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A comparison of the expression of lymphocyte activation markers in blood, bronchial biopsies and bronchoalveolar lavage: evidence for an enrichment of activated T lymphocytes in the bronchoalveolar space

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In this study healthy never-smoking subjects ($n = 18$) were recruited from a population study. Bronchoalveolar lavage (BAL), blood lymphocytes and bronchial biopsies, analysed both in the epithelium and lamina propria, were stained for T and B lymphocytes, natural killer (NK) cells and different subpopulations of T lymphocytes. In BAL, significantly higher proportions of T lymphocytes (CD3), T lymphocyte activation markers; HLA-DR, $CD26^+$, $CD49a^+$, $CD54^+$ and $CD69^+$, helper T ($CD3^+4^+$) and memory helper T lymphocytes ($CD4^+45RO^+29^+$) and memory T lymphocytes ($CD3^+45RO^+$) were found, compared to blood. However, the proportion of IL-2 receptor-positive T lymphocytes ($CD25^+$) was lower in BAL than in blood. A previously described higher ratio of $CD3^+4^+$/$CD3^+8^+$ in BAL than in blood (3·4 *vs* 1·7; $P = 0{\cdot}001$) was confirmed. In bronchial biopsies, we found significantly higher numbers of $CD8^+$ cell profiles per mm^2 in the epithelial compared to the lamina propria compartment.

We conclude that healthy never-smoking men have higher levels of activated memory T lymphocytes in BAL than in blood, and that the T-cell subpopulations differ in the epithelial compared to the lamina propria compartment in the bronchial mucosa and these compartments should be analysed separately. It is reasonable to think that there is a gradient from blood to the airway lumen where T cells are recruited from blood to take part in the defense towards damaging agents.

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Introduction

On the alveolar surface the external environment containing micro-organisms and other antigens is only a short distance from the blood. The major part of the antigenic load of the inhaled air is removed through the action of the mucociliary clearence of the conducting airways. A small fraction will reach the alveolus and interact with cells; alveolar macrophages, lymphocytes and alveolar epithelial cells. It has been suggested that pulmonary T cells mediate antigen-specific effector functions and, with other pulmonary immunocompetent cells, secrete cytokines and other biological response modifiers which favour the tissue localization and activation of newly recruited antigen-specific memory cells (1).

Most earlier studies have analysed the expression of lymphocyte surface markers in bronchial biopsies, bronchoalveolar lavage (BAL) or blood in patients with asthma or chronic bronchitis. Often only a relatively low number of healthy non-smokers were included as controls (2–4). However, no previous study has focused on life-long never-smokers and compared lymphocyte subpopulations in both bronchial biopsies, BAL and blood from each subject.

Furthermore, the important airway biopsy studies have described the lymphocyte subpopulations in lamina propria (2, 3, 5) only a few earlier studies have investigated lymphocyte subpopulations in the epithelium (6, 7).

Lymphocytes in the bronchial lamina propria have been shown to be mainly T lymphocytes and very low levels of B-lymphocytes and natural killer (NK) cells have been detected in the mucosa of central airways (3, 6). Both CD4- and CD8-positive T lymphocytes have been detected with a small dominance of CD8-positive cells.

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Lymphocytes in BAL have been shown to have a higher frequency of activation markers and memory cell markers than those in blood (4, 8).

Our hypothesis was that lymphocytes found in BAL are functional and act together with cells of the innate immune system as a first line of defence against inhaled damaging agents. Therefore, we studied activation and proliferation markers of T lymphocytes in the blood and BAL compartments and compared them with bronchial biopsy material divided into the epithelium and lamina propria compartments.

Materials and Methods

SUBJECTS

The subjects were recruited from the population study 'Men born 1933 in Göteborg' where 1016 men were studied in 1983 (9). In total, 879 men were called for a new evaluation in 1993. Of these, 532 were evaluated with spirometry. When the subjects were divided according to smoking habits, 112 were smokers, 198 were never-smokers and 222 ex-smokers. A random sample of 60 never-smokers was chosen for a lung examination. The result was that 34 never-smokers were included and, of these, 18 men agreed to undergo bronchoscopy, while 15 did not want to take part in the study.

Subjects were included if they were healthy and had not sought medical attention for any pulmonary problem. Subjects were excluded if they 1. had any pulmonary problem, 2. had scoliosis or other diseases with deformation of the thorax, 3. had a history of congestive heart failure or unstable angina pectoris, 4. had any other severe disease, 5. if they had any kind of infection during the 4 weeks preceeding the examination or 6. if they had been on cortocosteroid, *N*-acetylcysteine (NAC) or acetylsalicylic acid (ASA) treatment less than 4 weeks prior to blood tests or bronchoscopy (with the exception of those persons with a prior heart infarction). A total of 11 subjects were excluded.

The study was approved by the Ethics Committée at Sahlgrenska University Hospital, Göteborg, Sweden.

FIBRE-OPTIC BRONCHOSCOPY AND COLLECTION OF SAMPLES

Premedication with diazepam 5 mg orally was followed by 0.5 ml morphine–scopolamin i.m., except if the subject had a history of kidney or gall bladder disease in which case petidine 75 mg i.m. and atropine 0.5 mg s.c. were given instead. Additional i.v. diazepam was also given during the bronchoscopic procedure in some subjects.

All subjects were given terbutalin 0.25 mg dose^{-1} 2×3 in a nebulizer to avoid unexpected bronchoconstriction during the procedure. Local anaesthesia was achieved initially with 1% tetracaine spray in the mouth and laryngeal tract; additional anaesthesia was applied through the bronchoscope channel for the lower respiratory tract. A bronchoscopy was taken trans-orally with an Olympus flexible fibre-optic bronchoscope (Tokyo, Japan). The subject was examined in a supine position by one experienced bronchoscopist (A.E.J.). Two subjects were examined by another experienced bronchoscopist (C.G.L.). Oxygen saturation was measured with an Ohmeda Pulse Oximeter (Louisville, U.S.A.) during the bronchoscopy and supplemental oxygen at a rate of 2–3 l min^{-1} was given through a nasal catheter when needed.

All bronchoscopies were performed between 0830 and 1000 hours. Bronchoalveolar lavage (BAL) was performed as follows: 3×50 ml of phosphate- buffered saline (PBS) was instilled through the bronchoscope channel into the middle lobe with the bronchoscope in a wedged position and suction was performed after each portion of PBS into siliconized bottles placed on ice. The samples were then transported to the laboratory for analysis. Recovery was measured and the fluid filtered through a nylon web with a pore size of 100 μm for retention of mucus and cell debris. The lavage fluid was then centrifuged at 250 ***g*** for 10 min at 4°C. The supernatant was separated, and the cell pellet was resuspended in PBS. The total number of cells in BAL was determined by using a haemocytometer. Cell viability was estimated by means of trypan blue exclusion. Calculation of cell differentials was done on cytocentrifuged preparations (Cytospin 2; Shandon Southern Products Ltd., Runcorn, U.K.) stained with May–Grünwald–Giemsa and after counting 1000 cells. BAL was performed during all bronchoscopies, but in one case the BAL could not be evaluated due to a high amount of debris which made the flow cytometry analysis impossible to interpret.

Peripheral bronchial biopsies (3–4 biopsies per subject) were also taken in the lower left lobe with alligator forceps. The biopsies were gently removed from the forceps, immediately placed in a sterile, moist chamber and were transported to the laboratory for further processing. Ten biopsies were evaluable, while eight could not be evaluated. In one of the 18 subjects no biopsy material was obtainable, while in seven, no histologically evaluable biopsy material was obtained. Two more biopsies did not give an adequate CD69 staining in the epithelium and the results from both the epithelium and lamina propria were therefore excluded. The subjects in the eight unevaluable biopsies did not differ in sex, age or lung function, except for *D*LCO (% pred.), compared to the subjects giving evaluable biopsies (Table 1).

FLOW CYTOMETRY

Subpopulations of lymphocytes in BAL and blood were determined by flow cytometry. The samples were stained with combinations of murine monoclonal antibodies, directly conjugated with fluorochromes (fluorescein isothiocyanate, phycoerythrine or Per-CP) or with a biotinconjugated antibody and streptavidin conjugated with Per-CP. BAL cells (1×10^5 cells sample^{-1}) and whole blood (with EDTA as an anticoagulant in a volume corresponding to about 5×10^5 cells antibody staining^{-1}) were incubated at 4°C for 15 min with

TABLE 1. Lung function — descriptive statistics

	Evaluable biopsies ($n=10$)	Unevaluable biopsies ($n=8$)	P*
TLC (% pred.)	98 ± 13	98 ± 12	n.s.
RV (% pred.)	102 ± 36	86 ± 10	n.s.
VC (% pred.)	92 ± 9	102 ± 14	n.s.
FEV_1 (% pred.)	105 ± 9	114 ± 19	n.s
DLCO (% pred.)	88 ± 12	107 ± 14	0·01
DLCO VA^{-1} (% pred.)	88 ± 7	97 ± 14	n.s
N_2 test (% pred.)	108 ± 47	72 ± 30	n.s.

Data are given as mean ±SD.
*P according to Mann–Whitney«U-test; n.s. not significant

antibodies in the concentrations recommended by the manufacturer.

The following fluorescein isothiocyanate-conjugated antibodies were used: CD4, CD25, CD44, CD45RA, CD69, TCR$\alpha\beta$ (Becton-Dickinson Inc., Mountain View, CA, U.S.A.); CD26 (Coulter Corporation, Miami, FL, U.S.A.); CD29 (Dakppatts A/S, Glostrup, Denmark); CD49a and TCR $\gamma\delta$ (T Cell Diagnostics Inc., Cambridge, MA, U.S.A.). The following phycoerythrin-conjugated antibodies were used: CD8, CD25, CD28, CD38, CD45RO, CD54, $CD56^+$ CD16, HLA-DR and 62L (Becton-Dickinson Inc.). The Per-CP-conjugated antibodies CD3, CD4 and CD19 and Per-CP-conjugated streptavidin were used (Becton-Dickinson Inc.). The CDS biotinylated antibody was used: (Becton-Dickinson Inc.). Cell analysis was done on a fluorescence activated cell sorter (FACScan) flow cytometer (Becton-Dickinson Inc.), calibrated with CALIBRITE™ beads (Becton-Dickinson Inc.) and AutoCOMP™ software (Becton-Dickinson Inc.). A lymphocyte gate was set manually according to the location in the forward-scatter versus side-scatter diagram. Negative isotype controls (Becton-Dickinson Inc.) were used to set quadrant markers which delineated positive fluorescent staining from non-antigen specific staining. Dot plots and quadrant statistics from three-colour analysis were generated by Lysis II software (Becton-Dickinson Inc.). The absolute number of blood lymphocytes was determined using a haematological cell counter (Sysmex-K1000; TOA Medical Electronics Co, Japan). In a majority of the BAL samples it was difficult to discriminate events representing lymphocytes from events due to debris in the forward-scatter versus side-scatter diagram. To compensate for this, all percentages of lymphocyte subpopulations from BAL and blood from each individual were divided by the percentages of cells positive for CD45 and negative for CD14, which represent all major lymphocyte populations. These adjusted values were then used in all calculations. The results for each subpopulation were expressed as the percentage of lymphocytes or percentage of T lymphocytes in BAL and as the percentage of lymphocytes or percentage of T lymphocytes and as the number of cells $\times 10^9 l^{-1}$ in blood.

IMMUNOHISTOCHEMISTRY

Biopsies were oriented using a dissection microscope in order to obtain perpendicular sections including both epithelium and lamina propria. They were then embedded in Tissue Tek II OCT (Miles Scientific, Naperville, IL, U.S.A.), immediately frozen in isopenthane pre-cooled in liquid nitrogen and stored at -70°C. The biopsies were cut with a cryostat (Kryostat 1720 Digital, Leitz, Germany) into 5-μm thick sections and placed on gelatin-coated slides. One section from each biopsy was fixed in formalin alcohol and was stained by Mayer's haematoxylin, lithium carbonate and eosin. Only biopsies with intact epithelium and lamina propria were subject to immunohistochemistry. Five sections were taken 30–40 μm apart and were put on a gelatin-coated slide for each staining. The sections were stored at -20°C before immunohistochemistry staining was performed.

An indirect immunoperoxidase protocol was used and the optimal dilutions of reagents were determined using bronchial and tonsil tissue, as well as phythaemagglutinin (PHA) stimulated blood lymphocytes. Briefly, the sections were brought to room temperature, fixed in acetone and air-dried. Sections were rehydrated in PBS and incubated with the primary antibody. The following primary monoclonal antibodies and concentrations were used: CD3 (2·1 $\mu g\ ml^{-1}$), CD4 (1·7 $\mu g\ ml^{-1}$), CD8 (1 $\mu g\ ml^{-1}$) (DAKOPATTS A/S, Glostrup, Denmark) and CD69 (0·67 $\mu g\ ml^{-1}$, Caltag Laboratories, San Fransisco, CA, U.S.A.). After incubation for 1 h at room temperature and washing three times in PBS, the slides were further processed in an automatic staining machine (Cadenza; Shandon Scientific Ltd, Cheshire, U.K.). After washing in PBS the slides were incubated for 1 h at room temperature with a biotinylated rabbit anti-mouse immunoglobulin antibody (Fab E413, 4 $\mu g\ ml^{-1}$, DAKOPATTS A/S) diluted in PBS with 1% bovine serum albumin (BSA) (Sigma, St. Louis, MO, U.S.A.) and 1% human serum. After another washing in PBS, endogenous peroxidase activity was blocked with 0.3% H_2O_2 (E. Merck, Darmstadt, Germany) in PBS for 5 min in the dark. After washing in PBS, the slides were incubated with streptABComplex/HRP Duet (DAKOPATTS A/S) for 1 h. After a final wash in PBS, 3-amino-9-ethylcarbazole (AEC, Sigma), 33 $\mu g\ ml^{-1}$ diluted in 0.02M sodium acetate buffer (E. Merck), pH 5.5, with 12% dimethylsulphoxide (DMSO) and 0·5% H_2O_2 was added as substrate. The reaction was stopped after 8–15 min with distilled water for optimal colour and development. Counterstaining was performed with Mayer's haematoxylin. The slides were mounted in an aqeuous medium (Aquatex, E. Merck) .

The density of labelled cells, estimated as the number of positive cell profiles per mm^2 in each section, was determined using a light microscope (Axioplan, Carl Zeiss Jena GmgH, Jena, Germany), connected to a digital camera (Prog/Res 3012, Kontron Elektronik GmbH, Eching, Germany) and connected to a computer (Kontron Elektronik GmbH, Eching, Germany). Cell profiles per mm^2 were estimated using an interactive image analysis

system (IBAS version 2.5, Kontron Elektronik GmbH). Object magnification was ×40.

The sections were analysed separately in an epithelial and a lamina propria compartment. In the epithelial compartment the whole available area and in the lamina propria compartment the area between 0 and 100 μm below the basement membrane was analysed. Lymphocytes were counted as positive if they had a nuclear profile surrounded by a distinctly coloured cell membrane. Lymphocytes with only segments of a membrane were not counted and membranes without a nuclear profile were excluded.

TABLE 2. Recovery and cell viability in BAL. Cell populations in BAL

	Never-smokers (n=17)
Total number of cells in BAL ($\times 10^6$)	6·0 (0·2–18)
Cell concentration in BAL ($\times 10^4$cells ml^{-1})	8·5 (2·5–20·0)
Recovery in BAL (ml)	85 (50–120)
Cell viability in BAL (alive dead^{-1}, %)	76 (43–86)
Cell differential counts BAL (%)	
Macrophages	90 (75–96)
Lymphocytes	6 (0–23)
Neutrophils	2 (1–5)
Eosinophils	1 (0–3)

Data are given as median (range).

DATA ANALYSIS

As data for lymphocyte subpopulations are not normally distributed, group data are presented with their median and range. For lung function variables mean and standard deviation (SD) are presented. We assumed that the data were not evenly distributed, therefore we used the non-parametric Mann–Whitney U-test to analyse differences between the groups. We used Abacus Concepts Stat View 4.5® (Abacus Concepts, Inc., Berkeley, CA, U.S.A., 1994) for statistical calculations.

Results

COMPARISON OF LYMPHOCYTE SUBPOPULATIONS IN BAL AND BLOOD

Total cells, cell concentration, cell viability and cell differential counts in BAL are shown in Table 2. To

TABLE 3. Comparison of percentages of lymphocyte subpopulations in BAL and blood

Cell population	Surface antigen	BAL (n=17) Median (range)	Blood (n=34) Median (range)	P*
T lymphocyte	CD3$^+$	89 (79–97)	72 (57–92)	0·0001
B lymphocyte	CD19$^+$	0 (0–3)	8 (2–20)	0·0001
NK cell	CD3-56$^+$	3 (0–10)	13 (3–30)	0·0001
T lymphocyte subpopulations				
T cell receptor $\alpha\beta$	CD3$^+$TCR$\alpha\beta$	97 (84–103)	94 (70–104)	n.s.
T cell receptor $\gamma\delta$	CD3$^+$TCR $\gamma\delta$	2 (1–8)	3 (1–24)	n.s.
Unprimed cell	CD3$^+$45RA$^+$	29 (6–57)	58 (36–98)	0·0001
Memory cell	CD3$^+$45RO$^+$	96 (62–107)	66 (48–84)	0·0001
Memory helper T cell	CD4$^+$ 45RO$^+$29$^+$	75 (33–89)	43 (5–66)	0·0001
Helper/inducer cell	CD3$^+$4$^+$	75 (39–90)	62 (37–80)	0·001
Cytotoxic/supressor cell	CD3$^+$8$^+$	21 (10–59)	36 (16–66)	0·01
Helper/ cytotoxic ratio	CD3$^+$4$^+$/CD3$^+$8$^+$	3·4 (0·7–7·5)	1·7 (0·6–5·1)	0·001
Activated T cell	CD3$^+$DR$^+$	86 (65–104)	26 (5–96)	0·0001
Activated T cell	CD3$^+$25$^+$	11 (0–41)	25 (8–81)	0·002
Activated T cell	CD3$^+$26$^+$	12 (1–70)	2 (0–30)	0·0005
Activated T cell	CD3$^+$28$^+$	75 (10–95)	75 (50–96)	n.s.
Activated T cell	CD3$^+$38$^+$	17 (5–55)	52 (19–72)	0·0001
Activated T cell	CD3$^+$44$^+$	100 (94–106)	99 (90–105)	n.s.
Activated T cell	CD3$^+$49a$^+$	46 (22–72)	1 (0–36)	0·0001
Activated T cell	CD3$^+$ 54$^+$	82 (29–100)	30 (10–77)	0·0001
Activated T cell	CD3$^+$57$^+$	17 (0–50)	15 (8–39)	n.s.
Activated T cell	CD3$^+$69$^+$	80 (52–94)	2 (0–13)	0·0001
Activated T cell	CD3$^+$62L$^+$	18 (4–56)	70 (34–89)	0·0001
Activated T cell	CD3$^+$56$^+$16$^+$	2 (0–11)	2 (0–13)	n.s.

Data are give as median (range).
*P according to the Mann–Whitney U-test, n.s., not significant.

analyse possible differences in the distribution of major lymphocyte subpopulations in BAL and blood, the proportions in BAL and blood were compared (Table 3). The proportion of T lymphocytes (CD3) was significantly higher in BAL than in blood, while the proportions of B lymphocytes and NK cells were significantly lower in BAL than in blood .

The helper T lymphocytes ($CD3^+4^+$) were found in higher proportions in BAL than in blood (Table 3). The mean ratio of $CD3^+4^+/CD3^+8^+$ was 3·4 in BAL and 1·7 in blood and the difference was highly significant. The proportions of memory T ($CD3^+45RO^+$) and memory helper T ($CD4^+45RO^+29^+$) lymphocytes were significantly higher in BAL, and that of unprimed T lymphocytes

TABLE 4. Lymphocyte subpopulations in different compartments of bronchial biopsies from never-smokers

Lymphocyte subpopulations	Compartment	Density of labelled cells (profiles mm^{-2}) median (range)	P*	Number of counted cells (profiles/$subject^{-1}$) median (range)	Estimated tissue area (mm^2 $subject^{-1}$) median (range)
CD3	Epithelium	426 (160–1096)	n.s.	77 (9–144)	0·14 (0·06–0·21)
	Lamina prop.	460 (136–977)		111 (46–303)	0·22 (0·10–0·41)
CD4	Epithelium	217 (60–338)	n.s.	35 (2–234)	0·13 (0·05–0·27)
	Lamina prop.	138 (4–591)		30 (1–183)	0·23 (0·11–0·37)
CD8	Epithelium	518 (0–703)	0·04	62 (0–164)	0·13 (0·06–0·31)
	Lamina prop.	212 (6–591)		35 (2–234)	0·24 (0·12–0·70)
CD69	Epithelium	290 (16–575)	n.s.	41 (2–145)	0·12 (0·04–0·25)
	Lamina prop.	42 (1–773)		16 (1–245)	0·21 (0·10–0·39)

P according to Mann–Whitney *U*-test; n.s., not significant.

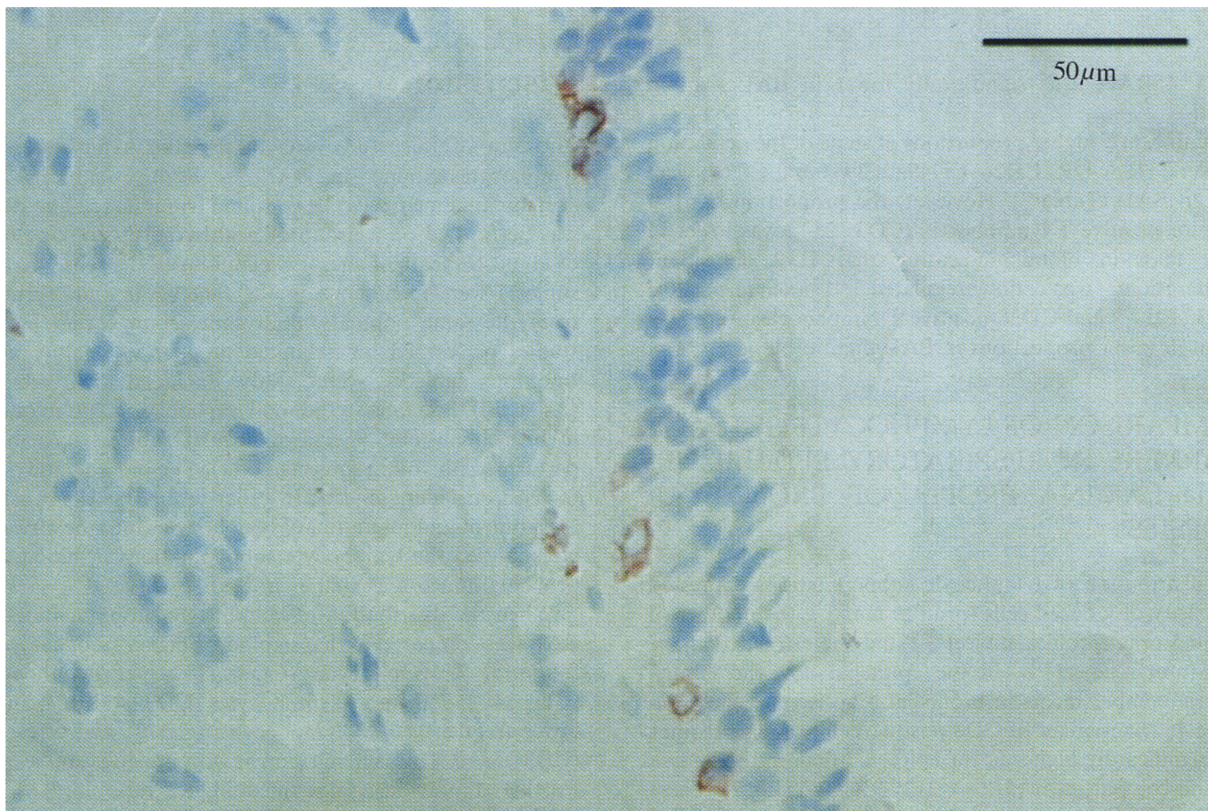

FIG. 1. Photomicrograph of a bronchial biopsy from a healthy never-smoking subject showing CD8-positive lymphocytes in the epithelial compartment.

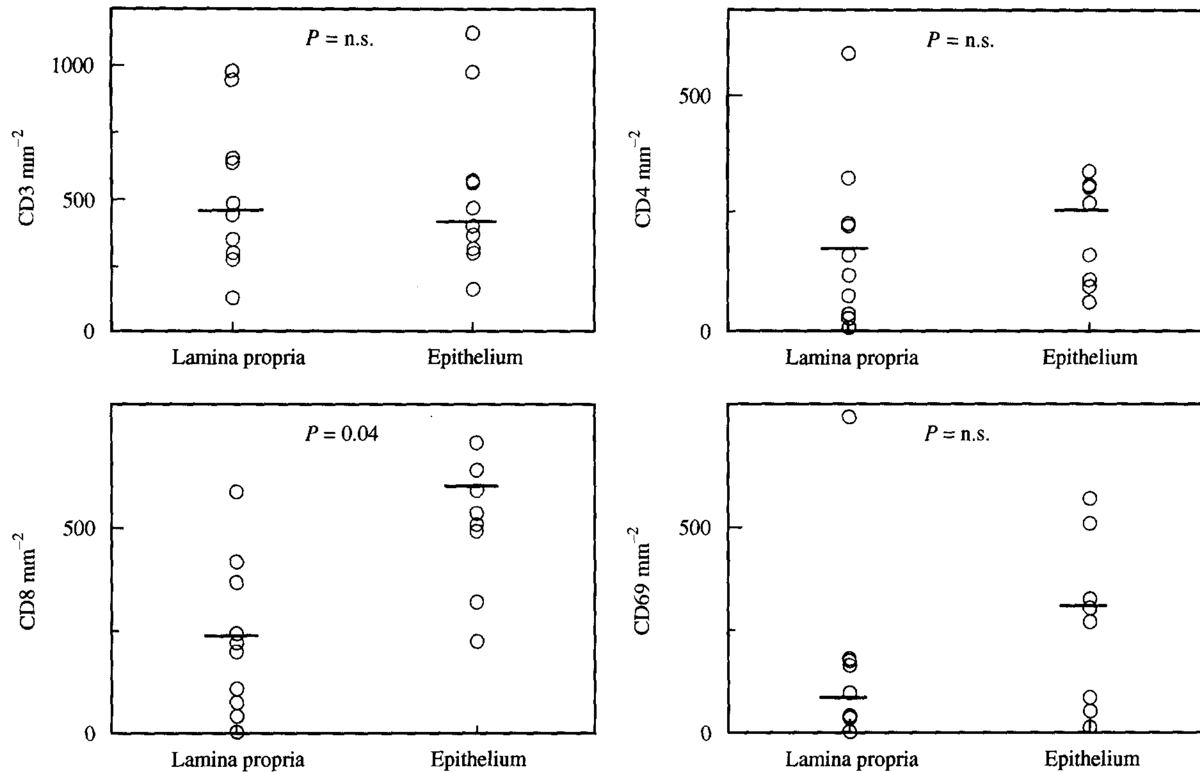

FIG. 2. Cell density/mm^{-2} of CD3$^+$, CD4$^+$, CD8$^+$ and CD69$^+$ cells in the epithelium and lamina propria compartment (*P* according to the Mann–Whitney *U*-test; n.s., not significant).

(CD3$^+$45RA$^+$) was significantly lower in BAL than in blood.

Significantly higher proportions of some of the activation markers, HLA-DR, CD26, CD49a, CD54 and CD69, were seen in BAL (Table 3). However, the proportion of IL-2 receptor-positive T lymphocytes (CD3$^+$25$^+$) was lower in BAL than in blood, indicating that IL-2 dependant proliferation was downregulated. L-selectin-positive (CD3$^+$62L$^+$) and CD38-positive T lymphocytes were also found in lower proportions in BAL than in blood.

COMPARISON OF LYMPHOCYTE SURFACE MARKERS IN RESPIRATORY EPITHELIUM AND LAMINA PROPIA OF BRONCHIAL BIOPSIES

The distribution of lymphocyte subpopulations expressed as density of labelled cells (profiles mm^{-2}), the number of counted cells (profiles subject^{-1}) and the estimated tissue area (mm^2 subject^{-1}), in the epithelial and the lamina propria compartments in bronchial biopsies, are shown in Table 4. The number of CD8 positive cell profiles per mm^2 was significantly higher in the epithelial than in the lamina propria compartment (518 *vs.* 212; $P = 0{\cdot}04$) (Fig.1). There were no significant differencies between the two compartments for CD3 positive, CD4 positive or CD69 positive cell profiles (Fig. 2).

Discussion

The aim of this study was to test the hypothesis that lymphocytes found in BAL in healthy subjects are accumulated memory cells recruited from blood. The study was performed on a group of healthy never-smoking men of about 60 years of age. It was unique in the sense that all subjects were men, born in the same year and recruited from the same population. In contrast to earlier biopsy studies performed on a limited number of healthy non-smoking subjects, our study included 18 subjects (2, 3, 6, 10). Previous studies on lymphocyte subpopulations in bronchoalveolar lavage of healthy subjects have been performed on young subjects (4, 11). The present study has focused on older subjects. In order to analyse the possible recruitment and migration of lymphocytes into the airways, we analysed the lymphocyte subpopulations in blood and bronchial mucosa as well as in BAL.

When the distribution of lymphocyte subpopulations in BAL was compared to that in blood a higher frequency was found of T lymphocytes (CD3), T helper lymphocytes (CD3$^+$4$^+$), T memory lymphocytes (CD3$^+$45R0$^+$), and a lower frequency of B lymphocytes (CD19) and NK cells (CD3$^-$56$^+$). This is in agreement with previous studies (4). Further a previously described higher ratio in BAL of CD3$^+$4$^+$/CD3$^+$8$^+$ was confirmed (8).

Earlier studies have described a higher frequency of memory helper T lymphocytes (CD4$^+$45RO$^+$29$^+$) in BAL

than in blood (4, 12). This was also seen in our study. After local immunization in the lung it has been shown that the main antibody production occurs locally and has the characteristics of a memory response (13, 14). It is reasonable to think that the high frequency of memory cells in BAL represent a local accumulation of lymphocytes directed against antigens frequently occurring in the airways. The blood population, on the other hand, has a high frequency of unprimed and unactivated naive lymphocytes.

In general, lymphocyte function can be analysed *in vitro* by direct stimulation and measurement of proliferation, surface marker expression or cytokine production. It is technically difficult to obtain enough pure lymphocytes from human lungs to perform a direct stimulation. In this study, *in vivo* cell surface marker expression was analysed to obtain indirect information about activation. Early (CD69) as well as later (HLA-DR, CD26, CD49a and CD54) activation markers were found in a much higher frequency on BAL lymphocytes than on blood lymphocytes in our healthy never-smoking subjects. These findings indicate that lymphocytes in the bronchoalveolar space are also recruited and activated in healthy never-smokers. Evidence for this was found in data from our bronchial biopsies.

In the biopsies, we chose to evaluate those T-lymphocyte markers which were different in BAL and blood, i.e. CD3, CD4, CD8, and the early activation marker CD69. An earlier study have described a predominance of CD8-positive over CD4-positive lymphocytes in both the epithelium and the lamina propria and our results agree with this (6). There was a higher number of cytotoxic T lymphocytes (CD8) profiles per mm^2 and a similar tendency for helper T lymphocytes (CD4) in the epithelial compartment compared to the lamina propria compartment in our study. A recent study by Kraft *et al.* (15) showed significantly greater numbers of, e.g., CD4 lymphocytes in the proximal airway lamina propria biopsies than in distal alveolar tissue transbronchial biopsies in patients with nocturnal asthma. Only alveolar tissue, not airway tissue, CD4-positive cells correlated inversely with lung function (FEV_1), however. We have not performed any comparison with distal alveolar tissue transbronchial biopsies. Our data do, however, support the hypothesis that there is a migration of lymphocytes from blood towards the bronchoalveolar space possibly induced by a chemotactic stimulus. Such a migration has been described in sensitized mice (16). Masuyama *et al.* (17) showed that T cells from human blood migrating through a monolayer of cultured human endothelial cells *in vitro* had signs of recent activation as well as of long term-activation. If a similar mechanism occurs in the bronchi, the expression of the early activation marker CD69 could be due to recent passage through the vessel endothelium. After *in vitro* stimulation, CD69 has been found on most haematopoetic cells, including lymphocytes. This has been described in the bronchial mucosa of mice, but the present study is the first to decribe it in humans (16).

A hypothesis has been presented suggesting that activation of pulmonary lymphocytes (e.g. increased CD69) does not lead to proliferation (18, 19). In an animal study, Paine *et al.* (19) and Holt *et al.* (20) showed that rat alveolar epithelial cells and alveolar macrophages, respectively, inhibited proliferation but not other functions of activated lymphocytes, e.g. cytokine production and upregulation of surface adhesion molecules. In a human study, Mukae *et al.* (18) detected signs of T-lymphocyte activation without signs of T-lymphocyte proliferation (unchanged levels of CD25) in bronchiolitis obliterans organizing pneumonia and chronic eosinophilic pneumonia patients. In our study, the frequency of the IL-2 receptor (CD25) was not higher but lower in BAL as compared to blood. As proliferating lymphocytes upregulate the expression of CD25, this agrees with the hypothesis that BAL lymphocytes can be activated without proliferation. This suggests that cells of the bronchoalveolar surface downregulate clonal expansion of local lymphocytes. A clonal expansion should not take place until the lymphocytes have migrated to a local lymphoid tissue where the expansion would not cause functional damage.

In summary, our study shows that healthy never-smokers have a higher frequency of $CD8^+$ T lymphocytes in the epithelial than in the lamina propria compartment in bronchial biopsies. BAL lymphocytes have a higher frequency of activated memory cells than blood lymphocytes. It is resonable to suggest that these lymphocytes have been recruited in response to exposure to local damaging agents. Thus, even in healthy never-smokers there are active T lymphocytes in the alveoli and broncial mucosa which can be part of a first line of defence against inhaled damaging agents.

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