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Grass Immunotherapy Induces Inhibition of Allergen-Specific Human Peripheral Blood Mononuclear Cell Proliferation

Key Words

Grass allergy
 Immunotherapy
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 Peripheral blood mononuclear cells
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Abstract

The peripheral blood mononuclear cells (PBMC) from humans allergic to grass pollens (GR+ subjects) show strong in vitro proliferative responses to purified allergens from *Lolium perenne* pollen Lol p 1, and to a lesser extent to Lol p 2 and Lol p 3. By contrast, PBMC from grass allergic patients undergoing immunotherapy (GR+IT subjects) exhibit a very poor Lol p-specific proliferative response, similar to that observed in nongrass allergic subjects (GR– subjects). Unlike GR– subjects, both GR+ and GR+IT subjects have high levels of antigen-specific serum IgG and IgE antibodies to Lol p 1, Lol p 2 and Lol p 3. While GR+ subjects exhibit a significant correlation between antigen-specific serum antibody and PBMC responses, GR+IT subjects do not show a correlation between the two responses. The possible mechanisms by which immunotherapy may modulate allergen-specific T cell proliferative response are discussed.

Introduction

Besides allergen avoidance and pharmacological intervention, the currently available therapeutic treatment for allergy involves repeated parenteral injection of allergen extracts [1, 2]. Preseasonal immunotherapy (IT) with pollens has been shown to significantly reduce spontaneous and allergen-stimulated histamine-releasing factor by mononuclear cells [3], asthma in children [4], late-phase skin reactions to ragweed [5, 6], and increases in inflammatory mediators and eosinophil migration into the nasal cavity following nasal provocation [7–9]. Specific IT in ragweed-sensitive patients decreased antigen-specific lymphocyte responses, decreased antigen-specific serum IgE and increased antigen-specific serum IgG [10]. However, the role of antigen-specific serum IgE and IgG antibodies in the clinical outcome of IT is not completely understood [11–

16]. IT has been shown to modulate the immune system in a variety of ways including induction of allergen-specific suppressor T cells [17, 18], generation of autoanti-idiotypic antibodies [19–21] suppression of IgE synthesis by IgE-binding factors [14] and changes in cytokine profile in allergen-specific T cells [22].

Although the perennial rye grass, *Lolium perenne* (Lol p) is one of the major grass species causing pollen allergy in humans [16], information on T cell responses to purified grass allergens is limited [23, 29–32]. To our knowledge, there has been no report on the effect of grass immunotherapy on the in vitro lymphocyte responses to purified Lol p allergens and their correlation with serum antibody responses. Three allergenic proteins from this grass pollen have been well characterized, namely Lol p 1, Lol p 2 and Lol p 3 [23–25]. Previous studies have shown that humans allergic to grass pollen have serum antibodies against Lol p

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proteins and suggested a possible association with certain HLA phenotypes [26–28]. In the present study, we have investigated the in vitro peripheral blood mononuclear cells (PBMC) proliferative responses to purified grass allergens Lol p 1, Lol p 2 and Lol p 3, and compared with the respective serum antibody responses in three groups of human subjects: those who are not allergic to grass pollen (GR–), those who are allergic to grass pollen but *not* on immunotherapy (GR+), and those who are allergic to grass pollen and undergoing IT for ≥ 2 years for grass pollen allergy (GR+IT). The results show that unlike GR– subjects, GR+ subjects exhibit significant Lol p-specific PBMC-proliferative responses which are concordant with corresponding Lol p-specific IgG and IgE serum antibody responses. By contrast, GR+IT subjects exhibit very poor Lol p-specific PBMC-proliferative responses despite higher levels of Lol p-specific serum antibody responses. Thus there is a significant correlation between the antigen-specific proliferative and antibody responses in GR+ subjects and a loss of such correlation in GR+IT subjects.

Materials and Methods

Subjects

Twenty-nine human subjects, ages between 25 and 52, were studied, and included 3 Asian Indians (in the GR+ group), 1 African American, 1 Chinese and 1 Japanese (in the GR– group); the remainder were Caucasians. The GR– and GR+ groups contained 5 males and 5 females each. The 9 GR+IT subjects included 4 males and 5 females from the Johns Hopkins Asthma and Allergy Center clinic. They have been under IT with timothy (*Phleum pratense*) and orchard (*Dactylis glomerata*) grass, which have extensive cross-reaction with the rye grass (*L. perenne*), for 2 years or more. They have also received other allergens according to their sensitivity. The GR+ and GR+IT subjects had 2 years or more of grass season (May and June) hay fever symptoms and were positive to one or more grass pollen extracts by skin test or radioallergosorbent test. The study was conducted during the months of July and August. This study was approved by the Joint Committee on Clinical Investigation of the Johns Hopkins University and each subject gave a written informed consent before participation.

Antigens

The grass pollen extracts employed to treat the GR+IT subjects were obtained in a lyophilized form (Greer Drug and Chemical Co., Lenoir, N.C.) and were standardized according to the US Food and Drug Administration in allergy units. The GR+IT subjects were on maintenance doses every 2 or 3 weeks. The Lol p 1, Lol p 2 and Lol p 3 proteins were purified from an aqueous extract of Lol p pollen by methods previously described [32, 33]. Each protein was homogeneous as shown by agarose gel electrophoresis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, isoelectric focussing and immunoelectrophoresis using hyperimmune goat antisera to Lol p pollen extract [33].

Lol p-Specific PBMC Proliferation

PBMC were isolated from heparinized blood by gradient centrifugation on Histopaque-1077 (Sigma Chemical Co., St. Louis, Mo.) as described [34]. After three washes with Dulbecco's phosphate-buffered saline (PBS; pH 7.2), the PBMC were resuspended to a concentration of 5×10^6 /ml in the culture medium RPMI-1640 supplemented with 5% human AB+ serum (heat-inactivated), 2 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 µg/ml gentamycin (GIBCO, Grand Island, N.Y.) and 50 µM 2-mercaptoethanol (Sigma Chemical Co., St. Louis, Mo.). PBMC (5×10^5) were cultured in triplicate at 200 µl/well in 96-well microtiter plates in the absence or presence of different concentrations of Lol p antigens at 37°C and 5% CO₂ in air. Five days later, 1 µCi/well [methyl-³H] thymidine (Dupont/New England Nuclear, Boston, Mass.) was added and its incorporation into DNA was measured after 16–18 h by liquid scintillation counter (Beckman, model LS 5000TD). The uptake of [methyl-³H] thymidine was used as an index of PBMC proliferative response to the Lol p antigens. The PBMC response to 5 µg/ml phytohemagglutinin (PHA-P, Sigma Chemical Co., St. Louis, Mo.) was measured as described above, with the exception that [methyl-³H] thymidine was added on day 2.

The PBMC response was determined in the antigen dose range of 0.2–25 µg/ml for the purified Lol p 1, 2 and 3 allergens. Maximum response was observed at 25 µg/ml Lol p 1, 2 and at 5 µg/ml Lol p 3. The PBMC response is depicted as Δ cpm and stimulation index (SI):

Δ cpm = cpm in the presence of antigen – cpm in the absence of antigen

SI = cpm in the presence of antigen/cpm in the absence of antigen.

Lol p-Specific Serum IgG

Lol p-specific serum IgG antibody was measured by a double antibody radioimmunoassay as described [32, 33]. Briefly, Lol p 1, Lol p 2, and Lol p 3 were radiolabeled with ¹²⁵I (Amersham, Arlington Heights, Ill.) by the chloramine T method [35]. The specific activities were 4.5×10^4 , 15.23×10^4 and 9.1×10^4 cpm/ng for iodinated-Lol p 1, 2, and 3, respectively. Serum and ¹²⁵I-Lol p 1, 2 or 3 were incubated overnight at 23°C. Polyclonal goat antihuman IgG was then added to precipitate IgG. After three washes with PBS (0.3 M, pH 7.2), bound ¹²⁵I was quantitated in a CAP-RIA 16 gamma counter (Capintec Instruments, Pittsburgh, Pa.). All determinations were performed in triplicate and each serum was analyzed in 2–4 assays. Each assay contained a standard serum with a known concentration of antigen-specific antibody. The concentration of specific antibody in the test sera were computed using a cubic spline-fit radioimmunoassay programme (Johns Hopkins University) and expressed in ng/ml.

Total and Lol p-Specific Serum IgE

Total serum IgE was measured using the IMX system according to the manufacturer's instructions (Abbott Laboratories, Abbott Park, Ill.). Lol p 1-, 2- and 3-specific IgE antibodies were quantified by solid phase radioimmunoassay [36]. Briefly, ¹²⁵I-Lol p 1, 2 or 3 (0.1 ml, 150,000 cpm) was incubated with various dilutions of rye grass-specific antibody-containing serum (0.1 ml, 1:1 to 1:10) in a manner similar to IgG antibody assay. After an overnight incubation, sepharose-bound mouse IgM monoclonal antihuman IgE Fc antibody (clone HP 6061, Hybridoma Reagent Laboratory, Baltimore, Md.) was added (0.5 ml, 3% v/v) to bind all IgE in the reaction mixture. After orbital rotation for 12–16 h at 23°C, unbound proteins were removed by four washes with PBS-1% BSA-0.05% Tween 20 buffer, and bound radioactivity was counted in a CAP-RIA 16 gamma counter. Multidilution dose-

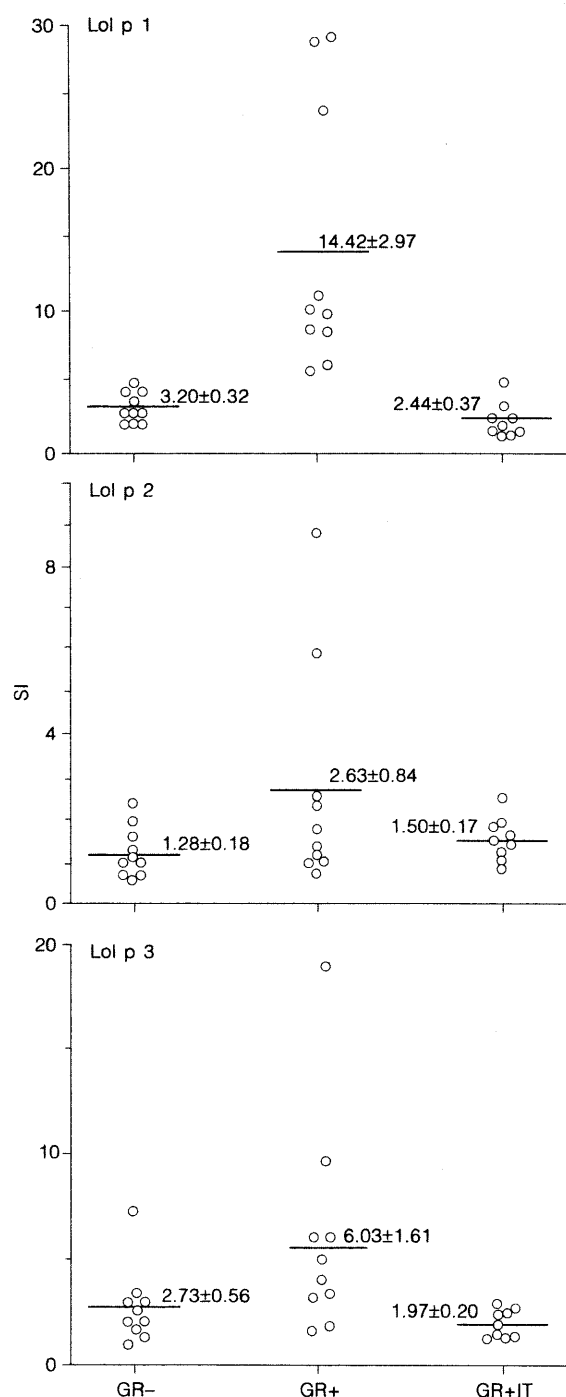


Fig. 1. Lol p-specific PBMC responses in GR-, GR+ and GR+IT subjects. The stimulation indices in PBMC response to the purified antigens, Lol p 1, Lol p 2, Lol p 3, are compared between GR-, GR+ and GR+IT subjects. Each group had 9–10 subjects as represented by the individual data points, some of which are superimposed, and hence not seen. The mean $\pm$ SE values are shown next to the small horizontal line.

response curves, each with a separate IgE anti-Lol p 1, Lol p 2 or Lol p 3 antibody-positive serum, were used to interpolate bound cpm into ng/ml IgE antibody in the test sera as described for the IgG assay.

Statistics

The serum antibody and PBMC responses to Lol p antigens in different groups were compared by parametric analysis using log serum antibody concentration and either Δ cpm or SI of the PBMC responses. The values depicted are arithmetic mean $\pm$ standard error (SE). Significance in the responses between groups was calculated by the Student two-tailed t test.

Results

Immunotherapy Abrogates PBMC Responses to Purified Lol p Antigens

It was of our interest to study the effect of grass IT on antigen-specific proliferative response. PBMC in vitro proliferative responses to purified Lol p antigens were investigated in GR-, GR+ and GR+IT subjects. PBMC from individual subjects were cultured with different amounts of purified Lol p 1, Lol p 2, Lol p 3 or a crude extract of Lol p pollen. Although the crude Lol p extract induced moderate response in PBMC from GR- subjects [data not shown], the purified Lol p antigens did not stimulate significant response in these subjects. By contrast, PBMC from GR+ subjects exhibited a significant dose-dependent response to Lol p extract, Lol p 1 and Lol p 3, and a relatively weak response to Lol p 2. The PBMC response to Lol p antigens in GR+IT subjects was similar to that observed in GR- subjects. Figure 1 compares the SI of PBMC responses in all the subjects in each group. The mean SI for Lol p 1 response in GR-, GR+ and GR+IT subjects was 3.2 ± 0.3 , 14.4 ± 3.0 and 2.4 ± 0.4 , respectively. Similarly, for Lol p 3 the values were 2.7 ± 0.6 , 6.0 ± 1.6 , and 2.0 ± 0.2 , respectively. The PBMC-proliferative responses to Lol p 1 ($p < 0.001$) and Lol p 3 ($p < 0.025$) were significantly higher in GR+ subjects than the responses in GR- subjects. Such an increase was not observed in GR+IT subjects. The responses to Lol p 2 were relatively low in all the three groups: 1.3 ± 0.2 (GR-), 2.6 ± 0.8 (GR+) and 1.5 ± 0.2 (GR+IT), and were not significantly different from each other ($p > 0.1$).

To determine whether the decreased Lol p-specific proliferative responses in GR+IT subjects is due to the inability of their PBMC to proliferate in vitro, we compared mitogen (PHA)-induced proliferative responses in all the three subject groups. The mean SI for PHA-induced proliferative responses were 99.3 ± 46.4 (GR-), 79.6 ± 27.7 (GR+) and 25.3 ± 9.6 (GR+IT) [data not shown]. Although the PHA response in GR+IT subjects was significantly lower when compared to GR- subjects ($p < 0.01$), the values were not

Table 1. Serum antibody responses to Lol p antigens

Group	IgG, ng/ml			IgE, ng/ml		
	Lol p 1	Lol p 2	Lol p 3	Lol p 1	Lol p 2	Lol p 3
GR-	29±5	6±1	9±1	0.4±0.2	0.6±0.2	1.4±0.5
GR+	1,491±1,004	348±173	1,715±1,172	28±11	45±20	77±42
GR+IT	4,295±1,230	1,025±406	1,842±723	19±12	69±50	29±24

Each group had 10 subjects and the values are the mean ± SE. Due to the use of different standard sera for each allergen, sensitivity of the radioimmunoassay varied for different allergens. For IgG antibodies, the sensitivities were Lol p 1, 9.3 ng/ml; Lol p 2, 4.3 ng/ml, and Lol p 3, 6.6 ng/ml. For IgE antibodies, the sensitivities were Lol p 1, 0.1 ng/ml; Lol p 2, 0.2 ng/ml, and Lol p 3, 0.9 ng/ml. Serum samples showing an undetectable antibody level were assigned an antibody level equal to the sensitivity of the corresponding assay. Thus, specific IgE level ≤ 0.9 ng/ml and specific IgG level ≤ 9.3 ng/ml cannot be considered significantly different.

statistically different between GR+ and GR+IT groups ($p > 0.1$). However, it should be noted that the response to PHA varied greatly between individuals (evident from large SE of the means) within each group, and the PHA response in many individuals overlapped among the three groups. Furthermore, a comparison of PBMC responses to PHA and Lol p within individual subjects revealed no direct correlation between the two responses. Nonetheless, these results indicate that IT resulted in a more pronounced decrease in antigen-specific PBMC proliferation *in vitro* in GR+IT subjects and this may not be a result of nonspecific dampening of PBMC responsiveness.

Serum Antibody Responses to Lol p Antigens

To compare the serum antibody response in GR-, GR+ and GR+IT subjects, we measured the total and Lol p-specific serum IgE and IgG levels. Total serum IgE levels were, in ng/ml, as follows: GR- subjects 32 ± 9 (range 3–85), GR+ subjects 338 ± 93 (range 56–811), and GR+IT subjects 591 ± 193 (range 2–1,914). Although both GR+ and GR+IT subjects had a higher amount of total serum IgE than GR- subjects, there was no significant difference in total serum IgE levels between GR+ and GR+IT subjects ($p > 0.2$). The presence of very low, but detectable levels of specific IgG in GR- subjects is not uncommon in nonallergic individuals (table 1). Whether this is a reflection of nonspecific binding of radiolabelled Lol p antigens with natural IgG or an immunologically specific response is not clear. On the other hand, GR+ and GR+IT subjects had 2- to 50-fold higher amounts of Lol p-specific IgG and IgE antibodies than GR- subjects (table 1, fig. 2). GR+IT subjects exhibit a slightly higher ($p > 0.05$) level of Lol p 1 and 2-specific IgG antibodies, but not Lol p 3, than GR+ subjects (table 1). Both GR+ and GR+IT subjects showed higher amounts of antigen-spe-

cific serum IgE antibody levels than GR- subjects, but there was no significant difference in the levels of antigen-specific IgE between GR+ and GR+IT subjects (table 1).

Loss of Correlation between Serum Antibody and PBMC Responses in GR+IT Subjects

In order to assess the relationship between antigen-specific serum antibody and PBMC proliferative responses, we analyzed the coefficient of correlations in the concordant occurrence of these two responses. Figure 2 shows the relationship between Lol p 1-specific serum IgG and IgE antibody responses and Lol p 1-specific PBMC-proliferative responses in the three groups of subjects. The GR+ subjects exhibit a significant correlation between these two parameters ($R = 0.84$, $p = 0.0025$ with IgE, and $R = 0.78$, $p = 0.007$ with IgG). By contrast, similar analysis of Lol p 1-specific responses in GR+IT subjects did not exhibit significant correlation ($R = 0.19$, $p = 0.6$ with IgE, and $R = 0.08$, $p = 0.8$ with IgG), indicating a loss of relationship between these two responses. Similar results are obtained when the log values of antigen-specific serum antibody concentrations were compared with the antigen-specific PBMC-proliferative responses (cpm and SI) to all the three Lol p antigens [data not shown].

Discussion

The present study compares PBMC and serum antibody responses to purified, well-characterized protein allergens from *L. perenne* pollen in three groups of human subjects: GR-, GR+ and GR+IT. The low PBMC-proliferative responses and serum IgG and IgE antibody responses to purified Lol p antigens in GR- subjects (fig. 1) agree with their

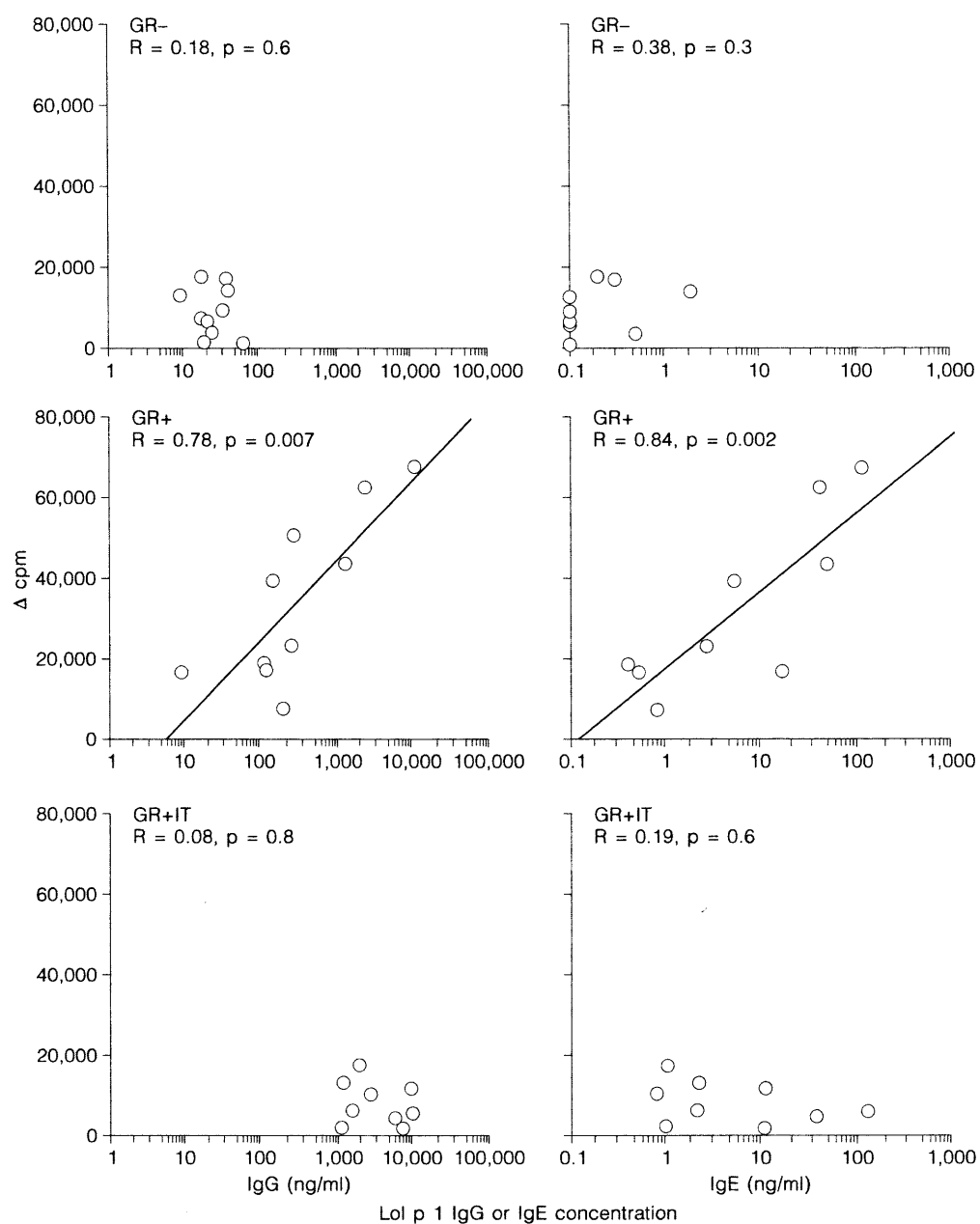


Fig. 2. Relationship between Lol p 1-specific PBMC and antibody responses. The Lol p 1-specific serum IgG and IgE antibody levels are compared with Lol p 1-specific PBMC proliferative responses in GR-, GR+ and GR+IT subjects. A significant correlation between these responses is seen only in GR+ subjects, as indicated by the regression line.

negative skin reactivity for grass allergens [data not shown]. The moderate PBMC response to Lol p extract observed in GR- subjects could be due to some mitogenic substance present in the crude pollen extract and/or cross-reactivity with some uncharacterized allergen to which they have been

sensitized. By contrast, the GR+ subjects exhibited significant dose-dependent Lol p-specific PBMC-proliferative responses. The magnitude of antigen-specific PBMC-proliferative responses fell in the following order: Lol p 1 > Lol p 3 > Lol p 2, and a similar pattern of proliferative responses

was observed in Lol p-specific T cell clones [31]. The elevated levels of Lol p-specific serum IgG and IgE antibody responses in the GR+ subjects (table 1, fig. 2) confirms previous studies [32]. Thus, the present study extends the previous observations which demonstrated antigen-specific serum antibody and PBMC-proliferative responses in patients allergic to short ragweed [10, 37], dust mite [38] and hen's egg or cow's milk [39].

Unlike the GR+ subjects, the GR+IT subjects exhibited very poor Lol p-specific proliferative responses (fig. 1). That the decreased PBMC responses to Lol p antigens in GR+IT subjects is influenced by the seasonal allergen exposure is unlikely because the study was performed during off-season for grass pollen allergy and there were no significant levels of spontaneous proliferation in all the subjects in this study. Several other possibilities could be speculated to play a role in causing this defect.

First, it is possible that the observed decrease in allergen-specific proliferation in GR+IT PBMC is due to a lack of appropriate major histocompatibility complex (MHC) class II molecule to present Lol p antigens to specific T cells in GR+IT subjects. However, such an MHC constraint seems unlikely for the following reasons. (1) All the GR+IT subjects have significant levels of serum IgG antibody to Lol p antigens (table 1), suggesting the presence and activation of Lol p-specific type 2 helper cells that were necessary to induce serum antibody response [22]. (2) Previous studies have shown a strong association between antibody responses to Lol p antigens and certain MHC class II molecules in grass allergic individuals; HLA-DR3, -DR5, -DR6, -DR11 and -DR52 [20, 26–28], and GR+IT subjects in the present study have one or more of these 'responder-type' MHC class II molecules [data not shown].

Second, the decreased proliferation of GR+IT PBMC to antigen stimulation in vitro could be a result of regulation of T cell activation by antigen-specific suppressor cells [17, 18] or due to the generation of anti-idiotypic antibodies [19–21]. Whether the elevated levels of Lol p-specific serum IgG in GR+IT subjects will have any such regulatory effects is not known.

Third, the prolonged treatment (IT) with a mixture of allergen extracts might have interfered with the pathways leading to antigen-specific T cell stimulation. It is possible that such a T cell nonresponsiveness was induced and maintained by the repeated injection of allergen extract (IT) in the GR+IT subjects. In this case it will be analogous to high (antigen) dose T cell tolerance requiring a certain level of antigen concentration in circulation, provided by the maintenance dose of allergen during IT. Two lines of evidence support this view. (1) The Lol p-specific PBMC response in

GR+ subjects is dampened during the peak pollen season compared to off-season response [Baskar, unpubl. observation]. (2) While we were readily able to derive Lol p-specific T cell clones from GR+ subjects [31], we were unsuccessful in our attempts to derive Lol p-specific T cell clones from several GR+IT subjects [Baskar, unpubl. observation].

Fourth, it remains to be known whether repeated antigen administration will interfere with the antigen-presenting functions of the specialized cells such as macrophages. Preliminary experiments show that exogenous addition of interleukin 1 (IL-1), a macrophage-derived cytokine, to GR+IT PBMC cultures stimulated significant proliferation response to Lol p 1 [Baskar, unpubl. observation]. This would suggest that the poor Lol p-specific proliferation in GR+IT PBMC in vitro may be due to the lack of a sufficient level of IL-1, and IL-1 has been shown to play a regulatory role in T cell activation [40].

In any event, the reduced allergen-specific proliferative responses in GR+IT subjects resulted in a loss of correlation between antigen-specific PBMC proliferation and serum antibody responses in these subjects (fig. 2). The presence of Lol p-specific serum antibodies in GR+IT subjects, despite a very low T cell response seen in vitro, remains unexplained. Additional experiments on the qualitative and quantitative differences in the cytokine responses in GR+ and GR+IT subjects upon antigen stimulation, and a comparison of these responses before and after IT will provide further insights into molecular mechanisms involved in grass allergy, and may also aid in the design of better immunotherapy strategies.

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