

Depletion of circulating allergen-specific T_{H2} T lymphocytes after allergen exposure in asthma

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Background: In allergic asthma, CD4⁺ T lymphocytes are a fundamental component of local chronic inflammation. Their cytokine profile is oriented toward a T_{H2} phenotype, characterized by production of IL-4, IL-5, IL-10, and IL-13. Egress of T cells from blood to airways after allergen challenge has been described.

Objective: We have studied a cohort of six patients with asthma who had multiple allergies to investigate how exposure to allergen affects the proliferation of peripheral CD4⁺ T lymphocytes with different allergen specificities and lymphokine profiles.

Methods: For each patient, CD4⁺ T-cell lines were generated by *in vitro* stimulation with sensitizing and with nonsensitizing allergens, and IL-4 and interferon- γ production by these lines was assessed. Proliferation of peripheral blood CD4⁺ T lymphocytes in response to the same allergens was measured before and 24 hours after inhalation challenge with a sensitizing allergen.

Results: We found that each single sensitizing allergen can deplete peripheral blood of T_{H2}-type CD4⁺ T lymphocytes specific for all sensitizing allergens, but not of T_{H1}-type CD4⁺ T lymphocytes.

Conclusions: Our results suggest the existence of mechanisms capable of sorting disease-associated antigen specificities together with defined lymphokine patterns into T lymphocytes that can migrate to target organs, in allergic asthma. (*J Allergy Clin Immunol* 1997;99:788-97.)

Key words: Allergy, bronchial provocation tests, asthma, T_{H2} lymphocytes

In airways of patients with allergic asthma, T_{H2} lymphocytes play a key role in modulating the local inflammatory processes that occur after exposure to allergen, in maintaining bronchial hyperresponsiveness,^{1,2} and in controlling specific IgE production.³

Data from our laboratory⁴ and other laboratories^{5,6} suggest that peripheral blood CD4⁺ T lymphocytes can actively recirculate to the airway lumen in the hours after allergen exposure when the late-phase asthmatic

Abbreviations used

AU:	Arbitrary units
BAL:	Bronchoalveolar lavage
EAR:	Early asthmatic reaction
IFN- γ :	Interferon- γ
LAR:	Late asthmatic reaction
PI:	Proliferation index
PBMCs:	Peripheral blood mononuclear cells
TT:	Tetanus toxoid

reaction develops. We reasoned that migration of antigen-specific T cells to the lungs should correspond to a depletion of the same cell population in blood. In this study we attempted to determine the effect of airway exposure to allergen on allergen-specific peripheral T cells. In particular, we investigated whether the allergen challenge could cause a specific or nonspecific depletion of circulating CD4⁺ T cells in asthmatic subjects with multiple IgE-dependent sensitizations, a common occurrence in the vast majority of allergic conditions. We also investigated the cytokine profile of T cells specific for sensitizing and nonsensitizing allergens in the same cohort of patients, as well as in nonatopic control subjects.

We found that the exposure to allergen causes depletion of circulating CD4⁺ T lymphocytes that are specific not only for the provoking allergen but also for the other allergens to which the subject is sensitized. We found that these T lymphocytes have a T_{H2}-polarized cytokine profile, as expected. In contrast, peripheral CD4⁺ T lymphocytes specific for antigens not involved in the generation of the allergen-dependent bronchospastic response are not depleted after inhalation of the sensitizing allergens. These T cells display a cytokine profile characterized by the prevalent production of interferon- γ (IFN- γ).

METHODS

Subjects

We studied six subjects with allergic asthma who had positive skin prick test (Lofarma, Milan, Italy) and RAST (Pharmacia, Uppsala, Sweden) responses to three or more allergens among the following: grass, *Parietaria*, olive, birch, and *Dermatophagoides pteronyssinus*. Anthropometric characteristics, lung function,⁷ and the profile of allergies are summarized in Table I. Six healthy nonsmoking volunteers (three men and three women) from whom allergen-specific T-cell lines were generated were also included in this study as nonatopic control subjects. The

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Received for publication Aug. 6, 1996; revised Dec. 5, 1996; accepted for publication Dec. 9, 1996.

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0091-6749/97 \$5.00 + 0 1/179719

TABLE 1. Characteristics of subjects

Subject No.	Sex	Age (yr)	Height (cm)	FEV ₁ * (%)	Total IgE (U/ml)	Sensitization†
1	F	48	160	98	66	G,P,B
2	F	27	159	80	164	G,P,O,(B)
3	F	40	161	95	670	G,P,D,(B)
4	F	23	158	75	28	G,P,D
5	M	49	174	108	680	G,P,(D)
6	F	56	170	88	343	G,D,B,(O)

*FEV₁ is expressed as a percent of predicted value.⁷

†Sensitization was defined on the basis of skin test and RAST results: G, grass (*Lolium perenne*); P, *Parietaria*; B, birch (*Betula verrucosa*); O, olive (*Olea europaea*); D, *Dermatophagoides*. Letters in parentheses indicate minor or doubtful sensitization.

mean age of these control subjects was 36 years (range, 20 to 54 years). These subjects had a negative anamnesis for asthma and atopic diseases and negative skin prick test results with a panel of the 12 most common allergens in Italy (*Lolium perenne*, *Parietaria*, *Olea*, *Betula*, *D. pteronyssinus*, *D. farinae*, *Cupressus*, cat dander, dog dander, *Alternaria*, *Cladosporium*, and *Aspergillus*).

All patients had been vaccinated with tetanus toxoid (TT) during childhood and had received a booster dose approximately every 5 years; therefore, as expected, they all displayed a memory antigen-specific T-cell response to this antigen. For this reason, TT was used as an intraassay control, because no modification in the repertoire of TT-specific T cells is expected in the absence of environmental exposure.

Protocol

On the screening day spirometry was performed, and blood was drawn for determination of serum levels of specific IgE and for purification and stimulation of T lymphocytes with sensitizing and nonsensitizing allergens (see below). The polysensitized subjects included in the study were free of symptoms at the time of the study, had not had respiratory infections during the previous 4 weeks, and had not received antiasthmatic treatment other than short-acting, β_2 -stimulants on an as-needed basis. These were discontinued at least 12 hours before challenges. All subjects were informed of the nature and aim of the study and gave written consent.

On the first study day, a control bronchial challenge was performed, and circadian variations of FEV₁ were determined. Peripheral lymphocytes were isolated 10 minutes before and 24 hours after the control challenge. Starting a week later, subjects underwent three bronchial challenges with the sensitizing allergens in a random sequence at 2-week intervals. Ten minutes before and 24 hours after each allergen challenge, blood was drawn into sterile plastic containers containing heparin, and peripheral lymphocytes were isolated.

Media, monoclonal antibodies, and allergens

Complete medium used for cell culture was prepared as follows: RPMI 1640 (PBI, Milan, Italy) was supplemented with gentamicin 5 μ g/ml, glutamine 2 mmol/L (PBI), and 5% normal human serum (Danish Red Cross Laboratories, Utrecht, Holland). Monoclonal antibodies used for purification of CD4⁺ T cells and macrophages were: UCHL-4 (anti-human CD8; IgG2a); BL-LGL/1 (anti-human CD56; IgM); SJ25-C1 (anti-human CD19; IgG1) (Sigma, Milan, Italy). Allergens were kindly provided by Lofarma-Allergeni (Milan, Italy) and consisted of commercial preparations of *Lolium perenne*, *Parietaria officinalis*, *Betula verrucosa*, *Olea europaea*, and *D. pteronyssinus*, the protein concentrations of which were calculated by using a

commercial kit (BioRad Laboratories, Segrate, Italy). TT was purchased from Connaught Laboratories (Willowdale, Ontario, Canada). The *Limulus* amoebocyte gelification test (PBI) was used to assess the presence of bacterial endotoxins in the allergen extracts. The assay was performed after the allergen extracts were reconstituted in culture medium at 1 mg/ml, according to the manufacturer's instructions. Endotoxin levels were 0.01 U/ml, similar to levels found in saline solution.

Preparation of CD4⁺ and CD14⁺ peripheral blood mononuclear cells

A cell preparation containing CD4⁺ T lymphocytes and macrophages (as professional antigen-presenting cells) was obtained from peripheral blood mononuclear cells (PBMCs) by negative immunobead selection with monoclonal antibodies, according to instructions from the manufacturer (Dynabeads; Unipath, Garbagnate, Italy). In brief, cells were incubated (20 minutes on ice) at a cell density of 1×10^7 /ml with normal human serum (10%) to saturate the Fc receptors and then with monoclonal antibodies to CD8, CD56, and CD19 (1 μ g/ 10^6 cells) to remove cytotoxic/suppressor T lymphocytes, natural killer cells, and B lymphocytes, respectively. Goat anti-mouse Ig-coated beads were reacted (20 minutes on ice with rotation) with monoclonal antibody-coated PBMCs; cells rosetting with beads were removed with a magnet. In the resulting cell population, CD4⁺ and CD14⁺ cells together accounted for more than 95% of the cells on analysis with a fluorescence-activated cell sorter (Becton-Dickinson).

Proliferation assay

CD14⁺, CD4⁺ cells were plated (5×10^4 /well) in 200 μ l in a 96-well flat-bottomed plate (Costar, PBI). Each antigen was added for 7 days, at 20 μ g/ml, to quadruplicate microcultures. Controls (cultures without antigen) were included. On day 7, 0.5 μ Ci/well of tritiated thymidine (Dupont, Cologno M, Italy) was added for 6 hours. Cells were then harvested (Skatron, Pharmacia LKB), and tritiated thymidine incorporation was counted with a β -plate counter (Pharmacia LKB). The proliferation index (PI) for each allergen was calculated as the ratio between the mean tritiated thymidine incorporation in test wells and that in control wells. Only basal PI values greater than 2 were retained for statistical analysis.

In vitro assay to test allergen extracts with specific T-cell lines

In preliminary experiments, five allergen-specific T-cell lines were derived from four asthmatic subjects (of the six studied) by using multiple cycles of restimulation with autologous irradiated PBMCs (as a source of antigen-presenting cells) and

pulsing with each of the allergens included in this study. PI values ranging from 3.4 to 11.0 were obtained by using the working concentration (20 $\mu\text{g/ml}$) of antigens in a standard 3-day proliferation assay (not shown). Stimulation of these lines with increasing concentrations of the specific antigen used to generate the line and with the other four nonrelevant antigens was carried out to assess possible cross-stimulation resulting from sharing of T-cell epitopes among the different allergen extracts. Nonrelevant antigens, at working concentrations, yielded PI values ranging from 0.8 to 1.2.

Allergen challenge

Allergens were aerosolized by means of a dosimetric technique. Aerosols were generated by an ampule-dosimeter device (MEFAR, Brescia, Italy). The dosimeter was set to deliver 10 μl of solution for 1 second at the beginning of each inspiration during quiet breathing. The particle median mass diameter ranged from 1.53 to 1.61 μm . The same set of identical ampules was used throughout the study. Allergen challenges were performed by using the same batches of allergen used for in vitro stimulation. Allergen extracts were predosed in arbitrary units (AU) by means of the RAST-inhibition technique against the corresponding in-house serum pools (Lofarma Allergeni) containing specific IgE of high titer. Double decreasing solutions from a stock solution of 10,000 AU/ml to 21 AU/ml were extemporaneously prepared by adding redistilled water to dried allergen. After administration of the saline control (50 breaths), the challenge started with a concentration of 21 AU/ml and continued with doubling allergen concentrations until the FEV_1 , measured 15 minutes after the inhalations, fell below 80% of the postsaline control value. Each concentration was inhaled during 50 quiet breaths, which corresponded to an inhaled volume of 0.5 ml. The maximum concentration of allergen was 2500 AU/ml, corresponding to an inhaled dose of 1250 AU. The FEV_1 was measured with a portable microspirometer (Micro Medical Limited, Rochester, England) 15, 30, and 45 minutes after the end of the inhalation challenge, and then hourly for the next 24 hours, except during sleep, to detect the occurrence of a late-phase asthmatic response. The early asthmatic response (EAR) was expressed as the maximum decrease in FEV_1 recorded within 1 hour after the last inhalation; the late asthmatic response (LAR) was expressed as the maximum decrease in FEV_1 recorded between 4 and 24 hours after the end of challenge. Allergen PD_{20} was also calculated by interpolation in the dose-response curve. It is worth noting that allergen PD_{20} values from different allergen challenges are not comparable because the biologic in vivo activities of AU for different allergens are not equal. Control challenge was performed by having the subjects inhale three series of 50 breaths of saline solution at 15-minute intervals and monitoring FEV_1 as described above. For details see Table II.

Generation of allergen-stimulated T-cell lines for the determination of IL-4 and IFN- γ production

T-cell lines for the determination of the cytokine profile on stimulation with sensitizing and nonsensitizing allergen in atopic subjects and with nonsensitizing allergen in nonatopic individuals were generated as described by Secrist et al.⁸ with some modification. In brief, PBMCs depleted of CD8^+ , CD56^+ , and CD19^+ cells were prepared as described earlier, cultured in 96-well plastic plates (Nunc, PBI, Milan) at $10^6/\text{ml}$ at 37°C , in 5% CO_2 in the presence of the appropriate antigen (see above). On day 7 of culture, cells were washed once with phosphate-buffered saline and recultured with irradiated autologous PBMCs as antigen-presenting cells, fresh antigen, and 10 U/ml

human recombinant IL-2. Between days 7 and 14 the microcultures were expanded as necessary. On day 14 of culture, cells were washed twice in phosphate-buffered saline and resuspended in complete medium at $2 \times 10^6/\text{ml}$ in 96-well plates (200 $\mu\text{l}/\text{well}$) with 10 $\mu\text{g/ml}$ phytohemagglutinin (PHA-P; Sigma) and 1 ng/ml phorbol myristate acetate (Sigma). After 18 hours of culture, supernatants were collected and frozen at -80°C until assayed for cytokine content. For analysis of IL-4 and IFN- γ , an in-house Roche ELISA was used (courtesy of Dr P. Panina, Roche Milano Ricerche, Milan, Italy).

Statistical analysis

Changes in the PI of peripheral CD4^+ T cells (before challenge vs after challenge) in the response to the provoking allergen were assessed by a paired t test. The differences in PI changes between the provoking allergen and each of the nonprovoking allergens and TT were separately tested by two-factor repeated-measures analysis of variance in which the factors were allergen (provoking and nonprovoking) and time (before and after challenge). With this test, the presence of significant interaction indicates that there is a significant difference between the provoking and nonprovoking allergens. The difference between the provoking allergen and the nonprovoking allergen and TT were also tested by analysis of variance on pooled data expressed as a percentage of the basal value. Single regression analysis was used to test correlations between RAST values, basal PI, change of PI, and LAR. Probability (p) values less than 0.05 were accepted as significant.

RESULTS

Results of the proliferation of CD4^+ T lymphocytes purified from peripheral blood in response to allergens that were both sensitizing and nonsensitizing in each individual, as well as to TT, are given in Tables II and III. In Table II individual incorporation values of assays measuring proliferation of CD4^+ T cells to each single antigen of our panel at baseline and after saline or allergen challenge are shown. In Table III the PI of CD4^+ T cells for each allergen is shown as the mean $\pm$ SD of values observed at baseline, that is, before control (saline) challenge and before each allergen challenge.

After control challenge with saline solution, the PI for each antigen expressed as a percentage of the basal value was not significantly changed from baseline (range of the means, 97% to 103%), and the maximum observed difference from the basal value was 16% (Fig. 1). This indicates that the bronchial challenge per se is not inducing changes in the PI of peripheral CD4^+ T cells.

All subjects were sensitized to grass and underwent a challenge with grass allergen (Fig. 2). Five subjects were also sensitized to *Parietaria* and underwent this challenge (Fig. 3). The remaining challenges were for *Dermatophagoides* ($n = 4$), olive ($n = 1$), and birch ($n = 1$). Two challenges with *Dermatophagoides* were discarded because subject 3 received steroid treatment to control a nocturnal LAR, and subject 5 had negative early and late asthmatic reactions. Challenges with olive, birch, and *Dermatophagoides* were grouped and indicated as "others" (Fig. 4). These allergens were also defined as "positive" or "negative," depending on whether they were sensitizing in that subject.

After the grass allergen challenge ($n = 6$), the PI for

TABLE II. Proliferation of CD4⁺ T cells to a panel of antigens at baseline and 24 hours after challenge with saline or sensitizing allergen

In vivo allergen challenge	Antigen used for in vitro proliferation assay						
	G	P	O	B	D	TT	NIL
Subject 1 (▲)*							
Con, baseline	11988	6318	4698	16362	1782	4860	1620
B, baseline	12668	8936	6533	10990	3949	3731	1804
P, baseline	12874	7065	6437	8164	2983	3611	1570
G, baseline	19411	8478	4844	29411	4592	3936	1514
Con, 24 hr	14821	8807	5800	24057	2150	6873	2148
B, 24 hr	3087	5005	5099	3364	2860	2907	1673
P, 24 hr	6392	5155	8248	5979	4330	5979	2062
G, 24 hr	7414	3888	6998	20111	4860	5832	1944
Subject 2 (▼)							
Con, baseline	15479	23123	5733	22358	10892	12803	1911
P, baseline	14450	27316	4807	22820	5733	9172	1911
G, baseline	7702	14546	2974	11130	4408	6280	1102
O, baseline	7586	15696	4054	12686	6670	7978	1308
Con, 24 hr	19078	30670	7486	26082	13041	17145	2415
P, 24 hr	2115	3678	2922	5332	8327	14160	2776
G, 24 hr	3368	5934	2245	8180	5934	9784	1604
O, 24 hr	4381	7706	2870	6195	7101	11107	1511
Subject 3 (●)							
Con, baseline	3374	2584	2154	2225	5097	5026	718
P, baseline	5370	3396	2958	2848	6576	6466	1096
G, baseline	3405	2556	2349	1702	4086	4215	691
Con, 24 hr	4758	3294	2746	3385	6862	5398	915
P, 24 hr	3010	1672	3122	2458	3679	5696	1115
G, 24 hr	1655	2045	2922	2922	3798	6233	974
Subject 4 (■)							
Con, baseline	2589	3393	1408	1524	3676	3585	688
P, baseline	1592	2388	796	835	3104	2149	398
G, baseline	3026	2740	1084	1713	5710	2855	571
D, baseline	3508	4280	1712	1712	8644	5220	856
Con, 24 hr	3001	3740	1615	1840	6320	3841	789
P, 24 hr	1541	924	873	975	2106	3236	513
G, 24 hr	865	1796	1531	1798	3996	3196	666
D, 24 hr	2852	4588	2356	2232	3348	9176	1240
Subject 5 (✦)							
Con, baseline	1284	4722	2450	1846	3070	10040	709
P, baseline	1492	6904	2736	2300	3918	12004	622
G, baseline	1440	5702	2936	1844	2994	10022	576
Con, 24 hr	1502	5915	2841	2200	3920	11740	887
P, 24 hr	1880	1880	2914	2776	5452	17390	940
G, 24 hr	1640	2965	2524	1893	2839	9591	631
Subject 6 (◆)							
Con, baseline	5125	1006	4020	3718	7738	3115	1005
D, baseline	5010	1402	4108	4508	8216	2004	1002
G, baseline	2326	649	2001	2272	4219	919	541
Con, 24 hr	5956	1242	4591	4343	9803	3598	1241
D, 24 hr	4283	1465	3382	2254	4170	2368	1127
G, 24 hr	1972	791	2130	1656	3313	1499	789

Results are expressed in counts per minute. The PI to antigens was obtained from these data as the ratio between antigen-specific incorporation and the respective spontaneous incorporation (NIL).

G, Grass (*Lolium perenne*); P, *Parietaria*; O, olive (*Olea europaea*); B, birch (*Betula verrucosa*); D, *Dermatophagoides pteronyssinus*; NIL, no antigen added; con, control (saline).

*Symbols in parentheses correspond to symbols for individual patients in Figs. 1 to 4.

grass was reduced from 6.1 ± 3.5 to 2.2 ± 0.9 ($p = 0.02$). This change was significantly different from the change for "others negative" (interaction = 0.03) and TT (interaction = 0.05) but not from the change of *Parietaria*

(interaction = 0.94), and "others positive" (interaction = 0.58). When expressed as a percentage of the basal values (Fig. 2), the change of PI for grass ($43\% \pm 21\%$ of basal value) was significantly different from that

TABLE III. Results in individual subjects

	Antigen					
	G	P	O	B	D	Con
Subject 1 (▲)*						
RAST (PRU)	14	15	0.3	6	0	
PD ₂₀ (AU)	46	38	nd	106	nd	
EAR (%)†	45	22		23		0
LAR (%)‡	48	37	nd	19	nd	2
PI:						
mean	8.9	4.7		7.1		
SD	2.7	0.7		2.6		
Subject 2 (▼)						
RAST (PRU)	14	17.5	5	2	0.2	
PD ₂₀ (AU)	332	229	13	nd	nd	
EAR (%)	20	26	45			2
LAR (%)	13	58	22	nd	nd	4
PI:						
mean	7	13	2.9	11.5	4.5	
SD	1	1	0.2	2.3	1.2	
Subject 3 (●)						
RAST (PRU)	>17.5	>17.5	0.2	7.6	>17.5	
PD ₂₀ (AU)	10	15	nd	nd	nd	
EAR (%)	47	27				0
LAR (%)	37	29	nd	nd	nd	3
PI:						
mean	4.9	3.5	3	2.8		
SD	0.15	0.3	0.4	0.4		
Subject 4 (■)						
RAST (PRU)	3.3	9.7	0	0.5	3.6	
PD ₂₀ (AU)	48	124	nd	nd	610	
EAR (%)	20	31			22	2
LAR (%)	12	10	nd	nd	13	0
PI:						
mean	4.5	5.3			9.3	
SD	0.7	0.6			1.3	
Subject 5 (✚)						
RAST (PRU)	7.1	>17.5	0.5	0.5	2.2	
PD ₂₀ (AU)	18	94	nd	nd	nd	
EAR (%)	22	29				3
LAR (%)	9	23	nd	nd	nd	4
PI:						
mean	2.5	5.3				
SD	0.1	0.6				
Subject 6 (◆)						
RAST (PRU)	>17.5	0.35	3.3	3.8	15.9	
PD ₂₀ (AU)	16	nd	nd	nd	15	
EAR (%)	29				26	3
LAR (%)	19	nd	nd	nd	26	5
PI:						
mean	4.8				7.9	
SD	0.4				0.3	

G, (*Lolium perenne*); P, *Parietaria*; O, olive (*Olea europaea*); B, birch (*Betula verrucosa*); D, *Dermatophagoides pteronyssinus*; TT, tetanus toxoid; Con, control (saline).

*Symbols in parentheses correspond to symbols for individual patients in Figs. 1 to 4.

†EAR: Early asthmatic response, expressed as percent of control value.

‡LAR: Late asthmatic response, expressed as percent of control value.

§PI: proliferation index, expressed as the mean of observed baseline values (i.e., before allergen challenge). nd, not done.

of "others positive" ($57\% \pm 5\%$, $p = 0.035$) and "others negative" ($92\% \pm 14\%$, $p = 0.01$), and it was highly significantly different from the change of TT (103 ± 10 , $p = 0.003$).

After *Parietaria* challenge ($n = 5$), the PI against *Parietaria* was reduced from 7.8 ± 4.7 to 1.8 ± 0.5 of basal value ($p = 0.05$). This change was not significantly different from that of grass (interaction = 0.25) and

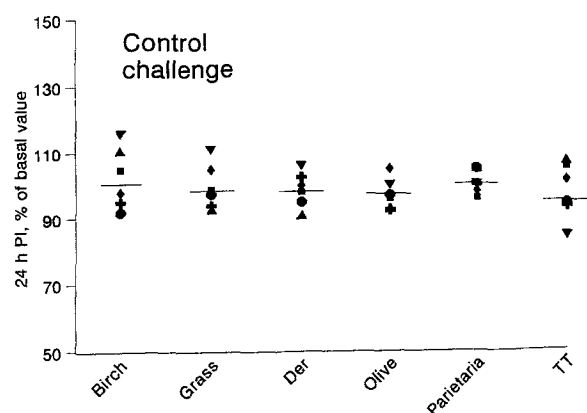


FIG. 1. Change of PI for each allergen and for TT after control (saline solution) challenge. Values observed 24 hours after challenge are expressed as percentage of basal values. Horizontal bars indicate the mean. For statistical differences see Results. *Der*, *Dermatophagoides*.

“others positive” (interaction = 0.44), whereas it was significantly different from the change of “others negative” and TT (interaction < 0.05 for both). When expressed as a percentage of the basal value (Fig. 3), the change of PI for *Parietaria* ($29 \pm 16\%$ of basal value) was not significantly different from the change of grass ($51\% \pm 29\%$, $p = 0.4$), was significantly different from change of “others positive” (49 ± 11 , $p = 0.025$), and was highly significantly different from the change of “others negative” ($99\% \pm 10\%$, $p = 0.0006$) and of TT (106 ± 15 , $p = 0.002$).

After challenges with “others positive” ($n = 4$), PI was reduced from 6.8 ± 2.9 to 2.6 ± 0.8 ($p < 0.05$). This change was not significantly different from the change of grass (interaction = 0.49) and *Parietaria* (interaction = 0.84), whereas it was significantly different from the change of “others negative” and TT (interaction < 0.05 for both). When expressed as a percentage of the basal value (Fig. 4), the change of PI for “others positive” ($41\% \pm 15\%$ of basal value) was not significantly different from the change of PI for grass ($52\% \pm 20\%$, $p = 0.4$) or for *Parietaria* ($58\% \pm 16\%$, $p = 0.4$), whereas it was highly significantly different from the change of “others negative” ($99\% \pm 10\%$) and TT ($106\% \pm 15\%$, $p = 0.007$ for both).

Basal PI of peripheral CD4⁺ T cells to nonsensitizing allergens was greater than 2 in all assays except one, even though it tended to be lower than the PI to sensitizing allergens for each subject (Table II).

In allergic and nonallergic individuals, cytokine production in response to specific stimulation revealed the presence of T cells producing both IL-4 and IFN- γ (Table IV). However, T cells derived from atopic individuals and stimulated with sensitizing allergens revealed a higher IL-4/IFN- γ ratio (Table IV and Fig. 5). On the contrary, T cells derived from atopic individuals and stimulated with nonsensitizing allergens, as well as T cells derived from nonatopic individuals, produced more IFN- γ than IL-4 (Table IV and Fig. 5).

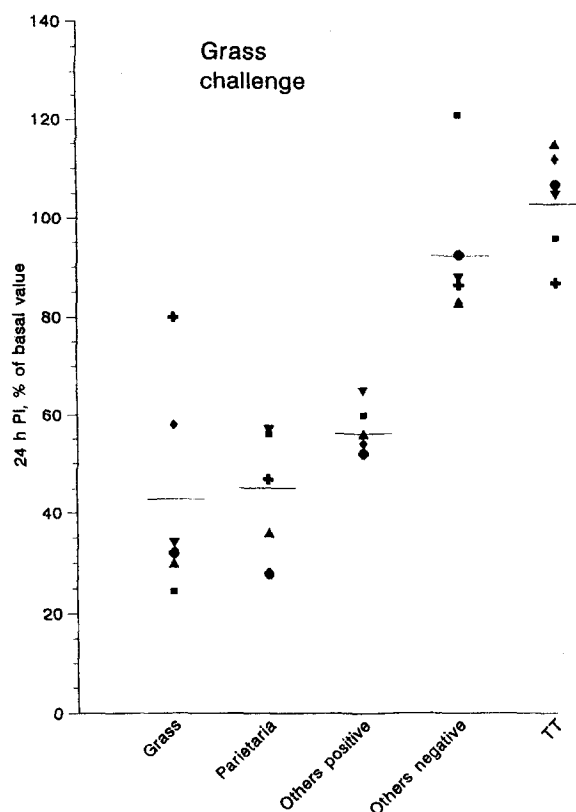


FIG. 2. In vitro proliferation of resting peripheral CD4⁺ T cells after inhalation of grass allergen. Change of PI for any allergen and for TT after challenge with grass (*Lolium perenne*). Values observed 24 hours after challenge are expressed as percentage of basal values. “Others positive” indicates the other allergens to which the subject is sensitized; “others negative” indicates allergens to which the subject is not sensitized. Horizontal bars indicate the mean. For statistical differences see Results.

DISCUSSION

The egress, on allergen challenge, of total^{5,6} and allergen-specific⁴ CD4⁺ T lymphocytes from the circulation to the lower respiratory tract in patients with allergic asthma has been described. This migration contributes to the inflammatory response that follows the exposure to allergen and can be functionally detected as the late-phase asthmatic response.⁹ Moreover, the ability of sensitized peripheral CD4⁺ lymphocytes to respond to allergen determines the magnitude of late airway responses,¹⁰ and the frequency of allergen-specific T cells contributes to the prediction of the extent of the EAR to allergen inhalation.¹¹ Therefore the study of circulating T cells in individuals exposed to experimental allergen inhalation provides information on the characteristics of the cells involved in airway inflammation in allergic asthma.

In this study we investigated whether airway exposure to an inhalant allergen, to which an asthmatic subject is sensitized, could cause a measurable depletion of peripheral T cells specific for the inhaled allergen and whether it could also cause a nonspecific depletion of T cells with other specificities. To this aim, we measured

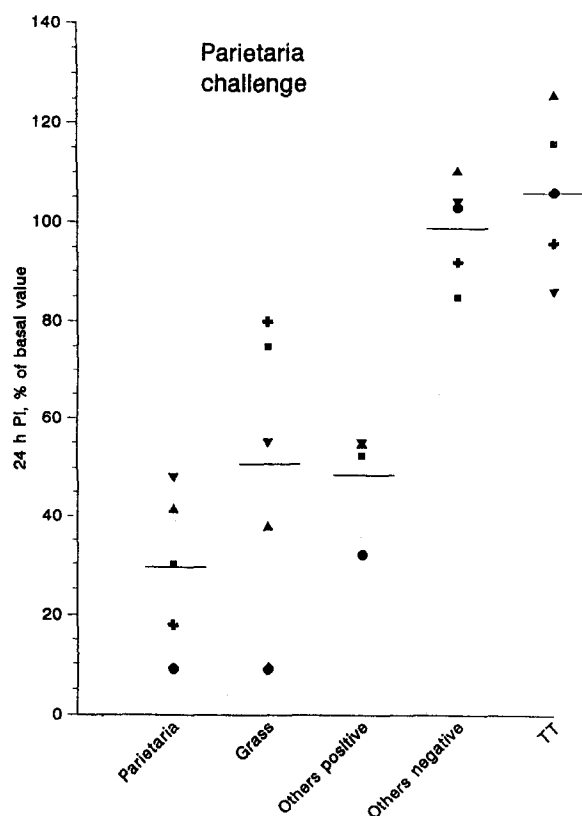


FIG. 3. In vitro proliferation of resting peripheral CD4⁺ T cells after inhalation of *Parietaria* allergen. Change of PI for any allergen and for TT after challenge with *Parietaria*. Other explanations are as in Fig. 2.

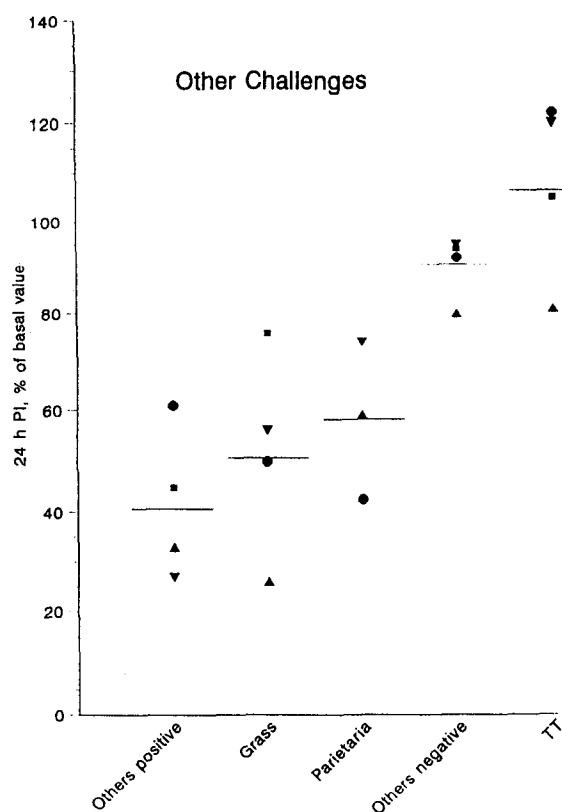


FIG. 4. Change of PI for any allergen and for TT after challenge with other allergens (*Dermatophagoides*, $n = 2$; *Olea europaea*, $n = 1$; *Betula verrucosa*, $n = 1$). Other explanations are as in Fig. 2.

the proliferation of CD4⁺ T lymphocytes from the peripheral blood in response to allergens that were both sensitizing and nonsensitizing in each individual, as well as to TT. Blood was taken 10 minutes before and 24 hours after allergen challenge. This time was chosen on the basis of our previous experience on recruitment of T cells to the lungs after allergen challenge,⁴ as well as on the basis of data from Gerblach et al.^{5,6}

After control challenge with saline solution, the PI for any antigen, expressed as a percentage of the basal value, was not significantly changed from baseline (range of the means was 97% to 103%), and the maximum observed difference from basal value was 16% (Fig. 1). This indicates that the bronchial challenge per se is not inducing changes in PI of peripheral CD4⁺ T cells. On the contrary, we show here that in polysensitized asthmatic subjects the inhalation of a single sensitizing allergen can remove from the circulation not only T cells specific for the allergen used in the challenge but also T cells specific for other allergens to which the patient is sensitized. Conversely, no mobilization of T cells specific for the nonsensitizing allergens or specific for a nonallergenic protein such as TT was observed (Table II and Figs. 2, 3, and 4). The clonal size of the T-cell population specific for TT, in the absence of environmental expo-

sure to this antigen by any route, was not altered by allergen inhalation. In fact, the PI to this antigen was consistent with the values observed at baseline after inhalation of any allergen (Table II and Figs. 2, 3, and 4).

The cytokine profiles of the same CD4⁺ T-cell preparations analyzed in these proliferation assays were studied by measuring IL-4 and IFN- γ production after in vitro allergen stimulation. We found that IL-4 production predominated over IFN- γ production in T cells stimulated in vitro with sensitizing allergens, as largely expected. On the contrary, T cells stimulated with nonsensitizing allergens displayed predominant IFN- γ production (Fig. 5). To our knowledge, this is the first time that this analysis has been performed in allergic subjects. A similar result was obtained with the internal control we included in this experiment, namely, T cells from nonallergic individuals stimulated in vitro with the same panel of allergens. This last observation is in agreement with a recent report by Ebner et al.,¹² who found a T_{H1}-oriented T-cell response in nonallergic subjects vaccinated with recombinant allergen from *Betula*. Taken together, these results suggest that the T-cell response to nonsensitizing allergens in allergic individuals is qualitatively comparable, in terms of cytokine profiles, to the T-cell response to allergens in nonallergic subjects, who

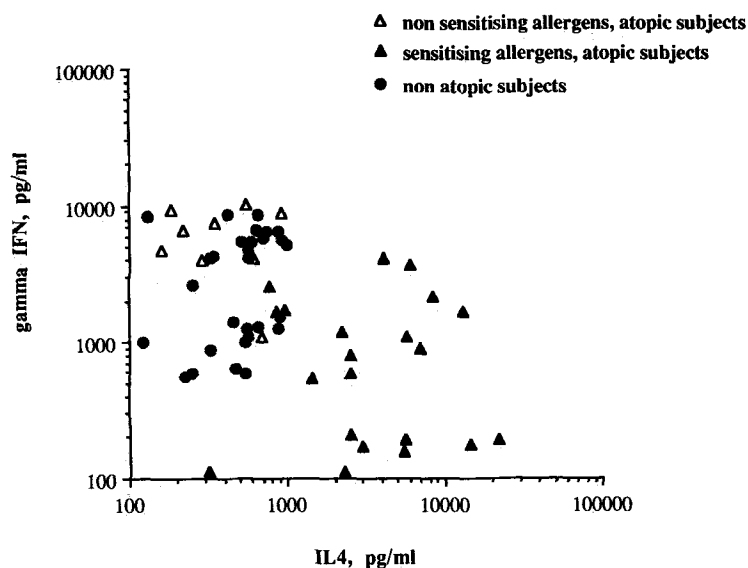


FIG. 5. IL-4 versus IFN- γ production in allergen-stimulated CD4⁺ T cells from allergic (*triangles*) and nonallergic (*circles*) individuals. *Open triangles* and *filled triangles* indicate T cells stimulated with nonsensitizing and sensitizing allergens, respectively. Individual data are listed in Table IV.

TABLE IV. IL-4 and IFN- γ production by T-cell lines from atopic and nonatopic subjects after stimulation with allergens

Subject*	Allergen†	IL-4 (pg/ml)	IFN- γ (pg/ml)	Subject*	Allergen	IL-4 (pg/ml)	IFN- γ (pg/ml)
S1	B	4121	4108	N1	<i>B</i>	558	1264
	G	5714	5111		<i>G</i>	nd	nd
	<i>D</i>	548	10111		<i>D</i>	698	5778
	<i>O</i>	915	8914		<i>O</i>	568	4724
	P	6911	914		<i>P</i>	nd	nd
S2	B	1414	541	N2	<i>B</i>	998	5141
	G	2498	206		<i>G</i>	nd	nd
	<i>D</i>	688	1114		<i>D</i>	254	2615
	<i>O</i>	2281	111		<i>O</i>	587	5411
	P	784	2565		<i>P</i>	564	4152
S3	B	2479	808	N3	<i>B</i>	926	5641
	G	958	1704		<i>G</i>	875	6542
	<i>D</i>	6027	3748		<i>D</i>	541	999
	<i>O</i>	348	7421		<i>O</i>	nd	nd
	P	8560	2140		<i>P</i>	541	998
S4	B	184	9473	N4	<i>B</i>	748	6542
	G	13,150	1658		<i>G</i>	514	5411
	<i>D</i>	2237	1186		<i>D</i>	899	1541
	<i>O</i>	284	3991		<i>O</i>	nd	nd
	P	2526	589		<i>P</i>	132	8465
S5	B	158	4720	N5	<i>B</i>	223	558
	G	5559	191		<i>G</i>	312	888
	<i>D</i>	14,520	175		<i>D</i>	123	998
	<i>O</i>	221	6746		<i>O</i>	542	587
	P	5515	155		<i>P</i>	nd	nd
S6	B	2959	171	N6	<i>B</i>	654	8572
	G	21,920	190		<i>G</i>	422	8641
	<i>D</i>	850	1681		<i>D</i>	245	585
	<i>O</i>	314	111		<i>O</i>	642	6587
	P	611	4151		<i>P</i>	465	652

B, *Betula verrucosa*; *D*, *Dermatophagoides pteronyssinus*; *G*, *Graminaceae*; *O*, *Olea europea*; *P*, *Parietaria*; nd, not done.

*Subjects S1 to S6 are atopic; subjects N1 to N6 are nonatopic.

†Nonsensitizing allergens are indicated in italics.

TABLE V. Comparison of changes in PI after addition of antigen to cultures

Subject	Allergy	Stimulus	Antigen added to culture at:									
			G		P		D		O		TT	
			day 0	day 7	day 0	day 7	day 0	day 7	day 0	day 7	day 0	day 7
3	G,P,D (B)	G	34	42	57	70	65	42	104	109	105	100
4	G,P,D	D	56	42	74	73	27	36	95	126	121	106
5	G,P (D)	P	83	88	18	16	92	93	nd	nd	96	95

Results are expressed as percentage of baseline PI. Comparison by *t* test for paired samples on pooled data (14 pairs) showed no significant difference between day 0 (73% $\pm$ 32%) and day 7 (74% $\pm$ 33%) ($p = 0.74$).

G, grass; P, *Parietaria*; D, *Dermatophagoides*; O, olive; B, birch; TT, tetanus toxoid; nd, not done.

have probably undergone environmental exposure to those allergens.

Clinical evidence indicates that a majority of allergic individuals are polysensitized. It is not known whether polysensitization is sustained by a nonspecific inflammatory background that is promoting the generation of progressively increasing numbers of single specificities for airborne antigens or by cross recognition by T lymphocytes of epitopes shared among different allergens.¹³ In both cases an important mechanism in the maintenance of the polysensitized status would be the availability of allergen-specific T lymphocytes at the site of allergen exposure. We suggest here that in polysensitized asthmatic individuals the migration of populations of T lymphocytes with different allergen specificities from the circulation to the airways may play a relevant role in sustaining a high level of inflammation at the disease site when exposure to a single sensitizing allergen takes place.

The basal PI of peripheral CD4⁺ T cells to nonsensitizing allergens was detectable in all assays but one, although it tended to be lower than the PI to the sensitizing allergens for each subject (Table II). A few comments can be made on this last point: (1) this result is expected because it has been reported that even T cells from nonallergic donors proliferate in response to stimulation with allergens¹⁴⁻¹⁶ (Burastero. Unpublished observations); (2) a lower level of basal proliferation to nonsensitizing allergens than to sensitizing allergens cannot be claimed to affect the results of our assay because statistical analysis was performed not only with the percentage variations of PI but also with the absolute values; (3) the evaluation of antigen-specific proliferation of peripheral lymphocytes with purified CD4⁺ T cells, which are the actual responders, and monocytes, which serve as antigen-presenting cells, increases the sensitivity of the test (Burastero. Unpublished observations); (4) expressing the antigen-specific response as the ratio between antigen-induced and baseline proliferation allows one to take into account possible changes in the level of in vivo activation of T cells, which has been described in asthmatic subjects after allergen challenge.^{5,6}

Although we did not carry out kinetics experiments to demonstrate that the depletion is a progressive process, we favor the hypothesis that the observed decrease in

the specific PI can be attributed to recruitment of allergen-specific T-cell clones to the lungs. In further experiments, we could directly demonstrate in one asthmatic subject that two allergen-specific T-cell clones were simultaneously expanded in the lungs and removed from peripheral blood on allergen challenge (Borgonovo et al. Submitted). A possible alternative explanation of the results of this study might be a decrease of the PI because of in vivo induction of hyporesponsiveness through T-cell receptor engagement.¹⁷ However, this would first imply that in our experimental system exposure to a single allergen could induce hyporesponsiveness to all sensitizing allergens, whereas these were different in the different patients. Second, this phenomenon would require a high degree of cross-reactivity among the allergen extracts we used, a fact we have evidence against, as described in the Methods section. Third, we designed experiments in three subjects to check the possible effect of hyporesponsiveness induced by T-cell receptor engagement on allergen-specific T cells. For this purpose we tested CD4⁺ T cells in proliferation assays performed by plating the cells in the absence of any stimulus and adding the antigen only after 7 days. This period was shown to allow recovery from hyporesponsiveness of in vitro-activated T cells,^{18,19} and it is widely used as a minimum time lag between restimulations in classical protocols for maintaining in vitro established T-cell clones.³ There are no data on the induction of anergy in experimental systems such as the one we used for our primary cultures, in which an antigen exposure in vivo is followed by an in vitro assay that requires restimulation with the same antigen used in vivo. In the modified experimental conditions we used to assay the effect of in vivo antigen exposure on in vitro antigen-specific proliferation, the same pattern (decrease of PI extended to all sensitizing allergen) was observed (Table IV). Taken together, these results favor the hypothesis that the phenomenon we describe can be explained by depletion of T cells from peripheral blood rather than by induction of hyporesponsiveness.

It is likely that the extent of the in vitro proliferation on antigen pulse roughly reflects the clonal size of T cells specific for that antigen. Indeed, we have previously demonstrated in a group of patients with allergic asthma

that higher PIs correspond to higher frequencies of allergen-specific T cells, as determined by limiting dilution analysis.¹¹

Our work shows that in allergic asthma the pattern of lymphokine production and the migration profile on allergen inhalation cosegregate in peripheral allergen-specific T lymphocytes. The following speculation on a possible coordinate mechanism underlying this finding can be envisaged: the acquisition of a "sensitizing" (T_{H2}) phenotype by CD4⁺ T lymphocytes primed in the lung immune system (presumably, in hilar lymph nodes)^{20, 21} could occur simultaneously with the acquisition of a homing phenotype that commits these cells to recirculate back to the periphery of the target organ. These homing receptors could be constitutively expressed on circulating CD4⁺ T cells, whereas the corresponding counterreceptors, which are likely to be located on endothelial cells, could be modulated as part of the reaction to allergen challenge, as described in the case of atopic and contact dermatitis.^{22, 23} Therefore in allergic asthma, mechanisms that are capable of sorting disease-associated antigen specificities together with defined lymphokine patterns into T lymphocyte subsets, which can migrate to target organs, are operating. This result is in agreement with recent reports on the regulation of homing to skin of cutaneous lymphocyte antigen-positive T cells in atopic dermatitis and contact dermatitis on antigen exposure.^{24, 25} This perspective may open the way to the design of new therapeutic approaches to allergic diseases.

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