

Recruitment of circulating allergen-specific T lymphocytes to the lung on allergen challenge in asthma

Barbara Borgonovo, PhD,^a Giulia Casorati, PhD,^a Emanuela Frittoli, BA,^a
Davide Gaffi, PhD,^a Emanuele Crimi, MD,^b and Samuele E. Burastero, MD^a
Milan and Genoa, Italy

Background: In allergic subjects with asthma, the migration of CD4⁺ T cells to the lungs in the hours after allergen exposure may contribute to allergic inflammation in the target organ.

Objective: We studied allergen-specific T cells from the peripheral blood and lungs of allergic subjects with asthma at baseline and after allergen challenge.

Methods: In each patient, blood samples were taken 10 minutes before and 24 hours after the inhalation of a major sensitizing allergen. In vitro proliferation of peripheral blood CD4⁺ T cells specific for the same allergen used in the in vivo challenge was assessed. In one patient two *Dermatophagoides pteronyssinus*-specific T-cell clones (TCCs) were derived from peripheral blood, and their T-cell receptors were sequenced to determine their clonotypic determinants on the β chains. The T-cell receptor determinants of the allergen-specific TCCs were sought in blood and bronchoalveolar lavage samples taken from this patient.

Results: We found that allergen inhalation is followed by a decrement in the specific proliferation of peripheral CD4⁺ T cells to the same allergen used for bronchial provocation. In one patient the clonotypic determinants of two allergen-specific TCCs diminished in the peripheral blood, whereas they were simultaneously expanded in the lower respiratory tract.

Conclusion: Our data suggest that allergen-specific T cells are recruited from the peripheral blood to the bronchial lumen after allergen challenge. (J Allergy Clin Immunol 1997; 100:669-78.)

Key words: T lymphocytes, migration, allergy, asthma, bronchial provocation tests

The immune system in the lower respiratory tract has several peculiar anatomic and functional features related to its chronic physiologic exposure to airborne antigens. Indeed, as a default mechanism, the immune response to any inhaled antigen is shut down. This characteristic prevents the establishment of a continuous inflammatory

Abbreviations used

APCs:	Antigen-presenting cells
AU:	Arbitrary units
BAL:	Bronchoalveolar lavage
EAR:	Early asthmatic reaction
LAR:	Late asthmatic reaction
mAbs:	Monoclonal antibodies
PBMCs:	Peripheral blood mononuclear cells
PCR:	Polymerase chain reaction
PHA:	Phytohemagglutinin
PI:	Proliferation Index
PMA:	Phorbol myristate acetate
TCC:	T-cell clone
TCR:	T-cell receptor
TT:	Tetanus toxoid

response. Although this could occur safely in other tissues, it could cause a relevant functional impairment in the respiratory exchange in the lung.¹ This inhibition is primarily maintained by alveolar macrophages.²

Immunocytes obtained by bronchoalveolar lavage (BAL) include a majority of T lymphocytes bearing the markers of activation-experienced cells,³ with a prevalent expression of late, rather than early, activation markers.⁴ These T cells home to the respiratory surface after being primed by encounter with antigen and proper antigen-presenting cells (APCs) in the hilar lymph nodes.⁵ Antigen-presenting function is provided by dendritic cells distributed in a continuous network, which spreads throughout the lung interstitium.⁶ These cells are capable of bringing antigens and proper costimulation to T lymphocytes in hilar lymph nodes.⁷ Therefore the absence, at least in human beings, of locally organized bona fide lymphoid structures (bronchus-associated lymphoid tissue⁸) is compensated in functional terms by a complex and dynamic system of specialized cell types migrating between different compartments. Although antigen receptors play no direct role in the selectivity of lymphocyte extravasation from blood,⁹⁻¹¹ antigens are critically important in regulating the microenvironmental homing properties of lymphocytes.¹¹ For example, antigen-specific T cells are cleared by the recirculating lymphocyte pool after antigen administration.⁹ We focused our investigation on the dynamic of peripheral blood and lung CD4⁺ T cells in a model of

From ^athe Department of Biotechnology, San Raffaele Scientific Institute, Milan; and ^bDipartimento di Scienze Motorie e Riabilitative, Facoltà di Medicina e Chirurgia, University of Genoa.

Supported by grant no. 930426 (VIII Progetto AIDS) from Istituto Superiore Sanità (Dr. Burastero) and the Associazione Italiana per la Ricerca sul Cancro (Dr. Casorati).

Received for publication Dec. 2, 1996; revised June 11, 1997; accepted for publication June 16, 1997.

Reprint requests: Samuele E. Burastero, MD, DIBIT, San Raffaele Scientific Institute, Via Olgettina, 58, 20132 Milan, Italy.

Copyright © 1997 by Mosby-Year Book, Inc.

0091-6749/97 \$5.00 + 0 1/1/84057

TABLE I. Subject characteristics

Subject	Sex	Age (yr)	Height (cm)	FEV ₁ * (%)	Total IgE (U/ml)	Sensitization†	Allergen	PD ₂₀ (AU)	EAR (%)	LAR (%)
q6	M	17	165	83	385	B,P,(G),(D)	B	212	35	50
q7	M	23	172	89	2060	B,D,O	O	90	19	0
q11	F	19	164	80	1200	D,P(B)	P	39	22	18
q12	M	22	175	85	220	B,D,G,P	B	54	20	20
qVAL	M	26	170	80	522	D,P	D	50	30	30

Sensitizations are defined on the basis of skin test and RAST results.

B, Birch (*Betula verrucosa*); G, grass (*Lolium perenne*); O, olive (*Olea europaea*); D, *Dermatophagoides pteronyssinus*; P, *Parietaria officinalis*.

*FEV₁ is expressed as a percent of predicted value.¹¹

†Allergens between brackets are minor or doubtful sensitizations.

asthma experimentally induced by controlled allergen inhalation. Our results are consistent with the hypothesis that antigen-specific mechanisms of T-cell recruitment that are operating in atopic asthma more than likely contribute to the maintenance of a pathologic response to allergen inhalation.

METHODS

Subjects

We studied five allergic nonsmokers (4 men, 1 woman) whose mean age was 21.4 years (range, 17 to 26 years). These subjects had asthma,^{12, 13} and their prevalent sensitizations were inferred from the results of skin prick tests and RASTs. Subject characteristics are summarized in Table I. Inclusion criteria were an FEV₁ greater than 60% of predicted value¹⁴ at the time of the study and no respiratory tract infections in the 4 weeks before the study. None of the subjects were, at the time of the study, receiving treatment for asthma other than short-acting β_2 stimulants on an "as needed" basis. These treatments were discontinued at least 12 hours before challenges. All subjects were informed of the nature and aim of the study, and each gave written consent.

Protocol

On the first day of the study, subjects q6, q7, q11, and q12 underwent blood sampling (40 ml, heparinized) and, 10 minutes later, spirometry and allergen challenge. Blood was taken again from these patients 24 hours after the challenge. Patient qVAL underwent BAL during an initial visit at baseline (i.e., without any allergen challenge). One month later he underwent blood sampling (40 ml, heparinized) and, 10 minutes later, spirometry and allergen challenge. Blood was taken again 24 hours after the challenge and 10 minutes before he performed a second BAL.

Aerosol device

Allergens were all aerosolized by means of dosimetric techniques. Aerosols were generated by an ampule-dosimeter device (MEFAR, Brescia, Italy). The dosimeter was set to deliver 10 μ l of solution for 1 second at the beginning of each inspiration during quiet breathing. The particle median mass diameter was 1.53 to 1.61 μ m. The same set of identical ampules was used throughout the study.

Media, monoclonal antibodies (mAbs), and antigens

Complete medium used for cell cultures was prepared as follows. RPMI-1640 medium (PBI, Milan, Italy) was supplemented with 5 μ g/ml gentamicin, 2 mmol/L glutamine (PBI), and 5% normal human serum (Danish Red Cross Laboratories, Utrecht, Holland). The mAbs used for purification of CD4 T cells and macrophages were UCHL-4 (anti-human CD8; IgG2a), NKH-1 (anti-human CD56; IgM), and SJ25-C1 (anti-human CD19; IgG1) (Sigma, Milan, Italy).

The allergens were generously provided by Dr. Paolo Falagiani (Lofarma-Allergeni, Milan, Italy). They were commercial preparations of *Parietaria officinalis*, *Betula verrucosa*, *Olea europaea*, and *Dermatophagoides pteronyssinus*, the protein concentrations of which were calculated with a commercial kit (BioRad Laboratories, Serrate, Italy). The same allergen preparations were used for both in vivo and in vitro studies. 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 a high titer of specific IgE. The Lymulus amoebocyte gelification test (PBI) was used to assess the presence of bacterial endotoxins in the allergen extracts. Briefly, the assay was performed on reconstitution of the allergen extract in culture medium at a concentration of 1 mg/ml according to the manufacturer's instructions. It measured 0.01 endotoxin units/ml. These levels were not different from those found in saline solution. Tetanus toxoid (TT) was purchased from Connaught Labs (Willowdale, Ontario, Canada).

Allergen challenge

Allergens were provided by the manufacturer in a lyophilized form obtained from a solution of the extract in saline. They were reconstituted just before administration by adding redistilled water. Twofold decreasing concentrations from 1000 AU/ml to 8 AU/ml were prepared. After a control measure with saline solution (50 breaths), the challenge started from the concentration of 8 AU/ml and continued with twofold increments of the allergen concentration until the FEV₁, measured 15 minutes after each inhalation, fell below 80% of the postsaline control value. Each concentration was inhaled for the duration of 50 quiet breaths, which corresponded to an inhaled volume of 0.5 ml. The maximum concentration of allergen was 1000 AU/ml, corresponding to an inhaled dose of 500 AU. At the end of the inhalation challenge, FEV₁ was measured at 15,

TABLE II. Cell differentials in BALs

Sample	BAL fluid recovered (ml)	Total cells $\times 10^6$	Macrophages (%)	Lymphocytes (%)	Neutrophils (%)	Eosinophils (%)
BAL (baseline)	80	2.3	78	18	2	2
BAL 1 (after challenge)	89	1.9	70	24	0	6
BAL 2 (after challenge)	70	2.9	68	25	1	6

BAL1 and BAL2 indicate bronchoalveolar lavage performed in the upper right middle lobe and in the lingula, respectively. Postchallenge bronchoalveolar lavage was performed 24 hours after allergen challenge with *Dermatophagoides pteronyssinus* extract.

TABLE III. Purification of CD4 lymphocytes and CD14 APCs from peripheral blood

Patient	Prechallenge		Postchallenge	
	CD4	CD14	CD4	CD14
q6	85	11	80	16
q7	67	30	60	17
q11	81	16	80	19
q12	74	22	70	29
qVal	65	30	57	39
mean $\pm$ SD	74.4 $\pm$ 8.6	21.8 $\pm$ 8.4	69.4 $\pm$ 10.8	24 $\pm$ 9.8

Numbers indicate the percentage of CD4 and CD14 cells after the purification procedure by negative immunoselection in the PBMCs isolated from patients at baseline (prechallenge) and after allergen inhalation (postchallenge).

30, and 45 minutes and then hourly until the BAL procedure was performed to determine whether a late asthmatic reaction (LAR) had occurred. An early asthmatic reaction (EAR) was expressed as the maximum decrease in FEV₁ recorded within 1 hour after the last inhalation, and LAR was expressed as the maximum FEV₁ decrease recorded starting 3 hours after the end of the challenge. EAR and LAR were considered positive if there was an FEV₁ decrement greater than 20% and greater than 15%, respectively. The allergen PD₂₀ was also calculated by interpolation in the dose-response curve. Subject characteristics are shown in Table I.

Fiberoptic bronchoscopy and BAL

Bronchoscopy with BAL in patient qVAL was performed as follows. After premedication with atropine (0.5 mg intramuscularly) and diazepam (10 mg intramuscularly), local anesthesia of the nostrils was obtained with 2% lidocaine and epinephrine 0.1/1000 ng/ml (1 ml each side). A fiberoptic bronchoscope (type P10; Olympus BF, Osaka, Japan) was then passed through the nose. The pharynx and large airways were anesthetized by instillation of 2% lidocaine, and the tip of the bronchoscope was wedged into a subsegmental branch of the right middle lobe and the lingula. Five aliquots (20 ml) of sterile, warm (37°C) saline solution were infused through the channel of the bronchoscope and recovered at a negative pressure of 50 to 120 mm Hg.

BAL sample processing

The BAL fluid recovered from patient qVAL was filtered through two layers of sterile gauze and centrifuged at 250 g for 5 minutes to separate the cells from the supernatants. The cell pellet was resuspended in ice-cold phosphate-buffered saline without Ca²⁺ and Mg²⁺, at a concentration of 10⁶ cells/ml. A small sample of the cell suspension was centrifuged by using a cytocentrifuge (Cytospin; Shandon Southern Instruments, Sewickley, Pa.), spinning approximately 100,000 cells at 500 rpm for 5 minutes onto a glass slide. BAL cells were air-dried and stained with Diff-Quick (Merz & Dade A.G., Duding, Switzerland). Cell differentials were determined by counting at least 300 cells per slide with a light microscope (Carl Zeiss, Oberkochen, Germany). Macrophages,

lymphocytes, neutrophils, and eosinophils were expressed as percent of total cells recovered. Results of cell differentials in the two BAL samples are shown in Table II. The remaining cells were immediately lysed in guanidium-isothiocyanate buffer and stored at -20°C until RNA extraction.

Preparation of CD4 T lymphocytes and APCs

A cell preparation containing CD4 T lymphocytes and macrophages (as professional APCs) was obtained from each patient, starting with peripheral blood mononuclear cells (PBMCs), by negative immunobead selection with mAbs according to the manufacturer's instruction (Dynabeads; Unipath, Gargagnate, Italy). Briefly, cells were incubated (20 minutes on ice) at a cell density of 1 $\times$ 10⁷ per milliliter with normal human serum (10%) to saturate the Fc receptors and then with mAbs to CD8, CD56, and CD19 (1 μ g/10⁶ cells) to remove cytotoxic or suppressor T lymphocytes, natural killer cells, and B lymphocytes, respectively. Goat anti-mouse immunoglobulin-coated beads were reacted (20 minutes on ice, rotating) 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 fluorescence-activated cell sorter analysis (Becton-Dickinson) (Table III).

Proliferation assay

CD14⁺, CD4⁺ cells isolated from each patient were plated (5 $\times$ 10⁴/well) in 200 μ l of complete medium in a 96-well flat-bottom plate (Costar, PBI). The same antigens used for bronchial provocation and TT were added for 7 days at 20 μ g/ml for each antigen to four microcultures. Control specimens (cells 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, Uppsala, Sweden), and tritiated thymidine incorporation was counted by using a β -plate counter (Pharmacia LKB). The proliferation index (PI) for each antigen was calculated as the ratio between the mean ³H-thymidine incorporation in test wells and that in control wells. Only PI

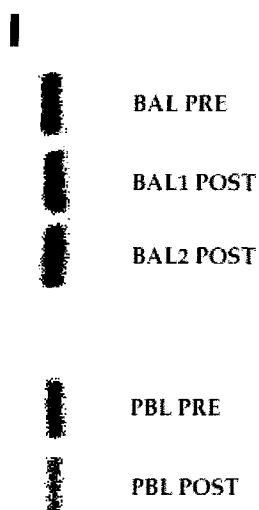


FIG. 1. PCR product of C α -C α amplification of cDNA from peripheral blood and BAL cells taken at baseline and 24 hours after allergen challenge from patient qVal. BAL1 and BAL2 indicate samples taken from lingula and right middle lobe, respectively. Bar corresponds to the 527 d molecular weight. This illustration shows negative film of ethidium bromide-stained gel used for densitometric analysis. Relative percentages of areas under the curve are (from left to right): 1.277, 1.892, 1.845, 1.0, and 0.821.

values greater than or equal to 2 were considered indexes of allergen-specific proliferation. The changes in PI for allergen and for TT were preliminarily tested in the patients included in this study after control challenge with saline solution. The maximum observed difference was 16% from basal value, thus indicating that bronchial provocation per se does not induce changes in the PI of peripheral T cells.

Establishment of allergen-specific T-cell clones (TCCs) and characterization of their cytokine profile

CD4⁺, CD14⁺ cells from patient qVAL were seeded (2×10^6 per well) in 1 ml of complete medium in 24-well plates (Costar, PBI). *Dermatophagoides* extract (Lofarma Allergeni) was added at 20 mg/ml on the day of seeding (day 0). On day 7, recombinant (r)IL-2 (20 IU/ml; Roche, Nutley, N.J.) was added. On day 15, a Ficoll-Hipaque gradient (Pharmacia) was used to discard cellular debris, and cells recovered on the gradient were used for cloning in 60-well Terasaki plates (PBI International) at 0.2 cells per well in the presence of 5000 allogeneic irradiated PBMCs, 10 μ g/ml phytohemagglutinin (PHA) (Sigma), and 100 U/ml rIL-2. Ten days after cloning, microcultures were transferred to 96-well flat-bottom plates in the presence of 100 U/ml rIL-2. Specificity for *Dermatophagoides* was determined as a stimulation index in a 48-hour proliferation assay performed with *Dermatophagoides* extract pulsed (20 μ g/ml, 4 hours at 37° C) with 3000 rad irradiated autologous PBMCs as APCs.

To expand TCCs, cells were restimulated with irradiated allogeneic PBMCs combined with rIL-2 and PHA at the above indicated concentrations. T cells were subsequently maintained in a medium containing rIL-2, added every third day, and restimulated every 2 weeks thereafter. To study the cytokine profile 7 days after last restimulation, TCCs were purified on a Ficoll gradient, washed, and cultured (10⁶/ml in a 24-well plate) in the presence of either PHA (10 μ g/ml) plus phor-

bol myristate acetate (PMA) (100 nm, Sigma) or autologous 3000 rad irradiated PBMCs (as the source of APCs) pulsed with *Dermatophagoides* extract (20 μ g/ml, 4 hours at 37° C). Supernatants were taken after 24 hours of culture from PHA-PMA-stimulated clones and after 48 hours of culture from antigen-stimulated clones and frozen at -80° C for analysis of IL-4 and interferon- γ . In-house Roche ELISAs were used for this analysis (courtesy of Dr. Paola Panina, Roche Milano Ricerche, Milan, Italy).

T-cell receptor (TCR) cloning and sequence

To clone the TCR V β genes expressed by TCC1 and TCC2 derived from the peripheral blood of patient qVAL, total RNA was extracted by using the procedure described by Chomczynski and Sacchi.¹⁵ One microgram was used for first-strand complementary DNA synthesis by using an oligo (dT) primer and Moloney murine leukemia virus reverse transcriptase (BRL, Gaithersburg, Md.).

Polymerase chain reaction (PCR) was then performed with a panel of V β -specific oligonucleotides,^{16,17} paired with a C β primer (5'-TGCTGACCCCACT-GTCGACCTCCTTCCCAIT-3') as previously described.^{18,19} Briefly, reactions were performed in 30 μ l of the final volume under the following conditions: 30 seconds of denaturation at 94° C, 30 seconds of annealing at 60° C, and 30 seconds for elongation at 72° C for 35 cycles preceded by 3 minutes of denaturation and followed by 5 minutes for final elongation. One third of the amplified product was run on a 2% agarose ethidium bromide-stained gel for analysis. V β 22 and V β 6.2 genes could be amplified from TCC1 and TCC2, respectively. The PCR products of the V genes amplified from the TCCs were then eluted from a preparative gel and directly sequenced as described by Casanova et al.²⁰ to determine the N-regions of the β chain-coding sequences by using the C β specific oligonucleotide, 5'-TGCTTCTGATG-GCTCAA.

Detection of TCR clonotypes of TCC1 and TCC2 among BAL and peripheral blood T lymphocytes

Total RNA and cDNA were prepared as described above from the following samples obtained from patient qVAL: (1) three samples of unfractionated BAL cells, namely, one sample from the lavage performed in a single lung segment (in the right middle lobe) at baseline (i.e., without any allergen challenge) and two samples taken from the lavage performed in the same patient in two different lung segments (in the right middle lobe and in the lingula, respectively) 24 hours after the allergen challenge; and (2) two samples of PBMCs (one sample taken before the allergen challenge and the other taken 24 hours later).

To compare the clonal size of TCC1 and TCC2 in each sample tested, a preliminary PCR was done to equilibrate the amount of template used in each single reaction. To this end, two primers were used, named C α upstream and C α downstream. The C α upstream oligonucleotide (5'-GAACCCTGAC-CCTGCCGTGTACC [forward primer]) corresponds to the sequence of the positive strain of the C α gene (bp 545 to bp 567). The C α downstream oligonucleotide (5'-ATCATAAAT-TCGGGTAGGATCC) corresponds to the sequence of the minus strain of the C α gene (bp 1112 to bp 1133 [reverse primer, numeration according to Rabbitts et al.²¹]). By using these primers and one thirtieth of each cDNA sample, we demonstrated that 25 cycles of amplification in each sample fell within the range of cycles, which yields an exponential increase in the amount of PCR product obtained (data not shown).²² The PCRs were performed in 30 ml of final volume with a

TABLE IV. Proliferation of CD4, CD14 cells to allergen and TT at baseline and after allergen challenge

Patient	Antigen	Before challenge			After challenge			PI (% of baseline)†
		Control*	Antigen*	PI†	Control*	Antigen*	PI†	
q6	<i>Betula spp.</i>	112 ± 12	1164 ± 160	10.4	189 ± 23	510 ± 33	2.7	25.9
	TT	112 ± 11	3696 ± 150	33.0	189 ± 11	5292 ± 140	28.0	84.4
q7	<i>Olea</i>	153 ± 15	1621 ± 154	10.6	208 ± 16	1060 ± 101	5.1	48.1
	TT	153 ± 9	4590 ± 73	30.0	208 ± 14	5844 ± 120	28.1	84.8
q11	<i>Parietaria</i>	201 ± 7	1286 ± 148	6.4	267 ± 21	560 ± 12	2.1	32.8
	TT	201 ± 14	3758 ± 111	18.7	267 ± 30	4031 ± 141	15.1	80.7
q12	<i>Betula</i>	195 ± 31	1111 ± 134	5.7	221 ± 40	685 ± 16	3.1	54.3
	TT	195 ± 25	2476 ± 130	12.7	221 ± 11	2895 ± 148	13.1	103.1
qVal	<i>Dermatophagoides</i>	134 ± 37	951 ± 70	7.1	201 ± 11	482 ± 36	2.4	33.8
	TT	134 ± 16	1527 ± 79	11.4	201 ± 19	2150 ± 110	10.7	93.8

*Mean ³H-thymidine incorporation (± SD) of quadruplicate cultures to the indicated antigens and in the control wells (medium only).

†PI, calculated as a ratio between mean proliferation to antigen and mean proliferation in control wells.

‡The percentage of PI to the antigen after challenge versus baseline (before challenge).

Thermal Cycler 9600 (Perkin Elmer, Weiterstadt, Germany) under the following conditions: 30 seconds of denaturation at 94° C, 30 seconds of annealing at 55° C, and 30 seconds for elongation at 72° C for 23 cycles preceded by 3 minutes of denaturation and followed by 5 minutes for final elongation. The amplified products were run on a 2% agarose gel, and ethidium bromide-stained products were densitometrically compared. On this basis, proper dilutions were made to normalize cDNA content. A second exponential α - α PCR was then performed. After normalization, DNA content in the compared samples, as judged from products of a further α upstream- α downstream-primed PCR, differed a maximum of 20% from each other on densitometric analysis (Fig. 1). Each sample was then amplified using a V β 6.2 or V β 22 family-specific oligonucleotide paired with the C β primer. PCRs were performed as described previously. One third of the amplified product was run on a 2% agarose ethidium bromide-stained gel for analysis.

Heteroduplex analysis

The remaining 20 μ l of PCR-amplified products was subsequently run on 12% native polyacrylamide gel for electrophoresis as previously described.^{18, 19} Briefly, 20 μ l of each amplified fragment was heated at 94° C for 5 minutes to denature DNA, reannealed for 1 hour at 50° C, and kept at 4° C until gel loading. This temperature allows reannealing between DNA strains with minor mismatches (heteroduplexes) and between homologous strains (homoduplexes). Gene fragments differing for single-point mutations in the N-region are detected with this assay.²³ V β -amplified fragments derived from peripheral blood, BAL, and allergen-specific TCCs were run in parallel lanes. Gels were run at 10 mA overnight at 4° C, stained with ethidium bromide, and photographed.

Hybridization with probes specific for N-region and C β of TCR

After electrophoresis, DNA was transferred from the acrylamide gel to nylon membranes (Hybond Plus, Amersham) by using capillary blotting with 20 $\times$ standard saline citrate buffer for 20 to 48 hours. Transferred DNA was denatured and fixed on the membrane, and the filter was incubated on 3-mm thick chromatography paper soaked in NaOH (0.4 mol/L) for 20 minutes. Membranes were prehybridized for 1 hour at 37° C in hybridization solution (a mixture of 6 $\times$ standard saline citrate,

1% blocking buffer, 5 mmol/L ethylenediamine tetraacetic acid, and 0.1% sodium dodecylsulfate) and then hybridized in the same hybridization solution with the proper oligonucleotide probe in a shaking bath for 16 hours at 37° C. The following oligonucleotides were used as probes: an internal C β -specific oligonucleotide, which hybridizes with all V β -C β PCR products obtained with this protocol (the same used for sequencing); a probe designed according to the N-region of TCC1 (5'-AGT-GAATTATGGCCTCTG); and a probe designed according to the N-region of TCC2 (5'-AGCTTAGCGCCAGCAGCGAG [see Results section]).

Probes were labeled as follows: 200 ng of oligonucleotide were labeled for 3 hours or overnight at room temperature with 50 μ Ci gamma phosphate 32-labeled adenosine triphosphate (Amersham), 1 μ l polynucleotide kinase (10 u/ μ l; Boehringer Mannheim, Germany) in 0.05 mol/L Tris (pH 7.6), 0.01 mol/L MgCl₂, 5 mmol/L dithiothreitol, 0.1 mmol/L spermidine HCl, and 0.1 mmol/L ethylenediamine tetraacetic acid in a 30 μ l reaction volume. The whole mixture was directly added to the hybridization solution. After hybridization, membranes were washed twice, for 30 minutes each, in a mixture of 6 $\times$ standard saline citrate and 0.1% sodium dodecylsulfate. The washing temperatures were 37° C for the C β probe and 50° C for the N-region probes. The filters were exposed between 1 hour and 6 days at -80° C. After the first hybridization with the N-regions, the probe filters were washed twice, for 30 minutes each, in a mixture of H₂O and 0.5% sodium dodecylsulfate at 70° C and hybridized with the C β probe.

Statistical analysis

A paired *t* test was used to compare the values of stimulation index before and after challenge.

RESULTS

Depletion of allergen-specific T lymphocytes after allergen challenge

Patients included in this study were challenged with the sensitizing allergens specified in Table I. They all had an EAR to the challenge, ranging from 19% to 35% fall of control (basal) value of FEV₁, and four of five had a measurable LAR (Table I). PD₂₀ values ranged from 39 to 212 AU of inhaled allergen (Table I). PI of peripheral

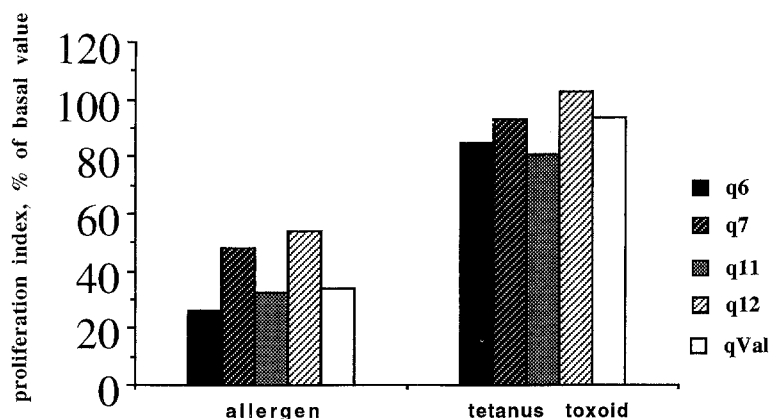


FIG. 2. PI (to allergen and TT) of CD4⁺ peripheral blood T cells taken from patients 24 hours after allergen challenge. Stimulation index is expressed as percent of value found before challenge; q6, q7, q11, q12, and qVal indicate patients. Allergens used for challenge and for determination of the stimulation index of CD4⁺ T cells were *Betula* (patients q6 and q12), *Olea* (patient q7), *Parietaria* (patient q11), and *Dermatophagoides* (patient qVal), respectively. Percent of basal PI to allergen was significantly lower than percent of basal PI to TT (mean value $\pm$ SD: 38.9% $\pm$ 11.7% vs 91.1% $\pm$ 8.7%, respectively; $p = 0.0007$).

CD4 T cells to the same allergen used for the challenge was measured in an initial sample taken just before the challenge and in a second sample taken 24 hours later. PI was significantly diminished 24 hours after the challenge, as compared with the value before challenge (mean value $\pm$ SD: 3.08 $\pm$ 1.2 vs 8.0 $\pm$ 2.3, respectively; $p = 0.004$). In contrast, PI of peripheral CD4 T cells to TT was not changed (mean value $\pm$ SD: 18.8 $\pm$ 8.6 vs 20.8 $\pm$ 10.2, respectively; $p = 0.12$) in the same samples. Individual results are expressed as ³H-thymidine incorporation and as PI in Table IV. When PIs in the samples taken 24 hours after challenge are expressed as a percent of PI in the respective prechallenge (baseline) samples, the percent of basal stimulation index to allergen was significantly lower than the percent of basal stimulation index to TT (mean value $\pm$ SD: 38.9% $\pm$ 11.7% vs 91.1% $\pm$ 8.7%, respectively; $p = 0.0007$) (Fig. 2).

Characterization of two *Dermatophagoides*-specific TCCs (TCC1 and TCC2)

In a 48-hour antigen-specific stimulation assay, TCCs derived from patient qVAL (TCC1 and TCC2) had PIs of 12.3 and 16.3, respectively. The cytokine profiles of both clones were compatible with a Th0/Th2 phenotype when they were stimulated with PHA-PMA. In contrast, stimulation with allergen yielded a strictly Th2 phenotype (Table V). TCRs of TCC1 and TCC2 were sequenced from the gene segments obtained by oligonucleotide-directed family-specific PCR amplification. Results are shown in Table VI.

Clonotypic determinants of TCC1 and TCC2 in peripheral blood and BAL samples

V β 6.2 and V β 22 gene segments from TCC1 and TCC2 were amplified, electrophoresed, and blotted on nylon membranes. In the PBMC and BAL samples, heteroduplex analysis of these gene segments yielded a smear type of pattern, with some emerging bands visible in the BAL samples taken after bronchial challenge

(Figs. 3 and 4, *top panels*). This is compatible with the polyclonality of T cells expressing these two V gene families, with single emerging clones in the BAL samples taken after challenge. The amount of total V β TCR DNA loaded in the lanes of samples taken before and after challenge were comparable (Figs. 3 and 4, *top panels*). Hybridization with N-region-specific probes yielded signals of the expected molecular weight in the lanes in which DNA from the two TCCs were loaded, respectively. Control samples (irrelevant PBMCs and BALs) were negative (Figs. 3 and 4, *top panels*). N-region-specific signals were also clearly visible in the prechallenge peripheral blood sample and in the two postchallenge BAL samples. In contrast, they were barely detectable in the postchallenge peripheral blood sample and in the two prechallenge BAL samples (Figs. 3 and 4, *top panels*). For both clones, the intensities of the N-region-specific signals were comparable in the two BAL samples taken after the challenge from two different lung segments (Figs. 3 and 4, *top panels*). Identity of peripheral blood-derived TCCs with BAL T cells was confirmed by direct sequencing of the N-regions from the respective PCR bands (N-region sequence is reported in the Methods section).

DISCUSSION

The main finding in this study is that single allergen-specific TCCs are increased in the lungs of allergic subjects with asthma on allergen challenge, whereas they are simultaneously decreased in peripheral blood. These data are compatible with the egress of these TCCs from the blood to the bronchial lumen after allergen challenge. In principle, these data could not be strictly allergen-specific.

We previously reported that oligoclonal expansion of single TCCs takes place in the BAL-derived T lymphocytes of subjects with asthma after allergen inhalation.¹⁸

TABLE V. Characterization of TCC1 and TCC2: lymphokine profile

	PHA + PMA (24 h)		Antigen (48 h)	
	IL-4 (pg/ml)	γ -IFN (pg/ml)	IL-4 (pg/ml)	γ -IFN (pg/ml)
TCC1	547	587	5840	<16
TCC2	657	894	4521	<16

Detection limit: 19 pg/ml for IL-4; 16 pg/ml for γ IFN. Antigen is *Dermatophagoides* extract, 20 μ g/ml. PHA, 10 μ g/ml; PMA, 100 nm.

In contrast, the baseline distribution of the lung TCR repertoire in both allergic subjects with asthma¹⁸ and control subjects without allergy¹⁹ is largely polyclonal. Other investigators observed the migration of total CD4⁺ T lymphocytes to the lungs in the model of provoked asthma.^{24, 25}

The decrease (24 hours after challenge) of the proliferation of peripheral CD4⁺ T lymphocytes specific for the allergen used in the bronchial provocation suggests the egress from circulation to the lung of allergen-specific T cells. Notably, proliferation to nonsensitizing allergens is not modified by bronchial provocation, indicating that not only the number of TT-specific T cells was left unaltered by allergen inhalation but also that sufficient numbers of efficient APCs were available. This result is in agreement with the relative numbers of CD14 monocytes and CD4 lymphocytes measured in three individuals before and after allergen challenge (Table III). Notably, the percentage of CD4 cells in cultures taken before challenge was lower than that in cultures taken after challenge (Table III). This is in agreement with previous reports^{24, 25} and might account, at least in part, for the observed lower proliferation in the samples taken after challenge. Moreover, TT-induced proliferation was reduced in four of five patients studied (Table IV). Although not statistically significant, this may reflect the fact that TT responses were much higher and therefore subject to greater variability when compared with allergen-induced responses.

In principle, this result could also depend on induction of allergy caused by TCR-mediated in vitro stimulation applied to specific T cells shortly after the in vivo challenge with the same allergen.²⁶ However, in further experiments performed in a panel of atopic subjects with asthma,²⁷ we observed that this phenomenon was still detectable in cultures "rested" in vitro for 7 days before addition of the antigen. This length of time has been shown to allow for recovery from hyporesponsiveness of in vitro activated T cells,^{28, 29} and it is widely used as a minimum time lag between restimulation in classical protocols for maintaining established TCCs.³⁰ On this basis we favor the hypothesis that this result can be explained by recruitment of T cells to the lung rather than by induction of hyporesponsiveness. In addition, we are confident that bronchial challenge per se does not induce changes in the PI of peripheral antigen-specific CD4⁺ T lymphocytes. In fact, the maximum observed

TABLE VI. Characterization of TCC1 and TCC2: TCR characterization

	V β	N-D-N					J β	V α
		GAA	TTA	TGG	CCT			
TCC1	22		Glu	Leu	Trp	Pro	2.6	17
TCC2	6.2	TTA	GCG	CCA	GCA	GCG	2.3	10
		Leu	Ala	Pro	Ala	Ala		

difference from the basal value of PI to sensitizing allergens, when the subjects with asthma included in this study were challenged with saline solution, was 16%. However, on the basis of our data we cannot exclude that T cells could have been inactivated by apoptosis or by retention in lymph nodes. Moreover, allergen challenge was performed with hypotonic saline solution at lower dilutions. We did not verify whether this could of itself result in reduction in the total or allergen-specific CD4 T-cell counts. However, we recently observed the reduction of peripheral blood allergen-specific proliferation to sensitizing, but not to nonsensitizing, allergen after an inhalation challenge²⁷ in subjects with asthma with different sensitization patterns. This makes it very unlikely that the aspecific effect of the inhalation procedure could per se induce the reduction of total or allergen-specific T-cell counts.

The lymphokine profiles of TCC1 and TCC2 are compatible with a classical Th2 phenotype when allergen stimulation is used to induce cytokine secretion. In contrast, a stronger and aspecific type of stimulation (PHA-PMA) induced a Th0 phenotype (Tables V and VI). This observation has been reported several times³¹ and indicates that Th2 lymphocytes are not intrinsically unable to produce Th1 lymphokines.

The results we observed with the two allergen-specific TCCs isolated from peripheral blood and traced back by N-region hybridization in BAL and peripheral blood samples taken at baseline and after allergen challenge deserve a few comments. First, PCR was first done in a quantitative way with oligonucleotides specific for the constant part of the TCR α gene to normalize the cDNA template content in the different samples (Fig. 1). Then V β -specific amplification followed with the standard (35 cycles) protocol.^{18, 19} The amount of total V family-specific DNA in the compared samples was similar (Figs. 3 and 4, *top*).²³ Second, this approach is semiquantitative and does not allow for the precise estimation of T-cell frequency in the compared samples (Burastero et al. Unpublished data). Despite this limitation, the difference we found in terms of N-region-specific signals was dramatically high between the samples taken before and after allergen challenge. This was true both for cells taken from BAL and for peripheral blood (Figs. 3 and 4, *bottom*). Therefore we are confident that the number of cells from these two clones is indeed greatly influenced in their compartmentalization by exposure to the sensitizing antigen. Third, formal proof of clonal identity between the peripheral blood-derived TCCs and those

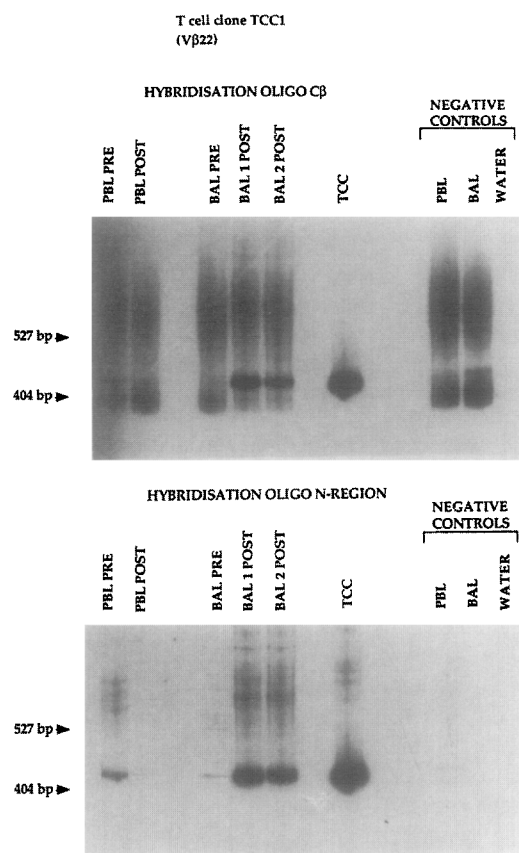


FIG. 3. Heteroduplex analysis of PCR-amplified gene segments from different samples hybridized with C β -specific (*top*) and TCC (N-region)-specific (*bottom*) oligonucleotides. V β family-specific PCR probes were used to amplify V β genes expressed by TCC1 (V β 22) from different samples (as indicated on top of lanes). The C β -specific oligonucleotide used for hybridization was 5'-TGCT-TCTGATGGCTCAA, and N-region-specific probes used for hybridization were designed according to the N-region TCR sequences of the β chain of TCC1 (see Methods and Table VI). TCC1 was used as a positive control. Water, PBMCs, and BAL from an irrelevant donor were included as negative controls. *PBL*, Peripheral blood lymphocyte.

traced in BAL and peripheral blood samples would imply isolation of cells from both sources. However, characterization of TCR clonotypes, where most of the TCR variability is localized, makes it very unlikely that TCCs with identical N-region sequences but different antigen specificity could be present in peripheral blood and BAL samples from patient qVAL by chance. On the other hand, the establishment of antigen-specific TCCs from BAL samples is extremely difficult. Fourth, it is noteworthy that the number of N-region-specific signals in the postchallenge BAL samples taken from two different lung segments is similar for both TCC1 and TCC2. This finding supports a previous observation of ours on the striking symmetry in the distribution of T-cell clonotypes in BAL samples taken after allergen challenge from different segments of the same or a contralateral lung of the same individual.¹⁸ Fifth, although a detectable proliferation of single allergen-

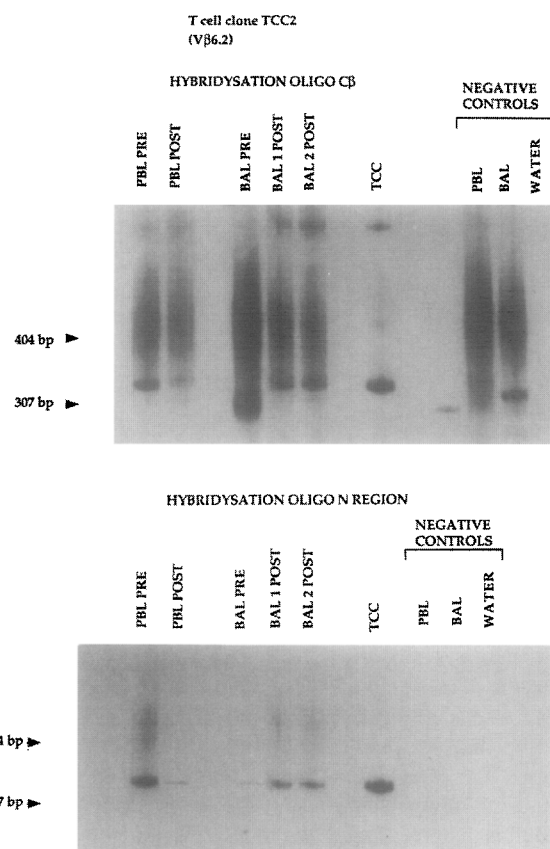


FIG. 4. Heteroduplex analysis of PCR-amplified gene segments from different samples hybridized with C β -specific (*top*) and TCC (N-region)-specific (*bottom*) oligonucleotides. V β family-specific PCR probes were used to amplify V β genes expressed by TCC1 (V β 22) from different samples (as indicated on top of lanes). The C β -specific oligonucleotide used for hybridization was 5'-TGCT-TCTGATGGCTCAA, and N-region-specific probes used for hybridization were designed according to the N-region TCR sequences of the β chain of TCC2 (see Methods and Table VI). TCC2 was used as a positive control. Water, PBMCs, and BAL from an irrelevant donor were included as negative controls. *PBL*, Peripheral blood lymphocyte.

specific TCCs in a 24-hour period seems unlikely, we cannot rule out the possibility that such a proliferation may play a role in maintaining and expanding the local T-cell response. Indeed, in an article presently in preparation, we could demonstrate that alveolar macrophages expressing the B7.1 costimulatory molecules can be identified in the lungs of subjects with asthma and that expression of this marker correlates with a higher capability to present soluble antigens, including allergens (Burastero et al. Unpublished data). Finally, we could not derive a TT-specific TCC to probe in parallel in BAL and in blood from patient qVal. This would have shown whether the phenomenon we describe is strictly antigen-specific or partly associated with a specific egress of CD4 T cells to the lung.

T lymphocytes represent a well-known component of allergic inflammation at disease sites. They not only contribute to the induction of IgE production by B

lymphocytes but also to the recruitment of eosinophils to target organs.^{32,33} Our results are compatible with the hypothesis that allergen exposure, besides triggering the EAR, can locally amplify allergen-specific T cells by recruiting them from peripheral blood in a clonotypic fashion, at times compatible with the development of the LAR to allergen inhalation. Notably, patient q7 did not have an LAR to allergen inhalation (Table I) but still displayed a reduction of PI on allergen inhalation (Fig. 2). This is compatible with a T-cell recruitment not quantitatively sufficient and/or qualitatively inadequate (e.g., low production of LAR-promoting cytokines) to trigger a functionally measurable bronchospastic reaction.

We have described a phenomenon compatible with a clonotypically specific recruitment of T lymphocytes. However, it is difficult to envisage a mechanism of clonotypic memory whereby signals carried by the TCR can make these T cells capable of recirculation to the lung. More reasonably, one can speculate that T cells that were primed in the lung by airborne antigens may acquire an array of adhesion molecules (homing receptors) that make them committed to recirculate to the lung. The induction of lung counterreceptors (likely on endothelial cells)¹¹ could explain the antigen-dependent triggering of this phenomenon. This was previously described in the case of atopic or contact dermatitis.³⁴ Indeed, we do not want to state that only antigen-specific T cells are recruited to the lung on challenge. In a recent article we showed that T lymphocytes specific for other sensitizing allergens are also depleted from the blood after inhalation of a single allergen.²⁷ However, T lymphocytes specific for nonsensitizing allergens (as is the case for TT-specific T cells in this investigation) were not depleted from the blood after challenge. Therefore the recruitment of allergen-specific TCCs is only one aspect of the overall picture emerging from our work on T-cell dynamics in subjects with asthma exposed to allergens. This picture is more compatible with an oligoclonal recruitment and possibly a local expansion of all Th2 cells, but not of T cells specific for nonallergenic molecules, than with a generalized egress of CD4 T cells.

In conclusion, these results, although extrapolated from a pathologic model, identify the lung immune system as a very efficacious system for rapid recruitment of a consistent number of antigen-specific T cells from the circulation. This most likely reflects the need to mount an efficacious immune response to inhaled pathogens in the absence of organized lymphoid tissue, which would hardly be compatible with an efficient alveolar gas exchange function.¹

It has been shown that in atopic individuals bronchial mucosa in rhinitis,³⁵ and even intestinal mucosa in asthma,³⁶ are sites of allergic inflammation with increased numbers of eosinophils and lymphocytes, despite not being sites of type I hypersensitivity reactions. It will be interesting to apply the approach of tracing TCCs at mucosal sites that we describe here to nontarget organs in sensitized individuals. This strategy should

shed light on the role of allergen-specific T cells and on the involvement of the whole mucosal immune system in allergy.

REFERENCES

1. Reynolds HY. Immunologic system in the respiratory tract. *Physiol Rev* 1991;71:1117-33.
2. Holt PG, Reynolds HY. Down-regulation of immune responses in the lower respiratory tract: the role of alveolar macrophages. *Clin Exp Immunol* 1986;63:261-70.
3. Saltini C, Kirby M, Trapnell BC, Tamura N, Crystal RG. Biased accumulation of T lymphocytes with "memory"-type CD45 leukocyte common antigen gene expression on the epithelial surface of the human lung. *J Exp Med* 1990;171:1123-40.
4. Saltini C, Hemler ME, Crystal RG. T lymphocytes compartmentalized on the epithelial surface of the lower respiratory tract express the very late activation antigen complex VLA-1. *Clin Immunol Immunopathol* 1988;46:221-33.
5. Harmsen AG, Muggenburg BA, Snipes MB, Bice DE. The role of macrophages in particle translocation from lungs to lymph nodes. *Science* 1985;230:1277-80.
6. Schon-Hegrad MA, Oliver J, McMenamin PG, Holt PG. Studies on the density, distribution, and surface phenotype of intraepithelial class II major histocompatibility complex antigen (Ia)-bearing dendritic cells (DC) in the conducting airways. *J Exp Med* 1991;173:1345-56.
7. Xia W, Pinto CE, Kradin RL. The antigen-presenting activities of Ia+ dendritic cells shift dynamically from lung to lymph node after an airway challenge with soluble antigen. *J Exp Med* 1995;181:1275-83.
8. Pabst R. Is BALT a major component of the human lung immune system? [see comments]. *Immunol Today* 1992;13:119-22.
9. Sprent J. Antigen-induced selective sequestration of T lymphocytes: role of the major histocompatibility complex. *Monogr Allergy* 1980;16:233-44.
10. Butcher EC. The regulation of lymphocyte traffic. *Curr Top Microbiol Immunol* 1986;128:85-122.
11. Butcher EC, Picker LJ. Lymphocyte homing and homeostasis. *Science* 1996;272:60-6.
12. National Asthma Education Program. Guidelines for treatment and management of asthma. Vol. 91. Bethesda (MD): National Heart, Lung, and Blood Institute; 1991. p. 3042.
13. American Thoracic Society. Chronic bronchitis, asthma and emphysema. *Am Rev Respir Dis* 1987;136:224-34.
14. Quanjer PH. Standardized lung function testing. Report on working party on standardization of lung function testing. European Community for Coal and Steel. *Bull Eur Physiopathol Respir* 1983;19:1-94.
15. Chomczynski P, Sacchi N. Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156-61.
16. Genevee C, Diu A, Nierat J, Caignard A, Dietrich PV, Ferradini L, et al. An experimentally validated panel of subfamily-specific oligonucleotides primers (V α 1-w29/V β 1-w24) for the study of human T cell receptor variable gene segment usage by polymerase chain reaction. *Eur J Immunol* 1992;22:1261-71.
17. Choi Y, Kotzin B, Herron L, Callahan J, Marrack P, Kappler J. Interactions of *Staphylococcus aureus* superantigens with human T cells. *Proc Natl Acad Sci U S A* 1989;243:217-27.
18. Burastero SE, Crimi E, Balbo A, Vavassori M, Borgonovo B, Gaffi D, et al. Oligoclonality of lung T lymphocytes following exposure to allergen in asthma. *J Immunol* 1995;155:5836-46.
19. Burastero SE, Borgonovo B, Gaffi D, Frittoli E, Wack A, Rossi GA, et al. The repertoire of T-lymphocytes recovered by bronchoalveolar lavage from healthy nonsmokers. *Eur Respir J* 1996;9:319-27.
20. Casanova J, Pannetier C, Jaulin C, Kourilski P. Optimal conditions for directly sequenced double-stranded PCR products with Sequenase. *Nucleic Acids Res* 1990;18:4028.
21. Rabbitts TH, Lefranc MP, Stinson MA, Sims JE, Schroder J,

- Steinmetz M, et al. The chromosomal location of T-cell receptor genes and a T cell rearranging gene: possible correlation with specific translocations in human T cell leukemia. *EMBO J* 1985;4:1461-5.
22. Wang AM, Mark DF. Quantitative PCR. In: PCR protocols. A guide to methods and applications. Vol. 1. Oxford: Oxford University Press; 1990. p. 70-5.
 23. Wack A, Montagna D, Dellabona P, Casorati C. An improved PCR-heteroduplex method permits high-sensitivity detection of clonal expansions in complex T cell populations. *J Immunol Methods* 1996;196:181-92.
 24. Gerblich AA, Campbell AE, Schuyler MR. Changes in T-lymphocyte subpopulations after antigenic bronchial provocation in asthmatics. *N Engl J Med* 1984;310:1349-52.
 25. Gerblich AA, Salik H, Schuyler MR. Dynamic T-cell changes in peripheral blood and bronchoalveolar lavage after antigen broncho-provocation in asthmatics. *Am Rev Respir Dis* 1991;143:533-7.
 26. Yssel H, Fasler S, Lamb J, de Vries JE. Induction of non-responsiveness in human allergen-specific type 2 T helper cells. *Curr Opin Immunol* 1994;6:847-52.
 27. Crimi E, Gaffi D, Frittoli E, Borgonovo B, Burastero SE. Depletion of circulating allergen specific T-h2 T lymphocytes after allergen exposure in asthma. *J Allergy Clin Immunol* 1997;99:788-97.
 28. Schwartz RH. A cell culture model for T lymphocyte clonal anergy. *Science* 1990;248:1349-56.
 29. LaSalle JM, Tolentino PJ, Freeman GJ, Nadler LM, Hafler DA. Early signaling defects in human T cells anergized by T cell presentation of autoantigen. *J Exp Med* 1992;176:177-86.
 30. Del Prete GF, De Carli M, D'Elios MM, Maestrelli P, Ricci M, Fabbri L, et al. Allergen exposure induces the activation of allergen-specific Th2 cells in the airway mucosa of patients with allergic respiratory disorders. *Eur J Immunol* 1993;23:1445-9.
 31. Yssel H, Johnson KE, Schneider PV, Wideman J, Terr A, Kastelein R, et al. T cell activation-inducing epitopes of the house dust mite allergen Der p I. Proliferation and lymphokine production patterns by Der p I-specific CD4+ T cell clones. *J Immunol* 1992;148:738-45.
 32. Robinson DS, Hamid Q, Ying S, Tsiopoulos A, Barkans J, Bentley AM, et al. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med* 1992;326:298-304.
 33. Robinson D, Hamid Q, Ying S, Bentley A, Assoufi B, Durham S, et al. Prednisolone treatment in asthma is associated with modulation of bronchoalveolar lavage cell interleukin-4, interleukin-5, and interferon-gamma cytokine gene expression. *Am Rev Respir Dis* 1993;148:401-6.
 34. Santamaria Babi LF, Moser R, Perez Soler MT, Picker LJ, Blaser K, Hauser C. Migration of skin-homing T cells across cytokine-activated human endothelial cell layers involves interaction of the cutaneous lymphocyte-associated antigen (CLA), the very late antigen-4 (VLA-4), and the lymphocyte function-associated antigen-1 (LFA-1). *J Immunol* 1995;154:1543-50.
 35. McFadden ER Jr, Gilbert IA. Exercise-induced asthma. *N Engl J Med* 1994;330:1362-7.
 36. Wallaert B, Desreumaux P, Copin MC, Tillie I, Benard A, Colombel JF, et al. Immunoreactivity for interleukin 3 and 5 and granulocyte/macrophage colony-stimulating factor of intestinal mucosa in bronchial asthma. *J Exp Med* 1995;182:1897-904.