Rye Grass Allergen Induced Lymphocyte Proliferation*

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SUMMARY The *in vitro* proliferative response of peripheral blood lymphocytes (PBL) to whole rye grass allergen and purified rye grass Group 1 allergen was studied. The PBL were obtained from 44 rye grass-positive (positive prick skin testing (PST) and positive RAST to rye grass) patients with seasonal allergic rhinitis and 17 rye grass-negative subjects. The response of the rye grass-positive group was significantly greater than that of the rye grass-negative group for both the whole rye grass allergen and the Group 1 allergen. The proliferative response of the rye grass-positive group correlated significantly with wheal diameter on PST but not with other parameters. The predominant responding cell type in the proliferative response was shown to be a T cell.

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Although atopy is characterised by a type 1 IgE-mediated reaction, it may also be associated with cell-mediated immunity. Late-onset skin reactions have been described, some of which may be IgE-mediated ('late phase response')[1,2] while others resemble the more classical delayed type of hypersensitivity (DTH) skin reaction on the basis of timing (maximal at 36-48 hours) and histology (mononuclear infiltrate).[3,4] Most individuals with seasonal allergic rhinitis do not give such DTH skin responses[4,5] to allergens, although cells transferred to non-atopic recipients often result in positive delayed skin reactions.[6]

Antigen-induced lymphocyte responses *in vitro* are considered to reflect cellular immunity and have been studied by blast transformation,[2,7-9] lymphokine production[2,10,11] and thymidine incorporation.[11-15] There is evidence that the cell responding to allergens is a T cell.[2,16,17] Clinical relationships are uncertain. In some studies,[7,8] but not all,[13-15] lymphocyte stimulation has correlated with immediate hypersensitivity.

In this study we describe a rye grass-allergen-induced T-lymphocyte proliferative response which is significantly greater in subjects with immediate hypersensitivity to rye grass than in non-sensitised subjects. Furthermore, we found a significant correlation between this response and the diameter of the wheal response demonstrated by prick skin testing (PST).

MATERIALS AND METHODS

Subjects

Forty-four subjects (14 males, 30 females) ranging in age from 15 to 60 years, with a history of seasonal allergic rhinitis, positive PST and positive radioallergosorbent (RAST) to rye grass *(Lolium perenne)* were compared with 17 subjects (3 males, 14 females), aged 20 to 54 years, with no history of rhinitis and negative PST and RAST. These are subsequently referred to as positive and negative subjects respectively. These patients were studied pre-seasonally and had not been hyposensitised to rye allergen, nor were they taking any therapy at the time of testing.

Skin tests and nasal challenge

PSTs[18] were performed using a commercial rye grass allergen preparation (allergenic extract of perennial rye grass pollen 1:20 w/v, Hollister Stier Laboratories), using the volar aspect of the forearm. Histamine monophosphate (1 mg/ml) (David Bull Ltd, Guildford, Australia) and saline were used as positive and negative controls respectively. The reaction was read at 15 minutes. The diameter (D) of the wheal was measured at its widest point and at right angles to this point (d), and the result expressed as the average of these two diameters ($\frac{D+d}{2}$). All positive subjects had a mean wheal diameter > 3 mm.

Nasal challenges were performed on the 44 rye grass-positive subjects. Subjects received one spray per nostril of the test solution using a nebuliser which delivered 0.1 ml of spray.

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The first solution used was saline which was used as a diluent. Following this, the subject received increasing concentrations of whole rye grass allergen. The initial concentration used was 0.01 μg/ml and this was increased by a factor of 10 at intervals of 10 minutes to a maximum concentration of 100 μg/ml. The nasal provocation dose (NPD) was that at which the subjects developed any three of the following symptoms or signs: nasal obstruction, itch, rhinorrhoea, sneezing and nasal mucosal swelling or pallor.

Antigens

Antigens were prepared from rye grass pollen (dry pollen, Greer Labs. Inc., Box 800, Levoir, NC 28654, U.S.A.) by the method of Marsh.[19] A whole allergen preparation (containing allergen Groups I, II, III) and a purified Group I allergen were prepared. Briefly, the pollen was defatted with anhydrous diethyl ether. The dry defatted pollen was extracted with 0.125 M NH_4HCO_3, centrifuged at 40,000x g for 20 minutes and dialysed extensively against 0.001 M NH_4HCO_3. This crude extract was lyophilised, dissolved in 0.01 M NH_4HCO_3 (to approximately 20 mg/ml) and loaded on a DEAE-sephacel (Pharmacia Fine Chemicals, Sweden) column (approximately 2 g of protein per 190 ml of column volume). To obtain the whole allergen preparation, the column was eluted with 0.25 M NH_4HCO_3. The eluted material was pooled, dialysed and lyophilised. To prepare the purified Group I allergen, the column was first eluted with 0.04 M NH_4HCO_3, which yielded impure group III allergen. Elution with 0.25 M NH_4HCO_3 then gave a fraction containing impure Group I and Group II allergens. This fraction was lyophilised, dissolved in 0.05 M NH_4HCO_3 and further fractionated on Sephadex G57 (Pharmacia Fine Chemicals, Sweden) to give purified Group I and purified Group II allergens. Samples of the crude extract, the whole allergen and the purified Group I allergen were run on SDS-polyacrylamide slab gel electrophoresis[20] (Fig. 1) using standard proteins to determine the molecular weight (Pharmacia low molecular weight standard kit). The whole allergen preparation contains two major bands, one MW ~33,000 (Group I allergen) and the other MW ~14,000 (Group II and Group III alergens). The purified Group I allergen has one major band. The whole allergen and the Group I allergen gave precipitin lines against goat anti-rye Group I antiserum (supplied by Dr. D. Marsh) in an Ouchterlony test (not shown).

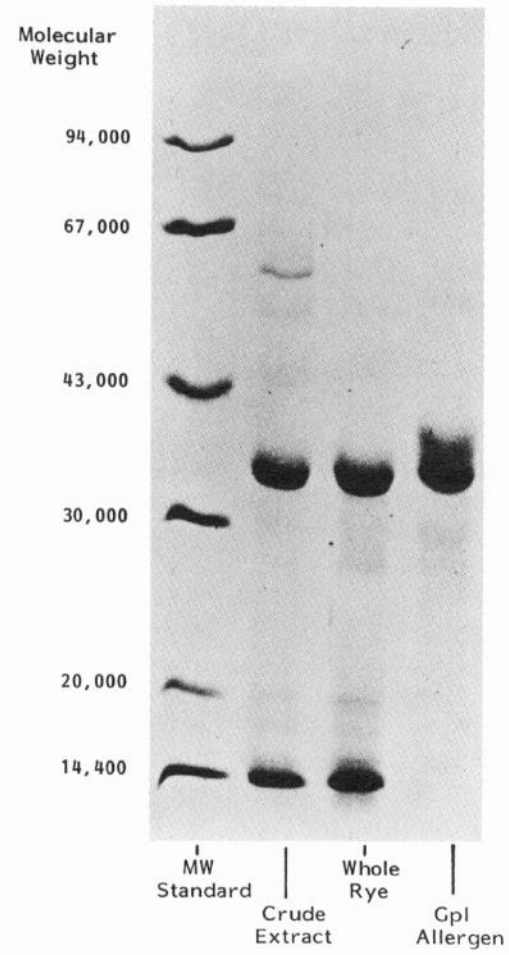

Fig. 1 Polyacrylamide slab gel electrophoresis of crude rye grass pollen extract, whole rye grass pollen extract and GpI allergen. Crude rye extract contains predominantly proteins of MW 33,00 and 14,000 and some higher MW proteins. Whole rye allergen contains predominantly proteins of MW ~33,000 and 14,000. GpI allergen contains only proteins of MW ~33,000.

Pokeweed mitogen (PWM) (Grand Island Biological Co., U.S.A.) was used as a positive control antigen.

In vitro stimulation of peripheral blood lymphocytes

Lymphocytes were separated from heparinised blood by centrifugation on Ficoll-Hypaque (Pharmacia Fine Chemicals, Sweden) by the method of Boyum.[21] The PBL were washed three times in Dulbecco's phosphate buffered saline (CSL, Australia) and resuspended in RPMI 1640 medium (Flow Labora-

tories, Australia) supplemented with 10% human AB serum (from a donor with negative rye grass RAST), 2 mM of L-glutamine, 100 units/ml of penicillin and 100 μg/ml of streptomycin sulphate. The PBL were cultured in triplicate in sterile microtitration plates with U-bottomed wells ('Nunclon', Nunc, Denmark) with no antigen, whole rye allergen (25 Ug of protein/ml), Group I allergen (25 Ug of protein/ml) or PWM (1:200 final dilution of reconstituted vial). After seven days, the cultures were pulsed for four hours with one microCurie of ^{3}H-thymidine (specific activity 2 mCi/mmol) and harvested on glass-fibre filters using a multiple automatic harvester. These were dried and counted in a toluenebased scintillant fluid using a β-scintillation counter. Results were expressed as mean cpm of triplicate cultures ± standard deviation.

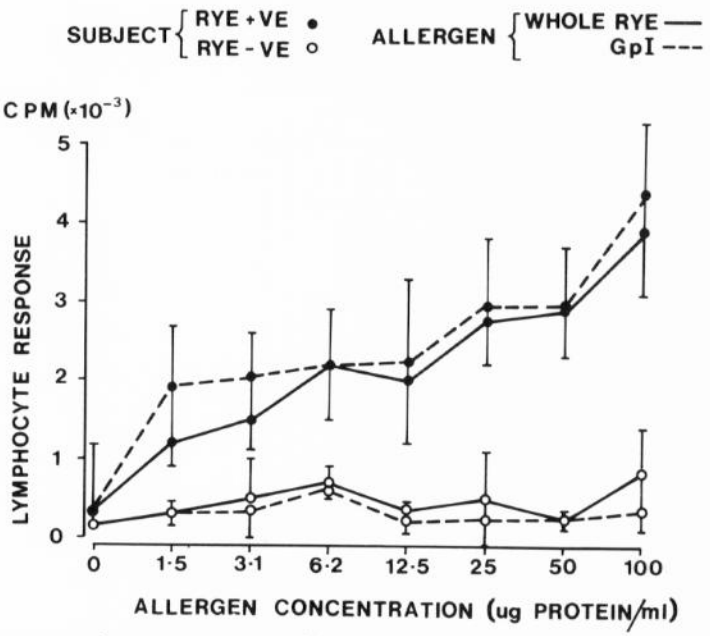

Fig. 2 Response of one rye-positive (●) and one rye-negative (○) individual to increasing doses of whole rye (–) and GpI (- - -) allergens. In subjects tested at doses > 100 μg/ml, the response plateaus (not shown).

RESULTS

1. Kinetics of lymphocyte response in positive and negative subjects.

Various concentrations of whole rye allergen or Group I allergen were cultured with lymphocytes from one rye-positive and one rye-negative subject (Fig. 2). The Group I and whole rye allergens gave a similar response. The response reached a plateau at doses above 100 μg/ml (not shown). In all subsequent cultures the concentration of allergens used *in vitro* was 25 μg/ml. This was considered to be a dose high enough for adequate response without consuming large quantities of purified allergen. The optimal response to the rye grass allergen occurs on day 7 of culture (Fig. 3). All subsequent cultures were therefore terminated on day 7.

2. Response of rye-positive subjects compared with that of rye-negative subjects

The proliferative response of PBL from 44 rye-positive subjects was compared with that of PBL from 17 rye-negative subjects. The response to whole allergen, Group I allergen and PWM was assessed (Fig. 4). The mean response of the rye-positive group was significantly higher than that of the rye-negative group for both the whole rye ($p < 0.001$) and the Group I allergen ($p < 0.001$). However, some rye-positive subjects gave little or no response and some rye-negative donors gave a reasonably high response.

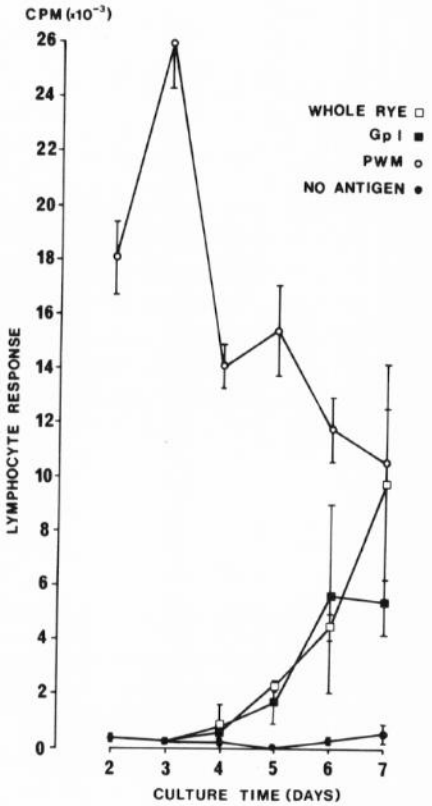

Fig. 3 Time course of response of a rye-positive donor to no antigen (●), whole rye allergen (□), Gpi allergen (■) and PWM (○). The concentration of allergens was 25 μg/ml and PWM 1/200 of reconstituted vial.

We found that 13 of the rye-positive subjects gave little or no response to Group I allergen and nine gave little or no response to whole rye allergen, while five rye-negative subjects responded to Group I allergen and six to whole rye allergen. The response of the two groups of subjects to PWM was not significantly different.

3. Characterisation of the responding lymphocyte

T cells, non-T cells or unseparated PBL were cultured with whole rye, Group I or PWM in two rye positive subjects. The PBL and T cells proliferated in response to grass allergens but the B-cell response was very low or absent. All three

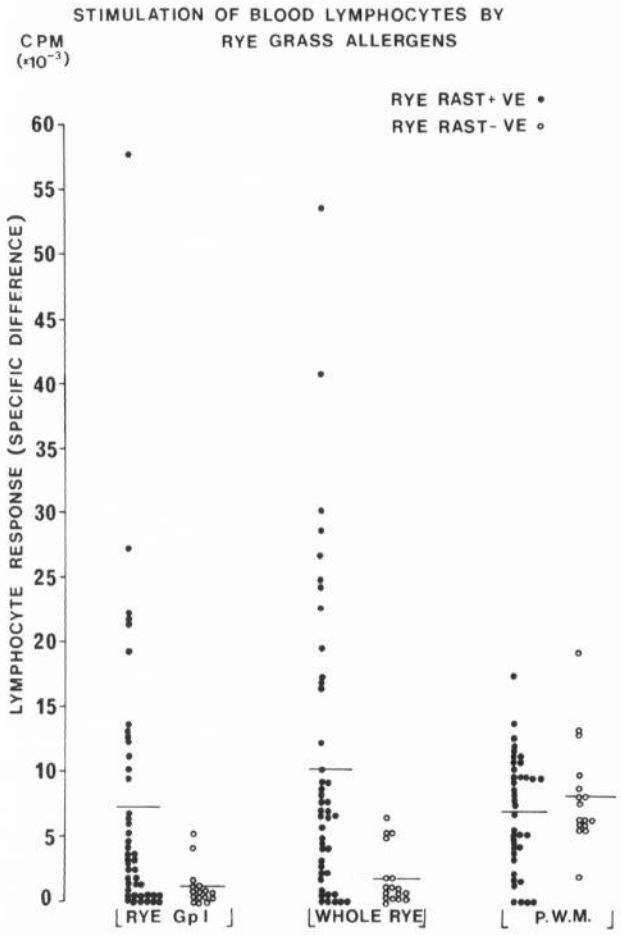

Fig. 4 Response of rye positive (●) and rye-negative (○) subjects to whole rye allergen (25 μg/ml), Gp I allergen (25 μg/ml) and PWM (1/200). Culture period was seven days. For Gp I allergen, the mean response of the rye-positive group (7,321 ± 10,646) was significantly greater ($P < 0.001$) than the mean response of the rye-negative group (1,078 ± 1,465). For whole rye allergen the mean response of the rye-positive group (10,636 ± 11,873) was also significantly higher ($P < 0.001$) than that of the rye-negative group (1,864 ± 2,152). For PWM, the mean response of the rye-positive group (6,928 ± 4,380) was not significantly different to that of the rye-negative group (8,191 ± 4,097).

Table 1 Proliferative response of separated lymphocytes

Donor	Cell type	Antigen	Proliferative response (cpm)			
			0	GpI	Whole rye	PWM
R.S.	non – T		708	918	1,091	9,951
	T		452	3,143	6,382	33,644
		PBL	861	35,115	26,555	16,951
S.L.	non – T		577	956	1,053	5,525
	T		650	3,124	1,638	6,389
		PBL	2,089	3,330	4,508	11,556

cell populations proliferated in response to PWM (Table 1).

Assay of culture supernatants for immunoglobulin was carried out for all the rye-positive subjects. Little or no IgG or IgA and about a ten-fold higher level of IgM were produced in response to Group I and whole rye allergens (Table 2). About 50 per cent of these had zero (or undetectable immunoglobulin levels. PWM stimulation gave levels of IgA and IgM about ten-fold higher, and IgG levels about twenty-fold higher than those produced by rye grass allergens. The culture supernatants of three subjects were assayed for total IgE (Table 3). There was a low spontaneous production of IgE which was unaltered by the addition of rye grass allergen or PWM.

4. Correlation between lymphocyte proliferation and skin test reactivity, RAST, total serum IgE and nasal provocation dose (NPD)

All rye-positive subjects had PST wheals greater than or equal to 3 mm in diameter and a NPD less than or equal to 100 μg/ml.

Linear regression analyses were carried out to test for correlation between *in vitro* lymphocyte proliferation and the above parameters. We found no significant correlation between the lymphocyte proliferation response *in vitro* in rye-positive subjects and total serum IgE, RAST score or NPD. However, we found significant correlation between this response and PST reactivity (wheal diameter) with both whole rye allergen ($n = 39$; $r = 0.5$; $p < 0.01$) and the Group I allergen ($n = 39$; $r = 0.52$; $p < 0.01$) (Fig. 5).

Separate cultures were set up (10 of each) from which the supernatants were pooled for assay of immunoglobulin levels. These cultures contained 10% foetal calf serum instead of human AB serum.

5. Separation of B and T cells from peripheral blood

Lymphocytes were separated from blood as previously described. T cells were purified by E-rosetting

Table 2 Immunoglobulin production *in vitro* in response to rye grass allergens

Antigen	Immunoglobulin (ng/ml) produced*		
	IgG	IgM	IgA
PWM	1,094±1,601 (n=31)	5,276±7,971 (n=30)	434±551 (n=39)
GpI allergen	21±59 (n=34)	615±1,120 (n=35)	41±74 (n=36)
Whole rye grass allergen	59±115 (n=36)	632±1,148 (n=37)	68±192 (n=37)

*Difference between immunoglobulin concentration in presence and absence of antigen.
n = number of rye grass-positive subjects assayed.

Table 3 IgE production *in vitro* in response to rye grass allergens

Subject	Cells	IgE (ng/ml)		
		0	rye	PWM
A.C.	PBL	0.45	0.30	0.25
H.S.	PBL	0.46	0.30	1.07
R.C.	PBL	0.56	0.51	0.66
	non – T	0.93	0.72	0.94
	T	0.08	< 0.06	0.08

with papain-treated sheep red blood cells (SRBC) and subsequent separation on Ficoll-hypaque.[22] In the T-lymphocyte population, more than 95 per cent were E-rosette-positive and less than one per cent were surface-immunoglobulin-positive. In the non-T cell population, 90 per cent were surface-immunoglobulin-positive and less than two per cent were E-rosette-positive; 85-90 per cent of the E-rosette positive cells were OKT_3-positive.

6. Radioimmunoassays

Serum rye grass-specific IgE was measured by the Nalebuff modified RAST assay.[23] Total serum and culture supernatant IgE was measured by Phadebas IgE PRIST[24] (Pharmacia Diagnostics, Sweden). For assay of IgE in culture supernatants, the sensitivity of the assay

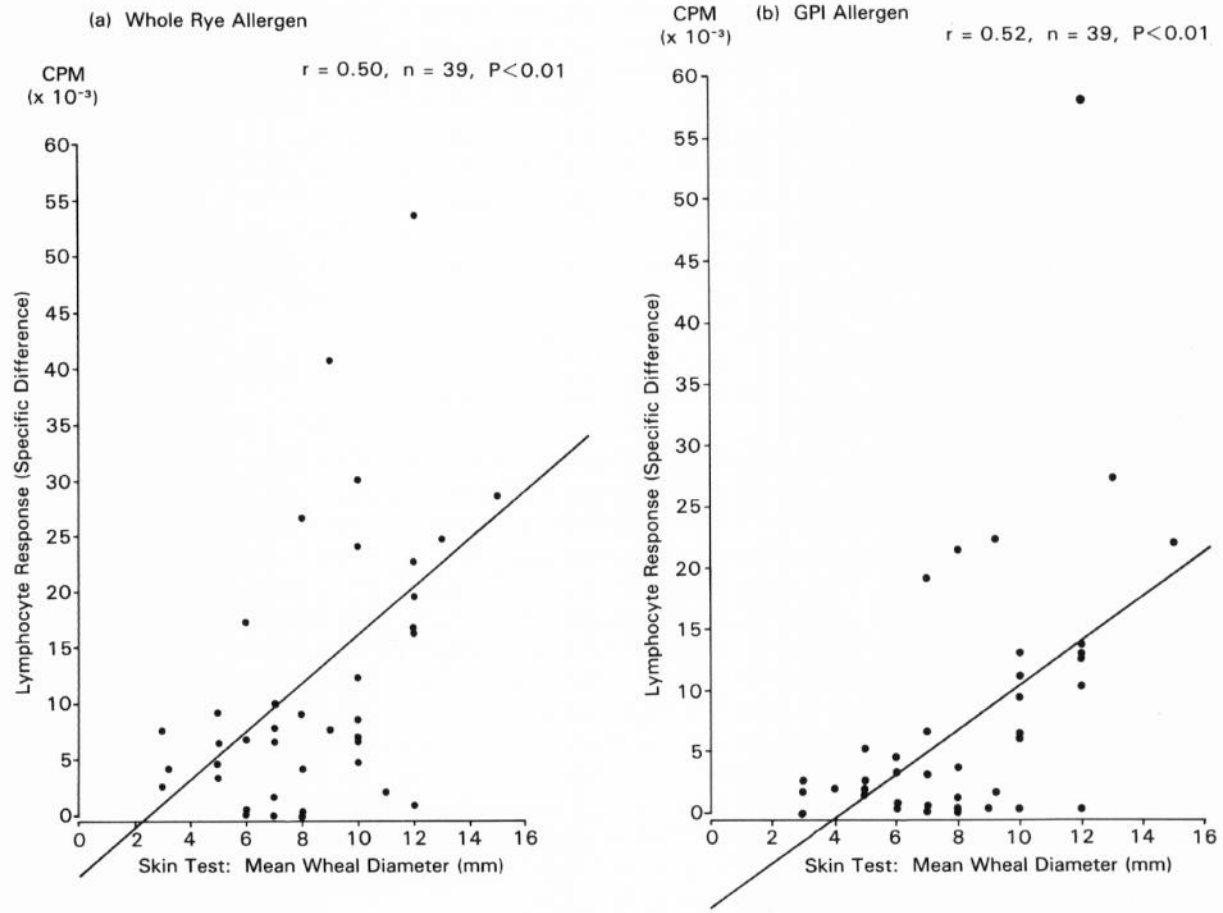

Fig. 5 Lymphocyte stimulation by rye grass allergen plotted against skin test. Linear regression analyses were carried out to test for correlation between lymphocyte proliferation and skin test reactivity. A significant correlation was found for both whole rye allergen and Gp I allergen.

was increased to 0.06 ng/ml by extending the incubation times and using radio-labelled anti-IgE of higher specific activity (Phageras RAST, Pharmacia Diagnostics, Sweden). The performance characteristics of this assay were similar to those observed in the unmodified IgE PRIST. Total IgG, IgM and IgA in culture supernatants were assayed by a solid-phase radioimmunoassay.[25]

7. Statistical analysis

Where appropriate, the data have been presented as the mean and standard error of the mean. Comparisons were evaluated, using the Student 't' test.

DISCUSSION

This study demonstrates a significant association between rye grass antigen-induced lymphocyte stimulation and immediate hypersensitivity to that allergen, and identifies the predominant responding cell as a T lymphocyte. These results were obtained using both crude rye grass extract and purified Group I allergen in carefully documented patients who were not taking therapy, and who had been studied pre-seasonally.

A quantitative correlation was found between the uptake of ^{3}H-thymidine by rye grass-stimulated lymphocytes and the wheal diameter following skin testing, though no such correlation could be demonstrated between the degree of lymphocyte stimulation and total serum IgE, specific IgE as detected by the RAST score, or the threshold concentration of rye grass antigen giving a positive nasal provocation test. A similar quantitative correlation with skin testing was demonstrated by Black and Marsh[15] for ragweed antigen E; they were also able to correlate lymphocyte response to the total serum IgE level. Their results, however, differed from ours in that they were unable to correlate lymphocyte stimulation with skin test positivity to rye grass Group I allergen.[15] The relatively low level of lymphocyte stimulation by rye grass antigens in patients, studied by Black and Marsh, may relate to previous hyposensitisation therapy,[12,14] to some differences in methodology of culture or skin testing, variation in antigens, or differences in the seasonal status of the patients at the time of testing. In general, our results support the findings of other studies of *in vitro* lymphocyte proliferation to allergens,[7,11,12,14,15] which demonstrated specific T-cell sensitisation in atopic subjects.

Our failure to demonstrate a correlation between lymphocyte responsiveness and serum IgE level indicates that the degree of lymphocyte response to rye grass antigens does not quantitatively reflect the atopic status of an individual. The failure of lymphocytes from most skin-test-negative subjects to respond to rye grass antigens supports a specific response for ragweed antigen E, rather than a polyclonal mitogenic effect of allergen as has been reported by some[13] but not all authors.[11,12,15] The proliferative effect of rye grass antigen in some non-atopic subjects does not deny specificity, as rye antigen is inhaled by all; moreover, the IgE response happens to be that particular balance of the immune response that characterises the atopic subject.

A dominant T-cell response to rye grass antigens was demonstrated by the failure to demonstrate significant levels of immunoglobulin or rye grass antibody in culture supernatants, and the restriction of proliferative response to the T-cell-enriched fraction. These results are supported by others who have failed to detect immunoglobulin in culture supernatants[7] though one group did claim to detect IgE production.[8] In general, T cells respond better than do B cells to soluble antigens including ragweed antigen E.[16,17]

The significance of our demonstration of a specific T-cell response to the inhaled allergen rye grass (essentially restricted to atopics demonstrating the same skin test specificity) is uncertain. Given that non-atopics have a similar exposure to rye grass yet usually do not develop a significant T-cell response, a broad based defect within mucosa-related lymphoid tissue may exist in the control of the immune response to mucosally presented antigen in atopic subject. T lymphocytes activated within the respiratory tract mucosa of man, may have a preferential intermucosal circulation, much as appears to occur in studies on animals.[26] Analysis of surface markers and the study of tissue localisation of T lymphocytes responding to rye grass antigen should better characterise the T-cell response to inhaled antigen in atopic subjects. Alternatively, the pronounced response of T cells may reflect the T-helper function, on which the IgE response is so dependent.[27-31] In this regard, it is of interest that hyposensitisation induces suppressor T cells capable of diminishing the T-cell response to allergen.[32] Further studies on the class and function of allergen-sensitive T cells are needed to answer these questions and to determine whether T-cell function tests have clinical value.

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