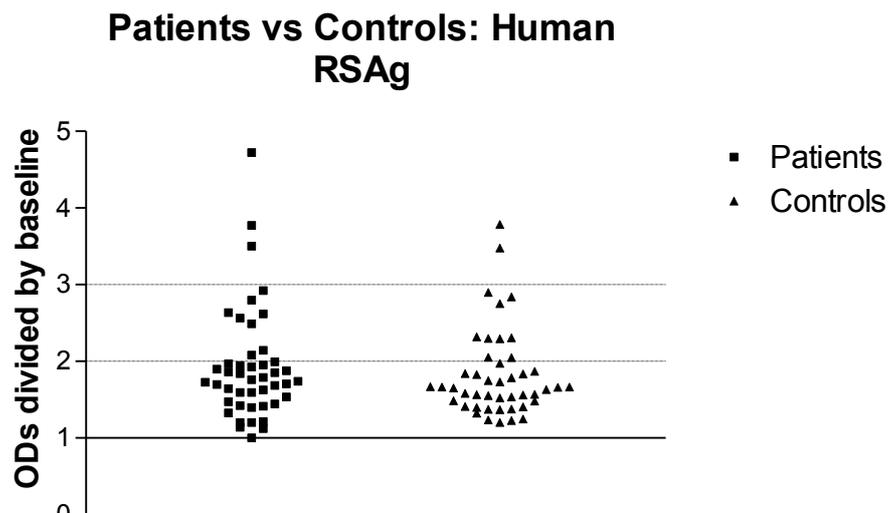


Figure 3.14 Comparison of bovine and recombinant human RSAg reactivity against patient and control sera, as tested by ELISA.

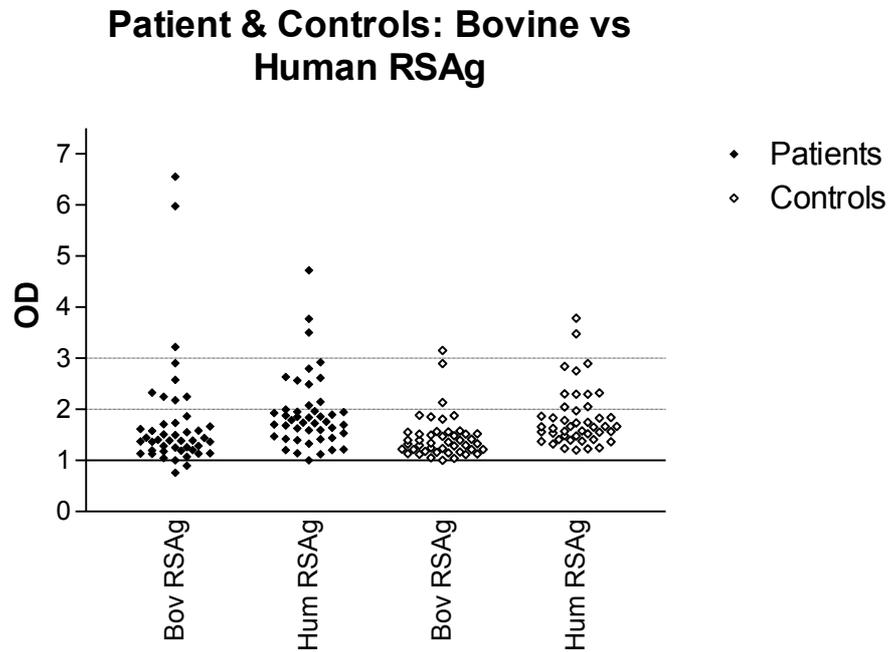


Figure 3.15 Comparison of reactivity of sera from active and inactive uveitis patients, against recombinant human RSAg by ELISA.

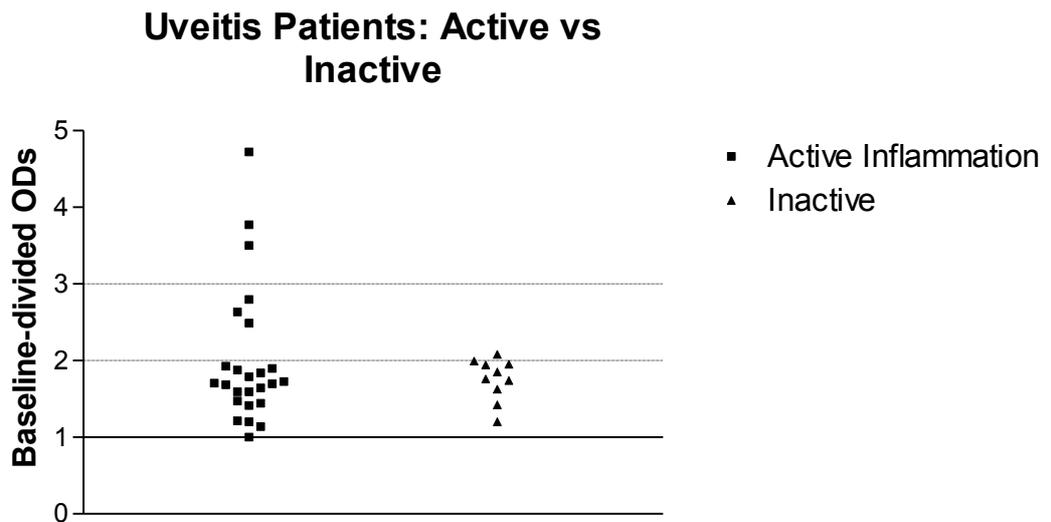


Figure 3.16 Correlation of corrected ELISA readings obtained from individuals using bovine and human recombinant RSAg.

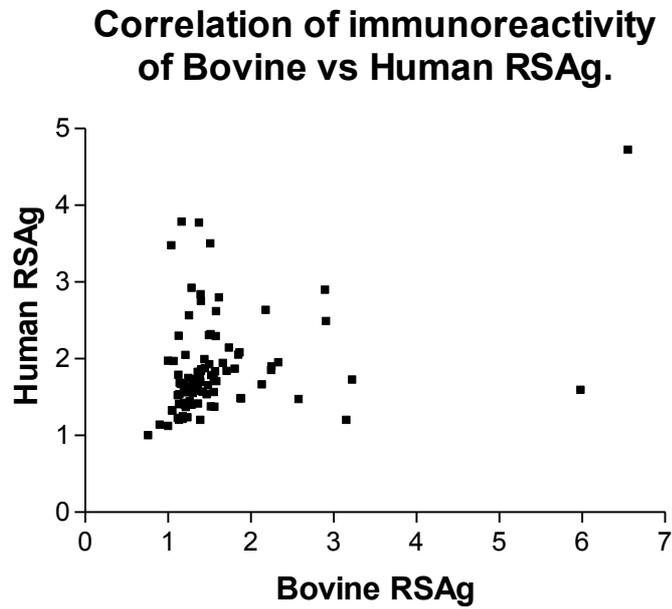


Figure 3.17 Correlation of readings from human recombinant RSAg and 58 kDa His-tagged control protein.

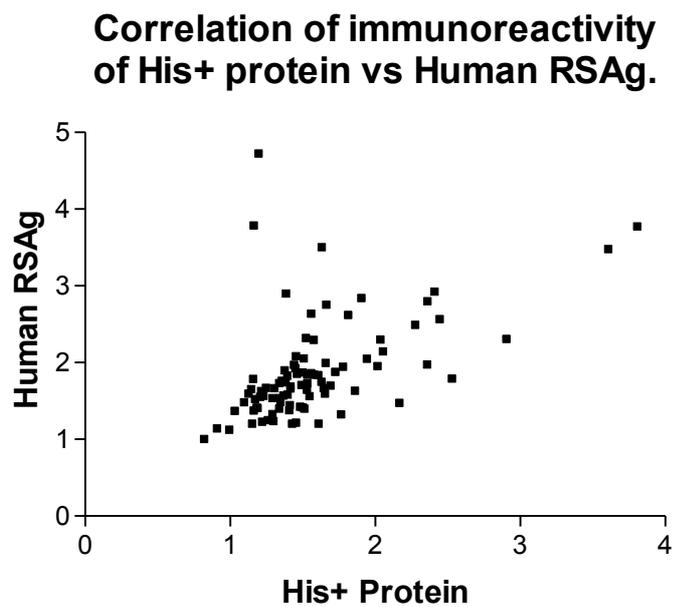
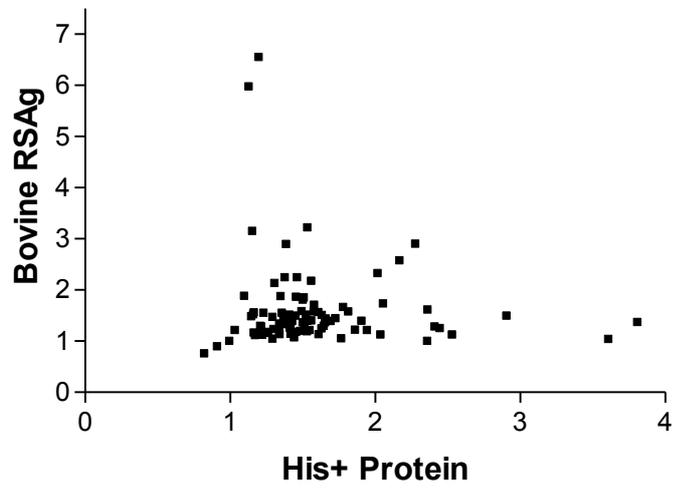


Figure 3.18 Correlation of readings from bovine RSAg and 58 kDa His-tagged control protein.

**Correlation of immunoreactivity
of His+ Protein vs Bovine RSAg**



3.3 Discussion

RSAg is probably the most widely studied of the candidate autoantigens for human autoimmune uveitis. Studies have demonstrated humoral and cellular immunoreactivity to RSAg, mainly in animal models but also in human subjects. However the bovine form of RSAg has been used in the great majority of these experiments, presumably because of lack of availability of the human form, and therefore experiments on humans using human antigen are relatively few. In this study I aimed to clone and express recombinant human RSAg, and then compare its performance to that of the bovine form in the measurement of autoantibody levels in uveitis patients and controls by ELISA.

Here we have demonstrated the successful cloning and expression of the human antigen as a fusion protein in both prokaryotic and eukaryotic cells. There are few reports of this being achieved previously, in particular of RSAg being expressed from human cells. Only moderate amounts of protein were produced from transfected eukaryotic cells, though yields could reasonably be expected to improve with fine-tuning of the optimal times for induction of these cells. Time constraints did not allow us to optimise the technique on this occasion. Antigen was expressed as a fusion protein incorporating a polyhistidine tag to aid in the purification process, which used a nickel chelate resin system. This tag was not cleaved in any of the following experiments because it was not felt necessary to do so. This however could have been done easily by cleaving the enterokinase site using a commercially available kit. Recombinant antigen was also expressed in bacterial cells. This resulted in higher yields and had the advantage of being easier and quicker to produce. As well as the advantage of being a theoretically unlimited supply of protein, recombinant antigen production also has advantages in terms of increased quality control, greater purity, and decreased likelihood of cross-contamination by other proteins of similar size. Given that the protein expressed in human cells would be more likely to contain eukaryotic post-translational modifications and therefore more closely mimic the native form, future efforts should probably concentrate on maximizing yields from this source. The successful production of large amounts of recombinant human RSAg, as demonstrated here, would

circumvent the need for the use of antigen from other species and would greatly increase the validity of future studies on humans using RSAg.

Previous ELISA studies, mostly but not exclusively using bovine RSAg, have failed to demonstrate a clear difference between uveitis patients and controls for either the presence of autoantibodies to RSAg (or indeed any other putative autoantigen) or their titres, as outlined above. In a preliminary experiment using bovine RSAg, again no differences were detected. In retrospect, the controls used in these ELISAs were not rigorous enough, and therefore the results will not be discussed further. The ELISAs were repeated on both bovine and human recombinant RSAg using individual sera from uveitis patients and healthy controls. Rigorous controls were used in these experiments, but yet again no differences in immunoreactivity between patients and controls as groups were demonstrated using either type of antigen. No significant differences were detected between active and inactive uveitis patients, but the small number of active patients in the group would have made this difficult to demonstrate. Analysis of uveitis patient results by clinical subgroup did not highlight any category with particularly high rates of immunoreactivity to RSAg. Again, as the numbers for each individual condition were small, it is possible that differences would be revealed if greater numbers were studied. Clinical details of patients (and controls) that displayed "high" titres of anti-RSAg were studied for possible causes. No particular association between high titres and disease type, state of activity, current disease duration, total disease duration or concurrent systemic immunosuppression could be found.

Immunoreactivity of bovine and human RSAg, as measured by ELISA, correlated significantly when analysed by Spearman correlation ($r = 0.3724$, $p = 0.0003$). Analysis of individual results however, revealed certain samples that were substantially more reactive to the human form of the antigen than the bovine, and a smaller number that were significantly more reactive to the bovine form. This demonstrates that there are species-specific epitopes on both forms of antigen, and that cross-reactivity is only partial. ELISA readings using human antigen were found to be significantly higher than those obtained with the bovine form. These results together suggest that only the human form of the

antigen should be used when investigating autoantibody responses, or other measures of immune response, in this (human) disease.

The recombinant human RSAg used in these ELISAs were fusion proteins attached to a His-tag sequence. Tags were not cleaved prior to use in ELISA, as this was thought to be unnecessary. The size of the tag was just over 6 kDa, compared with a mass of 48 kDa for the RSAg part. Certain checks were performed to minimise the chance of inadvertent reactivity to the His-tag portion of the molecule. Another His-tag containing protein (58 kDa protein kinase, Invitrogen) was used as an extra control when performing ELISA on each serum sample. This enabled identification of any possible anti-His reactivity, which should in theory have an almost identical reading to the RSAg protein (similar sized molecules). The baseline-corrected readings for all experimental samples (both patient and control) for recombinant RSAg and the 58-kDa protein were compared by correlation. While the readings were shown to correlate, by Pearson testing of log - transformed data ($P < 0.0001$), they had an R^2 value of 0.3145 showing that only 31 % of the variance in the 2 groups was shared. These results combined with the fact that the His-tags comprised only a small part of the total protein, and that immunoreactivity against such artificially engineered tags would be unlikely to occur in nature, justify the use of the whole, uncleaved molecule.

The finding of no difference between patients and controls in immunoreactivity to RSAg fits with and confirms the findings of previous studies. Given the interspecies variation of the forms of RSAg, it was particularly important to confirm this using human antigen. It seems that positivity for anti-RSAg antibodies is not a marker for disease in autoimmune uveitis. The finding of relatively few samples with truly high levels of anti-RSAg but many with low or moderate levels irrespective of disease state, might indicate that most of the activity detected on ELISA is due to non-specific immunoglobulin with quite low affinity for RSAg. There has been speculation that anti-RSAg antibodies may be protective for the disease (Dua et al. 1989b) but there is no firm evidence for this in humans at present. Analysis of the epitope specificities of anti-RSAg, perhaps in different stages of development of the disease, might be able to discriminate better between patients and controls, or active and inactive disease states.

In summary, this chapter demonstrates the successful cloning of human RSAg and its expression in prokaryotic and eukaryotic cells. Human antigen was found to be more sensitive than bovine for the detection of anti-RSAg activity by ELISA. ELISAs using bovine and human RSAg again demonstrated no differences between patient and control groups, but indicated substantial differences in epitope preferences between the two forms. I would therefore recommend the exclusive use of human RSAg, whether native or recombinant, for all future work on autoimmune uveitis.

**Chapter 4: B-cell epitope mapping of
RSAg with uveitis patient and control
sera.**

4.1 Introduction

Autoantibodies to RSAg have been detected equally in uveitis patients and healthy controls in previous studies (see Chapters 1 and 3). Although autoimmune uveitis is primarily a T-cell mediated disease, the role of autoantibodies is of interest for several reasons. Antibodies have been shown to have an influence on the course of a number of autoimmune diseases. These include anti-RSAg antibodies, which have been shown to have an inhibitory effect on EAU and may therefore have therapeutic potential. They could also potentially have a role as disease markers, either before disease onset or as a prognostic marker during treatment. Finally, analysis of the target epitopes of disease-associated antibody clones (were these to exist) could provide valuable data on the role of B-cell *and* T-cell mechanisms of disease in uveitis. However, these potential benefits are contingent on the delineation of disease-specific and/or protective B-cell epitopes. Currently, published data is lacking in this area.

Phage display was chosen as the technique to map the B-cell epitopes of RSAg. One of several types of combinatorial library, phage display has the advantage of being relatively inexpensive, versatile, straightforward to use, and capable of displaying a large number ($\approx 10^9$) of primary recombinants in a single library. This means there is a high chance of any particular target molecule (e.g. antibody, receptor, etc) affinity-selecting phage which display ligands that closely resemble the molecule's natural target *in vivo*. Phage display is well established in B-cell epitope mapping (Smith and Petrenko 1997). It was initially used to map the epitope preferences of monoclonal antibodies, but later adapted to the screening of polyclonal antibodies, including those derived from sera (Dybwad et al. 1993; Folgari et al. 1994; Motti et al. 1994; Sioud et al. 1994; Dybwad et al. 1995a; Meola et al. 1995; Felici et al. 1996; Mennuni et al. 1996; Prezzi et al. 1996; Sioud et al. 1996). It has been successfully used to map epitopes in rheumatoid arthritis, another T-cell mediated autoimmune disease, using sera (Dybwad et al. 1993; Sioud et al. 1994; Sioud et al. 1996) and synovial fluid (Dybwad et al. 1995b). Similarly, phage display has been successfully used in "epitope discovery" for polyclonal antibodies in the CSF of patients with multiple sclerosis (Rand et al. 1998), another disease closely

related to autoimmune uveitis. Phage display has been successfully used in identifying and then further refining the minimum epitope requirements of a monoclonal antibody against another uveitis candidate autoantigen IRBP (Tighe et al. 1996; Tighe et al. 1999).

In uveitis, the target epitope of a particular anti-RSAg monoclonal antibody has been located using cyanogen bromide digested antigen fragments (Donoso et al. 1986). To date, however, the target epitopes on RSAg to sera (polyclonal) from uveitis patients and controls have not been defined, and that was the aim of this study. I planned to use phage display libraries to define “public” epitopes that were common to both uveitis and control sera, and also “private” epitopes that were specific for one or other. The discovery of disease-specific epitopes could provide insights into disease mechanisms, as well as leading to the development of antagonist drugs or immunotherapies such as monoclonal antibodies or fusion proteins. Similarly, the identification of control-specific epitopes would provide avenues for the development of “protective” anti-RSAg monoclonal antibodies. One such approach could involve the direct use of selected phage clones in the generation of vaccines or monoclonal antibodies, by injecting them into laboratory animals.

4.2 Results

4.2.1 T7 library results

Separate preparations of affinity-purified anti-RSAg antibody were extracted from the pooled sera from 26 uveitis patients (P1-15, 17, 18-28) and 34 healthy controls (C2-5, 7-10, 13, 15-19, 22, 24, 26-33, 35-43, HS) (Table 3.1). Input phage from the constrained 9-mer (C9C) T7 phage library were affinity-selected through 4 rounds of biopanning against both types of anti-RSAg. Phage from the 4th round of amplification were plated out, and random plaques chosen for sequencing. Phage titres increased sequentially with each round of biopanning, for both types of anti-RSAg. Three clones (non wild-type) were isolated from the phage pool enriched using control sera (Table 4.1). However no recombinant clones were identified from among the plaques selected using uveitis patient sera.

Table 4.1 Clones affinity-selected from T7 library using pooled control sera.

Clone	Amino-acid sequence of phage displayed peptide	No. of clones identified
1	K-P-I-L-G-G-K-K-*	18
2	G-P-A-G-C-W-E-N-*	7
3	E-K-E-V-L-G-I-L-I	1

A possible motif, K- - LG, is shared between Clone 1 and 3. Apart from this however, the small number of clones derived from the control group and the absence of recombinant clones from the patient group makes further analysis impossible.

This experiment was repeated. Problems were encountered in amplifying affinity-selected phage between rounds of biopanning, and titres remained static or even fell despite varying a number of experimental parameters. Plaques derived from the 3rd and 4th rounds of biopanning were randomly sequenced. All were found to be wild-type phage and no recombinant clones were isolated for either patient or control sera.

4.2.2 Ph.D.-12 (M13) library

This linear 12-mer library was biopanned using affinity-purified anti-RSAg antibody derived from 27 uveitis patients (P1-15, 17, 18-28 and 30) and 34 controls (C2-5, 7-10, 13, 15-19, 22, 24, 26-33, 35-43, HS). Although numbers of affinity-selected phage were reasonable when titred immediately after biopanning (pre-amplification), diminished titres were observed after incubation of phage in bacterial cultures. This indicated a problem during amplification, possibly due to diminished infectivity of the affinity-selected phage. Various experimental parameters (incubation times, stringency of wash solutions, temperatures, etc) were varied without success. New anti-RSAg antibody was affinity purified, but this did not overcome the problem. Helper phage were introduced to enhance infectivity, but failed. Finally, the DNA was extracted from phage captured during the 1st round of biopanning, and directly introduced by electroporation into electrocompetent host cells. Even then, phage failed to replicate in adequate numbers, and use of this library was abandoned.

4.2.3 f88-4 linear and f88-4/Cys4 library results

These two 15-mer libraries were combined and used as input for the first of 3 rounds of biopanning against anti-RSAG antibody as described in Chapter 2. Affinity-purified antibody, extracted from the same uveitis patient and control sera as above, was used as target ligate. The total number of phage captured during each round of biopanning, prior to amplification, was estimated. Using patient-derived antibody, the total number of affinity-selected phage increased sequentially in each round (1st round 1.7×10^7 TUs, 2nd round 1.4×10^8 TUs, 3rd round 3.5×10^9 TUs), while using equal numbers of input phage. This probably indicates that phage displaying higher affinity peptides were being selected preferentially. Using control-derived antibody, there was an initial increase in phage yields (1st round 5.7×10^7 TUs, 2nd round 6.9×10^9 TUs), followed by a decrease (3rd round 1.4×10^8 TUs). This might indicate overgrowth of non-specific phage or represent a titration error. Post-amplification titres of patient and control phage solutions after the 3rd round of biopanning were (as expected) approximately equal: Patient 2.4×10^{11} TU/ml, Control 2.1×10^{11} TU/ml.

It is noteworthy that all clones isolated from both groups were derived from the constrained library, with none of the linear library clones being selected. After random screening of colonies from the 3rd round of panning, 16 recombinant clones were identified from the phage pool enriched using patient sera (Table 4.2). Most clones were represented by single copies but Patient Clone A (20 copies) and Patient Clone N (2 copies) were represented more than once. 24 clones were identified from the phage pool selected using control sera (Table 4.2). Several copies (6) of Control Clone A, a clone displaying the same 9-mer peptide as Patient Clone A, were identified. 2 copies of Control Clone G were identified, and all other clones were represented by single copies. Overall, no obvious consensus or motif was identified from within the groups of patient or control-derived clones.

Patient and control phage clones were tested against sera from their respective groups by 3-step ELISA. No clone stood out when groups of readings were compared, for either patient or control groups.

Patient and control-derived clones were analysed in terms of distribution of certain amino-acid residues, or residues with shared properties. It was

noticeable that most (14/15) of the patient-derived clones were full length (15 residues), but due to a large number of stop codons most of the control clones were truncated (17/24). There seemed to be a predominance of proline residues after the 2nd engineered cysteine in clones from the patient group (13 out of a possible 63 residues (20.6%) compared with 4 out of 34 (11.8%) for the controls – see Table 4.3). There also seemed to be a greater proportion of aromatic residues (FYWH) in the control group after the 2nd cysteine, where this part of the phage DNA was expressed (Table 4.4). In the patient group, 8 out of a possible 63 residues (12.7%) were aromatic, while the corresponding figure was 8 out of 34 (23.5%) for the controls. Amino acids were also grouped as polar, charged, positive, negative, small, tiny, hydrophobic and aliphatic, and differences in distribution between patient and control clones sought. No obvious differences between the groups were detected however.

Patient Clone K was noted to contain 6 amino acids of homology and one of similarity with RSAg. The sequence of homology was TANTCKKIK (N is the partial similarity) starting at amino acid 234 of human RSAg. Several other shorter similarities were also noticed.

Figure 4.2: Amino acid sequences of peptides affinity selected from GVIII phage library using uveitis patient- and control-derived antibody. Number of copies of each individual sequence shown in parentheses. Engineered cysteine residues are highlighted in red.

Patient Sequences

(A)	A-D-K-R-R-C-R-T-S-K-C-P-P-P-T	(20)
(B)	A-H-K-P-N-C-T-V-V-C-C-P-L-V-M	(1)
(C)	A-H-A-N-K-C-G-N-V-Q-C-N-L-S-N	(1)
(D)	A-E-K-K-V-C-T-K-S-S-C-L-Q-P-T	(1)
(E)	A-E-K-K-K-C-R-I-K-S-C-L-P-Q-A	(1)
(F)	A-P-R-D-P-C-S-THR-L-C-T-K-Q-P	(1)
(G)	A-M-E-T-T-C-T-S-L-Y-C-N-Y-P-R	(1)
(H)	A-A-K-V-T-C-L-S-T-P-C-T-K-M-N	(1)
(I)	A-R-Y-D-S-C-K-T-Q-A-C-G-H-T-M	(1)
(J)	A-G-L-R-E-C-E-Q-S-I-C-H-R-R-P	(1)
(K)	A-T-A-N-T-C-K-K-I-K-C-H-S-S-A	(1)
(L)	A-P-K-Y-G-C-V-M-N-Q-C-P-Q-D-I	(1)
(M)	A-N-K-P-I-C-K-P-K-A-C-E-P-H-V	(1)
(N)	A-T-K-N-G-C-P-A-N-T-C-H-P-S-G	(2)
(O)	A-T-S-L-S-C-S-G-Y-L-C-L-A-H-Y	(1)
(P)	A-N-T-H-A-C-N-H-M-K-C-T-S-P	(1)

Control Sequences

(A)	A-D-K-R-R-C-R-T-S-K-C-P-P-P-T	(6)
(B)	A-A-T-R-A-C-K-V-R-R-C-W-S-H	(1)
(C)	A-E-S-R-T-C-T-K-N-S-C-H-L-T-K	(1)
(D)	A-M-Q-K-S-C-V-E-S-P-C-Q-H-T	(1)
(E)	A-T-M-L-A-C-L-N	(1)
(F)	A-P-E-E-Y-C-R-K-H-W-C-R-K-Q-P	(1)
(G)	A-E-H-Q-T-C-G-V-F-G-C-W-I-Y-L	(2)
(H)	A-H-K-R-T-C-Q-T	(1)
(I)	A-T-K-N-L-C-E-H	(1)
(J)	A-E-A	(1)
(K)	A-E-W-E-E-C	(1)
(L)	A-K-S-E-T-C	(1)
(M)	A-N	(1)
(O)	A-S-D-L-T-C-D-P-W-T-C-W-D-S-N	(1)
(P)	A-A-N-D-K-C-P-R-I-K-C-Q-G-Q-S	(1)
(Q)	A-L-E-K-G-C	(1)
(R)	A	(1)
(S)	A-D-Q-G-Q-C	(1)
(T)	A-K-M-M	(1)
(U)	A-P-P-C-Q-C-L-S-K-S-C-D-T-H-K	(1)
(V)	A-D-T-D-K-C	(1)
(W)	A-L-S-N-T-C	(1)
(X)	A-A-P-A-K-C-R-S	(1)
(Y)	A-Q	(1)

Table 4.3 Distribution of proline residues (highlighted in green).

Patient Sequences – proline (P) residues

(A)	A-D-K-R-R-C-R-T-S-K-C-P-P-P-T	(20)
(B)	A-H-K-P-N-C-T-V-V-C-C-P-L-V-M	(1)
(C)	A-H-A-N-K-C-G-N-V-Q-C-N-L-S-N	(1)
(D)	A-E-K-K-V-C-T-K-S-S-C-L-Q-P-T	(1)
(E)	A-E-K-K-K-C-R-I-K-S-C-L-P-Q-A	(1)
(F)	A-P-R-D-P-C-S-THR-L-C-T-K-Q-P	(1)
(G)	A-M-E-T-T-C-T-S-L-Y-C-N-Y-P-R	(1)
(H)	A-A-K-V-T-C-L-S-T-P-C-T-K-M-N	(1)
(I)	A-R-Y-D-S-C-K-T-Q-A-C-G-H-T-M	(1)
(J)	A-G-L-R-E-C-E-Q-S-I-C-H-R-R-P	(1)
(K)	A-T-A-N-T-C-K-K-I-K-C-H-S-S-A	(1)
(L)	A-P-K-Y-G-C-V-M-N-Q-C-P-Q-D-I	(1)
(M)	A-N-K-P-I-C-K-P-K-A-C-E-P-H-V	(1)
(N)	A-T-K-N-G-C-P-A-N-T-C-H-P-S-G	(2)
(O)	A-T-S-L-S-C-S-G-Y-L-C-L-A-H-Y	(1)
(P)	A-N-T-H-A-C-N-H-M-K-C-T-S-P	(1)

Control Sequences

(A)	A-D-K-R-R-C-R-T-S-K-C-P-P-P-T	(6)
(B)	A-A-T-R-A-C-K-V-R-R-C-W-S-H	(1)
(C)	A-E-S-R-T-C-T-K-N-S-C-H-L-T-K	(1)
(D)	A-M-Q-K-S-C-V-E-S-P-C-Q-H-T	(1)
(E)	A-T-M-L-A-C-L-N	(1)
(F)	A-P-E-E-Y-C-R-K-H-W-C-R-K-Q-P	(1)
(G)	A-E-H-Q-T-C-G-V-F-G-C-W-I-Y-L	(2)
(H)	A-H-K-R-T-C-Q-T	(1)
(I)	A-T-K-N-L-C-E-H	(1)
(J)	A-E-A	(1)
(K)	A-E-W-E-E-C	(1)
(L)	A-K-S-E-T-C	(1)
(M)	A-N	(1)
(O)	A-S-D-L-T-C-D-P-W-T-C-W-D-S-N	(1)
(P)	A-A-N-D-K-C-P-R-I-K-C-Q-G-Q-S	(1)
(Q)	A-L-E-K-G-C	(1)
(R)	A	(1)
(S)	A-D-Q-G-Q-C	(1)
(T)	A-K-M-M	(1)
(U)	A-P-P-C-Q-C-L-S-K-S-C-D-T-H-K	(1)
(V)	A-D-T-D-K-C	(1)
(W)	A-L-S-N-T-C	(1)
(X)	A-A-P-A-K-C-R-S	(1)
(Y)	A-Q	(1)

Table 4.4 Distribution of aromatic (FYWH) residues (highlighted in blue).

Patient Sequences – aromatic residues (FYWH)

(A)	A-D-K-R-R-C-R-T-S-K-C-P-P-P-T	(20)
(B)	A-H-K-P-N-C-T-V-V-C-C-P-L-V-M	(1)
(C)	A-H-A-N-K-C-G-N-V-Q-C-N-L-S-N	(1)
(D)	A-E-K-K-V-C-T-K-S-S-C-L-Q-P-T	(1)
(E)	A-E-K-K-K-C-R-I-K-S-C-L-P-Q-A	(1)
(F)	A-P-R-D-P-C-S-T-H-R-L-C-T-K-Q-P	(1)
(G)	A-M-E-T-T-C-T-S-L-Y-C-N-Y-P-R	(1)
(H)	A-A-K-V-T-C-L-S-T-P-C-T-K-M-N	(1)
(I)	A-R-Y-D-S-C-K-T-Q-A-C-G-H-T-M	(1)
(J)	A-G-L-R-E-C-E-Q-S-I-C-H-R-R-P	(1)
(K)	A-T-A-N-T-C-K-K-I-K-C-H-S-S-A	(1)
(L)	A-P-K-Y-G-C-V-M-N-Q-C-P-Q-D-I	(1)
(M)	A-N-K-P-I-C-K-P-K-A-C-E-P-H-V	(1)
(N)	A-T-K-N-G-C-P-A-N-T-C-H-P-S-G	(2)
(O)	A-T-S-L-S-C-S-G-Y-L-C-L-A-H-Y	(1)
(P)	A-N-T-H-A-C-N-H-M-K-C-T-S-P	(1)

Control Sequence

(A)	A-D-K-R-R-C-R-T-S-K-C-P-P-P-T	(6)
(B)	A-A-T-R-A-C-K-V-R-R-C-W-S-H	(1)
(C)	A-E-S-R-T-C-T-K-N-S-C-H-L-T-K	(1)
(D)	A-M-Q-K-S-C-V-E-S-P-C-Q-H-T	(1)
(E)	A-T-M-L-A-C-L-N	(1)
(F)	A-P-E-E-Y-C-R-K-H-W-C-R-K-Q-P	(1)
(G)	A-E-H-Q-T-C-G-V-F-G-C-W-I-Y-L	(2)
(H)	A-H-K-R-T-C-Q-T	(1)
(I)	A-T-K-N-L-C-E-H	(1)
(J)	A-E-A	(1)
(K)	A-E-W-E-E-C	(1)
(L)	A-K-S-E-T-C	(1)
(M)	A-N	(1)
(O)	A-S-D-L-T-C-D-P-W-T-C-W-D-S-N	(1)
(P)	A-A-N-D-K-C-P-R-I-K-C-Q-G-Q-S	(1)
(Q)	A-L-E-K-G-C	(1)
(R)	A	(1)
(S)	A-D-Q-G-Q-C	(1)
(T)	A-K-M-M	(1)
(U)	A-P-P-C-Q-C-L-S-K-S-C-D-T-H-K	(1)
(V)	A-D-T-D-K-C	(1)
(W)	A-L-S-N-T-C	(1)
(X)	A-A-P-A-K-C-R-S	(1)
(Y)	A-Q	(1)

4.3 Discussion

Polyclonal anti-RSAg antibody from uveitis patients and controls was used to screen three different types of phage display library. In general the results were disappointing.

Using the T7 library a small number of clones were identified from the control group, 2 of which had a possible area of homology, but the number of clones was too small to draw firm conclusions. No clones were identified using patient antibody, which makes comparison impossible. Repeat experiments using the same library were not successful. Screening of the M13 library was similarly unsuccessful. Failure of phage to amplify after affinity-selection made completion of the experiment impossible. This may have been due to interference by the recombinant peptides (which are displayed on the surface of the Gene III protein in this phage system) in the process of infection of host bacteria. Attempts to overcome this problem with the use of helper phage or electroporation of phage DNA into host cells were unsuccessful. For both T7 and M13 libraries the experiments were repeated several times, while experimental conditions such as incubation times and temperatures, wash solutions and frequency of washes, age of host bacteria at time of inoculation and type of solid phase for immobilisation of target antibodies, were varied. None of these modifications produced positive results.

More success was had with the f88-4 system in that several phage clones were captured using antibody from both uveitis patients and controls. The fact that all clones identified originated from the constrained library emphasises the importance of epitope conformation in antigen/antibody interaction. Multiple copies of the same clone (Clone A) being affinity-selected by both types of anti-RSAg antibody is significant, but its meaning unclear. This may represent greater replicative performance (assembly or infectivity) over other library clones, or selection by a particularly prominent antibody. Nevertheless, it is impossible to draw conclusions from this in terms of RSAg antibody/epitope preferences. The prevalence of stop codons was higher in clones affinity-selected by control sera, and are more likely to represent non-specific binding. Because the engineered peptide is expressed at the N-terminal end of the fusion protein (unlike T7 fusions which are C-terminal), clones

containing stop codons will not express a fully functional GVIII protein. The bias towards selection of fully functional clones by uveitis patient sera could be explained in terms of specific antibody affinities, but the implications of this remain unclear. Patient-derived clones tended to express more proline residues at their C-terminus ends - proline can cause bends in a polypeptide and this could have conformational implications for anti-RSAg antibody/epitope interactions. The relevance of control-derived peptides being possibly more likely to contain aromatic residues is not clear. Significantly no consensus patterns or motifs emerged from clones in either patient or control group, and therefore the RSAg sequence could not be mapped in terms of disease-specific or protective epitopes. It is possible that screening and analysis of greater numbers of clones from the enriched phage pools would eventually identify relevant mimotopes, but time constraints did not permit this. Although one patient-derived clone (Clone K) showed partial homology to a short sequence of human RSAg, the lack of other clones bearing the same motif makes it impossible to determine if this really represents an epitope with preferential binding for anti-RSAg antibody derived from uveitis patients.

It is necessary to look at the reasons why many of the biopanning experiments were ultimately unsuccessful and what future changes would most likely provide improved results. It is possible that some of the patient serum samples, particularly those taken from patients in remission or on immunosuppression, contained low titres and/or low affinity anti-RSAg antibody. More meaningful results might be obtained if sera were taken from patients with active uveitis, preferably non-immunosuppressed. This, of course, would not have relevance for sera taken from control subjects. However, pre-screening of either type of sera and selective use of samples with high anti-RSAg titres might improve the quality of the target ligate, which is of particular importance for this technique. Experimental conditions were optimised over several repeat biopannings, but this did not appear to have any significant effects on the final results. It should be remembered that in the past, phage display libraries have yielded the best results when screened by well-defined monoclonal antibodies, and that screening with sera/polyclonal antibodies presents particular problems. Polyclonal sera by definition contain a wide range of immunoglobulins of unknown affinities and concentrations. This will result in a higher yield of non-

specific clones in comparison with monoclonal selection. In addition, whereas repeated rounds of selection with a monoclonal antibody will diminish the number of non-specific binders, this is not necessarily so when using polyclonal sera containing immunoglobulins of unknown concentrations and affinities.

Some researchers have modified the standard method of biopanning specifically to overcome these problems. Folgori et al (Folgori et al. 1994), affinity-selected binders from a phage library using only serum (non-affinity purified) from a seropositive patient. The large number of selected phage clones was then more narrowly defined by screening plaques on filters with the sera of other seropositive patients. A narrow range of “disease-specific” clones was eventually identified. Rodi et al (Rodi et al. 1999), also minimised the number of rounds of amplification and performed a statistical analysis on a larger number of sequences. Both these approaches would help to minimise the problem of high affinity binders being out-grown between rounds by lower-affinity clones with better growth rates. Finally, it is recognised that random phage display libraries work best when mapping continuous linear determinants, and not as well with discontinuous or conformation-dependent epitopes. Perhaps the use of a wider range of constrained random libraries, libraries displaying peptides on a protein scaffold, or even a RSAg cDNA display library would have been more successful here.

Overall, based on my experiences as described here, I would find it hard to recommend random phage display as an effective method for mapping B-cell epitopes of RSAg. Given that the experiments were repeated several times - under different conditions and using a range of reasonably diverse libraries - without significant success, it could be concluded that this technology does not lend itself well to this particular investigation. It is, however, a feature of using phage display technology that one library may fail to yield the expected results while another, for no apparent reason, is successful. It is therefore possible that future investigators, using a different approach will have more success.

**Chapter 5: Discovery and expression of
new autoantigens using a retinal cDNA
expression library**

5.1 Introduction

Identification of the target autoantigen(s) is important in autoimmune disease, both to understand the aetiology of the particular disease and for the development of specific immunotherapies. Autoimmune uveitis is one such disease, in which a number of organ-specific candidate autoantigens have been proposed. The main candidates, which are all retinal proteins, are RSAg, IRBP, phosducin, rhodopsin and recoverin. These were all initially identified by their ability to induce EAU in various types of laboratory animals. Subsequently cellular and humoral immunoreactivity to some of the antigens (RSAg, IRBP) (Gery et al. 1986a) has been demonstrated *in vitro* in animals and humans. However, the relationship between these autoantigens and human autoimmune uveitis remains uncertain.

The repertoire of candidate antigens studied so far is limited. Previous studies have concentrated on isolating a particular protein and testing it for its ability to induce EAU. Few studies have attempted to systematically define the complete group of uveitogenic antigens *in vivo* in humans (or indeed animals), or rank them in terms of importance to the initiation or maintenance of the human disease. The potential gap that exists in our knowledge of the extent of candidate uveitogenic molecules needs to be addressed before more detailed studies are carried out on any particular antigen.

Although the use of EAU experiments has yielded several candidate autoantigens, some of which later demonstrated human immunoreactivity, there are limitations to what can be established using this approach. Some of the established antigens demonstrate immunoreactivity to human tissues *in vitro*, but this may not be the case *in vivo*, and immunoreactivity might not be exclusive to that antigen. Reactivity to some or all of these proteins may therefore represent “bystander activity” secondary to tissue inflammation/destruction, or redundant inflammatory pathways. Different antigens, or even epitopes within a particular antigen, may be responsible for inflammation at different stages of the disease *i.e.* epitope spreading, and this needs to be addressed. In particular, there may be epitope spreading between the early disease stages and established disease. Obviously it would be unethical to try to induce uveitis *de novo* in humans with a candidate antigen,

but this is probably the only definitive way to confirm a protein's immunoreactivity in vivo. Conversely, because of interspecies amino acid variability, an antigen that may be highly relevant to human uveitis might fail to induce EAU, and therefore not come to the attention of investigators in this field.

The aim of this study was to search for novel human retinal antigens that display immunoreactivity in human uveitis patients. It is known that many T-cell mediated, organ-specific autoimmune diseases - of which autoimmune uveitis is one - induce autoantibodies (Roitt and Delves 2001). Here autoantibodies in the sera of uveitis patients were used as a "tool" to help select retinal proteins with potential as uveitis autoantigens. A recombinant cDNA expression library was constructed using purified mRNA from human retinas and screened through 3 rounds of biopanning against immobilised human antibodies (see Chapter 2). Affinity-selected clones were sequenced and identified. Selected protein fragments were cloned, expressed, purified and used in ELISA assays to measure immunoreactivity of uveitis patient and healthy control sera.

A similar method, serological analysis of recombinant cDNA expression libraries (SEREX) (Sahin et al. 1995), has been successfully used in the past to identify candidate autoantigens in T-cell mediated autoimmune diseases such as type 1 diabetes and SLE (Rabin et al. 1992; Whitehead et al. 1996). Two recent papers describe the successful use of this technique to screen a bovine uveal cDNA library with the sera of patients with Vogt-Koyanagi-Harada (VKH) disease, as well as sera from patients with other subcategories of autoimmune uveitis and healthy controls. One novel protein (UACA) appeared to be a target autoantigen shared by VKH, Behcet's and sarcoid uveitis (Yamada et al. 2001a), while another (LEDGF) appeared to be specific for VKH (Yamada et al. 2001b). The 2 main differences between these studies and the current work are (1) the use here of a human cDNA library and (2) the method of screening. The relative merits of biopanning versus SEREX screening will be compared in the Discussion.

5.2 Results

5.2.1 Construction of human retinal cDNA library

Separate neuroretina and choroid/RPE cDNA libraries were constructed as described in Chapter 2, using RNA extracted from both retinas of a 67 year-old male patient who died from “natural” causes. Retinas were extracted within 9 hours post-mortem and yielded approximately 62 μg of total RNA from the choroid/RPE tissue and 29 μg from the neuroretina. After 2 consecutive rounds of purification, each total RNA fraction yielded approximately 1 μg of highly purified mRNA. All neuroretina and RPE/choroid mRNA was converted to double-stranded cDNA. This was confirmed for the neuroretinal fraction by PCR amplification of the HPRT “housekeeping” gene (see Figure 5.1). cDNAs were ligated to directional linkers, digested with appropriate restriction endonucleases and size fractionated.

30% of the total cDNA from both sources was used in the first instance, for ligation into T7 vector arms and subsequent packaging into bacteriophage particles. This first packaging yielded 2.9×10^4 primary recombinants from neuroretinal cDNA, of which approximately 8% represented retinal inserts (around 2300 primary retinal recombinants). The number of retina-derived clones as a percentage of the whole library was estimated by PCR amplification across the cloning site of a sample of randomly selected clones. Those larger than 300 bps contained a retinal insert (Figure 5.2). Packaging of RPE/choroidal cDNA yielded 2.6×10^4 primary phage, but PCR analysis did not detect any clones expressing recombinant proteins among these.

The remaining 70% of the cDNA from both sources was concentrated and the ligation/packaging reactions repeated. This yielded another 1.4×10^4 primary recombinants from the neuroretinal cDNA of which approximately 9% were of retinal origin (1300 primary retinal recombinants). Unfortunately no viable phage particles were produced from the RPE/choroid cDNA fraction.

Both neuroretinal cDNA libraries were pooled, resulting in a composite library containing up to 3600 primary recombinants displaying retinal protein

fragments. Prior to use in biopanning experiments, this library was concentrated (by PEG precipitation and resuspension) to a titre of 2.2×10^{11} PFUs/ml.

Figure 5.1 Agarose gel of PCR products from amplification of the HPRT gene contained within double-stranded retinal cDNA.

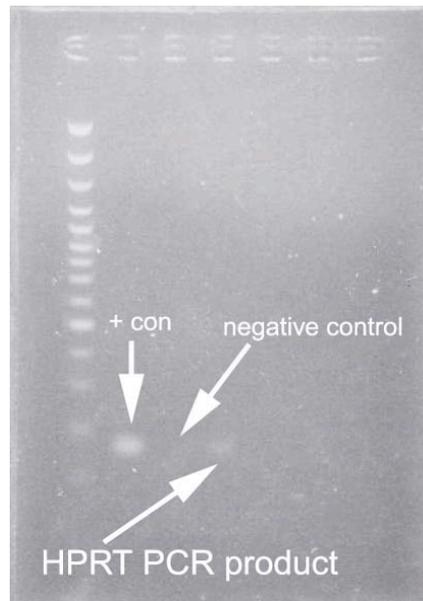
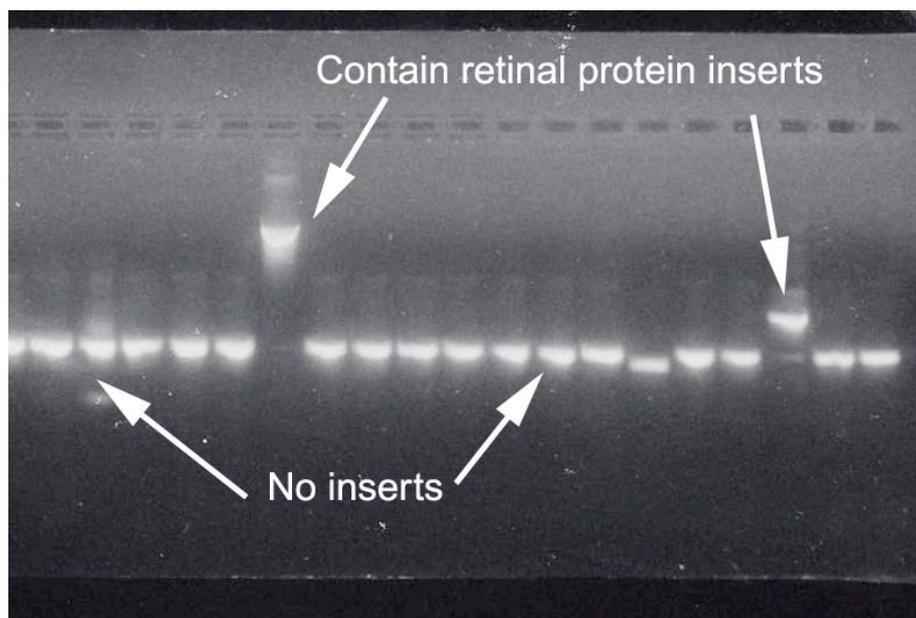


Figure 5.2 Retinal protein-containing clones within the cDNA library, identified by PCR amplification of the insert site.



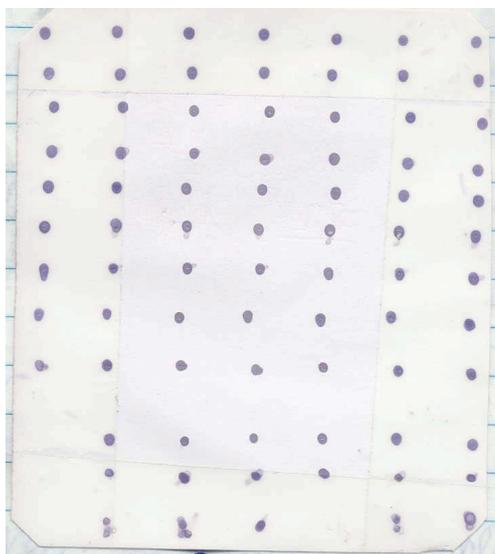
5.2.2 Affinity selection, isolation and analysis of high affinity cDNA library clones

5.2.2.1 Affinity selection, isolation and sequencing

Affinity selection of the neuroretinal cDNA library was carried out through 3 rounds using individual sera from 37 patients (P1-28, 30, 50-55, 58 and 59) and 42 controls (C1-33, 35-43 and HS), and analysed as described in Chapter 2. Two separate biopanning experiments were performed. Screening of phage pools produced in the first experiment (against sera from selected patients by dot-blot), failed to discriminate between specific and non-specific clones (Figure 5.3 of dot blots). The high noise:signal ratio was attributed to large numbers of residual non-specific phage in individual pools despite affinity selection. The second experiment therefore included an additional blocking step (with irradiated, non-replicative T7 phage), to enhance the specificity of resulting phage pools.

All phage pools from the 2nd and 3rd rounds of biopanning, from both experiments, were screened for positive (retinal-protein containing) clones (Figure 5.4). Various methods were of necessity used to separate and identify clones of interest from within the 79 affinity-selected phage pools in each round, as described previously. Isolated clones were then subjected to DNA sequencing and analysed further.

Figure 5.3 Analysis of phage pools by immunoscreening (“dot-blotting”) after 3 rounds of biopanning failed to differentiate the pools containing high-affinity phage clones.



The “gel separation method” proved useful in identifying phage pools that contained interesting clones (Figure 5.5), but this method used on its own proved inefficient at providing sufficiently pure (separated) DNA bands for sequencing and further analysis. Despite repeated attempts at separation, most extracted bands were contaminated with DNA from the same lane, which became apparent at re-amplification. Single bands distinct enough for DNA sequencing were impossible to obtain in the majority of cases. One clear band was isolated using this method (selected using serum C4), but when sequenced was found to contain a truncated T7 insert.

The method involving random screening of plaques by PCR was used in some cases to isolate clones from within phage pools already known to contain phage of interest. This technique produced several clones, 2 of which were eventually found to code for retinal protein fragments. One was selected using serum P22 (from 2nd round, 2nd experiment) and the other using serum P52 (from 3rd round, 2nd experiment). The technique had the advantage of producing homogenous DNA (PCR) samples corresponding to positive phage plaques, which gave clearer signals when sequenced (Figure 5.6). The overall process however, was found to be very time consuming and not suitable for

isolating large numbers of clones from multiple rounds of biopanning. Other methods were therefore adapted.

The method using EcoRV digestion of PCR products combined with amplification using modified T7 primers was designed to eliminate bands containing the default T7 vector DNA (no retinal insert). Used alone, this technique was successful in reducing the numbers of irrelevant bands, but it did not eliminate them and most of the corresponding DNA samples were not pure enough for successful sequencing. However, one band that proved of interest was isolated using this method. This clone was isolated from the phage pool produced by affinity-selection using serum P8 (3rd round, 2nd experiment) (Figure 5.7).

Figure 5.4 PCR amplification products of selected phage pools after 3 rounds of biopanning. Multiple bands within lanes indicate the presence of different phage clones within that pool.

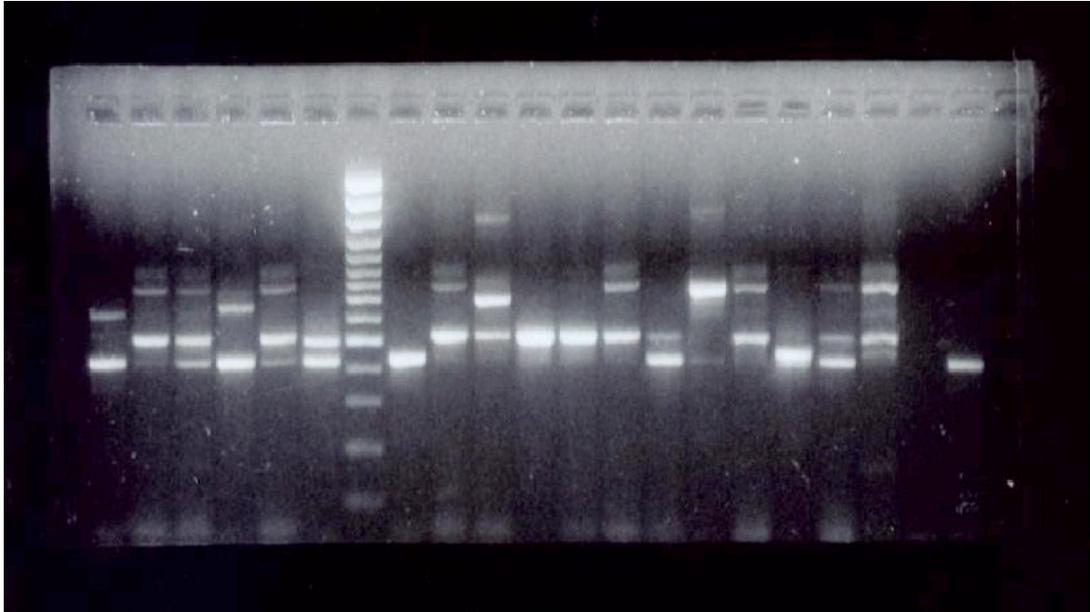


Figure 5.5 Isolation of DNA bands representing individual phage clones, by cutting bands out of gel after extended separation by agarose gel electrophoresis.

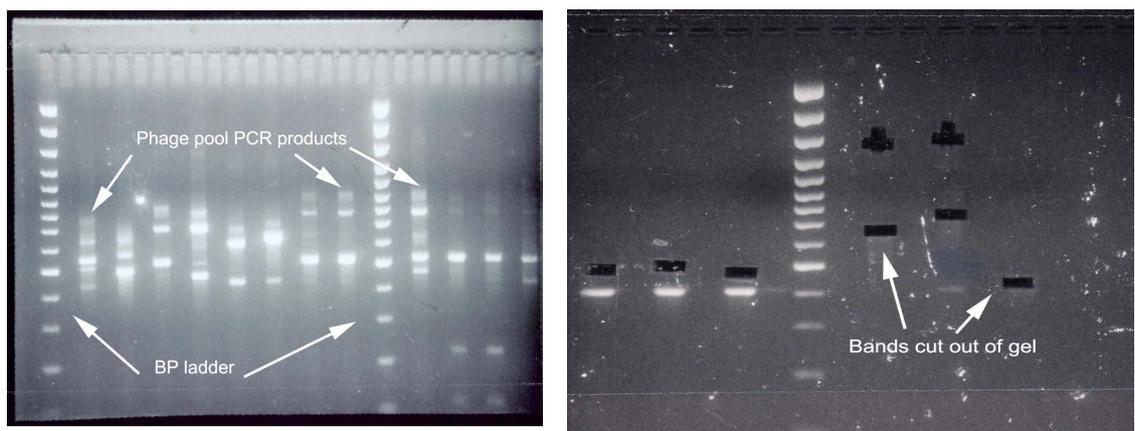


Figure 5.6 Isolation of clones by PCR screening of random plaques.

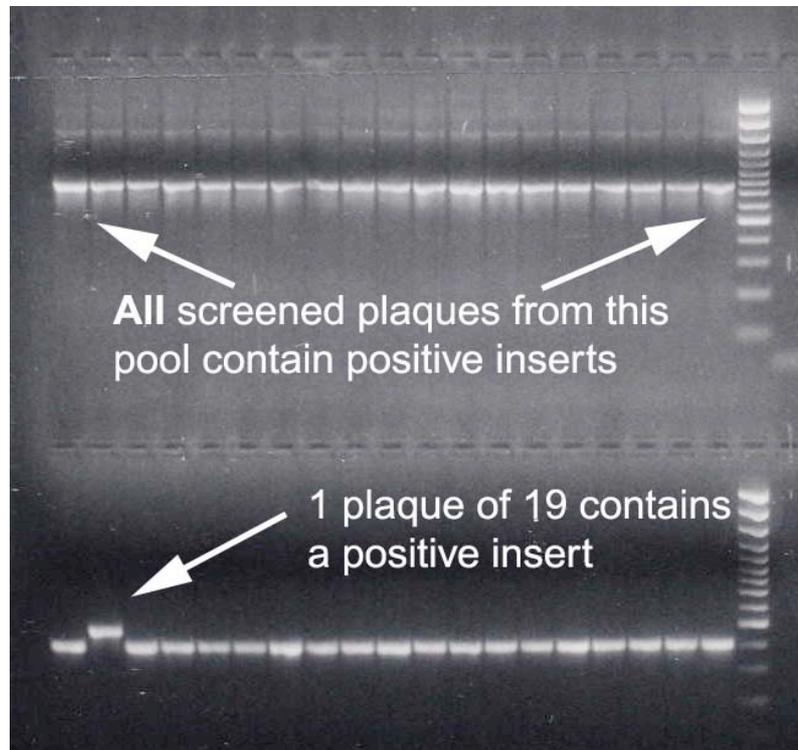
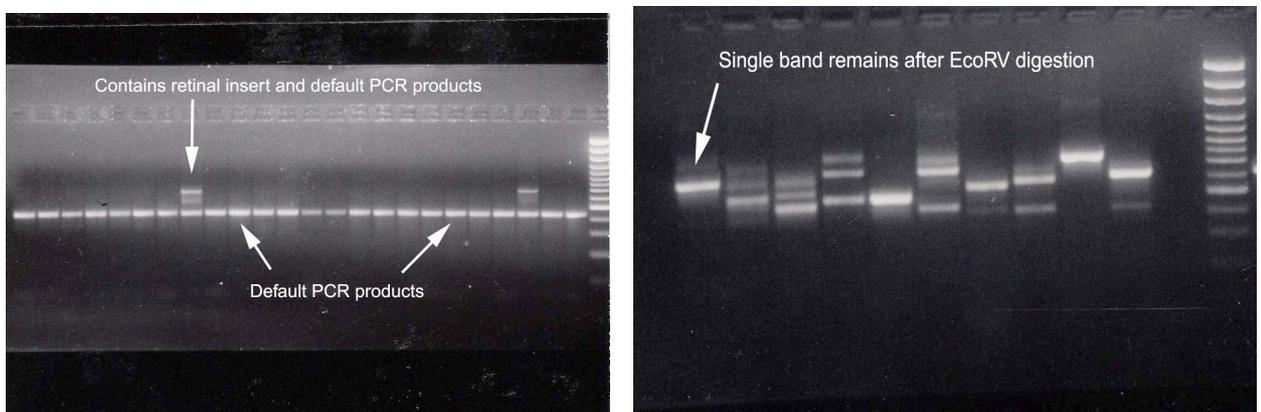


Figure 5.7 DNA bands separated by enzymatic (EcoRV) digestion of PCR products, followed by re-amplification.



The majority of bands that ultimately produced protein fragments of interest were isolated using a combination of the method described in the last paragraph followed by the “gel separation/extraction/re-amplification” method. This protracted process produced a significant number of bands that were subjectively judged to be clear/separate enough to undergo subsequent DNA sequencing (Figure 5.8). The drawback of this method was that some potentially interesting bands were not capable of being separated adequately and were therefore never analysed. In addition, some of the PCR products from “single bands” proved not pure enough for successful sequencing and gave corrupted readings. However, seven bands possibly worth further analysis and expression were identified using this hybrid method, and are listed in Tables 5.3 and 5.4.

Probably the most effective method for separation of the phage pools into their constituent clones and subsequent analysis, was the “shotgun cloning” technique, as described in Chapter 2. Unfortunately it was discovered after the majority of clones for analysis had already been sequenced and expressed, and therefore too late to have a major bearing on our findings. As well as producing pure PCR samples for sequencing from single colonies, clones that ultimately were shown to contain interesting sequences were already ligated into an expression vector and inserted into a host bacterium (Figure 5.9). This method identified and separated 2 clones, both selected using serum from patient P53 (3rd round, 2nd experiment).

PCR products isolated from clones using the various methods described above underwent automated DNA sequencing where possible. While most products produced readable sequences some did not, mainly because the DNA sample was not sufficiently homogenous. Successful sequence outputs were analysed further.

5.2.2.2 Sequence analysis

The DNA sequences of affinity-selected phage clone inserts were studied further and several unsuitable clones eliminated at this stage. For example, some clones were found to contain an incorrect leader sequence for the T7 vector and were therefore discounted, while several others obviously contained

early stop codons and were deemed irrelevant. The remaining sequences were analysed on electronic databases (using BLAST) at the DNA level. Sequences that contained high levels of homology to DNA from human retinal or neural tissue were earmarked. 12 clones (Table 5.1) were chosen for further analysis and possible expression. Their DNA sequences are displayed in Table 5.2.

Figure 5.8 Most bands were isolated by a combination of the enzyme digestion and gel separation/extraction/re-amplification methods. Bands clear enough to undergo sequencing were subjectively selected.



Figure 5.9 “Shotgun cloning” method of clone isolation and identification.

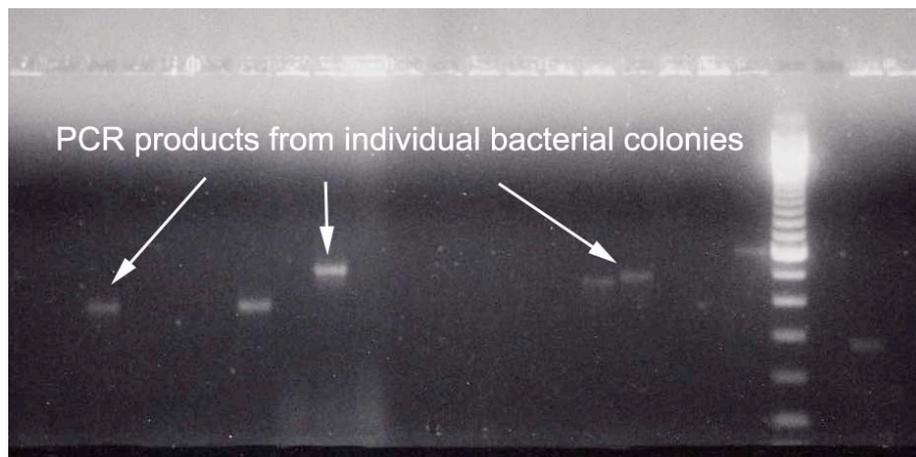


Table 5.1 Affinity-selected retinal cDNA library clones

Clone No.	Selecting Serum	Size of amplified PCR products.	Method used for separation	Round of biopanning	1st or 2nd experiment
1	C10	695	3	3 rd	2nd
2	C3	690	3	3 rd	2nd
3	C33	650	3	3 rd	2nd
4	P26	800	3	2nd	2nd
5	P54	475	3	3 rd	2nd
6	C38	525	3	3 rd	1st
7	P8	650	2	3 rd	2nd
8	P13, 14, 26 C2, 3, 4, 17, 19, 42	510	3	2nd & 3rd	1st & 2nd
9	P22	650	1	2 nd	2nd
10	P52	500	1	3 rd	2nd
11	P53	630	4	3 rd	2nd
12	P53	540	4	3 rd	2nd

1= Plaque plating + PCR screening with standard T7 primers. 2= EcoRV digestion + modified T7 primers. 3= "Gel separation method" + modified primers+ EcoRV digestion. 4= "Shotgun cloning" method.

Table 5.2 DNA sequences of affinity-selected clones from the retinal cDNA library. Endonuclease restriction sites: GAATTC=EcoR1, AAGCTT=Hind III.

1	ATCAATGGCAAAGTCCGAGAGATGAGTCAGATTACAGCGAACAA CGAAGAAGTTCTGGAGAGAATCTCAACCACACAGCGTTTAGTCT CGTCAGAAGTAGCCAAAGCTCAGCAGAATTTGCTCGAAACATCA GAAATATTGAAAAAATATCGTCCAGATGAAAGAACAGCAAAAG GAAATGGAAAAACCTCGACCTAAGCAAGATCGAGGTATGGATTT TGGGATGTAATTTTACTCAATTGTATATT AAGC TT
2	GAAT TCA AGC ATT CCA AGG CTT GCG ATA ATC TTG TTG AGA ATA CGC CGA GCG TTC GCT GCA TCT GCG TTA GCG TCA CCT TCC AGC GTT GAT ACC GGA GGT TCA CCG ATA GAC GCC AGA ATG TCG TTC ACA GCT GAT AAC TCA GCG GCA GTC TCA ACG TTC ATA TCG TAT GAG CGC ATA TAG TTC CTC CTT TCA GCA AAA AAC CCC TCA AGA CCC GTT TAG AGG CCC CAA GGG GTT AAC TAG TTA CTC GAG TGC GGC CGC AAG CTT
3	GAAT TCA AGC CAA TAA CAG GTC TGT GAT GCC CTT AGA TGT CCG GGG CTG CAC GCG CGC TAC ACT GAC TGG CTC AGC GTG TGC CTA CCC TAC GCC GGC AGG CGC GGG TAA CCC GTT GAA CCC CAT TCG TGA TGG GGA TCG GGG ATT GCA ATT ATT CCC CAT GAA CGA GGA ATT CCC AGT AAG TGC GGG TCA TAA GCT T
4	GAAT TCA AGC AAA GGT AAT TTG GAT TAG ATT ATG TCT CCT TGA ACC TGT TGT GAT TCC TAG TGA CAA CTG CTT TTT GTC TAT GTT TTA AAC GCT GGT GCA GTC ATT TTC AGG TGA TGC ATA TTG AGC TCA TGG GTC AGA TGC GGC CTT CTG GAA AAT TCC AAT AAT GTA TCC GGA TAT GGT TTC CTG GCA TTT TTC CTA TTC TGT ATG ATT TAA AAA TGT CTT TGT GTT TCC GCG ATC CCT TTT GCA TGT TGC CTT AGT TCA CTA GGT TGT GGT TTG TCT GGG ACT CAT TAT CTG GAG AGA AGC TT
5	GAAT TCA AGC GCC AGG TTC CCC ACG AAC GTG CGG TGC GTG ACG GGC GAG GGG GCG GCC GCC TTT CCG GCC GCG CCC CGT TTC CCA GGA CGA AGC TT
6	GAAT TCA AGC GCC TCA GCT CCA GGC TAT CTG GCA ATG ACA AAG AAA GTG GCA GTT CCT TAC AGC CCT GCT GCC GGG GTT GAT TTT GAA CTG GAG TCA TTT TCT GAA AGG AAA GAA GAG GAG AAG GAA GAA TTG ATG GAA TGG TGG AA GCT T

7	<p>GAAT TCA AGC GAC CTA CCC ATC CTA ATC CGC GAA TGT TCC GAT GTG CAG CCC AAG CTC TGG GCC CGC TAC GCA TTT GGC CAA GAG ACG AAT GTC CCT TTG AAC AAC TTC AGT GCT GAT CAG GTA ACC AGA GCC CTG GAG AAC GTT CTA AGT GGT AAA GCC TGA AGC CTC CAC TGA GGA TTA AGA GCA ACA GCC CCA GAG CCT GGG CTC TGC TGG ACT TAG TAT AAT GTG AAA AAA ATG TGT TCT CCT ACT CCT CAT AAA GCT T</p>
8	<p>GAAT TCA AGC GTG GTG GTG GTT GTG CCC AGC ACA GAC CTG GCA GGG TTC TTG CCG TGG CTC TTC CTC CTC CCT CAG CGA CCA GCT CTC CCT GGA ACG GGA GGG ACA GGG AAT TTT TTC CCC CTA AGC TT</p>
9	<p>GAAT TCA AGC GGG AAA GCT CTG GTG ACC CTG GGC TTC GCA GGG GTA GAT CCC AGG ACT CGG CAG TGG ATG GGA TGC AGC CAG TCA TGG GTT AGG GTC AGC AGA GAC TCA GAG TCC AGG GCA AGG TTC AAG GCA GAC TAA CCT CAT GCA TGG ATT GTA AAA AAC CAG CTC CCT TTG GAT CAA CCC AGC CTG GCA CCC TTG CCT GTC TGA GAG TGT CTC AAA GGG CTG ATG GCT TCC TGG TCC CCT TGA GTC ATC ACC AGC TTC CCC AAG AGA GTG TCA AGC TT</p>
1 0	<p>GAAT TCA AGC ACT GCT CCT GAT GCT CAT CCT CCT GGT GCG GCT GCC CTT CAT CAA GGA GAA GGA GAA GAA GAG CCC TGT GAT CAG GGC GCC CGC CCC GGC CAA CCC CGA AGC TT</p>
1 1	<p>GAAT TCA AGC AGC CAG CCA GTT TTG ACA AAG TAG CAA TTC CTG AAG TGA AGG AAA TTA TTG AAG GAT GCA TAC GAC AAA ACA AAG ATG AAA GAT ATT CCA TCA AAG ACC TTT TGA ACC ATG CCT TCT TCC AAG AGG AAA CAG GAG TAC GGG TAG AAT TAG CAG AAG AAG ACG ATG GAG AAA AAA TAG CCA TAA AAT TAT GGC TAC GTA TTG AAG ATA TTA AGA AAT TAA AGG GAT AAG CTT</p>
1 2	<p>GAAT TCA AGC CGA AGA CGA TCA GAT ACC GTC GTA GTT CCG ACC ATA AAC GAT GCC GAC CGG CGA TGC GGC GGC GTT ATT CCC ATG ACC CGC CGG GCA GCT TCC GGG AAA CCA AAG TCT TTG GGT TCC GGG GGG AGT ATG GTT GCA AAG CTT</p>

Table 5.3 Clones selected for expression of protein – analysis of sequences at the DNA level.

Clone No.	Size of cDNA insert (bp)	Source of clone /Molecules with homology to the cDNA library clone	Accession numbers of homologous sequences	Number of matching residues, % homology between clones and strand orientation
2	291	Chromosomal BAC clone Chromosome 2 Chromosome 6 Nuclear factor mRNA	AC21237 AC012085 AL117340 AC079777 AL451126 XM043750	20/20 (100%), + to – 20/20 (100%), + to – 19/19 (100%), + to – 18/18 (100%), + to -
5	84	Iodothyronine deiodinase mRNA “Proline rich protein” mRNA	S48220 AB048287	(100%), + to + (>90%), + to -
6	141	Carboxypeptidase E gene fragment	XM003479	(>95%), + to -
7	251	Ubiquinone gene fragment	XM003821	50/50 (100%)
8	119	SCAMP II mRNA	AF005038	38/38 (100%)
9	270	Neuronal Pentraxin Receptor mRNA	AL161974 NM058178	264/264(100%),+to+ 264/264(100%),+to+
10	102	G-protein coupled receptor BAC Chromos. 16q	NM016235 AC004131	71/71(100%), + to + 71/71(100%), + to -
12	139	Serine/threonine protein kinase mRNA	AF159295	135/135(100%),+to-

Table 5.4 Amino acid sequences of protein fragments for expression.

Sample No.	Name of protein/homologous molecule	Size of expressed fragment (a.a.)	Total size of fusion protein (Da)	Amino Acid Sequence (including vector leader sequence). • = stop.
Default protein	PCR-T7-NT-TOPO derived protein	33	N/A	MRGSHHHHHHGMASM TGGQQMGRDLYDDDD KDPSSRSAAGTMEFEA•
2	Unknown identity	56 (92)	10,120	MRGSHHHHHHGMASM TGGQQMGRDLYDDDD KDPNSSIPRLAILLRIRR AFAASALASPSSVDTGG SPIDARMSFTADNSAAV STFISYERI•
5	Small "proline-rich protein" like protein	48 (84)	9,240	MRGSHHHHHHGMASM TGGQQMGRDLYDDDD KDPNSSARFPTNVRCV TGEAAAFPAAPRFP RSLIRLLTKPERKLSWLL PPLS
6	Human Carboxypeptidase E	47 (83)	9,130	MRGSHHHHHHGMASM TGGQQ MGRDLYDDDD KDPNSSASAPGYLAMT KKVAVPYSPAAGVDFE LEFSERKEEEKEELME WWKA•
7	Ubiquinone (NADH dehydrogenase)	50 (86)	9,460	MRGSHHHHHHGMASM TGGQQMGRDLYDDDD KDPNSSDLPILIRECSDV QPKLWARYAFGQETNV PLNNFSADQVTRALENV LSGKA•
8	Secretory Carrier Membrane Protein (SCAMP) II	56 (92)	10,120	MRGSHHHHHHGMASMT GGQQMGRDLYDDDDKD PNSSVVVVVPSTDLAGF LPWLFLLPQRPALPGTG GTGNFFPLSLIRLLTKPE RKLSWLLP
9	Neuronal Pentraxin Receptor	42 (78)	8,580	MRGSHHHHHHGMASMT GGQQMGRDLYDDDDKD PNSSGKALVTLGFAGVD PRTRQWMGCSQSWVR VSRDSESRARFKAD•

10	Human G-coupled protein receptor	54 (90)	9,900	MRGSHHHHHHGMASM TGGQQMGRDLYDDDD KDPNSSTAPDAHPPGA AALHQGEGEEPCDQG ARPGQPRSLIRLLTKPE RKLSWLLPPLS
12	Human Serine/ Threonine protein kinase mRNA	65 (101)	11,110	MRGSHHHHHHMASMT GGQQMGRDLYDDDDK DPNSSRRRSDTVVVPT INDADRRCGGVIPMTRR AASGKPKSLGSGGSMV AKLDPAANKARKEELA AATA

5.2.3 Expression and purification of recombinant protein fragments

DNA was extracted from the phage clones identified above, and re-ligated into pCR-NT-T7-TOPO plasmids suitable for expression of the corresponding protein fragments in bacterial hosts. This was done to produce pure samples of recombinant protein fragments for further analysis, but without the polypeptides being attached to bacteriophage capsids, which might have interfered with downstream assays. Before expression, recombinant plasmids underwent PCR amplification across the restriction site (Figure 5.10) followed by confirmatory DNA sequencing, to ensure the inserts were properly inserted/orientated in the vector, were “in frame” and contained an open reading frame (ORF). Corresponding amino acid sequences were deduced using GeneJockey software and analysed on the BLAST database. Two previously identified clones (numbers (1) and (3) above) were found not to contain an ORF and were discounted from further analysis at this final stage. Two additional clones (numbers (4) and (11)) were found to code for very short protein fragments (in comparison with the polyhistidine tag part of the fusion protein), and were also discarded. The amino acid sequences of the polypeptides selected for expression are shown in Table 5.4.

Expression of the recombinant proteins proved very difficult. Plasmids were transfected into Top 10F` cells for maintenance and propagation, and “plasmid preps” from these then used to produce cell lines of BL21(DE3)pLysS. These cells were induced as described previously but did not produce detectable levels of protein over a range of induction times and different conditions. The reason for this is not immediately clear. The codon usage of the host bacteria was analysed (www.kazusa.or.jp/codon) but found to be compatible with expression of these particular polypeptides. However, it may have been related to cell toxicity to basal levels of recombinant protein expression (before induction with tetracycline). More success was had when the plasmids were transfected into BL21(DE3)pLysE hosts, which have tighter control over basal expression, although they produce diminished yields compared with BL21(DE3)pLysS. Pilot expression experiments (Figure 5.11)

were performed to determine the optimal induction times for expression of each of the 8 remaining recombinant proteins. Seven out of the eight clones eventually produced moderate amounts of fusion protein (containing a His tag), which was harvested, purified and stored (Figure 5.12) in preparation for subsequent analysis. Despite various modifications, it was impossible to express appreciable amounts of protein from Clone 2. Time ranges for viable expression, actual time used for substantive expression and total amounts of recombinant protein eventually produced are shown in Table 5.5.

Table 5.5.

Clone Number	Range of induction times for viable expression.	Induction time used for substantive expression	Total amount of protein produced
2	1 hour	1 hour	Nil
5	2-3 hours	2 hours	64 μg
6	1-3 hours	2 hours	167 μg
7	2-3 hours	3 hours	121 μg
8	1-2 hour	1 hour	103 μg
9	2-3 hours	3 hours	79 μg
10	1-3 hours	3 hours	147 μg
12	1-3 hours	3 hours	208 μg

Figure 5.10 PCR amplification of selected retinal inserts within recombinant pCR-NT-T7-Topo plasmids, prior to confirmatory sequencing. Plasmids containing inserts of interest were maintained within Top 10 F' cells, but expressed in BL21(DE3)pLysE hosts.

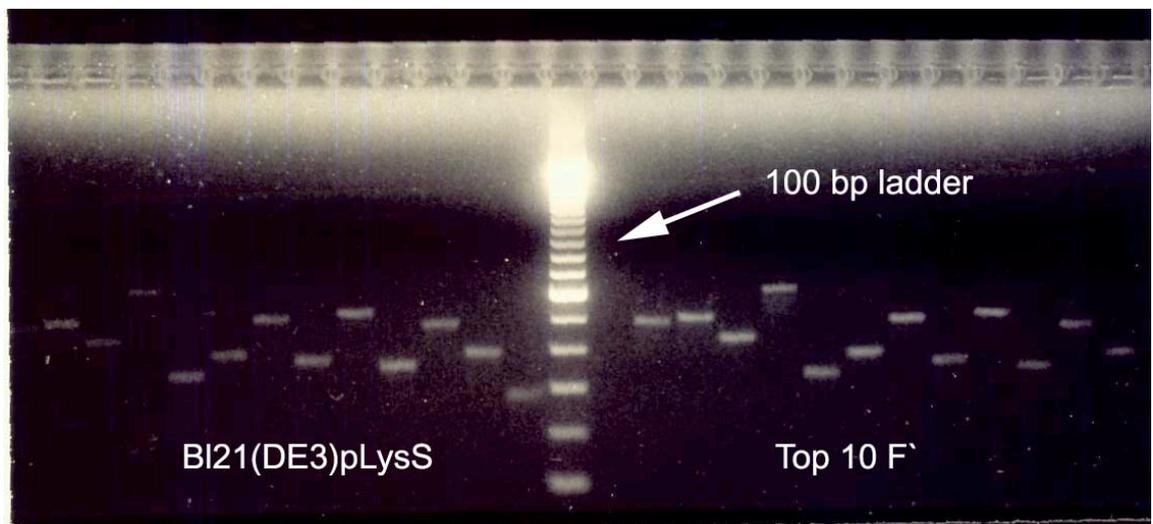
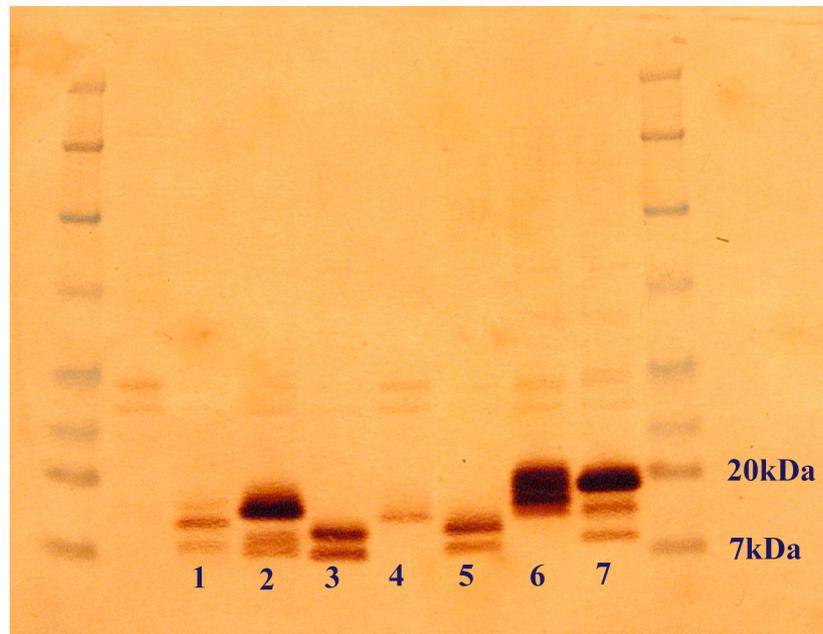


Figure 5.11 Western blot of protein expressions at different time points (in hours), probed with anti-His monoclonal antibody. Expression of Protein 6 peaked at 2-3 hours post induction and decreased thereafter, while Protein 7 expression peaked at 3 hours. Declining yields with increased induction times might be related to toxicity of the expressed recombinant proteins.



Figure 5.12 Western blot of successfully expressed recombinant protein samples from 7 out of the 8 clones of interest.



5.2.4 ELISA assays: cDNA polypeptides versus patient and control sera.

Human uveitis and control sera were tested for humoral immunoreactivity against the expressed and purified proteins by ELISA. A 4-layer ELISA, using anti-histidine monoclonal capture antibody to immobilise the recombinant protein, was tried first but proved unsuccessful in discriminating signal from noise. Significant amounts of antigen were used up during this process. A 3-step indirect ELISA, with antigen bound directly to solid-phase wells, was found to be more successful. Pilot experiments determined the optimum concentration for ELISA reagents to be 2.5 ug/ml test antigen, 1:25 serum diluted in PBS and 1: 20,000 secondary antibody. Control wells took the form of (1) negative wells blocked with 5% fat-free dried milk/ 0.1% Tween 20 in PBS and (2) 58-kDa polyhistidine-tagged control protein 2.5 ug/ml. Wells were tested in duplicate and “baseline-divided” (test reading divided by “blocked” well) readings used for data analyses. Four proteins (Samples 6, 7, 10 and 12) were tested against all 45 uveitis serum samples (P1-15, 17, 19-28, 30, 50-55, 58-69) and 47 control samples (C2-5, 7-10, 13, 15-19, 22, 24, 26-33, 35-43, HS, 50-62) (both from Table 3.1). Due to limited supplies of Proteins 5, 8 and 9, it was possible to test these only against a limited panel of 14 sera (P8, 13, 14, 22, 26, 52, 53, 54 and C2, 3, 4, 16, 18 and 36). Included in this group were the sera originally used to affinity-select the phage clone corresponding to each purified protein.

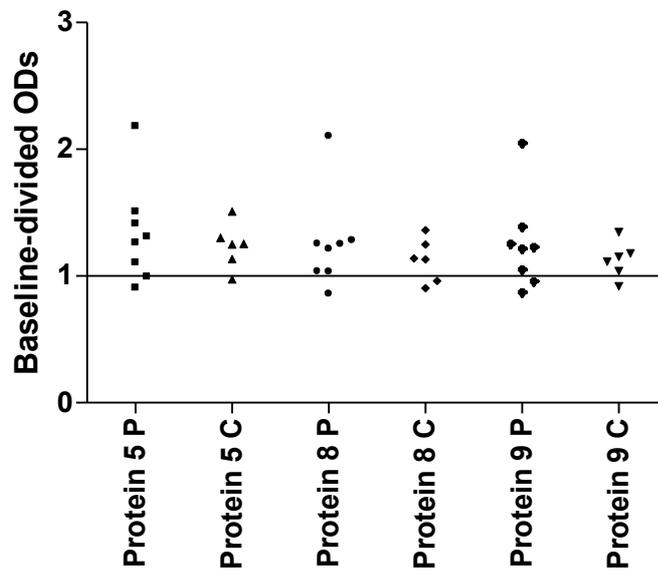
Averaged, baseline-divided OD readings were studied on an individual level and also analysed as groups. Isolated high readings were recorded for each of the 7 protein fragments against individual sera, mostly from the uveitis group. No unifying factor(s) could be identified among these individuals, however, to account for the high readings.

The small number of serum samples tested against Proteins 5, 8 and 9 rendered any comparison of uveitis patient and control groups meaningless for these proteins. There is a hint of slightly higher levels of reactivity in patient samples in Proteins 5 and 8 but without greater sample numbers this cannot be substantiated.

There were no distinguishing features for any of the serum samples that produced a high reading for a particular protein. Interestingly there was not a particularly high affinity between any of the purified proteins and the serum sample responsible for selecting its corresponding phage clone from the retinal cDNA library. Scattergrams of the above readings are shown in Figure 5.13

Figure 5.13

Proteins 5, 8 & 9: Patient vs. Control sera ELISA readings

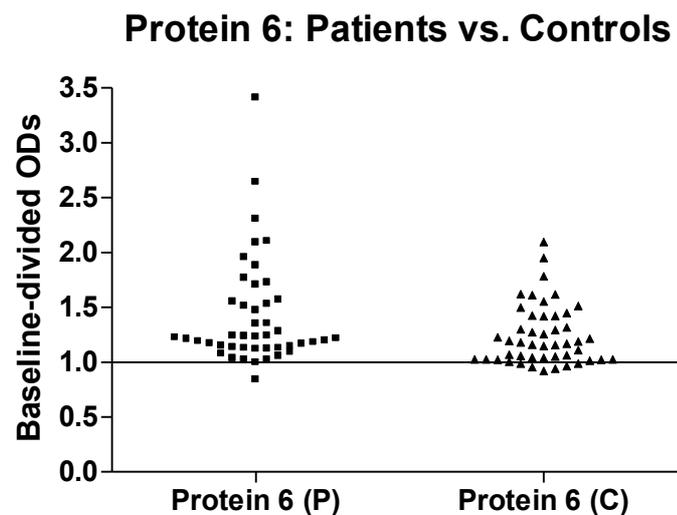


Baseline-divided ELISA OD readings were compared between patient and control groups for Proteins 6, 7, 10 and 12.

Protein 6

Data from Protein 6 readings followed a non-parametric distribution (even when log-transformed) and were therefore analysed using a 2-tailed, Mann-Whitney U test. The median value for the patient group was 1.227, with a minimum of 0.8450 and a maximum of 3.414. The median for the control group was 1.169 (minimum = 0.9210, maximum = 2.096). The 2 groups were found to be *significantly different*, $p=0.0443$. A scattergram comparing humoral immunoreactivity to Protein 6 in patient and control sera is shown in Figure 5.14

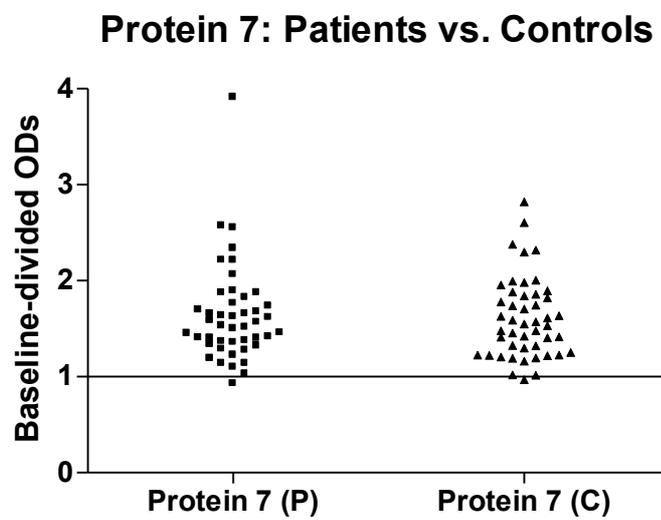
Figure 5.14



Protein 7

Protein 7 data groups followed a normal distribution. The patient group had a mean value of 1.642 (95% confidence intervals, 1.489 to 1.796) and the control group a mean of 1.610 (95% CI, 1.487 to 1.732). Analysis by unpaired, 2-tailed t-test showed there was no significant difference between the groups ($p=0.7354$). See Figure 5.15.

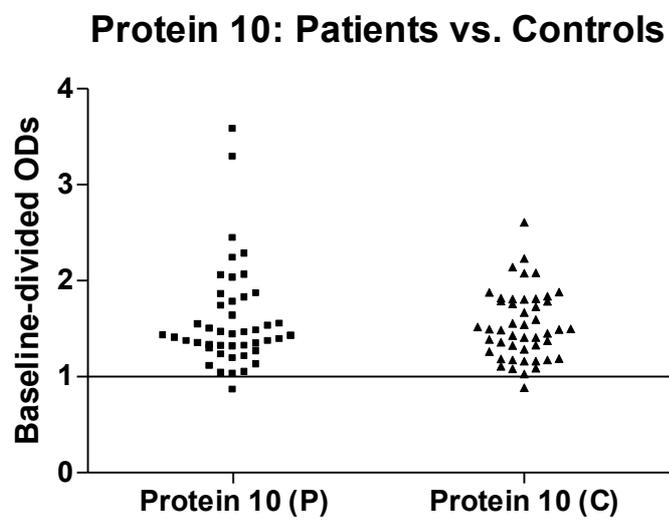
Figure 5.15



Protein 10.

The median value for the Protein 10 patient group was 1.427 (minimum = 0.8650, maximum = 3.582), and 1.492 for the control group (minimum = 0.8860, maximum = 2.610). Data were log-transformed to enable statistical analysis using parametric tests. A 2-tailed, unpaired t-test showed no significant difference between the 2 groups ($p = 0.7754$). See Figure 5.16.

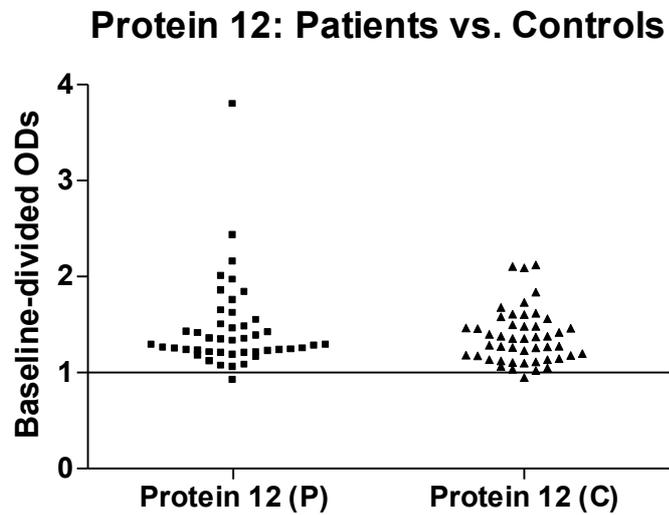
Figure 5.16



Protein 12

The median value for the patient group was 1.290 (minimum = 0.9250, maximum = 3.799), and for the control group was 1.356 (minimum = 0.9490, maximum = 2.121). Protein 12 data were also log-transformed to enable statistical analysis using parametric tests. A 2-tailed, unpaired t-test, with Welch's correction for unequal variances, showed no significant difference between the 2 groups ($p = 0.3922$). See Figure 5.17.

Figure 5.17



5.3 Discussion

The range of potential autoantigens for uveitis has not yet been fully defined, particularly in relation to the initiation and maintenance of the human disease. The use of animal models alone for this purpose risks missing potentially important antigens, given the interspecies differences that probably exist. This study sought to better define the set of potential autoantigens by constructing a human retinal cDNA library and screening it against the sera of both uveitis patients and healthy human controls. The objective was in the first instance, to identify any retinal protein fragments that demonstrated immunoreactivity against human uveitis or control sera.

This experiment achieved the successful construction of a retinal cDNA library, made using mRNA from human, predominantly neurosensory retina and packaged as a phage display library. It also demonstrated the feasibility of screening such a cDNA library with human sera using the technique of biopanning. A number of phage clones, whose DNA coded for several potentially interesting protein fragments, were affinity-selected from the retinal cDNA library during this process. This showed that the displayed protein fragments had at least some degree of affinity to antibodies in human sera, whether from uveitis patients or healthy subjects. It was possible to sub-clone some of these sequences into plasmids and express them in bacterial hosts, resulting in small but pure samples of 7 potentially autoantigenic polypeptides. These were screened against panels of human uveitis and normal sera by ELISA, and at least one protein (with homology to part of human carboxypeptidase E) showed a significantly higher level of immunoreactivity to the uveitis serum group. In addition, elevated serum antibody levels were demonstrated to all 7 proteins in at least some individuals. The highest titres tended to be found in sera from the uveitis group.

The experiments did not produce any protein that unequivocally demonstrated high levels of affinity to human uveitis sera, as determined by ELISA. The one protein fragment that showed higher readings in ELISA testing was in fact affinity-selected using serum from a control subject. In addition, the sera used to originally affinity-select the phage clones of interest, did not as

expected demonstrate particularly high ELISA readings to their corresponding purified protein fragments. The possibility therefore exists that clones were either non-specifically selected from the cDNA library, or that clones affinity-selected from the library, while originating from retinal tissue, were not specific for it. None of the previously well established putative autoantigens for uveitis were isolated, which was surprising.

It is possible to explain at least some of the above findings. While antibodies in human sera were used as target ligands to “capture” potentially uveitogenic protein fragments, it does not necessarily follow that high readings would be seen when these polypeptides were screened against human sera by ELISA. This is seen in the case of established autoantigens such as RSAg and IRBP, which although capable of provoking cell-mediated immune responses in human lymphocytes, do not generally demonstrate differences in autoantibody titres between groups of uveitis patients and controls (Doekes et al. 1987; Hoekzema et al. 1990; Doekes et al. 1992). It is therefore possible that some of the proteins isolated here would demonstrate uveitogenic potential if studied using cellular techniques.

Differences between patient and control groups might also have been demonstrated for some of the other 6 purified proteins if a larger, better-defined panel of uveitis sera had been available for ELISA screening. The panel of sera actually used, originated from a very heterogeneous group of uveitis patients, comprised of different clinical subtypes and of different disease durations, levels of activity and states of immunosuppression. Retesting the purified candidate proteins by ELISA against a larger group of sera, could possibly increase the power of any subsequent statistical analysis enough to demonstrate differences between patient groups or subgroups, and controls. Presumably there would be a greater chance of demonstrating high antibody titres to a given candidate antigen if sera were selected from patients with active disease of several weeks duration, who were not on immunosuppression.

Three of the seven purified fragments were tested only against 14 of a possible 92 sera. This was because most of the stocks had been depleted during either pilot experiments or during a series of ultimately unsuccessful ELISA assays. Repeat ELISA testing with these 3 antigens might demonstrate positive findings if the full panel of sera was tested. It would be particularly

interesting to see if Protein 8 produced a positive result, as the clone expressing this fragment was independently affinity-selected from wells using both patient and control sera.

The failure to isolate any of the established candidate autoantigens for uveitis might be explained by them being out-competed by higher affinity clones during the binding phases of the biopanning process (or out-grown during the amplification steps). If the former could be confirmed, it would further emphasise the need to use new approaches for the identification of new candidate antigens in uveitis. An alternative explanation is that the expected antigen fragments were among the number of clones that were not analysed further, due to technical difficulties encountered during the separation and sequencing processes. The third possibility is that the techniques employed here were not sufficiently robust to isolate these seemingly important antigens.

The experimental design would appear to be reasonably robust, but there are certain areas that could, with the benefit of hindsight, be strengthened. The diversity and representativeness of the retinal cDNA library could be increased. The library contained a maximum of 3600 clones, all of which were derived from the neurosensory part of the retina, and some of which were probably duplicates. A greater quantity of input mRNA, derived from different individuals, would probably increase the number of primary recombinants in the library. Greater quantities of cDNA, to allow empirical determination of the optimal proportions of cDNA molecules to vector arms during the ligation of recombinant T7 vectors, would also undoubtedly increase the number of primary clones. There is no obvious explanation for the non-production of clones from the non-sensory retina, other than sub-optimal quality of input mRNA, and this area of library construction needs to be revisited. The library should possibly have been negatively selected at some stage in its construction, to remove cDNA clones of elements not uniquely derived from ocular tissue. However, the possible existence of non-tissue-specific uveitogenic antigens was one factor that mitigated against doing this. Negative selection of the packaged library using monoclonal antibody against the vector system would have increased the percentage of recombinants containing retinal inserts in the library as a whole.

The affinity-selection technique could also possibly be improved, so as to increase the selection of high-affinity ligands rather than merely abundant clones. The technique used here, capture-antibody-coated microtitre wells used to immobilize the (polyclonal) immunoglobulin fraction of diluted sera, could have resulted in quite a small number of each specific antibody molecule being available for subsequent interaction with the cDNA library. Possible ways of overcoming this would be to screen using wells/plates with a much larger surface area for each individual serum, or carrying out successive rounds of affinity selection in liquid phase. It remains to be seen if any of these modifications would result in the isolation of higher affinity phage clones than before, as each method has its own individual strengths and weaknesses.

The technique of biopanning, as carried out here over 3 rounds, was only one possible method for screening a cDNA library with test sera. Alternative methods could have included single-round biopanning followed by immunoscreening of individual clones (Prezzi et al. 1996), or SEREX, which is discussed below.

The only work similar to this study in the literature is that by Yamada, as cited above. This group constructed a cDNA library using bovine retina and expressed it in bacteriophage. The library was screened by SEREX, in which phage plaques were grown on plates and nitrocellulose membrane replicas made of each plate. Replica membranes were immunoscreened against sera from 4 individual patients with VKH. $1-2 \times 10^5$ plaques were screened in this way and positive clones were isolated and then identified by sequencing. Clones representing fragments from a novel protein "UACA" and from lens epithelium derived growth factor (LEDGF) were identified. The human homologues of these proteins were cloned and then screened by ELISA against a larger panel of sera from VKH, other uveitides and controls. Anti-UACA antibodies were found to be relatively raised in a group of patients with pan-uveitis (including VKH, sarcoid and Behcet`s), while elevated anti-LEDGF titres were found to be specific for VKH.

There are advantages and disadvantages to both biopanning and SEREX in the screening of a cDNA library. SEREX benefits from relative simplicity, in that a single round of screening is used to identify positive clones. In the above examples it was successful in isolating library clones whose

human equivalents demonstrated increased immunoreactivity by ELISA. Isolation of positive clones was relatively straightforward, though this was achieved using the sera of only four patients. The use of a single round of screening is likely to produce many clones with low levels of affinity to the target sera, rather than a small number with high affinity. The latter is more likely to be achieved by the sequential re-amplification of selected clones over several rounds, as seen in biopanning. It is also easier to screen large numbers of sera (either pooled or individual) against a cDNA library using the biopanning approach and more feasible to screen larger numbers of library clones. In biopanning, however, it may be difficult to isolate individual clones from the resulting phage pools, as found here. In the future, it would therefore seem reasonable to re-screen this cDNA library using the SEREX approach.

As mentioned previously, many T-cell mediated diseases do not display significant differences between patient and control groups when tested for serum autoantibody levels against a known autoantigen. There is also evidence of abundant, low-affinity antibodies in many autoimmune diseases (Charlton and Lafferty 1995). It is therefore reasonable to question whether screening a cDNA library derived from a target tissue with sera, is a worthwhile method for identifying novel autoantigens? The answer is probably “yes”, as shown by the present study and those quoted. Both biopanning and SEREX would seem to have the potential to identify significant numbers of potential antigens for a specific disease. Further refinements to both techniques as outlined above, would probably further improve the yield of high-affinity, specific clones. Any such novel clones would need to be evaluated rigorously at the DNA/amino acid database level, before being expressed. It would be preferable to express the human form of any given protein in a eukaryotic expression system where possible. It would be essential to carry out further testing of any protein/fragment for evidence of humoral, or especially cell-mediated, immunoreactivity before designating it a candidate autoantigen for the disease. Since it is possible that a large number of clones would have to be analysed rapidly, ELISA screening against sera from well-defined patient and control groups would probably be the assay of choice for detection of humoral autoreactivity. Cytokine flow cytometry or ELISPOT would both be suitable

techniques for the rapid detection of antigen-specific, cell-mediated autoreactivity in patient and control samples.

In summary, in this experiment a cDNA expression library was successfully constructed from human retinal tissue and screened against sera from uveitis patients and controls using the technique of biopanning. A number of interesting clones were isolated and after analysis of their sequences, 7 protein fragments were expressed and purified. At least one of these potentially antigenic proteins, a fragment with homology to human carboxypeptidase E, demonstrated higher autoantibody levels by ELISA to a group of uveitis patients compared with controls. It would be interesting to see if cellular immunoreactivity could be demonstrated using this antigen. Some of the 6 remaining proteins might also have uveitogenic potential, but meaningful analysis of some of these was not possible, due to limited stocks of purified antigen and/or time constraints. This technique would seem to have great potential for the future identification of novel autoantigens in uveitis.

**Chapter 6: Retinal S antigen-specific
effector T-cell activation detected by
cytokine flow cytometry**

Much of the work described in this chapter has been included in a publication, a copy of which is contained in the Appendix.

6.1 Introduction

Autoimmune uveitis is a CD4⁺ T-cell mediated disease that is a good example of autoimmunity to organ-specific antigens in an immunologically privileged site. As discussed in Chapter 1, a number of retinal proteins have been proposed as the potential autoantigen(s) in autoimmune uveitis, mainly on the basis of their ability to induce EAU in certain strains of laboratory animal. Probably the most widely studied is RSAg.

In addition to inducing EAU in susceptible animal strains, studies have shown that RSAg is capable of stimulating T-cell proliferation in animal and human cells. Proliferative T-cell responses to RSAg have been seen using vitreous (Nussenblatt et al. 1984) and peripheral blood samples (Nussenblatt et al. 1980; Doekes et al. 1987) from autoimmune uveitis patients and also in healthy control humans (Hirose et al. 1988a; Hirose et al. 1988b). However, proliferation assays are mainly relevant to clonal expansion of memory T-cells, and tell us little about non-proliferative outcomes such as anergy, suppression and apoptosis, or activation in pre-primed, effector T-cells. T-cell “activation”, by which is meant the expression of cytokines and/or cell surface markers on effector cells, is particularly important because its effects can have a fundamental bearing on the whole future direction of the immune response. This is particularly so where the effector cell cytokine profile is predominantly of a Th1 or Th2 subtype.

To study RSAg-specific, effector T-cell activation in a rare cellular subgroup such as the autoreactive CD4⁺ population in autoimmune uveitis, a non-proliferation based technique for studying cytokine and surface marker expression at the single cell level is needed. Of existing single cell techniques T-cell cloning, single-cell PCR and in-situ hybridisation are either proliferation based or require laborious pre-selection of cell populations. Limiting dilution based assays have been successfully performed to study T-cell responses to RSAg (de Smet and Dayan 2000), but also are proliferation based and therefore not ideal for this task. ELISPOT has been used successfully in the study of

specific cytokine expression in other diseases, but is laborious and is limited in the number of cells capable of being analysed simultaneously. The technique of cytokine flow cytometry (CFC) could potentially circumvent many of these limitations.

This relatively new technique (Prussin and Metcalfe 1995) is a combination of 2 pre-existing methods: intracellular cytokine staining and multiparameter flow cytometry. It has emerged as an ideal technique for analysing cytokine production at the single cell level (Maino and Picker 1998) and therefore holds great potential for the study of lymphocytes activated by specific antigen. Because it is a single-cell technique, it enables the detection and enumeration of cytokine responses from rare T-cell subgroups in unselected cell populations that would be undetectable by more traditional methods. It is technically a relatively straightforward technique and the high throughput that is inherent in flow cytometry allows rapid analysis of very large numbers of cells whether they are activated or not. Multiparameter analysis, allowing simultaneous detection of several cytokines and/or surface markers, combined with electronic "gating" procedures, permits true Th1 vs. Th2 type cytokine differentiation for a specific T-cell subpopulation. Furthermore, CFC allows the study of individual T-cells directly *ex vivo*, minimising artefacts due to prolonged culture.

However, CFC has to date mainly been used to study T-cell responses to non-specific stimuli such as mitogens and ionophores (Prussin 1997). Antigen-specific responses have been elicited, mainly using highly immunogenic bacterial or viral coat proteins (Waldrop et al. 1997). It has also been used to study T-cell responses to epitopes in SLE, an antibody mediated autoimmune disease (Lu et al. 1999). However, CFC has not been widely utilised to study autoantigen-specific responses in T-cell mediated autoimmune uveitis. The potential of the technique in this disease however is great, particularly in the direct testing of T-cell cytokine/surface marker responses to stimulation by candidate autoantigens, and in analysing Th1 vs. Th2 cytokine differentiation. In this study, therefore, the aim was to apply CFC to the study of autoimmune uveitis and demonstrate antigen-specific effector T-cell activation in response to stimulation by RSAg.

6.2 Results

The main finding from this study is that antigen-specific effector T-cell activation in both uveitis patient and control groups was detectable using this highly sensitive and specific technique (see Chapter 2 for methods). Four out of 13 uveitis patient samples were seen to respond positively and specifically to the RSAg stimulus (50 μ g bovine RSAg/ml), 7 were negative and 1 patient's response was equivocal (see Table 6.1). Four out of 8 control subjects also responded positively to the antigen. In all positive samples, the preponderance of antigen-specific over negative-control events ranged from approximately 6:1 to 33:1. Responder frequencies for individual patients and controls are shown in Tables 6.2 and 6.3 respectively. Typical positive responses from uveitis patients are illustrated in Figures 6.1 and 6.2. A patient sample showing no significant response to RSAg, despite a good response to PMA (20 ng/ml)/ionomycin (1 μ M) stimulation, is shown in Figure 6.3.

Analysis of the values for baseline-subtracted positive events in the uveitis and healthy control groups showed there was no significant difference in responder frequency between the 2 groups ($p=0.779$). In the group of responding uveitis patients the percentage of positive events ranged from 0.015% to 0.047% of the overall CD4⁺ T-cell population. Analysis of the positive-control tube responses confirmed that the cell samples used had been immunologically competent. As noted previously (Anderson and Coleclough 1993), PMA and ionomycin stimulation results in substantial down-regulation of surface CD4 expression. Nevertheless, analysis of CD4⁺ positive-control lymphocytes stained with control-FITC antibody showed negligible levels of non-specific background staining.

Of the 4 positive "responders" in the uveitis group, 1 had intermediate uveitis, 2 (out of 2) had pan-uveitis and 1 had retinal vasculitis. All had at least some active inflammation at the time of sampling. One of the 4 was taking systemic immunosuppression. Total disease duration among responders ranged from 1 month to 15 years.

SDS-PAGE gel electrophoresis carried out on our RSAg preparation prior to starting the study, did not detect any contaminants. Dot-plots of negative

control tube samples showed remarkably clear backgrounds, which would mitigate against, for example, microbial contamination.

Limited supplies of antigen prevented us from carrying out repeat confirmatory testing on all positive results. However, we were able to reproduce positive results on individual samples while determining the optimum dose of antigen early in the study.

Table 6.1 Autoimmune uveitis patient details and responses to RSAg.

Case No.	IUSG Diagnosis	Activity	Duration of Current Episode	Total Disease Duration	Systemic Therapy	Response to RSAg
1	Intermediate Uveitis	Inactive	N/A	1 year	None	Negative
2	Panuveitis	Moderate	>3/12	6 years	Tacrolimus 7 mg/day Prednisolone 10 mg/day	Positive
3	Posterior Uveitis	Mild	>3/12	11 months	None	Negative
4	Posterior Uveitis	Severe	<3/12	1 month	None	Positive
5	Intermediate Uveitis	Mild	>3/12	11 years	None	Negative
6	Intermediate Uveitis	Mild	<3/12	13 months	None	Positive
7	Posterior Uveitis	Moderate	>3/12	17 years	None	Negative
8	Intermediate Uveitis	Mild	>3/12	8 months	None	Negative
9	Intermediate Uveitis	Mild	>3/12	2 years, 10 months	None	Negative
10	Panuveitis	Moderate	<3/12	18 years	None	Positive
11	Posterior Uveitis	Mild	>3/12	5 years	Cyclosporin 150mg/day Prednisolone 5mg/day	Equivocal
12	Intermediate Uveitis	Mild	>3/12	2 years	None	Negative
13	Intermediate Uveitis	Inactive	N/A	3 years, 3 months	None	Negative

Table 6.2. Responder frequencies from antigen-specific (50 µg RSAg + 1 µg anti-CD28 per ml) and negative control assays (1 µg anti-CD28 per ml) in uveitis patient group. Responses are adjusted per 100,000 CD4⁺ T-cells.

Patient No.	Responses to RSAg	Control Tube Responses
1	0.629	3.65
2	35.37	5.75
3	0.51	0
4	10.9	1.09
5	0.44	0
6	18.9	0.57
7	0	0
8	0.43	0.43
9	5.84	1.91
10	12.49	1.97
11	3.82	3.84
12	2.77	0
13	1.62	0

Table 6.3 Responder frequencies from antigen-specific (50 µg RSAg + 1 µg anti-CD28 per ml) and negative control assays (1 µg anti-CD28 per ml) in control group (responses per 100,000 CD4⁺ T-cells).

Control No.	Responses to RSAg	Control Tube Responses
1	15.06	0.75
2	5.17	1.7
3	8.45	1.65
4	5.27	4.7
5	7.27	15.3
6	6.13	9.24
7	30.12	7.24
8	0.46	0.46

Figure 6.1 Increased CD4⁺ responder levels are seen with extended incubation, without significant loss in overall CD4⁺ T-cell frequencies.

(A) and (B): Negative control samples of 6 and 18 hours incubation respectively.

(C) and (D): Antigen stimulated samples, 6 and 18 hours respectively.

CD4⁺ cells as a percentage of total events are as follows: (A) 35.5% (B) 31.8%

(C) 36.1% (D) 33.6%. Increased background-subtracted response rates

(CD69⁺, IFN- γ ⁺) are seen with extended (18 hour) antigen stimulation, without a significant decrease in the proportion of overall CD4⁺ T-cells.

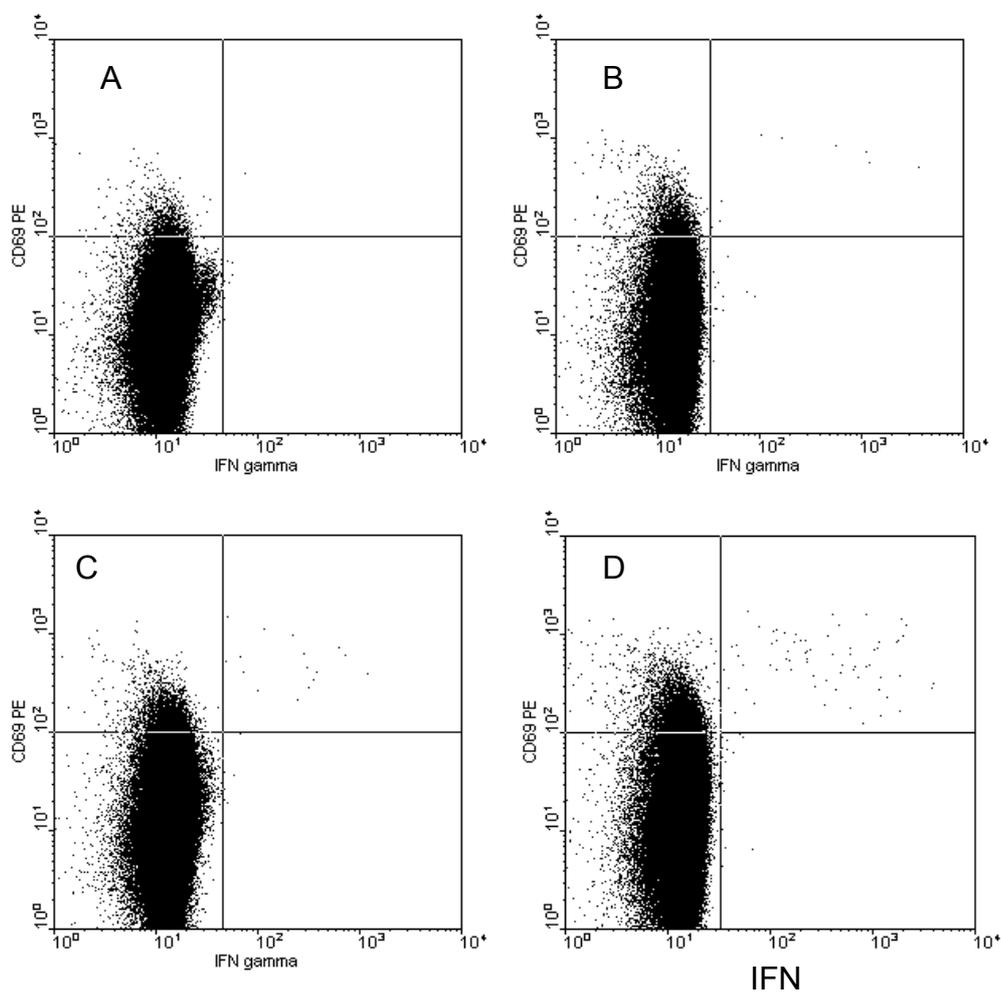


Figure 6.2 Typical antigen-specific response to RSAg in a uveitis patient sample (gated on CD4⁺ lymphocytes).

(A) PMA (20 ng/ml)/ionomycin (1 μM) stimulated sample.

(B) Same as (A), but with isotype matched negative control staining. Negligible levels of background staining noted.

(C) Negative control: Cells incubated in medium and anti-CD28 (1μg/ml) for 18 hours.

(D) Antigen stimulation: Cells incubated with RSAg (50μg/ml) and anti-CD28 (1μg/ml) for 18 hours. Positive response evident.

Total number of PBMCs analysed :A and B – 50,000; C and D – 500,000.

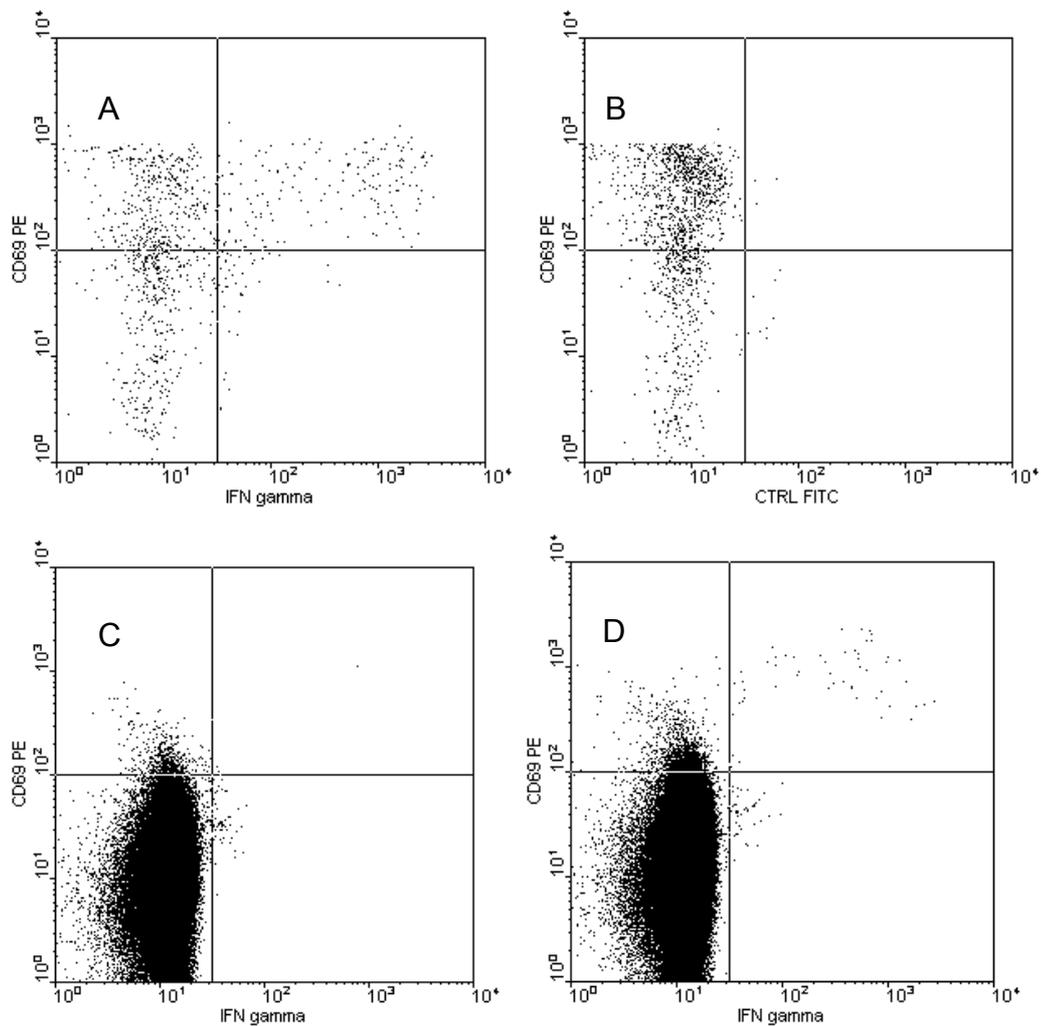
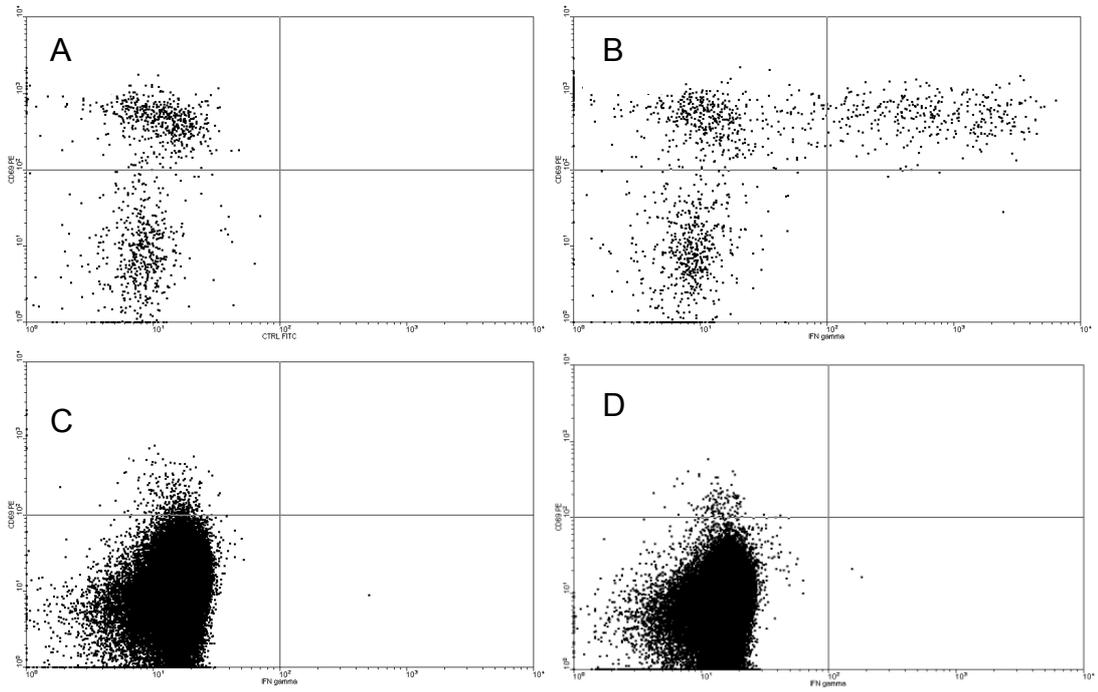


Figure 6.3 No response to RSAG stimulation in a uveitis patient sample (gated on CD4⁺ lymphocytes). A,B,C & D: as per Figure 6.2.



6.3 Discussion

These experiments constituted a pilot study, designed to establish the feasibility of detecting autoantigen-specific effector T-cell activation in autoimmune uveitis or control samples using the powerful new technique of cytokine flow cytometry. A RSAg-specific T-cell activation in samples from both groups of subjects has been demonstrated here. The high level of stringency employed in designating an individual patient/control sample as responding positively, the purity of the RSAg preparation and the low levels of responses in negative control samples support this. The response is possibly of a Th1 type, though further work targeting other cytokines needs to be undertaken to confirm this. I believe this study demonstrates the feasibility of applying cytokine flow cytometry to autoimmune uveitis and other T-cell mediated autoimmune diseases using appropriate modifications, and establishes it as the technique of choice for investigating cytokine responses in non-naïve effector T-cells.

This study was designed with the limited goal of demonstrating the feasibility of detecting effector cell activation. Calculations indicate that much higher patient and control numbers would be required to demonstrate a significant difference in the mean number of responding cells between the 2 groups. For example, to detect a difference of 0.25 standard deviations, using a significance level of 0.05 and a power of 0.8, would require the use of approximately 250 individuals in each group. The next logical progression is however to carry out such a study. In addition to detecting differences in the frequencies of responding cells between disease and control populations, higher numbers would also lend greater accuracy to calculations of the background responder frequency of non-stimulated, autoreactive T-cells in the peripheral blood compartment.

The use of modern multi-parameter flow cytometry would make it possible to expand the number of detectable intracellular cytokines, while retaining the same number of surface and activation markers. This would allow representation of at least one cytokine from each of the Th1 and Th2 subclasses and would allow accurate Th1, Th2 or Th0 classification of activated T-cells from various sources. It is highly likely that any differences in the cytokine profiles between patient and control groups would become apparent if

this approach were employed. Any qualitative differences detected between the 2 groups would possibly be more important than differences in the frequency of responding T-cells. Cytokine profiling of uveitis patient T-cells at different stages of disease activity, or comparison of cytokine responses from individuals on systemic immunosuppression to those without, might provide valuable insights. This technique could easily be harnessed to test the relevance to human disease of other uveitis candidate autoantigens. IRBP, phosducin, rhodopsin, recoverin, and any other yet to be discovered potential autoantigens could be tested, using animal, human or recombinant antigen sources. T-cell epitope mapping could be carried out using antigen fragments or synthetic peptides. Using the appropriate combination of surface marker and cytokine-specific labelled monoclonal antibodies, it would be possible to accurately determine the Th1 vs. Th2 cytokine response of autoreactive T-cells to various antigenic stimuli.

The 4 of 13 patients responding positively to RSAg is broadly comparable with the proportions reported previously in studies using proliferation assays (Nussenblatt et al. 1980; de Smet et al. 1990) or limiting dilution (Opremcak et al. 1991). Although these techniques are fundamentally different and measure different end-points (see above), it is further evidence that not all uveitis patients respond to the same antigenic stimulus. Four out of 8 healthy controls also responded positively. As mentioned already, responses to RSAg have been noted previously in normals using other techniques (Hirose et al. 1988a; Hirose et al. 1988b). While it would seem that CD4⁺ T-cell autoreactivity to RSAg plays an important role in the initiation of the uveitis disease state, these findings collectively suggest that other factors such as T-cell epitope specificity or Th1 vs. Th2 type cytokine expression, might also be relevant.

The potential of this technique for finally identifying the autoantigen(s)/autoepitope(s) and/or particular cytokine profiles that are responsible for the initiation and maintenance of human autoimmune uveitis is immense. This study demonstrates that a response to a particular retinal autoantigen is detectable using CFC, and that such responses can be both specific and sensitive.

Since the completion of the work described above, the use of multi-parameter flow cytometry has become ever more widespread in human PSII research. Flow cytometry has been used for many years as a tool for cell surface phenotyping, but sophisticated multi-parametric techniques such as CFC are increasingly being used to simultaneously detect multiple activation markers, chemokine receptors and intracellular cytokines in PSII. CFC has recently been successfully employed to demonstrate elevated expression of TNF- α and CD69 in peripheral CD4⁺ T-cells from patients with intermediate uveitis (Murphy et al. 2004). It has also been used to detect an increased level of IL-10 secreting CD4⁺ T-cells in response to anti-TNF- α therapy in patients with refractory PSII (Greiner et al. 2004), emphasising its potential role for studying responses to immunomodulatory treatments.

Chapter 7: General discussion

7.1 Introduction

Autoimmune uveitis is a complex disease, apparently caused by a combination of factors, both genetic and environmental. Despite substantial progress in the past, our knowledge of the precise mechanisms involved in its initiation, maintenance and resolution in humans remains limited. This in part reflects the ethical limitations and practical problems previously encountered when attempting to investigate the human disease directly, one example being the difficulty in obtaining biopsy material at crucial stages in the natural history of the disease. Consequently, most of our knowledge of the immune mechanisms in the initiation of autoimmune uveitis, and our knowledge of the candidate autoantigens implicated, is based on immunological, pathological and experimental studies involving animal models such as EAU. These studies have greatly advanced our general understanding of the mechanisms underlying the development of uveitis, and in many cases have delineated key immune processes in particular animal models. However, such findings cannot be directly extrapolated to human uveitis. Studies using biological material from human eyes or other human surrogate target tissues, both from uveitis patients and healthy controls, are therefore badly needed.

In this thesis, investigations into various cellular and humoral aspects of human autoimmune uveitis are described. The overriding theme is the direct application to human uveitis research of relatively new molecular and immunological techniques, some of which have already been successfully used to investigate other immunological diseases or animal uveitis models. A key goal was the evaluation of the suitability of these techniques for future research or clinical use. The serum and PBMC samples analysed were derived exclusively from human patients and controls. Assays were carried out using human test materials (or recombinant human materials) wherever possible.

In the following section, each of the four main results chapters (Chapters 3 to 6) are summarised and the suitability of each technique for human uveitis research commented upon. The possible impact of the overall findings on future uveitis research is assessed. Specific results from each chapter are not discussed as this has already been done in each Discussion section.

7.2 Analysis and verdict on individual experiments and techniques

7.2.1 Cloning and expression of human recombinant RSAg

In Chapter 3 the successful cloning and expression of human RSAg is described. A 1221 b.p. sequence for RSAg was amplified from mRNA by PCR, and ligated into pCR-T7-NT-Topo and pcDNA4-TO-myc-HisA vectors for expression in prokaryotic and eukaryotic systems respectively. Significant amounts of recombinant RSAg, produced as fusion proteins with polyhistidine tags, were relatively easily obtained from bacterial hosts after induction with IPTG. Small but quantifiable yields were obtained using the eukaryotic vector in human host cells. Recombinant protein from both systems was purified using a nickel chelate resin that binds to the His tag. The His tag could easily be cleaved enzymatically, leaving pure protein, but was unnecessary here for downstream applications. There are few previous reports of RSAg being cloned, and I was unable to find any reference to its successful expression from human cells in the literature.

I consider this experiment to be a success, and would recommend this approach where significant amounts of the antigen are needed for future research in human uveitis. Limited supplies of human antigen have undoubtedly hindered research in the past. The amounts produced using the eukaryotic system were small, but it is reasonable to expect yields to improve by adjusting the start time and total time of induction. The cultivation of larger cultures or use of "cell factories" would also increase output. Time constraints did not allow this during the current study. Given the advantages (post-translational modifications, etc) of expression from human cells, I would recommend the use of the eukaryotic over the prokaryotic system. The recombinant protein was purified under denaturing conditions, as this was found to be more efficient at the time. However, purification under native conditions is also possible. I believe the latter method is more appropriate where the antigen is being used for research purposes. This would be more likely to retain the conformation of the native

human protein, which may have a major bearing on key immunological processes e.g. antigen processing, B-epitope recognition.

7.2.2 Measurement of anti-RSAg antibodies in autoimmune uveitis patient and control sera

Using both bovine RSAg and recombinant human RSAg (expressed from bacterial hosts), sera from uveitis patients and controls were tested for levels of anti-RSAg antibody. No overall differences were detected between the 2 groups in terms of antibody titres, using either bovine or human RSAg. The small number of individuals representing distinct uveitis subcategories made statistical comparison of antibody titres from each group meaningless. However, there may have been a higher proportion of patients with intermediate uveitis and (possibly) sarcoid uveitis groups with "positive" titres. Interestingly, none of the 5 patients with Behcet's uveitis had a "positive" titre. Factors such as state of disease activity, duration of current disease episode or total disease duration, or use of systemic immunosuppression were not found to be significant, but again the numbers in each subgroup were very small. Individual subjects from both groups demonstrated "high" antibody titres, but no common factor could be identified. On occasion, markedly different antibody titres were found in the same individual to bovine and human RSAg, emphasising the importance of interspecies differences in epitope preference. ELISA readings using recombinant human RSAg were found to be significantly higher than bovine antigen, although a positive correlation existed between the 2 sets of readings.

The inability to demonstrate a difference in anti-RSAg antibody levels between uveitis patients and controls mirrors the findings of previous studies, and there seems little point in repeating such studies as described. The technique of indirect, 3-step ELISA is an effective method for detecting such antibodies, and the use of native or recombinant human RSAg is the most appropriate for reasons outlined above. There is no evidence at present for serum anti-RSAg antibodies being a marker for autoimmune uveitis. Perhaps this would become apparent for individual uveitis subcategories if larger groups were analysed. Alternatively, analysis of the epitope preferences of patients and controls might reveal differences. This was the subject of investigation in the

following section. It must also be remembered that the antibodies measured here were sampled from peripheral venous blood and that measurements obtained from intraocular fluids, particularly during disease episodes, would possibly give different and more relevant results. It is difficult to foresee adequate numbers of vitreous samples being routinely obtained, but sampling of aqueous humour from uveitis patients by paracentesis or from controls (during routine intraocular surgery) remains a possibility.

7.2.3 Analysis of polyclonal RSAg B-cell epitope preferences in autoimmune uveitis patients and controls

Polyclonal anti-RSAg antibodies, derived from the sera of uveitis patients and controls, were used to screen 3 different types of phage display library. In general the results were disappointing, and it was not possible to identify epitopes specific for either uveitis or control groups - the main goal of the study. Possible explanations for this failure are outlined in Chapter 4. One type of library (f88-4 Cys-4) yielded several clones for each group, including one clone common to both, and a number of other possible patterns were noted. However, the failure of a consensus pattern to emerge means that the relevance of this remains unclear. Again it is possible that analysis of greater numbers of clones from the final round of biopanning would eventually reveal a motif or consensus, but this is debatable. Comparison with previous studies is not possible, as B-cell epitope mapping of RSAg (or any other uveitis candidate autoantigen) had not previously been undertaken with random phage display libraries using polyclonal antibodies.

Overall I would not recommend random phage display technology for B-cell mapping using polyclonal anti-RSAg derived from sera. Several different libraries were screened here using various methods, and most experimental variables adjusted, but without success. Further modifications could be made, as discussed in Chapter 4, but it has previously been recognised that the biopanning of random phage libraries using polyclonal sera can be problematic. The technique has, however, been successfully employed using polyclonal

sera, where both the quality and titre of the antibody is high, in for example post-viral states (Prezzi et al. 1996).

In uveitis research, random peptide library screening has proven itself in determining the minimum epitope requirements of an anti-IRBP monoclonal antibody (Tighe et al. 1996) – perhaps the technique would be best suited to this kind of task in future.

7.2.4 Searching for novel uveitis autoantigens using a human retinal cDNA library

In Chapter 5 the successful construction of a cDNA library from human neuroretina is described. Screening the library with the sera of uveitis patients and controls by the biopanning method yielded several dozen affinity-selected phage clones. After DNA sequencing and subsequent analysis, 8 clones were highlighted for investigation, 7 of which were eventually expressed as recombinant protein fragments. Purified samples of these potential autoantigens were screened by ELISA against panels of uveitis and control sera for the presence of significant titres of specific antibodies. Depleted supplies of antigen meant 3 of these proteins being tested against a limited panel of sera, but the 4 remaining polypeptides were screened against 45 uveitis and 47 control serum samples. Raised antibody levels were demonstrated to all 7 proteins in at least some individuals, especially those from the uveitis group. No common factor could be identified among these individuals however. When patient and control readings were compared as groups, one peptide (a fragment with homology to human carboxypeptidase E) demonstrated significantly elevated readings to the patient group. Ironically, this peptide was originally affinity-selected using serum from a control subject. Interestingly, none of the main candidate autoantigens for uveitis were isolated during this process.

In my opinion, this experiment was a qualified success. In the short-term, the identification of carboxypeptidase E as a potential autoantigen warrants further investigation. Although not specific to the retina, this protein could nevertheless be relevant to uveitis pathogenesis, and is a known candidate autoantigen in a related condition, autoimmune diabetes. It was not possible to conclusively demonstrate a difference between uveitis and control ELISA

readings as groups, for any of the clones originally affinity-selected using uveitis sera – however, individual subjects demonstrated elevated readings to such clones, some markedly so. It would be worthwhile in the future screening the 3 remaining protein fragments against a full panel of uveitis and control sera. Similarly, screening each of the 7 proteins against sera from more tightly defined uveitis sub-categories might reveal interesting results. Stronger evidence of these candidate proteins' autoantigenicity could be demonstrated by their induction of experimental uveitis in animals, or perhaps even the demonstration of specific T-cell activation/proliferation directly in human samples. The technique of cytokine flow cytometry seems particularly attractive for this purpose.

The main advance from these experiments is the successful demonstration of the feasibility of the technique for directly identifying new human uveitis autoantigens. Various strategies for improving the outcome of future experiments are discussed in Chapter 5, and the refined method could be applied in repeat experiments to identify other uveitogenic proteins (which undoubtedly exist). A measure of the need to expand the pool of candidate antigens in humans, and rank them in terms of relevance, can be seen by studies with similar goals being carried out by other groups (Yamada et al. 2001b). The approach described here, however, which has certain advantages, has not been previously described. Confirmatory evidence of the relevance of the proteins identified here to human uveitis is needed, but the technique itself seems promising.

7.2.5 RSAg-specific T-cell activation demonstrated by cytokine flow cytometry

One of the most exciting techniques to emerge in recent years is cytokine flow cytometry (CFC), which allows the direct detection and identification of several cytokines and surface markers simultaneously in individual activated T-cells. Although used to detect antigen-specific responses in other diseases, mainly infectious, there were few reports of it being used in organ-specific autoimmune diseases, in particular autoimmune uveitis. In Chapter 6, the detection of an antigen-specific response (to stimulation with bovine RSAg) by CFC, in

peripheral T-cells is described. Antigen-specific responses were found in both uveitis patient and control T-cell populations, and there were no significant differences in baseline-corrected overall response rates between the 2 groups. Approximately equal proportions of individuals were judged as being "responders" in each group. Although the proportions of total T-cells responding specifically to the autoantigen were relatively low (as expected), the ability to produce clear backgrounds in negative control samples using this technique enabled recognition of a positive response if it existed. "Activation" of a CD4⁺ T-cell was pre-defined as the co-expression of the surface marker CD69 and the intracellular cytokine IFN- γ . Although the detection of IFN- γ expression might indicate a Th1 type response, this cannot be confirmed in the absence of markers for other cytokines. The detection of responses to RSAg in a significant proportion of healthy controls would appear not to support this antigen's role as a dominant autoantigen in autoimmune uveitis, but this small study needs to be repeated in greater detail and with greater numbers of samples.

The value of this study is the demonstration that CFC is applicable to the detection of antigen-specific T-cell responses in autoimmune uveitis. The technique was already known to be specific and sensitive in the study of cytokine responses at the single cell level in other disease states, but the very low frequency of autoreactive circulating Th-cells was potentially a limiting factor here. That doubt has now been removed. Not only can this technique identify new, potentially uveitogenic autoantigens by detecting generalised T-cell "activation", it can identify the cytokine profiles within individual cells and determine their overall numbers and frequencies. It is even possible to capture particular cellular subsets during analysis by using the FACS cell-sorting mode. The number of parameters capable of being simultaneously analysed is constantly increasing as flow cytometer technology advances, and it is now possible to simultaneously detect several intracellular cytokines as well as important surface markers such as CD4, CD8, CD69, CD25, etc. This enables accurate determination of the Th profile of responding cell types, or detect other antigen-specific responses such as those produced by regulatory T-cells. In my opinion, CFC is the technique of choice for detecting and analysing T-cell responses to potential autoantigens in future autoimmune uveitis research.

7.3 General recommendations for uveitis research

Specific recommendations for the improvement of individual experiments are contained in the Discussion sections of each "results" chapter. There are also lessons to be learnt in terms of the general strategy employed in autoimmune uveitis research, which have emerged during these experiments.

Uveitis is a very heterogeneous group of diseases, and debates concerning the most relevant definitions and classifications still continue. This is important as clinical uveitis entities are often included or excluded from a particular classification based on presumed aetiology of disease. Even within the group of conditions belonging to the category "autoimmune uveitis" there are questions to be resolved. For example, is anterior uveitis with no evidence of infection really a separate disease entity from (posterior) autoimmune uveitis? Should uveitis associated with systemic disease such as sarcoid or Behcet's be included in the same category as eye-specific autoimmune uveitis? Obviously these questions have great relevance to the inclusion/exclusion criteria used when recruiting a sample of patients for basic science research into the causes of the disease.

My experience has been that, because autoimmune uveitis is such a heterogeneous entity, even in a reasonably large group of patients the number of subjects representing individual disease categories tends to be small. While patients can be compared with controls as whole groups, statistical analysis of individual uveitis subtypes is often impossible, and important characteristic responses of disease subtypes may be overlooked. In addition, it is sometimes difficult to consign an individual to a uveitis sub-type with certainty, particularly early in the disease, and this leaves the analysis of results from these subtypes open to question. Another issue is that of disease activity at time of sampling. There is evidence here and elsewhere (de Smet and Dayan 2000) of greater immune responses, particularly cellular, being found during active disease episodes. The samples taken during this study were from a whole spectrum of disease durations, from first presentation to end-stage disease. It is now thought that immune responses vary with the stage of disease progression, and

this needs to be addressed in future research. Analysis of immunological parameters at disease initiation is probably of most interest. Some of the patients sampled were on systemic steroid or immunosuppressive therapy, and this could also have a bearing on results.

In future research into autoimmune posterior uveitis, I would therefore recommend more detailed, prolonged analysis of a single well-defined disease sub-category rather than cross-sectional studies on large heterogeneous groups. As the aetiology and classification of the various disease sub-categories is sometimes uncertain, I would focus on a clearly non-infectious, eye-specific, uveitis subtype with fairly typical features of the disease. Findings from in-depth studies (qualitative then quantitative) of the disease's immunopathogenic mechanisms could later be extrapolated to other uveitis sub-categories and verified experimentally. Sympathetic ophthalmia is the archetypical example of autoimmune uveitis, and would have many advantages as a disease model, but is probably too rare to recruit meaningful numbers. Instead I would perhaps study cases of idiopathic retinal vasculitis or pars planitis, both of which are reasonably common and representative of the disease category. Recruitment of patients at first presentation, coupled with repeat sampling at regularly spaced follow-up appointments as well as during active disease episodes, would be my preferred option. Patients and controls would be sampled for concurrent measures of cellular and humoral immune responsiveness, as these are often known to show an inverse relationship (Liew 2002). Episodes where patients were started on systemic immunosuppression would be clearly highlighted – perhaps it would be necessary to exclude such individuals from the study, or analyse their results separately if sufficient numbers were recruited. Such longitudinal studies would provide much information about the natural history of the disease in terms of immunopathogenic mechanisms, which is currently lacking. In future work, a multicentre study would have the advantage of increasing overall numbers, particularly those of rare uveitis entities, and thereby improve statistical validity. Such a study, coupled with a strictly controlled protocol for sampling and experimental procedure, would also provide cross-validation of results.

7.4 Future directions for uveitis research

The eventual goal of all research into autoimmune uveitis is the development of clinical therapies that treat the condition more effectively and with fewer side-effects than current treatments, or ideally abolish the disease completely. Immunological treatments potentially capable of treating or curing the condition include monoclonal antibodies/immunoadhesins, vaccines and the induction of mucosal tolerance to autoantigens. Most of these potential treatments would require the identification of the autoantigen(s) responsible for the disease. The delineation of pathogenic and possibly protective autoantigenic epitopes would also be essential. Because of the likelihood of epitope spreading as the disease process continued, any antigen-based treatment strategy would require a certain amount of flexibility. In autoimmune uveitis, there is still no definitive proof that any of the candidate autoantigens proposed so far are the actual cause of the disease in humans. This possibly remains the single biggest obstacle to future progress.

A number of the techniques described in this thesis could greatly facilitate the search for such key antigen(s) in future uveitis research. cDNA libraries of human retina, choroid or other ocular structures could be constructed and screened against antibodies from uveitis sera or ocular fluids, to identify the most relevant autoantigens. The uveitogenicity of putative autoantigens could be tested by stimulation of PBMC and the detection of T-cell activation by cytokine flow cytometry. The most reliable source of whole antigen is probably human recombinant expressed from human cells. CFC could also be used to detect responses in T-cell epitope mapping experiments using overlapping synthetic peptides. Combinatorial libraries other than random phage display, could potentially be used for B-cell epitope mapping studies.

Other technologies could also be of use in future uveitis research. The use of DNA microarrays could be used to identify autoantigens or other key target molecules (e.g. cytokines), by highlighting differential production of various mRNAs during the uveitis process. Tissue for this could be obtained in samples from vitrectomies (which are sometimes performed in severe cases of uveitis), or enucleated eyes in cases of sympathetic ophthalmia.

Although the goal of the research described here was to identify strategies that were independent of animal models, recently advances in the development of humanised transgenic animals are noteworthy. An example relevant to uveitis research is the development of a mouse model expressing humanised class II MHC (Pennesi et al. 2003). Experimental results from these animal models should be more directly applicable to human research than those obtained previously.

Autoimmune uveitis is a relatively little-known category of disease within the wider field of organ-specific autoimmunity, particularly in comparison with better known (and more widely researched) conditions such as autoimmune diabetes, rheumatoid arthritis and multiple sclerosis. It makes sense that techniques and strategies first found to work in these fields should then be extrapolated to uveitis research, and this will probably be the pattern for the foreseeable future. However, one area where uveitis research can lead the way in its wider field is in the direct visualisation of the target tissue i.e. retinal vessels and capillary beds (Becker et al. 2002). The eye is the only organ where this is directly possible, and using modern imaging systems, significant insights can be obtained into the disease process, particularly in terms of homing/targeting of activated immune cells. The use of fluorescent dyes as markers of immune cells, and the use of green and yellow fluorescent proteins in *in vivo* studies of animal models (including cytokine expression) is already established. Advances are currently being made in the field of *in vivo* human imaging, and we can look forward to further advances in this field.

A major problem remaining in human uveitis research is that there is still no satisfactory way of gaining access to biological samples from the structures most affected by the disease (i.e. the uvea and retina), except at surgery or as end-stage enucleated specimens. This limits our ability to perform immunohistological studies, or to serially sample intraocular structures for immune components such as antibodies, lymphocytes, cytokines, etc. Obviously any research would have greater validity if samples were actually taken from the eye, rather than using, for example, peripheral blood. *In vivo* imaging will hopefully circumvent some of the problems associated with tissue access, but clearly will not solve all of them. New ideas are needed in this area.

One area that is currently gaining much attention is the potential exploitation of regulatory T-cells to restore tolerance in autoimmune disease. Although the phenotypes of all regulatory cell subsets are not yet fully characterised, certain categories such as CD4⁺CD25⁺ cells, TH3 cells (expressing TGF- β) and Th2 cells expressing IL-10 have gained attention. The potential advantage of employing regulatory T-cells is that certain types of regulatory effects are non-antigen-specific once induced, and this could circumvent many of the potential problems encountered with epitope spreading in specifically targeted immunotherapies. If peptides from uveitic autoantigens were identified that could stimulate these regulatory cells, they could potentially be employed as immunotherapies, and this is another area for excitement in future uveitis research.

7.5 Conclusions

The overall aim of this series of experiments was to evaluate the potential for carrying out autoimmune uveitis research directly on human patients/samples using currently available immunological and molecular techniques. In my opinion the effectiveness of these methods has been demonstrated, and consequently more emphasis should now be placed directly on human research.

In the work described, several techniques have shown themselves to be capable of providing useful results. In particular, the production and use of recombinant antigens, the identification of new potential autoantigens using retinal cDNA libraries, and the use of CFC to detect antigen-specific cytokine responses at the single cell level, were identified as worthwhile techniques for human research. With further refinements and adaptations, much more can be achieved using these methods.

Animal models have served uveitis research well in the past, and will continue to do so as a basic source of new ideas and observations, and as tools for delineating general mechanisms involved in ocular immune responses. Animals will also continue to be essential in the initial testing of any new treatments, including immunotherapies. However, findings from animal models can never be fully extrapolated to humans, and applied human research will always therefore be necessary at a certain stage in the evolution of a study. It is hoped that the application of the techniques described in this thesis will allow direct human research to be carried out at an earlier stage in this process.

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Appendix