

Immunodeficiency and other clinical immunology

Selective early increases of bronchoalveolar CD8⁺ lymphocytes in a LEW rat model of hypersensitivity pneumonitis

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Background: The pathogenesis of hypersensitivity pneumonitis (HP) involves cell-mediated hypersensitivity; various bronchoalveolar T-cell subsets with uncertain roles in disease have been reported and implicated in the pathogenesis.

Objectives: Previous studies at 72 hours after initial antigen challenges showed proportionate increases in T-cell phenotypes. Therefore we tested the hypothesis that early events in response to inhaled antigen in a LEW rat model of HP would include a disproportionate appearance in bronchoalveolar lavage fluid (BALF) and lung parenchyma of a specific T-effector cell responsible for subsequent inflammation and that these events could be identified by phenotyping.

Methods: We double labeled BALF and parenchymal lung lymphocytes with monoclonal antibodies and used flow cytometry to quantitate CD4⁺ and CD8⁺ phenotypic subsets 4 and 24 hours after inhalation of antigen.

Results: We found disproportionate increases in BALF CD8⁺ phenotypes. The strongest correlation with pathologic findings was for a putative cytotoxic effector (CD8⁺CD45R⁻) T lymphocyte.

Conclusion: Meaningful interpretation of lung T-cell phenotype quantitation requires studies of kinetics of cellular influxes, timing after antigen challenge, and relative comparison with increases in other phenotypes. Any pathogenetic role assigned to a phenotype must also await functional studies, including cytokine generation and secretion and cell-cell interactions *in situ*. (*J ALLERGY CLIN IMMUNOL* 1995;96:113-21.)

Key words: Rat, lung, hypersensitivity, pneumonitis, T lymphocytes, CD4, CD8

The pathogenesis of immune-mediated damage to the lung and its resolution are incompletely understood.^{1,2} Pulmonary inflammation is readily induced in pre-sensitized animals given aerosolized antigen, but this typically wanes despite continued challenge.^{3,4} We previously reported an

Abbreviations used

BALF:	Bronchoalveolar lavage fluid
CFA:	Complete Freund's adjuvant
FITC:	Fluorescein-labeled
HP:	Hypersensitivity pneumonitis
mAb:	Monoclonal antibodies
PE:	Phycoerythrin-labeled

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Supported, in part, by SCOR grant HL37121 from the National Heart, Lung and Blood Institute, DHHS, and by the Flow Cytometry Facility, University of Iowa.

Received for publication May 23, 1994; revised Dec. 15, 1994; accepted for publication Dec. 21, 1994.

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0091-6749/95 \$3.00 + 0 1/1/63280

increase in numbers of lung T cells at the height of inflammation 3 days after the initiation of inhalational challenge in a LEW rat model of hypersensitivity pneumonitis.⁵ Although certain phenotypes in that study dominated in number, proportions among phenotypes were similar to controls. We considered then the possibility that the accumulation of selective effector T-cell phenotypes might

precede the accumulation of a nonselective population of inflammatory or modulating T cells. This report tests that notion by examining T-cell phenotypes 4 and 24 hours after initial inhalational challenge.

Increased numbers of lymphocytes in lung disease may be the result of attraction by gradient-dependent chemotaxis, immobilization of trafficking cells, or local proliferation.⁶ Phenotypic selection or preferential accumulation has been found in some human pulmonary conditions at various stages of disease.⁷⁻¹² Identification of effector cells accumulating early in lung inflammation is potentially an important step in the understanding of disease pathogenesis. Correlations of T-cell phenotype, activation, function, cytokine generation, and inflammatory or regulatory events may then be carried out to better understand pathogenetic mechanisms in hypersensitivity lung diseases, and eventually their prevention and treatment. Correlations of T-cell phenotypes and function in disease states are difficult in human disease, however, because kinetics are largely unknown and initial stages are subclinical. Therefore the use of animal models is justified to address the problem.

A relevant model should demonstrate inflammatory responses associated with an increase in identifiable candidate effector cells. CD4⁺ and CD8⁺ T cells in human beings, rats, and mice can be divided into subsets by monoclonal antibodies that recognize other phenotypic markers.^{13, 14} CD45, among the most abundant leukocyte cell surface glycoproteins and identified as a member of the protein tyrosine phosphatases, plays an important role in antigen-stimulated proliferation of T lymphocytes.¹⁵ OX22 monoclonal antibodies recognize CD45R epitopes dependent on exon 6, expressed on isoforms CD45 α , CD45 γ , CD45 δ and CD45 ϵ .¹⁵ Functional correlates and interspecies differences in respect to phenotypic subsets have been controversial and are not completely resolved. In the rat the CD4⁺CD45R⁺ T-cell subset contains those cells that proliferate in response to allogeneic cells and T-cell mitogens, produce high levels of interleukin-2, and are active in primary antibody responses.¹⁶ The CD4⁺CD45R⁻ subset contains memory cells that provide B-cell help for secondary immune responses and are reportedly derived from naive CD4⁺CD45R⁺ precursors.^{17, 18} Several functional activities may be present within single phenotypic subsets defined by available membrane markers, whose functional correlates remain putative for the most part. Cytotoxic precursors and suppressor T cells have been reported

to be contained in CD8⁺CD45R⁺ populations.¹⁶ Murine Th (CD4⁺) clones may be divided into Th1 and Th2 subtypes by patterns of lymphokine production and the expression of CD45 isoforms.^{15, 19} Present information indicates similar differentiation of murine suppressor (CD8⁺CD45R⁻) and cytotoxic (CD8⁺CD45R⁺) subpopulations: the suppressor phenotype produced IL-10 and the cytotoxic phenotype did not.¹⁹ Results in human peripheral blood-derived T cells suggest that CD45⁺ and CD45R⁻ indicate naive and memory CD45⁺ cells, respectively,^{20, 21} although this is challenged by demonstration of reversal in isoform switching.²² Data have also been presented that distinction between naive and memory T cells is questionable, as well for the CD8⁺ phenotype, in that the CD8⁺CD45R⁺ subpopulation contains some primed cells.²² In several inflammatory diseases of human beings there is a decrease in the number of T cells expressing CD45R isoforms.¹⁵ The CD8⁺ population in rats has been less well studied to date for functional differences related to CD45 isoform expression.

The rat RT6 antigen is present on most of the peripheral T cells and absent from B cells and thymus cells.²³ Effector cells for cutaneous delayed hypersensitivity and for allospecific cytotoxic reactions were shown to be confined to the CD4⁺RT6⁻ and CD8⁺RT6⁻ T-cell subsets, respectively.^{24, 25}

We used an established LEW rat model of experimental hypersensitivity pneumonitis (HP) to identify T-cell phenotypes present in bronchoalveolar lavage fluid (BALF) and lung parenchyma within the first 24 hours after initial aerosol challenge with antigen.⁵ We found a disproportionate accumulation in BALF of CD8⁺ phenotypes, and positive correlations occurred between pathologic scores and percentages of these phenotypes.

METHODS

These studies followed the guidelines given in the U.S. Public Health Services/National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Iowa Animal Care and Use Committee.

Animals

Female LEW rats (pathogen free) weighing 150 to 200 gm were purchased from Harlan Sprague-Dawley (Indianapolis, Ind.) and conventionally housed. They had free access to food and water.

Reagents

Ovalbumin, crystallized five times, was purchased from Miles Laboratories (Elkhart, Ind.). Complete

TABLE I. Selected putative functional correlations of T-cell subsets

mAb	Recognized membrane component	Phenotypic T-cell population	Functional correlates contained in T-cell subset*
W3/25	CD4	CD4 ⁺	
DS4.23	RT6	CD4 ⁺ RT6 ⁺	GVH; DH precursor
	—	CD4 ⁺ RT6 [−]	DH effector (T _{DH})
OX22 (T _{H1})	CD45R	CD4 ⁺ CD45R ⁺	Interleukin-2 producer
	—	CD4 ⁺ CD45R [−]	B-cell help (T _{H2})
OX8	CD8	CD8 ⁺	
DS4.23	RT6	CD8 ⁺ RT6 ⁺	Cytotoxic precursor/suppressor (T _s)
	—	CD8 ⁺ RT6 [−]	Cytotoxic effectors (T _c)
OX22	CD45R	CD8 ⁺ CD45R ⁺	Suppressor/cytotoxic precursor (T _s)
	—	CD8 ⁺ CD45R [−]	Cytotoxic effectors (T _c)

mAb, Monoclonal antibody; GVH, graft versus host; DH, delayed hypersensitivity.

*Functional correlates of the phenotypic T-cell populations are based on references 13-25.

Freund's adjuvant contained 5 mg/ml mycobacteria. Monoclonal antibodies (mAb) W3/25 anti-CD4, OX8 anti-CD8, OX22 anti-CD45R were purchased from Biological Products for Science (Indianapolis, Ind.), and DS4.23 anti-RT6.1 mAb was obtained from hybridoma culture supernatants and purified for use as described previously.^{23, 25} W3/25 and OX8 were purchased labeled with phycoerythrin, and DS4.23 and OX22 were fluoresceinated.²³ mAb controls included fluoresceinated murine IgG and phycoerythrin-labeled murine IgG (Becton Dickinson, Mountain View, Calif.). A summary of the mAbs, phenotypic markers, and putative functional correlates is given in Table I. Details of reagents and methods used in labeling and flow cytometry have been previously described.⁵

Preparation of rat models of HP

Experimental rats were sensitized with 1 mg ovalbumin emulsified in complete Freund's adjuvant in 0.1 ml total volume by means of footpad injections. Controls received identical volumes of saline solution emulsified in complete Freund's adjuvant. Inhalational challenges began 10 days later and were administered by means of ultrasonic nebulizers (DeVilbiss) in Plexiglas chambers. Acute challenges of 200 mg ovalbumin in 20 ml saline solution were given over approximately 45 minutes, either once or twice, to the experimental and control groups. Cells were collected, and histopathologic results were quantitated at 4 or 24 hours after the initial challenge. Results given in this report summarize several experiments necessarily done in tandem because of the time- and labor-intensive techniques required for cell preparation and flow cytometry. All experiments included experimental and control animals. All animals were used for simultaneous collections of cells from bronchoalveolar lavage fluid (BALF) and minced lung (parenchyma).

Cell recovery and histopathology

Animals were killed with an intraperitoneal overdose of pentobarbital. The chest was opened, and blood was

aspirated from the heart. The lungs were perfused until white with 6% dextran (Gentran 75; Travenol Laboratories, Deerfield, Ill.) through a blunt needle in the pulmonary artery with egress from the amputated left atrial appendage. The trachea was clamped and the lungs and mediastinum removed en bloc. The left mainstem bronchus was clamped, the trachea cannulated, and the right lung was lavaged with 6 aliquots of physiologic saline solution for recovery of BALF cells in a volume of 30 ± 4 ml (means ± SD) per animal. The volume of BALF recovery was about 75% of that delivered.

The left lung was inflated with 95% ethanol for fixation before the preparation of paraffin-embedded sections and histopathologic study. Entire cross sections of the whole left lung were stained with hematoxylin and eosin, blind-coded, and scored for pathologic condition on the basis of severity and extent of lesions on a scale of 0 to 3 (0 = no histopathologic abnormality; 1 = localized, scattered areas of mild alveolitis; 2 = larger, more generalized areas of moderate alveolitis; and 3 = areas of marked alveolitis throughout the section).

Single-cell suspensions were prepared from the lavaged right lung by mincing in phosphate-buffered saline 1% bovine serum albumin, pH 7.2, and pressing through a 60-gauge steel mesh screen. Cells were centrifuged and resuspended in hypotonic buffer to lyse erythrocytes, washed two times in PBS/BSA, resuspended in PBS/BSA/0.1% sodium azide (NaAz), and an aliquot was counted on a Coulter Densitometer (Coulter Electronics, Hialeah, Fla.). The cell suspension was adjusted to contain 10 × 10⁶ cells/ml.

Immunofluorescent staining

Cells and reagents were kept at 4°C throughout. Aliquots of 2 × 10⁶ cells from each cell source were dispensed into microtiter wells in round-bottom, 96-well plates (Corning Glassworks, Corning, N.Y.). Saturating concentrations of mAb were determined by using serial dilutions in PBS/1% BSA/0.1% NaAz with 1:100 heat-inactivated normal rat serum. In all reported experi-

ments, double labeling of cells was performed using premixed fluoresceinated (FITC) and phycoerythrin (PE)-labeled primary mAb. Cells were incubated 120 minutes with 50 μ l of the appropriate mAb and washed two times with PBS/BSA/NaAz. Wash with PBS/NaAz was repeated twice, the supernatant aspirated, 2% paraformaldehyde pipetted into each well for fixation, and the cell suspension transferred to tubes for flow cytometry.

Flow cytometry

Flow cytometry was performed with a Coulter EPICS 753 multiparameter fluorescence-activated cell sorter. Excitation was done at 488 nm by means of an argon ion laser (Coherent Innova 90-5). FITC and PE emissions were split by 550 LP and 600 SP dichroics. FITC emissions were collected through a 525 nm bandpass filter, and PE emissions were collected through a 595 nm bypass filter. The fluorescence signals were processed by logarithmic amplifiers. Forward and orthogonal light-scatter signals were processed by linear amplifiers. At least 3×10^4 events were collected in list mode. Data file management, graphics, and subpopulation analysis were performed with ELITE Software (Coulter Cytometry). On bivariate histograms of forward-angle light scatter versus orthogonal scatter, a bit-map was drawn around the lymphocyte population (small, nongranular cells) to exclude cell clumps, macrophages/monocytes, and neutrophils, and the program determined lymphocyte percentage of total cells. Bivariate FITC versus PE histograms were generated from this population. Subpopulations were then identified on the new histogram quadrants.

Data from the FITC versus PE histogram were calculated to determine percent and mean scale value. Background cells were determined similarly with cells stained with mAb controls. A computer program was used to determine numbers and percentages of lymphocytes stained specifically with FITC, PE, or both and to calculate phenotypes.

Statistical analysis

For comparison of responses of more than two groups of animals, one-way analysis of variance was performed for determination of overall differences among groups. When a significant overall difference was found, pairwise differences between individual groups were evaluated by using Tukey's Studentized range test.²⁶ Pearson's coefficient of correlation (r) was used to determine the degree of relationship between two dependent variables in paired samples. For these analyses we used an SAS program (SAS Institute, Cary, N.C.) at the University of Iowa Weeg Computer Center.²⁷

RESULTS

Histopathology and cell recovery

Acute alveolitis of mild to moderate degree was found in experimental groups A and B but not in control groups. Because no differences in histopatho-

logic finding, cell recovery, or phenotypic labeling were found between the two control groups, these were pooled for analysis. Mild to moderate infiltrates of mononuclear cells were seen at 4 and 24 hours after initial aerosol challenge in the experimental groups A and B (Fig. 1). Total numbers of cells were significantly increased in experimental compared with control animals; this was especially striking in BALF (Table II). The increased numbers were accounted for primarily by macrophages/monocytes with variable numbers of neutrophils (2% to 30%), especially in group B animals.

T-Cell phenotypes

Flow cytometry data were analyzed to quantitate T-cell subsets in BALF and parenchymal cells as identified by double labeling. Results for cell numbers and percentages for BALF cells are given in Fig. 2 and for parenchymal cells in Fig. 3.

Bronchoalveolar lavage cell phenotypes were increased in numbers of cells from controls to group A (4 hours) to group B (24 hours) (i.e., $C < A < B$) with the single exception of $CD4^+RT6^-$, and most noticeable in $CD8^+$ phenotypes. Statistical differences were limited, however, to increased numbers of group B compared with controls (denoted by asterisks in Figure 2, A). Percentages increased significantly in all $CD8^+$ phenotypes, with reciprocal decreases in $CD4^+RT6^-$ and $CD4^+CD45R^-$ populations (Fig. 2, B). Therefore, the shifts in BALF T-cell phenotypes occurred toward cytotoxic precursor and effector $CD8^+$ cells.

T-cell population shifts in parenchymal lung cells were much less apparent, with no statistically significant increases in numbers of cells among the groups (Fig. 3, A). On the other hand, a significant increase in percentages of phenotypes $CD8^+RT6^-$ and $CD8^+CD45R^+$ was significantly greater in group B than in controls (Fig. 3, B).

These results indicate a general shift in T-cell phenotypes toward cytotoxic populations and away from $CD4^+$ phenotypes in lung, especially in BALF cell populations, during the first 24 hours after initial encounter with inhaled antigen in systemically sensitized LEW rats. Although resident numbers of cells were much greater in parenchymal than in BALF cells, increases in cell numbers were largely seen in BALF, compared with lung parenchyma, suggesting rapid traffic from the intravascular compartment through the interstitium into the alveoli and ready recovery there by lavage.

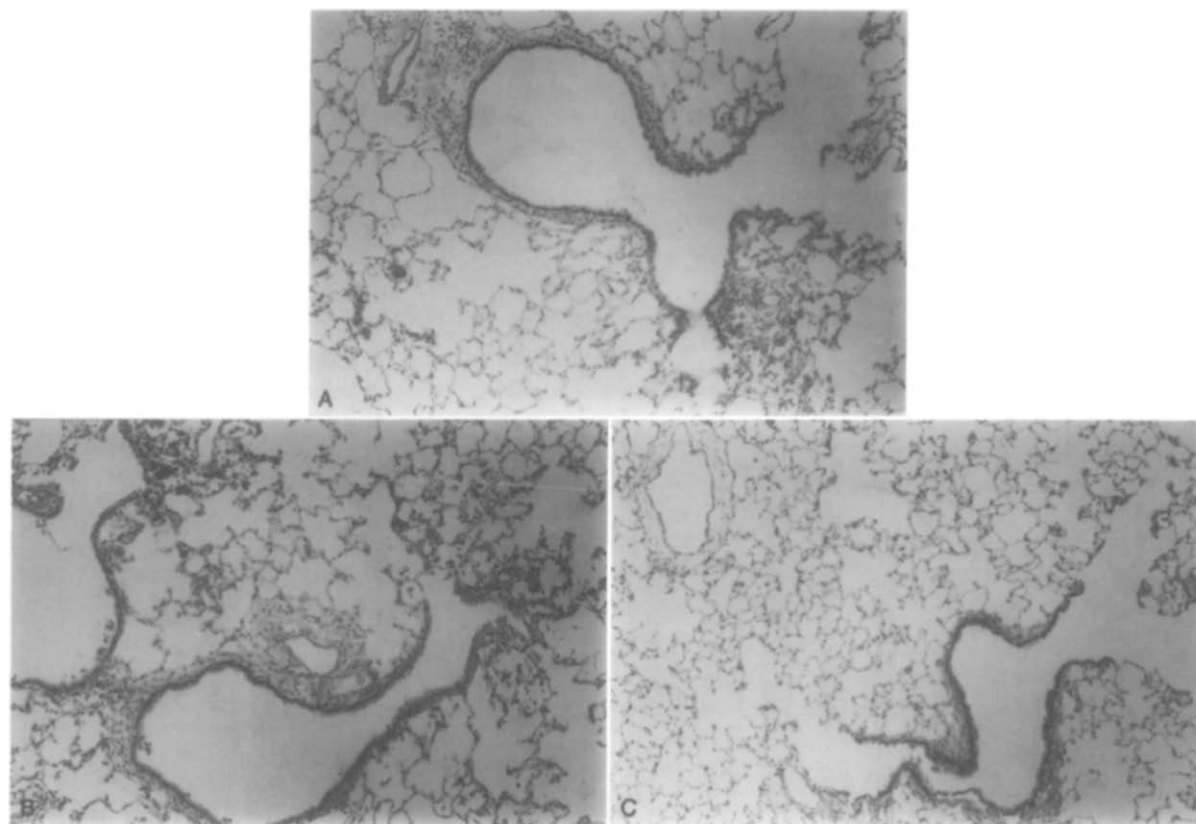


FIG. 1. Photomicrographs of representative pulmonary histopathologic conditions from experimental and control LEW rats. **A**, Section from animal in group A killed 4 hours after initial aerosol challenge with antigen. Small collections of mononuclear cells are seen in area of terminal bronchiole and alveolar ducts and around vessels. (Hematoxylin-eosin stain; original magnification $\times 80$.) **B**, Section from animal in group B killed 24 hours after initial aerosol challenge with antigen. Localized collections of mononuclear cells are seen in areas of terminal bronchioles, alveolar ducts, alveoli, interstitium, and vessels. (Hematoxylin-eosin stain; original magnification $\times 80$.) **C**, Section from control animal in group D. No cellular infiltrates are present. (Hematoxylin-eosin stain; original magnification $\times 80$.)

Correlations of pathologic score with presence of T-cell phenotypes

Pearson's coefficient of correlation (r) was used to evaluate associations of specific T-cell phenotype percentages with the extent of disease in individual animals (Table III). Positive correlations were limited to increases in CD8⁺ phenotypes, strongest for the CD8⁺CD45R⁻ (cytotoxic effector) population.

DISCUSSION

Previous studies of acute and chronic HP from 3 days to 14 weeks in LEW rats showed increased numbers of T cells without significant selective or disproportionate increases in individual phenotypes.⁵ Similar results were found in adoptive transfer experiments involving acute HP in the

TABLE II. Histopathologic condition and cell recovery

Rat group	Histopathologic condition	Total numbers of cells $\times 10^{-6}$	
		BALF	Parenchyma
A. 4-hour ($n = 9$)	$1.8 \pm 0.2^*$	54 ± 8	44 ± 7
B. 24-hour ($n = 8$)	2.0 ± 0.2	83 ± 15	40 ± 4
C and D. Controls ($n = 8$)	0.1 ± 0.1	8 ± 0.6	24 ± 1

*Numbers denote mean $\pm$ SEM. For grading of histopathologic condition, see text. Analysis of variance and Tukey's follow-up showed significant increases ($p < 0.05$) in experimental groups A and B compared with controls for all measures shown.

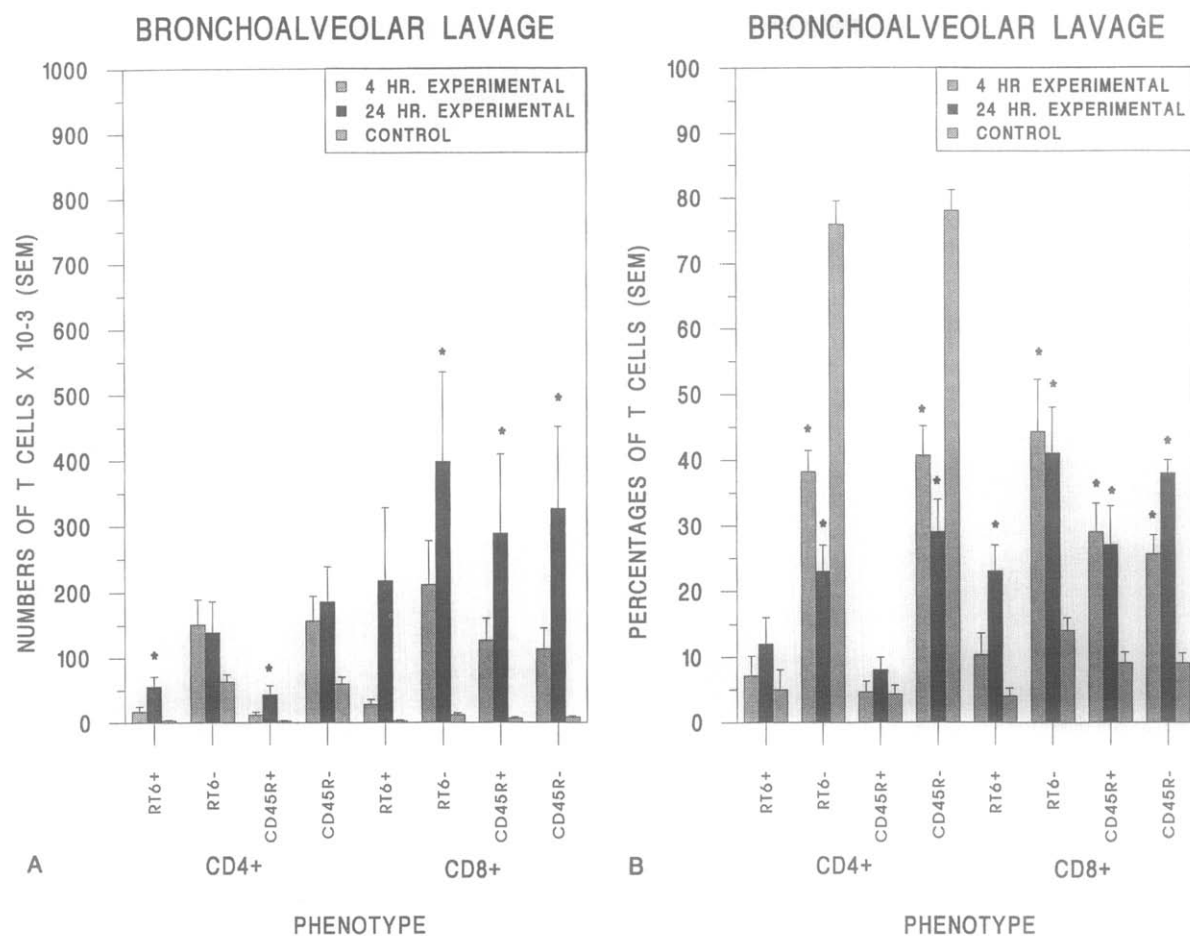


FIG. 2. Numbers (A) and percentages (B) of T-cell phenotypes obtained by bronchoalveolar lavage and evaluated by means of flow cytometric analysis. Increases are seen in numbers and shifts in percentages favoring CD8⁺ phenotypes in experimental groups. An asterisk denotes significant ($p < 0.05$) difference in experimental group phenotype compared with control numbers or percentages with Tukey's follow-up test.

LEW rat (Richerson HB, Coon JD, Lubaroff D. *Am J Respir Crit Care Med* 1995;151:in press). The present study was designed to look at early kinetics of T-cell changes in the lung after initial inhalational antigen challenge. We postulated that effector cells would be selectively increased during initial phases of inflammation with subsequent increases involving other phenotypes in willy-nilly fashion. Our data are consistent with that hypothesis, and suggest that initial increases in T-cell populations involve mainly CD8⁺ phenotypes of putative cytotoxic functions.

A popular viewpoint has been that different populations of lymphocytes selectively migrate to the site of antigen challenge during inflammatory responses involving cell-mediated hypersensitivity.^{6,28} Antigen-reactive T cells in lung have been demonstrated in animal models and human HP,²⁹⁻³² but little quantitative information is avail-

able on lymphocyte subset populations recruited to the lung during immune-mediated inflammation or on their state of activation.⁶ Studies of pulmonary viral infections have suggested that such recruitment depends to some extent on factors other than antigen specificity.³³⁻³⁵ T-cell influxes into rat lungs have been reported in experimental HP,⁵ as well as during injury induced by bleomycin and silica.^{36,37}

In human HP increased numbers of lymphocytes attributable in general to CD8⁺ cells have been reported by several investigators.^{7,8} Others, however, have observed no phenotypic aberrations of T-cell subsets but have found evidence of increased T-cell proliferation, activation, and function.^{9,10} Semenzato et al.³⁸ reported the presence of different types of cytotoxic cells, that is, natural killer cells and non-MHC-restricted cytotoxic lymphocytes, including lymphokine-activated killer cells. Trentin et al.^{11,12} found a high number of lymphocytes comprising a

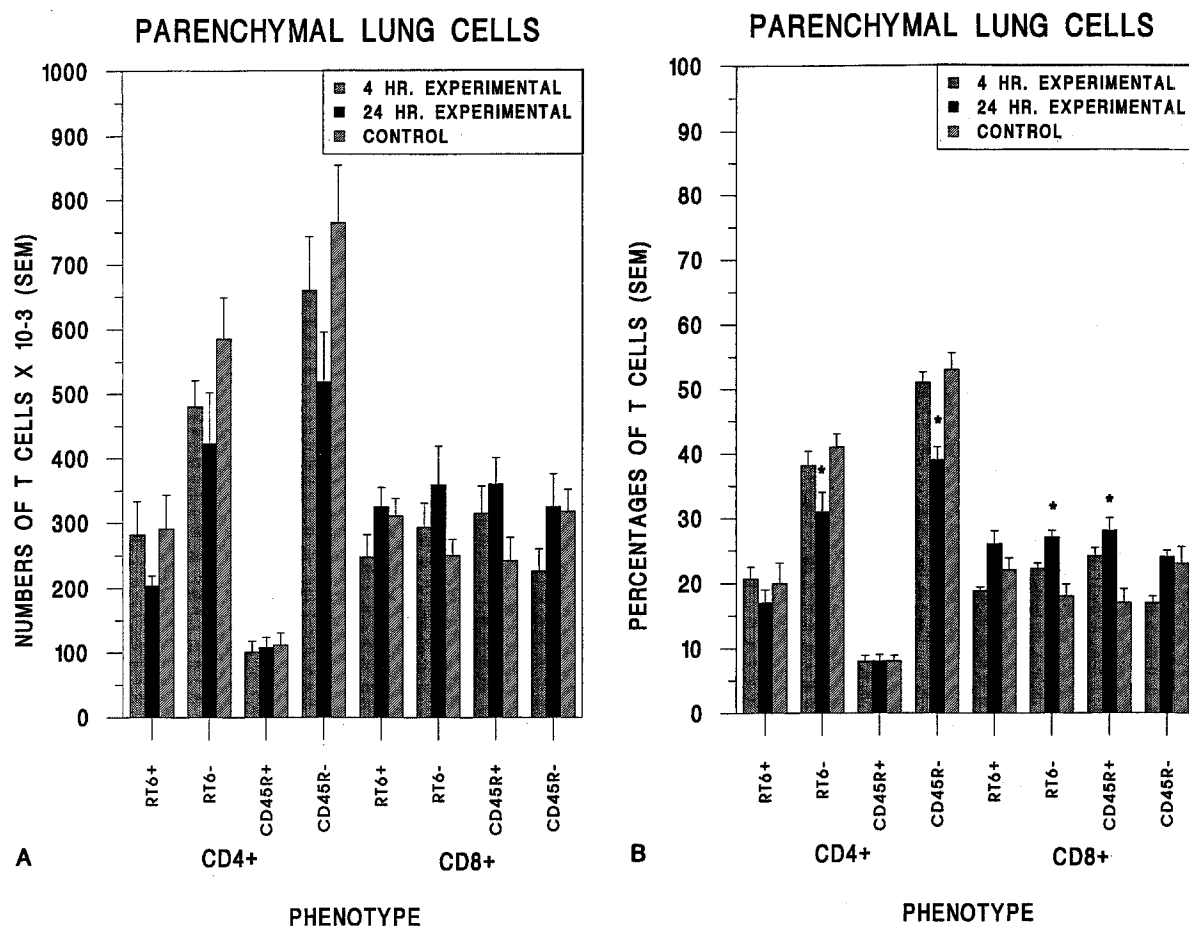


FIG. 3. Numbers (A) and percentages (B) of T-cell phenotypes obtained from minced, previously lavaged lungs and evaluated by means of flow cytometric analysis. Significantly increased percentages, but not numbers, of CD8⁺ RT6⁻ and CD8⁺CD45R⁺ phenotypes are seen in the 24-hour experimental group. An asterisk denotes significant ($p < 0.05$) difference in experimental group phenotype compared with control numbers or percentages with Tukey's follow-up test.

cytotoxic subset (CD3⁺, CD8⁺, CD16⁻, CD56⁺) in the BALF of patients with HP as well as an increase in γ/δ ⁺ lymphocytes. These mechanisms of human disease need further clarification.²

Our results may be interpreted as showing initial increases in T lymphocytes with phenotypes identified as cytotoxic precursors or cytotoxic T cells, although definitive identification of phenotype with function is not possible. Phenotypic studies of lung lymphocyte subpopulations as carried out here have been only recently pursued.⁵ BALF cells may be activated or memory cells, rather than the effector cells suggested in Table I; studies of T-cell phenotypes may reveal important differences in expression and function in the various compartments of the body.⁵

TABLE III. Pearson's coefficient of correlation*

Pathologic score vs	BALF	Parenchyma
%CD4 ⁺ RT6 ⁺	NS	NS
CD4 ⁺ RT6 ⁻	-0.78	-0.48
CD4 ⁺ CD45R ⁺	NS	NS
CD4 ⁺ CD45R ⁻	-0.75	-0.56
%CD8 ⁺ RT6 ⁺	0.43	NS
CD8 ⁺ RT6 ⁻	0.54	0.60
CD8 ⁺ CD45R ⁺	0.46	0.70
CD8 ⁺ CD45R ⁻	0.77	NS

NS, Not significant.

* r Value is given for significant positive or negative correlations ($p < 0.05$) of T-cell phenotype percentages with pathologic score.

Results of these experiments have several important implications for interpreting studies of the pathogenesis of hypersensitivity lung disease. Unless immune-mediated diseases are evaluated early after antigen encounter, the presence of cell phenotypes may reflect irrelevant, rather than selective, effector populations. Therefore different times for specimen collections in relation to antigen exposure may give variable results.

Another implication is the assurance from our findings that increases in BALF lymphocytes may be explained without enlisting cell proliferation,⁶ since little proliferation of antigen-stimulated cells would be expected within 4 to 24 hours of initial stimulation, as reported here. Of interest also is the relatively meager increase in parenchymal, compared with BALF, cells, suggesting a strong intraalveolar chemotactic stimulus or adhesion molecule activation, with little delay or perhaps even acceleration in trafficking through the interstitium from the circulation to alveolus under the conditions of these experiments.

A third implication is that the mere presence of cell phenotypes is an inadequate indication of their functional role, if any, in disease pathogenesis. Cell membrane markers may change in response to chemotactic stimuli, activation, or other environmental factors unrelated to effector function; additional information is needed concerning purposeful activation and function leading to tissue inflammation and its modulation or suppression. Studies of cytokine generation and production and interactions of cells with other cells and with adhesion and related molecules may be required, preferably in situ, to adequately explain effector and control mechanisms in immune-mediated inflammatory lung diseases.

We gratefully acknowledge Michael T. Suelzer, PhD, for his statistical expertise, Darla Bartels for her secretarial assistance, Larry Perkins for photography, and Justin Fishbaugh for performing flow cytometry.

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