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Nonpeptide ligands for human γδ T cells

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ABSTRACT **γδ T cells respond to a variety of microbial pathogens and transformed cells. Their limited receptor repertoire and activation by mycobacterial antigens resistant to proteases suggest that they may recognize nonpeptide antigens. We have tested a variety of nonpeptide molecules for stimulation of human γδ T cells. Synthetic alkyl phosphates, particularly monoethyl phosphate (MEP), selectively activated γδ T cells and stimulated their proliferation *in vitro*. All γδ T cells stimulated by MEP expressed Vγ2/Vδ2 receptors. The purified natural ligand of mycobacteria is chemically similar to, though distinct from, MEP and contains a phosphate residue that is critical for biological activity. Recognition and expansion of a specific T-cell receptor-bearing population to nonpeptide ligands is unprecedented among T cells. We suggest that MEP mimics small natural ligands capable of expanding one subset of γδ T cells and that this recognition of nonpeptide antigens may play an important role in human immunity to pathogens.**

T cells expressing γδ receptors represent a distinct population of T cells differing from αβ T cells in cell surface phenotype, in limited combinatorial diversity of their T-cell antigen receptors (TCRs), and in selective anatomical localization (1–3). It has been suggested that γδ T cells may play a unique role in the defense against pathogens (4, 5). Recent experimental evidence supporting a role for γδ T cells has been reported for *Listeria* infections in mice (6).

In humans, evidence is also accumulating for a role for γδ T cells in the immunity to microorganisms. γδ T cells bearing Vγ2/Vδ2 receptors undergo an extrathymic peripheral expansion in infancy that is not linked to major histocompatibility complex (MHC) or non-MHC genes but, instead, may be due to antigenic exposure (7). Furthermore, these same Vγ2/Vδ2-bearing T cells expand acutely with certain mycobacterial, bacterial, and parasitic infections (8–11) and respond to soluble mycobacterial and bacterial extracts *in vitro* (9, 12–15). Although the ligands recognized by Vγ2/Vδ2-bearing T cells have not been identified, the resistance of the mycobacterial ligands to proteases suggests that they may not be peptides (16). Widespread recognition of nonpeptide ligands by a T-cell subset would be unprecedented, given our present knowledge of antigens for αβ T cells. In the present study, we have identified monoalkyl phosphates, particularly monoethyl phosphate (MEP), as nonpeptide ligands for γδ T cells. These synthetic compounds have chemical similarities to the natural mycobacterial ligand. Recognition of monoalkyl phosphates is restricted to Vγ2/Vδ2-bearing T cells and appears to involve directly the γδ TCR.

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MATERIALS AND METHODS

Preparation of Monoalkyl Phosphates. Alkyl phosphates were prepared by allowing phosphorus pentoxide (Fluka) to react with the indicated alcohol (17). To obtain pure MEP, the crude mixture was purified by anion-exchange HPLC (Dionex), followed by gel-filtration chromatography (Bio-Gel P2). To verify the identity of the compound, fast atom bombardment mass spectral analyses were performed on a Finnigran MAT-90 mass spectrometer in the negative ion mode and gave the expected exact mass of MEP [124.999 g ($M - H^-$)] to within 8 ppm of its theoretical value. Diethyl phosphate and triethyl phosphate were purchased from Kodak and Aldrich, respectively. β-Hydroxyethyl phosphate was prepared from ethylene oxide by the action of aqueous disodium hydrogen phosphate (17) and purified by anion-exchange chromatography (QAE Sephadex A-25; Pharmacia). Phosphoglycolic acid was purchased from Sigma.

Derivation and Maintenance of T-Cell Clones. All T-cell clones were derived by limiting dilution. DG clones were derived from the synovial fluid of a rheumatoid arthritis patient by stimulation with *Mycobacterium tuberculosis*. The 12G12 γδ T-cell clone was derived from *M. tuberculosis* H37Ra-stimulated Ficoll/Hypaque-purified peripheral blood mononuclear cells (PBMC) of a patient with tuberculoid leprosy. γδ clones SD9, 10E12, 4H1, SE3, S58, PS7, 4B6, 24B1, 5C, and JF1 and αβ T-cell clones were derived from *M. tuberculosis*-stimulated PBMC of a normal donor. γδ T clones HF.2, HD.109, CP.2B2, HF.15, and HF.41 were derived from PBMC with phytohemagglutinin (18, 19), and γδ T clones T7A5, T7C6, T7C7, T5B9, MD16, MD21, MD22, and MD26 were similarly derived (20).

Proliferation and Cytotoxicity Assays. Assays were performed in triplicate using 4–10 × 10^4 cloned γδ or αβ T cells plus 5–10 × 10^4 irradiated allogeneic PBMC per flat or round bottom well of a 96-well plate. *M. tuberculosis* antigen was prepared by suspending 100 mg of heat-killed H37Ra (Difco) with 10 ml of distilled water followed by sonication. For some experiments, crude reaction mixtures of alkyl phosphates were diluted 1:200 for use. After 24–42 hr, the cells were pulsed with [^{3}H]thymidine (1 μCi/well; 1 Ci = 37 GBq), harvested at 48 hr, and assayed by liquid scintillation. For cytotoxicity assays, primary lymphocytes expanded for 7 days with MEP (30 μM) were added to Daudi or K562 cells (2 × 10^3) labeled for 90 min with 100 μCi of ^{51}Cr, and chromium release was measured at 6 hr.

MEP-Induced Expansion and Flow Cytometric Analysis of γδ T Cells. PBMC from healthy donors were cultured at 1.2 × 10^6 per ml in Yssel's medium together with 30 μM MEP or *M. tuberculosis* lysate (1:30 dilution). On day 7, viable cells

Abbreviations: MEP, monoethyl phosphate; PPD, purified protein derivative of tuberculin; PBMC, Ficoll/Hypaque-purified peripheral blood mononuclear cells; TCR, T-cell antigen receptor; MHC, major histocompatibility complex; FACS, fluorescence-activated cell sorting.

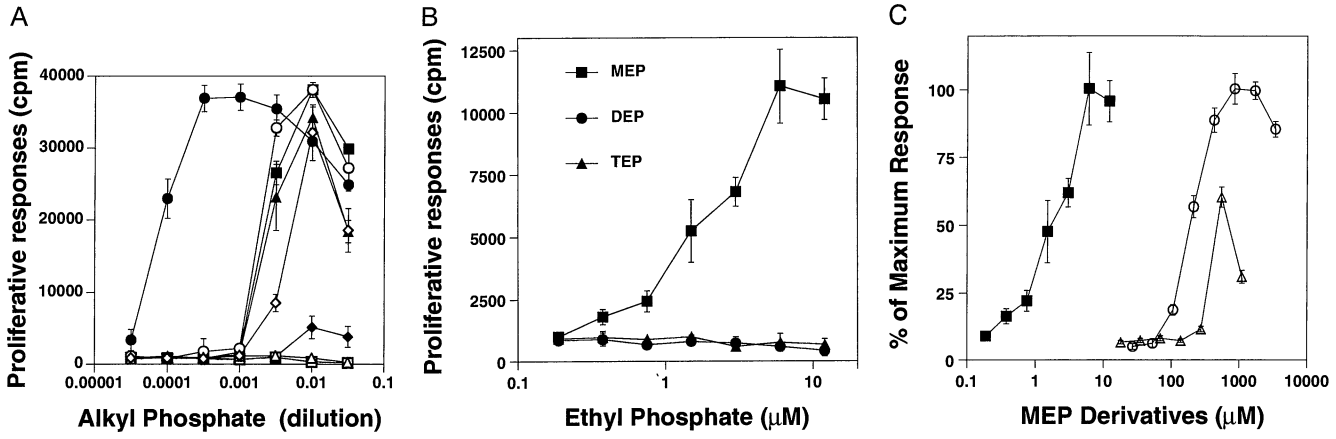

FIG. 1. γδ T cells respond to monoalkyl phosphates. (*A*) The γδ T-cell clone DG.SF68, which expresses Vγ2/Vδ2, proliferated when cultured with crude reaction mixtures of alkyl phosphates. ■, Methyl phosphate; ●, MEP; ▲, *n*-propyl phosphate; ◆, *n*-butyl phosphate; □, amyl phosphate; ○, isopropyl phosphate; △, isoamyl phosphate; ◇, *sec*-butyl phosphate. (*B*) Selective stimulation of the γδ T-cell clone SD9 by MEP. ■, MEP; ●, diethyl phosphate (DEP); ▲, triethyl phosphate (TEP). (*C*) Stimulation of the γδ T-cell clone SD9 by MEP derivatives. The percentage of the maximal proliferation response is shown. ■, MEP (shown for comparison from *B*); ○, hydroxyl derivative (β-hydroxyethyl phosphate); △, carboxyl derivative (phosphoglycolic acid).

were separated by Ficoll/Hypaque and then stained with antibodies and analyzed. Antibodies used included phycoerythrin-conjugated anti-TCR γ/δ-1, fluorescein isothiocyanate-conjugated anti-CD3 (Becton Dickinson), anti-Vδ2 chain (BB3; a generous gift of L. Moretta, Natl. Cancer Res. Inst., Genoa, Italy), anti-Vγ2 chain (TiγA; a generous gift of F. Triebel, Inst. Gustave-Roussy, Villejuif, France), and fluorescein isothiocyanate-conjugated anti-mouse IgG antibody (Ortho Diagnostic Systems Laboratories, Webster, TX). Stained cells were analyzed on a FACScan analyzer (Becton Dickinson). Controls with isotype-matched antibodies established the quadrants in such a way that >99% of the cells are in the double-negative region.

Partial Purification of the Major Natural Ligand from Mycobacteria. The major natural ligand was partially purified from *Mycobacterium fortuitum* or *Mycobacterium smegmatis* culture supernatant by ultrafiltration (Ultrasette 1K; Filtron), activated charcoal/Celite column (1:2, wt/wt; 2.5 × 1.5 cm) (21), barium precipitation, and reversed-phase and anion-exchange chromatography.

Enzymatic Treatment of MEP and the Natural Mycobacterial Ligand. For protease treatments, mycobacterial ligands and purified protein derivative of tuberculin (PPD; 500 μg/ml; Statens Serumsinstitut, Copenhagen) were treated with the endoprotease, pronase E (Sigma), subtilisin (Sigma), or proteinase K (Boehringer Mannheim) (all at 4 μg/400 μl). At 30 hr, the samples were heated at 65°C for 3 min and treated with either a second aliquot of endoprotease or an aliquot of the exopeptidase carboxypeptidase Y (Boehringer Mannheim) at 40 μg/400 μl. After an additional 20 hr, the samples were heated at 65°C for 3 min and used in proliferation assays with either the DG.SF68 clone for the samples or the mycobacteria-reactive $CD4^+$ αβ T-cell line, DG.1 (22), for the PPD samples.

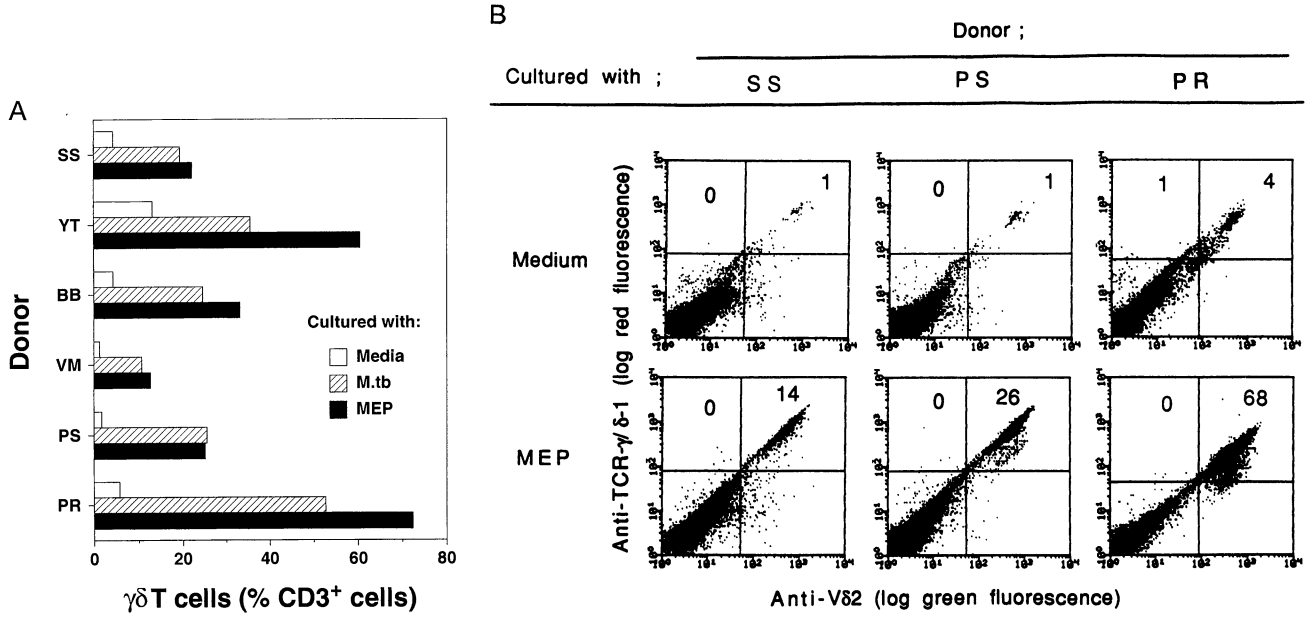

FIG. 2. Effect of MEP on primary PBMC and the specificity in the response of γδ T cells to monoalkyl phosphate ligands. (*A*) Expansion by MEP of γδ T cells in primary cultures of PBMC as determined by two-color FACS analysis. The increase of γδ T cells by MEP is comparable to that stimulated by *M. tuberculosis* (M.tb) lysates. (*B*) Two-color FACS analysis showing that γδ T cells expanded in primary culture by MEP exclusively expressed Vδ2. The percentages in the quadrants of interest are shown.

Mycobacterial ligands and MEP were treated with calf intestinal (New England Biolabs) or shrimp alkaline phosphatase (United States Biochemical) for 18 hr at 37°C, ultrafiltrated, and assayed for bioactivity. Toxicity to T cells was not observed in any samples (data not shown).

RESULTS AND DISCUSSION

MEP and Other Monoalkyl Phosphates Stimulate γδ T Cells. A wide variety of commercially available or synthetically derived carbohydrates, amino acids, nucleotides, and their chemically modified derivatives were examined for stimulation of a series of *M. tuberculosis*-specific human γδ T-cell clones derived from the lymphocytes of tuberculoid leprosy patients, a rheumatoid arthritis patient, or normal individuals. Most compounds (including a variety of phosphorylated amino acids, carbohydrates, nucleotides, and carboxylic acids) were inactive (data not shown). However, one class of compounds, the monoalkyl phosphates, induced strong proliferation in a mycobacteria-specific γδ T-cell clone, DG.SF68 (Fig. 1*A*). All alkyl phosphates tested with four or fewer carbons induced proliferation (Fig. 1*A*). One compound, MEP, was 50- to 100-fold more bioactive than the other compounds and was selected for further study. γδ T-cell clones proliferated in a dose-dependent manner to HPLC-purified MEP, reaching half-maximal proliferation at 2 μM, but failed to proliferate when exposed to the diethyl or triethyl derivatives (Fig. 1*B*). Neither αβ T-cell clones (Fig. 3*A*) nor NK clones (data not shown) proliferated in response to MEP. On the basis of these findings, a number of MEP derivatives were tested for γδ stimulation. Modification of C-2 by addition of a hydroxyl group or by conversion to a carboxyl group reduced the specific activity of MEP by 100- to 200-fold (Fig. 1*C*), whereas the addition of an amino group totally abolished biological activity (data not shown). Common three-carbon metabolites such as β-glycerophosphate, 2-phosphoglycerate, 3-phosphoglycerate, and phospho*enol*pyruvate were also inactive (data not shown).

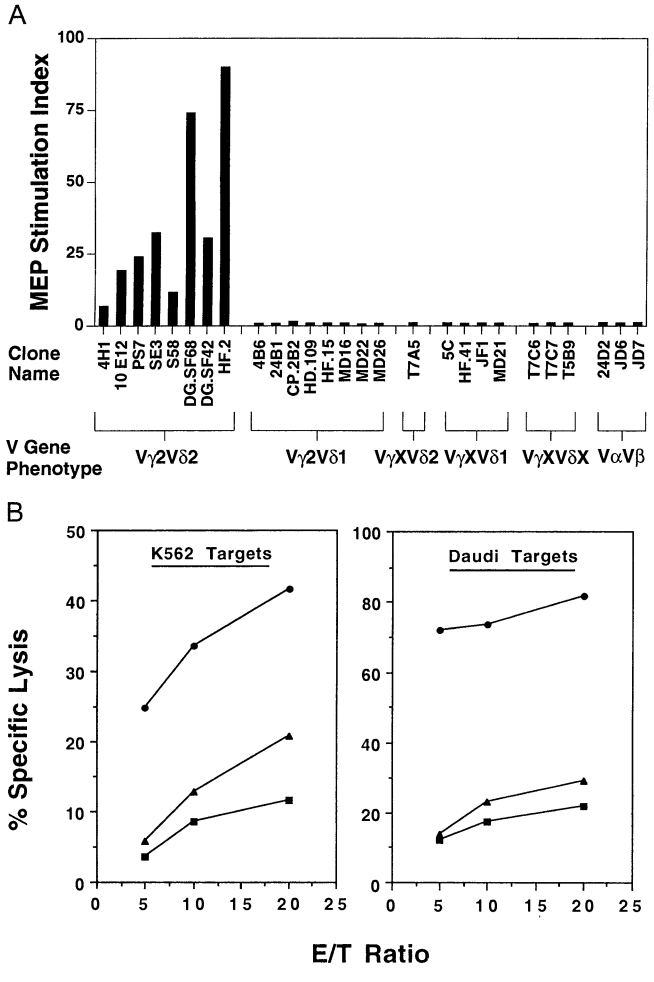

FIG. 3. Effect of MEP on γδ T-cell clones and heterogeneity in the response of Vγ2/Vδ2$^+$ T-cell clones to monoalkyl phosphate ligands. (*A*) Proliferation in response to MEP was restricted to γδ T-cell clones expressing the Vγ2/Vδ2 receptor. The stimulation indices for the response to phytohemagglutinin (positive control) were greater than 6 for all clones, with a mean value of 47.3. (*B*) Cytolytic activity of primary γδ T cells expanded by MEP. ●, Treated with 30 μM MEP for 7 days; ▲, treated with 30 μM MEP for 7 days and depleted of Vδ2 cells by BB3 antibody and magnetic beads (Dynal); ■, medium only. E/T, effector to target.

MEP Selectively Activates the Vγ2/Vδ2$^+$ Subset of γδ T Cells Through Cognate TCR Recognition. Previous studies showed that ligand(s) of *M. tuberculosis* and other mycobacterial species predominantly induce the expansion of γδ T cells from primary cultures of freshly isolated PBMC (8, 12–14, 23). A similar selective expansion of γδ T cells was observed after a 7-day period of exposure of PBMC from all healthy donors tested (12 individuals) to MEP (Fig. 2*A*). To determine if MEP expands the same Vγ2/Vδ2$^+$ subset of γδ T cells that is stimulated by mycobacteria, primary γδ T cells from normal individuals were expanded by MEP, and their V gene expression was determined by two-color fluorescence-activated cell sorting (FACS) analysis. The expanded γδ T-cell population was restricted to those that expressed the Vδ2 gene (Fig. 2*B*); no expansion of Vδ1-expressing γδ T cells was detected. To define the V gene requirement for activation further, a panel of γδ T-cell clones expressing a variety of γ and δ V genes was screened for proliferation to MEP. Strikingly, responsiveness to MEP was restricted to clones expressing Vγ2 in conjunction with Vδ2 (Fig. 3*A*). Clones expressing Vγ2 paired with Vδ1 or clones expressing Vδ2 paired with Vγ genes other than Vγ2 were not reactive to either MEP (Fig. 3*A*) or *M. tuberculosis* lysates (data not shown). DNA sequence analysis of seven MEP-reactive Vγ2/Vδ2$^+$ T-cell clones revealed extensive junctional diversity in the Vδ2 gene (data not shown), which is consistent with previous results obtained with mycobacterial-specific γδ T cells (13, 14, 24, 25). The proliferation of γδ T-cell clones to MEP was abolished by inclusion of anti-TCR-δ antibodies

Table 1. The MEP response is blocked by antibody to the γδ TCR

MEP	Antibody	Proliferative responses, cpm SD9	12G12
−	None	1,075	151
+	None	9,955	4578
+	Control mouse IgG	9,801	4760
+	Anti-MHC class I	6,827	3782
+	Anti-MHC class II	10,187	5347
+	Anti-TCR (Vδ2)	929	95

γδ clones SD9 and 12G12 were stimulated with MEP (30 μM) in the absence of antigen-presenting cells and in the presence of the indicated antibody. Antibodies used were mouse IgG1 plus IgG2a (2 μg/ml; Zymed) as a control, anti-HLA-A,B,C,E (W6/32, 1:1000; Sera-Lab, Crawley Down, Sussex, U.K.), anti-HLA-DR (MID3; 2 μg/ml; Sera-Lab), and anti-Vδ2 (BB3; 1:2000). All antibodies were dialyzed to remove sodium azide before assay. The proliferative responses to MEP in the presence of irradiated allogeneic PBMC were 947 (− MEP) and 10,430 (+ MEP) for SD9 and 204 (− MEP) and 6841 (+ MEP) for 12G12. Results shown are representative of three separate experiments and are means of triplicate values, with standard errors <10%.

(Table 1), which, together with the V gene specificity, suggests that the γδ TCR is directly involved in the recognition of MEP. To further confirm TCR involvement in the recognition of nonpeptide ligands, transfection of Vγ2 and Vδ2 genes from a mycobacteria-specific γδ T-cell clone into the TCR$^-$ Jurkat mutant, J.RT3-T3.5, conferred responsiveness to MEP and the natural mycobacterial ligand (J. Bukowski and C.T.M., unpublished results). Finally, MEP-stimulated Vγ2/Vδ2-bearing T cells have the capacity to lyse human transformed hematopoietic cells, illustrated in Fig. 3*B* (for Daudi and K562 cells). Thus, monoalkyl phosphate compounds appear to act as antigens or superantigens that stimulate Vγ2/Vδ2-bearing cells through their TCR in a manner similar to that of mycobacterial ligands.

MEP Shares Major Chemical Properties with the Natural Mycobacterial Ligand. To determine if the natural mycobacterial ligand for γδ T cells is chemically similar to MEP, the major natural ligand was partially purified from two representative mycobacterial species, *M. fortuitum* and *M. smegmatis*. These partially purified ligands had similar chromatographic properties and were able to expand primary Vγ2/Vδ2 T cells in a manner identical to crude mycobacterial preparations. On the basis of our purification protocols, the natural ligand has a molecular mass of <1 kDa, lacks significant hydrophobicity, and is anionic at pH 8. It is unlikely that the major ligand is a nucleotide or an oligosaccharide because an activated charcoal column failed to absorb the ligand (69–94% recovery), whereas almost all ribo- and deoxyribonucleotide compounds, as evidenced by the 0.15% recovery of the starting absorbance at 260 nm (Fig. 4*A* and refs. 26 and 27), and oligosaccharides (21) were bound. Underscoring the nonpeptide nature of the mycobacterial ligand, protease digestion with various endoproteases or a combination of an endoprotease followed by carboxypeptidase Y (which digests most peptides including those with blocked amino termini) (28) did not affect the bioactivity of the natural mycobacterial ligand, whereas the bioactivity of the PPD preparation for the mycobacteria-specific CD4$^+$ αβ T-cell line, DG.1, was completely abolished (Fig. 4*B*). In contrast to protease treatment, treatment with alkaline phosphatase completely abolished

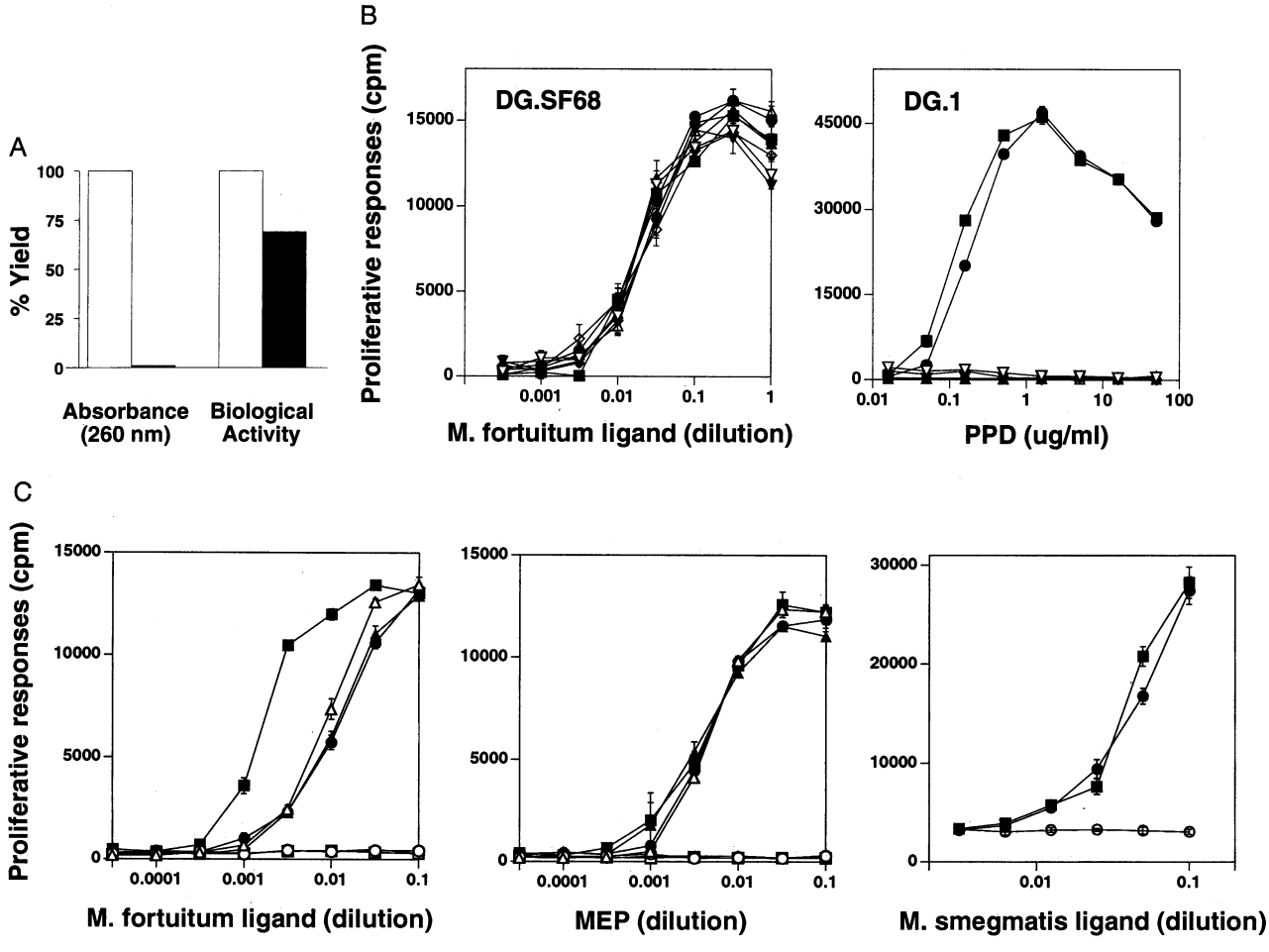

FIG. 4. Purification and enzymatic treatment of the natural mycobacterial ligand and MEP. (*A*) Selective binding, by an activated charcoal column, of nucleotides (which constitute the major compounds with absorbance at 260 nm) and oligosaccharides (21) but not the mycobacterial ligand from *M. smegmatis*. Open bar, before activated charcoal; solid bar, after activated charcoal. (*B*) The natural mycobacterial ligand was resistant to digestion with endo- and exoproteases, whereas the antigen for the mycobacteria-specific CD4$^+$ αβ T-cell line DG.1 was completely destroyed. Proliferative responses are shown for the DG.SF68 clone for the *M. fortuitum* ligand and the DG.1 line for PPD. Partially purified *M. fortuitum* ligand and PPD were untreated (■), mock-treated (●), treated with pronase E (▲), treated with subtilisin (◆), treated with proteinase K (△), or treated with a combination of carboxypeptidase Y and either pronase E (▼), subtilisin (◇), or proteinase K (▽). (*C*) The purified mycobacterial ligand and MEP were resistant to digestion with carboxypeptidase Y but were totally inactivated by treatment with alkaline phosphatase. Proliferative responses are shown for the DG.SF68 clone for *M. fortuitum* and MEP (*Left* and *Center*) and the SD9 clone for *M. smegmatis* (*Right*). Partially purified *M. fortuitum* ligand and crude MEP were untreated (■), mock-treated with the addition to 1 (●) or 10 (▲) mM $MgCl_2$, treated with calf intestinal alkaline phosphatase at 4 units/400 μl or 40 units/400 μl (□), treated with shrimp alkaline phosphatase at 20 units/400 μl (○), or treated with carboxypeptidase Y at 40 μg/400 μl (△). (*Right*) Partially purified *M. smegmatis* ligand was untreated (■), treated with heat-inactivated shrimp alkaline phosphatase (●), or treated with native shrimp alkaline phosphatase at 50 units/500 μl (○).

the biological activity of the purified natural ligand and MEP, indicating that the compounds contain a critical phosphate residue (Fig. 4*C*). The natural ligand appears to be significantly more bioactive on a molar basis than MEP (perhaps in the picomolar range), as it is bioactive at levels below our ability to detect phosphate groups. We conclude that in addition to their similar biological activities, MEP and the natural mycobacterial ligand are chemically similar; they are small, hydrophilic, anionic molecules with critical phosphate residues required for biological activity. Although similar, the natural ligands and MEP had distinct chromatographic properties on anion-exchange and thin-layer chromatography (data not shown) and are, therefore, distinct compounds.

Apart from the monoalkyl phosphates and the mycobacterial ligand, Vγ2/Vδ2-bearing T cells have been stimulated *in vitro* by other antigens including Gram-positive and Gram-negative bacteria (9, 14, 15), the malarial parasite (*Plasmodium vivax*; refs. 29 and 30), and the tumor cell lines Daudi (31) and RPMI 8226 (32). This same Vγ2/Vδ2$^+$ subset of $\gamma\delta$ cells expands *in vivo* in response to environmental stimuli, from a minor subpopulation in neonatal cord blood to dominate the $\gamma\delta$ population in adult peripheral blood (7). A similar acute *in vivo* expansion of Vγ2/Vδ2-bearing T cells (up to 40% of CD3$^+$ peripheral blood T cells) is noted with certain bacterial infection (9–11) and in leprosy skin lesions (8, 33). We propose that the monoalkyl phosphates mimic natural ligands (such as those present in mycobacteria) that are responsible for these *in vivo* expansions. As such, the monoalkyl phosphates may prove useful as immunotherapeutic agents to stimulate the Vγ2/Vδ2 subset of $\gamma\delta$ T cells. This subset of $\gamma\delta$ T cells may have been selected to respond to antigens entirely distinct from the peptide antigens to which $\alpha\beta$ T-cells respond. These antigens are low molecular mass, phosphorylated compounds produced by a variety of pathogens. By recognizing these nonpeptide ligands, $\gamma\delta$ T cells probably play a unique role in the human immunity to a wide spectrum of pathogenic bacteria and parasites.

Note Added in Proof. A recent report (34) suggests that the natural compound of mycobacteria able to stimulate $\gamma\delta$ T cells may be a derivative of thymidine triphosphate, although the structure of the active compound was not determined. The natural compound characterized in our report represents the major ligand in fast-growing mycobacteria (>99% of total activity) and is essentially devoid of OD_{260} absorbing nucleotides.

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