

Correlation between lymphocyte responses and immediate hypersensitivity to purified allergens*

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Seventy-nine unrelated subjects were selected for high allergic sensitivity to ragweed and/or grass pollens. Sensitivities to ragweed antigens E and Ra5 and rye grass group I were measured by intradermal skin testing and by histamine release in vitro. Lymphocyte responses were determined by incorporation of ^3H -thymidine in vitro in the presence of antigen. Skin-test and histamine-release sensitivities were significantly correlated with lymphocyte response in the case of antigen E, but not for group I and Ra5. In addition, lymphocyte response to antigen E was significantly correlated with total serum IgE level. Six antigen E-sensitive individuals had marked delayed, but no immediate, reactions to Ra5 and almost all showed lymphocyte responses to Ra5. We conclude that immediate sensitivity and lymphocyte responses of allergic individuals to antigen E are significantly correlated, and that lymphocyte responses and delayed reaction to an antigen without immediate sensitivity or IgG antibody are possible even in allergic subjects.

The role of cellular immunity (delayed hypersensitivity) in immediate hypersensitivity reactions has remained unclear despite many studies of possible relationships. While delayed skin reactivity has been used classically to establish the presence of cellular immunity, it has often been difficult to demonstrate such delayed reactions in hay fever patients. Some investigators^{1,2} have reported the occasional incidence of delayed skin reactions in allergic patients after conventional skin testing, and other investigators³⁻⁵ detected delayed reactions only with unconventional in-

jection procedures. However, others^{6,7} did not observe delayed reactions. To add further confusion, reports of late skin reactions (about 6 hr after injection) have also appeared, with the late reactions attributed to Arthus reactions⁸ and to IgE-mediated reactions.^{9,10}

Antigen-induced lymphocyte responses in vitro have often been considered as reflections of cellular immunity,¹¹ and provide another approach to the problem of assessing the relationship between cell-mediated immunity and immediate hypersensitivity responses. Several different assays of lymphocyte responses in vitro have been used; these include blast transformation,^{5,12-14} proliferation as measured by incorporation of ^3H -thymidine,^{7,11-15} and production of lymphokines.^{2,4,5,7} Furthermore, it has been shown that subpopulations of human lymphocytes enriched in thymus-derived lymphocytes (T cells) will proliferate in response to soluble antigens in vitro, while subpopulations enriched in bone marrow-derived lymphocytes (B cells) will not.¹⁶ Similar observations on the subpopulations of allergic individuals' lymphocytes proliferating in response to ragweed antigen E have been reported.¹⁷

Thus, it seems that lymphocytes, probably T cells, from allergic individuals will respond to pollen allergens in vitro. Therefore, in the present studies we sought to determine if a correlation exists between specific IgE-mediated sensitivity to highly purified al-

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TABLE I. Reproducibility of lymphocyte proliferative response to antigen E in 18 sensitive donors*

Comparison	T	p
Specific difference in experiment 1 vs Specific difference in experiment 2	73	0.30

T = Wilcoxon signed-ranks statistic.

*Donors were tested on two occasions, at least 2 wk apart.

lergens, as measured by quantitative intradermal skin testing or leukocyte histamine release, and lymphocyte proliferative response to the allergens. We also examined the possibility of correlation between total serum IgE level and lymphocyte response.

MATERIALS AND METHODS

Patients

Seventy-nine unrelated white subjects involved in clinical and immunologic studies of allergic diseases at this institution were chosen for high sensitivity to ragweed and/or grass pollen allergens.^{18, 19*} All patients had definite clinical histories of seasonal allergic rhinitis (hay fever) with or without asthma, and all gave positive intradermal skin-test reactions (see below) to ragweed and/or rye grass pollen allergens. At the time of the study, 12 patients (15%) were receiving conventional immunotherapy with both aqueous ragweed and grass pollen extracts, 30 (38%) with ragweed pollen extract only, 2 (3%) with grass pollen extract only, and 35 (44%) were not receiving immunotherapy. Those receiving therapy had been on treatment during the past 1 to 3 yr.

Antigens

Antigens purified from short ragweed (*Ambrosia elatior*) and rye grass (*Lolium perenne*) pollens were used in the present study. Ragweed antigen E²⁰ for skin testing and histamine release experiments and antigen E for the lymphocyte assays were supplied by Dr. T. P. King, Rockefeller University, and Dr. John Nutter, Research Resources Branch, National Institute of Allergy and Infectious Diseases, respectively. Ragweed allergen Ra5²¹ was kindly provided by Dr. Lawrence Goodfriend, McGill University. Rye group I was prepared in our laboratory by previously published procedures.^{22, 23} Each antigen possessed a similar high degree of immunologic and electrophoretic purity to the preparations described in the cited literature. Single precipitin lines were obtained for each antigen by double diffusion in agar gel with hyperimmune antisera raised against the purified antigens, as well as against whole ragweed pollen extract (for antigen E and Ra5) and against

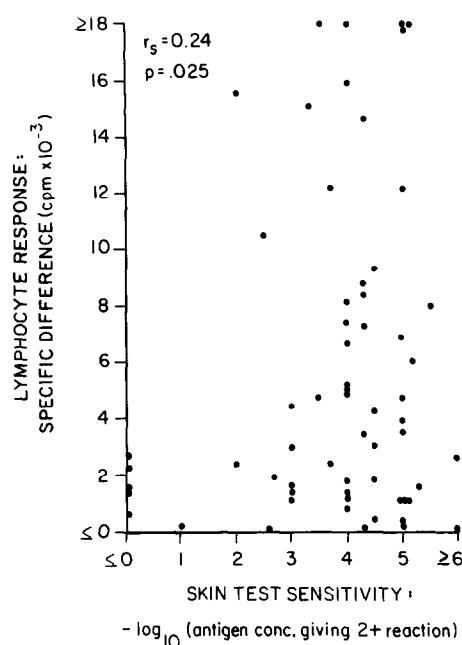


FIG. 1. Lymphocyte proliferative response (specific difference) plotted against skin-test sensitivity to antigen E in 69 allergic subjects. r_s = Spearman rank correlation coefficient. When individuals with negative or weak skin tests (2+ end points at $\geq 10^{-2}$ $\mu\text{g/ml}$) were excluded, $r_s = 0.21$, $0.025 < p < 0.05$ ($N = 63$). When individuals currently receiving immunotherapy with ragweed were excluded, $r_s = 0.35$, $0.025 < p < 0.05$ ($N = 30$). For treated individuals, $r_s = 0.04$, $p > 0.20$ ($N = 39$).

whole grass pollen extract (for group I). All three antigens differ widely in chemical composition and molecular size and show no immunologic cross-reactivity.^{23, 24}

Skin-test and histamine-release assays

Immediate skin reactivity was assayed by intradermal skin-test titration.¹⁸ Skin-test results were expressed as the antigen concentration giving a 2-plus reaction, defined as 8- to 10-mm wheal (with erythema) 15 to 20 min after injection. Data from in vitro leukocyte histamine-release experiments^{25, 26} were kindly provided by Dr. Lawrence Lichtenstein. Results were expressed as the allergen concentration causing release of 50% of total cellular histamine.

Radioimmunoassays

Total IgE levels were determined by competitive double-antibody radioimmunoassay.^{27, 28}

Serum IgG antibody to Ra5 was measured by a highly sensitive double-antibody radioimmunoassay²⁹ with modifications.³⁰ Arbitrary units of antibody per milliliter of patients' sera were obtained by comparison with a standard antigen-binding curve utilizing serum from a treated patient having a high IgG antibody titer to Ra5. These arbitrary units were converted into approximate nanograms antibody nitrogen per milliliter (ng AbN/ml) by studying the

*Note that not all patients were studied with all allergens (see figure legends for numbers of patients).

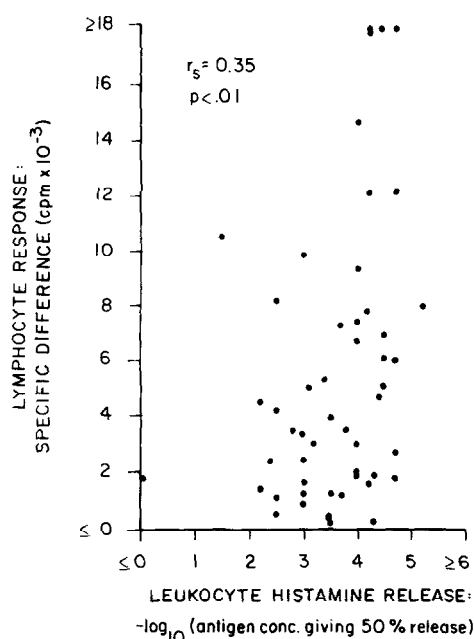


FIG. 2. Lymphocyte proliferative response plotted against leukocyte histamine-release sensitivity to antigen E in 51 allergic subjects. When individuals with negative or weak leukocyte histamine release (50% histamine-release end points at $\geq 10^{-2}$ $\mu\text{g/ml}$) were excluded, $r_s = 0.40$, $p < 0.005$ ($N = 49$). When individuals currently receiving immunotherapy with ragweed were excluded, $r_s = 0.37$, $0.05 < p < 0.10$ ($N = 14$). For treated individuals, $r_s = 0.27$, $p = 0.05$ ($N = 37$).

antigen-binding characteristics of the standard serum at a series of antigen concentrations, assuming that, in antigen excess, all antigen is in the form of Ag_2Ab complexes.³¹

Lymphocyte proliferative responses

Lymphocyte proliferative response assays were performed as described previously.³⁰ Briefly, lymphocytes were separated from heparinized whole blood by centrifugation in Ficoll-Hypaque. Washed cells were suspended at a concentration of 2 to $3 \times 10^6/\text{ml}$ in RMPI-1640 medium (Microbiological Associates, Rockville, Md.) containing L-glutamine (2 mM), penicillin-streptomycin (50 U/ml and 50 $\mu\text{g/ml}$, respectively) and 10% human AB serum from a single nonallergic donor possessing no detectable IgG antibodies to the antigens used. One milliliter of the cell suspension was cultured for 6 days with or without antigen in each round-bottomed 12×75 mm polystyrene tube (No. 2054, Falcon Plastics, Oxnard, Calif.). ^3H -Thymidine (1 μCi), specific activity = 1.9 Ci/mM (Schwartz-Mann, Orangeburg, N. Y.), was added to each tube 18 to 24 hr before harvesting. All cultures were performed at least in duplicate, usually in triplicate.

Antigens were used in the cultures at final concentrations of 10 $\mu\text{g/ml}$ except for rye group I, which was used at 15 $\mu\text{g/ml}$. Preliminary experiments in several allergic subjects showed that these antigen concentrations were optimal for

TABLE II. Comparison of the magnitude of lymphocyte responses to ragweed antigen E, rye grass group I, and ragweed Ra5

Patients	Antigen	Numbers of patients	Median specific difference (cpm)
All tested individuals	AgE	77	3,517
	Group I	44	1,245
	Ra5	42	636
Individuals with	AgE	64	4,677
skin-test sensitivity end points at $\leq 10^{-2}$ $\mu\text{g/ml}$	Group I	24	2,007
	Ra5	12	1,747

most individuals tested.³⁰ Higher concentrations (100 $\mu\text{g/ml}$) of Ra5 were found to give slightly better responses in a few cases, but the amount of time and effort involved in the preparation of highly purified pollen allergens precludes their routine use in such high concentrations. Antigen-dose responses have also been considered in detail in conjunction with a separate study³² using Microtiter plates (Linbro Scientific, Hamden, Conn.). In all tested donors, fairly broad dose-response curves were seen, with no large differences over a range of 10 to 100 μg Ag/ml. At antigen concentrations below 10 $\mu\text{g/ml}$ or above 100 $\mu\text{g/ml}$, responses decreased. In particular, higher antigen concentrations (≥ 200 $\mu\text{g/ml}$) caused a sharp drop in the response.

Negative control tubes contained no antigen. Positive control tubes containing streptokinase-streptodornase (SK-SD) mixture (25 U/ml) were included in most experiments. The same lot of SK-SD (Varidase; Lederle Labs., Pearl River, N. Y.) was used throughout. Lymphocyte proliferative response was expressed as the specific difference, defined as counts per minute in antigen-stimulated cells minus counts per minute in control cells. Student's *t* test was employed to assess the significance of the differences.

RESULTS

Reproducibility of lymphocyte proliferative responses

An important consideration when examining the relationships between various assays is their reproducibility. Clearly, one cannot meaningfully compare assays if they vary greatly from time to time. To check the reproducibility of the lymphocyte proliferative response assay, we compared the results from experiments in which the same donor was tested with antigen E on two different occasions. Eighteen donors were tested on two different occasions, with at least 2 wk between the first and second experiments. Results for the two experiments were compared by the Wilcoxon signed-ranks test.³³ As seen in Table I, the results from one experiment did not differ significantly from the results of the second experiment in

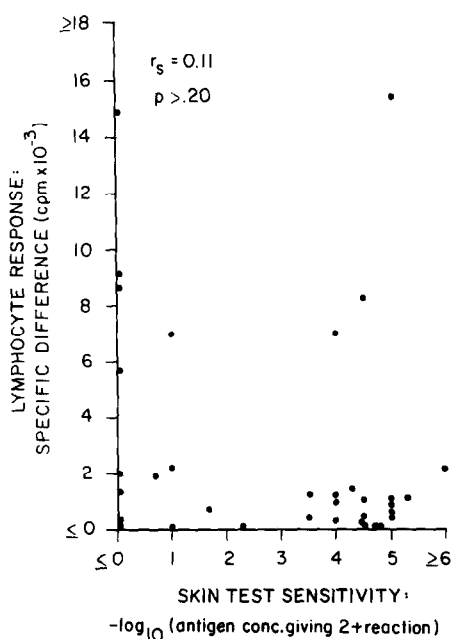


FIG. 3. Lymphocyte proliferative response plotted against skin-test sensitivity to group I in 39 allergic subjects. When individuals with negative or weak skin tests (2+ end points $\geq 10^{-2}$ $\mu\text{g/ml}$) were excluded, $r_s = 0.15$, $p > 0.20$ ($N = 24$). When individuals currently receiving immunotherapy with grass were excluded, $r_s = 0.03$, $p > 0.20$ ($N = 32$). For treated individuals, $r_s = 0.20$, $p > 0.20$ ($N = 7$). For lymphocyte response vs leukocyte histamine release, $r_s = 0.40$, $0.05 < p < 0.10$ ($N = 15$) (data not shown).

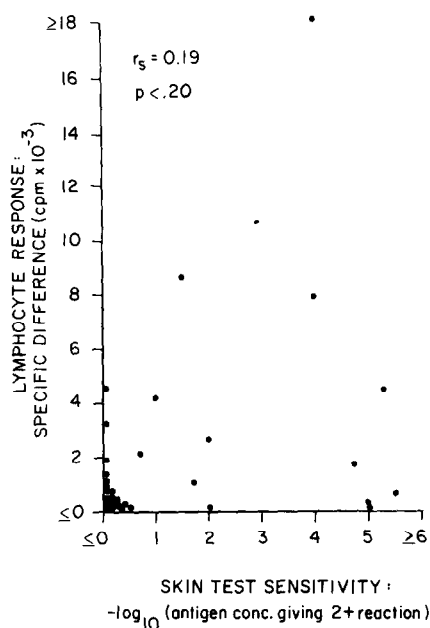


FIG. 4. Lymphocyte proliferative response plotted against skin-test sensitivity to Ra5 in 34 allergic subjects. When individuals with negative or weak skin tests (2+ end points $\geq 10^{-2}$ $\mu\text{g/ml}$) were excluded, $r_s = 0.13$, $p > 0.20$ ($N = 12$). When individuals currently receiving immunotherapy with ragweed were excluded, $r_s = 0.49$, $0.01 < p < 0.025$ ($N = 19$). For treated individuals, $r_s = 0.03$, $p > 0.20$ ($N = 15$). For lymphocyte response vs leukocyte histamine release, $r_s = 1.00$, $p < 0.05$ ($N = 3$) (data not shown).

the same donor. Previous experiments have shown that intradermal skin-test end points can be reproduced to within $\pm 0.5 \log_{10}$ (allergen concentration) in individuals tested over periods as long as 3 yr, even in patients receiving immunotherapy.¹⁸ Leukocyte histamine release shows a higher degree of reproducibility, except for patients on therapy, in which sensitivity is sometimes decreased.³⁴

Correlation between lymphocyte proliferative responses and specific IgE-mediated sensitivities

Almost all tested subjects responded to SK-SD, the positive control antigen. In Fig. 1, lymphocyte proliferative response (counts per minute in test minus control cultures) is plotted against skin-test sensitivity to antigen E. There is a weak but significant trend for the lymphocyte response to increase with increasing skin-test sensitivity ($r_s = 0.24$, $p = 0.025$). The results for lymphocyte response compared with leukocyte histamine release in vitro (Fig. 2) were similar ($r_s = 0.35$, $p < 0.01$). In the case of rye group I and Ra5, lymphocyte responses did not show significant correlations with either skin-test or histamine-release

TABLE III. Effect of immunotherapy on lymphocyte response to antigen E

Patients	Number	Median specific difference
Currently untreated	28	3,015
Currently treated with ragweed	42	5,139
		$p = 0.259^*$

*Values of p determined by Mann-Whitney U test.

sensitivities, except in the case of histamine release to Ra5 in which only three subjects were studied and in the case of skin test to Ra5 among untreated subjects (see legend to Fig. 4). The respective skin-test correlations, in which more data were available, are illustrated in Figs. 3 and 4. Immunotherapy had no large or consistent effect on the magnitude of the correlations, as shown in the legends of the individual figures. The overall magnitude of lymphocyte responses induced by antigen E, rye group I, and Ra5 in specifically allergic subjects was in the order of $\text{AgE} > \text{group I} > \text{Ra5}$ (Table II).

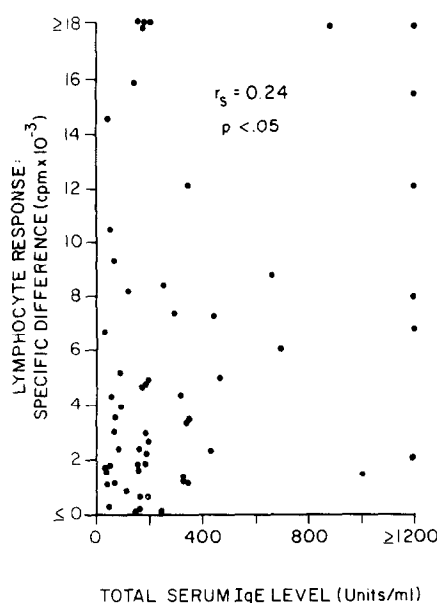


FIG. 5. Lymphocyte proliferative response induced by antigen E plotted against total serum IgE level in 67 allergic subjects. When individuals with negative or weak skin-test sensitivity ($2+$ end points $\geq 10^{-2}$ $\mu\text{g/ml}$) were excluded, results were similar, with $r_s = 0.24$, $0.025 < p < 0.05$ ($N = 64$). When individuals receiving immunotherapy with ragweed were excluded, $r_s = 0.20$, $0.10 < p < 0.20$ ($N = 28$). For treated individuals, $r_s = 0.27$, $p = 0.05$ ($N = 39$).

Correlation between lymphocyte responses and total serum IgE levels

We next considered the possibility that lymphocyte proliferative response may reflect some overall immune regulatory mechanism. It has been suggested by Willcox and Marsh³⁵ that an individual's overall ability to mount IgE antibody responses, reflected in the total basal IgE level, is controlled by an IgE-regulator gene that has been defined in two studies.^{28, 36} Since this gene may exert some generalized regulatory effect on immune function, we attempted to find a correlation between antigen-induced lymphocyte response and total serum IgE level. Antigen E was chosen for the analysis because responses to it were more widespread and of greater magnitude (see above) in our study subjects. The possible effects of putative *I*r genes are likely to be less in the case of antigen E than with the other antigens studied.¹⁹

There was a weak but significant correlation between lymphocyte response to antigen E and total serum IgE level ($r_s = 0.24$; $p < 0.05$; Fig. 5). When weakly sensitive individuals (skin sensitivities at $\geq 10^{-2}$ $\mu\text{g/ml}$) or subjects on immunotherapy were excluded from the analysis, the results were similar (see legend to Fig. 5). Skin-test sensitivity to anti-

gen E was not correlated with total IgE level ($r_s = 0.09$; $p > 0.20$).

Effect of immunotherapy on lymphocyte responses

Individuals currently receiving immunotherapy with ragweed extract had a higher median lymphocyte proliferative response to ragweed antigen E than did untreated individuals (Table III), but these differences were not significant ($p = 0.259$).

Delayed skin reactions in the absence of immediate reactions

Although we did not set out to study the question, it came to our attention that six individuals sensitive to ragweed reported marked delayed but no immediate skin reactions to the minor ragweed allergen Ra5 (Table IV). The delayed reactions consisted of greater than 2-cm-diameter swelling, usually with induration, in response to the highest doses of injected antigen (0.05 ml of 1 or 0.1 $\mu\text{g/ml}$). Reactions usually began at 12 to 16 hr and continued until about 48 hr after injection. Four of the six (J. A., C. S., J. S., and S. W.) had little or no detectable serum IgG antibody to Ra5 (≤ 1.7 ng AbN/ml). The other two (M. F. and S. G.), who were receiving immunotherapy with ragweed, had small amounts of IgG antibody. All subjects tested, except C. S., had significant lymphocyte responses to Ra5 ($p < 0.05$). The level of significance of these data was comparable to that seen in other experiments in which the magnitude of the response was similar. In other experiments in which the magnitude of the response was greater, the level of significance was generally higher ($p < 0.01$).

In addition, one individual (R. G.), who was sensitive to grass but not ragweed pollen, had a delayed skin reaction and a lymphocyte response in the absence of an immediate skin reaction to antigen E. These seven patients tended to have total serum IgE levels lower than most allergic individuals. Several patients besides R. G. showed selective sensitivities. C. S., while sensitive to grass, was only weakly sensitive to ragweed, trees, and dust. S. W. and S. G. were sensitive to ragweed and grass, and J. A. had multiple sensitivities. Thus, as a group, these seven seemed somewhat atypical of most allergic patients in that they tended to have selective sensitivities and lower total serum IgE levels.

DISCUSSION

The relationship between delayed hypersensitivity and immediate allergic reactions is not clear despite many studies of this problem. The present study is the

TABLE IV. Allergic individuals with weak or negative immediate skin reactivity to an antigen, but a marked delayed reaction

Subject	Current ragweed therapy	Total serum IgE (U/ml)	Antigen conc. tested (μg/ml)*	Skin reaction		Serum IgG antibody (ng AbN/ml)	Lymphocyte response	
				Immediate	Delayed		Stimulation index†	Specific difference
Antigen Ra5								
J. A.	—	176	1	±	+	<1.5	2.80	4,148
C. S.	—	55	1	—	+	1.7	1.00	0
J. S.	—	187	1	±	+	<1.5	2.07	3,327
S. W.	—	89	1	—	+	<1.5	2.42	1,337
M. F.	+	43	0.1	±	+	4.8	1.50	4,580
S. G.	+	158	1	—	+	53.7		ND
Antigen E								
R. G.	—	195	1	—	+	ND	1.96	2,244

ND = not determined.

All lymphocyte responses were measured in triplicate cultures and were significantly different from control ($p < 0.05$), except for C. S.

*0.05 ml injected.

†Stimulation index = counts per minute of ^3H -thymidine incorporated by antigen-stimulated cells divided by counts per minute of control cells without antigen.

first comprehensive investigation of highly allergic individuals to demonstrate that lymphocyte proliferative response induced by ragweed antigen E is correlated with specific IgE-mediated sensitivity (Figs. 1 and 2). Unlike a previous study,⁷ skin tests were performed by a semiquantitative and reproducible intradermal titration technique.¹⁸ Previous workers⁷ skin tested with crude rather than purified allergens, and it is impossible to determine which specific components of the mixture were causing a response. Such uncertainty confuses the analysis, and any correlations between assays would be reduced as a consequence. Therefore, the apparent discrepancy between the two studies is not altogether unexpected. Another interesting finding was that lymphocyte response to antigen E was correlated with total serum IgE level (Fig. 5). In addition, we examined the reproducibility of lymphocyte proliferative responses in the same donor tested on successive occasions with antigen E. We found that the results of one experiment did not differ significantly from the results of a successive experiment in the same patient (Table I). This question has been considered in greater detail elsewhere.³²

The correlations between lymphocyte proliferative responses to antigen E and both skin-test and histamine-release sensitivities (Figs. 1 and 2) are not striking ($r_s = 0.24$, $p = 0.025$, and $r_s = 0.35$, $p < 0.01$, respectively), but they are reasonable in view of the problems inherent in the lymphocyte-response assay. The correlations were significant at the $p < 0.05$ level for antigen E but not for group I or Ra5 (Figs. 3 and 4), both of which were, on average, much weaker

stimulators of lymphocyte proliferation than antigen E (Table II). Perhaps the lack of correlation for group I and Ra5 reflects their weaker potency.

It should be emphasized that our studies were performed only in highly allergic subjects. One might expect a reduction in the correlations had nonallergic individuals been included, due to an occasional incidence of proliferative responses to pollen allergens, as reported in our previous study.³⁰ Using different conditions and durations of culture, Buckley et al.³⁷ have reported that *all* normal individuals' lymphocytes (and even cells from cord blood) will proliferate in response to antigen E. We have not seen this apparent mitogenic activity using our purified antigen E. Occasional proliferative responses to antigen E by nonallergic individuals are not unexpected, since some individuals have the genetic capacity to respond to antigen E without expressing IgE-mediated sensitivity.³⁰ Thus, we see such responses as a *selective* rather than a universal phenomenon.

We also found that the antigen E-induced lymphocyte responses are weakly but significantly correlated with total serum IgE level ($r_s = 0.24$, $p < 0.05$; Fig. 5). However, skin-test sensitivity to antigen E was not correlated with total IgE. Thus, one cannot argue that the correlation between lymphocyte response and total IgE is an indirect reflection of a correlation between skin-test sensitivity and total IgE. These data are consistent with the view that a regulatory gene for IgE may exert a generalized immunoregulatory effect (see Results), but do not prove the point.

Allergic individuals currently on ragweed hypo-

sensitization therapy had somewhat higher lymphocyte responses to ragweed antigen E than did similar, untreated individuals, although this difference was not significant (Table III). It must be emphasized that the two groups were not matched, so that one cannot draw firm conclusions from these data. Conflicting reports about the effect of immunotherapy on lymphocyte responses have appeared previously. Some groups,^{3, 4} using emulsified antigen for immunization, have found increases in lymphocyte responses after therapy; others immunizing with aqueous antigen (as in the present studies), have found decreases^{17, 38} or no change.^{2, 12, 37} This whole question therefore remains unresolved and awaits more carefully controlled experiments.

An additional set of observations was quite surprising. Six ragweed-allergic individuals reported delayed skin reactions to Ra5 after having had weak or negative immediate skin reactions. The delayed skin reactions, all greater than 2-cm-diameter swelling, usually with induration, were maximal at 12 to 16 hr after injection and had faded away by 48 hr. It should be noted that the delayed reactions were induced by relatively high doses of antigen (0.05 μ g in most cases; see Table IV) routinely used as the upper test level. All the untreated subjects had little or no detectable serum IgG antibody to Ra5 (≤ 1.7 ng AbN/ml). All but one of those tested *did* have a lymphocyte response to Ra5. While these responses were not very strong, they were similar to those of individuals sensitive to Ra5 (Fig. 4; Table II).

The exact nature of these delayed skin reactions is unclear. They are not of the Arthus type, mediated by antigen-antibody complexes with the involvement of neutrophils and the complement system, as suggested by Pepys et al.,⁸ because most individuals had no detectable serum IgG antibody to Ra5. These delayed reactions are also not IgE-mediated, because immediate reactions to Ra5 were not seen in these otherwise allergic individuals. Our observations thus differ from reports^{9, 10} of IgE-mediated "late" skin reactions with peak responses at 6 to 12 hr—reports that emphasize that immediate reactions of sufficient intensity are absolute prerequisites for their delayed reactions. Although Solley et al.¹⁰ established elegantly that IgE-mediated reactions initiate a delayed-onset inflammatory process, it is not clear whether the delayed reaction was itself *directly* mediated by IgE or was the indirect result of the prerequisite immediate IgE-induced large wheal-and-flare response. In allergic subjects, there is also the further problem of uncertainties due to the use of crude allergenic extracts, where one component could be inducing the immediate and another the delayed response.

Our results do demonstrate clearly that it is possible, even for allergic individuals, to have a delayed skin reaction and positive lymphocyte proliferative response to an antigen without a significant IgE-mediated skin sensitivity or serum IgG antibody to the antigen. The presence of lymphocyte proliferative responses in most of these individuals is consistent with the possibility that these reactions are cell mediated, although we have no direct proof.

We wish to express our thanks to Drs. L. M. Lichtenstein and P. S. Norman for making their patients available for this study and for providing leukocyte histamine release and some skin-test data. We also wish to thank Drs. T. P. King and J. Nutter for supplying ragweed antigen E, and Dr. L. Goodfriend for ragweed antigen Ra5. We express our gratitude to Dr. G. J. Delespesse, who provided invaluable assistance in the formative stages of this work in helping set up the lymphocyte proliferation assay. We are also indebted to E. Siekierski and E. Weathers for expert technical assistance.

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