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Phenotypic characterization of T cells in bronchoalveolar lavage fluid (BALF) and peripheral blood of patients with diffuse panbronchiolitis; the importance of cytotoxic T cells

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SUMMARY

We investigated the contribution of T cells in diffuse panbronchiolitis (DPB) by identifying T cell subsets in BALF of 36 patients with DPB, before and after long-term treatment with macrolide antibiotics, and 16 healthy control subjects. The percentages of lymphocytes and $CD3^+$ $\gamma\delta^+$ cells in BALF of DPB patients and control subjects were similar, but the absolute number of these cells was higher in DPB patients. Treatment resulted in a significant reduction in the absolute number of these cells. A further two-colour analysis of T cell subsets in BALF showed a significantly higher ratio and number of $CD8^+$ HLA-DR^+ cells in DPB patients. Treatment resulted in a significant reduction of activated T cells. Most BALF $CD8^+$ cells were $CD8^+CD11b^-$ cytotoxic T cells. The number of these cells in BALF of DPB patients ($26{\cdot}69 \pm 5{\cdot}86 \times 10^3$/ml) was higher than the control ($2{\cdot}02 \pm 0{\cdot}38 \times 10^3$/ml; $P < 0{\cdot}001$), and a significant reduction was observed after treatment ($7{\cdot}69 \pm 2{\cdot}59 \times 10^3$/ml; $P < 0{\cdot}01$). The number of $CD4^+$ cells was also higher in DPB patients than in controls, and most were $CD4^+CD29^+$ memory T cells. However, treatment did not influence the number of these cells. The number of lymphocytes, $CD3^+$ $\gamma\delta^+$, $CD8^+CD11b^-$, $CD8^+$ HLA-DR^+, and $CD4^+CD29^+$ cells was higher in patients with bacterial infection than in those without bacterial infection, and interestingly, macrolide therapy reduced the number of lymphocytes, $CD3^+$ $\gamma\delta^+$, $CD8^+CD11b^-$ and $CD8^+$ HLA-DR^+ cells, irrespective of bacterial infection. In peripheral blood, the percentage of $CD8^+$ HLA-DR^+ cells was also higher in DPB patients than in healthy subjects, and significantly decreased after treatment. The percentage of $CD8^+CD11b^-$ cells in peripheral blood was similar in DPB patients and normal subjects, and treatment significantly reduced the percentage of these cells. Finally, the expression of the adhesion molecules CD11a/CD18 (α/β-chains of LFA-1) on lung $CD3^+$ cells and CD49d (α-chain of VLA) on lung $CD4^+$ cells was enhanced compared with that on peripheral blood in DPB patients. Our results suggest that elevation of memory T cells and activation of $CD8^+$ cells, mainly cytotoxic T cells, in the airway lumen of DPB patients may contribute to chronic bronchial inflammation, possibly through up-regulation of adhesion molecules. Our findings also indicate that macrolide antibiotics may have a direct or indirect suppressive effect on cytotoxic T cells, and as such, reduce inflammation and improve clinical condition.

Keywords diffuse panbronchiolitis memory T cells cytotoxic T cells bronchoalveolar lavage fluid macrolide antibiotics

INTRODUCTION

Diffuse panbronchiolitis (DPB) is a disease entity first described by Homma and coworkers [1]. The disease is characterized by chronic inflammation of the respiratory bronchioles and infiltration of inflammatory cells [1]. The main features of DPB include chronic sinopulmonary infection and inflammation. In 1984, Kudoh *et al.* [2] first reported that low-dose and long-term treatment with erythromycin was effective in DPB. Long-term treatment with roxithromycin and clarithromycin, two new 14-membered macrolide antibiotics derived from erythromycin, are also effective against DPB [3,4].

The pathogenesis of DPB is well described, but several aspects of the disease process remain obscure. In a recent study from our laboratory, we described a marked increase in neutrophils in BALF of DPB patients compared with healthy subjects. Furthermore, treatment with macrolide antibiotics significantly reduced the number of neutrophils in BALF. This finding suggested that

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neutrophils may play an important role in the pathogenesis of the disease [3,5–7]. The histological pattern in DPB is characterized by thickening of the walls of respiratory bronchioles, with infiltration of inflammatory cells, such as mononuclear and plasma cells [1]. In addition, hyperplasia of the bronchus-associated lymphoid tissue (BALT) is usually observed more frequently in DPB than in other respiratory diseases [8]. These findings confirm the important role of lymphocytes in bronchial inflammation in DPB.

In this study, we investigated the importance of lymphocyte accumulation within the lung by analysing T cell subsets and the expression of adhesion molecules on peripheral blood and BALF of DPB patients before and after treatment with macrolide antibiotics and compared the results with those of healthy subjects.

MATERIALS AND METHODS

Patient population

We studied 16 healthy volunteers (14 males and two females, mean age 24·8 ± 1·1 years) and 36 patients with DPB (21 males and 15 females, mean age 51·6 ± 2·2 years). Healthy volunteers had no previous history of pulmonary disease. The diagnosis of DPB was based on new criteria published by the Japanese Ministry of Health and Welfare in 1995. These included: (i) chronic cough, sputum, and dyspnoea on exertion; (ii) rales and rhonchi on physical examination; (iii) diffuse disseminated fine nodular shadows, mainly in the lower lung fields on chest x-ray or computed tomography (CT); (iv) two abnormalities of $FEV_1 < 70\%$ and $PaO_2 < 80$ mmHg; (v) cold haemagglutination > 64; (vi) complication or past history of chronic sinusitis. BAL was performed in all patients and healthy volunteers. In addition, a second BAL was performed in 27 patients after treatment with macrolide antibiotics. Erythromycin, roxithromycin or clarithromycin were administered to nine patients at 600 mg/day for 16·9 ± 4·4 months (range 1–39 months), to nine patients at 200 mg/day for 5·9 ± 1·7 months (1–14 months) or to nine patients at 150 mg/day for 7·9 ± 1·0 months (3–12 months). Sputum culture was performed in some patients at least on three occasions close to the day of BAL examination in order to detect, if any, the organism(s) causing airway infection.

Bronchoalveolar lavage

With informed consent, BAL was performed using a flexible fibreoptic bronchoscope (Olympus BF, Type P-20; Tokyo, Japan). After local anaesthesia of the upper airway with 4% lidocaine, the bronchoscope was wedged into the subsegmental bronchus of the right middle lobe, and 150 or 200 ml of sterile saline at body temperature were instilled in 50-ml aliquots. The fluid was immediately retrieved by gentle suction using a sterile syringe. BALF was passed through two sheets of gauze and centrifuged at 500 ***g*** for 10 min at 4°C. After washing twice with PBS, the cell pellets were suspended in PBS supplemented with 10% fetal calf serum (FCS), and counted with a haemocytometer. An aliquot was diluted to a concentration of 2×10^5 cells/ml, and 0·2 ml of the cell suspension was spun down onto a slide glass at 160 ***g*** for 2 min using a cytocentrifuge (Shandon Cytospin 2; Sewickley, PA). The slides were later dried, fixed, and stained by the May–Giemsa method and differential cell count was performed on 200 cells using a photomicroscope. The remaining BALF was centrifuged at 500 ***g*** for 5 min at 4°C. The cell pellets were suspended in PBS supplemented with 10% FCS, and incubated in plastic dishes for 60 min at 37°C in humidified 5% CO_2–air. More than 90% of non-adherent cells collected for flow cytometric analysis were viable by the trypan blue exclusion test.

Monoclonal antibodies

FITC-conjugated anti-CD3 (Leu-4), CD4 (Leu-3a), CD8 (Leu-2a), HLA-DR, γ/δ T cell antigen receptor (TCR-γ/δ-1), CD11a (leucocyte function-associated antigen 1α (LFA-1α)), and CD18 (LFA-1β) antibodies, and PE-conjugated anti-CD3 (Leu-4), CD4 (Leu-3a), CD8 (Leu-2a), CD11b (Leu-15), and CD49d (very late antigen-4α (VLA-4α)) antibodies were purchased from Becton Dickinson (Mountain View, CA). FITC-conjugated anti-CD45RA (2H4) and CD29 (4B4) antibodies were purchased from Coulter Immunology (Hialeah, FL). Mouse IgG1 conjugated with FITC or PE were purchased from Becton Dickinson and used to determine the borderline between stained and unstained cells in the flow cytometric analysis.

Two-colour direct immunofluorescence staining

A total of 100 μl whole blood collected by venipuncture with ethylenediamine tetraacetic acid (EDTA) was placed into a 12 × 15 mm polystyrene tube (Falcon Plastics, Oxnard, CA), and 5 μl of each MoAb were added. The tubes were incubated for 15 min at room temperature in the dark, and 2 ml of 1 × FACS lysing solution (Becton Dickinson) were added. Cells were mixed vigorously and incubated for 10 min at room temperature, and then washed once in cold PBS containing 0·1% sodium azide. Cells were finally resuspended in cold PBS containing 0·5% paraformaldehyde.

The concentration of BALF cells was adjusted to 1×10^7 cells/ml. A total of 5 μl of each MoAb was placed into a polystyrene tube, and 100 μl of the cell suspension were added. Then cells were incubated for 30 min on ice in the dark, washed once in cold PBS containing 0·1% sodium azide, and then resuspended in cold PBS containing 0·5% paraformaldehyde. Fixed cells were kept in the dark at 4°C until analysis.

Two-colour flow cytometry

Stained cells were analysed on a FACScan flow cytometer (FACS Division, Becton Dickinson). A computer system (Consort 30; Becton Dickinson) was used for data acquisition and analysis. List mode data for 10 000–20 000 events were stored. A cell gate containing lymphocytes was established on the basis of forward and side light scatter. To determine the borderline between stained and unstained cells, cells were also stained with mouse IgG1-conjugated FITC or PE. Most $CD4^+$ cells in peripheral blood are relatively CD29 antigen-'positive' according to control staining, and the distribution of the intensity is usually bimodal (for example, cells with 'low' and 'high' expression). Therefore, the determined $CD29^+$ cells were those with an FITC intensity greater than the calculated mean arbitrary unit for the midpoint between the peak intensity of 'low' and 'high' expression. CD11a and CD18 antigens or CD49d were assessed by the mean fluorescence intensity (MFI). The MFI of CD11a and CD18 antigens on cells was calculated after setting a marker to determine the $CD3^+$ area, and the MFI of CD49d was calculated after setting a marker to determine the $CD4^+$ area. Percentages were calculated on the basis of the number of lymphocytes found in each quadrant.

Statistical analysis

Data were expressed as mean ± s.e.m. The Mann–Whitney *U*-test or Wilcoxon signed-rank test were used to examine differences

Table 1. Characteristics of BALF cells in healthy volunteers and patients with diffuse panbronchiolitis (DPB) before and after macrolide therapy*

			DPB	
		Healthy volunteers	Before therapy	After therapy
Total cells	($\times 10^5$/ml)	1·56 ± 0·28	13·67 ± 2·61†	3·53 ± 0·89§
Macrophages	(%)	86·96 ± 2·15	20·70 ± 2·84†	53·81 ± 5·39§
Lymphocytes	(%)	10·73 ± 1·83	10·31 ± 1·28	16·98 ± 2·02‡
	($\times 10^5$/ml)	0·15 ± 0·03	1·05 ± 0·16†	0·39 ± 0·06§
Neutrophils	(%)	1·51 ± 0·89	68·14 ± 3·64†	28·52 ± 6·2§

* Values are expressd as mean ± s.e.m.
† $P < 0{\cdot}001$ *versus* healthy volunteers.
‡ $P < 0{\cdot}01$ *versus* before therapy.
§ $P < 0{\cdot}001$ *versus* before therapy.

between means of unpaired or paired samples. $P < 5\%$ was considered significant.

RESULTS

Characteristics of BALF cells

The characteristics of cells recovered on BAL are summarized in Table 1. The total number of cells was significantly higher in DPB patients before treatment than in healthy volunteers. The percentage of neutrophils was higher, although that of macrophages was lower in DPB patients compared with normal subjects. Macrolide therapy significantly reduced the total number of cells, percentage of neutrophils and recovered macrophages to near normal levels, but it did not change the percentage of lymphocytes. The mean absolute number of lymphocytes was significantly higher in DPB patients compared with healthy subjects, although treatment caused a significant reduction in the number of these cells (Table 1). As shown in Table 2, the percentage of $CD4^+$ cells was lower in DPB patients than in healthy volunteers, but in contrast, $CD8^+$ cells were higher in DPB patients than in healthy subjects, resulting in a low CD4/CD8 ratio in patients with DPB compared with controls. When $CD4^+$ or $CD8^+$ cells were expressed in absolute numbers, both were significantly higher than those of normal subjects. Macrolide therapy significantly reduced the number of these cells, particularly $CD8^+$ cells.

Two-colour analysis of T cells in BALF

There were no significant differences in the percentages of $CD3^+\gamma\delta^+$, $CD4^+CD45RA^+$, $CD4^+CD29^+$, $CD8^+CD11b^+$, $CD8^+CD11b^-$ cells in BALF of patients with DPB before or after treatment and healthy volunteers (data not shown). However, the absolute number of $CD3^+$ $\gamma\delta^+$ cells in DPB patients before treatment ($6{\cdot}08 \pm 1{\cdot}29 \times 10^3$/ml) was significantly higher than in healthy subjects ($0{\cdot}84 \pm 0{\cdot}18 \times 10^3$/ml; $P < 0{\cdot}001$), and treatment significantly reduced the number of these cells ($2{\cdot}51 \pm 2{\cdot}17 \times 10^3$/ml; $P < 0{\cdot}01$). The number of $CD4^+CD45RA^+$ and $CD4^+CD29^+$ cells in DPB patients before treatment was significantly higher than in healthy volunteers. Treatment reduced the number of $CD4^+CD45RA^+$ and $CD4^+CD29^+$ cells (Fig. 1a). Since the majority of $CD4^+$ cells in BALF coexpressed CD29, $CD4^+CD45RA^+$ cells were considered to be less important. Similarly, the number of $CD8^+CD11b^+$ and $CD8^+CD11b^-$ cells in DPB patients before treatment was significantly higher than in healthy volunteers, and treatment significantly reduced these subsets, although most $CD8^+$ cells in BALF were $CD8^+CD11b^-$ cells (Fig. 1b). We also examined the expression of HLA-DR antigen on

Table 2. T cell subsets in BALF of healthy volunteers and patients with diffuse panbronchiolitis (DPB) before and after macrolide therapy*

			DPB	
		Healthy volunteers	Before therapy	After therapy
$CD4^+$ cells	%	37·17 ± 3·13	30·51 ± 2·34	37·30 ± 3·70
Number	($\times 10^4$/ml)	0·63 ± 0·17	3·22 ± 0·55†	1·44 ± 0·36‡
$CD8^+$ cells	%	40·94 ± 3·11	47·44 ± 3·32	41·87 ± 3·83
Number	($\times 10^4$/ml)	0·55 ± 0·09	5·25 ± 0·77†	1·58 ± 0·31§
CD4/CD8		1·08 ± 0·13	0·82 ± 0·11	1·23 ± 0·28

* Values are expressed as mean ± s.e.m.
† $P < 0{\cdot}001$ *versus* healthy volunteers.
‡ $P < 0{\cdot}01$ *versus* before therapy.
§ $P < 0{\cdot}001$ *versus* before therapy.

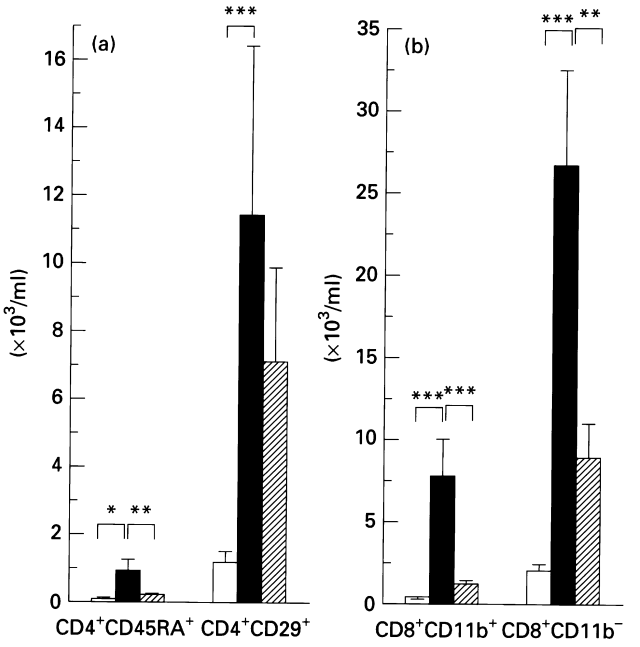

Fig. 1. Absolute number of $CD4^+$ and $CD8^+$ cell subsets in BALF of healthy subjects (□) and patients with diffuse panbronchiolitis (DPB) before (■) and after (▨) treatment with macrolide antibiotics. (a) $CD4^+CD45RA^+$ and $CD4^+CD29^+$ cells. (b) $CD8^+CD11b^+$ and $CD8^+CD11b^-$ cells. $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

$CD4^+$ and $CD8^+$ cells. The percentage of $CD8^+$ HLA-DR^+ cells in BALF was significantly higher in DPB patients before treatment than in control subjects (before, $41{\cdot}56 \pm 2{\cdot}96\%$; control, $22{\cdot}29 \pm 4{\cdot}84\%$; $P < 0{\cdot}01$), and it decreased significantly after treatment ($23{\cdot}46 \pm 4{\cdot}54\%$; $P < 0{\cdot}001$). The absolute number of $CD8^+$ HLA-DR^+ cells in BALF was significantly higher in DPB patients before treatment compared with healthy subjects (DPB, $23{\cdot}66 \pm 4{\cdot}62 \times 10^3$/ml; control, $1{\cdot}28 \pm 0{\cdot}27$; $P < 0{\cdot}001$). Treatment significantly decreased the number of $CD8^+$ HLA-DR^+ cells ($3{\cdot}81 \pm 1{\cdot}24 \times 10^3$/ml; $P < 0{\cdot}001$) (Fig. 2). There were no differences in the number of $CD4^+$ HLA-DR^+ cells among the three groups (Fig. 2).

Two-colour analysis of T cells in peripheral blood

In the next step, we examined subsets of $CD4^+$ and $CD8^+$ cells in peripheral blood. There were no significant differences in the percentage of $CD4^+CD45RA^+$ and $CD4^+CD29^+$ cells between DPB patients and healthy subjects (data not shown). The percentage of $CD8^+CD11b^-$ cells in DPB patients before treatment was higher, albeit insignificantly, than healthy subjects, but decreased significantly after treatment. There were no differences in the percentages of $CD8^+CD11b^+$ cells before and after treatment (Fig. 3). The percentages of $CD4^+$ HLA-DR^+ and $CD8^+$ HLA-DR^+ cells were significantly higher in DPB patients than in control subjects (DPB, $7{\cdot}71 \pm 1{\cdot}85\%$; control, $2{\cdot}25 \pm 0{\cdot}18\%$, $P < 0{\cdot}01$ for the former; DPB, $14{\cdot}3 \pm 1{\cdot}80\%$; control, $5{\cdot}53 \pm 0{\cdot}71\%$, $P < 0{\cdot}01$ for the latter). Treatment did not influence the number of $CD4^+$ HLA-DR^+ cells ($8{\cdot}31 \pm 2{\cdot}37\%$), but it significantly reduced the percentage of $CD8^+$ HLA-DR^+ cells to $7{\cdot}67 \pm 0{\cdot}84\%$ ($P < 0{\cdot}05$).

Influence of bacterial infection on BALF cells in DPB patients

As shown above, most BALF cells were $CD8^+CD11b^-$ or $CD4^+CD29^+$. We also examined the influence of bacterial infection on these cells in DPB patients. Before treatment, the number of lymphocytes in BALF of patients with bacterial infection was

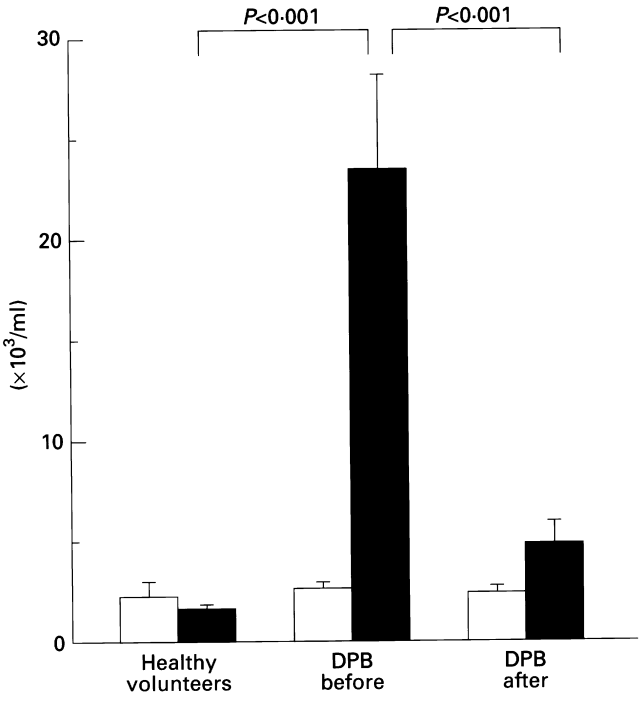

Fig. 2. Absolute number of $CD4^+$ HLA-DR^+ (□) and $CD8^+$ HLA-DR^+ cells (■) in BALF of healthy subjects and patients with diffuse panbronchiolitis (DPB) before and after treatment with macrolide antibiotics.

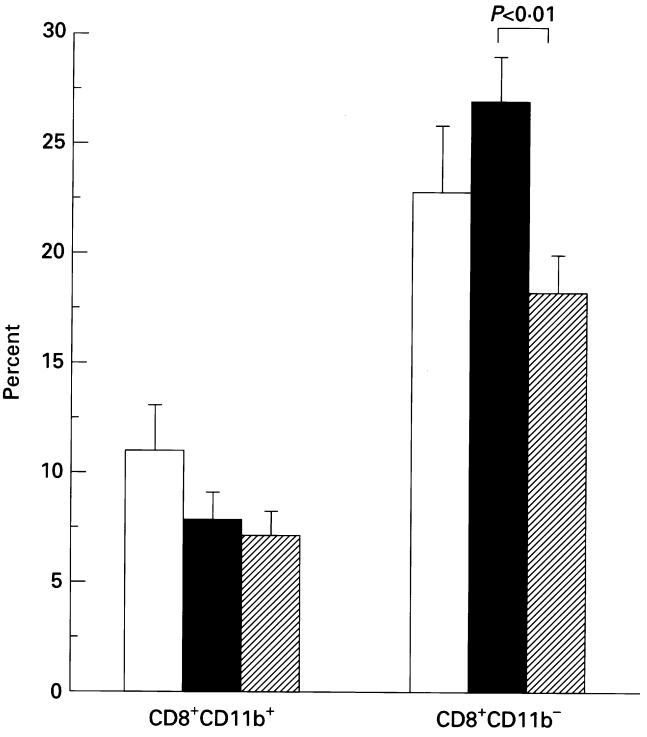

Fig. 3. Percentage of $CD8^+$ cell subsets in peripheral blood of healthy subjects (□) and patients with diffuse panbronchiolitis (DPB) before (■) and after (▨) treatment with macrolide antibiotics.

Table 3. Comparison in BALF cells of diffuse panbronchiolitis (DPB) patients before and after macrolide therapy, with and without bacterial infection*

			DPB	
			With bacterial infection (*n*)	Without bacterial infection (*n*)
Total cells	($\times 10^5$/ml)	Before	16·14 ± 3·86 (23)	8·27 ± 1·07 (6)
		After	3·82 ± 1·80 (10)†	2·38 ± 0·39 (15)†
Lymphocytes	($\times 10^5$/ml)	Before	1·24 ± 0·23 (23)	0·79 ± 0·29 (6)
		After	0·26 ± 0·06 (10)†	0·43 ± 0·06 (15)
$CD3^+\gamma\delta^+$ cells	($\times 10^3$/ml)	Before	6·72 ± 1·39 (14)	2·17 (1)
		After	3·21 ± 0·25 (4)	3·16 ± 1·22 (6)
$CD4^+CD29^+$ cells	($\times 10^3$/ml)	Before	7·52 ± 2·54 (10)	4·75 ± 1·37 (4)
		After	2·78 ± 0·98 (7)	5·26 ± 1·57 (9)
$CD8^+CD11b^-$ cells	($\times 10^3$/ml)	Before	25·74 ± 5·77 (17)	9·74 ± 1·84 (3)
		After	4·37 ± 1·26 (10)‡	6·89 ± 1·55 (13)
$CD4^+$HLA-DR^+ cells	($\times 10^3$/ml)	Before	2·90 ± 0·41 (15)	2·65 ± 0·97 (3)
		After	2·17 ± 0·98 (8)	2·34 ± 0·92 (10)
$CD8^+$HLA-DR^+ cells	($\times 10^3$/ml)	Before	22·82 ± 4·56 (15)	11·73 ± 5·24 (3)
		After	2·15 ± 1·04 (8)†	4·35 ± 1·75 (10)

* Values are expressd as mean ± s.e.m.
† $P < 0{\cdot}001$ *versus* before therapy.
‡ $P < 0{\cdot}05$ *versus* before therapy.

higher than that in patients without bacterial infection (Table 3). The number of $CD3^+$ $\gamma\delta^+$, $CD8^+CD11b^-$ and $CD4^+CD29^+$ cells showed the same change (Table 3). Moreover, we investigated the relationship between the expression of HLA-DR antigen on BALF cells and the presence of bacterial infection. The number of $CD4^+$ HLA-DR^+ cells was unaffected by the bacterial infection. The number of $CD8^+$ HLA-DR^+ cells in patients with bacterial infection was higher than in patients without bacterial infection (Table 3). Macrolide therapy reduced the total number of lymphocytes, $CD3^+$ $\gamma\delta^+$, $CD8^+CD11b^-$ and $CD8^+$ HLA-DR^+ cells, but this reduction was unaffected by bacterial infection (Table 3). There was a significant correlation between the number of lymphocytes in BALF and the number of $CD3^+$ $\gamma\delta^+$ cells ($r = 0{\cdot}713$, $P < 0{\cdot}0001$) and $CD8^+CD11b^-$ cells ($r = 0{\cdot}718$, $P < 0{\cdot}0001$) in BALF, suggesting that the reduction was due to diminished total number of lymphocytes.

Adhesion molecule expression on T cells in peripheral blood and BALF

We assessed the MFI of CD11a and CD18 on $CD3^+$ cells or CD49d on $CD4^+$ cells in peripheral blood and BALF of DPB patients and in peripheral blood of control subjects. The expression

Table 4. Mean fluorescence intensity (MFI) of CD11a and CD18 on $CD3^+$ cells or CD49d on $CD4^+$ cells in perpheral blood (PB) and BALF of diffuse panbronchiolitis (DPB) patients and healthy volunteers*

		MFI		
		CD11a	CD18	CD49d
Healthy volunteers	BALF	79·07 ± 9·36‡¶ ($n = 7$)	104·29 ± 10·62†§ ($n = 7$)	65·55 ± 6·23 ($n = 4$)
	PB	47·17 ± 5·64 ($n = 7$)	57·86 ± 5·82 ($n = 7$)	44·46 ± 4·91 ($n = 4$)
DPB	BALF	74·94 ± 7·41‡§ ($n = 18$)	92·94 ± 9·20‡§ ($n = 18$)	89·91 ± 7·76†§ ($n = 10$)
	PB	47·64 ± 7·48 ($n = 18$)	57·16 ± 6·08 ($n = 18$)	63·28 ± 7·33 ($n = 10$)

* Values are expressed as mean ± s.e.m.
† $P < 0{\cdot}01$ *versus* PB of healthy volunteers.
‡ $P < 0{\cdot}05$ *versus* PB of healthy volunteers.
§ $P < 0{\cdot}01$ *versus* PB of DPB.
¶ $P < 0{\cdot}05$ *versus* PB of DPB.

of adhesion molecules CD11a and CD18 on BALF $CD3^+$ cells in patients was significantly enhanced over that of peripheral blood $CD3^+$ cells in both groups, although there was no significant difference in the MFI of CD11a and CD18 on peripheral blood $CD3^+$ cells between patients and normal subjects (Table 4). In DPB patients, the MFI of CD49d on BALF $CD4^+$ cells was significantly enhanced compared with that on peripheral blood $CD4^+$ cells of DPB patients and normal subjects. Furthermore, there was a trend towards an enhancement compared with that on BALF $CD4^+$ cells of normal subjects (Table 4).

DISCUSSION

The presence of a high percentage of neutrophils and associated factors, such as neutrophil chemotactic and elastolytic activities, has been described in BALF of patients with DPB [3,5–7]. Previous studies of chronic bronchitis and bronchiolitis also demonstrated the presence of a high percentage of neutrophils in BALF [9,10]. Thus, neutrophils may play an important role in establishing the pathogenesis of these chronic airway diseases. However, most cells in lung biopsies of patients with DPB, bronchiectasis and chronic bronchitis are mononuclear cells [1,11–13]. In patients with bronchiectasis and chronic bronchitis, $CD8^+$ T cells outnumber $CD4^+$ cells in intraepithelial layers [11,12]. In the present study, we described a high number of lymphocytes and a predominance of $CD8^+$ over $CD4^+$ cells in BALF of patients with DPB compared with those in healthy subjects. Furthermore, a large number of $CD8^+$ cells carried HLA-DR antigen, while only a few $CD4^+$ cells carried this antigen, but the ratio of each type of these cells expressing HLA-DR antigen in peripheral blood was higher in DPB than in control subjects.

The CD11b antigen has been used to subdivide $CD8^+$ cells into suppressor ($CD8^+CD11b^+$) and cytotoxic ($CD8^+CD11b^-$) subsets. Our results demonstrated that the population of $CD8^+CD11b^-$ cells was higher in BALF of DPB patients compared with controls, while a much smaller difference in the numbers of $CD8^+CD11b^+$ cells between patients and normal subjects was present. Thus, these results suggest that most $CD8^+$ cells in BALF of DPB patients are activated cytotoxic T cells. This observation confirms our earlier findings [14] and similar findings in patients with bronchiectasis and chronic bronchitis [11,12]. However, it differs from another study showing a predominance of $CD4^+$ over $CD8^+$ cells in the parafollicular area of BALT in open lung biopsies of DPB patients [8]. The discrepancy in T cell subsets between bronchiectasis or chronic bronchitis and DPB may be due to differences in the examined area. The former diseases were assessed in the lamina propria just below the epithelial basement membrane, while the latter was assessed in BALT, a follicular collection containing mainly B lymphocytes overlaid by specialized epithelium and probably involved in antigen sampling [15,16]. These observations suggest that the predominance of $CD8^+$ cells in the present study may reflect events occurring in the lamina propria just beneath the epithelial basement membrane in the airway of patients with DPB. Thus, we speculate that activated $CD8^+$ cells, especially cytotoxic T cells, are important cellular components defining the pathogenesis of DPB as well as bronchiectasis and chronic bronchitis. However, in this study, we could not elucidate the reason for the increased cytotoxic T cells. Further studies on, for example, chemokines which are chemoattractant for cytotoxic T cells are necessary to define better the role of the cells.

$CD4^+$ cells can also be divided into two phenotypic and functionally distinct types, including $CD4^+CD45RA^+$ suppressor inducer (naive) cells, and $CD4^+CD29^+$ helper inducer (memory) cells [17,18]. The present results demonstrated a similar composition of peripheral blood $CD4^+CD29^+$ cells or $CD4^+CD45RA^+$ cells in DPB patients and healthy subjects. However, the number of $CD4^+CD29^+$ cells in BALF was higher in patients than that of $CD4^+CD45RA^+$ cells in the same patients as well as normal subjects. The number of $CD4^+CD45RA^+$ cells was also higher in patients compared with controls, but these naive T cells represented a small proportion of BALF $CD4^+$ cells. As stated above, the parafollicular area in BALT of DPB patients can be considered as equivalent to a T cell-dependent zone, as verified by the presence of T cells with the predominance of helper T cells, while BALT formation is likely to occur as a result of repeated and prolonged antigen stimulation, transported from the airway lumen [8]. Since a typical activation of naive T cells following exposure to an antigen may convert these cells to memory T cells [19,20], an increased proportion of memory T cells in BALF of DPB patients may mirror the conversion of naive to memory T cells in the epithelium following stimulation by the organisms, which are often isolated from the sputum of patients with DPB. *Haemophilus influenzae* and *Streptococcus pneumoniae* are present early in the course of the disease, and this can change to *Pseudomonas aeruginosa* as the disease progresses [1]. In this context, the present study also demonstrated the effect of coexisting bacterial infection on BALF cells such as $CD3^+ \gamma\delta^+$, $CD8^+CD11b^-$, $CD8^+$ $HLA\text{-}DR^+$ and $CD4^+CD29^+$ cells. This indicates that bacterial infection may contribute to the accumulation or activation of lymphocytes in the lung of DPB. However, we cannot completely rule out a possible contribution of a latent viral infection to the increased number of memory T cells, since the precise etiology of DPB is still unknown.

The attachment of T cells to endothelial cells is a critical event for lymphocyte migration into inflammatory sites. CD11a/CD18 (α/β-chains of LFA-1) is expressed on human T and B lymphocytes, natural killer (NK) cells, monocytes and macrophages, and plays an important role in the firm adhesion of intercellular adhesion molecule-1 (ICAM-1) on endothelial cells followed by migration into the affected tissue [21,22]. Furthermore, CD49d/CD29 (α/β-chain of VLA) also exhibits cell–cell and cell–matrix adhesion functions, and may play a major role in mediating mononuclear leucocyte migration during inflammation [23]. The present study revealed a significantly high expression of $CD3^+CD11a^+$, $CD3^+CD18^+$ and $CD4^+CD49d^+$ cells in BALF of DPB patients. While LFA-1-mediated adhesion of resting T cells to ICAM-1 is reportedly minimal, activation of T cells with phorbol ester or cross-linking of the T cell receptor within minutes results in LFA-1-mediated adhesion, with no change in the level of cell surface LFA-1 [24]. Similar results have been shown for the VLA-4 integrin [25], suggesting a common mode of activation-dependent regulation of integrins on human T cells. It has also been reported that the conversion of T cells from naive to memory subset increases the level of expression of LFA-1 [26]. These observations suggest that our findings regarding the high expression of integrins may result from activation and differentiation of memory T cells by antigen stimulation in the epithelium rather than its contribution to the migration of T cells into the inflammatory sites of DPB patients. However, further qualitative studies are necessary to investigate the relation of such integrins to T cell migration in this disease.

Several groups have investigated the therapeutic mechanisms of macrolide antibiotics in DPB. These studies suggest that the mechanism of action of these drugs is through their anti-inflammatory properties rather than antibacterial effect [5–7,27]. However, the exact mechanism of action is not fully understood, particularly the effect of macrolides on lymphocytes. Japanese investigators have maintained that administration of erythromycin for more than 1 month reduces the number of highly activated $CD8^+$ cells in peripheral blood of DPB patients [28,29]. The present results are in agreement with these earlier studies. Thus, long-term macrolide treatment reduced the expression of HLA-DR on $CD8^+$ cells in peripheral blood only and not on $CD4^+$ cells. Our results also demonstrate a new effect for macrolide antibiotics, a reduction in the number of lymphocytes and activated $CD8^+$ cells, mainly cytotoxic T cells, in BALF of patients with DPB, while very little reduction was observed in memory T cells. Although the exact mechanism of such action was not investigated, we believe that erythromycin suppressed the proliferative response of lymphocytes stimulated with mitogens and antigens [30], since the suppressive effect of macrolide therapy was not due to bacterial infection. Nevertheless, our findings indicate that the suppressive effect of macrolide antibiotics on activated T cells may lead to the clinical improvement in DPB patients.

To the best of our knowledge, the demonstration in the present study of the increased number of memory and cytotoxic T cells possibly carrying LFA-1, VLA-4 or HLA-DR antigen constitutes the first detailed report of lymphocyte activation in the airway lumen of patients with DPB. In addition, the effect of macrolide antibiotics on lymphocytes supports the involvement of these cells, mainly activated cytotoxic T cells, in the pathogenesis of DPB.

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REFERENCES

1 Homma H, Yamanaka A, Tanimoo S *et al.* Diffuse panbronchiolitis: a disease of the transitional zone of the lung. Chest 1983; **83**:63–69.
2 Kudoh S, Uetake T, Hagiwara K *et al.* Clinical effect of low-dose long-term erythromycin chemotherapy on diffuse panbronchiolitis. Jpn J Thorac Dis 1987; **25**:632–42.
3 Kadota J, Sakito O, Kohno S *et al.* Roxithromycin treatment in patients with chronic lower respiratory tract diseases—its clinical efficacy and effect on cytokine. J Jpn Assoc Inf Infect Dis 1994; **68**:27–33.
4 Mikasa K, Sawaki M, Kita E *et al.* Long-term chemotherapy using erythromycin (EM) for chronic lower airway infection: effectiveness of clarithromycin in EM ineffective cases—the fourth report. J Jpn Assoc Infect Dis 1992; **66**:1097–104.
5 Ichikawa Y, Ninomiya H, Koga H *et al.* Erythromycin reduces neutrophils and neutrophil-derived elastolytic-like activity in the lower respiratory tract of bronchiolitis patients. Am Rev Respir Dis 1992; **146**:196–203.
6 Kadota J, Sakito O, Kohno S *et al.* A mechanism of erythromycin treatment in patients with diffuse panbronchiolitis. Am Rev Respir Dis 1993; **147**:153–9.
7 Mukae H, Hirota M, Kohno S *et al.* Elevation of tumor-associated carbohydrate antigens in patients with diffuse panbronchiolitis. Am Rev Respir Dis 1993; **148**:744–51.
8 Sato A, Chida K, Iwata M *et al.* Study of bronchus-associated lymphoid tissue in patients with diffuse panbronchiolitis. Am Rev Respir Dis 1992; **146**:473–8.
9 Kindt GC, Weiland JE, Davis WB *et al.* Bronchiolitis in adults: a reversible cause of airway obstruction associated with airway neutrophils and neutrophil products. Am Rev Respir Dis 1989; **140**:483–92.
10 Thompson AB, Daughton D, Robbins RA *et al.* Intraluminal airway inflammation in chronic bronchitis: characterization and correlation with clinical parameters. Am Rev Respir Dis 1989; **140**:1527–37.
11 Silvia LE Jr, Jones JAH, Cole PJ *et al.* The immunological component of the cellular inflammatory infiltrate in bronchiectasis. Thorax 1989; **44**:668–73.
12 Fournier M, Lebargy F, Ladurie FLR *et al.* Intraepithelial T-lymphocyte subsets in the airways of normal subjects and of patients with chronic bronchitis. Am Rev Respir Dis 1989; **140**:737–42.
13 Saetta M, Stefano AD, Maestrelli P *et al.* Activated T-lymphocytes and macrophages in bronchial mucosa of subjects with chronic bronchitis. Am Rev Respir Dis 1993; **147**:301–6.
14 Mukae H, Kadota J, Kohno S *et al.* Increase in activated $CD8^+$ cells in bronchoalveolar lavage fluid in patients with diffuse panbronchiolitis. Am J Crit Care Respir Med 1995; **152**:613–8.
15 Bienenstock J. Bronchus-associated lymphoid tissue. In: Bienenstock J, ed. Immunology of the lung and upper respiratory tract. New York: McGraw Hill, 1984:96–118.
16 Bienenstock J, Befus D. Gut- and bronchus-associated lymphoid tissue. Am J Anat 1984; **170**:437–45.
17 Morimoto C, Letvin NL, Boyd AW *et al.* The isolation and characterization of the human helper inducer T cell subset. J Immunol 1985; **134**:3762–9.
18 Morimoto C, Letvin NL, Distaso JA *et al.* The isolation and characterization of the human suppressor inducer T cell subset. J Immunol 1985; **134**:1508–15.
19 Clement LT, Yamashita N, Martin AM. The functionally distinct subpopulations of human $CD4^+$ helper/inducer T lymphocytes defined by anti-CD45R antibodies derive sequentially from a differentiation pathway that is regulated by activation-dependent post-thymic differentiation. J Immunol 1988; **141**:1464–70.
20 Sanders ME, Makgoba MW, Shaw S. Human naive and memory T cells: reinterpretation of helper-inducer and suppressor-inducer subsets. Immunol Today 1988; **9**:195–9.
21 Pilewski JM, Albelda SM. Adhesion molecules in the lung. Am Rev Respir Dis 1993; **148** (Suppl.):S31–S37.
22 Arnaout MA. Structure and function of the leukocyte adhesion molecules CD11/CD18. Blood 1990; **75**:1037–50.
23 Hemler M, Elices M, Parker C *et al.* Structure of the integrin VLA-4 and its cell–cell and cell–matrix adhesion functions. Immunol Rev 1990; **114**:45–65.
24 Dustin ML, Springer TA. T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. Nature 1989; **341**:619–24.
25 Shimizu Y, Seventer GAV, Horgan KJ *et al.* Regulated expression and binding of three VLA (β1) integrin receptors on T cells. Nature 1990; **345**:250–3.
26 Sanders ME, Makgoba MW, Sharrow SO *et al.* Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (UCHL1, CD29w, and Pgp-1) and have enhanced IFN-γ production. J Immunol 1988; **140**:1401–7.
27 Nagai H, Shishido H, Yoneda R *et al.* Long-term low-dose administration of erythromycin to patients with diffuse panbronchiolitis. Respiration 1991; **58**:145–9.
28 Suwabe A, Yakuwa N, Nakamura H *et al.* Effects on blood leukocyte functions of long term therapy with low doses of erythromycin in diffuse panbronchiolitis. Jpn J Thorac Dis 1988; **26**:1284–90.
29 Sugiyama Y, Sugawa Y, Takeuchi K *et al.* Analysis of peripheral lymphocyte subsets and changes due to erythromycin therapy in patients with diffuse panbronchiolitis. Jpn J Thorac Dis 1990; **28**:1574–80.
30 Keicho N, Kudoh S, Yotsumoto H *et al.* Antilymphocytic activity of erythromycin distinct from that of FK506 or cyclosporin A. J Antibiot (Tokyo) 1993; **46**:1406–13.