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Characterization of a 10- to 14-Kilodalton Protease-Sensitive *Mycobacterium tuberculosis* H37Ra Antigen That Stimulates Human γδ T Cells

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γδ T-cell receptor-bearing T cells (γδ T cells) are readily activated by intracellular bacterial pathogens such as *Mycobacterium tuberculosis*. The bacterial antigens responsible for γδ T-cell activation remain poorly characterized. We have found that heat treatment of live *M. tuberculosis* bacilli released into the supernatant an antigen which stimulated human γδ T cells. γδ T-cell activation was measured by determining the increase in percentage of γδ T cells by flow cytometry in peripheral blood mononuclear cells stimulated with antigen and by proliferation of γδ T-cell lines with monocytes as antigen-presenting cells. Supernatant from heat-treated *M. tuberculosis* was fractionated by fast-performance liquid chromatography (FPLC) on a Superose 12 column. Maximal γδ T-cell activation was measured for a fraction of 10 to 14 kDa. Separation of the supernatant by preparative isoelectric focusing demonstrated peak activity at a pI of <4.0. On two-dimensional gel electrophoresis, the 10- to 14-kDa FPLC fraction contained at least seven distinct molecules, of which two had a pI of <4.5. Protease treatment reduced the bioactivity of the 10- to 14-kDa FPLC fraction for both resting and activated γδ T cells. Murine antibodies raised to the 10- to 14-kDa fraction reacted by enzyme-linked immunosorbent assay with antigens of 10 to 14 kDa in lysate of *M. tuberculosis*. In addition, γδ T cells proliferated in response to an antigen of 10 to 14 kDa present in *M. tuberculosis* lysate. γδ T-cell-stimulating antigen was not found in culture filtrate of *M. tuberculosis* but was associated with the bacterial pellet and lysate of *M. tuberculosis*. These results provide a preliminary characterization of a 10- to 14-kDa, cell-associated, heat-stable, low-pI protein antigen of *M. tuberculosis* which is a major stimulus for human γδ T cells.

The T-cell response to intracellular bacterial pathogens such as *Mycobacterium tuberculosis*, *Listeria monocytogenes*, and *Salmonella* spp. is characterized by the activation of both αβ T-cell receptor (TCR)-bearing T cells (αβ T cells) and γδ TCR-bearing T cells (γδ T cells) in vitro and in vivo (11, 17, 20, 27). The relative role and functions of these two T-cell subsets in the immune response to these pathogens remain to be determined. In murine models, both αβ and γδ T cells protect mice from challenge with *L. monocytogenes*, although the kinetics of their activation and mechanism(s) used for protecting the host appear to differ (23, 39, 42). As effector cells, both T-cell subsets secrete gamma interferon and are cytotoxic for antigen-pulsed antigen-presenting cells (APC) in vitro (24, 31, 38).

The αβ T-cell response to *M. tuberculosis* in humans is characterized predominantly by the activation of $CD4^+$ T cells (29). Secreted mycobacterial antigens, the source of tuberculin (purified protein derivative [PPD]), are particularly effective in stimulating $CD4^+$ T-cell responses but not γδ T cells. These $CD4^+$ T cells respond to a large number of distinct mycobacterial protein antigens presented by class II major histocompatibility complex-encoded molecules on APC (4, 41). When intact or live *M. tuberculosis* is used to stimulate human T cells, activation of both γδ T cells and $CD4^+$ αβ T cells is observed (3, 14, 20). The major antigen(s) of *M. tuberculosis* responsible for activation of γδ T cells and the manner in which they are presented by APC are unknown (13). A number of protein and nonprotein mycobacterial antigens for γδ T cells have been proposed, but none have been characterized definitively. In humans, the 65-kDa mycobacterial heat shock protein can activate some γδ T cells but was found not to be a major antigen for γδ T cells activated by mycobacteria (3, 14, 20). There is evidence that a small (<1 kDa) nonprotein antigen from lysates of *M. tuberculosis* stimulates γδ T cells (7, 33, 34). A subset of neonatal cord blood γδ T cells is activated by trehalose dimycolate (cord factor), a nonprotein antigen also known as cord factor (43).

The possibility that γδ T cells are activated by cross-reactive bacterial antigens was suggested by the finding that some cloned γδ T-cell lines were activated by *M. tuberculosis* as well as *Escherichia coli* and *L. monocytogenes* (8). The TCRs of γδ T cells activated by *M. tuberculosis* are predominantly $V\gamma9^+$ and $V\delta2^+$ (19). Sequencing of these γδ TCR chains demonstrated marked junctional diversity consistent with a T-cell response to a superantigen (8, 28, 30). The cross-reactivity of some γδ T cells for microbial antigens, their presence at sites of infection, and the large number of infectious diseases in which γδ T cells are activated have led to speculation that there are a limited number of (super)antigens for γδ T cells. Activation by these antigens would allow γδ T cells to play a role in the early immune response to many infectious pathogens. Antigens for γδ T cells, therefore, could be potential vaccine candidates or immunoadjuvants, since in immunodeficiency states such as human immunodeficiency virus infection, γδ T-cell responses appear to remain intact (2, 9).

The current study was undertaken to characterize antigens of *M. tuberculosis* which activate γδ T cells. We had demonstrated earlier that live *M. tuberculosis* bacilli readily activated

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$V\gamma9^+$ γδ T cells, that γδ T-cell activation by *M. tuberculosis* was dependent on APC in a non-class I or class II major histocompatibility complex-restricted manner, and that macrophages were efficient APC for γδ T cells (3, 14). When mycobacteria were heat treated, the ability of the bacilli to stimulate stimulate γδ T cells was greatly reduced. Rather than destroying the antigen for γδ T cells, we have found that heat treatment of *M. tuberculosis* efficiently released the antigen(s) for γδ T cells from the bacterial cell mass into the supernatant. The supernatant from the heat-treated mycobacteria was found to contain a protein antigen of 10 to 14 kDa, which was a potent activator of resting and activated human γδ T cells.

MATERIALS AND METHODS

Culture of *M. tuberculosis* and preparation of antigens. *M. tuberculosis* H37Ra was cultured in Middlebrook 7H9 medium with ADC enrichment, and frozen stocks were prepared as described previously (14). Bacterial counts and viability were performed by light microscopy and by counting CFU on 7H10 medium. *M. tuberculosis* H37Ra stocks were tested periodically for viability and with an *M. tuberculosis* complex-specific DNA probe (AccuProbe; Gen-Probe, San Diego, Calif.) to ensure the purity of the *M. tuberculosis* stocks.

Soluble γδ T-cell-stimulating antigen (γδ antigen) from *M. tuberculosis* H37Ra was prepared from mycobacterial cells grown in aerated liquid cultures to the late log phase. Cells were washed (three times), resuspended in H_2O, and heated to 120°C for 30 min, and the supernatant (SN) was harvested. The SN was filtered (with a 0.2-μm-pore-size filter), lyophilized, and reconstituted in H_2O at a concentration of 1.0 mg of protein per ml (Bio-Rad Laboratories, Richmond, Calif.). The yield was 10 to 60 μg of protein per ml of late-log-phase *M. tuberculosis* culture.

Cytosolic antigens of *M. tuberculosis* H37Ra were prepared by sonicating washed mycobacteria on ice (30 min, three times) and then passaging them through a French press (three times). Soluble lysate of *M. tuberculosis* was centrifuged for 2 h at 145,000 × *g* and 4°C. The soluble material was harvested and designated *M. tuberculosis* cytosol (35). Cytosol was concentrated and stored at −70°C. Protein content was estimated by the Bio-Rad method and stored at 1.0 mg/ml. No protease inhibitors were added to either the SN or the cytosolic antigen preparations. PPD of *M. tuberculosis* was a gift from Lederle Laboratories (Pearl River, N.Y.). Monoclonal antibody SA12 specific for the 10- to 12-kDa GroES homolog of *M. tuberculosis* and purified *M. tuberculosis* GroES were kindly provided by John Belisle and Patrick Brennan (through NIH contract NO1-AI-25147).

Gel filtration on Superose 12 HR 10/30 by fast-performance liquid chromatography (FPLC). Antigen preparations were concentrated to 2 to 6 mg of protein per ml in H_2O and injected (0.2- to 0.5-ml aliquot) into a Superose 12 HR10/30 column (Pharmacia LKB, Uppsala, Sweden). The column was equilibrated with phosphate-buffered saline (PBS; pH 7.4), and PBS was used to elute 0.5- to 1-ml fractions at a flow rate of 0.5 ml/min. Elution from the column was monitored by A_{280}. Fractions were concentrated by lyophilization and dialyzed against H_2O with a 3.5-kDa-molecular-weight-cutoff membrane (Spectra/Por; Spectrum, Houston, Tex.). Molecular mass standards from 440 to 6.5 kDa (Pharmacia LKB; Sigma Chemical Co., St. Louis, Mo.) were used to calibrate the column with PBS as buffer.

Isoelectric focusing. γδ antigen-containing SN underwent preparative isoelectrical focusing with a Rotofor Cell apparatus (Bio-Rad). SN (5 to 10 mg) was resuspended in H_2O with 2% CHAPS {3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate}, 10% glycerol, and 2% Bio-Lyte ampholytes (pH 3.5 to 9.5) and placed in a Rotofor Cell device. After 4,000 V · h, the fractions were harvested, their pH was measured, and they were dialyzed in H_2O and concentrated by lyophilization.

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was performed by the method of O'Farrell by Kendrick Labs, Inc. (Madison, Wis.) (26). Sample was resuspended in a mixture of 5% β-mercaptoethanol, 9.5 M urea, and 2% Nonidet P-40. Isoelectric focusing was carried out in glass tubes (2-mm diameter) with 2% Resolytes (pH 2.5 to 5; Hoefer Scientific Instruments, San Francisco, Calif.) for 9,600 V · h. Tube gel was sealed to a stacking gel on a 12.5% acrylamide slab gel, and sodium dodecyl sulfate (SDS) gel electrophoresis was carried out for 4 h at 12.5 mA per gel. Gels were stained with silver stain.

Protease treatment. FPLC fractions containing γδ T-cell antigen were treated with pronase (Boehringer GmbH, Mannheim, Germany) at a 50:1 protein-to-pronase ratio at 37°C for 2 h in 0.1 M ammonium bicarbonate buffer (pH 7.4). This treatment was repeated twice, after which the reaction mixture was lyophilized and resuspended in H_2O before being used in the bioassay. Controls included mock treatment (i.e., no pronase) of γδ antigen to control for the treatment conditions and pronase alone to test for nonspecific activation or inhibition.

Isolation of PBMC and monocytes. Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation over sodium diatrizoate-Hypaque gradients, and monocytes were obtained from PBMC by adherence as described previously (3, 14). Briefly, PBMC were incubated on plastic tissue culture dishes precoated with pooled human serum, nonadherent cells were removed, and plastic-adherent cells (≥90% monocytes by Wright's, peroxidase, and nonspecific esterase staining) were collected by scraping with a plastic policeman.

PBMC were isolated from healthy volunteers (18 to 45 years old) who were either tuberculin skin test positive or responded to tuberculin in vitro. They were selected for consistency of resting γδ T-cell expansion (20 to 60% γδ TCR^+ T cells) after stimulation with live *M. tuberculosis* or γδ antigen-containing SN.

Immunofluorescence analysis. The monoclonal antibodies used were OKT3 (anti-CD3, immunoglobulin G2a; Ortho Diagnostics, Raritan, N.J.), BMA031 (anti-αβ TCR), and TCR-δ1 (anti-γδ TCR) (T Cell Sciences, Cambridge, Mass.). Unless indicated otherwise, cells were stained by indirect immunofluorescence for single-color analysis. For two-color fluorescence-activated cell sorter analysis, phycoerythrin-conjugated OKT3, fluorescein isothiocyanate (FITC)-conjugated BMA031 and FITC-conjugated TCRδ-1 (T Cell Sciences) were used with FITC- and phycoerythrin-conjugated isotypic controls.

Cells were analyzed by flow cytometry on a FACScan (Becton Dickinson) with LYSYS II software. Cells were gated on a two-parameter plot of 90° versus forward-angle scatter. The gate for lymphocytes was set widely. Ten thousand gated events were recorded for each cell surface marker. The position of the cutoff marker for positive and negative fluorescence was set manually on the basis of the distribution of cells stained with secondary antibody alone and was kept constant for the experiment. The percentage reported for a given cell surface marker represents the proportion of gated cells with a positive signal minus the percentage of cells staining positive with secondary antibody alone.

Expansion of resting γδ T cells from PBMC. Resting γδ T cells were defined as γδ T cells present in freshly isolated

PBMC and did not express HLA-DR or interleukin-2 receptor (CD25) as determined by flow cytometry. For γδ T-cell expansion, PBMC (2×10^6/2-ml well) were incubated in 24-well plates (Costar, Cambridge, Mass.) with either live mycobacteria (5×10^6/2-ml well) or SN from heat-treated mycobacteria. Culture medium consisted of RPMI 1640 supplemented with 10% pooled human serum, 20 mM HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid), 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. After 7 to 9 days of coculture, viable cells were harvested, counted, and stained before the percentage of γδ T cells was determined by flow cytometry (see above). The percentage of γδ T cells was expressed as a percentage of total viable cells. In freshly isolated PBMC, γδ T cells were less than 6% of cells, and after 7 to 9 days of culture in medium alone, the percentage of γδ T cells was either the same or less than the starting percentage.

γδ T-cell lines and proliferation assay. T cells were harvested after 7 to 9 days of stimulation with live *M. tuberculosis* or γδ antigen, Ag, and viable cells were purified by density sedimentation over sodium diatrizoate-Hypaque gradients. Expansion of γδ T cells was confirmed by flow cytometry analysis (see above), and γδ T cells were enriched by negative selection with magnetic beads coated with antibodies (Dynal, Great Neck, N.Y.) as described previously (3). For γδ T-cell enrichment, cells were treated simultaneously with anti-CD4- and anti-CD8-coated beads. Generally, one cycle of treatment was sufficient for depletion of the αβ T cells, although in some experiments, two cycles were performed. The purity of the negatively selected T-cell populations was assessed by two-color flow cytometry and was generally ≥90% positive for the selected T-cell marker. γδ T-cell lines were maintained by biweekly stimulation with γδ antigen, interleukin-2, and irradiated PBMC as APC. These are considered activated γδ T cells and are HLA-DR$^+$ and interleukin-2R$^+$.

Purified γδ T cells (2×10^4 to 2.5×10^4/200-μl well) were cocultured with irradiated monocytes as APC and antigen for 72 h in 96-well plates. Cells were pulsed with 1 μCi of [^{3}H]thymidine (ICN, Costa Mesa, Calif.) for 12 to 16 h before being harvested on glass fiber filters with a PHD harvester (Cambridge Technology, Watertown, Mass.). [^{3}H]thymidine incorporation was measured by liquid scintillation counting and expressed as the mean counts per minute.

ELISA. BALB/c mice were injected at the base of the tail every 2 weeks for a total of three immunizations with 3 μg of protein from FPLC fractions with γδ T-cell bioactivity in adjuvant. Adjuvant consisted of 1% (vol/vol) Pluronic 121 (BASF Wyandotte, Parsippany, N.J.), 10% squalene (Aldrich Chemical Co., Milwaukee, Wis.), and 0.4% Tween 80 (Fisher Scientific, Fair Lawn, N.J.) in PBS (5, 32). Mice were bled 7 days after the last immunization. For the enzyme-linked immunosorbent assay (ELISA), 96-well polystyrene plates were coated with 5 μg of SN per ml and blocked with bovine serum albumin, and sera were added at 1:50 to 1:1,600 dilutions. Peroxidase-conjugated goat anti-mouse immunoglobulin G was used as the detecting antibody. Normal mouse serum served as a control.

RESULTS

Antigen of *M. tuberculosis* which stimulates γδ T cells is released into the SN by heat treatment. In earlier studies, we had shown that live *M. tuberculosis* stimulated the proliferation of peripheral blood γδ T cells from healthy tuberculin-positive donors. Upon heat treatment, the ability of *M. tuberculosis* bacilli to activate γδ T cells was lost (14). We next determined whether heat treatment destroyed the antigen for γδ T cells or caused its release from the mycobacteria. In these experiments, a fixed number of *M. tuberculosis* bacilli known to stimulate γδ T cells was heat treated, and both the pellet and the SN were tested for the expansion of resting γδ T cells and for proliferation of activated γδ T-cell lines. As shown in Fig. 1, the SN contained an antigen which stimulated two γδ T-cell lines. In contrast, the pellet did not stimulate γδ T cells but was immunogenic for the cloned CD4$^+$ αβ T-cell line 2P4A7 (4). As shown in earlier studies, PPD stimulated the CD4$^+$ T cells and not the γδ T-cell lines (3). APC were required for γδ T-cell proliferation, but HLA matching was not, as shown for γδ T-cell line 2 (Fig. 1). When the SN was used to stimulate PBMC for resting γδ T-cell expansion (measured by flow cytometry as the percentage of γδ T cells after 7 days of stimulation), the same results as those seen with the γδ T-cell lines were obtained and the percentage of γδ T cells was similar to that seen with live *M. tuberculosis* (38.5% for SN versus 44.1% γδ T cells for *M. tuberculosis*). The γδ T cells activated by SN were Vγ9$^+$ by flow cytometry, which was the same as observed when live *M. tuberculosis* was the stimulus (3). These results demonstrated that an antigen for γδ T cells was released by heat treatment of *M. tuberculosis* and that this antigen was heat stable, H_2O soluble, and associated with the mycobacterial cell, either as cell wall or intracellular antigen. After these initial experiment, SN was prepared from larger numbers of mycobacteria, and γδ T-cell-activating activity was

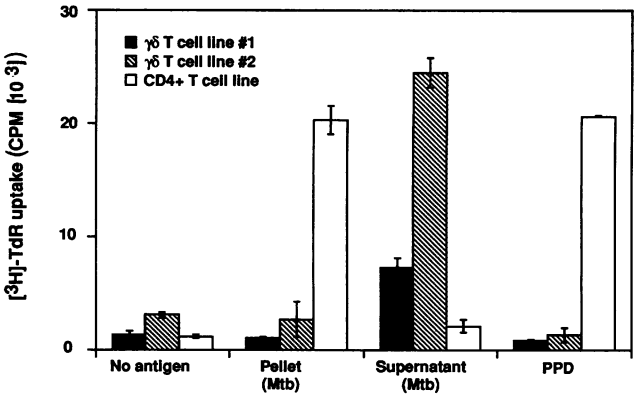

FIG. 1. Release of antigen of *M. tuberculosis* that stimulates γδ T cells into SN by heat treatment of mycobacteria. *M. tuberculosis* H37Ra bacilli (10^8) in 100 μl of H_2O were heat treated at 120°C for 30 min. Mycobacterial pellet and SN were separated by centrifugation. One hundred microliters of SN was filtered, and the pellet was resuspended in 100 μl of H_2O. SN (5 μl/200-μl well) and pellet (5 μl or 5×10^6 bacterial equivalents per 200-μl well) were tested in a proliferation assay with two γδ T-cell lines (2.5×10^4 cells per 200-μl well) and one CD4$^+$ αβ T-cell line (2.5×10^4 cells per 200-μl well). PPD was used at 10 μg/ml. No exogenous interleukin-2 was added to the proliferation assay. Irradiated monocytes (5×10^4 cells per 200-μl well) were used as APC and obtained from the same donor from whom the γδ line 1 and CD4$^+$ T-cell line were derived. γδ T-cell line 2 was derived from another, unrelated donor. γδ T-cell line 1 was a long-term line maintained by repeated stimulation with live *M. tuberculosis* and irradiated PBMC as APC. γδ T-cell line 2 was a short-term line obtained by stimulating PBMC with live *M. tuberculosis* for 10 days and sorting for γδ T cells by negative selection with antibody-coated magnetic beads. CD4$^+$ T-cell line was the cloned T-cell line 2P4A7 specific for PPD which has been described previously (4). Results are shown in counts per minute (mean ± standard deviation of triplicate cultures). Results are shown for one experiment representative of three performed. TdR, thymidine; Mtb, *M. tuberculosis*.

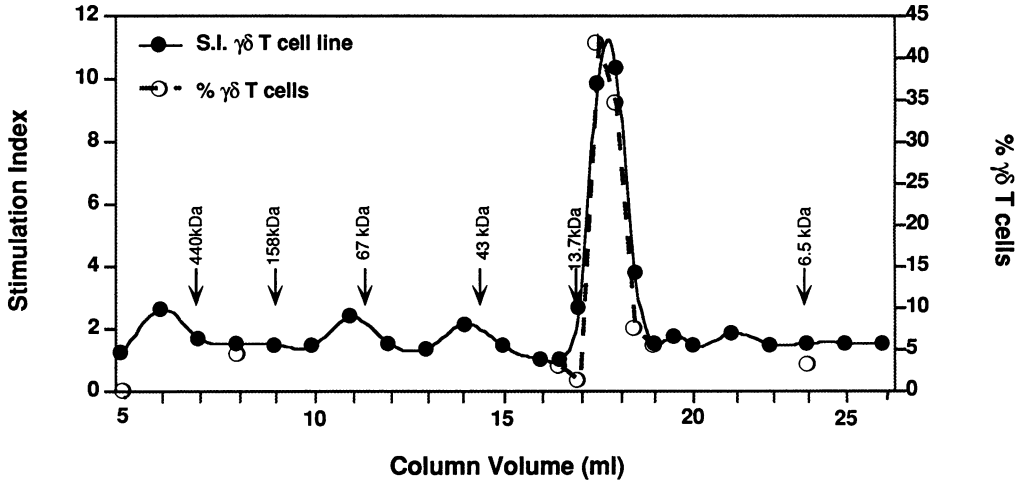

FIG. 2. Measurement of molecular mass of bioactive material for γδ T cells in SN of heat-treated *M. tuberculosis* by gel filtration. Concentrated SN from heat-treated *M. tuberculosis* was fractionated on a Superose 12 HR column by FPLC. Fractions were tested in a 3-day proliferation assay with a γδ T-cell line and in the resting γδ T-cell expansion assay. Results are presented as a stimulation index (S.I.; mean counts per minute of the fraction/mean counts per minute with no antigen) for the γδ T-cell line (left *y* axis) and as a percentage of γδ T cells measured by flow cytometry for the induction assay (right *y* axis). Results of proliferation are the mean of duplicate cultures. The column was calibrated with proteins of known molecular masses, which are indicated by the arrows. For starting SN, the stimulation index was 9.5, and the percentage of γδ T cells by flow cytometry was 55.2%.

found consistently in SN after heat treatment of 15 different *M. tuberculosis* cultures. Peak bioactivity for γδ T cells was observed at protein concentrations in SN between 2.5 and 8 μg/ml.

The heat-stable γδ T-cell antigen of *M. tuberculosis* has a molecular mass of 10 to 14 kDa. To determine the molecular mass of the γδ T-cell antigen, concentrated SN was fractionated by gel filtration over a Superose 12 column equilibrated in PBS and tested for bioactivity. In the experiment shown in Fig. 2, all fractions were tested first in a proliferation assay with the γδ T-cell line. All positive fractions and selected negative fractions then were tested in a γδ T-cell induction assay in which PBMC were stimulated with the fractions and the percentage of γδ T cells was determined after 7 days by flow cytometry. A defined peak of bioactivity; which corresponded to a molecular mass of 10 to 14 kDa, was observed at a column volume of 17 to 18 ml in both bioassays. This molecular mass estimate was reproducible for three different batches of SN in five experiments. Peak bioactivity for γδ T cells was measured for protein concentrations of 0.3 to 0.5 μg/ml, an approximately 10-fold enrichment compared with that of the starting SN. Independent confirmation of the approximate molecular mass was obtained from two experiments. First, extensive dialysis in H_2O with 3.5- and 12- to 14-kDa-molecular-mass cutoff membranes did not remove bioactivity (data not shown). Second, on SDS-polyacrylamide gels, both the starting SN and the fraction with peak bioactivity after gel filtration had material migrating at a molecular mass of 10 to 14 kDa (Fig. 3a and b). The bioactive fraction was enriched for 10- to 14-kDa material compared with the starting SN. These results indicate that the γδ T-cell antigen in SN of heat-treated *M. tuberculosis* was 10 to 14 kDa in size.

The low-molecular-weight γδ antigen of *M. tuberculosis* has a low isoelectric point (<4.5). When SN was fractionated by preparative isoelectric focusing under nonreducing and nondenaturing conditions, peak bioactivity for γδ T cells was observed for material migrating to a pI of <4, as shown in Fig. 4. In contrast, antigens for a $CD4^+$ T-cell line specific for PPD from the same donor were found in fractions with a pI of 4 to 6. The same low-pI fractions induced resting γδ T-cell expansion (data not shown). When low-pI fractions with bioactivity for γδ T cells from three preparative isoelectric focusing runs were combined, concentrated, and run on SDS-polyacrylamide gels, a dominant band of 10 to 14 kDa as well as two to three lesser low-molecular-mass bands was again observed (Fig. 3c). The 60- to 65-kDa material was likely carryover albumen from the medium enrichment added to Middlebrook 7H9 medium

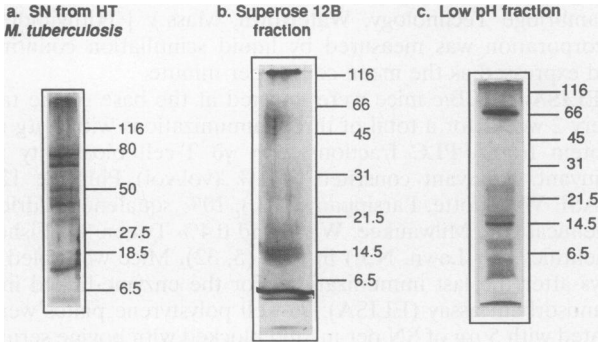

FIG. 3. Analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of SN from heat-treated (HT) *M. tuberculosis* (a), the bioactive fraction after gel filtration over a Superose 12 HR30/60 column (b), and the bioactive low-pH fraction after preparative isoelectric focusing (c). (a) Ten micrograms of heat-treated SN from *M. tuberculosis* was loaded on an SDS–12% polyacrylamide gel, transferred to a nylon membrane, and stained with AuroDye (Amersham). (b) The bioactive fraction after gel filtration was dialyzed and concentrated, and 4 μg was loaded on an SDS–15% polyacrylamide gel. (c) Low-pH bioactive fractions after preparative isoelectric focusing were pooled, and 5 μg was loaded on an SDS–15% polyacrylamide gel. Gels shown in panels b and c were stained with silver stain (Bio-Rad). Locations of molecular weight markers (in thousands) are indicated to the right of each gel.

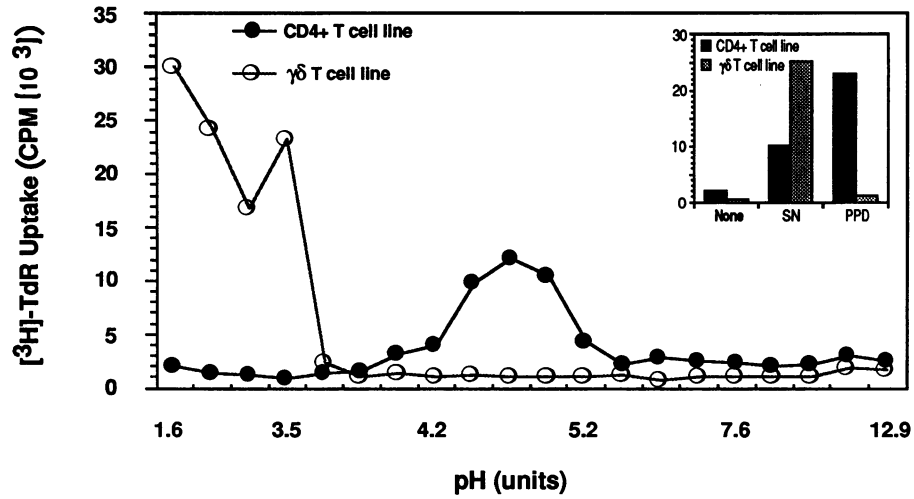

FIG. 4. Response of γδ and $CD4^+$ T-cell lines to fractions of heat-treated *M. tuberculosis* SN separated by pH by preparative isoelectric focusing. Five milligrams of SN was mixed with broad-range ampholytes and separated by pH value by preparative isoelectric focusing. The pH was measured, and the fractions were dialyzed and reconstituted to 1 ml. Ten microliters of each fraction was added in proliferation assays (final volume, 200 μl) with γδ and $CD4^+$ T-cell lines. γδ and $CD4^+$ T-cell lines were derived from the same donor. Live *M. tuberculosis* was the stimulus for the γδ T-cell line, and PPD was the stimulus for the $CD4^+$ T-cell line. Irradiated autologous monocytes were used as APC. Results are expressed as the means of duplicate cultures. The pH range is shown along the *x* axis. Responses of both T-cell lines to PPD and heat-treated *M. tuberculosis* SN (SN) are shown in the inset box. TdR, thymidine.

in which the mycobacteria were grown. By Western blot (immunoblot), there was no reactivity with antibodies to the 65-kDa mycobacterial heat shock protein in the low-pH material shown in Fig. 3c (data not shown).

We then analyzed the 10- to 14-kDa bioactive fraction after FPLC by two-dimensional gel electrophoresis under reducing and denaturing conditions with ampholytes of low pH (2.5 to 5). As shown in Fig. 5, the low-molecular-mass FPLC fraction

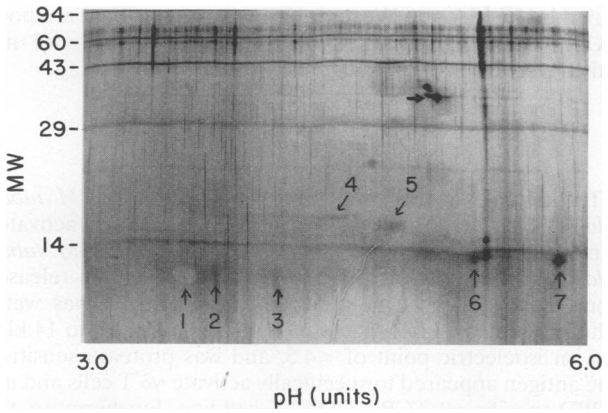

FIG. 5. Analysis by two-dimensional gel electrophoresis of 10- to 14-kDa bioactive fraction of SN from heat-treated *M. tuberculosis*. Twenty micrograms of the 10- to 14-kDa bioactive fraction shown in Fig. 3b was separated first by pI under reducing and denaturing conditions in the presence of low-pH ampholytes. Proteins were then separated by molecular weight in an SDS–12.5% polyacrylamide gel and stained with silver stain. The isoelectric focusing standard tropomyosin (molecular mass, 33 kDa; pI 5.2) is indicated by a short thick arrow. The pH gradient of the gel is indicated at the bottom of the gel. Molecular weight standards were included within the gel. They appear as the horizontal lines with their molecular weights (in thousands) marked on the side. The long thin arrows numbered 1 to 7 indicate distinct spots in the 10- to 14-kDa range separated by pI.

contains approximately seven spots of 10 to 14 kDa, of which two have pIs greater than that of the internal standard tropomyosin (pI 5.2; molecular mass, 32.7 kDa). There is a dominant negatively staining spot with a pI of 4.5, and there are two additional spots with pIs of 4.5 to 5. These findings on a two-dimensional gel do not exclude the possibility of more than one antigen for γδ T cells. However, these results do indicate that there is at least one low-pI (<4.5) antigen of 10 to 14 kDa from *M. tuberculosis* responsible for the activation of human γδ T cells.

The γδ T-cell antigen of *M. tuberculosis* is protease sensitive. In light of reports that γδ T cells might be activated by a small (1- to 3-kDa) protease-insensitive antigen(s) of *M. tuberculosis*, we investigated the protease sensitivity of the γδ antigen in SN (7, 33, 34). The low-molecular-weight bio-active fraction after FPLC (Fig. 3b) was treated with pronase and tested in both γδ T-cell activation assays. As shown in Fig. 6A, treatment with pronase reduced the expansion of γδ T cells from 36.2 to 8.0% after 7 days. Pronase itself was not inhibitory, as tested by adding it exogenously to untreated γδ antigen in the bioassay. The same results were observed when pronase-treated fractions were tested with γδ T-cell lines, as shown in Fig. 6B. Similar results were obtained when the antigen was treated with pepsin and then proteinase K (data not shown).

Cytosol of *M. tuberculosis* contains an antigen of 10 to 14 kDa which stimulates human γδ T cells. We next determined whether an antigen with a similar molecular weight was present in *M. tuberculosis* cytosol to ensure that heat treatment had not substantially altered the immunogenicity and molecular weight of the native antigen. First, *M. tuberculosis* was cultured for 3 weeks in nonenriched medium (Proskauer-Beck) to avoid the excess protein of the medium enrichment. Culture SN was harvested, filtered, and concentrated to 1 mg of protein per ml by lyophilization. The bacterial pellet was disrupted by sonication and passage through a French press. The protein contents in the lysate and pellet were normalized to 1 mg/ml, and all three antigen preparations were tested in the γδ T-cell induction assay. Bioactivity for γδ T cells was found in both the

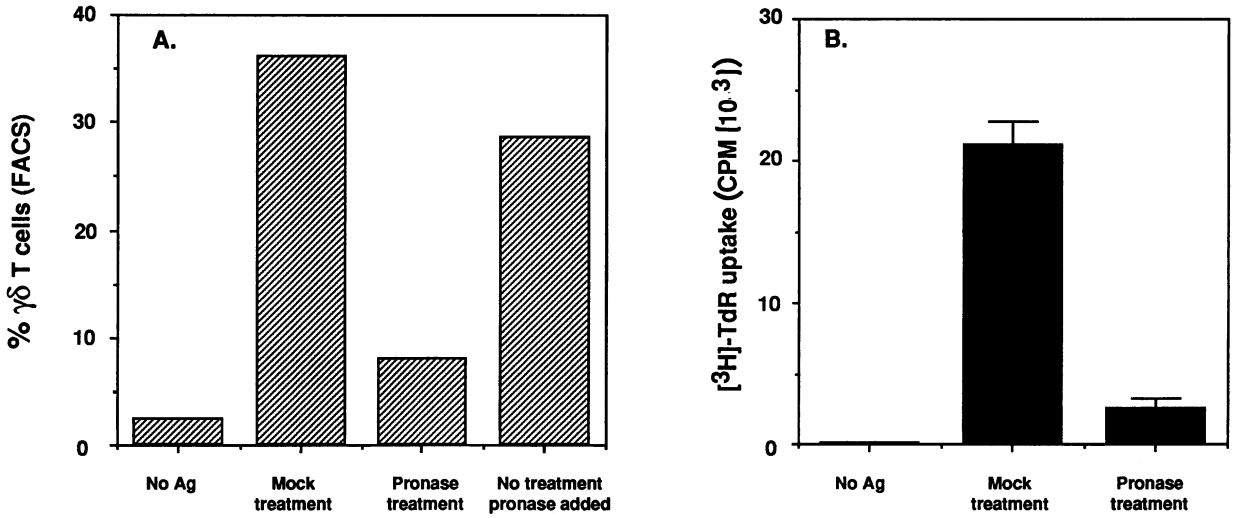

FIG. 6. Protease sensitivity of the 10- to 14-kDa γδ T-cell antigen. The 10- to 14-kDa bioactive FPLC fraction shown in Fig. 3b was treated with pronase and tested in the resting γδ T-cell induction assay (A) and in a proliferation assay with a γδ T-cell line (B). Irradiated monocytes from an unrelated donor served as APC for the γδ T-cell line. Controls included mock treatment of the 10- to 14-kDa fraction and addition of pronase at the time of bioassay. Pronase alone did not activate γδ T cells (3.9%). Results shown were representative of five experiments. TdR, thymidine. Ag, antigen.

lysate and the bacterial pellet but not in the culture filtrate. Mycobacterial lysate was then ultracentrifuged to obtain the soluble cytosol. Cytosol was fractionated by FPLC over Superose 12 by use of the same protocol as that described above for γδ antigen-containing SN of heat-treated *M. tuberculosis*. FPLC fractions were tested for proliferative responses with short-term γδ T-cell lines which had been generated by stimulating PBMC with the SN from heat-treated *M. tuberculosis*. As shown in Fig. 7, γδ T cells proliferated in response to an antigen in cytosol of the same molecular weight range as that of the antigen found in the SN of heat-inactivated *M. tuberculosis*. Independent confirmation of the presence of a 10- to 14-kD antigen in sonicate was provided by antibody studies. Murine antibodies raised against the 10- to 14-kDa fraction from SN reacted with *M. tuberculosis* lysate in an ELISA. These findings suggest that heat treatment had not substantially altered the immunogenicity and molecular weight of the native γδ antigen. The relationship between the 10- to 14-kDa antigen for γδ T cells and mycobacterial GroEs was explored by using purified *M. tuberculosis* GroES and monoclonal antibody SA12 specific for mycobacterial GroES. Both SN and cytosolic antigen preparations contained GroES, as determined by ELISA and Western blot with monoclonal antibody SA12; however, purified GroES (1 to 10 μg/ml) did not activate γδ T cells.

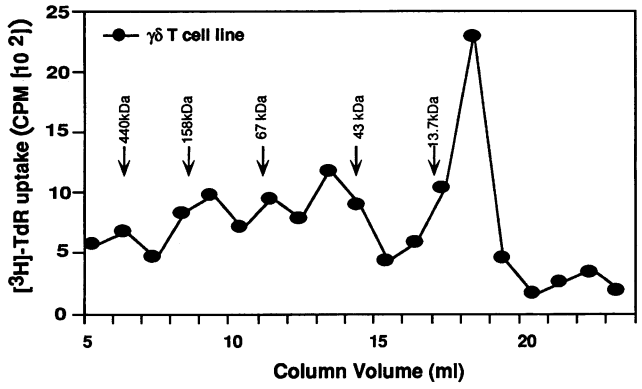

FIG. 7. Proliferative response of a γδ T-cell line to cytosolic antigens of *M. tuberculosis* fractionated by gel filtration on a Superose 12 HR10/30 column. One-half milligram of *M. tuberculosis* cytosol was fractionated by FPLC on a Superose 12 column as described in Materials and Methods. Fractions (10 μl/200-μl well) were tested in a proliferation assay with a γδ T-cell line derived by stimulating PBMC with the γδ antigen-containing SN from heat-treated *M. tuberculosis* and sorting γδ T cells by negative selection. Irradiated autologous monocytes served as APC. The results shown are the mean of duplicate cultures. Response without antigen was 177 cpm; response to live *M. tuberculosis* was 8,555 cpm. TdR, thymidine.

DISCUSSION

The findings of this study describe an antigen of *M. tuberculosis* which is a major activator of both resting and activated human γδ T cells. The antigen was associated with *M. tuberculosis* bacilli, was not secreted, and was efficiently released from the bacilli by heat treatment. The antigen was water soluble and heat stable, had a molecular mass of 10 to 14 kDa and an isoelectric point of <4.5, and was protease sensitive. The antigen appeared to specifically activate γδ T cells and not a PPD-specific αβ TCR^+ $CD4^+$ T-cell line. Furthermore, the 10- to 14-kDa antigen for γδ T cells was distinct from mycobacterial GroES.

In two separate bioassays for γδ T-cell activation, the γδ antigen was found to have the same physicochemical properties. The possibility that heat treatment of the mycobacteria had altered the native γδ antigen was addressed and excluded by two sets of experiments. First, γδ T-cell lines derived by stimulating PBMC with the γδ antigen-containing SN responded to an antigen of 10 to 14 kDa in lysates from *M. tuberculosis*. Second, murine antibodies raised against the 10- to 14-kDa antigen reacted in a Western blot with an antigen of the same molecular mass in the lysate. It is likely that heat

treatment of *M. tuberculosis* bacilli was simply an efficient means of releasing the γδ T-cell antigen from the cells.

The antigen was distinct in its molecular mass and protease sensitivity from other mycobacterial antigens which are known to activate γδ T cells. These include the 65-kDa mycobacterial heat shock protein and the 1- to 3-kDa, lectin-binding, nonprotein antigen of *M. tuberculosis* and the recently described phosphorylated thymidine-containing compound (7, 25, 33, 34). Trehalose dimycolate, a mycobacterial glycolipid, was shown to stimulate human cord blood but not peripheral blood γδ T cells and therefore is likely limited to the Vδ1 subset (43). The γδ antigen stimulated the $V\delta2^+$ subset of γδ T cells, the dominant subset activated by *M. tuberculosis* (8, 19, 28).

The γδ antigen was dependent on APC for γδ T-cell activation but did not require HLA matching of T cells and APC. The degree of resting γδ T-cell expansion and γδ T-cell proliferation by the 10- to 14-kDa antigen was similar to that seen when whole *M. tuberculosis* was used as the antigen. This suggested that the 10- to 14-kDa antigen was a major antigen for human γδ T cells. Furthermore, in our hands, *M. tuberculosis* and the 10- to 14-kDa antigen were most efficient in activating γδ T cells in individuals previously sensitized to mycobacterial antigens, consistent with a recall response. Since the molecule(s) on APC responsible for antigen presentation to γδ T cells is unknown and since few distinct antigens or epitopes for γδ T cells have been described, the mechanism(s) through which mycobacterial antigens activate γδ T cells remains to be defined. Indirect evidence that γδ T cells activated by *M. tuberculosis* might be responding to a superantigen was provided by studies in which the sequences of expressed γδ TCRs were determined. Extensive junctional diversity was observed even though the Vγ and Vδ usage was limited (predominantly Vγ9 and Vδ2), consistent with a superantigen (8, 28, 30). Others have suggested that γδ T cells respond to host antigens, presently undefined, expressed by cells exposed to microbial antigens or otherwise stressed. Our results do not exclude the possibility that the 10- to 14-Da antigen could be a superantigen for γδ T cells or induced expression of host antigens on APC.

The hypothesis that γδ T cells might respond to nonprotein antigens of microbes is of interest, although the biochemical characteristics of these antigens and their epitope(s) remain to be fully clarified. In earlier studies, we had shown that *M. tuberculosis*-activated γδ T cells did not respond to lipoarabinomannan, the major mycobacterial polysaccharide (3). There is substantial evidence that some γδ T cells respond to microbial protein antigens, which include the 65-kDa mycobacterial heat shock protein, a 70-kDa *Leishmania chagasi* antigen, *Herpes simplex* glycoprotein I, and tetanus toxoid (16, 18, 25, 40). The γδ T-cell response to infectious pathogens, therefore, could be characterized by the recognition of a large number of different microbial antigens. Unlike those for αβ T cells, antigens for γδ T cells may include nonprotein ligands. The potential for great diversity of γδ TCRs through the rearrangement of J, D, and N segments would allow for recognition of many different microbial antigens (36).

Although *M. tuberculosis* was one of the first infectious pathogens found to activate γδ T cells, their activation has been observed in response to a large number of microbial pathogens. These include intracellular bacteria such as *L. monocytogenes*, *Salmonella* spp., and *Mycobacterium leprae*, parasites such as *Plasmodium falciparum* and *Leishmania* spp., and viruses such as herpesviruses, influenza virus, and human immunodeficiency virus (2, 6, 9–12, 15, 21, 22, 27, 37, 40). Whether the γδ T cells activated by these diverse pathogens are responding to a limited number of cross-reactive microbial antigens or to a large diversity of microbial antigens remains to be defined. αβ T cells also are activated by these infectious agents, and it is likely that αβ and γδ T cells have different roles in the immune response to these pathogens. Studies in the murine model of *L. monocytogenes* infection have defined distinct and complementary roles for αβ and γδ T cells (23, 39, 42). Both T-cell subsets were found to have a role in the protective immune response to *L. monocytogenes*. γδ T cells were protective in mice lacking αβ T cells, did not induce delayed-type hypersensitivity responses, and appeared to regulate the αβ T-cell response responsible for granuloma formation in the liver. In contrast, αβ T cells were found to be more effective in conferring protection when mice were immunized, i.e., as memory T cells. In addition, γδ T cells often are found in epithelial and mucosal surfaces and therefore may act as a first line of defense against foreign antigens (13).

αβ and γδ T cells are likely to have complementary roles in the human immune response to *M. tuberculosis* as well. The alveolar surfaces of the lung are the first place of contact between *M. tuberculosis* and the host. γδ T cells can be activated by mycobacterial antigens in the lung or by *M. tuberculosis* when alveolar macrophages are the APC and therefore could be activated in the primary immune response to *M. tuberculosis* (1, 2a). In the immune response to *M. tuberculosis*, $CD4^+$ αβ T cells respond to a large number of different protein antigens and epitopes and function not only as memory cells and a source of macrophage-activating cytokines but also as cytotoxic effector cells. Whether γδ T cells will have the same diversity of function and antigen recognition remains to be determined. The identification of antigens for γδ T cells will provide the tools to elucidate the role of γδ T cells in host defense against pathogens such as *M. tuberculosis*. In addition, antigens for γδ T cells may have a role in vaccine development, in diagnosis of early infection, or possibly in immunotherapy for *M. tuberculosis* infection.

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