

# Immunocompetent Cells from the Lower Respiratory Tract of Normal Human Lungs

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**ABSTRACT** Subpopulations of lymphocytes in the broncho-alveolar air spaces of normal human lungs were compared with those in peripheral blood. Bone marrow-derived (bursal-equivalent) cells (B cells) were identified by complement receptors (EAC rosettes) and by surface immunoglobulin. Thymus-derived lymphocytes (T cells) were identified by their proliferative response to mitogens and the E rosette technique.

Cells in lung air spaces were recovered from eight healthy nonsmoking volunteers by segmental lavage with the flexible bronchofiberscope. On the average, macrophages constituted 78% and lymphocytes 17% of the cells in the aspirates. B cells detected by surface immunoglobulin and complement receptors equaled 22% and 15% of lung lymphocytes, respectively. The distribution of lung B cells into heavy chain immunoglobulin classes revealed IgM and IgG to be the predominant classes, with mean values of 14.5% and 9.3%, respectively; the corresponding value for IgA was 5%. A comparable order of frequency (IgM > IgG > IgA) was observed for purified peripheral blood lymphocytes in the same and other control subjects. T cells comprised the majority (47%) of identifiable lung lymphocytes by the E rosette method. The presence of lung T cells was also corroborated by their proliferative response to mitogens (phytohemagglutinin and concanavalin A), but the response was less than that of equal numbers of peripheral blood lymphocytes from the same subjects.

The B/T cell ratio for lung lymphocytes was comparable to results with peripheral blood lymphocytes in

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the same subjects, but a higher proportion of lung lymphocytes could not be identified as either T or B cells.

It is postulated that lung lymphocytes participate in the local immune defenses of the lung.

## INTRODUCTION

The search for mechanisms that allow the lung to cope with potentially injurious airborne substances has focused primarily on the mucociliary blanket and the phagocytic and metabolic activities of the alveolar macrophage (1, 2). Relatively little is known about local immune mechanisms and how they participate in the overall design of the lung's defense mechanisms. Central to this understanding is some knowledge as to the deployment and functional identity of lymphoid cells within the respiratory tree.

It is generally accepted that antigens (including bacteria and viruses) introduced into the respiratory tract stimulate the secretion and transport of antibody, primarily IgA, by lymphocytes and plasma cells located beneath the lamina propria along the tracheobronchial tree (3). More recently, it has been shown that in certain experimental animals, lymphocytes obtained by lavage from the lower respiratory tract constitute a significant fraction (10-30%) of the cells and are capable of both cellular and humoral responses to antigens introduced intranasally or intratracheally (4-8). In guinea pigs, Waldman and Henney demonstrated that antigenic stimulation via the respiratory tract elicited local production of antibody in bronchial secretions and cell-mediated responses, as measured by production of macrophage inhibition factor by lymphocytes within lung air spaces (4). Using the single cell method for antibody assay (Jerne plaque technique) (9), others have demonstrated that lymphoid cells recovered from the lungs of rabbits and dogs by lavage were capable of in vitro secretion of antibody specific for the sheep red blood

cells (SRBC)<sup>1</sup> previously introduced into the respiratory tract (5-8). Thus, there is increasing evidence to suggest that a population of lymphoid cells that resides or is mobilized into the broncho-alveolar air spaces is capable of both humoral and cellular immunity and forms part of the local immune system of the lung.

In immune tissues such as lymph node and spleen, phagocytic cells and lymphocytes interact or collaborate in the generation of immune responses (10, 11). These lymphocytes are separable into at least two functional subpopulations: thymus-derived lymphocytes (T cells), which control cell-mediated immunity, and bone marrow-derived (bursal-equivalent) or B cells, which are the precursors of antibody-secreting cells (plasma cells). Although they may be morphologically similar, T and B cells possess unique membrane characteristics that permit their identification in peripheral blood and lymphoid tissues (12): T cells in human blood are detected by their adherence to sheep red blood cells (E rosettes) (13, 14); the corresponding B cells can be identified by surface immunoglobulins (15) and receptors for modified components of complement (16).

As part of experiments designed to gain insight into the immune system in the human lung, we have examined the composition of cells obtained by segmental bronchial lavage of healthy volunteers. The studies were aimed at determining whether a stable population of immunocompetent cells resides in the lower respiratory tract of normal human lungs; and, if such a population exists, to identify and quantitate the cells as subpopulations of T and B lymphocytes. Some indication of the functional capacity of cells recovered from the airways was also assessed by studying their response to soluble mitogens, phytohemagglutinin (PHA) and concanavallin A (Con A). These immunologic parameters were compared in the lung and peripheral blood of the same subjects.

## METHODS

*Subjects.* Healthy male volunteers, ages 21-35, were included in this study according to the following criteria: (a) freedom from chronic pulmonary disease; (b) complete abstinence from smoking (including pipe and cigar) from childhood; (c) no industrial dust exposure; (d) a normal chest X ray; (e) normal pulmonary function tests including those designed to detect small airway disease (closing volumes and flow volume loops); and (f) no medication at the time of study. All subjects were fully informed as to the nature and purpose of each aspect of the study in which they were involved.<sup>2</sup>

<sup>1</sup> *Abbreviations used in this paper:* B cells, bursal equivalent or bone marrow-derived lymphocyte; Con A, concanavallin A; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution (with Ca<sup>++</sup> and Mg<sup>++</sup>); PHA, phytohemagglutinin; SIg, surface immunoglobulin; SRBC, sheep red blood cell; T cell, thymus-derived lymphocytes.

<sup>2</sup> Approved by the Hospital of the University of Pennsylvania Committee on Human Experimentation.

*Bronchoscopy and collection of specimens.* Segmental bronchial lavage was performed with an Olympus BF-5B bronchofiberscope (Olympus Corporation of America, New Hyde Park, N. Y.). The subjects were first premedicated with 25-50 mg meperidine and 0.1 mg atropine (i.m.). After topical anesthesia of the pharyngeal and nasal mucosa with a solution of lidocaine (2%) in isotonic saline, the bronchoscope was passed via the nose into the lower lobes of the segmental bronchus. While the bronchoscope tip was wedged in place, 100-200 ml of sterile isotonic saline was introduced into the isolated bronchial segment. The bronchial contents were then aspirated into a sterile trap on ice containing two volumes of Hanks' balanced salt solution (HBSS) (Grand Island Biologic Co. [GIBCO], Grand Island, N. Y.) supplemented with 20% (vol/vol) fetal calf serum (FCS) (Microbiological Associates, Cockeysville, Md.), 10 U of heparin/ml (Lipo-Hepin, Riker Laboratories, Inc., Northridge, Calif.), 100 U of penicillin/ml, and 100 µg streptomycin/ml GIBCO).

*Separation of lymphocytes from lung washings.* The aspirated cell suspension was first passed through six layers of sterile surgical gauze and a 250-µm stainless steel mesh to remove mucous clumps and cell debris. The suspension was then washed three times in HBSS (400 g at 4°C). For cell counting, small macrophages and monocytes were distinguished from large lymphocytes by the capacity of the former to ingest latex particles (1.1 µm, Dow Chemical USA, Midland, Mich.). This was accomplished by first incubating a suspension of lung cells, 5 × 10<sup>6</sup> cells/ml, with 0.1% latex particles (in HBSS plus 20% FCS) for 30 min at 37°C in a shaker bath. Cytocentrifuge (Shandon Southern Instruments Inc., Sewickley, Pa.) preparations were then made and stained with Wright-Giemsa stain for differential counting. Monocytes and macrophages were identified by size, the presence of large lipid vacuoles, or the presence of ingested latex particles.

Lymphocytes were separated from the remaining washed cells by one of two methods. In the first, a suspension of lung cells, 5 × 10<sup>6</sup> cells/ml, in HBSS solution with 20% FCS, was incubated with carbonyl iron particles (10 mg/ml) (powder W, GAF Corp., New York) in a shaker bath for 30 min. 8-ml portions of the cell suspension were then layered onto 1.5 ml of a Ficoll-Hypaque gradient<sup>3</sup> (specific gravity 1.077) and centrifuged in siliconized test tubes (RTU, No. 7816, Beckman Instruments, Spinco Div., Palo Alto, Calif.) at 400 g for 20 min at 4°C (17). Cells (at least 90% lymphocytes) were aspirated from the interface of the mixture and washed twice in HBSS. The second method was used to obtain larger numbers of cells. It used direct magnetic sedimentation of phagocytic cells after the incubation step (37°C, 30 min) with carbonyl iron (10 mg/ml), thus omitting the Ficoll-Hypaque gradient. Yields were 50% greater with the latter method. By both methods, lymphocytes constituted more than 90% of the cells and viability was greater than 95% by the 0.1% trypan blue test. Purity of the lymphocyte preparation was examined by incubating a portion of separated cells with latex particles as described above. Contamination with phagocytic cells was less than 10%. It was noteworthy that residual phagocytic cells, identified by morphology and ingested latex particles, also bound several carbonyl iron particles on the cell surface. Thus, the use of car-

<sup>3</sup> Prepared by mixing 24 parts of 9 g/100 g Ficoll (Pharmacia Fine Chemicals, Piscataway, N. J.) with 10 parts of a 33.0 g/100 g Hypaque (Winthrop Laboratories, New York).

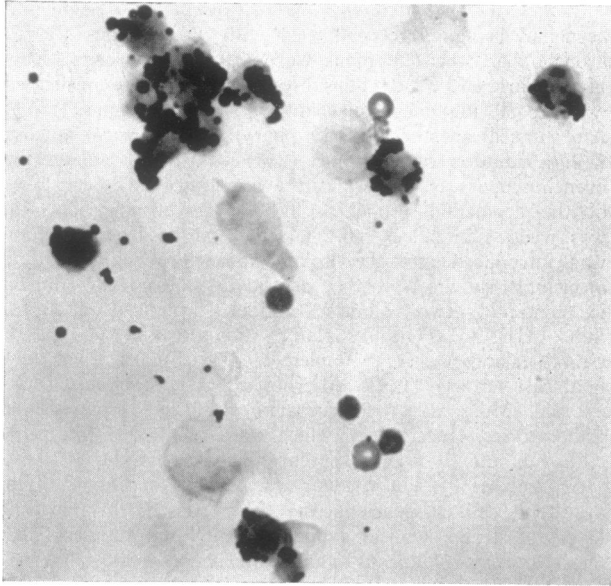


FIGURE 1 The identification of alveolar macrophages by the adherence of carbonyl iron particles to the cell membrane. Also shown in photomicrograph are three lymphocytes that fail to bind iron particles.  $\times 1,000$ .

bonyl iron particles provided a means of both identifying and separating phagocytic cells from lymphocytes (Fig. 1).

*Separation of lymphocytes in peripheral blood.* Before bronchoscopy, 20 ml of peripheral or arterial venous blood was collected in tubes containing 1,000 U of heparin. Lymphocytes were separated from heparinized blood by a modification of the Ficoll-Hypaque technique that included preincubation with carbonyl iron particles ( $37^{\circ}\text{C}$  for 30 min) to remove phagocytic cells (17). Purified lymphocytes (greater than 95%) were suspended in HBSS at a concentration of  $5 \times 10^6$  cells/ml for assays of membrane receptors.

*Preparation of lymphocytes with surface immunoglobulin (SIg).* B cells were identified by detecting SIg with fluorescein-conjugated antisera to human immunoglobulins. For fluorescence studies of SIg, 0.01-ml samples of purified lung and peripheral blood lymphocytes at a concentration of  $5 \times 10^6$  cells/ml were incubated for 30 min at  $4^{\circ}\text{C}$  with 0.1 ml of rabbit or goat fluoresceinated anti-human serum immunoglobulins (Meloy Laboratories Inc., Springfield, Va.). These preparations were then washed three times, mounted in 9:1 glycerol:buffered saline solution and examined with a Leitz fluorescent microscope (E. Leitz, Inc., Rockleigh, N. J.) with a Ploem incident light attachment. To detect the total number of cells bearing SIg, polyvalent antisera specific for human heavy and light chains were used ( $\mu$ ,  $\gamma$ ,  $\alpha$ ,  $\kappa$ ,  $\lambda$ ). For B cell heavy-chain classes ( $\mu$ ,  $\alpha$ , and  $\gamma$ ), monospecific antisera was used. Purity and specificity of each batch of antisera were tested by immunoelectrophoresis before use.

*Detection of B cells bearing complement receptors—EAC rosettes.* B cells also possess receptors for modified components of complement that allow them to form rosettes with sheep red blood cells (E) sensitized with antibody (EA) and complement (EAC rosettes) (16, 18). SRBC were obtained from the Veterinary School of the University of Pennsylvania. Each batch was collected in Alsever's solution (GIBCO) and used within 1 wk of collection.

The indicator cells (EAC) were prepared with SRBC-sensitized with rabbit anti-Forssman serum (GIBCO) diluted 1:1,000 (subagglutinin titer) with HBSS. The sensitized SRBC were then incubated ( $37^{\circ}\text{C}$ , 30 min) with a 1:10 dilution of serum from normal  $\text{C}_3\text{H}$  mice (Jackson Labs, Bar Harbor, Maine) as a source of complement, washed, and resuspended to a final concentration of 0.5% in HBSS. Indicator cells were used within 48 h of preparation. Equal volumes (0.1 ml) of these indicator cells (EAC) and lymphocytes ( $4 \times 10^6$  cells/ml) were mixed in duplicate and centrifuged at 200 *g* for 5 min at  $37^{\circ}\text{C}$ . The pellets were resuspended immediately and the percentage of lymphocytes forming rosettes was recorded by counting at least 300 lymphocytes; rosettes were defined by the adhesion of at least three SRBC to a lymphocyte. Controls consisted of testing lymphocytes as described above with unsensitized SRBC (E) at  $37^{\circ}\text{C}$  and SRBC sensitized without complement (EA); nonspecific rosette formation never exceeded 2%.

*Detection of T cells by E rosettes.* E rosettes were formed by mixing equal volumes of a 0.5% suspension of SRBC in HBSS and a suspension of lymphocytes,  $5 \times 10^6$  cells/ml (18). The cells were mixed in duplicate in U-microtiter plates (#1-220-24, Cooke Laboratory Products Div., Dynatech Laboratories Inc., Alexandria, Va.) and human AB (Hospital of the University of Pennsylvania Blood Bank) serum was added to each well to give a final concentration of 10% (vol/vol). The cell suspension was then centrifuged at 200 *g* for 5 min at room temperature ( $22^{\circ}\text{C}$ ). The cell pellets were incubated for 1 additional h at room temperature, resuspended, and then examined microscopically in a hemocytometer; rosettes were counted as described above.

The human AB serum used in this assay was previously heat-inactivated ( $56^{\circ}\text{C}$ , 30 min) and absorbed twice with SRBC. We and others (19, 20) have shown that AB serum increases the number of E rosettes formed and permits 90–95% of normal human blood lymphocytes to be identified as either B or T.

*Mitogenic response of blood and lung lymphocytes.* The presence of T cells was also tested by their capacity to undergo blastogenesis and proliferation in the presence of certain plant mitogens, phytohemagglutinin (PHA) and concanavallin A (Con A) (21, 22).

Lung and peripheral blood lymphocytes were used. 50  $\mu\text{g}$  of Con A (Calbiochem, San Diego, Calif.) and PHA-M (Difco Laboratories, Detroit, Mich.) were added to cultures containing either  $5 \times 10^5$  or  $5 \times 10^4$  lymphocytes in 1 ml of modified Eagle's medium (MEM) (GIBCO) supplemented with glutamine (2.5  $\mu\text{g}/\text{ml}$ ) (GIBCO), penicillin (100 IU/ml), streptomycin (100  $\mu\text{g}/\text{ml}$ ), and 10% (vol/vol) human AB serum. Cultures were incubated at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  and air for 72 h. In two experiments, cultures were terminated after 48, 72, 120, and 168 h.

Survival of control (without mitogen) cultures was measured at 48 and 72 h by trypan blue test. No significant difference in viability was observed between lung and peripheral blood lymphocyte cultures.

For studies involving cellular proliferation, 0.25  $\mu\text{Ci}$  of tritiated thymidine ( $[^3\text{H}]\text{TdR}$ ) (sp act 7.7 Ci/mmol, New England Nuclear, Boston, Mass.) was added to cultures 16 h before termination of the experiment. After exposure to tritiated thymidine, the supernate was removed from the cell pellet, and the mixture was agitated on a Vortex mixer (Scientific Industries, Inc., Queens Village, N. Y.). Cellular proteins and nucleic acids were precipitated with 5 ml of chilled 3 M trichloroacetic acid solution. The pre-

TABLE I  
Percent of Lymphocytes Identified as B Cells in  
Peripheral Blood and Lung Washings

Exp.	Surface immunoglobulin* (polyvalent)		
	Arterial	Venous	Lung
	%	%	%
1	—	18	22
2	—	22	15
3	27	23	20
4	25	28	21
5	28	30	16
6	—	31	20
7	20	24	25
8	—	22	16
Mean ± SEM	25 ± 1.8	25 ± 1.6‡	19 ± 1.2‡

\* Detected by fluorescein-conjugated antisera to human immunoglobulins ( $\gamma$ ,  $\mu$ ,  $\alpha$ ,  $\lambda$ ,  $\kappa$ ).

‡ Difference between these results significant,  $P < 0.05$ , Student's  $t$  test.

precipitate was centrifuged at 1,000  $g$  at 0°C for 20 min. The supernate was discarded and the precipitate dissolved in a 2 M ammonium hydroxide solution (0.5 ml). Aliquots of the dissolved samples were transferred to scintillation vials. Scintillation fluid<sup>4</sup> was added and radioactivity was measured with a liquid scintillation spectrometer (Packard Tri Carb, Packard Instrument Co., Inc., Downers Grove, Ill.).

## RESULTS

After introduction of 100–200 ml of sterile isotonic saline into a segmental bronchus via the bronchoscope, aspiration recovered a mixture of 50–100 ml of saline and bronchial secretions. The number of cells ranged between 4 and 16 million/ml (mean of  $9.5 \times 10^6$  cells/

<sup>4</sup> Toluene 3.78 liter; POPOP (1,4-bis[2-(5-phenyloxazol)]benzene) 1.14 g; PPO (2,5-diphenyloxazole) 15.1 g (Packard Instrument Co., Inc., Downers Grove, Ill.).

lavage, 12 experiments). The paucity of red blood cells in the aspirates indicated that cells were obtained from the bronchoalveolar air spaces and not from blood.

A Wright-Giemsa stain of the aspirated cells incubated with latex particles indicated that on the average, macrophages constituted 78% of the cells (range 60–88%); lymphocytes were 17% (11–30%).

*B cell markers—SIg.* The fraction of purified lymphocytes identified by SIg in peripheral blood and lung washings are compared for the same subjects in Table I. The mean percentage of lung lymphocytes bearing SIg for eight experiments was 19% (range of 15–25%). By comparison, the mean percentage of lymphocytes in peripheral venous blood with SIg was significantly greater, equaling 25% for the same eight subjects (range 18–31%). The proportion of lymphocytes bearing SIg was greater in peripheral venous blood than in lung in six out of eight subjects. A similar difference existed in three out of four experiments where the fraction of lymphocytes having SIg was compared in lung and peripheral arterial blood (Table I). Indeed, in four experiments, no significant difference in the fraction of cells bearing SIg existed between arterial and venous blood ( $P > 0.5$ ).

The distribution of B cells bearing SIg into cells with heavy chain classes,  $\mu$ ,  $\gamma$ , and  $\alpha$ , is shown for four subjects in Table II. For each experiment, the percentage of lung lymphocytes bearing a specific class of immunoglobulin is compared to that of peripheral venous blood lymphocytes in the same subjects and in eight other age-matched controls. Also in the table are the corresponding values (percentage) of lymphocytes bearing SIg detected by polyvalent antisera for venous blood and lung. For peripheral blood lymphocytes, IgM and IgG were the predominant classes in four experiments; the mean values were 12.5% and 11%, respectively; the corresponding mean value for IgA was 6.5%. A compar-

TABLE II  
B Cell Classes in Peripheral Blood and Lung Washings

Exp.	IgM		IgG		IgA		Polyvalent‡	
	Blood*	Lung	Blood	Lung	Blood	Lung	Blood	Lung
	% of purified lymphocytes							
1	14	18	10	12	9	4	28	21
2	16	13	11	6	6	7	30	16
3	12	16	13	12	4	5	24	25
4	8	11	10	7	7	4	22	16
Mean	12.5	14.5	11	9.3	6.5	5.0	26	19.5
Control§	(6–16)		(4–11)		(4–10)		(14–38)	

\* Peripheral venous blood.

‡ Polyvalent = antisera to  $\mu$ ,  $\gamma$ ,  $\alpha$ ,  $\lambda$ ,  $\kappa$  determinants.

§ Other controls from comparable age range (range of values,  $n = 10$ ).

TABLE III  
B Cells Identified by Complement Receptors in  
Peripheral Blood and Lung Washings

Exp.	EAC rosettes		
	Arterial	Blood	Lung
	% of purified lymphocytes		
1	—	18	10
2	—	24	16
3	21	24	18
4	16	15	13
5	30	26	16
6	—	36	28
7	15	16	11
8	—	20	11
Mean±SEM	21±3.4	22±2.4*	15±2.7*

\* Difference between these results significant ( $P < 0.001$ , paired Student's *t* test).

able order of frequency was observed in four experiments involving lung lymphocytes: IgM > IgG > IgA, with mean values of 14.5%, 9.3%, and 5%, respectively. The values for lung and blood lymphocyte classes were within the range of values for other controls. However, in each experiment, the sum of lung lymphocytes possessing heavy-chain Ig classes exceeded the percentage of lung B cells detected by polyvalent antisera: for lung lymphocytes, the total of cells with SIg classes equaled 28.8%, compared to 19.5% for the cells detected by polyvalent antisera (mean values for four experiments). In peripheral blood, the discrepancy was less striking, 30% and 26%, respectively.

*Identification of B cells—complement receptors.* Table III indicates the fraction of lung and peripheral

TABLE IV  
Lymphocytes Identified as T Cells in Peripheral  
Blood and Lung Washings

Exp.	E rosettes		
	Arterial	Venous	Lung
	% of purified lymphocytes		
1	—	48	30
2	—	64	45
3	72	74	61
4	75	72	58
5	74	77	66
6	—	58	44
7	70	64	52
8	—	75	20
Mean±SEM	73±1.1	67±3.5*	47±5.6*

\* Difference between lung and venous blood results significant,  $P < 0.01$ , Student's paired *t* test.

TABLE V  
Partition of T and B Cells in Lung and Peripheral  
Blood (Eight Subjects)

	Lung	Peripheral blood
	%±SEM	%±SEM
T cells*	47±5.6	67±3.5
B cells†		
EAC rosettes	15±2.1	22±2.4
SIg	19±1.2	25±1.6
Null cells‡		
(100% - T + SIg)	34±5.6	10±4.3
B/T ratio¶	1/2.5	1/2.7

\* Determined by the percentage of purified lymphocytes capable of forming E rosettes.

† B cells identified as percentage of cells possessing complement receptors (EAC) rosettes and surface immunoglobulin identified by fluorescein-tagged antisera to human immunoglobulins.

‡ Null cells defined as the percentage of lymphocytes that could not be identified as T cells by their capacity to form E rosettes or B cells by the presence of surface immunoglobulin.

|| Difference between these results is significant ( $P < 0.01$ , Student's *t* test).

¶ B/T ratio defined by the percentage of cells bearing surface immunoglobulin/percentage of each capable of forming E rosettes.

blood lymphocytes possessing complement receptors and capable of forming EAC rosettes. As for cells bearing SIg, the fraction of lymphocytes forming EAC rosettes was less for lung than for peripheral blood in eight experiments (15% versus 22%). Similarly, no significant difference was noted in the percentage of lymphocytes forming EAC rosettes between arterial and venous blood ( $P > 0.5$ ).

*Identification of T cells—E rosettes.* The percentage of lung lymphocytes identified as T cells by their capacity to form rosettes with unsensitized SRBC (E rosettes) is shown in Table IV. T cells comprised the majority of identifiable lung lymphocytes, equaling 47% compared to 67% T lymphocytes in peripheral blood (mean values for eight experiments). As for the B cell markers, the percentage of T cells obtained from lung was less in every experiment than the percentage of T cells among peripheral blood lymphocytes. Again, no significant differences in the percentage of T cells were observed between arterial and venous blood ( $P > 0.5$ ).

*Null cells.* Although the B/T cell ratio for lung lymphocytes (1/2.5) was comparable to results with peripheral blood lymphocytes (1/2.7), the proportion of cells that apparently lacked membrane markers ("null cells") was greater for lung (34%) than peripheral blood (10%) (Table V).

*Identification of T cells—response to mitogens.* The in vitro response of lung cells to soluble mitogens, PHA and Con A, was also assessed. Because of the relatively small numbers of lymphocytes recovered from each lavage, it was necessary to reduce the cell numbers per culture from  $5 \times 10^5$  cells/ml (standard culture) to  $5 \times 10^4$  cells/ml. Preliminary experiments were performed to examine the effect of mitogens on reduced numbers of cells. Fig. 2 compares the response of lung and peripheral blood lymphocytes for two subjects to PHA at cell concentrations of  $5 \times 10^4$  cells/ml over a 168-h period.

Each point represents the mean value of duplicate samples for the incorporation of radiolabeled thymidine, measured in disintegrations per minute. Peak responses of 6,000 and 9,000 dpm were observed for lung lympho-

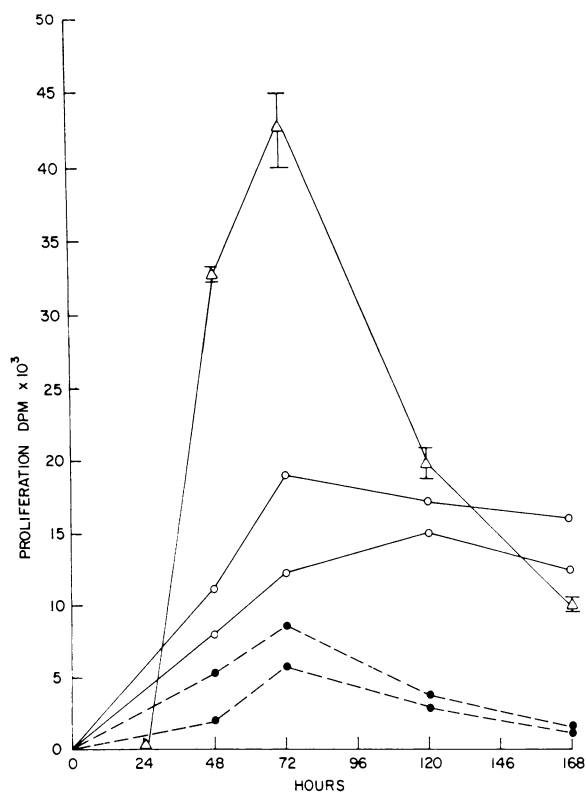


FIGURE 2 Response of peripheral venous blood and lung lymphocytes to PHA. Ordinate indicates incorporation of radiolabeled thymidine into replicating cells and measured as disintegrations per minute. ( $\Delta$ — $\Delta$ ) A representative response for a standard culture for peripheral blood lymphocytes containing  $5 \times 10^5$  cells/ml (20); vertical bar indicates SEM. ( $\circ$ — $\circ$ ) The response of peripheral blood lymphocytes of two normal subjects to PHA at a concentration of  $5 \times 10^4$  cells/ml. Each point represents the mean value for duplicate cultures. ( $\bullet$ — $\bullet$ ) The response of lung lymphocytes of two normal subjects to PHA at a concentration of  $5 \times 10^4$  cells/ml. Each point indicates the mean values for duplicate cultures.

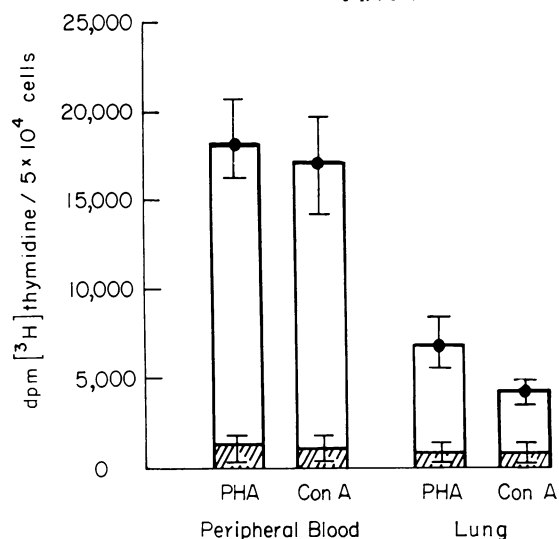


FIGURE 3 Response of peripheral blood and lung lymphocytes to PHA and Con A. Lymphocytes from lung and peripheral blood were obtained simultaneously from same subjects and cultured at a concentration of  $5 \times 10^4$  cells/ml with optimal doses ( $50 \mu\text{g}$ ) of PHA and Con A (20).  $\bar{x}$  represents mean  $\pm$  SEM for four experiments. Shaded area represents control cultures lacking mitogens. Difference between results for lung and blood lymphocyte responses to PHA is significant ( $P < 0.005$ , Student  $t$  test). Difference between results for lung and blood lymphocyte responses to Con A is significant ( $P < 0.01$ , Student  $t$  test). The proportion of T cells present in each culture (% T cells in lung/% T cells in peripheral blood) was 61/74, 58/72, 44/58, and 52/64.

cytes at 72 h and declined to the abscissa by 168 h. By comparison, equal numbers of peripheral blood lymphocytes exhibited greater peak responses of 19,000 and 12,000 dpm by 72 h and sustained these levels throughout the 7-day culture period. Also indicated in Fig. 2 is a representative response curve for normal peripheral blood lymphocytes cultured at a standard cell concentration of  $5 \times 10^5$  cells/ml. Peak responses of 43,000 dpm were observed between 48 and 72 h and declined rapidly between 5 and 7 days (20).

Fig. 3 summarizes the results of four experiments comparing the response to PHA and Con A of lung and peripheral blood lymphocytes obtained simultaneously from the same subjects and cultured in vitro for 72 h. Lung lymphocytes had a response of  $6,700 \pm 1,300$  dpm (mean  $\pm$  SEM) and  $4,300 \pm 850$  dpm to PHA and Con A, respectively. However, equal numbers of peripheral blood lymphocytes gave significantly greater responses to PHA and Con A, equaling  $18,500 \pm 2,400$  and  $17,000 \pm 2,700$  dpm, respectively. The proportion of T cells present in mitogen cultures for the four experiments was 61/74, 58/72, 52/64, and 44/58 (percentage of lung T cells/percentage of peripheral blood T cells). There was no significant difference in the viability be-

tween lung and peripheral blood lymphocytes after 72 h in culture.

Since local anesthetics in relatively high doses have been shown to inhibit mitogen stimulation of lymphocytes (23), additional experiments were performed to examine the possibility that lidocaine, used for topical anesthesia of the upper airway, might account for the diminished proliferative response of lung lymphocytes compared to peripheral blood lymphocytes. Purified blood lymphocytes ( $5 \times 10^5$  cells/ml) were first incubated for 30 min at 37°C with various concentrations of lidocaine-HCl ( $10^{-2}$ – $10^{-6}$  M) (Astra Pharmaceutical Products, Inc., Worcester, Mass.) and then incubated with PHA for 72 h (lidocaine remained in cultures throughout). Lidocaine at  $1 \times 10^{-3}$  M (230  $\mu$ g/ml) resulted in a 70% decrease in PHA stimulation compared to untreated cells. However, complete reversibility of inhibition occurred when cells were first incubated with lidocaine ( $10^{-3}$  M, 37°C for 30 min) and then washed three times in HBSS (as for lung) before culturing with PHA. Significant toxicity for lymphocytes was observed at  $1 \times 10^{-2}$  M lidocaine (less than 70% viability by trypan blue test). Controls consisted of cells incubated in the absence of lidocaine but washed and cultured with PHA as for test cultures. In addition, lidocaine ( $10^{-3}$  M) had no significant effect on the capacity of peripheral blood lymphocytes to form E and EAC rosettes.

## DISCUSSION

Our findings indicate that a significant fraction of cells (11–30%) recovered by segmental bronchial lavage in normal human lungs are viable lymphocytes consisting of T and B cells.

It should be emphasized that the population of normal subjects used in this study was a group of men, ages 21–35, who have never smoked, gave no history of acute or chronic lung disease, and have lived in an urban environment for at least 1 y. Furthermore, all subjects included in this study had completely normal pulmonary function tests, including more sensitive tests to evaluate small airway disease (closing volume). This definition of a normal population is relevant to our results since we and others (24–25) have observed marked changes in the total number and distribution of cells obtained by lavage from smokers who would otherwise be considered free of lung disease. In an age-matched group of subjects who had smoked for at least 5 y, the number of cells recovered by lavage was 10–20-fold greater than in the normal nonsmokers; also, in the smokers, alveolar macrophages constituted more than 95% of the cells (R. P. Daniele, unpublished data).

Cells obtained by lavage in this study are assumed to be representative of the distal airways to at least four to five generations of bronchi. These cells are

thought to be either free in the broncho-alveolar air spaces or loosely invested within the tracheobronchial mucosa so as to be dislodged by gentle irrigation with saline. The possibility that white cells obtained by lavage were derived from blood in the airways was considered unlikely because of the scarcity of red blood cells in the aspirates.

*Lung T cells.* Our findings differ in certain respects from that of Kaltreider and Salmon (26), who examined lung cells obtained by bronchopulmonary lavage in the dog. Although they showed that normal canine lungs had a population of B cells identified by *in vitro* IgG synthesis, they judged T cells to be absent from the lack of response of the cultured lung cells to PHA; a membrane marker for T cells was not employed. However, they did detect T cells as defined by the PHA response after tracheobronchial irritation. One explanation for the discrepancy between the study of Kaltreider and Salmon (26) and the present study may be species difference in the tracheobronchial response to irritation, i.e., in the same environment, the threshold for recruiting T cells into the canine lung air spaces may be higher than for the human lung.

In our study, using the E rosette method, T cells comprised 47% of identifiable lung cells. Although the nature and function of the receptor for SRBC is unclear, there is now considerable evidence to support the functional identity of thymus and thymus-derived lymphocytes with those cells having the capacity to form E rosettes (10, 12). Furthermore, we have recently shown that the total number of cells responding to PHA agrees closely with the number of cells capable of forming E rosettes (20).

The presence of T cells in human lungs was also tested by the response of lung lymphocytes to soluble Con A and PHA. Although the specificity of these mitogens for T cells has been questioned (27), the greater weight of current evidence suggests that PHA and Con A stimulate primarily T cells, particularly when proliferative responses are measured at 72 h (27–30). Although not quantitative, the response of lung lymphocytes to these mitogens is of particular importance since this phenomenon apparently mimics antigen recognition and stimulation of specific clones of T cells, and thus may be a valid model for cellular immunity (22).

The proliferative response at 72 h of lung lymphocytes to PHA and Con A was significantly less than that of peripheral blood lymphocytes obtained simultaneously from the same donors and cultured under identical conditions. The reason for this discrepancy is not clear. It cannot be ascribed to differences in viability between lung and peripheral blood lymphocytes (at 72 h). The likelihood that lidocaine might have been responsible for the diminished proliferative response of lung lymphocytes is argued against by additional experiments show-

ing reversibility of mitogen stimulation of peripheral blood lymphocytes incubated with lidocaine and then washed by the same procedure used for the preparation of lung lymphocytes. The possibility that the difference in mitogen response is due to the smaller proportion of lung T cells placed in culture compared to peripheral blood lymphocytes (maximum difference = 14%, Fig. 3) cannot be ruled out.

*Lung B cells.* B cells were demonstrated in human lungs by two independent membrane markers, the complement receptor and SIg. The proportion of lung cells identified with SIg by polyvalent antisera was 19% (mean value for eight experiments). With Ig class-specific antisera, IgM-positive cells were predominant, comprising 15% of lung lymphocytes (mean value for four experiments); IgG- and IgA-positive cells equaled 9% and 5%, respectively. It is noteworthy that in each experiment, the sum of the values for cells of each Ig class exceeded the proportion of cells bearing SIg identified by polyvalent antisera. Although we have no explanation for this consistent finding, one possibility is that a portion of lung lymphocytes may bind exogenous Ig (i.e., antigen-antibody complexes) via the Fc receptor for immunoglobulin (IgG); such a phenomenon has been shown to occur for B cells (31) as well as certain activated T cells (32).

Using the single cell assay of Jerne for antibody synthesis, Ford and Kuhn demonstrated in vitro production of antibody by cells obtained from bronchial lavage after antigenic stimulation via the respiratory route (6, 7). 75–90% of these cells produced IgM and 10–20% produced IgG, with 80% of the cells having typical lymphocyte morphology. Kaltreider et al. (8) have made similar observations on the local immune response to SRBC in canine lung, detecting primarily IgM- and IgG-producing cells; interestingly, they were unable to find IgA-producing cells in the bronchial aspirates. Thus, the relative order of frequency (i.e., IgM > IgG) of antibody-producing cells obtained by lavage in these studies is in agreement with our data showing the predominance of cells with IgM and IgG on their surface.

15% of the lung lymphocytes also possessed complement receptors. This fraction was less than the percentage of B cells identified by SIg in both lung and blood. Although the complement receptor is present on the majority of cells bearing SIg, the binding of antigen-antibody complement complexes to some lung cells could inhibit their capacity to form EAC rosettes (33) and account for the observed differences in the proportion of lung cells identified by complement receptors and SIg. Further studies are required to reconcile these differences.

*The presence of null cells.* As mentioned above, the fraction of lung lymphocytes identified as T and B cells by membrane markers was less than corresponding

values for peripheral blood. Table V summarizes the percentages of lymphocytes identified as T cells by E rosettes and as B cells by SIg and complement receptors. It is apparent that for lung lymphocytes, the sum of T and B cells identified by E rosettes and SIg leaves 34% of the lymphocytes unclassified. Cells having lymphocyte morphology but lacking T or B membrane markers have been tentatively called null cells by several investigators (34, 35). However, the incidence of null cells may depend on the methods used. With present techniques, we detected a null population in peripheral blood lymphocytes of about 10%. This value agrees roughly with that of Dickler et al. (36), who showed, using multiple markers on the same cells, that a small fraction of lymphocytes from human peripheral blood (2–13%) lack identifying surface markers. Thus, the consistently large fraction of cells in bronchial aspirates that lack surface receptors in the present study suggests that null cells in lungs represent more than imperfect methodology. Hypothetically, null cells could consist of one or several unique populations of cells. For example, they could represent a lymphocyte population at an early developmental stage when there is an insufficient density of membrane markers to be detected by the techniques described (37). Alternatively, they could represent mature effector cells that have lost membrane markers (i.e., plasma cells) (38, 39). A third possibility is that membrane markers may be blocked by exogenous factors such as antigen-antibody-complement complexes (33).

If the broncho-alveolar air spaces are a compartment possessing active immunologic cells that act as a first-line defense against air-borne antigens, then one might assume that there is a sufficiently dynamic mechanism to supply and replenish the required cellular elements. Little is known concerning the origin, traffic, and life history of lymphoid cells present in the lung airways. The presence of lung T and B cells in the same relative distribution as found in peripheral blood suggests that blood may be an important source for lung lymphocytes. Indeed, recent studies in the rat using radiolabeled lymphocytes indicate that a significant number of recently divided lymphocytes migrate from blood into the lung's air spaces (C. Beacham and R. P. Daniele, manuscript in preparation). Furthermore, the possibility that the lung may constitute a preferential trap for either T or B cells or both from blood is not excluded by our data showing an insignificant difference in the fraction of T or B cells between venous and arterial blood. Large arteriovenous differences would only be expected if trapping of either T or B cells across the alveolar capillary bed were massive, since the large blood flow would allow considerable trapping in the face of small differences in the number of T or B cells across this vascular network.



It should be emphasized, however, that it is unclear as to what relative contribution blood, tracheobronchial lymph nodes, and the submucosal secretory system make towards the composition of broncho-alveolar lymphocytes.

To our knowledge, this is the first demonstration that relatively stable populations of T and B lymphocytes reside in the lower respiratory tract of normal human lungs. The presence of viable T and B cells in the broncho-alveolar air spaces in a ratio (about 1/2.5 for B/T) comparable to what is found in peripheral blood, lymph node, and spleen (10) suggests that these cells may participate in the local immune defenses of the lung. However, to ascribe such a function to the lung lymphocytes identified in this study will require more direct evidence that they correspond or are related to the sensitized cells lavaged from experimental animals and shown to express humoral and cellular immune responses.

Should such a relationship prove valid, then cells sampled from the lower respiratory tract may provide some measure of the local immunocompetence of the lung, much as sampling of peripheral blood is used to assess systemic immunity.

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#### REFERENCES

- Green, G. M. 1970. In defense of the lung. *Am. Rev. Respir. Dis.* **102**: 691-703.
- Nelson, D. S. 1969. Macrophages and immunity. *Front. Biol.* **11**: 335 pp.
- Tomasi, T. B., Jr. 1972. Secretory immunoglobulins. *N. Engl. J. Med.* **287**: 500-506.
- Waldman, R. H., and C. S. Henney. 1971. Cell-mediated immunity and antibody responses in the respiratory tract after local and systemic immunization. *J. Exp. Med.* **134**: 482-494.
- Holub, M., and R. E. Hauser. 1969. Lung alveolar histiocytes engaged in antibody production. *Immunology.* **17**: 207-226.
- Ford, R. J., Jr., and C. Kuhn. 1973. Immunologic competence of alveolar cells. I. The plaque-forming response to particulate and soluble antigens. *Am. Rev. Respir. Dis.* **107**: 763-770.
- Ford, R. J., Jr., and C. Kuhn. 1973. Immunologic competence of alveolar cells. II. Modification of the plaque-forming response by inhibitors, tolerance and chronic stimulation. *Am. Rev. Respir. Dis.* **107**: 771-775.
- Kaltreider, H. B., L. Kyselka, and S. E. Salmon. 1974. Immunology of the lower respiratory tract. II. The plaque-forming response of canine lymphoid tissues to sheep erythrocytes after intrapulmonary or intravenous immunization. *J. Clin. Invest.* **54**: 263-270.
- Jerne, N. K., A. A. Nordin, and C. Henry. 1963. The agar plaque technique for recognizing antibody-producing cells. In *Cell-Bound Antibodies*. B. Amos and H. Koprowski, editors. The Wistar Institute Press, Philadelphia, Pa. 109-125.
- Greaves, M. F., J. J. T. Owen, and M. C. Raff. 1973. T and B Lymphocytes. Origin, Properties and Role in Immune Responses. Excerpta Medica Amsterdam, American Elsevier Publishing Co., Inc., New York.
- Raff, M. C. 1973. T and B lymphocytes and immune responses. *Nature (Lond.)*. **242**: 19-23.
- Jondol, M., H. Wigzell, and F. Auiti. 1973. Human lymphocyte subpopulations: classification according to surface markers and/or functional characteristics. *Transplant. Rev.* **16**: 163-195.
- Coombs, R. R. A., B. W. Gurner, A. B. Wilson, G. Holm, and B. Lindgren. 1970. Rosette-formation between human lymphocytes and sheep red cells not involving immunoglobulin receptors. *Int. Arch. Allergy Appl. Immunol.* **39**: 658-663.
- Lay, W. H., N. F. Mendes, C. Bianco, and V. Nussen-zweig. 1971. Binding of sheep red blood cells to a large population of human lymphocytes. *Nature (Lond.)*. **230**: 531-532.
- Rabellino, E., S. Colon, H. M. Grey, and E. R. Unanue. 1971. Immunoglobulins on the surface of lymphocytes. I. Distribution and quantitation. *J. Exp. Med.* **133**: 156-167.
- Bianco, C., and V. Nussen-zweig. 1971. Theta-bearing and complement-receptor lymphocytes are distinct populations of cells. *Science (Wash. D. C.)*. **173**: 154-156.
- Böyum, A. 1968. Separation of leukocytes from blood and bone marrow. *Scand. J. Clin. Lab. Invest. Suppl* **97**: 21: 1-109.
- Mendes, N. F., M. E. A. Tolnai, P. A. Nelson, N. P. A. Silveira, R. B. Gilbertson, and R. S. Metzgar. 1973. Technical aspects of the rosette tests used to detect human complement receptor (B) and sheep erythrocyte-binding (T) lymphocytes. *J. Immunol.* **111**: 860-867.
- Bentwich, Z., and H. G. Kunkel. 1973. Specific properties of human B and T lymphocytes and alterations in disease. *Transplant. Rev.* **16**: 29-50.
- Nowell, P. C., R. P. Daniele, and L. Winger. 1975. Kinetics of human lymphocyte proliferation: Proportion of cells responsive to PHA and correlation with E rosette formation. *RES. J. ReticuloEndothel. Soc.* **17**: 47-56.
- Nowell, P. C. 1960. Phytohemagglutinin: an initiator of mitosis in cultures of normal human leukocytes. *Cancer Res.* **20**: 462-466.
- Greaves, M., and G. Janossy. 1972. Elicitation of selective T and B lymphocyte responses by cell surface ligands. *Transplant. Rev.* **11**: 87-130.
- Cullen, B. F., P. B. Chretien, and B. G. Leventhal. 1972. The effect of lignocaine on PHA-stimulated human lymphocyte transformation. *Br. J. Anaesth.* **44**: 1247-1251.
- Golde, D. W., L. A. Byers, and T. N. Finley. 1974. Proliferative capacity of the human alveolar macrophage. *Nature (Lond.)*. **247**: 373-375.
- Harris, J. O., E. W. Swenson, and J. E. Johnson, III. 1970. Human alveolar macrophages: comparison of phagocytic ability, glucose utilization and ultrastructure in smokers and non-smokers. *J. Clin. Invest.* **49**: 2086-2096.

26. Kaltreider, H. B., and S. E. Salmon. 1973. Immunology of the lower respiratory tract. Functional properties of bronchoalveolar lymphocytes obtained from the normal canine lung. *J. Clin. Invest.* **52**: 2211-2217.
27. Chess, L., R. P. MacDermott, and S. F. Schlossman. 1974. Immunologic functions of isolated human lymphocyte subpopulations. I. Quantitative isolation of human T and B cells and response to mitogens. *J. Immunol.* **113**: 1113-1121.
28. Lohrmann, H.-P., L. Novikovs, and R. G. Graw, Jr. 1974. Cellular interactions in the proliferative response of human T and B lymphocytes to phytomitogens and allogeneic lymphocytes. *J. Exp. Med.* **139**: 1553-1567.
29. Geha, R. S., and E. Merler. 1974. Response of human thymus-derived (T) and non-thymus-derived (B) lymphocytes to mitogenic stimulation *in vitro*. *Eur. J. Immunol.* **4**: 193-199.
30. Greaves, M., G. Janossy, and M. Doenhoff. 1974. Selective triggering of human T and B lymphocytes *in vitro* by polyclonal mitogens. *J. Exp. Med.* **140**: 1-18.
31. Dickler, H. B. 1974. Studies of the human lymphocyte receptor for heat-aggregated or antigen-complexed immunoglobulin. *J. Exp. Med.* **140**: 508-522.
32. Yoshida, T. O., and B. Andersson. 1972. Evidence for a receptor recognizing antigen complexed immunoglobulin on the surface of activated mouse thymus lymphocytes. *Scand. J. Immunol.* **1**: 401-408.
33. Eden, A., C. Bianco, and V. Nussenzweig. 1973. Mechanism of binding of soluble immune complexes to lymphocytes. *Cell. Immunol.* **7**: 459-473.
34. Williams, R. C., Jr., J. R. DeBord, O. J. Mellbye, R. P. Messner, and F. D. Lindström. 1973. Studies of T- and B-lymphocytes in patients with connective tissue diseases. *J. Clin. Invest.* **52**: 283-295.
35. Stobo, J. D., N. Talal, and W. E. Paul. 1972. Lymphocyte classes in New Zealand mice. II. Decreased frequency of immunoglobulin-bearing lymphocytes and increased frequency of lymphocytes lacking detectable  $\theta$  or immunoglobulin determinants. *J. Immunol.* **109**: 701-710.
36. Dickler, H. B., N. F. Adkinson, Jr., and W. D. Terry. 1974. Evidence for individual human peripheral blood lymphocytes bearing both B and T cell markers. *Nature (Lond.)* **247**: 213-215.
37. Gelfand, M. C., G. J. Elfenbein, M. M. Frank, and W. E. Paul. 1974. Ontogeny of B lymphocytes. II. Relative rates of appearance of lymphocytes bearing surface immunoglobulin and complement receptors. *J. Exp. Med.* **139**: 1125-1141.
38. Basten, A., N. L. Warner, and T. Mandel. 1972. A receptor for antibody on B lymphocytes. II. Immunological and electron microscopy characteristics. *J. Exp. Med.* **135**: 627-642.
39. Paraskevas, F., S-T. Lee, K. B. Orr, and L. G. Israels. 1972. A receptor for Fc on mouse B-lymphocytes. *J. Immunol.* **108**: 1319-1327.