INFECTION AND IMMUNITY, Apr. 1995, p. 1211–1217 Vol. 63, No. 4
0019-9567/95/$04.00+0
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# Expansion of Mycobacterium-Reactive γδ T Cells by a Subset of Memory Helper T Cells

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Received 5 October 1994/Returned for modification 18 November 1994/Accepted 4 January 1995

**Human γδ T cells expressing the Vγ9/Vδ2 T-cell receptor have been previously found to proliferate in response to certain microorganisms and to expand throughout life, presumably because of extrathymic activation by foreign antigens. In vitro expansion of Vγ9/Vδ2 cells by mycobacteria has been previously shown to be dependent on accessory cells. In order to gain an insight into the mechanisms involved in the expansion of these cells, we have undertaken to identify the peripheral blood subset of cells on which proliferation of Vγ9/Vδ2 cells in response to mycobacteria is dependent. Contrary to their role in antigen presentation to αβ T cells, professional antigen-presenting cells, such as monocytes, B cells, and dendritic cells, were unable to provide the cellular support for the expansion of Vγ9/Vδ2 cells. Selective depletion of T-cell subsets, as well as the use of highly purified T-cell populations, indicated that the only subset of peripheral blood cells that could expand Vγ9/Vδ2 cells were $CD4^+$ $CD45RO^+$ $CD7^-$ αβ T cells. These cells underwent distinct intracellular signaling events after stimulation with the mycobacterial antigen. Expansion of Vγ9/Vδ2 cells by αβ T cells was dependent on cell-cell contact. This is the first evidence that a small subset of the memory helper T-cell population is exclusively responsible for the peripheral expansion of Vγ9/Vδ2 cells. These data illustrate a unique aspect of antigen recognition by γδ T cells and provide new means to study their immune defense role.**

The role of γδ T cells in immune defense against pathogens is incompletely understood. A growing body of evidence indicates that these cells expand in vivo in response to certain bacteria, viruses, and parasites (3, 6, 8, 18, 22, 30, 31, 38). The reactivity of γδ T cells to mycobacteria, in particular, has attracted considerable attention. Proliferation of human peripheral blood γδ T cells in response to mycobacteria defines a subset of cells expressing a T-cell receptor (TCR) with a uniform set of V genes, namely Vγ9 and Vδ2. In vitro exposure of these cells to mycobacteria in the presence of accessory cells results in the expansion of the entire Vγ9/Vδ2 subset, regardless of γ and δ chain V-(D)-J junctional sequences (14, 23). This nonclonal activation superficially resembles the reactivity of αβ T cells to bacterial superantigens. Recent data indicate, however, that the two reactivities differ in two aspects. (i) Reactivity of Vγ9/Vδ2 cells to mycobacteria is independent of the major histocompatibility complex (11). (ii) Different from those of the bacterial superantigens, which are 20- to 30-kDa polypeptides, the chemical structure of the mycobacterial component which activates the Vγ9/Vδ2 subset is consistent with a low-molecular-mass phosphorylated, nonprotein moiety (5).

One of the intriguing aspects of the biology of Vγ9/Vδ2 cells is their in vivo expansion throughout life (24). These cells, which constitute a small minority of $CD3^+$ cells at birth, gradually expand to become the major subset in adults, accounting for 70 to 90% of all peripheral γδ T cells. The expression of activation markers by peripheral Vγ9/Vδ2 cells and the absence of parallel postnatal intrathymic expansion suggest that the peripheral expansion of this subset of γδ T cells is the result of extrathymic activation by foreign antigens (24).

To gain a better insight into the mechanisms underlying peripheral expansion of Vγ9/Vδ2 cells, we have undertaken to determine the cellular requirements for their proliferation in the peripheral blood. Here we report that proliferation of Vγ9/Vδ2 cells in response to mycobacteria is dependent on accessory αβ T cells bearing the phenotype $CD4^+$ $CD45RO^+$ $CD7^-$, a recently described subpopulation of normal human peripheral blood T lymphocytes.

## MATERIALS AND METHODS

**Antigens and antibodies.** The acetone-precipitable fraction of *Mycobacterium tuberculosis* (AP-MT) was prepared as described previously (9) from strain $H_{37}$ Ra, purchased from Difco Laboratories, Inc., Detroit, Mich. AP-MT constitutes 20% of the dry weight of the bacterium. Recent studies (5) have characterized the active mycobacterial ingredient which is responsible for the expansion of γδ T cells. This moiety appears to be a small (500 to 600 Da) nonpeptidic 5′-triphosphorylated thymidine-containing compound. Tetanus toxoid (TT) was purchased from Massachusetts Department of Public Health, Jamaica Plains, Mass. Staphylococcal enterotoxin A was purchased from Toxin Technology, Sarasota, Fla.

The following purified monoclonal antibodies (MAbs) were used. OKT4 (anti-CD4), OKT8 (anti-CD8), and OKT11 (anti-CD2) were obtained from Ortho Pharmaceuticals, Raritan, N.J. Anti-Leu-4 (anti-CD3), anti-TCR-γδ-1, anti-TCR-1 (WT31), anti-Leu-9 (anti-CD7), anti-Leu-16 (anti-CD19, a B-cell marker), and anti-Leu-18 (anti-CD45RA, a marker of naive T cells) were purchased from Becton Dickinson, Mountain View, Calif. UCHL-1 (anti-CD45RO, a marker of memory T cells) was obtained from Dako A/S, Carpinteria, Calif., and TCRδ1 (pan-δ chain antibody) was purchased from T Cell Sciences (Cambridge, Mass.). MAbs MY4 (anti-CD14, a monocyte/macrophage marker) and B1 (anti-CD20, a B-cell marker) were purchased from Coulter, Hialeah, Fla. MAb 3A1 (anti-CD7) was purchased from Sigma, Saint Louis, Mo. The OKT3 hybridoma was purchased from the American Type Culture Collection and used in ascites form. Anti-Mo1 (anti-CD11b, an integrin subunit expressed on monocytes) was kindly provided in ascites form by K. Cooper, University of Michigan.

**T-cell clones.** Isolation and characterization of the $CD4^-$ $CD8^-$ Vγ9/Vδ2 T-cell clones 1.2 and 1.4 from the synovial fluid of a patient with rheumatoid arthritis (10) and BC1.54 and BC1.62 from peripheral blood of a healthy indi-

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vidual (12) have been previously described. The TT-specific $CD4^+$ αβ T-cell clone TT44G was isolated from peripheral blood of a healthy individual (29) and was kindly provided by B. Richardson, University of Michigan.

**Cell depletion.** Depletion of cell populations from peripheral blood mononuclear cells (PBMCs) was performed by immunomagnetic separation. PBMCs were isolated from heparinized peripheral blood of healthy individuals by gradient centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). PBMCs ($20 \times 10^6$) were incubated with one of the following MAbs: 1:40 anti-Mo1 for monocyte depletion, 2.5 μg of anti-Leu-16 per ml for B-cell depletion, 2.5 μg of WT31 per ml for αβ T-cell depletion, 2.5 μg of anti-TCR-γδ-1 per ml for γδ T-cell depletion, 10 μg of OKT4 per ml for CD4 cell depletion, 10 μg of OKT8 per ml for CD8 cell depletion, or 20 μg of OKT11 per ml for CD2 cell depletion. The cells and MAbs were incubated at 4°C for 45 min and mixed gently by a rotator wheel. At the end of the incubation, cells were washed three times with supplemented modified Eagle's medium and resuspended in 1 ml of supplemented modified Eagle's medium containing $10^8$ Dynabeads M-450 (Dynal, Oslo, Norway). The mixture was incubated at 4°C for 45 min and mixed by a rotator wheel. Cell rosettes and beads were separated from the nonrosetted cells by application of a magnet on the side of the culture tube.

**Purified cell populations.** Monocytes were isolated from PBMCs by gradient centrifugation on Nycodenz-Monocytes (Nyegaard, Oslo, Norway). PBMCs ([20 to 100] $\times 10^6$) were suspended in 3 ml of RPMI 1640 containing 0.25% EDTA and 10% human serum and incubated at room temperature for 40 min. Cell suspensions were layered onto 3 ml of Nycodenz-Monocytes and centrifuged at $650 \times g$ for 15 min. The low-density cells in the interface were collected and washed twice in supplemented RPMI 1640 containing 10% fetal calf serum. The cells obtained were greater than 95% Mo1 positive.

B cells were positively selected from peripheral blood by immunomagnetic selection as described previously (7) with Dynabeads coated with anti-CD19 MAb AB1 (Dynal), which binds to a functionally inert epitope of CD19. B cells separated by this technique have been previously found to maintain their proliferative response to various mitogens (17). Briefly, $50 \times 10^6$ PBMCs were incubated at 4°C for 45 min with $10^7$ Dynabeads M-450 Pan-B (Dynal). Rosetted cells were washed six times with supplemented modified Eagle's medium and thereafter resuspended in 1 ml of RPMI 1640 containing 1% fetal calf serum. Cells were incubated overnight at 37°C in a 5% $CO_2$ incubator. After incubation, most of the beads detached from the cells and bead-free cells were collected as described above. The cells obtained were greater than 95% B cells.

Dendritic cells were separated from PBMCs as described previously (2) by gradient centrifugation of transiently adherent cells on Nycodenz-Monocytes. The cells obtained were greater than 80% dendritic cells as determined by cell morphology.

To avoid potential receptor-mediated in vitro activation or inhibition of cells, purified $CD4^+$ and $CD8^+$ T cells, $CD4^+$ $CD45RA^+$ T cells, and $CD4^+$ $CD45RO^+$ T cells were obtained by negative selection with immunomagnetic separation techniques. $CD4^+$ T-cell purification was achieved by incubating PBMCs with a mixture of anti-Mo1, anti-Leu-16, anti-TCR-γδ-1, and OKT8. The cells obtained were over 98% $CD4^+$ αβ TCR T cells. $CD8^+$ T cells were isolated by incubating the PBMCs with anti-Mo1, anti-Leu-16, anti-TCR-γδ-1, and OKT4. The cells obtained were over 90% $CD8^+$ αβ T cells. Isolation of $CD4^+$ $CD45RA^+$ and $CD4^+$ $CD45RO^+$ T cells was achieved by incubation of PBMCs with a mixture of MAbs, as described above for $CD4^+$ T-cell separation, and with either 30 μg of UCHL-1 or 10 μg of anti-Leu-18, respectively, per ml. The purity of the isolated cells was over 98%.

In some experiments, $CD45RA^+$ T cells and $CD45RO^+$ T cells were negatively selected by the MACS immunomagnetic method (17). PBMCs were first subjected to E rosetting by a standard technique with sheep erythrocytes purchased from Colorado Serum Company, Denver, Colo. $CD14^+$ and $CD20^+$ cells were further depleted from the $E^+$ population by incubating them with a combination of MY4 and B1 antibodies, both at 1 μg/$10^6$ cells on ice for 15 min. The cells were then washed three times in MACS buffer (phosphate-buffered saline with 1% bovine serum albumin and 5 mM EDTA) and resuspended in MACS buffer containing 20 μl of goat anti-mouse immunoglobulin G microbeads per $10^7$ cells (Miltenyi Biotech, Inc., Sunnyvale, Calif.) and incubated on ice for 15 min. The cells were again washed three times, resuspended in MACS buffer, and applied to an iron wool column against a high-power magnet. The flowthrough (nonadherent) cells were collected. This method yielded a purity of over 99% $CD3^+$ cells. $CD3^+$ cells were then depleted of $CD45RO^+$ or $CD45RA^+$ cells by incubation in either of the anti-CD45RO (UCHL1) or anti-CD45RA (Leu-18) MAbs, respectively. This was followed by the addition of goat anti-mouse microbeads as described above. The cells were applied to fresh MACS columns and the flowthrough cells were collected, yielding a purity of 99% $CD45RA^+$ T cells and over 97% $CD45RO^+$ T cells.

To obtain purified $CD7^+$ and $CD7^-$ T cells, a two-stage MACS separation technique was used. First, $CD3^+$ cells were isolated from PBMCs on the MACS column as described above with anti-CD3 antibodies (anti-Leu-4, 1 μg per $10^6$ cells). The isolated $CD3^+$ cells were kept at 4°C overnight to allow detachment of the antibodies. This population was over 99% $CD3^+$. At the second stage, $CD7^+$ T cells were isolated by MACS with anti-Leu-9 or 3A1 MAbs. Purification of $CD7^-$ T cells was obtained by negative separation with anti-CD7 antibody-coated Dynabeads (Dynal, Great Lake, N.Y.). Contamination of both $CD7^+$ cells in the $CD7^-$ preparation and $CD7^-$ cells in the $CD7^+$ preparation was less than 3%.

**Proliferation assays.** Proliferative responses were determined as described previously (10, 12) by incubation of $2 \times 10^4$ cloned T cells with irradiated (3,000 R) mononuclear cells per well in 96 microtiter plates. The number of accessory cells per well in each experiment was adjusted to match the calculated number of cells of that particular subset per $10^5$ PBMCs. Cultures were grown in triplicate for 72 h in the presence or absence of 20 μg of AP-MT per ml in supplemented RPMI 1640 containing 10% human serum. [$^3$H]thymidine (1 μCi per well) was added 18 h before harvesting. Results are expressed as mean counts per minute ± standard deviation.

In some experiments, Transwell 24-well plates (Costar, Cambridge, Mass. [catalog no. 3413]) were used. Cloned γδ T cells ($2 \times 10^5$ per well) were put in the inner chamber and irradiated PBMCs ($10^6$ per well) or purified αβ T cells ($5 \times 10^5$ per well) were placed either in the outer chamber, separated from the γδ T cells by a semipermeable membrane, or in the inner chamber, together with the γδ T cells. Cultures were grown in triplicate in the presence or absence of AP-MT (10 μg/ml) in RPMI 1640 supplemented with 10% human serum. Cultures were pulsed with [$^3$H]thymidine (Dupont, NEN, Wilmington, Del.) at 1 μCi per well after 54 h, harvested 18 h later, and counted. These results are expressed as mean counts per minute ± standard deviation.

**$^{32}$P incorporation studies.** Purified T-cell populations were washed three times in phosphate-free modified RPMI 1640 containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and resuspended in phosphate-free medium containing 1% Nutridoma HU (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), 25 mM HEPES, 200 mM glutamine, and 1% penicillin-streptomycin. $^{32}P_i$ (Amersham, Arlington Heights, Ill.) at 250 μCi/$5 \times 10^6$ cells, was added for 2 h prior to antigen stimulation. Cells were stimulated with AP-MT (25 μg/ml) or OKT3 (as ascites at 1:100 dilution) over a time course, lysed after 1, 5, 10, and 15 min of antigen stimulation, and resolved by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) as described below.

**2-D PAGE.** Cell pellets were lysed in 40 μl of a detergent lysate containing 8 M urea, 2% (vol/vol) Nonidet P-40 surfactant (Hoefer Scientific Instruments, San Francisco, Calif.), 20 mM ampholytes (pH 3.5 to 10) (Pharmacia, Piscataway, N.J.), 2% (vol/vol) 2-mercaptoethanol, 50 mM NaF, and 0.1 mM sodium vanadate (Sigma) in distilled deionized water. 2-D PAGE was performed as previously described (36). Twenty-five-microliter aliquots containing approximately 70 μg of protein were applied to isofocusing gels. First-dimension gels contained 50 ml of ampholytes per liter (pH 3.5 to 10). Isofocusing was performed at 1,200 V for 16 h and 1,500 V for the last 2 h. For 2-D separation, an acrylamide gradient of 11.4 to 14.0% was used. After the 2-D separation, the gels were fixed by incubation in 50% ethanol and 5% acetic acid for 2 h. The gels were washed for 15 min with 2% glycerol and 20% methanol and dried. The dried gels were then exposed to storage phosphorimaging plates (Molecular Dynamics, Sunnyvale, Calif.) for 48 to 72 h. Digitized images were obtained with a PhosphorImager system as described previously (13). Twenty-nine phosphoprotein spots were quantitated with ImageQuant software (Molecular Dynamics). Seven neighboring reference spots were used to adjust the phosphor signal for gel-to-gel variation. Results are expressed as fold change of phosphorylation (PhosphorImager signal per hour in the phosphorylated state of a spot divided by the signal obtained in its unphosphorylated state). Positive values greater than +2 represent significant hyperphosphorylation changes compared with baseline signals, and negative values smaller than −2 indicate significant dephosphorylation.

## RESULTS

Previous studies have shown that proliferation of Vγ9/Vδ2 cells in response to AP-MT is dependent on the presence of accessory cells (11). In an attempt to better define the cellular requirements for activation of Vγ9/Vδ2 cells by AP-MT, we have studied the role of distinct subpopulations of peripheral blood cells. Figures 1 to 4 and Table 1 show representative experiments with one of the following Vγ9/Vδ2 γδ T-cell clones: BC1.54, BC1.62, 1.4, or 1.2. Each of the experiments was reproduced at least three times.

The ability of professional antigen-presenting cells to expand Vγ9/Vδ2 cells was assessed by using purified populations of monocytes, B cells, and dendritic cells (Fig. 1). As can be seen, monocytes isolated by gradient centrifugation on Nycodenz (over 95% pure as determined by flow cytometry and by Wright-Giemsa staining) were capable of presenting the nominal antigen TT to an antigen-specific αβ T-cell clone, TT44G (Fig. 1B), but could not provide the cellular requirement for the proliferation of Vγ9/Vδ2 cells in response to AP-MT (Fig. 1A). Purified B cells could efficiently present staphylococcal enterotoxin A to peripheral blood αβ T cells (Fig. 1D) but

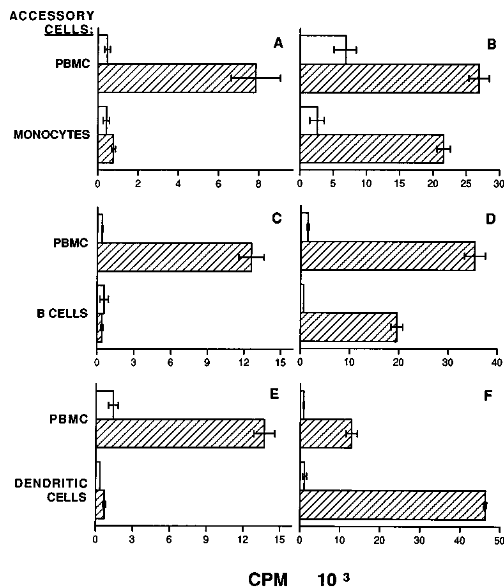



FIG. 1. Professional antigen-presenting cells do not support proliferation of Vγ9/Vδ2 cells. [$^3$H]thymidine incorporation assays with (hatched bars) or without (open bars) antigens. Proliferation of Vγ9/Vδ2 cells in response to AP-MT in the presence of purified PBMCs (A), B cells (C), and dendritic cells (E) is compared with proliferation of TT-specific αβ T-cell clone TT44G in response to TT in the presence of monocytes (B) or dendritic cells (F) and the proliferation of peripheral blood αβ T cells to staphylococcal enterotoxin A in the presence of peripheral blood B cells (D). In each experiment, the proliferative response in the presence of PBMCs is shown for comparison. Differences in the magnitude of the proliferation of αβ T cells (right panels) compared with γδ T cells (left panels) are expected and reflect the different proliferative capacities of the two T-cell subsets.

failed to support the proliferation of Vγ9/Vδ2 cells in response to AP-MT (Fig. 1C). Peripheral blood dendritic cells were also tested (Fig. 1E and F). These cells could efficiently present TT to αβ T cells but did not provide the stimulus for the expansion of the γδ T cells in response to AP-MT. Thus, competent professional antigen-presenting cells cannot provide the cellular support necessary for the proliferation of Vγ9/Vδ2 cells in response to the mycobacterial antigen. Collectively, these data indicate that the expansion of Vγ9/Vδ2 cells is independent of professional antigen-presenting cells.

T cells make up the largest subpopulation of PBMCs. In order to investigate the role of T cells in the activation of Vγ9/Vδ2 cells in response to AP-MT, we first depleted those cells and examined the ability of the remaining PBMC population to support the proliferation of the γδ T cells. Figure 2 shows that depletion of $CD4^+$ cells completely inhibited proliferation of Vγ9/Vδ2 T cells in response to AP-MT. Depletion of $CD8^+$ cells, B cells, or monocytes did not significantly reduce the proliferation.

CD4 is expressed primarily on helper T cells; however, monocytes can also express this molecule. In order to better identify the $CD4^+$ population whose depletion resulted in loss of AP-MT-induced proliferation of Vγ9/Vδ2 cells, we depleted $CD2^+$ cells. CD2 is expressed on the majority of T cells, but there is no CD2 expression on either monocytes or dendritic cells. As can be seen in Fig. 2A, depletion of $CD2^+$ cells resulted in a complete loss of the ability of PBMCs to support the proliferation of Vγ9/Vδ2 cells. These results strongly suggest that the $CD4^+$ T-cell population in the peripheral blood is playing a role in the mediation of AP-MT-induced proliferation of Vγ9/Vδ2 T cells.

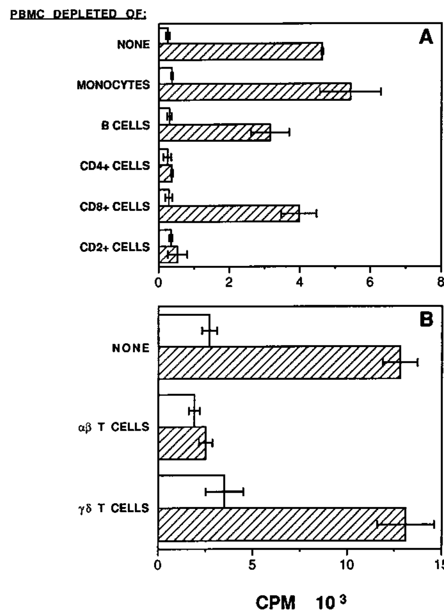



FIG. 2. Proliferation of Vγ9/Vδ2 cells in response to AP-MT in the presence of subset-depleted PBMCs. Subsets of cells were depleted from PBMCs as described in Materials and Methods, and the remaining cells were used in proliferation assays with Vγ9/Vδ2 cells in the presence (hatched bars) and absence (open bars) of AP-MT. A and B represent two different experiments.

The vast majority of peripheral $CD4^+$ T cells express αβ TCRs. However, CD4 can be expressed on rare γδ T cells. In order to exclude the possibility that peripheral blood $CD4^+$ γδ T cells are playing the accessory role for Vγ9/Vδ2 AP-MT-induced proliferation, we examined γδ T-cell-depleted versus αβ T-cell-depleted PBMCs. As Fig. 2B shows, depletion of the αβ T cells abolished the ability of PBMCs to support the proliferation of Vγ9/Vδ2 cells. Depletion of the γδ T cells had no measurable effect.

To directly address the role of $CD4^+$ T cells, highly purified $CD4^+$ and $CD8^+$ T-cell populations were prepared by negative separation techniques. PBMCs were first depleted of monocytes, γδ T cells, and B cells by immunomagnetic beads and were subsequently subjected to depletion of either $CD4^+$ or $CD8^+$ cells. As can be seen in Fig. 3, the $CD4^+$-enriched population (CD8 depleted) but not the $CD8^+$-enriched (CD4 depleted) population could support the proliferation of Vγ9/Vδ2 cells in response to AP-MT. Taken together, these results

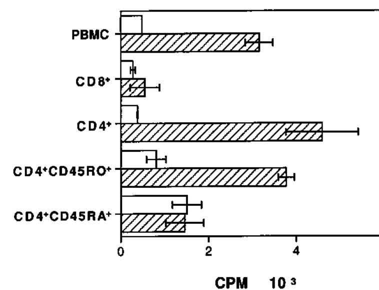



FIG. 3. Proliferation of Vγ9/Vδ2 cells in response to AP-MT in the presence of purified T cells. The ability of PBMCs and purified $CD4^+$ T cells, $CD8^+$ T cells, $CD4^+$ $CD45RO^+$ T cells, and $CD4^+$ $CD45RA^+$ T cells to function as accessory cells for Vγ9/Vδ2 cell proliferation was compared in the presence (hatched bars) and absence (open bars) of AP-MT.

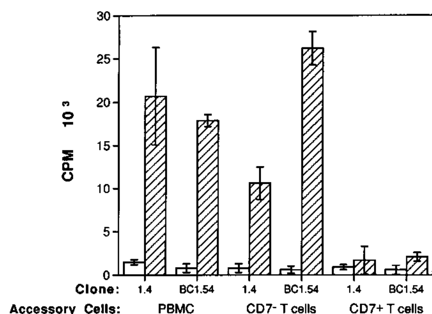



FIG. 4. Expansion of Vγ9/Vδ2 cells by CD7$^{-}$ T cells. Purified CD7$^{+}$ T cells and CD7$^{-}$ T cells were compared as accessory cells for the proliferation of Vγ9/Vδ2 cells in the presence (hatched bars) and absence (open bars) of AP-MT.

TABLE 1. Cell contact requirement for γδ T-cell expansion

| Accessory cells | Cell contact | Proliferative response (cpm [$10^3$] ± SD) to: No antigen | AP-MT (10 μg/ml) | Phytohemagglutinin (0.25 μg/ml) |
|---|---|---|---|---|
| Expt 1 | | | | |
| PBMCs | − | 0.2 ± 0.01 | 0.6 ± 0.1 | ND[a] |
| | + | 0.6 ± 0.01 | 3.9[b] ± 0.2 | ND |
| T cells | − | 0.1 ± 0.05 | 0.2 ± 0.01 | ND |
| | + | 1.0 ± 0.06 | 6.8[b] ± 0.2 | ND |
| Expt 2, PBMCs | − | 0.7 ± 0.1 | ND | 37.1[b] ± 8.7 |
| | + | 0.7 ± 0.01 | ND | 112.5[b] ± 0.6 |

[a] ND, not done.
[b] Positive proliferative response.

indicate that the subset of peripheral blood cells that can provide the cellular support for the expansion of Vγ9/Vδ2 T cells in response to mycobacterial antigens is a CD4$^{+}$ CD8$^{-}$ CD2$^{+}$ αβ TCR$^{+}$ T cell.

CD4$^{+}$ T cells can be divided into two functionally distinct subsets. CD4$^{+}$ T cells expressing the RO isoform of CD45 display memory cell functions, and CD4$^{+}$ T cells expressing the RA isoform of CD45 are considered naive cells. We carried out experiments to address the role of these two subsets in supporting the proliferation of γδ T-cell clones in response to AP-MT. We found that PBMCs depleted of CD45RA could support the proliferation of Vγ9/Vδ2 cells, whereas CD45RO-depleted PBMCs were unable to provide such support (not shown). To directly define the role of these two subsets, we purified CD4$^{+}$ CD45RA$^{+}$ T cells and CD4$^{+}$ CD45RO$^{+}$ T cells by negative immunomagnetic separation. Figure 3 shows that only the cells of the helper memory (CD4$^{+}$ CD45RO$^{+}$) subset but not the helper naive (CD4$^{+}$ CD45RA$^{+}$) cells were capable of supporting AP-MT-induced proliferation of the γδ T cells.

The majority of mature T cells express the CD7 glycoprotein. A small subset of CD7$^{-}$ T cells was recently identified as a distinct subset in the peripheral blood (28). Most of CD3$^{+}$ CD7$^{-}$ cells express the αβ TCR$^{+}$ CD4$^{+}$ CD45RO$^{+}$ phenotype. In order to examine the role of this subset in the expansion of γδ T cells, we tested highly purified populations of CD7$^{+}$ and CD7$^{-}$ T cells. Both populations were 99 to 100% pure T cells and contained less than 3% CD7$^{+}$ or CD7$^{-}$ cross-contamination. As can be seen in Fig. 4, only the CD7$^{-}$ T cells could support the proliferation of γδ T cells.

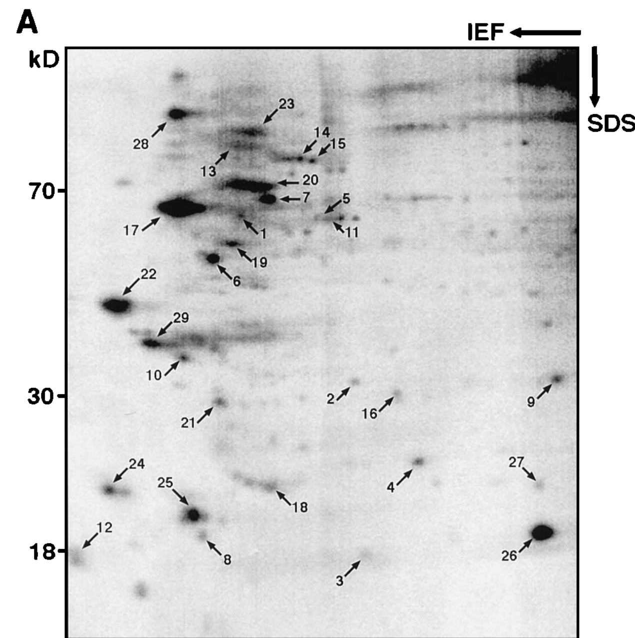
To examine the possibility that cytokines secreted by αβ T cells account for their ability to expand Vγ9/Vδ2 cells, we tested whether proliferation of the latter could be achieved in the absence of cell-cell contact with the Transwell tissue culture system. Cloned Vγ9/Vδ2 cells were placed in the inner chamber. Accessory cells were placed either in the outer chamber, separated from the γδ cells by a semipermeable membrane, or in the inner chamber, in close contact with the γδ cells. As Table 1 shows, proliferation of Vγ9/Vδ2 cells in response to AP-MT could be seen only when physical interaction between them and the accessory cells (either PBMCs or purified T cells) was allowed. In contrast, proliferation of the γδ T cells in response to phytohemagglutinin was not dependent on cell-cell contact, because Vγ9/Vδ2 cells showed proliferative responses even when they were separated from accessory cells (Table 1). Thus, the expansion of cloned Vγ9/Vδ2 cells by αβ T cells in response to AP-MT is uniquely dependent on cell-cell contact and could not be ascribed to a released cytokine.

To gain a better understanding about the mechanisms underlying the differential support that subpopulations of T cells can provide to Vγ9/Vδ2 cells, we compared intracellular signaling events in response to AP-MT in different subsets. Highly purified populations were incubated with AP-MT or OKT3 in the presence of $^{32}P_i$. Cellular proteins were separated by 2-D PAGE, and $^{32}P$ incorporation into individual protein spots was quantitated with a phosphorimaging technique. Figure 5 shows data from a representative experiment, one of three repetitions with CD45RA$^{+}$ and CD45RO$^{+}$ T cells. Consistent phosphorylation changes were noticed in 29 spots, shown in Fig. 5A for orientation. Figure 5B shows quantification of phosphorylation changes in each of the 29 spots in purified CD45RO$^{+}$ and CD45A$^{+}$ T cells stimulated with either AP-MT or with OKT3. As can be seen, stimulation of CD45RO$^{+}$ T cells with AP-MT resulted in substantial phosphorylation changes. Twenty-one of the 29 spots showed a greater than twofold change (hyperphosphorylation in 19 spots and dephosphorylation in 2 spots) upon stimulation with AP-MT, compared with only 7 spots in the CD45RA$^{+}$ subset. Only seven phosphoproteins (spots 4, 5, 7, 10, 11, 14, and 28) showed similar changes in the CD45RA$^{+}$ and CD45RO$^{+}$ subsets. Phosphorylation changes induced by AP-MT in CD7$^{-}$ T cells (Fig. 6) were remarkably similar to those seen in CD45RO$^{+}$ T cells (Fig. 5B). Thus, the subpopulation of CD45RO$^{+}$ T cells which supports the expansion of Vγ9/Vδ2 cells and shows distinct phosphorylation changes in response to AP-MT carries the CD7$^{-}$ phenotype.

The phosphorylation changes induced by AP-MT in CD45RO$^{+}$ T cells were distinct from those induced by the T-cell mitogen OKT3. As can be seen in Fig. 5B, only 8 of the 21 phosphoproteins which showed significant changes after stimulation with AP-MT showed the same changes upon stimulation of this subset with OKT3. Thus, the unique ability of CD45RO$^{+}$ T cells to support the expansion of Vγ9/Vδ2 cells is associated with distinct signaling events in these accessory cells.

## DISCUSSION

Reactivity of γδ T cells to mycobacteria has been previously shown to differ from the reactivity of αβ T cells to nominal antigens and superantigens, with respect to the role of major histocompatibility complex molecules and the structure of the stimulating antigen (5, 11). The present study provides evidence for another distinguishing feature. In this study, we have identified the accessory cells necessary for peripheral expansion of Vγ9/Vδ2 cells. Our results show that the αβ TCR$^{+}$ CD4$^{+}$ CD45RO$^{+}$ CD7$^{-}$ T-cell population is solely responsible



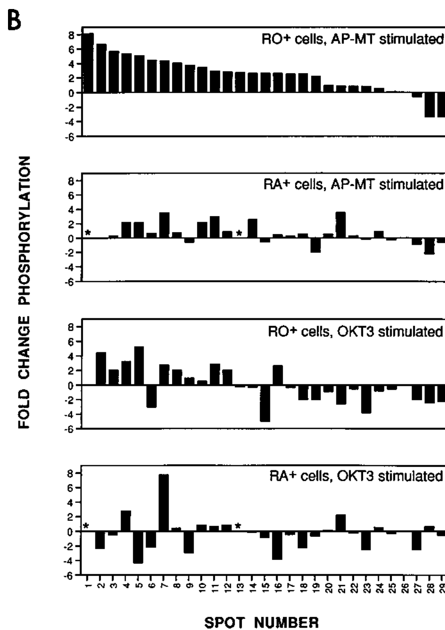




FIG. 5. 2-D PAGE of phosphoproteins. Purified CD45RO$^+$ T cells or CD45RA$^+$ T cells ($5 \times 10^6$) were stimulated with AP-MT or OKT3 and metabolically labeled as described in Materials and Methods. Phosphorylated polypeptides were separated by 2-D PAGE and visualized with a PhosphorImager. (A) For orientation, a 2-D gel with 29 quantifiable phosphoproteins is shown. Gels were run from pH 4.0 (left) to pH 7.0 (right). Approximate molecular masses are shown on the left. IEF, isoelectric focusing; SDS, sodium dodecyl sulfate-PAGE. (B) Quantitation of phosphorylation changes of individual spots in CD45RO$^+$ and CD45RA$^+$ T cells stimulated with AP-MT or OKT3. Phosphorylation changes are expressed as fold change in the phosphor signals for each spot upon stimulation, as described in Materials and Methods. Values greater than +2 represent significant phosphorylation, and values smaller than −2 represent significant dephosphorylation changes. Asterisks identify phosphoprotein spots that were absent in CD45RA$^+$ T cells.

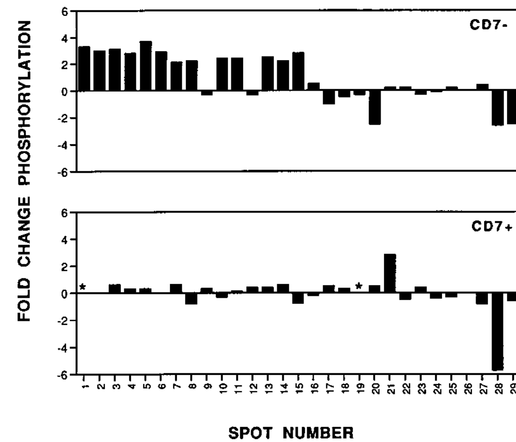



FIG. 6. AP-MT-induced phosphorylation changes in CD7$^-$ T cells. Purified CD7$^-$ T cells (top) and CD7$^+$ T cells (bottom) were stimulated with AP-MT over a time course, and phosphorylation changes were quantitated in 29 spots. Spot numbers correspond to those shown in Fig. 5A. Asterisks identify phosphoprotein spots that could be found only in CD7$^-$ T cells but not in CD7$^+$ T cells.

for this function. Purified professional antigen-presenting cells such as monocytes, dendritic cells, and B cells were unable to expand Vγ9/Vδ2 cells.

Our data are the first indication that highly purified T cells support the expansion of Vγ9/Vδ2 cells in the absence of professional antigen-presenting cells. Some of the T-cell preparations studied were 100% purified T cells, as determined by flow cytometry analysis. This fact excludes the possibility that non-T-cell contaminants have contributed to the accessory role. In addition, our previous data have shown that long-term T-cell leukemia lines can serve as accessory cells for the expansion of Vγ9/Vδ2 cells (11). The fact that pure T cells can support the proliferation of Vγ9/Vδ2 T cells in response to AP-MT is consistent with the observation that activation of γδ T cells does not require antigen processing (33).

In previous reports, a role for CD4$^+$ cells in the expansion of γδ T cells in response to pathogens has been implied both in mice (34, 40) and in humans (26). In vivo expansion of splenic γδ T cells in the course of malaria in mice was recently shown to be dependent on CD4$^+$ cells. Transfer of enriched populations of CD4$^+$ cells but not CD4$^-$ cells from BALB/c mice into SCID mice resulted in increased numbers of γδ T cells and led to resolution of parasitemia (40). In another study, in vitro expansion of γδ T cells from *Listeria monocytogenes*-immune mice was found to be dependent on αβ T cells. A recent study has suggested a similar situation in humans. Depletion of CD4$^+$ cells from PBMCs abolished their ability to expand Vγ9/Vδ2 cells in response to mycobacteria. Reconstitution of CD4$^+$ cells restored the ability of E$^-$ PBMCs to expand the γδ T cells (26). No information is provided in that study about whether purified CD4$^+$ T cells alone could support the expansion of Vγ9/Vδ2 cells or about the phenotype of the accessory cells. That study also suggested that the expansion of Vγ9/Vδ2 cells was mediated by a soluble factor, because help for proliferation of Vγ9-bearing cells by accessory cells which were reconstituted with CD4$^+$ cells could be observed across semipermeable membranes (26). Our data, however, indicate that a soluble factor is unlikely to play a major role in the expansion of cloned Vγ9/Vδ2 cells, because no significant proliferative response could be observed when Transwell membrane systems were used to separate the γδ T cells from the accessory cells. Our previous studies (11) showing that accessory cells preincubated with AP-MT and then fixed can expand Vγ9/Vδ2 cells strongly support this conclusion.

One possible reason for the apparent discrepancy between

the two studies is related to the use of unstimulated, partially purified polyclonal populations of γδ T cells in the study by Pechhold et al. (26). It is conceivable that quiescent γδ T cells are more amenable to an exocrine effect of accessory cells, while activated clones are dependent on an autocrine-mediated expansion which can be triggered upon contact with the accessory cells. Other differences, such as the use of irradiated accessory cells in our study versus the use of nonirradiated accessory cells by Pechhold et al. (26) and the different readout systems used to determine cell expansion in these two studies, can also account for the disparate conclusions.

The differential ability of $CD4^+$ T cells expressing distinct CD45 isoforms to activate Vγ9/Vδ2 cells is also of interest. The CD45 glycoprotein is expressed on leukocytes in various isoforms which arise from alternate mRNA splicing (37). Most peripheral T lymphocytes belong to either one of two functionally distinct subsets expressing either the RA isoform or the RO isoform of CD45. These two populations contain naive and memory T cells, respectively. Both naive and memory $CD4^+$ T cells proliferate in response to allogeneic cells, but only the $CD4^+$ $CD45RO^+$ subset is capable of providing help for B cells to produce immunoglobulins (20, 21, 32, 39). The two subsets have recently been shown to develop distinct intracellular signaling events upon activation (25). To the best of our knowledge, the results reported here are the first evidence that $CD4^+$ $CD45RO^+$ and $CD4^+$ $CD45RA^+$ subsets are also distinguishable in their ability to activate other subsets of T cells.

CD7 is a 40-kDa glycoprotein which appears early in ontogeny and is thought to be involved in T-cell activation and signal transduction (4). The $CD7^-$ population has been recently identified as a distinct T-cell subset in the peripheral blood of normal individuals (28). Most of the peripheral blood $CD7^-$ cells express the αβ $TCR^+$ $CD4^+$ $CD45RO^+$ phenotype. The function of this small subset (less than 10% of $CD3^+$ cells [28]) is currently unknown. To the best of our knowledge, the data reported here provide the first evidence that this subset has a distinct functional role.

T cells of the $CD45RO^+$ and $CD7^-$ phenotypes are found preferentially in nonlymphoid and inflammatory sites, such as the rheumatoid joint and inflamed skin (15, 16, 19, 27). It is noteworthy that γδ T cells have also been found in high frequencies in various inflammatory sites such as the lungs of patients with sarcoidosis (1), the joints of patients with rheumatoid arthritis (10), and skin lesions of patients with leprosy (18). The coaccumulation of these two subsets of T cells in inflammatory sites suggests that their interaction in situ may play a role in the pathogenesis of these diseases. Interestingly, the Vγ9/Vδ2 subset has also been found to express high levels of CD45RO (24). This might suggest a homotypic interaction between the two cell populations. However, our preliminary data (unpublished) do not support the possibility that the CD45RO glycoprotein is involved directly in the expansion of Vγ9/Vδ2 cells, because anti-CD45RO MAbs failed to inhibit their proliferation. It is possible that αβ-γδ cell interaction is mediated by a surface molecule coexpressed with CD45RO. Experiments to directly study the role of the CD45RO glycoprotein in the expansion of Vγ9/Vδ2 cells in response to AP-MT are under way in our laboratory.

Stimulation of the accessory T cells with AP-MT resulted in distinct intracellular signaling changes. The patterns of phosphorylation in the purified $CD45RO^+$ and the $CD7^-$ T-cell preparations were remarkably similar. This finding indicates that the subpopulation which accounts for the ability CD45 $RO^+$ T cells to expand Vγ9/Vδ2 cells carries the $CD7^-$ phenotype. The identities of most of the phosphoproteins which are selectively activated by AP-MT are unknown. Future attempts to decipher the AP-MT-triggered signaling pathway will focus on those phosphoproteins that show both subset-specific and antigen-specific changes. Interestingly, the location of one of the spots which is selectively phosphorylated in accessory cells with expansion capability (spot 3) is consistent with Op 18c, an 18-kDa cell cycle-dependent phosphoprotein (35). This phosphoprotein, whose activation pathway is partially characterized, may provide insight about the signaling events that lead to the ability of $CD45RO^+$ $CD7^-$ T cells to expand Vγ9/Vδ2 cells.

In summary, we have identified a small subpopulation of memory helper T cells as the subset in the peripheral blood on which the expansion of Vγ9/Vδ2 cells is exclusively dependent. Future studies with this phenotypically and functionally defined subpopulation of cells may facilitate our understanding of the mechanism by which this unique immune activation is mediated.

## ACKNOWLEDGMENTS

We thank Rork Kuick for gel imaging advice and Robert Hinderer and Yi-Feng Jia for technical assistance. We also thank David Markovitz for critical reading of the manuscript.

This work was supported by grants AR 40544, AR 41703, AR 20557, and AR 08220 from the National Institutes of Health, as well as grants from The Arthritis Foundation and The Searle Scholar Program/Chicago Community Trust.

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