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Limited Clonal Heterogeneity of Antigen-Specific T Cells Localizing in the Pleural Space during Mycobacterial Infection

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To detect possible differences in phenotype and fine specificity for mycobacterial antigens between CD4-positive T cells from peripheral blood (PB) and from inflammatory sites, we identified four patients presenting with a mycobacterial pleural exudate (PE) rich in PPD-specific lymphocytes and with a negative skin test to tuberculin purified protein derivative (PPD) and a negative proliferative response of PB lymphocytes to PPD at the same time. Several weeks after chemotherapy, these patients converted to PPD responsiveness in the periphery, and PPD-specific clones could be generated from PB at this stage. The phenotypic comparison of PE lymphocytes and concomitant PB lymphocytes obtained before treatment showed an increase of CD8 cells and a high frequency of HLA-DR-positive activated T cells in PE. The frequency of tetanus toxoid-specific and *Candida albicans*-specific proliferating T cells was lower than that of PPD-specific cells in PE but not in PB. PPD-specific clones were derived initially from PE and from PB once the patients had converted to PPD responsiveness. The two sets of clones from each patient were compared for proliferative response to mycobacterial antigen clusters of defined molecular weight ranges. A large number of PE-derived clones (36%) responded to a fraction of 27 to 35 kDa, whereas only one clone from PB responded to the same fraction. The purified antigen P32 (32 kDa), a soluble mycobacterial protein, stimulated PE-derived clones that were responsive to the 37- to 27-kDa fraction but did not stimulate PB-derived clones. The data demonstrate that PE- and PB-derived lymphocytes differ both in phenotype and in fine specificity, suggesting a limited clonal heterogeneity of T cells localizing at the inflammatory site in tuberculous patients without a PPD response in the periphery. Therefore T cells compartmentalized at inflammatory sites provide information that is different from that provided by T cells in the periphery.

The T-cell response plays a critical role in protective immunity against mycobacterial infections (5, 11, 17). For a better understanding of the mechanisms that lead to protection and for a better design of new vaccines, it is important to define and characterize the fine specificities of activated T cells, which can be related to protective or nonprotective responses (10). Extensive studies have been performed on the different antigen specificities identified by peripheral blood (PB) lymphocytes (PBL) (8, 38) and by T-cell clones generated from the same source (25, 39). However the concepts of lymphocyte recirculation and of regional immunity (45, 48) cast some doubts on the reliability of information gained only from T cells derived from PB. Sites of inflammation, in fact, may also contain specific cells that are relevant for defenses and that are different from those found in the periphery.

Phenotypic characterization of T cells in inflammatory sites in mycobacterial diseases has been performed with monoclonal antibodies (36, 55). More recently, detailed information on functional properties of infiltrating cells in lepromatous lesions has been gained by in situ hybridization (9), suggesting that a similar approach can be extended to other granulomatous diseases.

To test for possible differences in phenotype and specificity between antigen-specific T cells from the periphery and from inflammatory sites, we focused on patients with mycobacterial infection of the pleural space associated with a lymphocyte-rich exudate, in which response to purified protein derivative (PPD) was absent in the periphery (negative skin test to PPD, negative proliferative response of peripheral lymphocytes to PPD) (44). Although it is not clear at this stage whether these patients represent a different group or a given stage in the evolution of the disease, they provide a unique opportunity to study antigen-specific T lymphocytes that are exclusively confined to the pleural space. The same patients converted to a positive PPD skin test several weeks later, after antimycobacterial chemotherapy and clinical resolution. Therefore, PPD-specific T cells could be obtained also from the periphery and compared with T cells from the exudate.

In this study we present evidence that T-cell clones generated at a later stage from PB exhibit a specificity pattern for mycobacterial antigens that is distinct from the pattern of T-cell clones from pleural exudate (PE). This suggests that PPD-reactive T cells initially sequestered in the pleural space do not repopulate ipso facto the PB compartment.

MATERIALS AND METHODS

Patients. The tuberculous patients described in this study were admitted to the First Department of Lung Diseases, San Martino Hospital in Genoa. Patients 1, 2, 3, and 4 had a clinical presentation suggesting a tuberculous pleural effusion, with (i) PE containing large numbers of lymphocytes

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TABLE 1. CD4/CD8 ratios in PBL and PEL[a]

Patient no.	CD4/CD8 ratio	
	PB	PE
1	1.7	0.4
2	2.0	0.5
3	1.9	0.4
4	1.3	0.9
Mean ± SD	1.72 ± 0.27	0.55 ± 0.20
5	1.8	0.6
6	1.8	0.4
7	1.6	0.4
8	1.3	2.5
Mean ± SD	1.62 ± 0.24	0.97 ± 1.02

[a] The CD4/CD8 ratio is shown for patients 1, 2, 3, and 4, who had a negative skin test to PPD, and for patients 5, 6, 7, and 8, who had a positive skin test to PPD.

and few mesothelial cells, (ii) pleural cultures negative for viral or bacterial pathogens, (iii) absence of malignant cells, (iv) no antibodies for viruses causing PE or for *Mycoplasma pneumoniae*, and (v) negative skin test to PPD. Clinical follow-up, response to chemotherapy, and positive cultures of pleural fluid confirmed the diagnosis of mycobacterial infection. Patients 5, 6, 7, and 8 were chosen as controls and presented with clinical features similar to patients 1, 2, 3, and 4 but showed a positive skin test to PPD and in vitro response to PPD. Lymphocytes from the PE (PEL) were used for the experiments or frozen for later assays. Heparinized venous PB was collected for the expansion of the T clones. Alternatively, lymphocytes were frozen and thawed when needed. Informed consent was obtained from the patients before thoracentesis and venipuncture.

Antigens. PPD was purchased from Statens Seruminstitut (Copenhagen, Denmark) and used at 25 μg/ml (final concentration). Tetanus toxoid (TT) was purchased from the Massachusetts Public Health Laboratories (Boston, Mass.) and used at 5 μg/ml (final concentration). *Candida albicans* antigen was prepared by autoclaving saline-washed yeast bodies and used at 10^6 bodies per ml. P32 is a mycobacterial antigen purified from a Sauton zinc-deficient culture filtrate of *Mycobacterium bovis*. Its purification and biochemical characterization have been described in detail (12).

Antigenic fractions from mycobacteria separated by gel electrophoresis and transferred on nitrocellulose were prepared by published procedures (1, 26, 57), with minor modifications. A soluble extract was prepared by sonication of an autoclaved suspension of *Mycobacterium hominis* (kindly provided by V. Colizzi, University of Rome). After centrifugation at 12,000 × *g* for 20 min, the clear supernatant containing 1 mg of protein (determined by the Lowry method [31]) per ml was defined as mycobacterial soluble extract. Fifty micrograms of mycobacterial soluble extract was run on a reducing sodium dodecyl sulfate–7.5% acrylamide gel in a mini gel Bio-Rad apparatus in a single well. The gel was electroblotted on a nitrocellulose membrane. Nine horizontal strips (5 by 40 mm) were cut from the membrane. By using β-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (42 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (22 kDa), and lysozyme (14 kDa) as markers, the following molecular mass ranges were estimated for each strip: 1 (>110 kDa), 2 (88 to 110 kDa), 3 (70 to 88 kDa), 4 (55 to 70 kDa), 5 (44 to 55 kDa), 6 (35 to 44 kDa), 7 (27 to 35 kDa), 8 (22 to 27 kDa), and 9 (14 to 22 kDa). The strips were solubilized in 4 ml of dimethyl sulfoxide, and nitrocellulose was precipitated under sterile conditions by dropwise addition of carbonate buffer (0.05 M, pH 8.6) with vigorous mixing. The particles were washed with Hanks solution and stored as a 10% suspension at 4°C for no longer than 2 weeks.

Cell preparation. PBL and PEL were purified on a Ficoll-Hypaque gradient by established methods and suspended at 2 × 10^6/ml in complete medium as described below. Heparinized plasma was saved for addition to culture medium. Cells were frozen in fetal calf serum containing 10% dimethyl sulfoxide and stored in liquid nitrogen.

Phenotyping. Fresh cells or cultured lines and clones (2 × 10^5) were typed for surface markers with a B-D fluorescence-activated cell sorter and monoclonal antibodies purchased from Becton Dickinson. CD3, CD4, CD8, and

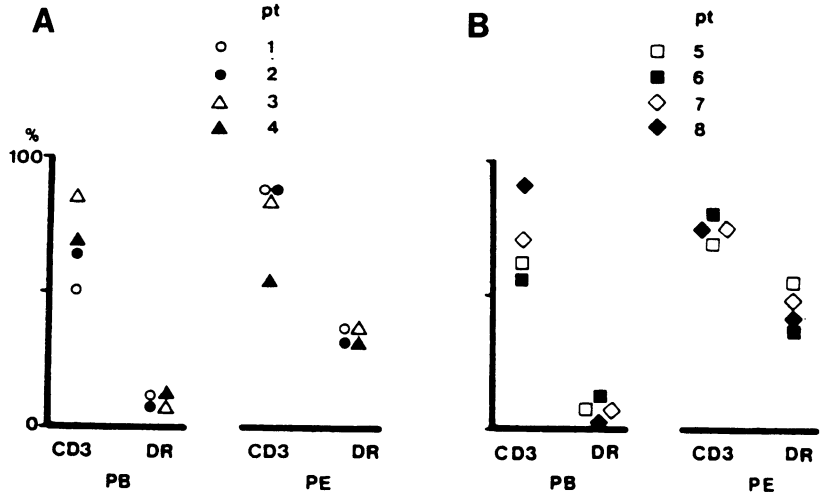

FIG. 1. Percentage of activated DR-positive T cells from PB and from PE in patients (pt) 1, 2, 3, and 4 (A) and from patients 5, 6, 7, and 8 (B). CD3-positive T cells are shown as a percentage of total mononuclear cells. DR-positive, CD3-positive cells are shown as a percentage of total CD3-positive cells.

TABLE 2. Effect of IL-2 on proliferative response and phenotype of PBL and PEL

Patient no.	Response to IL-2[a]		CD4/CD8 ratio after IL-2 stimulation[b]		Proliferative response to PPD[c]	
	PB	PE	PB	PE	Before IL-2	After IL-2
1	2.4	12.8	1.8	0.3	12	9
2	4.4	69.5	1.9	0.5	7	9
3	8.9	24.3	1.9	0.3	14	12
4	5.1	40.9	1.5	0.6	8	9
Mean ± SD	5.2 ± 2.7	36.9 ± 24.6	1.8 ± 0.2	0.42 ± 0.1	10.2 ± 3.3	9.7 ± 1.5

[a] PBL and PEL from patients 1, 2, 3, and 4 were stimulated with IL-2 for 3 days and pulsed with thymidine for 4 h. Results are given as 10^3 cpm of thymine incorporation.

[b] After IL-2 expansion in 24-well plates for 5 days, PBL and PEL were tested for phenotype.

[c] A titrated proliferation to PPD was performed on PEL diluted 1:10 with irradiated autologous PBL before and after expansion of activated T cells with IL-2 for 7 days. Results are given as 10^3 cpm of thymine incorporation after a 5-day culture with antigen.

HLA-DR were identified by direct immunofluorescence; anti-WT31 was used in indirect immunofluorescence. Standard procedures were used for cell staining (23).

Proliferation assays on PBL and PEL. Purified lymphocytes were suspended at 2×10^6 cells per ml in RPMI 1640 supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 2-mercaptoethanol (10^{-5} M), and L-glutamine (10 mM) and enriched with autologous heparinized plasma at 2.5% (final concentration). The cell suspensions were dispensed in 96-well flat-bottom microtiter plates (Costar) at 0.2 ml per well. The plates were pulsed with tritiated thymidine (0.5 μCi per well; specific activity, 5 Ci/mmol; Amersham Corp., Amersham, United Kingdom) on day 5 and harvested 12 h later.

Limiting dilution assays. Responding PBL and PEL were plated in round-bottom microtiter plates (Nunc, Roskilde, Denmark) in 24 replicates for each concentration, as indicated in Results. In addition, each well received 10^5 irradiated autologous PBL and antigen. No exogenous interleukin-2 (IL-2) was added. The plates were pulsed on day 7 and harvested on day 8. The frequency of antigen-specific cells was calculated with a computer program (14). Wells were defined as positive when thymidine incorporation was higher than the mean value plus 3 standard deviations as determined from negative replicates containing the same cell concentrations, in the absence of antigen (29).

Generation of T-cell lines. PBL and PEL were dispensed in 24-well plates (Costar) at 1.5 ml per well. PPD was added at 25 μg/ml (final concentration). Five days later the cells from one well were split in six wells in 1.5 ml of medium containing 30 U of recombinant IL-2, kindly provided by Hoffman-La Roche (Basel, Switzerland), per ml. After 4 or 5 days, the cells were further split under the same conditions and cultured for 4 days. At this stage antigen-specific stimulation was performed. T cells (2×10^5) in 1.5 ml of medium were cocultured with 5×10^5 autologous PBL irradiated with 2,500 rads from a cesium source (Gammacell; Atomic Energy of Canada) in the presence of antigen alone. IL-2 (30 U/ml, final concentration) was added after 2 days. Proliferative assays on the lines were performed by transferring 0.2 ml of cell suspension from the large wells into microtiter plate wells. The cultures were pulsed after 44 h and harvested 4 h later.

Generation of T-cell clones. T-cell clones were obtained by limiting dilution cloning of T-cell lines from PBL and PEL at the same stage of in vitro expansion after two cycles of stimulation with PPD. T cells were plated at 0.5 cell per well plus 5×10^4 autologous irradiated PBL, PPD, and IL-2 in round-bottom microtiter plates. After 10 to 14 days, the positive wells were transferred to 24-well plates with the addition of 4×10^5 irradiated autologous PBL, antigen, and IL-2. After 1 week the wells were split and expanded with

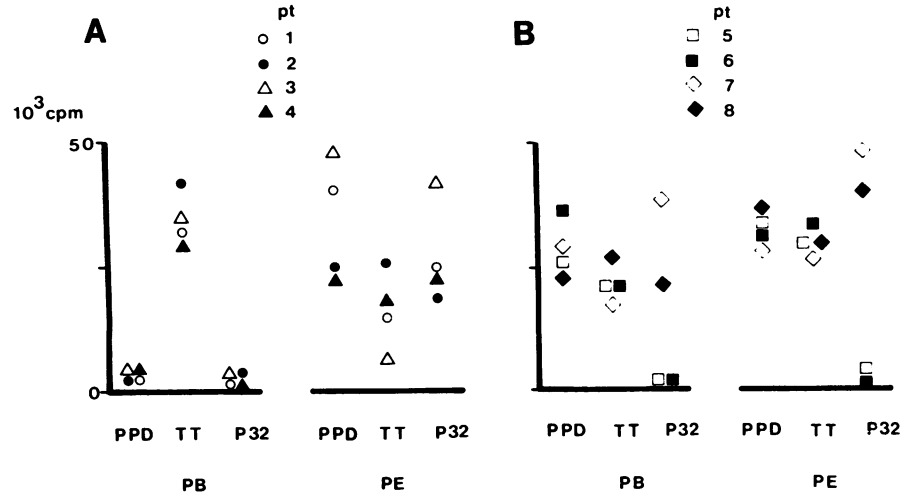

FIG. 2. Proliferative response of PBL and PEL to PPD, TT, and P32 from patients (pt) 1, 2, 3, and 4 (A) and patients 5, 6, 7, and 8 (B). Results are given as thymidine incorporation.

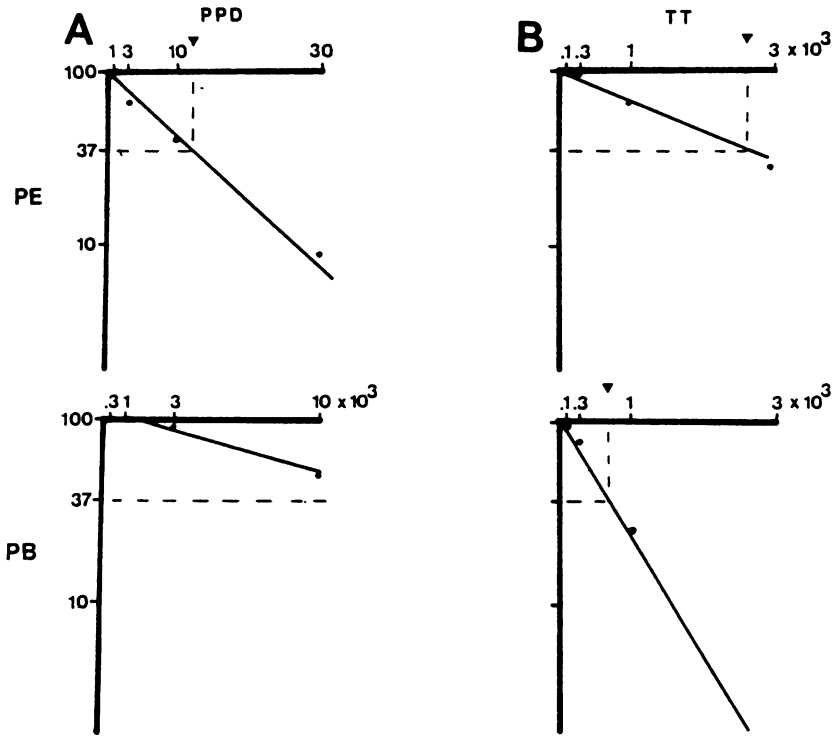

FIG. 3. Frequency of PPD-specific (A) and TT-specific (B) cells from the PE and PB of patient 1. The ordinates show the different cell concentrations used in the assays. The abscissae indicate the fraction of negative culture determined on 24 replicates for each dilution. The frequency for PPD-specific cells was 1 in 12 in PE and <1 in 10^4 in PB, and for TT-specific cells it was 1 in 2,700 in PE and 1 in 800 in PB, as shown by the arrowheads.

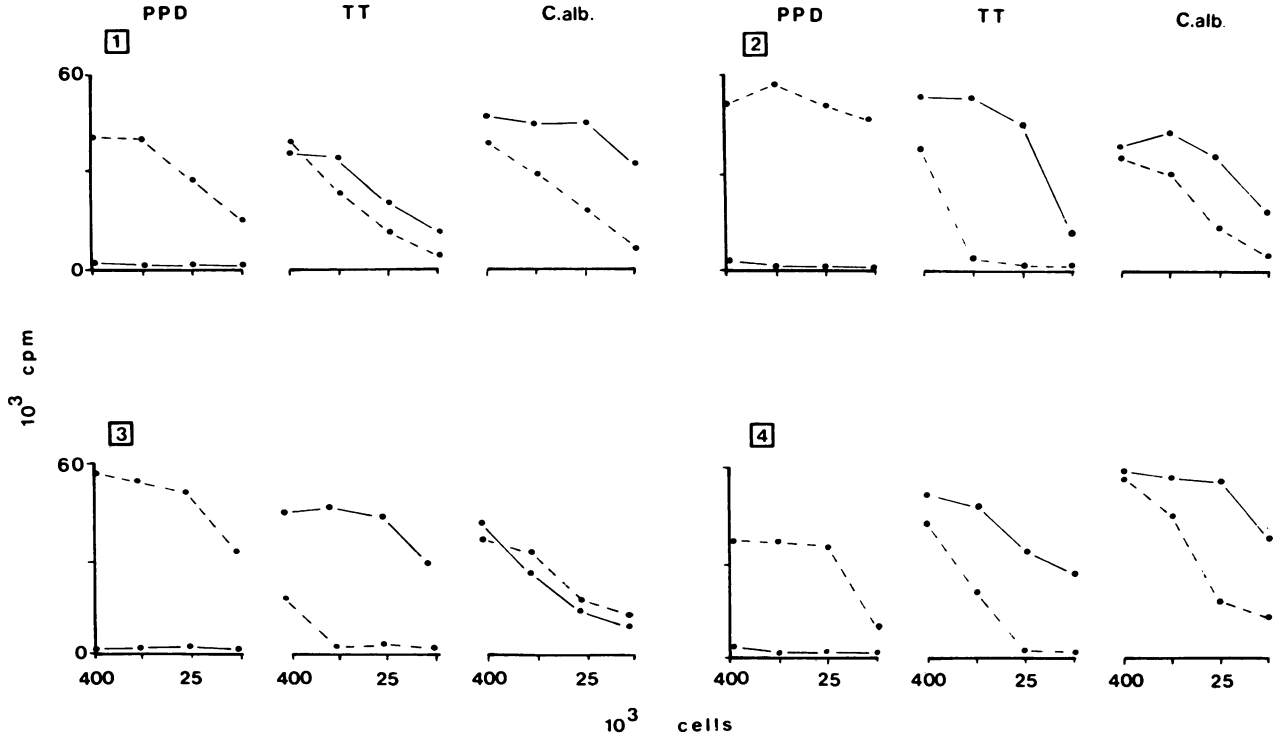

FIG. 4. Titrated proliferation assay of antigen-specific cells. Cells from PB (———) and from PE (– – –) from patients 1, 2, 3, and 4 were cultured at various concentrations (ordinate) with PPD, TT, or *C. albicans*, with the addition of irradiated autologous PBL to maintain a total number of 4×10^5 cells in all wells. Results on the abscissa indicate the proliferative response as thymidine uptake.

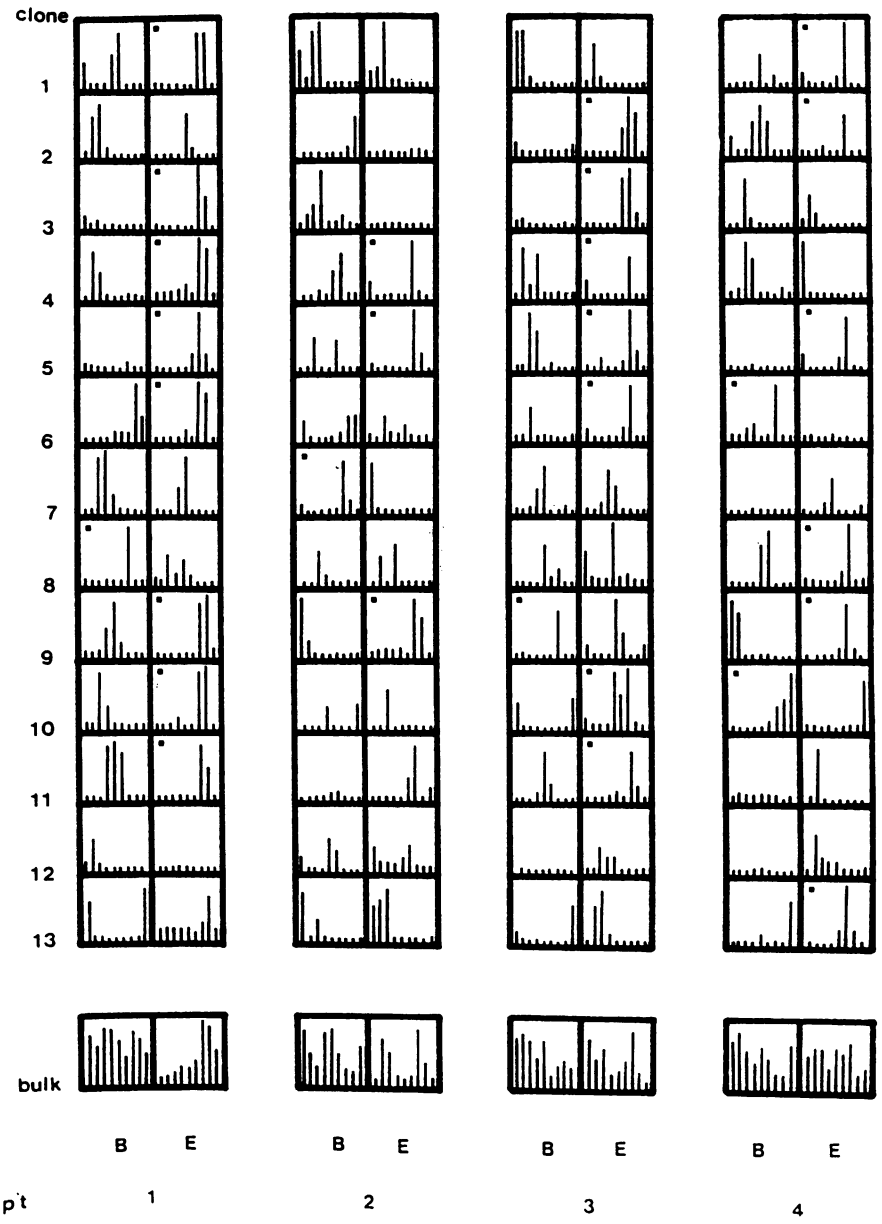

FIG. 5. Screening of clonal specificities with solid-phase antigenic fractions in patients (pt) 1, 2, 3, and 4. Thirteen clones and the bulk lines from which the clones were derived were obtained from PB (B) and from PE (E) and tested in a proliferative assay with nitrocellulose particles carrying fractions of a mycobacterial sonicated soluble extract separated according to molecular weight. Each square represents one clone. The nine bars in each square indicate the proliferative response (range of ordinate, 0 to 10 × 10^3 cpm of thymidine incorporated) to the nine fractions. Clones marked with a solid square responded to fraction 7.

IL-2 alone for several days to provide enough cells for the specificity screening.

The proliferative response of individual clones was determined by culturing 2 × 10^4 T cells with 5 × 10^4 irradiated autologous PBL in 200 μl of medium with 20 μl of particulate antigenic fractions as a 2% nitrocellulose suspension or with soluble antigens. Cultures were pulsed and harvested as described above for antigen-specific T-cell lines.

RESULTS

Phenotype of PBL and PEL in skin-test-negative patients. The phenotype of PBL and PEL shows that lymphocytes in the exudate do not represent a spillover of cells from the periphery to the inflammatory site. A higher frequency of CD8 cells over CD4 cells was seen among PEL (Table 1), with a mean ratio of 0.55 ± 0.20 as opposed to a ratio of 1.7 ± 0.27 among PBL. This result is not characteristic of patients with a negative skin test, since patients 5, 6, and 7, with a peripheral response to PPD, showed a similar distribution of T-cell subsets. Although there was no remarkable difference in the percentage of CD3 cells between PBL and PEL (Fig. 1), the frequency of DR-positive, activated T cells was higher in PEL (mean 30.0% ± 3.2% for patients 1, 2, 3, and 4; 47.0% ± 6.8% for patients 5, 6, 7, and 8) than in PBL (7.5% ± 2.9% and 8.0% ± 2.8%, respectively) in all of the

TABLE 3. Number of T-cell clones from patients 1, 2, 3, and 4 responsive to different nitrocellulose particle fractions[a]

Antigenic fraction	Size range (kDa)	No. of T-cell clones from patient: 1		2		3		4		Total		P
		PB	PE	PB	PE	PB	PE	PB	PE	PB	PE	
1	>110	3	0	3	4	2	2	2	2	10	8	0.30
2	88–110	3	0	1	3	2	2	1	3	7	8	0.40
3	70–88	4	1	5	4	2	2	2	2	13	9	0.20
4	55–70	5	2	2	1	3	2	3	1	13	6	0.05
5	44–55	4	2	2	2	3	5	3	1	12	10	0.30
6	35–44	4	1	2	2	1	4	2	2	9	9	0.50
7	27–35	1	8	2	4	2	6	2	6	7	24	0.01
8	22–27	1	8	1	2	0	2	1	1	3	10	0.10
9	14–22	2	0	3	0	2	1	2	1	9	2	0.01

[a] Each clone was scored as a responder to one or more of the nine fractions when the proliferative response was three times above the background. The Student *t* test was applied to determine which antigenic fraction used for stimulation yielded a statistically significant discrimination between PB- and PE-derived clones from patients 1, 2, 3, and 4. The difference between the two populations is highly significant ($P = 0.01$) with fractions 7 and 9.

patients with a mycobacterial PE, irrespective of the PPD response in the periphery.

The activation state of cells from PEL identified by the presence of DR antigens was confirmed functionally by evaluating the proliferative response to exogenous IL-2 (Table 2). The response to exogenous IL-2 was higher in PEL than in PBL (Table 2). In addition, IL-2 expansion for 5 days did not alter the CD4/CD8 cell ratio in PBL and in PEL (Table 2), suggesting that activated T cells functionally responsive to IL-2 are evenly distributed among CD4 and CD8 subsets. Furthermore, when PEL diluted with irradiated PBL (to provide the optimal number of antigen presenting cells but a limiting number of responding T cells [see below]) were tested before and after IL-2 expansion for response to PPD, similar proliferative responses were obtained (Table 2), suggesting that there was no selective increase in the frequency of antigen-specific cells upon IL-2 expansion. This indicates that both antigen-specific and antigen-nonspecific cells present in PE are in an IL-2-responsive state.

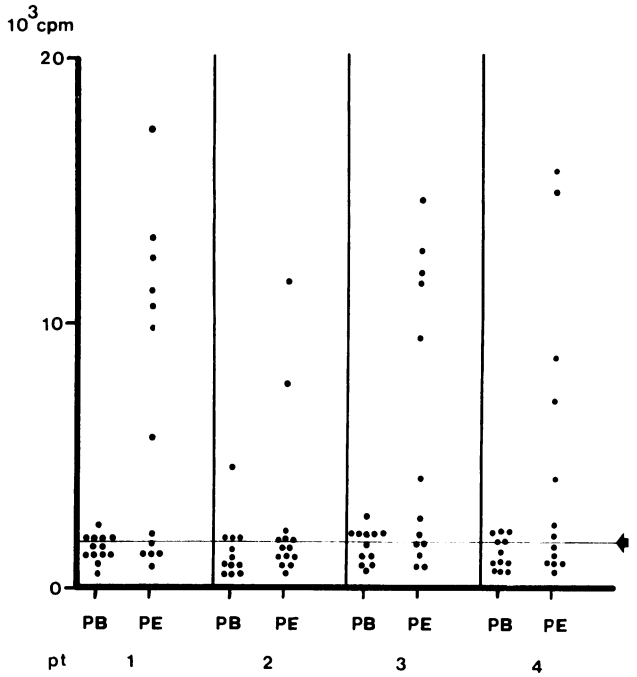

FIG. 6. Proliferative response of PB and PE clones from patients (pt) 1, 2, 3, and 4 to purified antigen P32. The same clones described in the legend to Fig. 5 were tested. Results are given as thymidine uptake. The line indicated by an arrow shows mean proliferation of the clones in the absence of antigen plus 1 standard deviation.

Proliferative responses of PBL and PEL to soluble antigens. PPD and P32 were stimulatory for PEL only and not for PBL in patients 1, 2, 3, and 4 (Fig. 2A), in agreement with the negative skin test to PPD. In contrast, the response to TT was higher in PBL than in PEL in all cases. The proliferative responses to PPD of PBL from patients 5, 6, 7, and 8 confirmed the positive skin test to PPD (Fig. 2B). In these patients the response to P32 was absent or present concordantly in both sites.

Frequency of antigen-specific T cells in PBL and PEL. A limiting dilution assay was performed to determine the frequency of PPD-specific and TT-specific proliferating cells in PBL and in PEL from patient 1. In this assay no exogenous IL-2 was added. As suggested by our previous experience, exogenous IL-2 increased the frequency of positive cultures in the wells with or without antigen, without a significant change in the frequency of antigen-responsive, positive wells. In this limiting dilution assay there was no need to use T-depleted cells as antigen-presenting cells, since PBL from patient 1 did not contain detectable PPD-responsive cells. Therefore, lymphokine production affecting the outcome of the experiment by antigen-responsive, irradiated PBL was unlikely. A remarkably high frequency of 1 PPD-specific cell out of 12 was found in PEL (Fig. 3). The frequency of PPD-specific cells could not be defined in PBL (<1 in 10^4) because of unresponsiveness to PPD. As predicted by the proliferative assay, the frequency of TT-specific cells was higher in the periphery (1 in 700) than in the exudate (1 in 2,700). Since limiting dilution experiments demand large numbers of irradiated PBL as antigen-presenting cells, we also used an alternative assay that estimates the relative frequency of specific cells in different populations. By using a titrated proliferation assay, we enhanced the differences in relative frequencies of antigen-specific cells between PBL and PEL. Each panel in Fig. 4 shows the proliferative response of PBL and PEL diluted with irradiated PBL to maintain an optimal number of antigen-presenting cells. Differences in response to the various antigens between PBL and PEL could be enhanced upon dilution. It

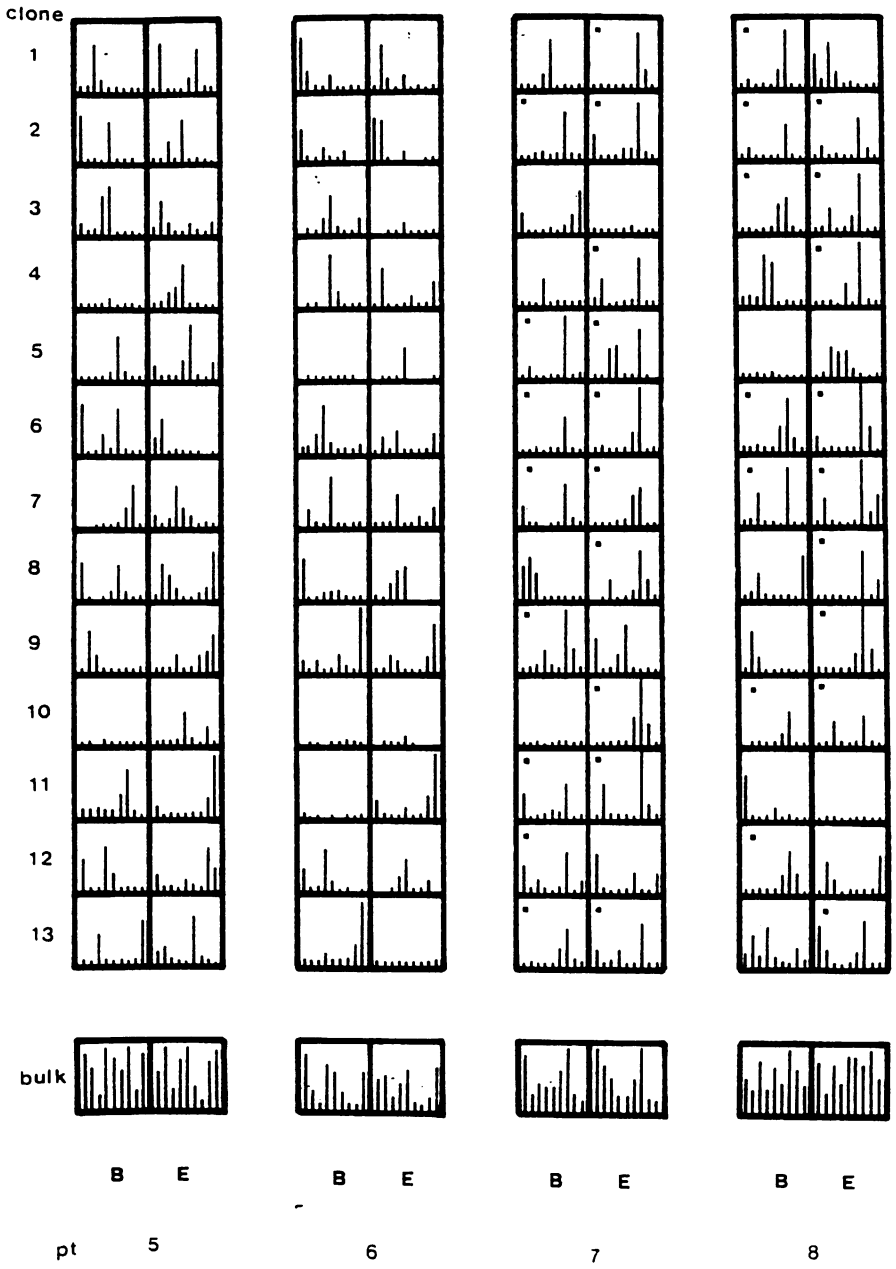

FIG. 7. Screening of clonal specificities with solid-phase antigenic fractions in patients (pt) 5, 6, 7, and 8. See legend to Fig. 5 for details.

was confirmed that PPD-specific cells were at higher frequency in the exudate, whereas TT- or *C. albicans*-specific cells were at higher frequency in the periphery in the four patients tested.

Response of PBL and PEL clones from PPD skin-test-negative patients to antigenic fractions. Clones were derived by limiting dilution from antigen-specific T-cell lines established initially from PEL and from PBL once the patients had converted to PPD-responsive status in the periphery. The cells from all of the clones were CD4 positive (>96%) and positive with monoclonal antibody WT31 (>90%), which is specific for the alpha/beta T-cell receptor (49) (data not shown). To screen PPD-responsive clones for specificity to different antigenic components of mycobacteria, we used clusters of antigenic components of defined molecular weight separated by sodium dodecyl sulfate-gel electrophoresis, blotted, and used as nitrocellulose particles in proliferation assays. With nine fractions ranging in molecular mass from 14 to >110 kDa we probably included most of the antigens present in a soluble sonicate extract. It should be noted that the lower limit of 14 kDa was defined by the lysozyme band on the gel. Since lysozyme comigrated with smaller molecules not retained on the gel, fraction 9 also included components of the mycobacterial extract with a molecular mass lower than 14 kDa. It is possible that additional antigenic components of PPD were not present in the soluble extract; nevertheless, 183 (88%) of 208 clones generated and maintained with PPD recognized one or more antigenic fractions.

Each of the 13 clones generated from PB and PE of

TABLE 4. Number of T-cell clones from patients 5, 6, 7, and 8 responsive to different nitrocellulose particle fractions[a]

Antigenic fraction	Size range (kDa)	No. of T-cell clones from patient:										P
		5		6		7		8		Total		
		PB	PE	PB	PE	PB	PE	PB	PE	PB	PE	
1	>110	4	2	4	2	4	3	1	2	13	9	0.15
2	88–110	1	4	2	4	1	2	2	3	6	13	0.025
3	70–88	3	2	2	2	2	2	2	4	9	10	0.35
4	55–70	4	3	3	3	3	2	2	2	12	10	0.15
5	44–55	4	4	3	4	1	1	1	2	9	11	0.35
6	35–44	4	3	2	1	1	4	3	2	10	10	0.5
7	27–35	2	2	1	1	8	10	7	9	18	21	0.40
8	22–27	1	3	1	3	2	4	2	2	6	12	0.025
9	14–22	1	5	2	5	1	1	1	3	5	13	0.05

[a] Each clone was scored as responder to one or more of the nine fractions when the proliferative response was three times above the background. The Student *t* test was applied to determine which antigenic fraction used for stimulation yielded a statistically significant discrimination between PB- and PE-derived clones from patients 5, 6, 7, and 8.

patients 1, 2, 3, and 4 was tested with the nine fractions of nitrocellulose particles carrying the blotted antigens and compared with the response pattern of the corresponding bulk lines (Fig. 5). The estimated molecular weight range for each fraction is indicated in Materials and Methods. Table 3 summarizes data obtained from the screening of the clones. Altogether, the clones from PBL recognized most of the different fractions. In contrast, 24 of 52 PEL-derived clones responded to fraction 7 (27 to 35 kDa). This suggests that clones from the inflammatory site have a heterogeneity in specificity pattern that is more limited than that of clones derived from PBL at a later stage.

Response of PBL- and PEL-derived clones from PPD skin-test-negative patients to purified P32. P32, purified from mycobacterial cultures as a secreted protein, is a good candidate as an antigen contained in fraction 7. T-cell clones were tested for response to P32 in a proliferative assay. For patient 2, only 1 clone (2%) of 52 PBL-derived clones specific for PPD responded to P32 (Fig. 6). In contrast, P32 stimulated 20 (38%) of 52 clones derived from PEL. All of the clones that responded to P32 gave a proliferative response to fraction 7 in the previous experiments. On the contrary, 6 clones of 7 from PBL and 4 clones of 24 from PEL that did respond to fraction 7 were not stimulated by P32, suggested that in fraction 7 antigenic components other than P32 can be recognized by T-cell clones.

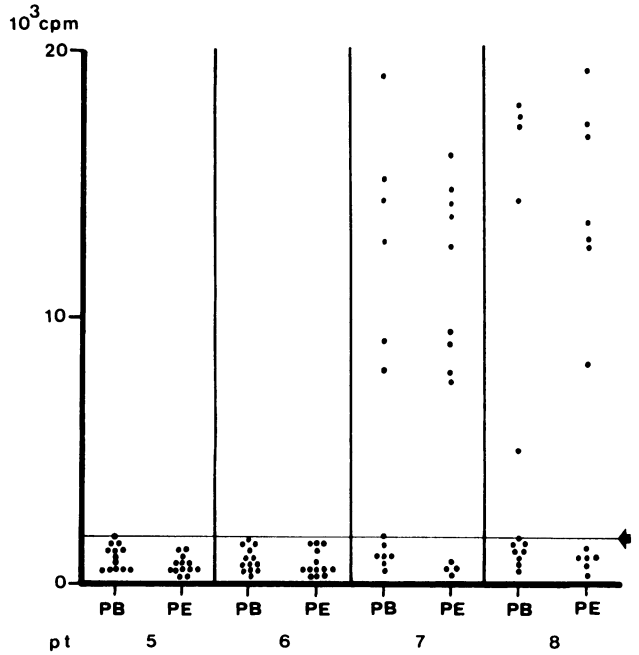

FIG. 8. Proliferative response of PB and PE clones from patients (pt) 5, 6, 7, and 8 to purified antigen P32. The same clones described in Fig. 7 were tested. Results are given as thymidine uptake. The line indicated by an arrow shows mean proliferation of the clones in the absence of antigen plus 1 standard deviation.

Specificity patterns of clones from patients with a concomitant PPD response in PEL and PBL. Clones were also derived from patients that exhibited a proliferative response to PPD in both PEL and PBL at the same time (patients 5, 6, 7, and 8). Patients 5 and 6 showed no response to P32, whereas patients 7 and 8 showed a vigorous response to P32 both in the periphery and in the exudate (Fig. 2). Clones derived from PBL and from PEL were tested for proliferation to the nitrocellulose fractions and to P32. Figure 7 shows similar patterns of reactivity between clones derived from PEL and clones derived from PBL. Patients 7 and 8, in particular, exhibited a large number of clones that were reactive to fraction 7, whereas only a few clones from patients 5 and 6 responded to this fraction.

The data from Fig. 7 are summarized in Table 4. It is evident that when PPD-responsive lymphocytes are present at the same time in the exudate and in the periphery, similar specificity patterns are present among clones derived from the two sites. In particular, the response to fraction 7 is not a characteristic feature of pleural lymphocytes, as seen in patients 1, 2, 3, and 4, with unique cells localizing in the pleural space and with no response to PPD in the periphery.

Figure 8 shows the response of the same clones to P32. None of the clones from patients 5 and 6 recognized P32, as predicted by the lack of response by PBL and PEL to P32 (Fig. 2). In contrast, similar numbers of clones from PBL (11 of 26) and PEL (16 of 26) of patients 7 and 8 recognized P32.

DISCUSSION

The analysis of the T-cell response to mycobacterial antigens has developed with two approaches that are often combined. The first is the dissection of the responding T-cell population by using cloned cells (27); the second is the

dissection of the complex antigenic spectrum of mycobacteria by using purified (44) and recombinant cloned antigens (25, 27) or antigen clusters separated according to molecular weight (28, 38, 57). Finally, synthetic peptides (56) and overlapping minimal peptides (54) allow accurate mapping of T-cell epitopes.

Because of the similarities in pathogenetic mechanisms (53) and because of sharing of numerous antigens between the pathogens (30), comparable studies have been performed in lepromatous and tuberculous patients. In addition to immunogenicity, mycobacterial components should also be analyzed for induction of protective response from the pathogen. This type of information can be gained by studying the human system in addition to experimental models of animals infected with mycobacteria (30, 53) or primed with antigens (6). In particular, attention must be paid to the T cells that are present exclusively in the sites of inflammation.

We studied tuberculous patients presenting with a mycobacterial infection of the pleural space associated with a lymphocyte-rich exudate, in whom absence of a skin response to PPD, confirmed by the absence of PPD-responsive T cells in the periphery, indicates that the exudate cells are unique to the inflammatory site and not exchanged with the periphery (44). These patients developed a peripheral PPD response after chemotherapy and clinical response, as confirmed by the appearance of PPD-specific cells in PB. Patients 1, 2, 3, and 4 represent a homogeneous group according to the immunological parameters we have considered. PEL differed from PBL according to phenotype. A higher frequency of CD8-positive cells was found in the exudates, whereas a normal ratio was seen in the periphery. This is in contrast with previous reports that have described a higher percentage of CD4 over CD8 cells in inflammatory pleural fluids (4, 47). At the present time we have no satisfactory explanation to account for this discrepancy. Furthermore, a high frequency of activated T cells defined by the expression of DR antigens was present in the exudates. The activation state of T cells in the exudates may be due to antigenic stimulation, although it is unlikely that all of the activated T cells are antigen specific. The frequency of PPD-specific cells tested by limiting dilution assay has been reported for PE and PB, with a higher frequency in the PE (15). The limiting dilution experiment in patient 1, in fact, shows that the frequency of PPD-specific T cells (8%) is lower than that of activated T cells (32%). A similar increase in CD8 cells over CD4 cells and in activated T cells in the exudate was seen in patients 5, 6, and 7, who presented with PPD reactivity in the periphery. Even though the predominant phenotype of pleural exudate lymphocytes was CD8, we focused in the present study on CD4 cells, which become the dominant population after in vitro stimulation. Human CD8 clones with specificity for mycobacterial antigens have been generated from pleural effusions, provided that a preliminary step of selection of CD8 cells is performed in advance (42). The recognition of discrete antigenic fractions and the use of major histocompatibility complex class I as restriction elements (42) suggest that also antigen-specific CD8 cells should be examined and compared with their peripheral counterparts to obtain a more complete picture.

The absolute and relative frequencies of PPD-specific cells do not reflect the frequency of T cells that are responsive to mycobacterial antigens. PPD, in fact, does not contain all of the antigens of mycobacteria. Nevertheless, most of the clones generated and maintained by repeated stimulation with PPD were stimulated by one of the antigenic fractions obtained by blotting on nitrocellulose (a sonicated mycobacterial extract). Nitrocellulose blotting of antigenic mixtures separated according to molecular weight has been described previously for mycobacterial extracts (25–27) and more recently for the analysis of human T-cell clones specific for *Leishmania* (32) and *Schistosoma* (43) antigens.

It was evident from our study that PPD-responsive T-cell clones generated from the periphery at a later stage of the disease recognize different antigenic fractions when compared with clones generated from the exudate. The latter clones, in fact, react mostly with fraction 7 (27 to 35 kDa). Most of the clones reacting with fraction 7 are also specific for the purified antigen P32. P32 is a protein secreted by mycobacteria (12), but the response of clones to fraction 7 indicates that P32 is also present in association with mycobacteria, in agreement with data previously reported (12). The response of PPD-specific clones to P32 is not unexpected, since sharing of epitopes with PPD can be demonstrated at least at the B-cell level with monoclonal antibodies that bind to both PPD and P32 in a solid-phase assay (10a). P32 may be present as a soluble product at a high concentration in the inflammatory site where the pathogens are, accounting for its dominant antigenicity in patients with compartmentalization of PPD-specific cells in particular. The high frequency of clones that are specific for P32 suggests a limited clonal heterogeneity at the inflammatory site. The concept of limited clonal heterogeneity (or clonal dominance or oligoclonality) has already been proposed in other pathological conditions, such as sites of autoimmune inflammation (40) and in autoimmune encephalomyelitis in mice (2), in synovial exudates from patients with rheumatoid arthritis (33, 50), in cerebrospinal fluid and brain lesions from patients with multiple sclerosis (16, 37), and in infiltrates from patients with kidney allograft rejection (7). In all of these papers limited clonal heterogeneity was defined by the analysis of T-cell receptor gene rearrangements rather than by analysis of the specificity patterns of specific T cells as described in this report.

The presence of antigen-specific T cells in the pleural space in a given stage of tuberculous infection in patients without a PPD response in the periphery may be due (i) to localization of circulating specific cells, (ii) to local proliferation and expansion of few specific precursors, or (iii) to a combination of the two mechanisms. The mechanisms that lead to compartmentalization in cellular hypersensitivity (19) are antigen specific and antigen nonspecific. The former are mediated by the T-cell receptor for antigen that allows localization of the relevant T cells in sites where antigen is presented by antigen-presenting cells (46). The latter involve surface markers that discriminate between naive and memory T cells, allowing differential interactions with the lymphoid tissues (52) and adhesion molecules that control and regulate lymphocyte trafficking (13).

The appearance of specific T cells in the periphery may be due either to recirculation of T cells that spill over from the inflammatory site or to delayed availability of antigen(s) in the periphery, which eventually stimulates the infrequent specific precursors to expand clonally. The different specificity patterns between pleural and peripheral clones as defined by response to antigenic fractions and to P32 suggest that the two T-cell populations develop independently of each other and not as the result of exchange of cell recirculation in some patients. An argument against this possibility is the fact that PB clones from patients 1, 2, 3, and 4 were derived several months after pleural clones were obtained, in contrast to patients 5, 6, 7, and 8, from whom specific clones were derived from the two compartments at the same time.

What can be safely concluded is that the T-cell response to fraction 7 is high in patients with pleuritis during the active stage of the disease, in spite of a lack of response to PPD in the periphery.

The analysis of the fine specificity of different clones for particulate fractions showed that several clones recognize two or more noncontiguous fractions, suggesting that the relevant epitope is present in more than one fraction. Alternatively, since cloning at 0.5 cell per well results in 75% of the positive cultures arising from a single precursor, one-fourth of the wells may still contain more than one precursor cell (51). This accounts for a positive response of certain "clones" to more than one antigenic fraction.

It is of interest to note that several clones respond to fraction 4 (55 to 70 kDa), which probably encompasses within its molecular mass range the heat shock protein HSP65, a major antigenic component of mycobacteria. Since HSP65 bears epitopes shared by numerous bacterial pathogens and by human HSP65 (35), it has been suggested that, although it is of biological relevance (58), the immune response to HSP65 cannot be taken as an indicator of mycobacterial infection, unless epitopes restricted to a given pathogen are identified (24).

Patients 5, 6, 7, and 8 showed PPD-specific cells in both sites and similar specificity patterns between the periphery and the inflammatory site, irrespective of recognition of P32. Data from numerous antigens (46), in particular on the 65-kDa mycobacterial antigen (58), and from the H2-linked control of gamma interferon production in vitro in response to P32 (21) suggest that the immune response to P32 may be related to major histocompatibility complex haplotypes also in humans. The number of pleural samples we have examined so far is too limited to allow a possible correlation with HLA class II antigens, but studies on PBL have shown that a large fraction of patients respond to P32 (22). CD4-positive cells that express the alpha/beta T-cell receptor have been selected by the culture conditions we used. It should be noted that CD4-negative cells bearing the gamma/delta receptor have been detected in the lungs of mice (3). Gamma/delta cells specific for mycobacterial antigens have been isolated (18, 20, 34), and a limited receptor repertoire has been described in a subset of such cells (41). The CD8 component, which is largely represented in the exudates, was selected against. The CD8 population, nevertheless, may play a relevant role in defense, and it has been shown that mycobacterial antigen-specific proliferating CD8 cells can be isolated by using culture conditions that positively select for such cells (42).

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REFERENCES

1. **Abou-Zeid, C., E. Filley, J. Steele, and G. A. W. Rook.** 1987. A simple new method for using antigens separated by polyacrylamide gel electrophoresis to stimulate lymphocytes in vitro after converting bands cut from Western blots into antigen-bearing particles. J. Immunol. Methods **98**:5–10.
2. **Acha-Orbea, H., D. J. Mitchell, L. Timmermann, D. C. Wraith, G. S. Taush, M. K. Waldor, S. S. Zamvil, H. O. McDavitt, and L. Steinman.** 1988. Limited heterogeneity of T cell receptors from lymphocytes mediating autoimmune encephalomyelitis allows specific immune intervention. Cell **54**:263–273.
3. **Augustin, A., R. T. Kubo, and G.-K. Sim.** 1989. Resident pulmonary lymphocytes expressing the gamma/delta T cell receptor. Nature (London) **340**:239–241.
4. **Barnes, P. F., S. D. Mistry, C. L. Cooper, C. Pirmez, T. H. Rhea, and R. Modlin.** 1989. Compartmentalization of a CD4+ T lymphocyte subpopulation in tuberculous pleuritis. J. Immunol. **142**:1114–1119.
5. **Bloom, B. R., and V. Mehra.** 1984. Immunological unresponsiveness in leprosy. Immunol. Rev. **80**:5–28.
6. **Brett, S. J., J. R. Lamb, J. H. Cox, J. B. Rothbard, A. Mehlert, and J. Ivanyi.** 1989. Differential pattern of T cell recognition of the 65-kDa mycobacterial antigen following immunization with the whole protein or peptides. Eur. J. Immunol. **19**:1303–1310.
7. **Carrie Miceli, M., and O. J. Finn.** 1989. T cell receptor beta chain selection in human allograft rejection. J. Immunol. **142**: 81–86.
8. **Converse, P. J., T. H. M. Ottenhoff, N. Gebre, J. P. Ehrenberg, and R. Kiessling.** 1988. Cellular, humoral and gamma interferon responses to mycobacterium leprae and BCG antigens in healthy individuals exposed to leprosy. Scand. J. Immunol. **27**:515–525.
9. **Cooper, C. L., C. Mueller, T. Sinchraisri, C. Pirmez, J. Chan, G. Kaplan, S. M. M. Young, I. L. Weissman, B. R. Bloom, T. H. Rea, and R. L. Modlin.** 1989. Analysis of naturally occurring delayed-type hypersensitivity reactions in leprosy by in situ hybridization. J. Exp. Med. **169**:1565–1581.
10. **Crowle, A. J.** 1988. Immunization against tubercolosis: what kind of vaccine? Infect. Immun. **56**:2769–2773.

10a. **Damiani, D.** Unpublished data.

11. **Dearborn, E., and C. H. Kirkpatrick.** 1986. The immunology of mycobacterial diseases. Am. Rev. Respir. Dis. **134**:1062–1071.
12. **De Bruyn, J., K. Huygen, R. Bosmans, M. Fauville, R. Lippens, J.-P. Van Vooren, P. Falmagne, M. Weckx, H. G. Wiker, M. Harboe, and M. Turneer.** 1987. Purification, characterization and identification of a 32 kDa protein antigen of *Mycobacterium bovis* BCG. Microb. Pathogen. **2**:351–366.
13. **Duijvestijin, A., and A. Hamann.** 1989. Mechanisms and regulation of lymphocyte migration. Immunol. Today **10**:23–28.
14. **Fazekas de St. Groth, S.** 1982. The evaluation of limiting dilution assay. J. Immunol. Methods **49**:R11–R36.
15. **Fujiwara, H., and I. Tsuyuguchi.** 1986. Frequency of tuberculin reactive T lymphocytes in pleural fluid and blood from patients with tuberculous pleurisy. Chest **89**:530–532.
16. **Hafler, D. A., A. D. Duby, S. Jin Lee, D. Benjamin, J. G. Seidman, and H. L. Weiner.** 1988. Oligoclonal T lymphocytes in the cerebrospinal fluid of patients with multiple sclerosis. J. Exp. Med. **167**:1313–1322.
17. **Hahn, H., and H. E. Kaufmann.** 1981. The role of cell-mediated immunity in bacterial infections. Rev. Infect. Dis. **3**:1221.
18. **Haregewoin, A., G. Soman, R. C. Hom, and R. W. Finberg.** 1989. Human gamma/delta$^+$ T cells respond to mycobacterial heat-shock protein. Nature (London) **340**:309–312.
19. **Hay, J. B.** 1983. Lymphocyte migration in cellular hypersensitivity and chronic granulomatous inflammation. Surv. Synth. Pathol. Res. **1**:229–240.
20. **Holoshitz, J., F. Konig, J. E. Coligan, J. De Bruyn, and S. Strober.** 1989. Isolation of CD4$^-$ CD8$^-$ mycobacteria-reactive T lymphocyte clones from rheumatoid arthritis synovial fluid. Nature (London) **339**:226–229.
21. **Huygen, K., K. Palfliet, F. Jurion, J. Hilgers, R. Ten Berg, J. P. Van Vooren, and J. De Bruin.** 1988. H2 linked control of in vitro gamma interferon production in response to a 32-kilodalton antigen (P32) of *Mycobacteria bovis* bacillus Calmette-Guerin. Infect. Immun. **56**:3196–3200.
22. **Huygen, K., J. P. Van Vooren, M. Turneer, P. Dierckx, and J. De Bruin.** 1988. Specific lymphoproliferation, gamma interferon production and serum immunoglobulin G directed against a purified 32 kDa mycobacterial protein antigen (P32) in patients with active tuberculosis. Scand. J. Immunol. **27**:187–194.

23. **Jackson, A. L., and N. L. Warner.** 1986. Preparation, staining and analysis by flow cytometry of peripheral blood leukocytes, p. 226–235. *In* N. R. Rose, H. Friedman, and J. H. Fahey (ed.), Manual of clinical laboratory immunology. American Society for Microbiology, Washington, D.C.
24. **Kaufmann, S. H. E.** 1990. Heat shock proteins and the immune response. Immunol. Today **11**:129–136.
25. **Lamb, J. R., J. Ivanyi, A. D. M. Rees, J. B. Rothbard, K. Howland, R. A. Young, and D. B. Young.** 1987. Mapping of T cell epitopes using recombinant antigens and synthetic peptites. EMBO J. **6**:1245–1249.
26. **Lamb, J. R., R. E. O'Hehir, and D. B. Young.** 1988. The use of nitrocellulose immunoblots for the analysis of antigen recognition by T lymphocyetes. J. Immunol. Methods **110**:1–10.
27. **Lamb, J. R., and D. B. Young.** 1987. A novel approach to the identification of T-cell epitopes in *Mycobacterium tubercolosis* using human T-lymphocyte clones. Immunology **60**:1–5.
28. **Lee, S. P., N. G. Stoker, K. A. Grant, Z. T. Handzel, R. Hussain, K. P. McAdam, and H. M. Dockrell.** 1989. Cellular immune response of leprosy contacts to fractionated *Mycobacterium leprae* antigens. Infect. Immun. **57**:2475–2480.
29. **Lefkovits, I., and H. Waldmann.** 1979. Limiting dilution analysis of cells in the immune system. Cambridge University Press, Cambridge.
30. **Leveton, C., S. Barnass, B. Champion, S. Lucas, B. De Souza, M. Nicol, D. Banerjee, and G. Rook.** 1989. T-cell-mediated protection of mice against virulent mycobacterium tubercolosis. Infect. Immun. **57**:390–395.
31. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
32. **Melby, P. C., and D. L. Sacks.** 1989. Identification of antigens recognized by T cells in human leishmaniasis: analysis of T-cell clones by immunoblotting. Infect. Immun. **57**:2971–2976.
33. **Miltenburg, A. M., J. M. Van Laar, M. R. Daha, R. R. De Vries, P. J. Van Den Elsen, and F. C. Breedveld.** 1990. Dominant T cell receptor beta chain gene rearrangiaments indicate clonal expansion in the rheumatoid joint. Scand. J. Immunol. **31**:121–125.
34. **Modlin, R. L., C. Pirmez, F. M. Hofman, V. Torigian, K. Uyemura, T. H. Rea, B. R. Bloom, and M. B. Brenner.** 1989. Lymphocytes bearing antigen-specific gamma/delta T cell receptors accumulate in human infectious diseases lesions. Nature (London) **339**:544–548.
35. **Munk, M. E., B. Schoel, S. Modrow, R. W. Karr, R. A. Young, and S. H. Kaufmann.** 1989. T lymphocytes from healthy individuals with specificity for self epitopes shared by the mycobacterial and human 65 kilodalton heat shock protein. J. Immunol. **143**:2844–2849.
36. **Narayanan, R. B., K. Bhutani, A. K. Sharma, and I. Nath.** 1983. T cell subsets in leprosy lesions: in situ characterization using monoclonal antibodies. Clin. Exp. Immunol. **51**:421–429.
37. **Oksenberg, J. R., S. Stuart, A. B. Begovich, R. B. Bell, H. A. Erlich, L. Steinman, and C. C. Bernard.** 1990. Limited heterogeneity of rearranged T cell receptor V alfa transcripts in brains of multiple sclerosis patients. Nature (London) **345**:344–346.
38. **Ottenhoff, T. H. M., P. J. Converse, N. Gebre, A. Wondimu, J. P. Ehrenberg, and R. Kiessling.** 1989. T cell response to fractionated *Mycobacterium leprae* antigens in leprosy. The lepromatous nonresponder defect can be overcome in vitro by stimulation with fractionated *M. leprae* components. Eur. J. Immunol. **19**:707–713.
39. **Ottenhoff, T. H. M., P. R. Klatser, J. Ivanyi, D. G. Elferink, M. Y. L. de Wit, and R. R. P. de Vries.** 1986. *Mycobacterium leprae*-specific protein antigens defined by cloned human helper T cells. Nature (London) **319**:66–68.
40 **Padula, S. J., D. C. Sgroi, A. G. Lingenheld, J. T. Love, C. J. Chou, and R. B. Clarke.** 1988. T cell receptor beta chain gene rearrangement shared by murine T cell lines derived from a site of autoimmune inflammation. J. Clin. Invest. **81**:1810–1818.
41. **Pat Happ, M., R. T. Kubo, E. Palmer, W. K. Born, and R. L. O'Brien.** 1989. Limited receptor repertoire in a mycobacteria-reactive subset of gammadelta T lymphocytes. Nature (London) **342**:696–698.
42. **Rees, A., A. Scoging, A. Mehlert, D. B. Young, and J. Ivanyi.** 1988. Specificity of proliferative response of human CD8 clones to mycobacterial antigens. Eur. J. Immunol. **18**:1881–1887.
43. **Reynolds, S. R., S. L. Kunkel, D. W. Thomas, and G. I. Higashi.** 1990. T cell clones for antigen selection and lymphokine production in murine schistosomiasis mansoni. J. Immunol. **144**: 2757–2762.
44. **Rossi, G. A., B. Balbi, and F. Manca.** 1987. Tubercolosis infection of the pleural space: evidence for selective migration of PPD specific T lymphocytes to the site of inflammation. Am. Rev. Respir. Dis. **136**:575–579.
45. **Rouse, R. V., R. A. Reichert, W. H. Gallatin, I. L. Weissman, and E. C. Butcher.** 1984. Localization of lymphocyte subpopulations in peripheral lymphoid organs: directed lymphocyte migration and segregation into specific microenvironments. Am. J. Anat. **170**:391–396.
46. **Schwartz, R. H.** 1985. T-lymphocyte recognition of antigen in association with gene products of the major histocompatibility complex. Annu. Rev. Immunol. **3**:237–262.
47. **Shiratsuchi, H., and I. Tsuyuguchi.** 1984. Analysis of T cell subsets by monoclonal antibodies in patients with tuberculosis after in vitro stimulation with purified protein derivative of tuberculin. Clin. Exp. Immunol. **57**:271–277.
48. **Smith, M. E., and W. L. Ford.** 1983. The recirculating lymphocyte pool of the rat: a systematic description of the migratory behaviour of recirculating lymphocytes. Immunology **49**:83–94.
49. **Spits, H., J. Borst, W. Tax, P. J. A. Capel, C. Terhorst, and J. De Vries.** 1985. Characteristics of a monoclonal antibody (WT31) that recognizes a common epitope on the human T cell receptor for antigen. J. Immunol. **135**:1922–1926.
50. **Stamenkovich, I., M. Stegagno, K. A. Wright, S. M. Krane, E. P. Amento, R. B. Colvin, R. J. Duquesnoy, and J. T. Kurnick.** 1988. Clonal dominance among T lymphocyte infiltrates in arthritis. Proc. Natl. Acad. Sci. USA **85**:1179–1183.
51. **Steinberg, C., and I. Lefkovits.** 1985. Table for evaluating limiting diluting experiments. Immunol. Methods **3**:317–373.
52. **Stoolman, L. M.** 1989. Adhesion molecules controlling lymphocyte migration. Cell **56**:907–910.
53. **Takashima, T., and F. M. Collins.** 1988. T-cell-mediated immunity in persistent *Mycobacterium intracellulare* infections in mice. Infect. Immun. **56**:2782–2787.
54. **Van der Zee, R., W. Van Eden, R. H. Meloen, A. Noordzij, and J. D. A. Van Embden.** 1989. Efficient mapping and characterization of a T cell epitope by simultaneous synthesis of multiple peptides. Eur. J. Immunol. **19**:43–47.
55. **Van Voorhis, W. C., G. Kaplan, E. N. Sarno, M. A. Horwitz, R. M. Steinman, W. R. Levis, N. Nogueira, L. S. Hair, C. R. Gattass, B. A. Arrick, and Z. A. Cohn.** 1982. The cutaneous infiltrates of leprosy: cellular characteristics and the predominant T-cell phenotypes. N. Engl. J. Med. **307**:1593–1597.
56. **Watson, J. D.** 1989. Leprosy: understanding protective immunity. Immunol. Today **10**:218–221.
57. **Young, D. B., and J. R. Lamb.** 1986. T lymphocytes respond to solid-phase antigen: a novel approach to the molecular analysis of cellular immunity. Immunology **59**:167–171.
58. **Young, R. A., and T. J. Elliott.** 1989. Stress proteins, infection and immune surveillance. Cell **59**:5–8.