

Lymphocyte proliferation to antigen E: Demonstration of the restriction of antigen E-specific T cells to ragweed-allergic donors

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Allergic sensitivity to ragweed is common among atopic individuals in North America and can be associated with symptoms of seasonal hay fever and increased airway reactivity in asthma. This sensitivity is mediated by IgE antibody to ragweed antigens that in turn is presumed to be the product of B-lymphocytes regulated by various T cell subsets. Proliferation in vitro by lymphocytes obtained from individuals allergic to ragweed and cultured in the presence of ragweed antigen E (AgE) has been repeatedly described, but a comprehensive study of this proliferation has questioned the specificity of this response. We have examined this question and found that in the first week of culture, the specific lymphocyte proliferation to AgE may be obscured by high background and mitogen-like proliferation. However, by carrying the cells for a longer period of time in culture and providing a second in vitro boost with AgE, specific proliferation could be clearly documented. Lymphocytes from atopic ragweed-allergic donors proliferated at levels 20 to 50 times beyond background in the presence of AgE. Cells from nonragweed-allergic donors (either nonatopic or atopic) did not do so. The AgE-responsive cells could be expanded in culture and demonstrated to be T cells. Moreover, AgE-responsive T cells could only be cloned from AgE-allergic donors and, after expansion and subcloning, demonstrated to respond to AgE but not partially purified dust mite antigen. In contrast, a clone of T cells from a dust mite-sensitive individual proliferated in response to the dust mite antigen but not AgE. (J ALLERGY CLIN IMMUNOL 1987;79:933-40.)

The modern understanding of the allergic response owes much to the description of IgE antibody.¹ Since that time, increasing attention has been devoted to the cellular events responsible for the production of IgE^{2,3} and to that which may underlie the atopic state. In animal models, it has been demonstrated that B cell production of IgE is T cell dependent^{4,5} and can be modified by suppressor T cells.⁶ A complex

Abbreviations used

AgE: Antigen E
IL-2: Interleukin-2
PBM: Peripheral blood mononuclear
BSA: Bovine serum albumin
HSA: Human serum albumin
APC: Antigen-presenting cells

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series of regulatory factors has been proposed to mediate these T cell effects,⁷ leading to either enhancement or suppression of IgE production.^{8,10}

In the human, the regulatory events controlling IgE synthesis are less well understood but are also believed to be T cell mediated.¹⁰⁻¹⁷ Regulatory factors homologous to those in the rodent appear to exist.^{18,19} In vitro proliferation by lymphocytes from atopic donors cultured in the presence of appropriate allergens has been described for the last two decades.²⁰⁻³⁷

Nevertheless, controversy surrounds the proliferative response to AgE, the major allergen in rag-

TABLE I. In vitro proliferation to ragweed AgE: Time course of primary response*

Donor	Antigen (10 µg/ml)	Proliferative response (cpm)		
		Day 5	Day 6	Day 7
Atopic				
J. P.	AgE	4,515	2,571	1,505
	BSA	1,835	2,088	1,832
B. J.	AgE	15,432 ± 4,905	22,205 ± 4,474	ND
	BSA	7,760 ± 2,865	14,784	ND
	HSA	4,168 ± 189	9,042	ND
Nonatopic				
J. E.	AgE	5,142 ± 1,929	7,510 ± 2,203	11,190 ± 2,555
	BSA	3,454 ± 2,419	9,848 ± 2,229	6,762
	HSA	5,869 ± 1,388	7,620 ± 2,568	9,760
B. A.	AgE	2,455	1,519	1,340
	BSA	2,176	587	1,626

*PBM cells (0.5×10^6 cells per well) were cultured for varying numbers of days with 10 µg/ml of AgE and pulsed with isotope 20 to 24 hours before harvesting. Cells were cultured in triplicate (mean cpm ± standard deviation) or duplicate (average cpm). (Cpm in all tables were cpm for the sample minus a cpm blank for the isotope counter.)

weed sensitivity. Several articles have reported specific T cell proliferation to AgE by cells from atopic ragweed-sensitive donors and not by cells from non-atopic donors.^{29, 31-33, 37} In contrast, a study of a comprehensive series of experiments has suggested that proliferation of a similar magnitude occurred with cells from both atopic and nonatopic individuals.³⁴ Similarly, although a recent study demonstrated significantly greater proliferation by cells from ragweed-sensitive individuals to AgE compared to control subjects,³⁷ cells from control individuals also proliferated above background levels in the presence of AgE. However, there appeared to be an increase in the proliferative response during ragweed season, occurring only with cells from sensitive individuals.³⁷

We have explored the issue of antigen specificity by expanding the AgE-responsive cells in vitro with techniques of repeated antigen presentation,³⁸ long-term T cell culture, and cloning.^{17, 39} Our results demonstrate that AgE-specific T cells could be recovered only from ragweed-sensitive individuals. The ability to clearly identify such cells was improved by expanding the cells through two rounds of AgE presentation in vitro before pulsing with isotope. Furthermore, these cells could be expanded into AgE-specific lines and cloned lines.

METHODS

IL-2 source

Purified IL-2 was obtained from Electro-Nucleonics Inc. (Silver Springs, Md.). IL-2 was used at the time of cloning and in the maintenance of cloned cells in culture at a concentration of 1% to 2%.

Media

CRPMI was RPMI 1640 with 10% fetal bovine serum (Gibco, Grand Island, N. Y.), 1% L-glutamine (Gibco), 1% antibiotics/antimycotics (Gibco), and 0.1% 2-mercaptoethanol (5×10^{-5} mol/L, Sigma Chemical Co., St. Louis, Mo.).

Source of lymphocytes and donor characterization

Human PBM cells were isolated by Ficoll-Hypaque centrifugation^{17, 39} from donors who had been skin tested for reactivity to ragweed extract and a variety of other common allergens, including three molds, three pollens, dust, dust mite, and two animal danders (Bencard Ltd., Toronto, Canada). Atopy was assessed by results of skin test, RAST, and patient history. Based on these findings, the donors were then designated as atopic and ragweed allergic, atopic but ragweed nonallergic, or nonatopic and therefore ragweed nonallergic. None of the cell donors had been treated with immunotherapy.

Proliferation assay

PBM cells were isolated as above and washed three times in phosphate-buffered saline, and viable cells were counted by trypan blue exclusion (1:1 dilution). The cells were resuspended in RPMI 1640 supplemented with 5% fetal bovine serum (Gibco), 1% L-glutamine, and 1% penicillin-streptomycin at 5×10^6 cells per milliliter. One hundred microliters of this cell suspension was added per well to tissue culture plates that contained 100 µl of AgE (generally 10 µg/ml) or control proteins (BSA, HSA, or dust mite antigen) (the latter from Pharmacia Diagnostics AB, Uppsala, Sweden) and pulsed at various times with ³H-thymidine (25 µl of 1/25 dilution stock added per well) (Sigma Chemical Co.). Twenty to 24 hours after pulsing, cells were harvested

TABLE II. In vitro proliferation to ragweed AgE: Time course of secondary response*

Donor	Antigen (10 µg/ml)	Proliferative response (cpm)			
		Day 8	Day 10	Day 12	Day 14
Atopic (ragweed sensitive)					
B. J.	AgE	11,462 ± 1,062	15,711 ± 2,101	24,162 ± 3,284	17,109 ± 2,615
	BSA	2,162 ± 741	3,167 ± 1,096	2,611 ± 642	1,849 ± 866
J. K.	AgE	10,321	29,036	67,273	18,262
	BSA	875	2,395	2,357	1,745
Atopic (nonragweed sensitive)					
L. P.	AgE	3,642 ± 976	2,711 ± 471	2,164 ± 941	2,110 ± 463
	BSA	2,116 ± 1,012	3,024 ± 1,629	1,932 ± 260	2,436 ± 523

*Cells were cultured with 10 µg/ml of AgE as described in Table I, but on day 7, 0.1 ml of fresh medium and 10 µg/ml of AgE were added.

TABLE III. In vitro proliferation to ragweed AgE: Proliferation response after varying antigen doses*

Donor	Antigen concentration		Isotope incorporation (cpm)
	Initial (µg/ml)	Boost (µg/ml)	
Atopic (H. M.)	0.00	0.0	435 ± 106
	0.01	10.0	13,227 ± 7,364
	0.10	10.0	15,614 ± 4,035
	1.0	10.0	20,329 ± 3,435
	10.0	10.0	13,674 ± 2,709
	50.0	10.0	10,423 ± 4,023
	100.0	10.0	657 ± 179
Nonatopic (A. G.)	0.00	0.0	295 ± 86
	0.01	10.0	266 ± 96
	0.10	10.0	191 ± 19
	1.0	10.0	302 ± 73
	10.0	10.0	306 ± 63
	50.0	10.0	321 ± 51
	100.0	10.0	368 ± 132

*Cells were cultured in triplicate as described but in varying concentrations of AgE. On day 7, 0.1 ml of fresh medium with 10 µg/ml of AgE was added.

or plates were frozen, and cells were harvested later. Filters were suspended in scintillation fluid and counted in a beta counter, window 0 to 440 (Beckman Ltd., Fullerton, Calif.). Seven days after the initiation of culture, fresh media were added to cells plus 10 µg/ml of AgE or control protein. Similarly, when it was necessary, fresh media were added at days 14 and 21.

Source of allergens

Purified AgE and partially purified dust mite antigen P₁ (from *Dermatophagoides pteronyssinus*) were kindly provided by Drs. S. Allstedt and B. Björkstén, Pharmacia Di-

agnostic AB. The AgE was estimated to be >90% purity, and this was confirmed by electrophoresis on polyacrylamide. The dust mite antigen(s) was still relatively crude. Both were provided in a lyophilized form.

Cloning of reactive cells

Cloning was done by limiting dilution. PBM cells were isolated as above and set up in bulk culture for 2 weeks at 2×10^5 cells per milliliter in 96-well plates with 10 µg/ml of AgE or BSA/HSA control. Fresh antigen was added at day 7. At day 14, cells were resuspended, viable cells were counted and seeded at a concentration of 1000

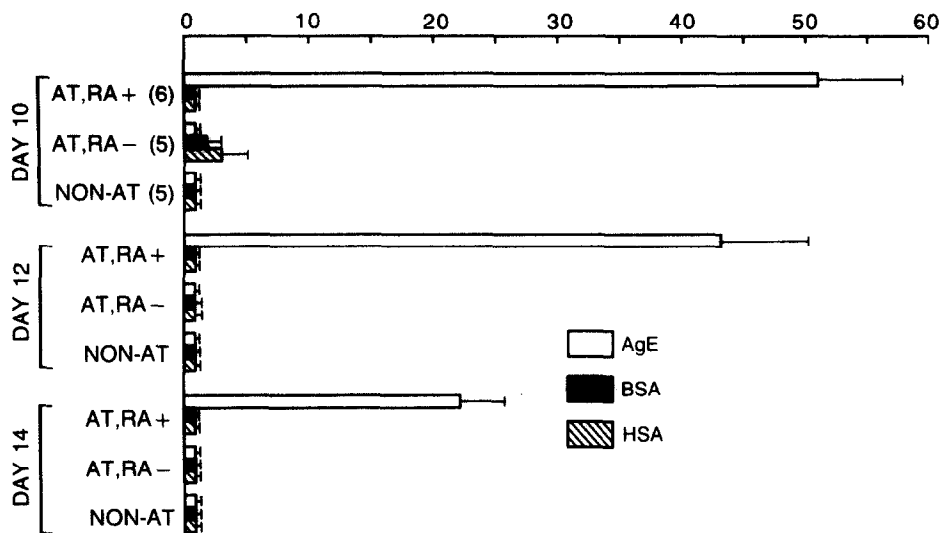


FIG. 1. In vitro proliferation to AgE. Response of cells from atopic and nonatopic donors. Data expressed as mean stimulation index $\pm$ standard error of mean = cpm antigen stimulation/cpm control medium.

cells per well, 100 cells per well, 10 cells per well, and one cell per well in the presence 1% IL-2, 10 μ g/ml of AgE, and irradiated PBM cells from the original donor (3000 R) at a concentration of 10^5 cells per well. Wells were screened at day 4 onward for growth. Seven days after cloning, wells plated at one cell per well had additional IL-2 and antigen added. Cells were expanded in 24-well plates in the presence of irradiated donor PBM cells, 10 μ g/ml of AgE, and 1% to 2% IL-2 and CRPMI. Immunofluorescent staining to characterize the lymphocyte surface phenotype was performed as previously described.¹⁷

Proliferation assay of clones

Cloned cells were counted and plated into 96-well plates at a concentration of 2×10^4 cells per well with 2×10^4 irradiated (3000 rads) PBM cells from the original donor, 10 μ g/ml of AgE or control protein, and CRPMI. Twenty to 24 hours after initiation of assay, wells were pulsed with ^{3} H-thymidine (same as above), and the cells were harvested and counted 18 to 24 hours after pulse.

RESULTS

In vitro lymphocyte proliferation to AgE: Varied length of culture

RBM cells from donors sensitive to ragweed and other donors not sensitive to ragweed were cultured in vitro with antigen for 7 days, and the proliferative response was assessed at several time points by ^{3} H-thymidine incorporation (Table I). Under the conditions of assay, we found that specific proliferation during the first week of culture was often obscured by a high background (Table I, donor B. J.) or poor proliferation (Table I, donor J. P.). Manipulations that

reduced the background did not improve the system, and under some assay conditions, we found a high incidence of apparent proliferation to AgE by cells from nonatopic donors (data not presented).

In contrast, when the cells were held in culture for longer than 1 week, the nonspecific background dropped substantially. Moreover, by adding an additional 10 μ g of AgE to the cultures on day 7 and feeding with fresh complete medium, the specific proliferation to AgE was enhanced (Table II). Specific proliferation from 10 to 20 times background was demonstrated with cells from patients sensitive to ragweed AgE (Table II, donor B. J. and J. K.) but not from nonragweed-sensitive individuals (Table II, donor L. P.).

In vitro lymphocyte proliferation to AgE: Varied dose of antigens

We next examined the effect of varying the concentration of AgE used in the first stimulation while we were keeping the second boost constant at 10 μ g/ml. As can be observed from the results in Table III, specific proliferation was obtained over a wide range of initial antigen doses with the cells from a ragweed-sensitive donor but not with the cells from the nonatopic donor. In this experiment with cells from ragweed-sensitive donor H. M., a stimulation index of 10 to 20 times background was obtained with an initial concentration of AgE varying from 0.1 to 50 μ g of AgE. No proliferation was obtained over the same range of antigen concentrations with cells from nonatopic donor A. G. (Table III). Other experiments

were performed, exploring a range of antigen doses for the second boost, and 10 $\mu\text{g/ml}$ was found to be optimal (data not presented).

Proliferation obtained with multiple donors

To confirm the observation that proliferation only occurred with cells from ragweed-sensitive donors, we tested several donors from three categories: atopic, ragweed sensitive, six donors; atopic, ragweed non-sensitive, five donors; and nonatopic, ragweed non-sensitive, five donors. Proliferation was assessed at three time points (10, 12, and 14 days) after the second antigen boost administered at day 7. The results summarized in Fig. 1 as proliferation indices clearly demonstrate proliferation to AgE by cells from the ragweed-sensitive individuals at all three time points (20 to 50 times background) but not by cells from the two groups of nonsensitive individuals. Proliferation by cells from the three groups of donors did not differ from background in the presence of either HSA or BSA (Fig. 1).

T-lymphocyte nature of the proliferating cell

In order to examine the lineage of the proliferating cells, we established a line from the ragweed-sensitive donor H. M. by repeated stimulation with AgE during several weeks of culture. Viable cells were then expanded with exogenous growth factor alternating with AgE and irradiated autologous PBM cells. Cells from this line were demonstrated to proliferate in response to AgE but only in the presence of irradiated autologous APC (Table IV). AgE in the absence of the APC failed to increase proliferation beyond background levels, whereas the addition of such cells with the AgE increased proliferation to six to eight times background. Culture of the irradiated APC with cells from the line failed to increase proliferation beyond control levels (Table IV).

When these cells were examined for surface phenotype, >90% of the viable cells carried the T cell markers OKT3 and OKT4. None were positive for OKT8. The results suggested that cells proliferating in response to AgE were T cells and that this particular cell line was of the helper phenotype.

Cloning of proliferating cells

As further confirmation that the proliferative response to AgE was specific, we grew cells by seeding them at limiting dilution in the presence of AgE, BSA, or HSA, plus irradiated autologous APC and a source of exogenous growth factor. Wells seeded with one cell were examined daily for 3 weeks, and the wells containing a developing colony were identified (Table V). Colonies developed in the presence of AgE

TABLE IV. In vitro proliferation to ragweed AgE: Cell line

Cells (H. M.)	Irradiated APC (H. M.)	AgE ($\mu\text{g/ml}$)	Proliferation (cpm)
+	—	—	492 $\pm$ 160
+	+	—	507 $\pm$ 290
+	—	1.0	288 $\pm$ 74
+	—	10.0	452 $\pm$ 260
+	—	50.0	366 $\pm$ 133
+	—	100.0	477 $\pm$ 199
+	+	1.0	3704 $\pm$ 761
+	+	10.0	3273 $\pm$ 94
+	+	50.0	3578 $\pm$ 667
+	+	100.0	3256 $\pm$ 474

only when the cells originated from atopic, ragweed-sensitive donors (36 colonies in 72 wells, donor B. J.; 20 colonies in 72 wells, donor J. P.). No colonies developed in AgE with cells from ragweed non-sensitive donors (L. P., J. E., and B. A.; Table V). Moreover, only two wells of 96 wells developed colonies in the presence of BSA with one donor, B. J., but these could not be expanded.

Proliferation to antigen by cloned T cells

The efficiency with which cell colonies formed in the presence of AgE with cells from ragweed-sensitive donors was very high (Table V, 50% efficiency for donor B. J. and just under 30% for donor J. P.). In order to document the antigen specificity of these clones, we attempted to expand the clones and then subclone them to ensure clonality. Five clones were expanded sufficiently to test, and three of these clones (B. J. 4, B. J. 5, and B. J. 15, Table VI) were subcloned at one cell per well twice. As a further control for specificity, a preparation of partially purified dust mite antigen(s) was obtained. Cells from a dust mite-sensitive, ragweed-nonsensitive donor were tested for specific proliferation to mite antigen, and a cloned cell line was successfully established from this donor (Table VI). Each of the five clones obtained by stimulation with AgE proliferated to a greater extent in the presence of AgE than control HSA (Table VI). One of these clones, B. J. 5, was also tested for proliferation in the presence of 10 μg of mite antigen. This clone proliferated 60 times background in the presence of 10 μg of AgE, but only at background levels in the presence of mite antigen. By contrast, the clone L. P. established by stimulation with mite antigen failed to proliferate beyond background levels in the presence of AgE but had a proliferation index 15 times background in the presence of mite antigen.

TABLE V. Cloning efficiency*

	Atopic (RA +)		Atopic (RA -)	Nonatopic (RA -)	
	B. J.	J. P.	L. P.	J. E.	B. A.
AgE	36/72	20/72	0/96	0/96	0/96
BSA	2/96	0/96	0/96	0/96	0/96
HSA	0/96	0/96	0/96	0/96	0/96

RA + = ragweed-sensitive donors; RA - = nonragweed-sensitive donors.

*Cells were seeded at one cell per well with irradiated autologous PBM cells and AgE as described in Methods. Colony development was scored visually by microscopy during the course of 3 weeks after cloning.

TABLE VI. In vitro proliferation to ragweed AgE or dust mite antigen isotope incorporation (cpm)

AgE clones	AgE	Dust mite	HSA
B. J. 5*	60,020 $\pm$ 2,391	1,026 $\pm$ 313	917 $\pm$ 43
B. J. 15*	30,163 $\pm$ 4,292	ND	4,119 $\pm$ 1,093
B. J. 4*	40,196 $\pm$ 3,318	ND	1,162 $\pm$ 101
J. P. 1	60,112 $\pm$ 9,346	ND	15,041 $\pm$ 1,091
J. P. 2	23,164 $\pm$ 6,943	ND	4,293 $\pm$ 2,997
Dust mite antigen			
L. P. (bulk cells)	601 $\pm$ 112	50,106 $\pm$ 1,926	596 $\pm$ 141
L. P. (clone)	1,062 $\pm$ 203	15,691 $\pm$ 460	977 $\pm$ 155

ND = not done.

*Subcloned twice.

DISCUSSION

Ragweed sensitivity is an important human model for investigating and understanding the nature of atopy. The important allergens in crude ragweed pollen have been characterized,^{40, 41} and the major allergen, AgE, has been identified.⁴⁰ Ragweed-pollen sensitization is common in North America. The season is well defined, and the characterization of rising and falling IgE antibody to the major allergen, AgE, is well studied.⁴²⁻⁴⁴ Pollen sensitivity has been demonstrated to be associated with increased airway reactivity among pollen-sensitive individuals with asthma during the pollen season.⁴⁵⁻⁴⁷ Sensitivity to ragweed pollen has been demonstrated to result in considerable immunologic activity during ragweed season including (1) rising IgE antibody titers,⁴²⁻⁴⁴ (2) appearance of circulating B cells that spontaneously secrete IgE in vitro,¹³ and (3) increased allergen-specific T cell proliferation in vitro.³⁷ Thus, increased antiragweed antibody results from T and B cell interactions driven by the allergen. This immunologic activity can be presumed to occur in the airways of allergen-sensitive individuals with asthma, resulting in the activation of a broad spectrum of the immunologic network, including APC, T cells, B cells, and various nonantigen-specific factors, such as interleukin-1 and II-2.¹⁷ Con-

firmation of this hypothesis would broaden the concept of the "allergic" reaction in atopic individuals from simple IgE-initiated mediator release to an active immune response.

The data in this article demonstrate that T cells that proliferated in vitro to AgE could be grown from the atopic donors who were sensitive to ragweed AgE but not from donors who were not allergic to ragweed. Although the specificity of this proliferation was often obscured by high nonspecific background proliferation in the first week of culture (Table I), by culturing the cells for longer time periods and restimulating with AgE and fresh medium on day 7, the specificity of the response became apparent (Tables II and III).

The T cell nature of the responding cells was suggested by expanding and growing a bulk culture of AgE-specific cells into a line carried for several months. This line demonstrated specific proliferation to AgE when irradiated APC were provided and was uniformly of the helper T cell phenotype, OKT4. However, with time in culture, the line became autoreactive, proliferating in response to the restricting HLA-D antigen and promoting immunoglobulin production by B cells of the correct HLA-D determinant.*

*Chambers C, Zimmerman B. Data to be published.

In the present experiments, we have not formally proven the histocompatibility-restricted nature of this APC function; however, Meuer et al.⁴⁸ have described an AgE-specific T cell clone, and in their experiments, they documented the restricted nature of the antigen presentation.

The data presented in this article and data of Meuer et al.⁴⁸ and Rawle et al.⁴⁹ for house dust-mite antigen confirm the work of earlier investigators, suggesting the presence of allergen-specific T cells in donors sensitive to the appropriate allergen.^{21, 22, 24, 29, 31-33, 37}

At first glance, these data appear to conflict with data of Buckley et al.³⁴ who found that in vitro proliferation of lymphocytes in the presence of AgE occurred with cells from both atopic and nonatopic donors, suggesting a mitogen-like activity of the AgE. Indeed, we were led to formally evaluate the specificity of in vitro proliferation to AgE when we found in preliminary experiments that the response occurring in the first 10 days of culture (without the second boost of antigen) was subject to extreme variability* with a third of the nonatopic donor cells demonstrating proliferation to AgE at eight to 10 times background in the first 8 to 10 days of culture. Our strategy, although it was awkward, was to expand the cells over time in vitro to demonstrate specificity of the response. Gatien et al.³¹ described a biphasic peak of proliferative responses to AgE at low and high doses of antigen, but Buckley et al.³⁴ did not observe a biphasic response and generally explored the proliferative response at high antigen doses (50 to 100 µg/ml). We postulated that specificity might be enhanced by use of lower doses of antigen and expanding the more avid cells by repeated antigen presentation. Subsequently, cell lines and cloned cell lines were grown in T cell growth factor and then demonstrated to proliferate in the presence of AgE but not control antigens.

The methods currently available for growing and cloning of allergen-specific T cells should allow a more precise definition of such cells and their function than has been possible in the past, allowing a more precise dissection of regulatory events, including antigen-specific suppressor cells such as described by Rocklin et al.^{29, 35}

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