Scand. J. Immunol. **47**, 496–501, 1998

Characterization of Local Memory Cells in Stage-Classified Pulmonary Tuberculosis: Preliminary Observations

E. URDANETA*, E. FEO-FIGARELLA*, C. MONTALVO*, C. TÁLAMO†, Y. CASTILLO‡, D. CARRASCO‡, H. RIVERA*, I. BLANCA*, I. MACHADO*, G. ECHEVERRÍA DE PÉREZ*, J. B. DE SANCTIS* & N. E. BIANCO*

**Institute of Immunology, Faculty of Medicine and †The Pulmonary Diseases Department, Central University Hospital, Central University of Venezuela; and ‡TB Clinic of the Jose I Baldo Hospital, Caracas, Venezuela*

(Received 17 October 1997; Accepted in revised form 8 January 1998)

Urdaneta E, Feo-Figarella E, Montalvo C, Tálamo C, Castillo Y, Carrasco D, Rivera H, Blanca I, Machado I, Echeverría de Pérez G, De Sanctis JB, Bianco NE. Characterization of Local Memory Cells in Stage-Classified Pulmonary Tuberculosis: Preliminary Observations. Scand J Immunol 1998;47:496–501

Immunophenotype analysis and proliferative responses were investigated in bronchoalveolar lavage (BAL) cells from 21 patients with stage-classified tuberculosis: six with localized pulmonary infiltrate (LPI); seven with diffuse pulmonary infiltrate (DPI); and eight with pleural effusions (PE). Bronchoalveolar lavage cells from these patients contained a high number of cells/ml. The macrophage number was significantly lower in the DPI group ($P < 0.05$) compared to the LPI or PE groups. Conversely, neutrophils were markedly increased in DPI patients compared to LPI ($P < 0.01$) and PE ($P < 0.01$) patients. Lymphocyte infiltration ($97.7 \pm 2.3\%$ $CD3^+$, $> 83\%$ $\alpha\beta^+$ and $CD4^+ > CD8^+$) was observed in the three groups. A significant increase in the number of total lymphocytes ($P < 0.01$) and $CD4^+$ cells ($P < 0.05$) was observed in the LPI group compared to the PE group. In the LPI group $CD4^+$ $CD45RO^+$ cell infiltration was higher than $CD4^+$ $CD45RA^+$ cells ($P < 0.001$), contrasting to similar numbers of these subpopulations in the DPI group. Lymphocytes from three out of three LPI patients ($\alpha\beta^+$ $CD4^+$ $CD45RO^+$) responded against tuberculin purified protein derivative contrasting to the unresponsiveness of five patients with either DPI or PE. This impaired response was reverted in two out of five patients by using peripheral blood monocytes instead of alveolar macrophages. It is suggested that, in humans, $\alpha\beta CD4^+CD45RO$ cells are the main lymphocyte type involved in the initial local cell-mediated immune response against *Mycobacterium tuberculosis*.

Dr Nicolás E. Bianco, Instituto de Immunología, Universidad Central de Venezuela, AEROCAV no. 1216, PO Box 02–5304, Miami, Florida 33102–5304, USA

INTRODUCTION

Mycobacterium tuberculosis (*Mt*) reaches the lower airways of a host being engulfed by alveolar macrophages (MAC) by C3b and mannose receptors [1, 2]. Inside the phagosome, the bacilli can be destroyed by diverse bactericidal mechanisms. Otherwise, cytolytic $CD4^+$ and $CD8^+$ T cells may contribute to eradicate infected macrophages [2–6], generating a granuloma lesion surrounding the site of infection, and formed by activated T cells and MAC. Cell-mediated immunity (CMI), then, seems to be responsible for the control and eradication of *Mt* infection. Despite this fact, 10% of infected hosts may develop progressive disease or the disease may be reactivated after a latent period [7].

Most studies in mice and humans have suggested that the $CD4^+$ lymphocytes may play a key role in both CMI and effector functions against the infected macrophages (MAC) [5, 6, 8, 9]. tuberculin purified protein derivative (PPD) was shown to be a strong stimulus for peripheral $CD4^+$ cells and cloned $CD4^+$ cells, either from peripheral blood or from the pleural space predominantly secreted interferon (IFN)-γ upon stimulation [8]. These cells resemble Th1 CD4 cells and may show cytotoxic activity against *Mt*-infected macrophages [9, 10]. Recently Robinson *et al.* [11] demonstrated that both $CD4^+$ T cells and MAC from bronchoalveolar lavage (BAL) obtained from patients with pulmonary tuberculosis (TB) secrete significant amounts of IFN-γ.

Despite numerous reports [1–11], there remains to be established an integral immunophenotype analysis in parallel to a complete assessment of antigen-specific proliferative responses in BAL of stage-classified pulmonary TB. Thus the aim of the

Table 1. Distribution of BAL fluid cell populations in stage-classified pulmonary tuberculosis

Group	cases	Cells $\times 10^5$/ml	MAC $\times 10^5$/ml	PMN $\times 10^5$/ml	LYM $\times 10^5$/ml
LPI	6	3.9 ± 1.5	1.89 ± 1.15	0.66 ± 0.56	1.37 ± 0.96
DPI	7	3.9 ± 2.8	0.69 ± 0.6 *	2.42 ± 1.95**	0.97 ± 0.93
PE	8	1.5 ± 0.9	1.08 ± 0.73	0.24 ± 0.05	0.35 ± 0.21

The table represents the amount of cells recovered form BAL and the different cell populations assessed by the May-Grunwald-Giemsa dye as described in Materials and Methods. The amount of MAC and PMN recovered in DPI patients was significantly lower (* $P<0.05$, ** $P<0.01$) than those recorded in LPI and PE groups. LYM, lymphocytes; PMN, polymorphonuclear.

present report was to study, using BAL, flow cytometry and proliferative responses, the type and the response of the infiltrating lymphocytes in stage-classified TB patients.

MATERIALS AND METHODS

Subjects. Twenty-one patients (16 males and five females, age range 18–60 years, median 34) with different forms of active TB, evaluated at the Central University Hospital and at the Jose I Baldó Hospital TB Clinic, Caracas, Venezuela, were selected. Routine blood tests were performed and concurrent infectious diseases including human immunodeficiency virus (HIV) were ruled out. Tuberculosis infection was investigated by sputum and/or pleural fluid analysis (Koch bacilli identification and cultures) and X-rays in each of the subjects. In some cases, transbronchial or pleural biopsies were performed. The patients were further classified according to the tuberculosis classification of the American Thoracic Society [7] and Lenzini *et al.*'s report [12].

The patients were classified as follows. Six showed localized pulmonary infiltrate (LPI). Lesions were limited to one lobule (superior right or left lobule) without consolidation or cavities. All patients were positive for AFB (acid-fast bacilli assessed by smears and cultures) and PPD skin tests.

Seven demonstrated diffuse pulmonary infiltrate (DPI) with more than one lobule affected and bilateral compromise. The infiltrates were dense and showed pneumonic appearance and cavities. All had positive AFB and six showed positive PPD.

Eight patients were found to have pleural effusions (PE) without evidence of radiological pulmonary infiltrate. They were AFB negative and seven of them showed a positive PPD.

Only five patients (two LPI and three PE) were mild smokers (less than one packet a day). No difference was observed in the clinical evolution and PPD response between smokers and non-smokers.

In contrast to the practice in many countries, the Ethical Committee of the TB National Institute did not approve bronchoscopy and BAL in normal volunteers. For reference purposes, we followed the BAL Cooperative Group Steering Committee of the National Heart, Lung and Blood Institute [13] as well as the report of Merchant *et al.* [14].

Cells from BAL. Bronchoalveolar lavage was performed with a flexible bronchoscope after local anaesthesia with 1% xylocaine with the patient previously sedated. A total of five aliquots of 50 ml sterile 0.9% saline solution was instilled into the affected segment or in the lingula or in the middle lobe in the pleural TB cases. The fluid was immediately retrieved by gentle suction and collected in sterile tubes containing 100 ml of Hank's balanced salt solution (HBSS: Grand Island Biological, NY, USA) and 2% penicillin/streptomycin (Grand Island Biological). Samples were transported at 4°C and processed within 1 h. The BAL fluids were filtered through a sterile gauze, placed in 50-ml plastic tubes and centrifuged at 400 *g*. The cell pellet was washed once with phosphate-buffered saline (PBS) and the erythrocytes were lysed with ammonium chloride lysing (ACK) solution (NH_4Cl–$KHCO_3$–EDTA); then the cells were washed and resuspended in RPMI-1640 medium (Grand Island Biological) containing 25 mM glutamine, 100 IU/ml penicillin, 50 IU/ml streptomycin and 10% normal human serum (NHS: complete medium). Total cell count and cell viability was measured using Trypan Blue and differential cell count was assessed either using toluidine (cell suspension) or May-Grunwald-Giemsa dye (cytospin slide).

The cell suspensions (3×10^6 cells/ml) were incubated on plastic Petri dishes (12 × 100 mm: Falcon Labware, Oxnard, CA, USA) at 37°C for 1 h in a 5% CO_2 humidified incubator and both non-adherent cells (mostly lymphocytes) and adherent cells (macrophages) were recovered. Viability of both cell populations was >95% as determined by Trypan Blue exclusion.

BAL lymphocyte phenotype. The expression of CD3, CD4, CD8, CD14, CD16, CD19, CD45RA and CD56 (antibodies purchased from Coulter Immunology, Hialeah, FL, USA), and TCR$\alpha\beta$, TCR$\gamma\delta$, CD45RO and HLA-DR (antibodies purchased from Becton Dickinson, San José, CA, USA), were assessed using fresh or precultured (18 h) lymphocytes as previously standardized in our laboratory [15, 16]. The expression of TCR$\alpha\beta$ and TCR$\gamma\delta$ was optimal after a preculture period of 48 h. Single and double colour analysis was assessed using an EPICS 753 flow cytometer (Coulter Corporation, Hialeah, FL, USA) after a previous calibration with DNA beads. Immunoglobulin (Ig) isotype controls conjugated with the corresponding fluorochrome were used for non-specific fluorescence.

Proliferative studies. The cell proliferative assays were carried out as previously reported [15, 16] using precultured BAL T cells with autologous adherent BAL cells or adherent peripheral blood cells as antigen-presenting cells (10^4 antigen-presenting cells per 10^5 responders lymphocytes). Phytohaemagglutinin (PHA: Wellcome Research Lab., UK) and PPD (Batch RT 47: Statems Serum Institute, Copenhagen, Denmark) were employed as lymphocyte activators. Optimal doses for PHA and PPD were 1 μg/ml and 10 μg/ml, respectively.

Statistical analysis. Since normal distribution of BAL cells is not widely standardized, the Kolmogorov Smirnov non-parametric test and the Box Plot analysis were employed.

RESULTS

Table 1 summarized the different cell distributions of the

Table 2. Percent of T lymphocyte subsets in BAL fluid in stage classified pulmonary tuberculosis

Subset	LPI group ($n=6$)	DPI group ($n=4$)	PE group ($n=5$)
$CD4^+$	74.1 ± 14.4*	60.1 ± 26.5	49.5 ± 14.4
$CD8^+$	25.3 ± 12.1	31.3 ± 17.1	28.6 ± 14.8
Ratio CD4:CD8	3.5 ± 1.8	2.0 ± 0.6	2.0 ± 0.9
CD56	3.9 ± 2.3	4.2 ± 2.4	4.7 ± 3.3
$CD4^+$:$CD45R0^+$	70.8 ± 21**	54.5 ± 28	ND
$CD4^+$:$CD45RA^+$	19.8 ± 19	45.6 ± 38	ND
HLA-DR	21 ± 12.6	10.3 ± 9.2	23.6 ± 23
$\alpha\beta$ TCR	84.5 ± 6.4	88.8 ± 5.4	83.3 ± 10.9
$\gamma\delta$ TCR	4.7 ± 2.5	6 ± 4.8	6.3 ± 6

The different subsets of T lymphocytes encountered in BAL of the different patients is depicted. The values expressed are the mean ± SD of the percentage of total subpopulations studied. Significant differences were encountered in the $CD4^+$ cell population between the LPI and PE group (* $P<0.05$) and between the number of $CD4^+/CD45RO^+$ and $CD4^+/CD45RA^+$ cells in the LPI group (** $P<0.001$). ND represents not done.

BAL cell composition among the studied groups. Results are expressed in total cell count/ml. The number of macrophages was significantly lower in the DPI group ($P<0.05$) compared to the LPI group or PE groups. On the other hand, neutrophil numbers were markedly increased in DPI patients compared to a mild increase in LPI ($P<0.01$) and PE ($P<0.01$) patients. Lymphocyte infiltration (97.7 ± 2.3% $CD3^+$) was increased in the three pulmonary presentations, being significantly higher in the LPI group compared to the PE group ($P<0.01$).

The influence of age, sex and cigarette smoking was assessed on cell distribution in the different groups. No difference between smokers and non-smokers was encountered in the number of infiltrating lymphocytes.

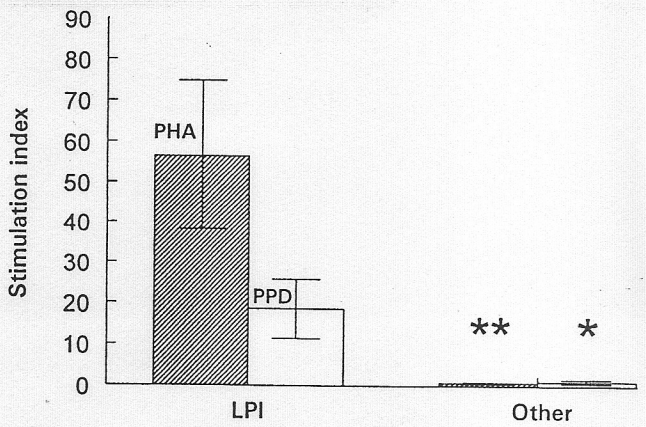

Fig. 1. Proliferative response of BAL cells. Cells recovered from BAL of the LPI patients ($n=3$), and from DPI ($n=3$) and PE patients ($n=2$), named 'others' in the figure, were cultured with optimal concentrations of PHA (3 days) and PPD (6 days) as described in Materials and Methods. The bars represent the stimulation index observed. The proliferative response observed in LPI patients is significantly higher (* $P<0.05$, ** $P<0.01$) than the response observed in the non-responders.

In the LPI, DPI and PE groups, $CD3^+$ T cells (97.7 ± 2.3) were predominant when compared to $CD19^+$ B cells (2.3 ± 2.3). Most of the cells that express the CD56 marker were $CD3^+$ in the three groups contrasting with the scarce presence of $CD3^-$ $CD16^+$ $CD56^+$ lymphocytes (found in low numbers in only four patients). As depicted in Table 2, $CD4^+$ cells were significantly higher than $CD8^+$ in LPI, DPI and PE patients ($P<0.05$). The LPI group showed the highest proportion of $CD4^+$ cells, reaching significance when compared to the PE group ($P<0.05$). Furthermore, in the LPI group, $CD45RO^+$ lymphocytes were significantly higher than the $CD45RA^+$ ($P<0.001$) compared to similar numbers of naive and memory cells encountered in the DPI group.

In order to identify the lymphocyte population further, HLA-DR and TCR expression were assessed (Table 2). Expression of HLA-DR was variable from one sample to the other, but lower in the DPI group. On the other hand, the percentages of T cells expressing $\alpha\beta$ TCR were similar in the three groups and significantly higher than $\gamma\delta$ TCR expression ($P<0.001$ for each group). These values were obtained using 48-h precultured BAL cells since, in fresh or 18-h precultured cells, a low and unpaired expression of the TCR chains was observed in comparison to the total amount of CD3 observed.

The proliferative studies were performed on 18-h precultured cells. The BAL T cells were challenged in the presence of BAL macrophages. Figure 1 depicts the proliferative response of the three LPI patients in comparison to non-responsive DPI ($n=3$) and PE ($n=2$) patients (named 'others' in the figure). Significant differences ($P<0.01$ for PHA and $P<0.05$ for PPD) were observed. The responsive cells were mostly $CD4^+/CD45RO^+$ cells as represented in Fig. 2 (90% $CD4^-$ CD45RO and 10% $CD4^-$ CD45RA).

In those non-responding patients peripheral blood monocytes

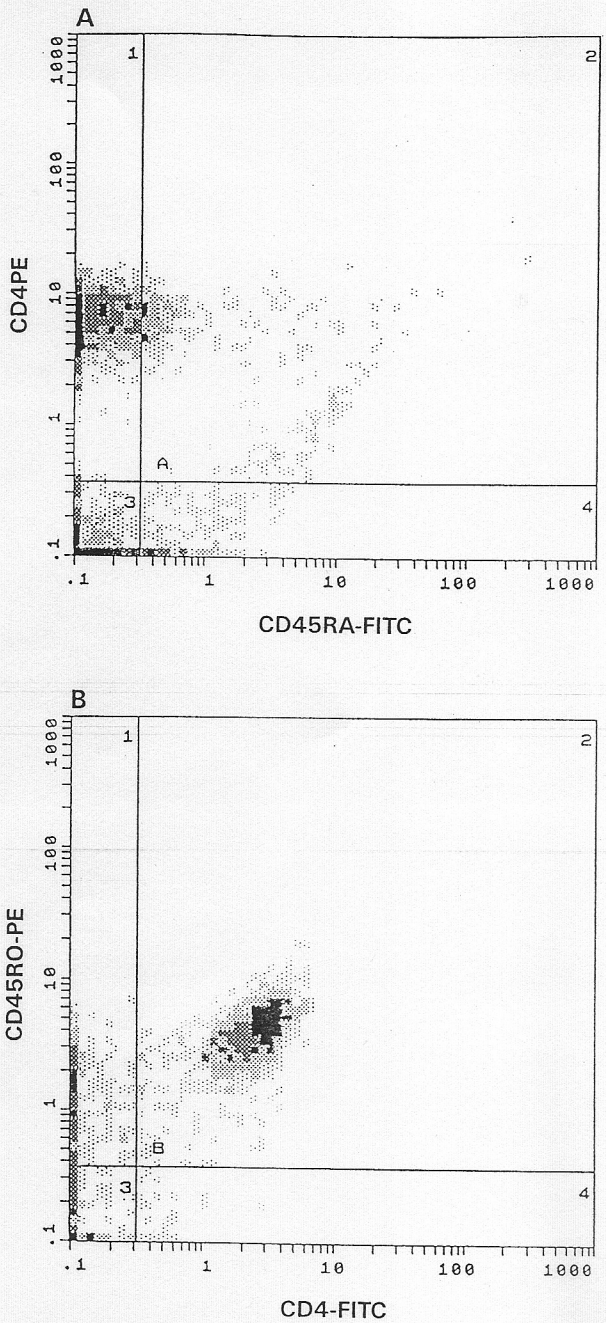

Fig. 2. Flow cytometry analysis of CD4 cells of an LPI-responsive patient. A typical flow cytometry is represented in the figure. The double colour analysis of $CD4^-CD45RO$ and $CD4^-CD45RA$ cell populations is presented. A: The expression of $CD4^-$PE and CD45RA cells. Most of the cells express CD4 (box 1), but a low number (10%) express both markers (box 2). B: The expression of $CD45RO^-$ PE and $CD4^-$ FITC. Most of the $CD4^+$ cells (90%) express the CD45RO phenotype (box 2).

were used as antigen-presenting cells, and in two cases, one DPI patient and one PE patient, an antigen-specific proliferative response was observed (mean 38 615 stimulation index 96 and 5636 cpm stimulation index 10, respectively).

Peripheral blood mononuclear cells (PBMC) from all the studied patients showed an adequate proliferative response to PHA in comparison to age-matched controls. In 12 out of 15 patients tested, antigen-specific proliferative responses ranged from 4577–121 571 cpm (mean ± SEM 21 966 ± 12 500 cpm). However, these antigen-specific proliferative responses contrast with the results depicted in Fig. 1 (patients with DPI and PE responded to antigen-specific stimuli).

DISCUSSION

Tuberculosis may evoke different clinical events depending on the resistance or susceptibility of the host and/or other factors. It is generally agreed that the *Mt* infiltrates progress from the initial infiltrate described by Assmann [17] and Hinshaw & Garland [18], to the cavity formation, and up to a fibrous scar if left untreated. Nevertheless, some patients develop progressive disease with pleural and/or extrapulmonary organ involvement.

Alterations of cellular immune response may be the key issue in TB. In our laboratory, Andrade *et al.* [15] showed the existence of several immunomodulatory factors (membrane-bound immune complexes, high levels of soluble interleukin-2R and suppressor MAC) in patients with current active pulmonary TB which may contribute to a perturbed CMI reaction. Nonetheless, in order to understand the mechanisms of disease installment and progression, efforts have to be focused on the assessment of the local cell infiltrate and its functional characteristics. Lenzini *et al.* [12] suggested a bipolar immunopathological spectrum in active pulmonary TB. The 'reactive pole' shows small and localized infiltrates, characterized by mononuclear cells (MAC and lymphocytes), strong PPD responses, absence of anti-PPD antibodies and few bacilli, while the 'non-reactive (anergic)' pole exhibits diffuse cell infiltrates, neutrophils, cutaneous anergy to PPD, high titres of anti-PPD antibodies, large quantities of bacilli and poor response to treatment.

In order to address this issue, our research protocol was designed to include untreated TB subjects at different stages. The amount of cells recovered in BAL in the three groups was higher than those reported in controls: fourfold in LPI and DPI and 1.5-fold in PE [13]. Even though MAC in patients with LPI (50 ± 22%) and PE (60 ± 13%) was the predominant cell type, its amount was reduced when compared to normal controls (70–95%). This was due to an increase of lymphocyte (33 ± 17% in LPI, 26 ± 13% in PE, 5–6% in controls) and neutrophil (33 ± 17% in LPI, 14 ± 1% in PE, < 1% in controls) infiltration. As the lung inflammation progresses, there is a significant increase of neutrophil infiltration (61 ± 20%) in severe DPI cases (Table 1). Even though neutrophil infiltration is an important event in TB infection, its role is limited and controversial [2, 19, 20].

Lymphocytes were considered to be the most important cell type recruited. First of all, these cells were increased in all the patients studied. Second, they were primarily $CD3^+CD4^+$ T lymphocytes. Third, the CD4:CD8 ratios were higher than the

values reported in controls [13], resembling the early stages of Sarcoidosis infection [22–24] . Fourth, the highest lymphocyte concentrations were found in the LPI group when compared to DPI and particularly to the PE group. These observations support previous reports of local lymphocyte infiltration in TB [3–6, 21–24] and the hypothesis of Lenzini *et al.* [12].

In the LPI group and in DPI patients, $CD4^+ CD45RO^+$ memory cells were the predominant subset encountered, with few naive, $CD4^+ CD45RA^+$, infiltrating cells. These cells expressed mostly $\alpha\beta$ TCR after a preculture period of 48 h and low numbers of $\gamma\delta$ TCR^+ cells. Furthermore, $CD8^+$ T cells were detected in similar amounts in LPI, DPI and PE patients. It may be proposed that $\alpha\beta^+$, $CD4^+$, $CD45RO^+$ memory cells are the main infiltrating lymphocyte type and these cells exhibit a regulatory and effector function against *Mt.*

Specific and mitogenic proliferative responses indicate that these $\alpha\beta$, $CD4^+$, $CD45RO^+$ memory T cells are sensitized to *Mt* antigens. This is better illustrated if we consider the responses obtained in the LPI group which represents the early and most efficient effector phase when the bacilli are encountered. Furthermore, when adherent PBMC were employed as antigen-presenting cells [15, 25–27], we were able to elicit a specific PPD response in two out of five patients who were unresponsive to antigen presented by alveolar MAC. The PBMC differed from local responses to specific antigens suggesting that the specific response is crucial for disease progression.

In conclusion, sensitized $\alpha\beta^+$, $CD4^+$, $CD45RO^+$ memory T-helper cells are the initial and main cell type involved in the local CMI response against *Mt.* We also suggest that this type of cell may be present in more advanced stages of pulmonary and extrapulmonary TB infection, probably subjected to immunomodulatory influences which may render them anergic to the specific antigen, as has been previously proposed [28]. Future studies should address the issue of cell types and the progression of TB.

ACKNOWLEDGMENTS

We are very grateful to the nursing staff of both the Jose I Baldó and University Hospitals and to Lucila Blanco for reviewing the statistical analysis. Supported by the GENIC program from CONICIT.

REFERENCES

1 Schlesinger L. Macrophage phagocytosis of virulent but not attenuate strains of *Mycobacterium tuberculosis* is mediated by mannose receptors in addition to complement receptors. J Immunol 1993;150:2920–6.
2 Kaufmann S. Immunity to intracellular bacteria. Annu Rev Immunol 1993;11:129–63.
3 Dunlap N, Briles D. Immunology of tuberculosis. Med Clin North Amer 1993;77:1235–51.
4 Orme I, Andersen P, Boom H. T Cell Response to *Mycobacterium tuberculosis*. J Infect Dis 1993;176:1481–7.
5 Andersen P. Host responses and antigens involved in protective immunity to *Mycobacterium tuberculosis*. Scand J Immunol 1997;45:115–30.
6 Ottenhoff THM. Putting numbers on Mycobacterium activated T cell subsets. Clin Exp Immunol 1996;104:381–3.
7 American Thoracic Society. Diagnostic standards and classification of tuberculosis. Am Rev Resp Dis 1990;142:725–35.
8 Nathan C, Murray C, Wiebe M, Rubin B. Interferon gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. J Exp Med 1983;158:670–8.
9 Mutis T, Cornelisse Y, Ottenhoff T. Mycobacteria induce $CD4^+$ T cells that are cytotoxic and display Th1-like cytokine secretion profile: heterogeneity in cytotoxic activity and cytokine secretion levels. Eur J Immunol 1993;23:2189–293.
10 Tsukaguchi K, Balaji K, Boom H. $CD4^+$ $\alpha\beta$ T cell and $\gamma\delta$ T cell responses to *Mycobacterium tuberculosis*. J Immunol 1995;154:1786–96.
11 Robinson D, Ying S, Taylor I *et al.* Evidence for Th1-like bronchoalveolar T cell subset and predominance of interferon-gamma gene activation in pulmonary tuberculosis. Am Rev Resp Care Med 1994;149:989–93.
12 Lenzini L, Rottoli P, Rottoli L. The spectrum of human tuberculosis. Clin Exp Immunol 1977;27:230–7.
13 The BAL Cooperative Group Steering Committee. Bronchoalveolar lavage constituents in healthy individuals, idiopathic pulmonary fibrosis and selected comparison groups. Am Rev Res Dis 1990;141:S169–202.
14 Merchant R, Schwartz D, Helmers R, Dayton C, Hunninghake G. Bronchoalveolar lavage cellularity. Distribution in normal volunteers. Am Rev Res Dis 1992;146:448–53.
15 Andrade R, Machado I, Fernández B, Blanca I, Ramírez R, Bianco NE. Cellular immunity in current active pulmonary tuberculosis. Am Rev Res Dis 1991;143:496–500.
16 Feo E, Morillo F, Blanca I, Bianco NE. Failure of cell mediated effector mechanisms in lung cancer. J Natl Cancer Inst 1984;73:1–13.
17 Assmann H. Uber die infraklavikularen lungeninfiltrationen im beginn der tuberkulose jugendlicher erwachsener und ihr schicksal. Deutsche Med Wchnschr 1927;53:781.
18 Hinshaw C, Garland H. Tuberculosis. In: Hinshaw C, Garland H, eds, Diseases of Chest. Philadelphia: Saunders, 1956:393–450.
19 Strieter R, Lukacs N, Standiford T, Kunkel S. Cytokines and lung inflammation: mechanisms of neutrophil recruitment to the lung. Thorax 1993;48:765–71.
20 Rogers H, Tripp C, Unanue E. Different stages in the natural and acquired resistance to an intracellular pathogen. The Immunologist 1995;3:152–6.
21 Drent M, Wagenaar S, Mulder P, van Velzen-Blad H, Diamant M, van den Bosch J. Bronchoalveolar lavage fluid profiles in sarcoidosis, tuberculosis and non-Hodgkin's and Hodgkin's disease. Chest 1994;105:514–9.
22 Hoheisel G, Tabak L, Teschler H, Erkan F, Kroegel C, Costabel UB. Bronchoalveolar lavage, cytology and immunocytology in pulmonary tuberculosis. Am Rev Res Dis Crit Care Med 1994;149:460–3.
23 Ozaki T, Nakahira S, Tani K, Ogushi F, Yasuoka S, Ogura T. Differential cell analysis in bronchoalveolar lavage fluid from pulmonary lesions of patients with tuberculosis. Chest 1992;102:54–9.
24 Sharma S, Pande J, Singh Y *et al.* Pulmonary function and immunologic abnormalities in milliary tuberculosis. Am Rev Res Dis 1992;145:1167–71.

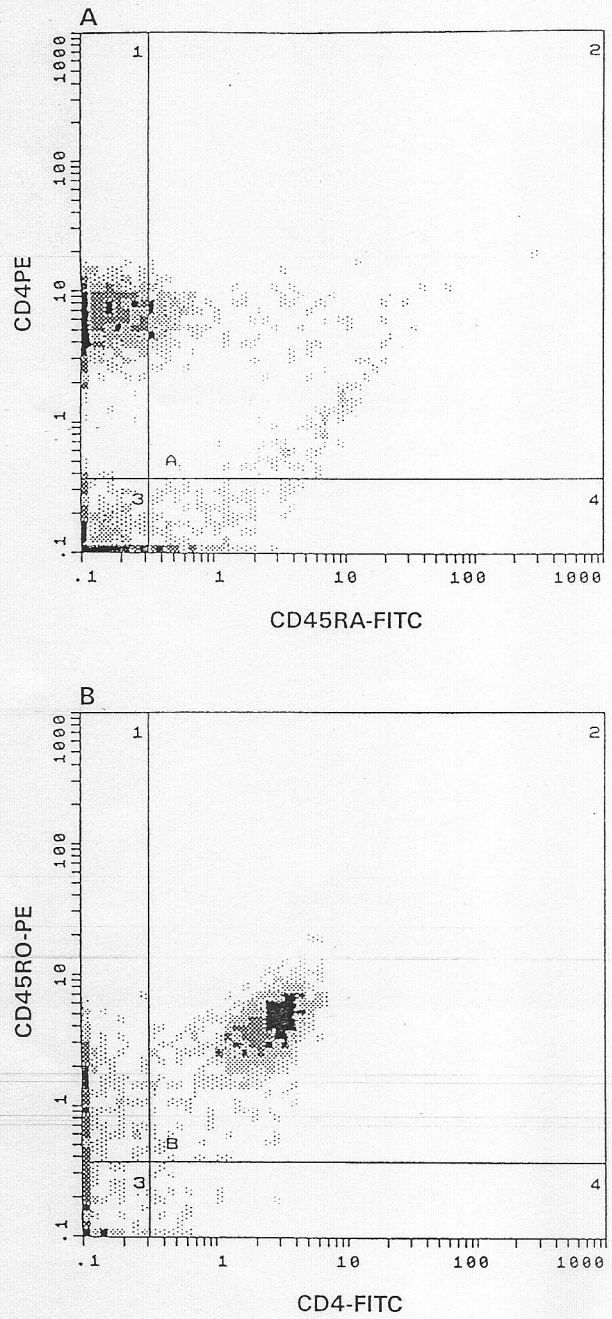

Fig. 2. Flow cytometry analysis of CD4 cells of an LPI-responsive patient. A typical flow cytometry is represented in the figure. The double colour analysis of $CD4^-CD45RO$ and $CD4^-CD45RA$ cell populations is presented. A: The expression of $CD4^-PE$ and CD45RA cells. Most of the cells express CD4 (box 1), but a low number (10%) express both markers (box 2). B: The expression of $CD45RO^-$ PE and $CD4^-$ FITC. Most of the $CD4^+$ cells (90%) express the CD45RO phenotype (box 2).

were used as antigen-presenting cells, and in two cases, one DPI patient and one PE patient, an antigen-specific proliferative response was observed (mean 38 615 stimulation index 96 and 5636 cpm stimulation index 10, respectively).

Peripheral blood mononuclear cells (PBMC) from all the studied patients showed an adequate proliferative response to PHA in comparison to age-matched controls. In 12 out of 15 patients tested, antigen-specific proliferative responses ranged from 4577–121 571 cpm (mean ± SEM 21 966 ± 12 500 cpm). However, these antigen-specific proliferative responses contrast with the results depicted in Fig. 1 (patients with DPI and PE responded to antigen-specific stimuli).

DISCUSSION

Tuberculosis may evoke different clinical events depending on the resistance or susceptibility of the host and/or other factors. It is generally agreed that the *Mt* infiltrates progress from the initial infiltrate described by Assmann [17] and Hinshaw & Garland [18], to the cavity formation, and up to a fibrous scar if left untreated. Nevertheless, some patients develop progressive disease with pleural and/or extrapulmonary organ involvement.

Alterations of cellular immune response may be the key issue in TB. In our laboratory, Andrade *et al.* [15] showed the existence of several immunomodulatory factors (membrane-bound immune complexes, high levels of soluble interleukin-2R and suppressor MAC) in patients with current active pulmonary TB which may contribute to a perturbed CMI reaction. Nonetheless, in order to understand the mechanisms of disease installment and progression, efforts have to be focused on the assessment of the local cell infiltrate and its functional characteristics. Lenzini *et al.* [12] suggested a bipolar immunopathological spectrum in active pulmonary TB. The 'reactive pole' shows small and localized infiltrates characterized by mononuclear cells (MAC and lymphocytes), strong PPD responses, absence of anti-PPD antibodies and few bacilli, while the 'non-reactive (anergic)' pole exhibits diffuse cell infiltrates, neutrophils, cutaneous anergy to PPD, high titres of anti-PPD antibodies, large quantities of bacilli and poor response to treatment.

In order to address this issue, our research protocol was designed to include untreated TB subjects at different stages. The amount of cells recovered in BAL in the three groups was higher than those reported in controls: fourfold in LPI and DPI and 1.5-fold in PE [13]. Even though MAC in patients with LPI (50 ± 22%) and PE (60 ± 13%) was the predominant cell type, its amount was reduced when compared to normal controls (70–95%). This was due to an increase of lymphocyte (33 ± 17% in LPI, 26 ± 13% in PE, 5–6% in controls) and neutrophil (33 ± 17% in LPI, 14 ± 1% in PE, < 1% in controls) infiltration. As the lung inflammation progresses, there is a significant increase of neutrophil infiltration (61 ± 20%) in severe DPI cases (Table 1). Even though neutrophil infiltration is an important event in TB infection, its role is limited and controversial [2, 19, 20].

Lymphocytes were considered to be the most important cell type recruited. First of all, these cells were increased in all the patients studied. Second, they were primarily $CD3^+CD4^+$ T lymphocytes. Third, the CD4:CD8 ratios were higher than the

values reported in controls [13], resembling the early stages of Sarcoidosis infection [22–24] . Fourth, the highest lymphocyte concentrations were found in the LPI group when compared to DPI and particularly to the PE group. These observations support previous reports of local lymphocyte infiltration in TB [3–6, 21–24] and the hypothesis of Lenzini *et al.* [12].

In the LPI group and in DPI patients, $CD4^+ CD45RO^+$ memory cells were the predominant subset encountered, with few naive, $CD4^+ CD45RA^+$, infiltrating cells. These cells expressed mostly $\alpha\beta$ TCR after a preculture period of 48 h and low numbers of $\gamma\delta$ TCR^+ cells. Furthermore, $CD8^+$ T cells were detected in similar amounts in LPI, DPI and PE patients. It may be proposed that $\alpha\beta^+$, $CD4^+$, $CD45RO^+$ memory cells are the main infiltrating lymphocyte type and these cells exhibit a regulatory and effector function against *Mt.*

Specific and mitogenic proliferative responses indicate that these $\alpha\beta$, $CD4^+$, $CD45RO^+$ memory T cells are sensitized to *Mt* antigens. This is better illustrated if we consider the responses obtained in the LPI group which represents the early and most efficient effector phase when the bacilli are encountered. Furthermore, when adherent PBMC were employed as antigen-presenting cells [15, 25–27], we were able to elicit a specific PPD response in two out of five patients who were unresponsive to antigen presented by alveolar MAC. The PBMC differed from local responses to specific antigens suggesting that the specific response is crucial for disease progression.

In conclusion, sensitized $\alpha\beta^+$, $CD4^+$, $CD45RO^+$ memory T-helper cells are the initial and main cell type involved in the local CMI response against *Mt.* We also suggest that this type of cell may be present in more advanced stages of pulmonary and extrapulmonary TB infection, probably subjected to immunomodulatory influences which may render them anergic to the specific antigen, as has been previously proposed [28]. Future studies should address the issue of cell types and the progression of TB.

ACKNOWLEDGMENTS

We are very grateful to the nursing staff of both the Jose I Baldó and University Hospitals and to Lucila Blanco for reviewing the statistical analysis. Supported by the GENIC program from CONICIT.

REFERENCES

1 Schlesinger L. Macrophage phagocytosis of virulent but not attenuate strains of *Mycobacterium tuberculosis* is mediated by mannose receptors in addition to complement receptors. J Immunol 1993;150:2920–6.

2 Kaufmann S. Immunity to intracellular bacteria. Annu Rev Immunol 1993;11:129–63.

3 Dunlap N, Briles D. Immunology of tuberculosis. Med Clin North Amer 1993;77:1235–51.

4 Orme I, Andersen P, Boom H. T Cell Response to *Mycobacterium tuberculosis*. J Infect Dis 1993;176:1481–7.

5 Andersen P. Host responses and antigens involved in protective immunity to *Mycobacterium tuberculosis*. Scand J Immunol 1997;45:115–30.

6 Ottenhoff THM. Putting numbers on Mycobacterium activated T cell subsets. Clin Exp Immunol 1996;104:381–3.

7 American Thoracic Society. Diagnostic standards and classification of tuberculosis. Am Rev Resp Dis 1990;142:725–35.

8 Nathan C, Murray C, Wiebe M, Rubin B. Interferon gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. J Exp Med 1983;158:670–8.

9 Mutis T, Cornelisse Y, Ottenhoff T. Mycobacteria induce $CD4^+$ T cells that are cytotoxic and display Th1-like cytokine secretion profile: heterogeneity in cytotoxic activity and cytokine secretion levels. Eur J Immunol 1993;23:2189–293.

10 Tsukaguchi K, Balaji K, Boom H. $CD4^+$ $\alpha\beta$ T cell and $\gamma\delta$ T cell responses to *Mycobacterium tuberculosis*. J Immunol 1995;154:1786–96.

11 Robinson D, Ying S, Taylor I *et al.* Evidence for Th1-like bronchoalveolar T cell subset and predominance of interferon-gamma gene activation in pulmonary tuberculosis. Am Rev Resp Care Med 1994;149:989–93.

12 Lenzini L, Rottoli P, Rottoli L. The spectrum of human tuberculosis. Clin Exp Immunol 1977;27:230–7.

13 The BAL Cooperative Group Steering Committee. Bronchoalveolar lavage constituents in healthy individuals, idiopathic pulmonary fibrosis and selected comparison groups. Am Rev Res Dis 1990;141:S169–202.

14 Merchant R, Schwartz D, Helmers R, Dayton C, Hunninghake G. Bronchoalveolar lavage cellularity. Distribution in normal volunteers. Am Rev Res Dis 1992;146:448–53.

15 Andrade R, Machado I, Fernández B, Blanca I, Ramírez R, Bianco NE. Cellular immunity in current active pulmonary tuberculosis. Am Rev Res Dis 1991;143:496–500.

16 Feo E, Morillo F, Blanca I, Bianco NE. Failure of cell mediated effector mechanisms in lung cancer. J Natl Cancer Inst 1984;73:1–13.

17 Assmann H. Uber die infraklavikularen lungeninfiltrationen im beginn der tuberkulose jugendlicher erwachsener und ihr schicksal. Deutsche Med Wchnschr 1927;53:781.

18 Hinshaw C, Garland H. Tuberculosis. In: Hinshaw C, Garland H, eds, Diseases of Chest. Philadelphia: Saunders, 1956:393–450.

19 Strieter R, Lukacs N, Standiford T, Kunkel S. Cytokines and lung inflammation: mechanisms of neutrophil recruitment to the lung. Thorax 1993;48:765–71.

20 Rogers H, Tripp C, Unanue E. Different stages in the natural and acquired resistance to an intracellular pathogen. The Immunologist 1995;3:152–6.

21 Drent M, Wagenaar S, Mulder P, van Velzen-Blad H, Diamant M, van den Bosch J. Bronchoalveolar lavage fluid profiles in sarcoidosis, tuberculosis and non-Hodgkin's and Hodgkin's disease. Chest 1994;105:514–9.

22 Hoheisel G, Tabak L, Teschler H, Erkan F, Kroegel C, Costabel UB. Bronchoalveolar lavage, cytology and immunocytology in pulmonary tuberculosis. Am Rev Res Dis Crit Care Med 1994;149:460–3.

23 Ozaki T, Nakahira S, Tani K, Ogushi F, Yasuoka S, Ogura T. Differential cell analysis in bronchoalveolar lavage fluid from pulmonary lesions of patients with tuberculosis. Chest 1992;102:54–9.

24 Sharma S, Pande J, Singh Y *et al.* Pulmonary function and immunologic abnormalities in milliary tuberculosis. Am Rev Res Dis 1992;145:1167–71.

25 Leucossier D, Valeyre D, Loiseau A, Battesti J, Soler P, Hance A. T-lymphocytes recovered by bronchoalveolar lavage from normal subjects and patients are refractory to proliferative signals. Am Rev Res Dis 1988;137:592–9.

26 Leucossier D, Valeyre D, Loiseau A *et al.* Antigen-induced proliferative response of lavage and blood T lymphocytes. Am Rev Res Dis 1991;144:861–8.

27 Ettensohn D, Roberts N. Human alveolar macrophage support of lymphocyte responses to mitogens and antigens. Am Rev Res Dis 1983;128:516–22.

28 Bianco NE. The immunopathology of systemic anergy in infectious diseases: a reappraisal and new perspectives. Clin Immunol Immunopathol 1992;62:253–7.