

Allergens, IgE, mediators, inflammatory mechanisms

Preferential recruitment of activated, memory T lymphocytes into skin chamber fluids during human cutaneous late-phase allergic reactions

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To determine whether activated memory T lymphocytes ($CD3^+CD4^+CD45RO^+CD25^+HLA-DR^+$) preferentially accumulate during the cutaneous IgE-dependent late-phase response, we challenged 10 atopic patients with allergen and monitored the cellular influx for 12 hours with a skin blister chamber model. Four patients with allergy were also challenged with irrelevant allergen in control experiments. Histamine release and the size of the cutaneous late-phase response were measured. Light microscopic analysis and phenotyping of recruited and blood lymphocytes were performed with immunofluorescence and flow cytometry. Antigen challenge induced significant histamine release, a macroscopic late-phase response, and significant cellular influx in the appropriately challenged patients with allergy but not in control subjects. In the control group, only limited phenotypic analyses could be performed, which demonstrated an equivalent percentage of $CD3^+CD4^+$ cells in the chamber fluids compared with blood. In contrast, a higher proportion of $CD4^+$ T lymphocytes and a lower proportion of $CD8^+$ T lymphocytes accumulated in chamber fluids during the late-phase response compared with that present in blood. The vast majority of the recruited T lymphocytes expressed a memory phenotype ($CD45RO^+$) with enhanced percentages of $CD25^+$ and $HLA-DR^+$ cells. Also, these cells had increased levels of very late antigen-4 ($CD49d/CD29$) and reduced levels of L-selectin compared with the same cells in blood. These results demonstrate that allergen challenge of the skin in patients with allergy leads to the preferential accumulation of activated, memory T lymphocytes. The mechanism by which these cells are selectively recruited during cutaneous allergic inflammation remains to be determined. (J ALLERGY CLIN IMMUNOL 1995;96:57-65.)

Key words: Lymphocyte homing, memory T lymphocyte, skin chamber, late-phase response, cutaneous allergic inflammation, CD25, HLA-DR, CD45RO

Intradermal allergen challenge of patients with allergy frequently results in a biphasic reaction that is clinically characterized by a pruritic wheal-and-

flare response within 15 minutes and a late-phase response (LPR), consisting of erythema, induration, pruritus, and tenderness, which peaks between 8 and 12 hours and generally resolves within 24 hours.¹ The LPR is IgE-dependent, and histologic analysis reveals a mixed cellular infiltrate enriched with eosinophils, basophils, and mononuclear cells.^{2,3} The LPR, which occurs after allergen challenge in the skin and lungs,^{4,5} has become a useful model for the study of the inflammatory disease processes occurring in chronic allergic inflammation, because there are many similarities in the pattern of mediator release and cell influx in these responses.

Recently, the role of T-lymphocyte infiltration in

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Abbreviations used

CLA:	Cutaneous lymphocyte antigen
FITC:	Fluorescein isothiocyanate
IL:	Interleukin
LPR:	Late-phase response
mAb:	Monoclonal antibody
MFI:	Mean fluorescence intensity
PE:	Phycoerythrin
PNU:	Protein nitrogen units
RL:	Ringer's lactate solution
RLH:	Ringer's lactate solution containing 30 U/ml heparin
VCAM:	Vascular cell adhesion molecule
VLA:	Very late antigen

allergic and other dermatologic reactions has been the subject of numerous studies, in part because these cells are able to produce proinflammatory mediators including cytokines and therefore may play an important role in modulating the disease process. T-lymphocyte infiltration is found in a variety of different inflammatory skin diseases. For example, CD4⁺ T lymphocytes are found to be the dominant cell type in purified protein derivative-induced delayed-type hypersensitivity lesions in human skin.^{6,7} The same cell type predominates in biopsy specimens from both involved and uninvolved psoriatic skin^{8,9} and in lesional skin from patients with atopic dermatitis.^{10,11} How these CD4⁺ lymphocytes are preferentially recruited is unknown, but it is likely that selective lymphocyte recruitment is influenced by chemoattractants and various adhesion molecules expressed on lymphocytes and within local tissue sites.¹²⁻¹⁵

Several studies show that CD4⁺CD45RO⁺ lymphocytes move into inflamed tissue *in vivo*.¹⁴ The CD45RO marker is believed to represent the so-called memory CD4⁺ T lymphocyte phenotype, whereas naive T lymphocytes express the CD45RA antigen. The different isoforms of CD45 are the result of alternative splicing of three exons encoding for the extracytoplasmic domain of the molecule.¹⁶ CD4⁺CD45RO⁺ cells show an overall increase in expression and function of adhesion molecules, including β 1 integrins.¹⁷ Indeed, 80% to 90% of the infiltrating T lymphocytes found in most chronic dermatoses are CD45RO⁺.⁷ After intradermal allergen injection, analysis of skin biopsy specimens at 24 and 48 hours revealed that the majority of CD3⁺ cells were CD4⁺ and expressed an increased level of interleukin (IL)-2 receptors (CD25) and HLA-DR antigens.¹⁸ We

therefore hypothesized that T lymphocytes expressing the activated, memory phenotype are selectively recruited during the IgE-dependent cutaneous LPR. To test this hypothesis, the mononuclear cells accumulating in skin chamber fluids during the 12-hour period after allergen challenge were characterized. Our findings demonstrate that activated T lymphocytes of the helper-memory type are preferentially recruited during the allergen-induced LPR.

METHODS

Reagents and monoclonal antibodies

Reagents were obtained from the sources indicated: pyrogen-free Ringer's lactate solution (RL) and dextran (Kendall McGaw Laboratories, Irvine, Calif.), RPMI-1640 medium, L-glutamine, penicillin, and streptomycin (Gibco Laboratories, Grand Island, N.Y.), fetal bovine serum (Hyclone Laboratories, Inc., Logan, Utah), heparin (Elkin-Sinn, Cherry Hill, N.J.), phosphate-buffered saline (Quality Biologicals Inc., Gaithersburg, Md.), bovine serum albumin, erythrosin B dye, and paraformaldehyde (Sigma Chemical Co., St. Louis, Mo.).

Monoclonal antibodies (mAbs) directly conjugated with the fluorochromes fluorescein isothiocyanate (FITC) or phycoerythrin (PE) were purchased from the sources indicated: CD2-FITC (IgG_{2a}), CD3-PE (IgG₁), CD4-FITC (IgG₁), CD8-FITC (IgG₁), CD45-FITC (IgG₁), CD45RO-PE (IgG₁), CD45RA-FITC (IgG₁), CD25-FITC (IgG_{2a}), HLA-DR-PE (IgG_{2a}), and Leu-8-PE (anti-L-selectin, IgG_{2a}) (Becton-Dickinson, Mountain View, Calif.); CD14-PE (IgG_{2a}), CD19-PE (IgG₁), and very late antigen (VLA)-4-PE (IgG₁) (AMAC, Inc., Westbrook, Maine); CD56-PE (IgG₁) (Coulter Corp., Hialeah, Fla.). Isotype-matched control mAbs were also purchased (Becton-Dickinson, Coulter).

Allergens

Stock solutions of ragweed, oak, and mixed grass (timothy and orchard) diluted in saline solution with 0.3% human serum albumin and 1% phenol were obtained from Miles Pharmaceuticals (West Haven, Conn.) in concentrations of 100,000 to 150,000 protein nitrogen units (PNU)/ml. The same solutions used for skin testing were also used for allergen challenge. Before allergen challenge, stock solutions were diluted in RL and were used at final concentrations ranging from 0.1 to 1000 PNU/ml, depending on skin test sensitivity (see below).

Subjects

The study group consisted of 14 adult atopic subjects (7 men and 7 women) ranging in age from 26 to 52 years. At the time of the study, none were receiving any medications. Before entry into the study, all subjects signed a consent form, and the protocol was approved by the Francis Scott Key Institutional Review Board for

Human Subjects Research. All subjects were previously screened for immediate wheal-and-flare skin test reactions to ragweed, mixed grass, or oak. A concentration of antigen known to induce an LPR was used (ranging from 100 to 1000 PNU/ml) at four separate chamber challenge sites simultaneously so that adequate numbers of recruited cells would be available for study. Preliminary studies with saline control challenges yielded an insufficient number of lymphocytes for cell surface marker analysis. However, challenge of patients with allergy with an irrelevant antigen (at a concentration of 1000 PNU/ml), to which the subject had no skin test reactivity, permitted a limited analysis of a few markers in some patients. A total of 10 subjects were challenged with relevant allergen, and four were challenged with irrelevant allergen. Induration and flare diameters were measured and recorded hourly.

Blister formation and allergen challenge

Approximately 12 to 18 hours before the allergen challenge was performed, four blisters were induced by gentle heat and suction on the anterior aspect of both forearms as described previously.^{19,20} The bullae were left overnight and unroofed the next morning. Sterile aluminum chambers with entry and exit ports were attached over the denuded dermis,²⁰ and chambers were washed twice with 0.5 ml RL containing 30 U/ml heparin (250 µg/ml, RLH). The challenges of the blister bases with relevant allergen were performed at a concentration 10 times the "optimal challenge dose," ranging from 100 to 1000 PNU/ml. The optimal challenge dose was previously defined as the allergen concentration inducing a wheal of 15 mm or greater in diameter (or 10 mm with pseudopods) 15 minutes after intradermal injection on the upper arm and an induration greater than 30 mm in diameter at 8 hours and has been used to study allergen-induced cell influx in this model.^{20,21} The four atopic subjects in the control group were challenged with a concentration of 1000 PNU/ml of irrelevant allergen at each of the four blister sites. Antigen extract (500 µl) was introduced into each separate blister site for the cutaneous challenge and was allowed to remain in the chamber for 60 minutes. The fluid overlying the denuded dermis was collected hourly for 12 hours, and each time was replaced with 500 µl of fresh RLH. Because preliminary studies demonstrated no effect of skin chamber challenge on the percentages and phenotype of circulating T lymphocytes at several different time points throughout the 12-hour study, blood sampling was performed by venipuncture at hour 9 for comparison of cells with those obtained from blister chamber sites. Blood leukocytes were isolated by dextran sedimentation, and any remaining red blood cells were removed by hypotonic lysis.²²

Analysis of blister fluid mediators and cytologic findings

Collected fluid was centrifuged (3000 rpm, 5 minutes), and the cell pellet was resuspended in 300 µl of RPMI

supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin and stored at 4° C. The blister fluid obtained at each hour was stored at -70° C and assayed for histamine levels with a radioenzymatic assay (sensitivity: 0.05 ng/ml²³). Viability was determined by erythrosin B dye exclusion, and cell counts were performed with a hemocytometer and light microscopy. To generate a sufficient number of cells for analysis, four separate blister chambers were challenged simultaneously, and cells from hourly washes were combined into two pools for control subjects (hours 1 through 6 and hours 7 through 12) or three pools for patients with allergy challenged with relevant allergen (hours 1 through 4, hours 5 through 8, and hours 9 through 12). This approach also permitted a rough analysis of the kinetics of T-lymphocyte recruitment, although the number of surface markers studied was limited by the total numbers of cells that could be recovered from the chamber fluids. Cyto centrifugation slides were prepared (Shandon Southern Instruments, Inc., Sewickley, Pa.) and staining with Diff Quik, a modified Wright-Giemsa stain (American Scientific Products, MacGraw Park, Ill.), was done as previously described.²¹ A differential count of 200 cells was performed by two blinded investigators.

Flow cytometry

To analyze the phenotype of recruited T lymphocytes and those in peripheral blood, cell surface antigens were labeled with FITC- and PE-conjugated mAbs. Blood and pooled blister chamber cells were analyzed by dual-color flow cytometry (EPICS Profile flow cytometer; Coulter Corp.) after excitation at 488 nm. At least 30,000 cells per tube per mAb were stained, and at least 500 events per sample were analyzed. Percentages and mean fluorescence intensity (MFI) for surface molecules were determined on lymphocytes after appropriate gating on the basis of light scatter characteristics.²⁴ Fluorescent signals were compared with those observed with fluorochrome-conjugated isotype-matched irrelevant antibody.

Statistical analysis

Data are presented as means ± SEM. Nonparametric analysis (Wilcoxon signed-rank test) was applied to determine significant differences between the blister chamber pools and blood. Nonparametric Spearman's rank correlation was used to test for correlations between various cellular and clinical measurements. The Mann-Whitney U test was done to describe significant differences between results from the control and allergen-challenged groups of subjects.

RESULTS

All allergic patients challenged with relevant allergen ($n = 10$) had an early wheal-and-flare response followed by late-phase induration. Maxi-

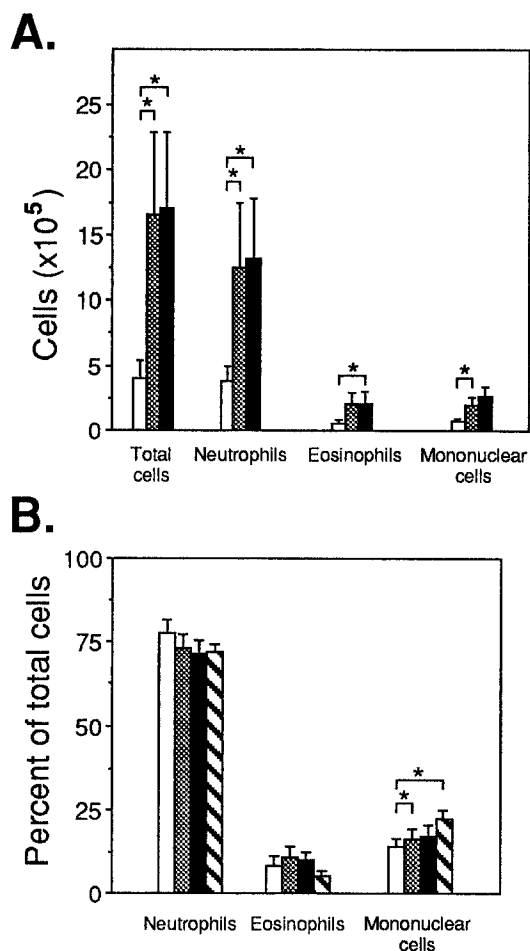


FIG. 1. Total cell counts (**A**) and percentages of cells (**B**) in chamber fluids from allergen-challenged sites pooled from hours 1 to 4 □, hours 5 to 8 ▨, hours 9 to 12 ■, and in peripheral blood ▤ ($n = 10$). Data are presented as means $\pm$ SEM. In the flow cytometry experiments, in which we used mAbs, there was not always enough leukocytes recruited into the skin to study all markers in every patient, so the number varies between 6 and 10. * $p < 0.05$; ** $p < 0.002$.

mal mean induration diameter was reached by hours 6 to 7 (44 ± 3 mm, mean $\pm$ SEM). Histamine levels measured hourly in the blister fluid peaked sharply during the first hour (39 ± 6 ng/ml) and then fell to a nadir by hours 5 to 6. Only two subjects showed a secondary increase in histamine during the LPR hours 6 to 12. None of the control subjects (patients with allergy challenged with irrelevant allergen, $n = 4$) had a wheal-and-flare response or demonstrated histamine release (data not shown).

The accumulation of cells in the chamber fluid sites after allergen challenge was evaluated by light microscopy and is reported as total cell numbers (Fig. 1, A) and percentages for each cell type (Fig. 1, B). Subjects challenged with relevant antigen

showed an influx of leukocytes into the chamber fluids (hours 1 to 4: $4.1 \pm 1.3 \times 10^5$ total cells; hours 5 to 8: $16.5 \pm 6.3 \times 10^5$; hours 9 to 12: $17.1 \pm 5.8 \times 10^5$, $n = 10$). Significantly more cells accumulated during the later periods (hours 1 to 4 versus hours 5 to 8, $p < 0.007$; hours 1 to 4 versus hours 9 to 12, $p < 0.02$, $n = 10$). Control challenged subjects had significantly lower total cell counts in pooled chamber fluids (hours 1 to 6: $0.8 \pm 0.5 \times 10^5$ and hours 7 to 12: $1.5 \pm 0.7 \times 10^5$, $n = 4$) for the entire 12-hour period compared with those challenged with relevant allergen ($p < 0.005$).

As with the total leukocytes, the total number of neutrophils in pooled chamber fluids from the group challenged with relevant antigen increased significantly during the later periods (hours 1 to 4 versus hours 5 to 8, $p = 0.04$; hours 1 to 4 versus hours 9 to 12, $p < 0.02$, $n = 10$). The percentages of neutrophils did not change significantly during the 12-hour study and were not different from their percentages in peripheral blood. In control challenged subjects neutrophils were $68\% \pm 10\%$ in pools obtained during hours 1 to 6 and $56\% \pm 11\%$ in pools obtained during hours 7 to 12. The total number of eosinophils recruited over the 12-hour period was greater at sites challenged with relevant allergen than at those challenged with irrelevant allergen (relevant allergen group, $4.6 \pm 1.6 \times 10^5$, $n = 10$, versus control group $0.06 \pm 0.06 \times 10^5$, $n = 4$, $p = 0.007$). The percentage of eosinophils was greater at sites challenged with relevant allergen than at those challenged with irrelevant allergen for the 12-hour study ($p < 0.02$). The percentage of eosinophils at sites challenged with relevant allergen was also greater than that in peripheral blood, although this did not reach statistical significance. The percentage of mononuclear cells at sites challenged with relevant allergen was significantly lower than that in blood during hours 1 to 4 and increased significantly from hours 1 to 4 and 5 to 8 (Fig. 1, B). The total number of mononuclear cells accumulating over 12 hours at the sites challenged with relevant allergen was higher than that observed at control challenged sites (relevant allergen group: $5.4 \pm 1.2 \times 10^5$ versus control group: $0.9 \pm 0.5 \times 10^5$, $p < 0.02$). For mononuclear cells, the percentages in cell pools from sites challenged with relevant allergen (hours 5 to 8: $14\% \pm 2.6\%$; hours 9 to 12: $17\% \pm 3\%$, $n = 10$) were significantly lower than those in cell pools from control challenged sites (hours 1 to 6: $31\% \pm 10\%$ and hours 7 to 12: $43\% \pm 12\%$, $n = 4$, $p < 0.05$).

The skin chamber fluids from hours 5 to 8 and 9

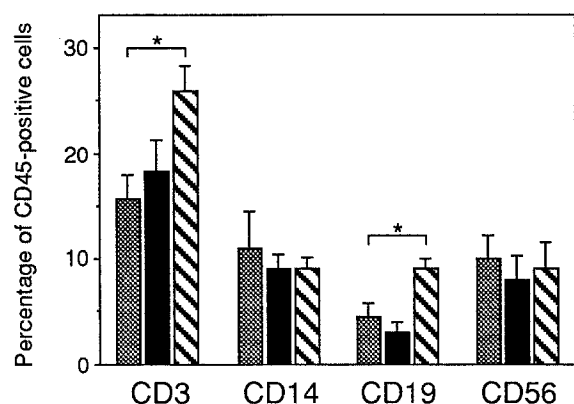


FIG. 2. Co-expression of CD3, CD14, CD19, or CD56 on CD45⁺ cells in chamber fluids from allergen-challenged sites pooled from hours 5 to 8 (■), hours 9 to 12 (■), and in peripheral blood (▨) ($n \geq 7$); * $p < 0.05$ for CD3 and CD19.

to 12 yielded consistently sufficient numbers of cells to permit further characterization of the mononuclear cells by immunofluorescence and flow cytometry. The number of mAbs studied at different time points in individual subjects varied depending on the number of leukocytes recruited into the cutaneous allergen challenge sites. Leukocytes (CD45⁺ cells) were examined for co-expression of CD3 (T lymphocytes), CD14 (monocytes), CD19 (B lymphocytes), and CD56 (natural killer cells). Although CD3⁺CD45⁺ cells dominated the three other cell types in blister fluid, the percentage of CD3⁺ leukocytes in blister fluid was consistently less than that in peripheral blood. The percentage of B lymphocytes (CD19) in blister fluid was also less than that in peripheral blood (Fig. 2).

Additional gating was performed by combining fluorochrome-conjugated CD2 or CD3 antibodies with conjugated antibodies recognizing CD4, CD8, CD45RO, CD45RA, CD25, or HLA-DR to determine the percentages of T lymphocytes representing activated, memory cells. A greater percentage of CD4⁺ than CD8⁺ T lymphocytes was seen in both blood and chamber fluids. However, the percentages of CD4⁺ cells were significantly higher in chamber fluids than in peripheral blood (hours 5 to 8 versus blood, $p < 0.005$; hours 9 to 12 versus blood, $p < 0.02$). In contrast, the percentages of CD8⁺ cells were significantly lower in blister fluids versus blood ($p < 0.05$ for both comparisons) (Fig. 3, A). A greater percentage of T lymphocytes in blood expressed CD45RA (naive phenotype) than CD45RO (memory phenotype) (Fig. 3, B). In contrast, approximately 90% of skin-infiltrating T lymphocytes expressed CD45RO, so that the percentages of CD45RO⁺

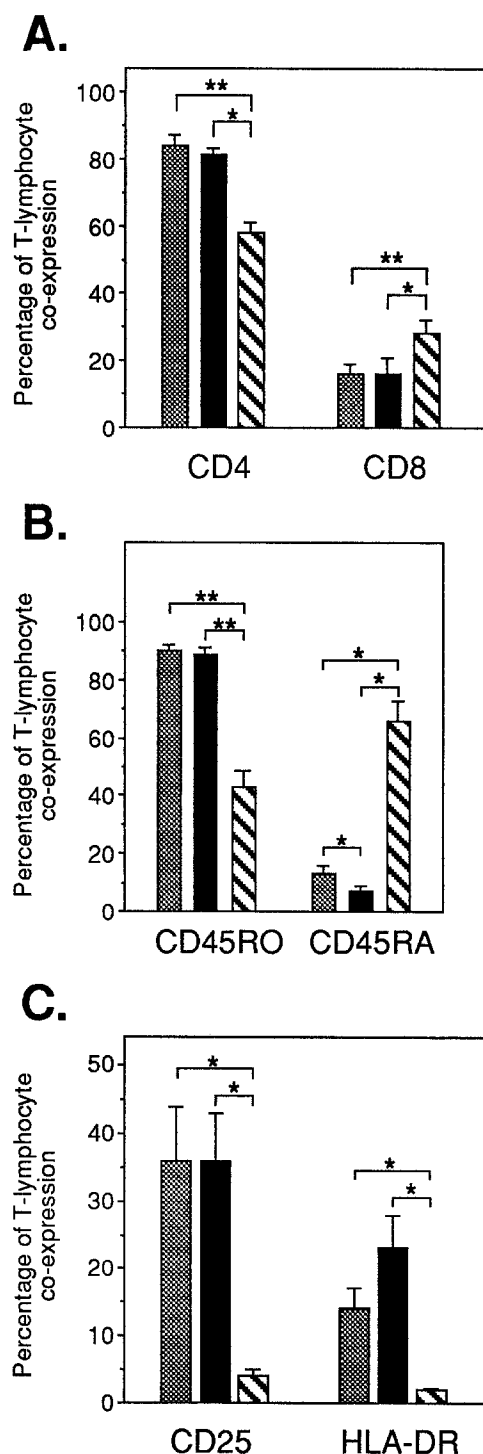


FIG. 3. T-lymphocyte co-expression of CD4 or CD8 (A), CD45RO or CD45RA (B), and CD25 or HLA-DR (C) in chamber fluids from allergen-challenged sites pooled from hours 5 to 8 (■), hours 9 to 12 (■), and in peripheral blood (▨) ($n \geq 6$); * $p < 0.05$, ** $p < 0.002$.

and CD45RA⁺ cells were significantly different than those in peripheral blood. That the CD45RO⁺ cells represented memory T lymphocytes was further supported by the finding of

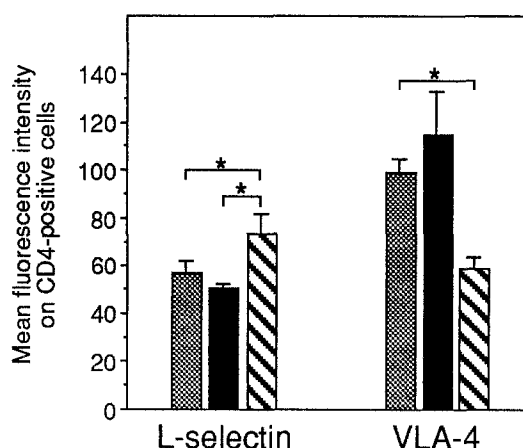


FIG. 4. Comparison of levels of expression of L-selectin and VLA-4 α (CD49d) on T lymphocytes in chamber fluids from allergen-challenged sites pooled from hours 5 to 8 (■), hours 9 to 12 (■), and in peripheral blood (▨) ($n \geq 6$); * $p < 0.05$.

significantly higher percentages of T lymphocytes in chamber fluids expressing CD25 and HLA-DR compared with peripheral blood (Fig. 3, C). Additionally, the level of expression of CD25, characterized by the MFI of the fluorochrome marker, was significantly higher on skin blister T lymphocytes than on blood T lymphocytes (MFI for cells in hours 5 to 8: 10 ± 0.8 ; hours 9 to 12: 9 ± 1.5 ; blood: 3.2 ± 0.4 , $n = 6$, $p < 0.05$ for both comparisons). The phenotypes of infiltrating T lymphocytes in chamber fluid pools from hours 5 to 8 and 9 to 12 were identical, except for a slightly higher percentage of CD45RA $^{+}$ cells in the earlier pool (Fig. 3, B). Finally, two additional markers of T-lymphocyte activation were examined. VLA-4 α (CD49d) was expressed by more than 90% of CD4 $^{+}$ cells in chamber fluids and blood (data not shown). However, the levels of expression were higher on skin than on blood lymphocytes (hours 5 to 8 versus blood, $n = 7$, $p = 0.02$) (Fig. 4). In contrast, levels of L-selectin were significantly lower on skin than on blood lymphocytes ($p < 0.05$ for both pools, Fig. 4).

Because of the limited numbers of cells at the control challenged sites, only T-lymphocyte CD4 expression was examined. In contrast to what was seen at sites challenged with relevant allergen, the percentages of CD4 $^{+}$ T lymphocytes in the sites challenged with irrelevant allergen were lower, but still higher than in peripheral blood in this group (hours 1 to 7: $58\% \pm 3.8\%$, 8 to 12: $62\% \pm 7\%$, blood: $37\% \pm 1.9\%$, $n = 3$).

DISCUSSION

Using a blister chamber system, we have studied the cutaneous LPR for a 12-hour period after allergen challenge. The allergen-induced immediate reaction and LPR were verified macroscopically by the presence of an immediate wheal-and-flare response, late induration, and erythema and by evidence of histamine release, findings that were not observed in control patients with allergy challenged with irrelevant allergen. The total number of cells recovered over the 12-hour period was approximately 16 times higher in patients with allergy challenged with relevant allergen compared with control subjects. The influx of many different cell types was observed in both groups of challenged subjects, but only in the appropriately allergen-challenged group was eosinophil recruitment prominent. Previous studies have shown that the cytologic findings of the blister fluid resemble the histologic findings of the blister base.²¹

We have focused on the accumulation of lymphocytes during this inflammatory reaction. The mononuclear cell response was compared with the response elicited in control patients with allergy by irrelevant allergen challenge. In addition, the percentages and phenotype of recruited cells were compared with populations of T lymphocytes present in peripheral blood. It is interesting to note that the percentages of CD14 $^{+}$ and CD56 $^{+}$ cells were similar in blood and in blister fluids, whereas the numbers of CD3 $^{+}$ and CD19 $^{+}$ cells were significantly higher in peripheral blood (Figs. 1 and 2). These data suggest that the mechanisms involved in recruitment of T and B lymphocytes may differ from that of monocytes and natural killer cells, especially at earlier time points. The percentages of mononuclear cells recruited to allergen-challenged blister sites have been shown to range from below 10% to as high as 40%,^{1,25} which is consistent with results presented here. In our study among the mononuclear cells examined, CD3 $^{+}$ T lymphocytes represented the largest subpopulation, regardless of the hourly pool studied (Fig. 2). CD4 $^{+}$ cells were the predominant T-lymphocyte type in both blood and blister fluids, although the percentage of CD4 $^{+}$ T lymphocytes in blister chamber fluids was significantly higher than that in peripheral blood, whereas the percentage of CD8 $^{+}$ T lymphocytes was significantly lower than that in blood (Fig. 3). Approximately 90% of the T lymphocytes recruited to the skin co-expressed CD45RO, a surface marker associated with the memory phenotype; very few naive T lymphocytes (CD45RA $^{+}$) could be identified, which is consis-

tent with a previous report.²⁶ In this study the accumulation of eosinophils was not preceded by an influx of memory T lymphocytes. In fact, both cell types accumulated with a similar time course. These data therefore suggest that recruitment of activated T lymphocytes may not be a prerequisite for eosinophil recruitment into the LPR in skin chambers. A significantly higher percentage of T lymphocytes from skin chamber fluids expressed the activation markers CD25 and HLA-DR than those in peripheral blood. Levels of expression of VLA-4 α (CD49d) and L-selectin were significantly altered on cells recovered from skin chambers (the former being increased, the latter being significantly decreased compared with their expression on peripheral blood T lymphocytes, Fig. 4).

The finding of a predominant CD4⁺ population among recruited T lymphocytes is consistent with previous reports demonstrating preferential recruitment of this cell type into the airways of patients with asthma^{24, 27} and during the LPR in the lungs.²⁸ In some of these studies, especially those involving lung challenge, a transient decrease in the number of circulating CD4⁺ T lymphocytes was seen before their influx into the lungs.²⁹ In our study a decrease in numbers of CD4⁺ T lymphocytes in blood was not seen (data not shown). This might be because a lower dose of antigen and a lower surface area of allergen challenge were used in this study. Our results also agree with those of Frew and Kay,¹⁸ demonstrating a higher percentage of cutaneous lymphocytes that express the activation markers CD25 and HLA-DR. One additional aspect of our study was that by pooling cells from various periods, we were able to analyze the kinetics of recruitment of T lymphocytes. Interestingly, cells pooled from hours 5 to 8 and hours 9 to 12 were essentially identical in both total number and cell surface marker expression. These data suggest that the same type of leukocyte was being recruited throughout the study.

Induction of CD25 and HLA-DR expression occurs within 24 to 48 hours of activation *in vitro*,³⁰ and conversion of T lymphocytes from the naive to the memory phenotype takes 3 to 6 days.¹⁶ Therefore our data strongly suggest that the T-lymphocyte phenotype observed in skin chamber fluids is the result of selective recruitment of this subpopulation rather than activation during the recruitment process *per se*. Indeed, this activated, memory subpopulation of T lymphocytes has been reported to have an enhanced capacity to migrate from blood into tissues compared with naive, resting T cells^{31, 32} and to have increased surface levels

of many β 1 integrins, including VLA-3, VLA-4, VLA-5, and VLA-6, as well as the β 2 integrin leukocyte function associated antigen-1.³³ In addition, CD25⁺ T lymphocytes are found in increased numbers in the lungs of patients with sarcoidosis³⁴ and in both blood and bronchoalveolar lavage fluids during acute severe asthma attacks where levels of this marker correlate with asthma severity.^{24, 27, 35}

The biologic significance of enhanced expression of HLA-DR, VLA-4, and CD25 on skin-infiltrating lymphocytes remains speculative. VLA-4 (α 4 β 1, CD49d/CD29) is a ligand for the cytokine-inducible vascular cell adhesion molecule-1 (VCAM-1) and the alternatively spliced CS-1 region of fibronectin³⁶ and therefore may play a role in adhesion and recruitment. Issekutz³⁷ has shown that blockade of VLA-4 in the rat inhibits antigen-stimulated migration of peripheral lymphocytes into inflamed or cytokine-injected skin sites. Studies in guinea pigs have shown that VLA-4-dependent eosinophil recruitment can occur very rapidly in response to chemotactic factors, presumably under conditions in which VCAM-1 expression is absent.³⁸ Whether this is due to interaction of VLA-4 with fibronectin was not determined. In human beings VCAM-1 can be detected at sites of cutaneous allergen challenge,³⁹ but it is expressed relatively late, and therefore its role in mediating lymphocyte (and eosinophil) recruitment into the cutaneous LPR remains unclear.

Levels of L-selectin were reduced on lymphocytes harvested from skin chamber fluids. L-selectin is an adhesion molecule that mediates binding of T lymphocytes and other cells under conditions of shear stress.⁴⁰ Ligands include GlyCAM-1 and CD34, both of which are present on lymph node postcapillary venules.^{41, 42} The exact ligand for L-selectin on postcapillary venule endothelium remains unknown. Expression of L-selectin has been shown to decline during cell activation both *in vitro* and *in vivo*.^{43, 44} It is presumed that the shedding of L-selectin is a prerequisite for cell emigration and permits leukocyte extravasation. Whether a lower level of L-selectin expression on cells recruited into the skin is due to the recruitment of cells expressing lower levels of the molecule or whether the molecule is shed during emigration into the skin cannot be determined from these studies.

It has recently been reported that the ability of T lymphocytes to migrate into skin sites is associated with expression of a carbohydrate ligand termed *cutaneous lymphocyte antigen* (CLA).¹⁷ CLA was found to be expressed on lymphocytes in inflamed

skin in association with a number of dermatologic diseases, despite the fact that CLA⁺ lymphocytes represent a very small proportion (<15%) of circulating lymphocytes.¹⁷ Because antibody to CLA was not available in time for this study, it will be interesting in future studies to determine whether CLA expression is also associated with T-lymphocyte recruitment during the allergic LPR.

Other studies have demonstrated a preferential accumulation of T_H2-type lymphocytes during the LPR^{45, 46} (i.e., those expressing mRNA for cytokines such as IL-3, IL-4, and IL-5 but not interferon- γ ⁴⁷). The specific role of the recruited lymphocyte in the allergic LPR, as well as in chronic allergic inflammatory diseases in the skin and other tissues, remains uncertain. It has been postulated that cytokines produced by recruited lymphocytes may further regulate local responses, including that of the dermal microvascular endothelium and keratinocytes, by altering their expression of cell adhesion molecules.⁴⁸⁻⁵⁰ For example, the presence of intraepidermal lymphocytes correlates well with keratinocyte intercellular adhesion molecule-1 expression in psoriasis, atopic dermatitis, lichen planus, and mycosis fungoides.⁵¹ However, the exact role of the recruited T lymphocyte in the LPR and in chronic allergic diseases of the skin and other tissues remains to be determined.

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