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Increase of γ/δ T Cells in Hospital Workers Who Are in Close Contact with Tuberculosis Patients

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γ/δ T cells are likely to participate in the immune response to tuberculous infection in humans. In this study, we carried out an investigation to characterize the responsiveness of γ/δ T cells from tuberculous patients and healthy individuals to mycobacterial stimulation in vitro. Healthy subjects were assigned to the following two groups: those who had been exposed to tuberculosis (contacts) and those who had not been exposed (noncontacts). The percent γ/δ T cells in fresh peripheral blood obtained from health care workers who were tuberculin skin test positive and who had constant contact with patients with active tuberculosis (healthy contacts) was significantly higher, whereas healthy noncontacts showed the normal range of γ/δ T cells. Patients with active pulmonary tuberculosis also had low levels of γ/δ T cells. HLA-DR antigen-bearing activated γ/δ T cells were observed in higher percentages among healthy contacts than among healthy noncontacts or patients with pulmonary tuberculosis. In healthy contacts, γ/δ T cells increased as a percentage of peripheral blood mononuclear cells after in vitro stimulation with purified protein derivative (PPD) tuberculin compared with the percentage of fresh peripheral blood mononuclear cells that they made up, whereas no such increase was observed in patients with tuberculosis or in healthy noncontacts. Phenotypic analysis of the γ/δ T cells in healthy contacts, which increased in number in vitro in response to PPD, revealed the preferential outgrowth of $CD4^+$ $V\gamma2^+$ γ/δ T cells. This expansion of γ/δ T cells by PPD required accessory cells, and it was inhibited by the addition of an antibody against HLA-DR in culture. Proteolytic digestion of PPD showed that γ/δ T cells increased in number in response to peptide, but not nonpeptide, components of PPD. These findings suggest that γ/δ T cells, especially $CD4^+$ $V\gamma2^+$ γ/δ T cells, may participate in the immune surveillance of tuberculous infections in humans.

It is estimated that *Mycobacterium tuberculosis* causes three million deaths annually worldwide, a burden of mortality greater than that of any other infectious pathogen (3). A major effort toward the development of a potent vaccine against tuberculosis is under way. To reach this goal, a precise definition of the mechanisms of immunological resistance to tuberculous infection is required. In the case of tuberculous infection in humans, only a small percentage of individuals infected with *M. tuberculosis* develop overt disease and most of those infected remain disease-free. In a minority of infected individuals, weakening of the immune system at a later time tips the balance in favor of the microbe and the disease develops after reactivation of foci harboring *M. tuberculosis* organisms. In other words, in an individual who has been infected with tubercle bacilli, reinfection with an exogenous strain of tubercle bacilli seems unlikely because of the immunity conferred by the initial infection. On the other hand, Romeyn (30) described the importance of reinfection in situations involving high levels of exposure. Although declining tuberculosis case rates make reinfection less likely in developed countries, exogenous reinfection has nevertheless been described in several case reports (22, 28, 31).

Protective immunity against mycobacteria, which are intracellular pathogens, is dependent on antigen-specific T cells. Most T cells recognize foreign antigenic peptides bound to major histocompatibility complex (MHC) class I or class II molecules via T-cell receptor (TCR) α/β. Several years ago, T cells expressing TCR γ/δ (γ/δ T cells) were identified (5). γ/δ T cells represent a minor subpopulation in the peripheral blood. γ/δ T cells proliferate in response to mycobacterial extracts or to heat-shock protein (4, 9, 11, 23). An increased number of γ/δ T cells were observed in the lungs of mice after exposure to aerosols containing *M. tuberculosis* (1) and in draining lymph nodes of mice after immunization with *M. tuberculosis* in the limbs (13). γ/δ T cells appear in mice at a relatively early stage of primary infection with *Listeria monocytogenes* (10). In visceral leishmaniasis, one of the human infectious diseases caused by an intracellular pathogen, the percent γ/δ T cells in the peripheral blood increased significantly (29). Taken together, these reports indicate that γ/δ T cells may have a role in the generation of protective immunity against infection with intracellular pathogens, including *M. tuberculosis*.

Despite numerous studies, it remains uncertain to what extent γ/δ T cells participate in the immune response to tuberculous infection in humans. In this study, we intended to see whether γ/δ T cells might contribute to the antituberculous immunity in healthy individuals who were in close contact with patients with active tuberculosis (healthy contacts). Nurses and doctors who work in tuberculosis wards and are frequently exposed to sputum-smear-positive patients are a high-risk group with regard to infection with *M. tuberculosis* (8, 19). Our study shows that numbers of activated γ/δ T cells among peripheral blood mononuclear cells (PBMC) of healthy contacts were increased compared with those among PBMC of healthy persons not exposed to tuberculosis (healthy noncontacts) or patients with tuberculosis and that these numbers were expanded further in vitro by mycobacterial stimulation, whereas no such increase in numbers of γ/δ T cells was observed in cases of active tuberculosis. Frequent exposure to

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TABLE 1. Profile of study groups

Subject group	No. of subjects	Age of subjects (yr)		Sex of subjects (no. M/no. F)[a]
		Mean ± SD[b]	Range	
Healthy noncontacts	18	36.4 ± 13.1	21–59	8/10
Healthy contacts	17	35.6 ± 8.5	24–53	7/10
Patients with tuberculosis	19	34.5 ± 10.7	21–58	11/8

[a] M, male; F, female.
[b] SD, standard deviation.

mycobacterial organisms may induce the expansion of the numbers of γ/δ T cells in vivo in healthy subjects, and the additional γ/δ T cells may be protecting the host from the development of tuberculosis following reinfection with mycobacterial organisms.

MATERIALS AND METHODS

Subjects. Healthy subjects consisted of 35 hospital staff members, and they were assigned to the following two groups: healthy contacts and healthy noncontacts. Healthy contacts included 10 nurses and seven physicians who were usually working in the tuberculosis wards in our hospital and who had constant contact with patients with active tuberculosis. The healthy noncontact group included five nurses, four pharmaceutists, five laboratory workers, and four physicians who were usually working in other sections remote from tuberculosis wards and who had few chances for close contact with patients with tuberculosis. All the healthy subjects (contacts and noncontacts) had no history of tuberculosis and reacted positively to a tuberculin skin test. Even the healthy noncontacts tested tuberculin positive, probably because of the high level of coverage of *Mycobacterium bovis* BCG vaccination in the case of tuberculin-negative individuals in Japan. Patients included 19 people who were diagnosed as having pulmonary tuberculosis on the basis of chest radiographs and the presence of acid-fast bacilli in the sputum. They were patients with newly diagnosed tuberculosis. They showed a positive delayed-type skin reaction (erythema, induration, and in some cases, blister formation) to the standard dose (5 TU) of tuberculin injection. Blood samples were collected after hospitalization and just before the start of antituberculosis chemotherapy. The age and sex distributions of the subjects are shown in Table 1.

Mononuclear cell separation and preparation of T cells and monocytes. PBMC were isolated by Ficoll-Hypaque density gradient centrifugation (32) from heparinized venous blood obtained after informed consent was received. The resultant mononuclear cell suspension was separated into adherent and nonadherent cells as follows. A 5-ml sample of 2×10^6 PBMC per ml of RPMI 1640 medium supplemented with 10% fetal calf serum (M.A. Bioproducts, Walkersville, Md.) was placed in a plastic petri dish (60 by 15 mm; Kotai Kagaku Co. Ltd., Tokyo, Japan) and incubated at 37°C in 5% CO_2 in air for 2 h. Adherent cells were then extensively washed and were removed by adding 3 ml of cold EDTA solution and further incubating the dish at 4°C for 30 min. Adherent cells thus prepared were used as the monocyte source. In the experiments in which adherent cells were added to T-cell proliferation culture, irradiated (3,500 rads) adherent cells were used. T cells were obtained by passing the nonadherent cells through a nylon-wool column as described previously (33). The cellular composition of adherent cells and T cells thus prepared was examined by fluorescence-activated cell sorter analysis. The adherent cell population contained more than 95% $LeuM3^+$ cells. The T-cell population contained less than 0.5% $LeuM3^+$ cells and more than 85% $Leu4^+$ cells.

Antigens. Purified protein derivative (PPD) tuberculin from culture filtrates of *M. tuberculosis* was obtained from the Institute for Microbial Disease, Osaka University, Osaka, Japan. Proteolytic digestion of PPD was performed by the method described by Pfeffer et al. (26). Digestion was carried out by the successive treatment of PPD with proteinase K and pronase.

Lymphocyte proliferation assay. PBMC were cultured in RPMI 1640 medium supplemented with 10% pooled human serum, 100 U of penicillin, and 100 μg of streptomycin per ml. Mononuclear cells were cultured at a density of 2.5×10^5 cells per ml with an appropriate concentration of PPD in flat-bottom tissue culture plates (Microtest II; Falcon catalog no. 3072). Cultures were maintained in humidified 5% CO_2 in air at 37°C. Cells were harvested after 6 days. No exogenous cytokines were added. Eighteen hours before culture termination, 0.2 μCi of [^{3}H]thymidine was added. At the end of the culture, cells were harvested and washed with a semiautomated microharvester (Laboscience Co. Ltd., Tokyo, Japan), and then [^{3}H]thymidine incorporation was evaluated. Each determination was performed in triplicate, and the data were expressed as mean counts per minute ± standard error of the mean (SEM). In the experiment to evaluate whether PPD-induced expansion of numbers of γ/δ T cells requires an MHC class II antigen, graded amounts of anti-HLA-DR monoclonal antibody (MAb) (anti-HLA-DR immunoglobulin G2B [IgG2B]; Cosmo Bio. Co. Ltd., Tokyo, Japan) were added to the culture at the initiation of cultivation. Normal mouse IgG2B (mouse myeloma protein; Cappel, West Chester, Pa.) and anti-MHC class I MAb [anti-HLA(ABC) class I IgG2A, clone W6/32; Serotec, Oxford, United Kingdom] were employed as the controls.

Immunofluorescence staining and two-color analysis of mononuclear cells. Fresh or PPD-stimulated mononuclear cells were resuspended at 2×10^6 to 5×10^6 cells per ml in staining buffer (phosphate-buffered saline–2% human serum–1% bovine serum albumin–0.02% sodium azide) containing a saturated amount of antibodies and incubated for 30 min at 4°C. After three washes with staining buffer, the cells were analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.). MAbs used were as follows: phosphatidylethanolamine (PE)-conjugated anti-Leu-4 (anti-CD3), anti-Leu-3a (anti-CD4), anti-Leu-2a (anti-CD8), and anti-HLA-DR MAbs. These MAbs were purchased from Becton Dickinson. Fluorescein isothiocyanate (FITC)-conjugated anti-TCR δ1 (anti-Cδ), anti-δTCS1 (anti-Vδ1-Jδ1/2), and anti-TiγA (anti-Vγ2; Vγ2 is also referred to as Vγ9 in the alternative nomenclature) MAbs were purchased from T Cell Science, Inc. (Cambridge, Mass.). Data were represented as two-dimensional contour maps. To obtain the percentage of a subpopulation, total counts were integrated in selected areas of contour plots. Background staining with negative control Ig was subtracted from experimental values.

Statistical analysis. For continuous variables, the difference between groups was assessed by Student's *t* test or the Wilcoxon rank sum test. For categorical variables, the Scheffe F test was used.

RESULTS

Proportion of γ/δ T cells among PBMC in healthy subjects and patients with tuberculosis. The percentage of freshly isolated PBMC expressing TCR γ/δ was determined by using two-color direct immuno-flow cytometry. The summarized

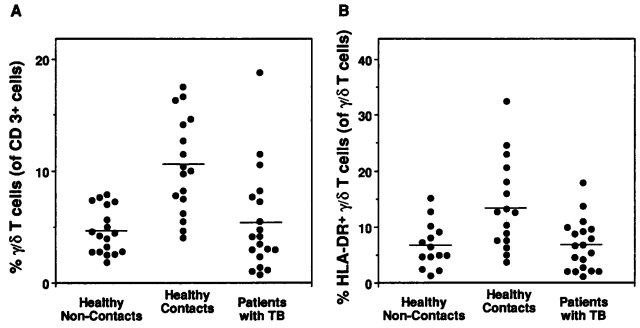

FIG. 1. The percents peripheral blood γ/δ T cells and HLA-DR-bearing activated γ/δ T cells in healthy subjects and patients with tuberculosis. (A) Freshly isolated PBMC were stained with FITC-conjugated anti-TCR δ1 MAb and PE-conjugated anti-CD3 MAb. (B) Freshly isolated PBMC were stained with FITC-conjugated anti-TCR δ1 MAb and PE-conjugated anti-HLA-DR MAb. The horizontal bars represent the mean levels.

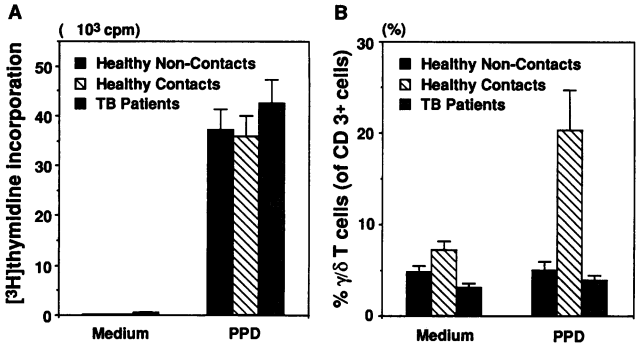

FIG. 2. (A) Proliferative responses of PBMC after in vitro stimulation with PPD. The extent of proliferation was assessed by measuring the radioactivity of [^{3}H]thymidine incorporated into the cultured cells. (B) Expansion of numbers of γ/δ T cells after in vitro stimulation with PPD. After in vitro stimulation with PPD, PBMC were stained with PE-conjugated anti-CD3 MAb and FITC-conjugated anti-TCR δ1 MAb. The percent γ/δ T cells after in vitro stimulation with PPD was analyzed. In both panel A and panel B, values are means ± SEM. For healthy noncontacts, n was 18; for healthy contacts, n was 17; and for patients with tuberculosis, n was 19.

data are shown in Fig. 1. In healthy contacts, the percent γ/δ T cells in blood was significantly higher than that in healthy noncontacts or that in patients with tuberculosis ($P < 0.001$). No difference in the percent γ/δ T cells between healthy noncontacts and patients with tuberculosis was observed (Fig. 1A). On average, γ/δ T cells in blood in healthy contacts (n = 17), in healthy noncontacts (n = 18), and in patients with tuberculosis (n = 19) made up 10.4% ± 4.4%, 4.7% ± 2.2%, and 5.3% ± 4.5% of the CD3$^+$ cells, respectively, and α/β T cells in blood made up 90.2% ± 1.1%, 95.4% ± 0.5%, and 94.7% ± 1.0% of the CD3$^+$ cells, respectively (all values are means ± SEM). The percent HLA-DR-bearing activated cells among total γ/δ T cells in healthy contacts was also higher than that in healthy noncontacts or patients with tuberculosis (Fig. 1B; $P < 0.05$).

Expansion of numbers of γ/δ T cells in vitro in response to PPD. To determine whether blood γ/δ T cells respond to mycobacterial organisms, PBMC from each subject were cultured in vitro with PPD. No exogenous interleukin 2 (IL-2) was added in culture. The extent of proliferation was assessed by measuring the radioactivity of [^{3}H]thymidine incorporated into the cultured cells. Cultures were carried out for 6 days in the presence of 10 μg of PPD per ml. PBMC from all groups proliferated in vitro to similar extents in response to PPD. There was no difference in the extent of [^{3}H]thymidine incorporation among three groups (Fig. 2A). The stimulation indexes for healthy contacts, healthy noncontacts, and patients with tuberculosis were 144.0 ± 18.8, 166.8 ± 30.3, and 135.7 ± 23.3, respectively (means ± SEM). Cultured cells were stained with FITC-conjugated anti-TCR δ1 MAb and PE-conjugated anti-CD3 MAb, and the percent γ/δ T cells among CD3$^+$ cells was determined. Healthy contacts had preferential expansion of γ/δ T-cell numbers after in vitro stimulation with PPD, whereas healthy noncontacts and patients with tuberculosis did not (Fig. 2B). The mean percent γ/δ T cells in healthy contacts after in vitro stimulation with PPD was also greater than the percent in healthy noncontacts or that in patients with tuberculosis ($P < 0.001$). After in vitro stimulation with PPD, γ/δ T cells in healthy contacts (n = 17), in healthy noncontacts (n = 18), and in patients with tuberculosis (n = 19) made up 20.3% ± 4.4% (mean ± SEM; range, 2.6% to 65.8%), 5.0% ± 1.0% (mean ± SEM; range, 0.6% to 17.5%), and 3.3% ± 0.6% (mean ± SEM; range, 0.5% to 9.9%) of the CD3$^+$ cells, respectively. In healthy contacts, the percent γ/δ T cells increased significantly in response to PPD compared with the percent among fresh PBMC (from 10.4% ± 4.4% to 20.3% ± 4.4%; $P < 0.05$). In patients with tuberculosis, on the other hand, the percent γ/δ T cells after in vitro stimulation with PPD was significantly lower than the percent in fresh PBMC (a decrease from 5.3% ± 4.5% to 3.3% ± 0.6%; $P < 0.01$). No significant change was observed in healthy noncontacts before and after PPD stimulation (an increase from 4.7% ± 2.2% to 5.0% ± 1.0%). The increase in the percent γ/δ T cells in healthy contacts after in vitro stimulation with PPD was due to an increase in the absolute number of γ/δ T cells and not to a decreased number of non-γ/δ T cells. For example, the increases in numbers of γ/δ T cells in three healthy contacts were 45-fold (from 4.4×10^4 to 200.0×10^4), 32-fold (from 2.1×10^4 to 66.3×10^4), and 3-fold (from 8×10^4 to 20×10^4) after in vitro stimulation with PPD. Stimulation with concanavalin A or phytohemagglutinin did not induce a preferential expansion of γ/δ T-cell populations in vitro, although concanavalin A or phytohemagglutinin stimulated PBMC to proliferate to an extent similar to that of the proliferation stimulated by PPD (Table 2).

Effect of proteolytic digestion of PPD on stimulation of γ/δ T cells. As shown in Table 3, after the treatment with proteinases, PPD lost the capacity to activate γ/δ T cells in healthy contacts, indicating that γ/δ T cells increased in number in response to peptide components but not to nonpeptide components of PPD. The impaired response of PBMC to PPD after proteinase treatment was not due to the digestion with residual enzyme of the surface receptors of T cells and/or antigen-presenting cells, because a control experiment showed that PBMC incubated in the presence of both treated and untreated PPD responded to the same extent as PBMC incubated with untreated PPD alone.

Phenotypic analysis of γ/δ T cells which expanded in number in vitro in response to PPD. As shown in Fig. 3, flow cytometric analysis with MAbs against Vδ1 (δTCS 1), Vγ2 (TiγA), CD4 (Leu-3a), and CD8 (Leu-2a) revealed an exclusive expansion of the numbers of Vγ2-bearing T cells, as had been reported previously (14). Surprisingly, γ/δ T cells expressing CD4 antigen on their surface formed 22.8% (n = 8) of the total γ/δ T cells in healthy contacts after in vitro stimulation

TABLE 2. Proliferation and percent γ/δ T cells after in vitro stimulation with concanavalin A, phytohemagglutinin, or PPD

Stimulator (concn)	Culture period (days)	Expt 1		Expt 2	
		Amt of [^{3}H]thymidine incorporation (10^3 cpm ± SEM)	γ/δ T cells/ $CD3^+$ cells (%)	Amt of [^{3}H]thymidine incorporation (10^3 cpm ± SEM)	γ/δ T cells/ $CD3^+$ cells (%)
Medium	3	0.2 ± 0.0	3.1	0.5 ± 0.1	1.8
	6	0.3 ± 0.0	4.7	0.6 ± 0.1	1.8
Concanavalin A (10 μg/ml)	3	19.0 ± 0.6	4.4	23.1 ± 0.5	1.9
Phytohemagglutinin (1 μg/ml)	3	38.6 ± 2.6	5.7	63.1 ± 4.3	1.7
PPD (10 μg/ml)	6	29.0 ± 1.4	65.8	54.5 ± 3.8	15.9

with PPD (Table 4). In particular, $CD4^+$ γ/δ T cells made up 38.0% ± 4.3% of the γ/δ T cells in the blastoid form proliferating in response to PPD tuberculin. The mean proportion of $CD4^+$ cells among total γ/δ T cells in fresh PBMC was less than 2% for each of three groups examined. The γ/δ T cells expressing CD8 antigen (although weakly positive) were about 40% of the total γ/δ T cell population. No significant increase in the percent $CD8^+$ γ/δ T cells was observed after PPD stimulation. Although the proportion of $CD4^-$ $CD8^-$, double-negative γ/δ T cells was as high as 65% of the total γ/δ T cells in culture with medium alone, the proportion of double-negative γ/δ T cells was about 40% after in vitro stimulation with PPD, indicating that the γ/δ T cells proliferating in response to PPD were not double-negative γ/δ T cells. Results of a representative two-color flow cytometric analysis of the γ/δ T cells proliferating after in vitro stimulation with PPD are shown in Fig. 4. PBMC were obtained from a healthy contact.

Requirement for adherent cells in PPD-stimulated expansion of numbers of γ/δ T cells. To see whether antigen-presenting cells are required for the expansion of γ/δ T-cell populations in response to PPD, we carried out adherent-cell depletion–cell-mixing experiments with T cells. Nonadherent, nylon-wool column-passed peripheral blood T cells were cultured in the presence of autologous adherent cells (3,500-rad-irradiated) and stimulated with PPD. A representative result from three independent experiments is shown in Table 5. Purified T cells alone did not respond to stimulation with PPD. When adherent cells were added to the culture of 10% of the T cells, a vigorous proliferative response was observed. The expansion of γ/δ T-cell numbers after in vitro stimulation with PPD was also restored by the addition of adherent cells. These results indicate that the adherent cells were required for the expansion of γ/δ T-cell numbers among PBMC in response to PPD.

Next, we evaluated whether PPD-induced expansion of γ/δ T-cell numbers, like the expansion of $CD4^+$ α/β T-cell numbers in response to a specific antigen, required an MHC class II antigen. Graded amounts of anti-HLA-DR MAbs were added to the cultures. As shown in Fig. 5, both the proliferative responses of PBMC and the expansion of γ/δ T cells in response to PPD stimulation were effectively inhibited by the addition of anti-HLA-DR MAbs in a dose-dependent manner. Control mouse Ig, IgG2B, had no effect on the blastogenesis and the expansion of γ/δ T cells in response to PPD stimulation. The viability of cells cultured with anti-HLA-DR MAb was the same as that of the cells treated with control mouse Ig. An independent experiment showed that anti-MHC class I MAb caused no significant suppression of either the blastogenesis or the expression of γ/δ T cells in response to PPD stimulation (data not shown). These results indicate that the expansion of γ/δ T cells after in vitro stimulation of PBMC with PPD required an MHC class II antigen.

TABLE 3. Effect of protease treatment of PPD on expansion of γ/δ T-cell numbers[a]

Stimulator (concn)	Amt of [^{3}H]thymidine incorporation (10^3 cpm ± SEM)	γ/δ T cells/ $CD3^+$ cells (%)
Medium	0.2 ± 0.0	3.1
PPD (10 μg/ml)	27.8 ± 1.4	48.3
Protease-treated PPD	0.8 ± 0.2	7.3
Protease-treated PPD + untreated PPD (10 μg/ml)	23.4 ± 1.5	44.9

[a] A representative result from three independent experiments is shown.

DISCUSSION

Here we demonstrated that in the case of healthy individuals who were frequently exposed to mycobacterial organisms (healthy contacts), the percentage of freshly isolated peripheral blood γ/δ T cells was significantly higher than the percentage for healthy noncontacts or patients with tuberculosis. The in vitro study indicated that the percent γ/δ T cells in healthy contacts increased further in response to PPD in the presence of adherent cells. The major subset of γ/δ T cells whose numbers were expanded in vitro in response to PPD stimulation were $CD4^+$ $V\gamma2^+$ but not $CD4^-$ $CD8^-$ double-negative γ/δ T cells. In contrast, peripheral blood of patients with tuberculosis and healthy noncontacts contained a smaller number of γ/δ T cells than that of healthy contacts. PBMC from these three groups, however, proliferated vigorously and equally after in vitro stimulation with PPD, suggesting that in patients with tuberculosis and healthy noncontacts, α/β T cells were the major T cells responding to PPD stimulation, whereas in healthy contacts γ/δ T cells were also proliferating in addition to α/β T cells. We were not able to evaluate the γ/δ T cells in subjects who were tuberculin skin test negative, because of the high level of coverage of BCG vaccination in Japan. Kabelitz et al. (15) reported the unresponsiveness of γ/δ T cells to stimulation with PPD without exogenous addition of recombinant IL-2 in normal subjects who reacted negatively to a tuberculin skin test. While most γ/δ T cells lack both CD4 and CD8, that is, they are doubly negative, a few γ/δ T cells do express either CD4 or CD8 (18). Analysis of human γ/δ T-cell clones indicated that $CD4^+$ γ/δ T cells resemble $CD4^+$ α/β T cells in that activated cells produce lymphokines at high levels but little cytolytic activity (21, 32). Lymphokines produced by $CD4^+$ γ/δ T cells included gamma interferon, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor alpha. These lymphokines may activate monocytes to eliminate phagocytosed mycobacteria.

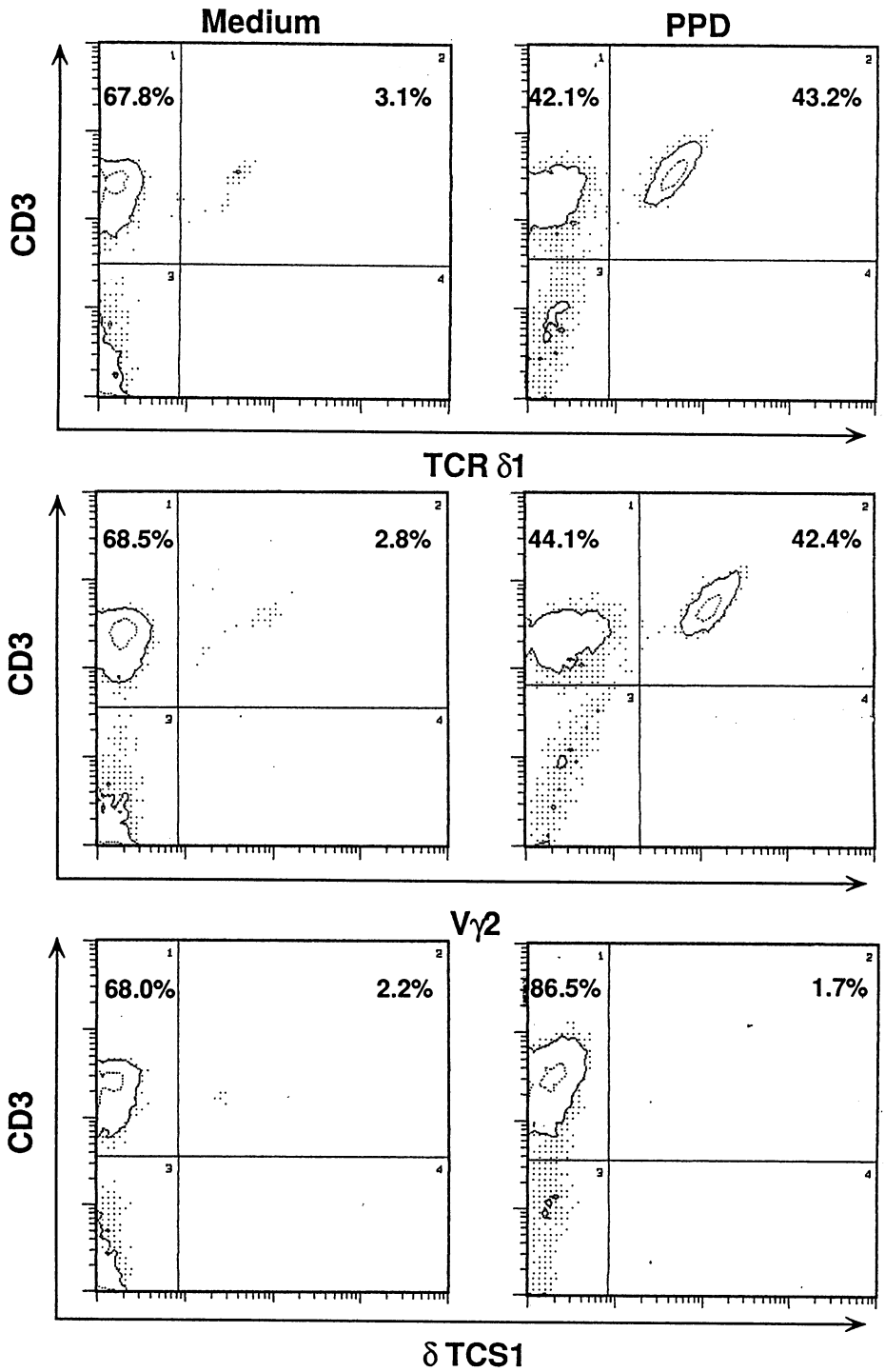

FIG. 3. Two-color fluorescence-activated cell sorter analysis of T-cell subsets after in vitro stimulation with PPD. The TCR phenotype of PBMC after in vitro stimulation with PPD was analyzed. After in vitro stimulation with PPD, PBMC were stained with PE-conjugated anti-CD3 MAb and with FITC-conjugated anti-TCR δ1 MAb, FITC-conjugated anti-Vγ2 MAb, or FITC-conjugated anti-δTCS1 MAb.

We showed that the numbers of γ/δ T cells in the peripheral blood of healthy contacts who had constant contact with sputum-smear-positive patients with tuberculosis were increased. Frequent exposure to the mycobacterial organisms might have induced the expansion of the γ/δ T-cell populations in vivo in the healthy contacts. The expanded γ/δ T-cell populations may contribute to the immune resistance against tubercle bacillus invasion. The question, then, is why this is not the case in active tuberculosis. In our study, no significant increase of the percent γ/δ T cells was observed in PBMC from patients with active tuberculosis after in vitro stimulation with mycobacterial antigen, as had been reported previously (2).

TABLE 4. Expression of CD4, CD8, Vδ1-Jδ1, and Vγ2 on the surfaces of γ/δ T cells after in vitro stimulation with PPD[a]

Stimulator (concn)	% of CD3$^+$ cells with the following phenotype:			% of γ/δ T cells with the following phenotype:	
	TCR δ1$^+$	δ TCS1$^+$	Vγ2$^+$	CD4$^+$	CD8$^+$
Medium	7.4 ± 0.8	2.2 ± 1.0	5.4 ± 1.0	4.8 ± 0.7	30.0 ± 3.7
PPD (10 μg/ml)	29.7 ± 6.2	3.1 ± 1.3	23.7 ± 6.0[b]	22.8 ± 2.3[b]	36.3 ± 8.4

[a] Values are means ± SEM; cells were from eight healthy contacts.
[b] $P < 0.05$ compared with culture in medium.

Increased numbers of γ/δ T cells in response to mycobacterial organisms might be playing a role in preventing the host from developing tuberculosis following frequent mycobacterial infections. The absence of this increase of γ/δ T-cell numbers in response to the exposure to mycobacterial organisms might drive the individual into the disease, tuberculosis. Another plausible explanation for the absence of an increase of γ/δ T-cell numbers in peripheral blood of patients with tuberculosis is the sequestration of γ/δ T cells in a pathological tissue lesion. Falini et al. (7) reported an increased number of γ/δ T cells in a lesion of tuberculous lymphadenitis. γ/δ T cells have accumulated in granulomatous skin lesions of patients with leprosy (20). Alternatively, the γ/δ T cells do exist but might be in a tolerant form in the peripheral blood of patients with tuberculosis because of large amounts of mycobacterial antigens, including the self-stress protein of the host. Kawabe and Ochi (16) reported that in vivo priming with *Staphylococcus* enterotoxin B (SEB) induced extrathymic tolerance in Vβ8$^+$ CD4$^+$ T cells. They also reported that this tolerance can be achieved by both deletion and functional inactivation of antigen-reactive T cells (17). The human γ/δ T cells that respond to mycobacteria use Vγ2 and Vδ2 chains, both of which exhibit considerable junctional diversity, suggesting that mycobacteria may contain a superantigen for human Vγ2$^+$ T cells. The γ/δ T cells in patients with tuberculosis may be made tolerant in a fashion reminiscent of that in which SEB-induced extrathymic tolerance was produced.

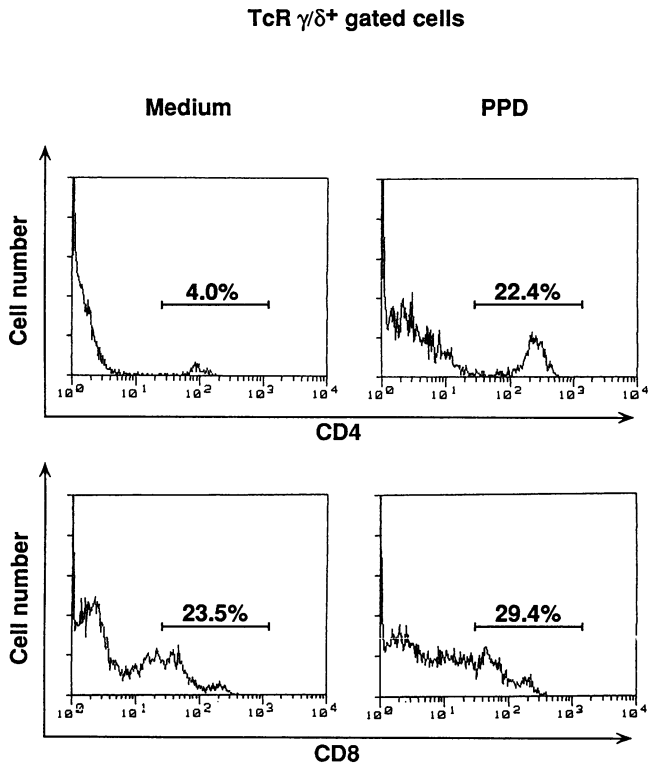

FIG. 4. Histograms of CD4- or CD8-positive cells among total γ/δ T cells after in vitro stimulation with PPD. After in vitro stimulation with PPD, PBMC were stained with FITC-conjugated anti-TCR δ1 MAb and with PE-conjugated anti-CD4 MAb or PE-conjugated anti-CD8 MAb. Expression of CD4 or CD8 on the surfaces of γ/δ T cells after in vitro stimulation with PPD tuberculin was analyzed. The histograms illustrate the levels of CD4 and CD8 on the surfaces of TCR δ1 gated cells.

To define the contribution of γ/δ T cells toward protection against mycobacterial infection, it will be important to characterize the antigens recognized by γ/δ T cells. γ/δ T cells proliferate in response to a crude lysate of mycobacteria regardless of prior exposure to mycobacterial organisms (15, 24, 34). γ/δ T-cell clones specific to mycobacterial heat shock protein (hsp65) have been reported. Most mycobacterial antigen-reactive γ/δ T cells, however, did not react to hsp65 (15). Pfeffer et al. (26, 27) reported that a major component of mycobacteria which stimulates γ/δ T cells was a lectin-binding, protease-resistant mycobacterial ligand but not protein antigens of mycobacteria, including hsp65. Recently, Constant et al. (6) described a human γ/δ T-cell clone responding to a protease-resistant, phosphorylated thymidine-containing compound of mycobacterial extracts. Our results, however, indicate that CD4$^+$ γ/δ T cells may respond specifically to a protein antigen (protease-sensitive component) of PPD. Most CD4$^-$ CD8$^-$ double-negative γ/δ T cells may, on the other hand, proliferate in response to protease-resistant mycobacterial ligands. A significant increase in numbers of double-negative γ/δ T cells was observed when cord blood lymphocytes from newborn babies in which no PPD-reactive T cells seemed to exist were cultured in vitro with protease-resistant mycobacterial lysate (34).

It remains unclear whether TCR γ/δ recognizes mycobacterial antigen in the context of MHC antigen (4, 12, 23). In our study, we demonstrated that the PPD-induced expansion of γ/δ T cells required adherent cells and that the response was

TABLE 5. Requirement of adherent cells for expansion of γ/δ T-cell numbers in response to PPD

Type(s) of cells cultured	Amt of [^{3}H]TdR incorporation (10^3 cpm ± SEM)[a]		γ/δ T cells/CD3$^+$ cells (%)	
	Medium[b]	PPD[c]	Medium[b]	PPD[c]
PBMC	0.2 ± 0.0	13.7 ± 1.3	3.4	38.5
T cells	0.1 ± 0.0	0.3 ± 0.1	0.9	2.2
T cells + adherent cells[d]	0.9 ± 0.1	13.0 ± 0.9	2.5	24.7

[a] TdR, thymidine.
[b] Cells in culture medium alone.
[c] Cells in culture medium with added PPD.
[d] Adherent cells were previously irradiated with 3,500 rads.

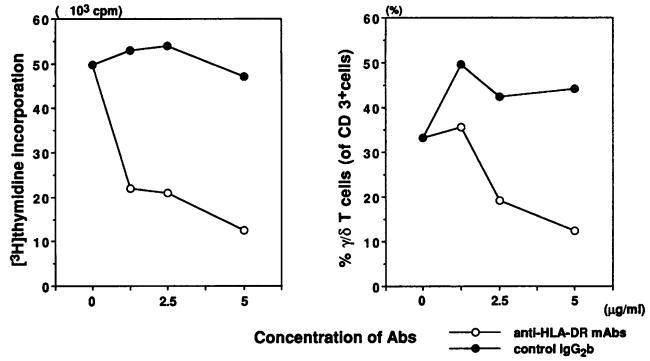

FIG. 5. Effect of anti-HLA-DR MAb on the PPD-induced expansion of γ/δ T-cell numbers in healthy contacts. A representative result from three independent experiments is shown. PBMC were cultured with PPD in the presence of a diluted amount of MAb against HLA-DR antigen throughout the culture period. As the control of MAb, normal mouse IgG2B was used. (A) The extent of proliferation was assessed by measuring the radioactivity of [^{3}H]thymidine incorporated into the cultured cells. (B) PBMC were stained with FITC-conjugated anti-TCR δ1 MAb and PE-conjugated anti-CD3 MAb.

inhibited by the addition of anti-HLA-DR MAbs. These results indicate that CD4$^+$ Vγ2$^+$ γ/δ T cells may recognize mycobacterial antigen in the context of MHC class II. Activation of CD4$^+$ Vγ2$^+$ γ/δ T cells and CD4$^+$ α/β T cells may both be driven by interaction between CD4 and MHC class II molecules. An alternative explanation is that the expansion of γ/δ T-cell numbers was due to the growth factor produced by the PPD-induced activated CD4$^+$ α/β T cells coexisting in the culture. Activation of CD4$^+$ α/β T cells by PPD tuberculin requires MHC class II antigen. Activated CD4$^+$ α/β T cells produce IL-2, which then may stimulate γ/δ T cells to proliferate. Recently, Pechhold et al. (25) showed that helper factors delivered by CD4$^+$ α/β T cells were required for the primary in vitro response of γ/δ T cells to *M. tuberculosis*. Our preliminary data also show that γ/δ T cells among PBMC expanded in number in vitro in response to recombinant human IL-2. However, most of the γ/δ T cells thus proliferating after IL-2 stimulation were CD4$^-$ CD8$^-$ cells and not CD4$^+$ γ/δ T cells. We are now trying to establish CD4$^+$ γ/δ T-cell clones that respond to mycobacteria to further examine the MHC class II restriction in the PPD response of human CD4$^+$ γ/δ T cells.

Our present study, when taken together with others, suggests that γ/δ T cells, CD4-positive γ/δ T cells in particular, may play an important role in the defense against the invasion of tubercle bacilli, irrespective of primary infection or exogenous reinfection. In this context, it seems very important for the development of a potent antituberculosis vaccine to search out the most relevant epitope of the mycobacterial organism that stimulates γ/δ T cells in healthy subjects. This issue is currently under investigation.

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