

T cell response to grass pollen allergens: Correlation with skin test reactivity and serum IgE levels

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Summary T cell proliferative responses to rye and Bermuda grass pollen allergens have been studied in a series of 51 atopic and 18 non-atopic subjects. Mean T cell responses were higher in the atopic group than in the non-atopic group ($P < 0.001$), and there was a strong correlation between the magnitude of reaction in the T cell assay and in the skin test (rye $P < 0.01$, Bermuda $P < 0.05$). A similar association was shown between T cell reactivity and serum levels of allergen-specific IgE (rye $P < 0.05$, Bermuda $P < 0.05$), but no relationship was found between serum allergen-specific IgG levels and any other parameter studied. T cell reactivity was not found in three cord blood samples tested. Discordance between positivity for T cell responses and skin test reactions in some cases might reflect reactivity by T cell subsets that promote IgG antibody or cell-mediated responses without IgE antibody production. A precise knowledge of T cell recognition of grass pollen allergens will provide exciting new prospects for more effective and safer immunotherapy strategies for allergic diseases including asthma.

Key words: allergens, Bermuda, hypersensitivity, IgE, *Lolium*, lymphocyte transformation, pollen, T lymphocytes, skin tests.

Introduction

The pivotal role of T cells in controlling the initiation and type of immune response to most antigens is well accepted. However, the exact molecular basis for this T cell control is not yet clearly understood. The immune response to allergens represents the commonest immunological disorder, and may be associated with life-threatening conditions such as asthma and anaphylaxis. Thus, elucidation of the mechanisms underlying these reactions would lead to significant improvement in the diagnosis and treatment of these disorders.

Current diagnostic tests for reactivity to allergens usually include skin prick tests and radio-immunoassays for total and allergen-specific IgE, but these test results do not always correlate with clinical symptoms of allergy. Moreover, widely used protocols for down-regulation of the allergic response by antigen-specific immunotherapy are empirical and there is no reliable test for monitoring the efficacy of this treatment. A more detailed knowledge of the mechanisms for T cell control of allergic responses could provide solutions to these problems.

Initiation of the immune response to allergens requires T cell recognition of processed antigenic peptides in the context of MHC class II molecules on the surface of antigen presenting cells.¹ *In vitro* studies have shown that subsequent activation of a subset of helper T cells (Th2)

secreting appropriate cytokines, especially IL-4, results in the promotion of an IgE antibody response.^{2,3} For example, house dust mite (HDM)-specific T cell clones isolated from an atopic individual have been shown to promote IgE synthesis by autologous B cells *in vitro* and this helper activity was IL-4-dependent.^{4,5} However, studies investigating the relationship between T cell reactivity and IgE-mediated allergic responses have produced mixed results. Reactivity to HDM allergens has been most studied in this respect. Several groups have reported a correlation between antigen-specific T cell reactivity and skin test reactivity,^{6–12} but others found similar T cell reactivity for both HDM-atopic and non-atopic subjects.^{5,13–15} Only limited studies have been performed using pollen allergens, again with conflicting results.^{16–20}

Grass pollens are the major aeroallergens during spring and summer (when most of the grasses flower), correlating with the seasonal severity of asthma and rhinitis in many patients.^{21,22} A study in Melbourne, Australia, found that 84% of asthmatics were atopic and that grass pollens were among the commonest allergens recognized (M. Abramson pers. comm. 1992). Rye and Bermuda grass pollens are clinically important aeroallergens in temperate and sub-tropical Australia respectively.^{22,23} Thus, in order to gain further insight into the regulation of this allergic immune response by T cells, we have established assays for T cell reactivity to rye and Bermuda grass pollen allergens, and in this study describe the relationship between peripheral blood T cell response, skin test reactivity and allergen-specific serum IgE levels in a series of atopic and non-atopic donors.

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Materials and methods

Subjects

Subjects studied were patients attending the Asthma and Allergy Clinic of the Alfred Hospital in the course of their routine diagnosis and treatment, or laboratory staff. Entry into the study followed informed consent by the subject and approval of the hospital's Ethics Review Committee. The subjects were divided into two groups: group A comprising 51 subjects who were skin test positive to rye and/or Bermuda grass pollen extract, and group B comprising 18 subjects who were skin test negative to these allergens (Table 1). The mean age and male:female ratio were not significantly different between the two groups.

Forty-six subjects in group A had clinical symptoms of allergic disease (e.g. rhinitis, asthma, eczema), while five subjects in this group had never experienced such symptoms. In group B, 15 subjects were asymptomatic, one had seasonal rhinitis but no skin test reactivity to any common allergen, one was being desensitized to HDM and another to bee venom.

In addition, three cord blood samples (group C) were tested in the T cell proliferation assay to provide a measure of 'base-line' reactivity, that is, T cell reactivity for subjects unlikely to have been exposed to grass pollen allergens.

Skin testing

Skin test reactivity was tested against several common allergens, including rye and Bermuda grass pollen extracts (1:20, w/vol; Hollister Stier, Elkhart, IN, USA). The skin prick test was performed by placing a small drop of each test extract, 0.9% saline (negative control) and histamine (10 mg/mL, positive control) on to the volar aspect of the forearm. A sterile 1 mm lancet was then used to scratch the skin beneath the drop and excess extract was blotted off. Wheal diameters were measured after 15 min, and reactivity was graded on a scale of 0 to 4 where a diameter of <2 mm was graded as 0 and the histamine response for an individual was graded as 3. At the time of skin testing, no subject was taking any medication that might have interfered with the test.

T cell proliferation assays

Allergen preparations Dry, non-defatted rye and Bermuda grass pollen extracts (Greer Laboratories, Lenoir, NC, USA) were further extracted in 1 mmol/L NH_4HCO_3 , centrifuged and supernatants dialysed against PBS pH 7.2 overnight. The supernatants were then passed through a 0.2 µm pore size filter and protein concentrations determined using the Bio-Rad Microassay (Bio-Rad, Richmond, CA, USA).

Table 1. Study groups A and B.

	Group A	Group B
Skin test response to rye and/or Bermuda grass pollen extract	Positive	Negative
No. subjects	51	18
Clinical symptoms of allergic disease	46	3
Mean age $\pm$ s.d.	26 $\pm$ 9	34 $\pm$ 13
Male:female ratio	1:1.6	1:1.3

Cell culture and proliferation assay PBMC were separated from heparinized blood samples by centrifugation on a Ficoll-Paque density gradient (Pharmacia, Uppsala, Sweden), and resuspended at a concentration of 5×10^5 cells/mL in RPMI-1640 tissue culture medium (ICN Biomedicals Inc., Costa Mesa, CA, USA) supplemented with 1% L-glutamine, 0.125% gentamycin and 10% heat-inactivated screened human A+ serum. The cells were then cultured at 200 µL/well in 96-well U-bottomed microtitre plates (Linbro, Flow Laboratories, McLean, VA, USA) with rye or Bermuda grass pollen extract (0.5–1000 µg/mL) for 7 or 8 days, respectively. Control cultures included cells incubated with no allergen (negative control) and with phytohaemagglutinin (1–4 µg/mL; PHA, Wellcome, Dartford, England; positive control). *In vitro* proliferation was then assessed by pulsing with 1 µCi/well [^{25} I]-5-iodo-2-deoxyuridine (Amersham, Buckinghamshire, England) for 18–20 h prior to harvesting on to glass-fibre filters. The counts associated with each filter were determined using a gamma counter (LKB Wallace, Turku, Finland), and the mean counts per minute (ct/min) $\pm$ s.d. for triplicate cultures was determined. Since background incorporation varied significantly between donors, to compare proliferation results between donor groups, responses were converted to a stimulation index by dividing the mean ct/min for cultures containing the optimal concentration of antigen by the mean ct/min for the same cells cultured without antigen.

Allergen-specific IgE

Serum samples were analysed for IgE antibodies specific for rye or Bermuda grass pollen allergens using Phadebas RAST kits (Pharmacia, Uppsala, Sweden) which generated results as grades (0–4). Mean values for duplicate serum analyses are presented.

Allergen-specific IgG

An ELISA was used to detect rye and Bermuda grass pollen-specific IgG. Flat bottomed 96-well plates (Nunc, Kamstrup, Ruskilde, Denmark) were coated for 4 h with 100 µL/well rye or Bermuda grass pollen extract (10 µg/mL) diluted in 0.25 mol/L carbonate-bicarbonate buffer, pH 9.5. The plates were washed with 5% Tween 20/PBS and blocked with 5% BSA/PBS overnight at 4°C. After washing the plates, serum (neat, 100 µL/well) was added and incubated for 1 h. The plates were then washed and further incubated for 45 min with a 1:200 dilution of rabbit anti-human IgG horse radish peroxidase conjugate (Dakopatts, Glostrup, Denmark; 100 µL/well). Plates were then washed, incubated with the substrate *o*-phenylenediamine (Sigma Chemical Co., St Louis, MO, USA) in the dark for 30 min, and the $\text{OD}_{492\text{ nm}}$ were measured using a Titertek Multiskan ELISA reader (Flow LabSystems and Laboratories, Finland). Wells which served as blanks were those subjected to all incubations and washing procedures except addition of test serum. Mean values for duplicate samples were determined.

Statistics

Student's unpaired *t*-test was employed to assess the level of significance of differences between mean values for a particular parameter for any two groups, and a Chi-squared or linear regression analysis was performed to assess the degree of correlation between two parameters.

Results

Optimization of the T cell proliferation assay

Optimal culture conditions for the T cell proliferation assay were established in preliminary experiments using PBMC from several atopic donors. Variables examined included cell concentration, antigen dose, incubation time and serum supplement. In these pilot studies, optimal T cell reactivity was found after 7 days culture with rye grass pollen extract and after 8 days with Bermuda grass extract, and these times were therefore used for assessing T cell reactivity in the larger series of donors studied. PHA stimulated cultures were included in every assay as a positive control. After the 7 or 8 day culture period, the PHA response was sub-optimal, but the stimulation index was always greater than 4.0.

Greatest T cell response was obtained at different doses of allergen for different donors, therefore testing of subjects in this study was performed using a wide range of allergen concentrations (0.5–1000 µg/mL). The dose-response relationship obtained for a T cell reactive subject is shown in Fig. 1. When comparing stimulation indices between subjects, the peak values were used. For example, in the case shown in Fig. 1, the maximal stimulation indices for T cell response to rye and Bermuda grass pollen extracts were 8.0 and 8.9, respectively.

For the three cord blood samples, mean stimulation indices $\pm$ s.d. were 1.3 ± 0.3 and 1.0 ± 0.2 for rye and Bermuda grass pollen extracts, respectively. Therefore in tests on group A and B subjects, a stimulation index of greater than or equal to 2 (i.e. greater than 2 s.d. above the mean

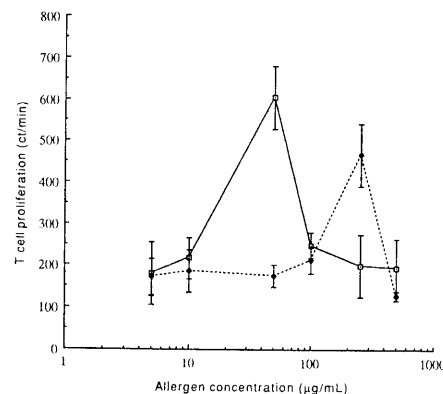


Fig. 1. Dose-response relationship for peripheral blood T cell proliferation to rye (—) and Bermuda (---) grass pollen allergens. PBMC from an atopic donor were cultured with rye or Bermuda grass pollen extracts for 7 or 8 days, respectively, and proliferation was assessed by [^{25} I]-iodo-deoxyuridine uptake. Mean ct/min $\pm$ s.d. for triplicate cultures are shown. Mean background values, i.e. cultures with no added allergen, were 75 and 52 ct/min for days 7 and 8, respectively.

stimulation index for the cord blood samples) was considered positive.

Comparison of T cell responses between atopic and non-atopic donors

T cells from subjects skin test positive to rye and Bermuda grass pollen allergens (group A) showed a wide range of responses to these allergens, but the mean stimulation index was significantly higher than that for subjects skin test negative to these allergens (group B; $P < 0.001$) and that for the cord blood samples (group C; $P < 0.01$; Fig. 2). This effect was not due to differences in background incorporation as shown by the mean counts for each group presented in the legend of Fig. 2.

The mean T cell response for subjects skin test negative to the allergens (group B) was higher than that for the cord blood samples (group C) but this difference did not reach statistical significance. Within each of the three groups, the mean T cell responses to the two grass pollen extracts were similar, reflecting general agreement between T cell reactivity to the two grasses for most subjects in this study.

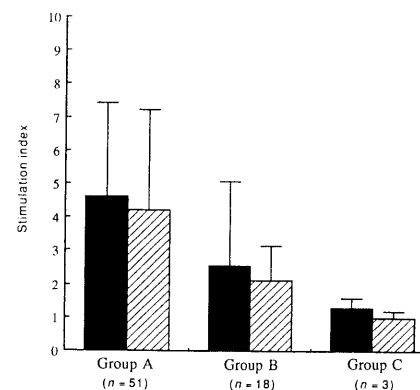


Fig. 2. T cell reactivity against rye (solid bars) and Bermuda (hatched bars) grass pollen extracts for PBMC from 51 subjects skin test positive to rye and/or Bermuda grass pollen extract (group A) compared with 18 subjects skin test negative to these allergens (group B). T cell reactivity for three cord blood samples (group C) is also shown. Tests for each subject were performed in triplicate and the mean stimulation index $\pm$ s.d. for each group is shown. The mean T cell response for group A was significantly higher than that for group B ($P < 0.001$) and that for group C ($P < 0.01$). Mean background incorporation ($\pm$ s.d.) for each group: A, 98 ± 60 at 7 days and 100 ± 60 at 8 days; B, 73 ± 36 at 7 days and 95 ± 42 at 8 days; C, 102 ± 8 at 7 days and 126 ± 18 at 8 days. Mean maximal counts ($\pm$ s.d.): A, 452 ± 410 with rye at 7 days and 422 ± 413 with Bermuda at 8 days; B, 174 ± 136 with rye at 7 days and 215 ± 174 with Bermuda at 8 days; C, 132 ± 27 with rye at 7 days and 131 ± 42 with Bermuda at 8 days.

Correlation between T cell responses and skin test reactivity to rye and Bermuda grass pollen allergens

When T cell and skin test reactivity to the grass pollen allergens were compared for each of the 69 individuals in groups A and B, there was a significant positive correlation (rye $P < 0.001$, Bermuda $P < 0.01$, χ^2 test), that is, subjects who were skin test positive to an allergen generally showed a positive T cell response to that allergen and those skin test negative showed no T cell reactivity (Fig. 3). Moreover, there was a correlation between the magnitude of reaction in these assays (rye $P < 0.01$, Bermuda $P < 0.05$, Pearson's correlation; Fig. 4).

However, in 25 of the 69 cases (36%) exceptions to this agreement were noted. There was a slightly higher proportion of discordant cases for reactivity to Bermuda (28%) than to rye grass pollen (20%), and a higher proportion of T cell positive/skin test negative cases (29%) than vice versa (19%). For seven of the eight cases which were T cell reactive but skin test negative to both grass pollens, T cell responses were weak (mean stimulation index = 3.1). In contrast, for the four subjects T cell negative but skin test positive to both grasses, skin test reactivity was strong.

Correlation of T cell responses with rye and Bermuda grass pollen-specific IgE

Serum samples were available from 37 of the 51 cases in group A and all of those in group B for testing for allergen-specific IgE by RAST. There was a strong association ($P < 0.001$, Pearson's correlation) between skin test and RAST results. A significant correlation between T cell response and RAST results was observed (rye $P < 0.05$, Bermuda $P < 0.05$, Pearson's correlation; Fig. 4), al-

Skin test T cell response	Skin test			
	Rye + Bermuda +	Rye + Bermuda -	Rye - Bermuda +	Rye - Bermuda -
Rye + Bermuda +	32	7	1	4
Rye + Bermuda -	3	2	0	3
Rye - Bermuda +	2	0	0	1
Rye - Bermuda -	4	0	0	10

Fig. 3. Correlation between T cell response and skin test reactivity to rye and Bermuda grass pollen allergens for 69 subjects (rye $P < 0.001$, Bermuda $P < 0.01$, χ^2 test).

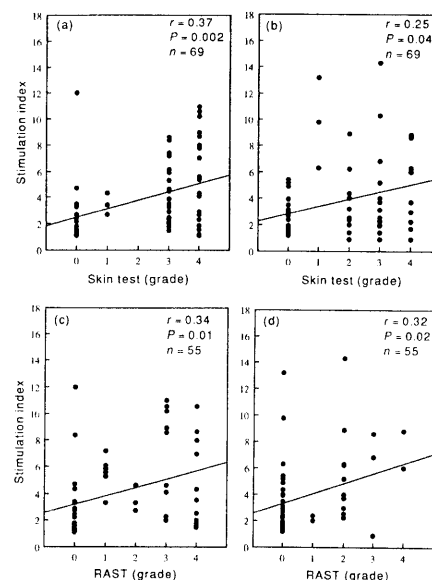


Fig. 4. Relationship between T cell stimulation index and skin test grade against rye (a) and Bermuda (b) grass pollen extract, and between T cell stimulation index and RAST grade against rye (c) and Bermuda grass pollen extract (d).

though less pronounced than that between T cell and skin test reactivity.

Allergen-specific IgG

A range of allergen-specific IgG reactivities was detected in the samples tested (28 from group A, 12 from group B and all three of group C), with no significant difference in mean values between the three groups of subjects. Moreover, there was no correlation between IgG levels and any other parameter tested. In particular, IgG levels were not higher in those examples which were T cell positive and skin test and/or RAST negative.

Discussion

We have established assays for T cell proliferation in response to stimulation with grass pollen extracts, following optimization of antigen dose range, duration of assay and culture conditions. In the present study, these assays have been used to determine peripheral blood T cell reactivity to rye and Bermuda grass pollen allergens in a series of atopic and non-atopic donors. The finding that T cell responses were in general higher for individuals with positive skin tests to the grass pollen allergens (group A) than for those with negative skin tests (group B) is consistent with T cell reactivity being required for the development

of allergen-specific IgE and thus positive skin tests. This concept is supported by the correlation observed between T cell response and allergen-specific IgE levels in this study and also in that by Black and Marsh¹⁷ using a rye group I allergen.

Interestingly, most individuals showing T cell reactivity to one of the grass pollens showed T cell reactivity to the other pollen, a relationship also seen for the skin test results. However, aerosolized rye grass pollen is present in far greater abundance than Bermuda grass pollen in the community sampled,²⁴ suggesting either stronger immunogenicity of Bermuda grass allergens or cross-reactivity between the grasses. Since only very limited cross-reactivity has been demonstrated between these two pollens in antibody binding studies,²⁵⁻²⁷ the intriguing possibility remains that cross-reactivity may occur at the T cell level. In support of this concept, we observed a weak proliferative response of a rye grass pollen T cell line when stimulated with Bermuda grass pollen extract.

T cell reactivity to grass pollen allergens was detected in 44% of the skin test negative donors, although in all cases except one the responses were low in magnitude. These responses were not due to non-specific mitogenic activity of the pollen extracts since not all donors tested in this study responded and cord blood samples showed virtually no reactivity to the extracts. It was of interest that while T cell responses of the cord blood samples were minimal, detectable levels of allergen-specific IgG were found in these samples, presumably due to maternal transfer of serum IgG. Others have reported non-atopic donor T cell reactivity to rye grass,¹⁷ ragweed¹⁸ and HDM allergens^{5,6,8,9,11-15} but, in contrast, some authors report proliferative responses to HDM¹⁰ and ragweed allergen^{16,19} in skin test positive cases only. The number and clinical status of patients studied, and the purity of allergen preparations used are likely sources of difference between these studies. In general, where studies have included large numbers of clinically well characterized subjects, a correlation between T cell reactivity and skin test reactivity is found, with some non-atopic cases showing positive, albeit weak T cell responses in agreement with our findings.

Several explanations can be proposed to explain the lack of complete correlation observed in our study between T cell response and skin test reaction. First, a positive T cell reaction would not only reflect an IgE antibody response but could alternatively be associated with an IgG antibody response. In the limited series tested, we found no evidence of higher pollen-specific IgG levels in subjects T cell positive, skin test negative compared with those T cell negative, skin test negative. However, reports of different allergen-specific immunoglobulin class and subclass production in allergic compared with non-allergic donors^{28,29} suggest that this possibility should be explored more thoroughly. A further possibility is that the T cell response reflected reactivity by CD8 positive T cells rather than the CD4 positive T cells required for IgE immune responses. Studies have found that proliferating blood mononuclear cells in HDM-stimulated cultures comprised up to 31% CD8 positive cells for some individuals,¹³ and CD8 T cells have been shown to play

an important immunoregulatory role in the airways.^{30,31} Thus, the possibility of these cells influencing the allergic immune response to grass pollens cannot be excluded.

Pollen extracts from different commercial companies were used in skin testing, T cell proliferation assays and the RAST in this study. Rye and Bermuda grass pollen have each been shown to contain 17 different allergens.^{32,33} Although it would be expected that all major pollen components were present in the three different preparations, and the general agreement between assays found in this study supports that, differences in the amount of minor allergens or chemical alterations due to different extraction procedures may account for inconsistencies between assays in some cases.

There have been very few studies of the specificity of grass pollen allergen reactive T cells. In one such study,³⁴ cross-reactivity between the rye grass pollen allergens Lol p 1, II and III was shown for T cell clones prepared from atopic donors (consistent with significant sequence similarity between these allergens), and each of four T cell clones raised against Lol p I were found to react with a single 20-mer peptide of Lol p I.³⁵ Of functional significance are studies on the cytokine profile of these T cells: they have been found to be predominantly of the Th2 type, that is, producing IL-4 rather than IL-2 and IFN- γ , and thus able to provide help for IgE-producing B cells.³⁶ We are currently generating grass pollen specific T cell lines and clones from atopic and non-atopic subjects and testing their specificity using a panel of synthetic peptides based on the known sequences of the major allergens of rye and Bermuda grass pollen. One such T cell clone from an atopic donor has been found to react with a single 12 amino acid peptide, with restriction to HLA-DR4. Further studies to elucidate the role of T cells in the allergic immune response and identification of the precise T cell epitopes of grass pollen allergens will lead to improved screening and more rational and safer therapy for grass pollen allergic reactions including asthma.

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Expression and immune recognition of stress proteins in sarcoidosis and other chronic interstitial lung diseases

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Summary Stress proteins (SP) are major immunogens in a number of microbial infections and have been implicated in some autoimmune diseases. The aetiology of sarcoidosis, a non-caseating granulomatous disease, remains unknown, but mycobacteria as well as autoimmunity have been considered. In the present study, patients diagnosed with sarcoidosis and other interstitial lung diseases (ILD), as well as healthy volunteers were studied to determine: (i) the level of expression of SP in alveolar macrophages and blood monocytes; (ii) the serum levels of antibodies specific for mycobacterial SP65 and SP70; and (iii) the reactivity of peripheral blood and alveolar lymphocytes to mycobacterial SP65. Our results suggest that SP are expressed constitutively at high levels in alveolar macrophages, retrieved by bronchoalveolar lavage, from all individuals regardless of health status. In contrast, freshly isolated blood monocytes express low levels of SP, which are, however, readily upregulated following exposure to IFN- γ and TNF- α . Lymphocyte reactivity and presence of antibodies against mycobacterial SP may reflect the current state of *in vivo* inflammation rather than the cause of inflammation.

Key words: alveolar macrophages, blood monocytes, lung diseases, sarcoidosis, stress proteins.

Introduction

The stress response is a rapid, complex and transient reprogramming of cellular activities, consisting of induction of gene transcription and synthesis of stress proteins (SP) and post-transcriptional inhibition of normal protein synthesis.¹ The stress response is induced by exposure to supra-optimal temperatures ('heat shock', hence also 'heat shock protein'), as well as many forms of cellular injury including oxidative injury, viral and bacterial infections, exposure to heavy metals, ethanol, or amino acid analogues, nutrient deprivation and changes in pH.^{2,3}

Many SP are constitutively expressed in both eukaryotic and prokaryotic cells and are involved in normal cellular functions; for example, SP have been shown to interact with newly made proteins, assisting in their folding and translocation across membranes.³ In addition, stress induces a modest increase in the cell surface expression of MHC class II antigens,⁴ and SP expression may be transiently upregulated during lymphocyte activation and proliferation.⁵ These and other findings suggest a possible role for SP in antigen processing, presentation and cellular activation during an immune response.^{4,6}

Proteins from a diverse range of pathogenic and commensal micro-organisms have been identified which share sequence homologies with members of the five known

families of vertebrate SP.³ A frequent finding in studies of the immune responses to bacteria have been that the microbial SP often contain the immunodominant epitopes presented to the host immune system during infection.⁷ This has led to the hypothesis that cross-recognition of SP by T cells could be the link between the immune response to infectious agents and autoimmunity.^{3,7} Evidence to support this hypothesis includes the finding that human T cell clones specific for a mycobacterial 65 kDa SP sequence also respond to a peptide corresponding to a similar, but not identical, sequence in the human SP65,⁸ while other studies have suggested a role for anti-SP immunoreactivity in juvenile chronic rheumatoid arthritis.^{9,10} In murine models of mycobacterial immunity it has also been shown that antigen presenting cells (macrophages) stressed by viral infection or heat can become targets for cytotoxic CD8⁺ T cells, specific for the 65 kDa mycobacterial SP.¹¹ Autoimmune diseases in animals with anti-SP reactivity as an important component can be induced with preparations containing mycobacteria, for example, adjuvant arthritis and diabetes.^{10,12}

The above hypothesis, however, begs a number of questions before the involvement of SP in autoimmunity can be fully contemplated and explored: (i) what is the level of expression of the various SP under normal steady-state conditions in organ systems most often involved in autoimmune reactions; (ii) how is the expression of these SP regulated during acute and chronic inflammatory processes; and (iii) are the SP, at constitutive and upregulated (inflammation) levels, recognized by the immune system,

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