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Direct Presentation of Nonpeptide Prenyl Pyrophosphate Antigens to Human γδ T Cells

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Summary

Human Vγ2Vδ2$^+$ T cells recognize mycobacterial nonpeptide antigens, such as isopentenyl pyrophosphate, and their synthetic analogs, such as monoethyl phosphate, through a TCR-dependent process. Here, we examine the presentation of these antigens. Vγ2Vδ2$^+$ T cells recognized secreted prenyl pyrophosphate antigens in the absence of other accessory cells but, under such conditions, required T cell–T cell contact. Recognition required neither the expression of classical MHC class I, MHC class II, or CD1a, CD1b, and CD1c molecules, nor MHC class I or class II peptide loading pathways. Fixed accessory cells also presented the prenyl pyrophosphate antigens to γδ T cells. Thus, in contrast with the presentation of conventional peptide antigens, protein antigens, and superantigens to αβ T cells, prenyl pyrophosphate antigens are presented to γδ T cells through a novel extracellular pathway that does not require antigen uptake, antigen processing, or MHC class I or class II expression. This pathway allows for the rapid recognition of bacteria by γδ T cells and suggests that γδ T cells play a role in the early response to bacterial infection.

Introduction

Two distinct CD3-associated antigen receptors, T cell receptor αβ (TCRαβ) and TCRγδ, are expressed on separate populations of T lymphocytes. αβ T cells predominantly recognize foreign and self-peptides presented by MHC class I and class II molecules (Germain and Margulies, 1993; Yewdell and Bennink, 1992). This recognition is mediated by antigen receptors encoded by a variety of Vα and Vβ gene segments with diverse V(D)J junctional sequences. In contrast, the antigen receptors of γδ T cells use only a few Vγ and Vδ gene segments with the δ chain exhibiting marked diversity in the length and amino acid sequence of its V(D)(D)(D)J joining region (Porcelli et al., 1991; Rock et al., 1994). Most γδ T cells do not express CD4 or CD8 (Groh et al., 1989), but are capable of effector functions such as cytolysis and secretion of multiple cytokines, including interleukin-2 (IL-2), IL-4, IL-5, interferon-γ (IFNγ), tumor necrosis factor-α, and granulocyte/macrophage colony-stimulating factor (Morita et al., 1991).

Antigen recognition by γδ T cells is not well understood (Haas et al., 1993; Porcelli et al., 1991). Few γδ T cells have been reported to respond to peptide antigens in a major histocompatibility complex (MHC)-restricted fashion (Holoshitz et al., 1992; Kozbor et al., 1989) or to be alloreactive (Kabelitz et al., 1990a). However, recent evidence suggests that large numbers of γδ T cells do respond to foreign microbial pathogens. In mice, γδ T cells expand in response to mycobacteria, listeria, and salmonella infections (Augustin et al., 1989; Emoto et al., 1992; Fu et al., 1993; Hiromatsu et al., 1992; Inoue et al., 1991; Janis et al., 1989). Mice lacking γδ T cells develop liver abscesses in response to listeria infections, and mice that have γδ T cells but lack αβ T cells can survive listeria and malaria infections that kill pan T cell–deficient mice (Mombaerts et al., 1993; Tsuji et al., 1994). In humans, γδ T cells dramatically expand in vivo (up to 45% of all T cells in the peripheral blood) following infections with bacteria such as Mycobacterium tuberculosis, Salmonella typhi, Brucella melitensis, and Francisella tularensis (Balbi et al., 1993; Barnes et al., 1992; Bertotto et al., 1993; Hara et al., 1992; Sumida et al., 1992), and following infections with parasites such as Plasmodium vivax and Plasmodium falciparum (Ho et al., 1990; Perera et al., 1994). Similarly, γδ T cells expand upon in vitro culture of lymphocytes with soluble extracts of mycobacteria (Holoshitz et al., 1989; Kabelitz et al., 1990b; Modlin et al., 1989), salmonella (Hara et al., 1992), and other pathogens (Behr and Dubois, 1992; Bender and Kabelitz, 1992). The human γδ T cells expanded by these pathogens express Vγ2 and Vδ2 TCR gene segments. Responses to pathogens by these cells are relatively independent of their junctional region sequences, a characteristic in common with the Vβ-related activation of αβ T cells by superantigens.

The mycobacterial antigens that expand Vγ2Vδ2$^+$ T cells are small (<500 Da), protease resistant, and contain critical phosphate moieties (Constant et al., 1994; Pfeffer et al., 1990; Schoel et al., 1994; Tanaka et al., 1994). Recently, we have determined the structure of these natural antigens as isopentenyl pyrophosphate (IPPP) and a related prenyl pyrophosphate, important intermediates for many critical biosynthetic pathways based on isoprenoid subunits in both prokaryotes and eukaryotes (Tanaka et al., 1995). Isoprenoid subunits are required for the synthesis of dolicol phosphates (membrane components important for carbohydrate attachments), carotenoids (vita-

min A compounds), ubiquinones (respiratory proteins in bacteria), and cholesterol and sterol compounds. Nucleotide conjugates of IPPP and related compounds also stimulate Vγ2Vδ2$^+$ T cells (Constant et al., 1994; Tanaka et al., 1995). A synthetic analog of IPPP, monoethyl phosphate (MEP), precisely mimics the ability of IPPP to stimulate human γδ T cells (Tanaka et al., 1994). Thus, this group of nonpeptide antigens recognized by γδ T cells are fundamentally distinct from conventional protein antigens or protein superantigens recognized by αβ T cells.

The unusual chemical structure of these nonpeptide antigens suggests that they may be presented to γδ T cells in a distinct manner. Conventional protein antigens are presented to αβ T cells through two major presentation pathways (Germain and Margulies, 1993). In the MHC class I pathway, peptides generated from endogenous cytoplasmic proteins are transported into the endoplasmic reticulum, and associate with MHC class I heavy chains and β_2-microglobulin (β_2m). In the MHC class II pathway, peptides generated from extracellular proteins associate with the MHC class II molecules in the acidic endosomal environment. In contrast, superantigens do not require processing, because they are proteins that bind directly to MHC class II molecules on the cell surface and to the TCR β or TCR γ chain of the TCR (Herman et al., 1991).

In this study, we show that the recognition of secreted prenyl pyrophosphate antigens by human γδ T cells could be accessory cell independent and did not require processing. Unlike presentation pathways for αβ T cells, prenyl pyrophosphate antigen presentation did not require the expression of known antigen-presenting molecules, including classical MHC class I, MHC class II, CD1a, CD1b, CD1c, or other β_2m-dependent molecules. Antigen internalization also was not required as fixed accessory cells were able to present the prenyl pyrophosphate antigens. Moreover, these antigens could not be "pulsed" onto accessory cells and needed to be present continuously during T cell stimulation. Thus, we propose that prenyl pyrophosphate antigens are presented through a novel extracellular pathway of presentation that does not require antigen internalization or processing. This pathway allows for the rapid recognition of bacteria by γδ T cells and suggests that γδ T cells play a role in the early response to bacterial infection.

Results

γδ T Cells Respond to Secreted Mycobacterial Antigens

To determine whether the prenyl pyrophosphate antigens of mycobacteria were secreted or were sequestered in the cytoplasm of the bacterium, M. fortuitum was cultured in vitro for varying periods of time, harvested, and sonicated. The amounts of biological activity in the mycobacterial extracts and the culture broth were quantitated by stimulation of the Vγ2Vδ2$^+$ clone, DG.SF68. The level of biological activity in the culture broth increased with time until constituting 75% of the total biological activity (Figure 1A). Since the logarithmic phase of mycobacterial growth was tested, it was unlikely that significant bacterial death was oc-

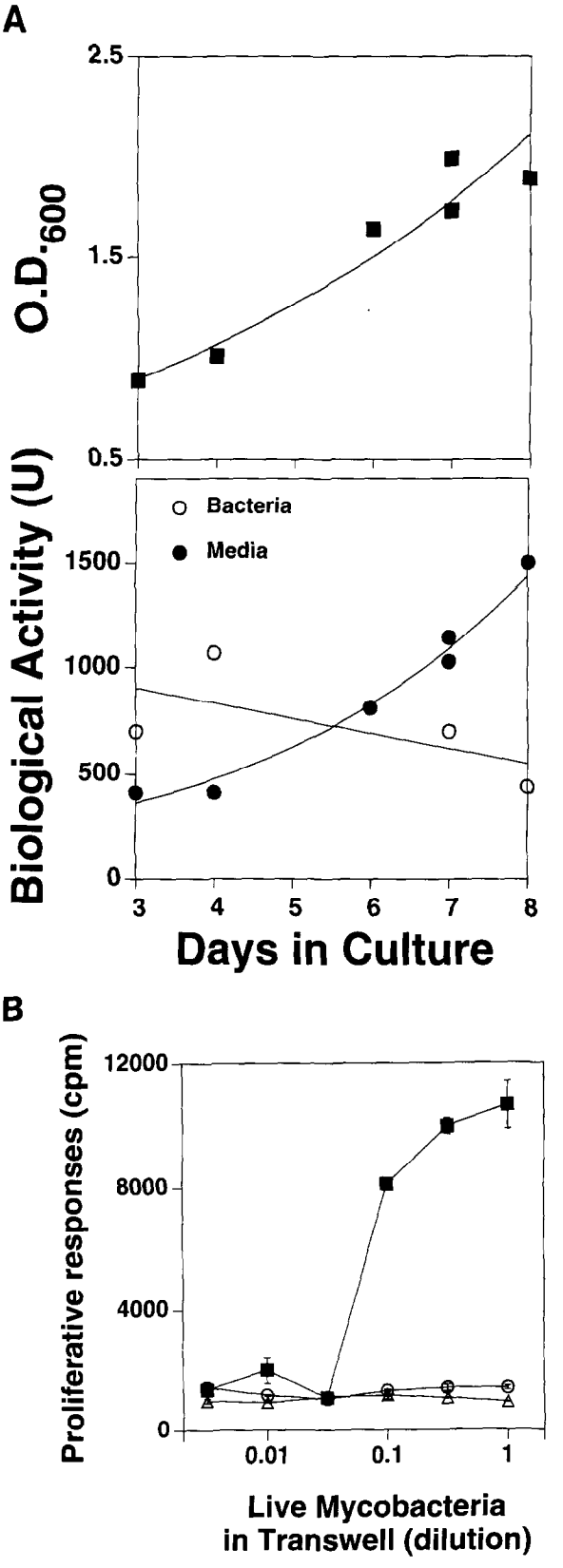
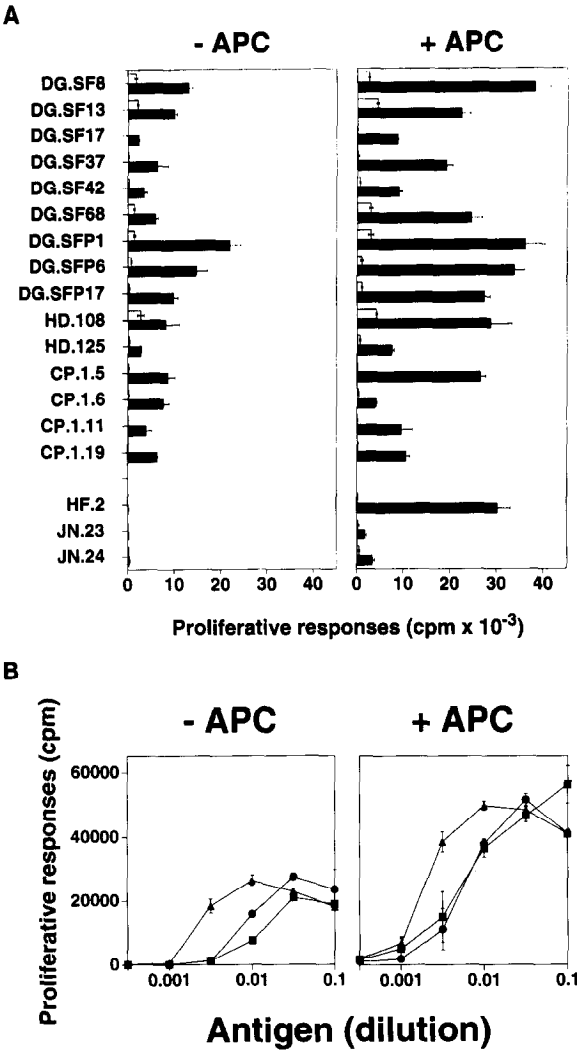


Figure 1. Mycobacterial Secretion of Prenyl Pyrophosphate Antigens for γδ T Cells

(A) Secretion of prenyl pyrophosphate antigens by mycobacteria. M. fortuitum was cultured for the indicated period in Middlebrook's media. The optical density of the bacteria was measured at 600 nm at each



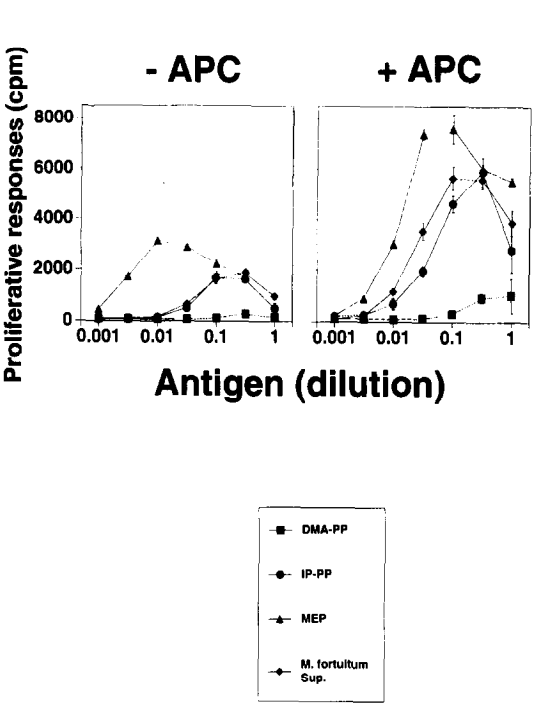


Figure 2. Response of Vγ2Vδ2+ T Cell Clones to Prenyl Pyrophosphate Antigens in the Absence of Other Accessory Cells

(A) Vγ2Vδ2+ T cell clones were cultured in the absence (open bars) or presence of M. tuberculosis extract (closed bars) with (right) or without (left) PBMC as a source of APC and accessory cells. Note that although $CD4^-CD8^-$ and $CD8^+$ γδ clones proliferated without the requirement for PBMC, the $CD4^+$ γδ clones, HF.2, JN.23, and JN.24, required PBMC for proliferation.

(B) Purified populations of the γδ T cell clone, DG.SF68, proliferate to prenyl pyrophosphate antigens in the absence of other cell types. Resting T cell cultures were stained with MAbs against the CD14 macrophage surface marker and the complement receptor 1 (CD35) B cell surface marker and then residual macrophages and B cells were negatively deleted with magnetic beads. A portion of the resulting purified T cell population was stained with FITC–control or FITC–anti-δ chain MAbs and analyzed by flow cytometry. The purified DG.SF68 cells were used in a proliferation assay in the absence (left) or presence (right) of PBMC with MEP (closed triangle), MMP (closed circle), or mycobacterial extract (closed square).

(C) The CP.1.15 γδ T cell clone proliferates in the absence of accessory cells to natural antigens from mycobacterial supernatant or to synthetic isopentenyl pyrophosphate. T cells were cultured in the absence (left) or presence (right) of PBMC with mycobacterial supernatant (closed diamond), isopentenyl pyrophosphate (31.6 μM) (closed circle), dimethylallyl pyrophosphate (31.6 μM) (closed square), or MEP (closed triangle).

curring. Other mycobacterial strains, including M. tuberculosis and M. smegmatis, as well as Gram-negative bacteria, such as Escherichia coli and Xanthamonas maltiphilia, also secreted antigenic activity (data not shown). This secretion of natural prenyl pyrophosphate antigens into the extracellular environment suggested that γδ T cells may encounter free antigen in vivo.

To extend these observations, the recognition of secreted prenyl pyrophosphate antigens of mycobacteria was assessed in a transwell system. For these experiments, live mycobacteria were placed in an inner well separated by a 0.4 μm membrane from an outer well containing Epstein–Barr virus (EBV)-transformed B cells and either αβ or γδ T cells. The membrane prevented the passage of whole bacteria but allowed soluble molecules to pass through. As expected, a mycobacteria-specific αβ T cell line (that reacts with sonicated M. smegmatis ex-

timepoint from separate cultures set up in parallel. The bacteria from 50 ml of media then were harvested by centrifugation and probe sonicated. The total bioactivity was determined for the spent media and the bacterial lysates by bioassay.

(B) Recognition of mycobacteria by γδ T cells does not require direct contact between the mycobacteria and the T cells or APCs. Live M. smegmatis mycobacteria were serially diluted and added to the inner wells of transwells, where they were separated from the outer wells by a 0.4 μm membrane. The γδ T cell clone, CP.1.15 (closed square), the mycobacteria-specific $CD4^+$ αβ T cell line, DG.1 (open circle), or the tetanus toxin–specific $CD4^+$ αβ T cell clone, SP-F3 (open triangle) were added to the outer wells (5×10^6 cells/well) with mitomycin C-treated DG.EBV cells (5×10^6 cells/well). After culture for 24 hr in the presence of 0.03 nM rIL-2, the cells in the outer wells were resuspended and 0.2 ml of the cell suspension added to three wells of a 96-well plate. Each well was pulsed with [^{3}H]thymidine, and harvested 24 hr later. Positive controls included stimulation with phytohemagglutinin for CP.1.15 (5,426 mean cpm) and SP-F3 (27,315 mean cpm) and with M. smegmatis extract for DG.1 (5,223 mean cpm).

tracts presented by the B cell line), and a tetanus toxin-specific αβ T cell clone did not proliferate when physically separated from the mycobacteria (Figure 1B). In contrast, the Vγ2Vδ2$^+$ T cells proliferated strongly despite the physical separation of the mycobacteria and the γδ T cells and the B cell line (Figure 1B). Thus, neither phagocytosis of whole mycobacteria or its fragments by antigen-presenting cells (APCs) nor direct contact with mycobacteria by either APC or γδ T cells was required for antigen recognition. Instead, Vγ2Vδ2$^+$ T cells could respond to secreted prenyl pyrophosphate antigens from live mycobacteria.

Vγ2Vδ2$^+$ T Cell Clones Can Respond to Prenyl Pyrophosphate Antigens in the Absence of Accessory or APCs

We next sought to determine whether accessory or APCs were required for the recognition of the mycobacterial antigens. A panel of Vγ2Vδ2$^+$ T cell clones derived from adult blood or synovial fluid was tested for responsiveness to antigens present in M. tuberculosis extracts in the presence or absence of other cells. As expected, all Vγ2Vδ2$^+$ T cell clones responded to the M. tuberculosis extract, including those that were CD4$^-$CD8$^-$, CD8$^+$, and CD4$^+$ (Figure 2A). While all of the clones responded in the presence of peripheral blood mononuclear cells (PBMCs) as APCs and low levels of human IL-2 (Figure 2A, right), surprisingly, most clones also responded in the absence of PBMC, although at lower levels (Figure 2A, left). To ensure that no macrophages or B cells remained in the T cell populations, the DG.SF68 clone was treated to remove potential accessory cells by negative magnetic bead deletion with monoclonal antibody (MAb) to CD14 (a macrophage marker) and to the complement receptor, CR2 (a B cell marker). FACS analysis confirmed that non-T cells comprised less than 0.3% of the purified DG.SF68 population. These purified T cells responded strongly to the M. tuberculosis extract as well as to the synthetic monoalkyl phosphates, MMP and MEP (Figure 2B, left). Similarly, a second γδ T cell clone, CP.1.15, proliferated when cultured in the absence of other cells with either mycobacterial supernatant, synthetic isopentenyl pyrophosphate, or MEP (Figure 2C, left). The low level of proliferation of some CD4$^-$CD8$^-$ γδ T cell clones when exposed to prenyl pyrophosphate antigens in the absence of other cells was augmented significantly by the addition of rIL-2 (data not shown) or by the addition of accessory cells (Figures 2A, 2B, and 2C). Thus, accessory cells may play a role in prenyl pyrophosphate recognition by providing IL-2, by increasing the synthesis of IL-2 by γδ T cells, or by providing other undefined signals. However, γδ T cell recognition of the prenyl pyrophosphate antigens clearly occurred in the absence of these signals.

To confirm further that the recognition of prenyl pyrophosphate antigens could occur in the absence of other cells, we tested the ability of the Vγ2Vδ2 TCR transfectant, DBS43, to recognize prenyl pyrophosphate antigens (Bukowski et al., 1995). This tumor cell line was cultured continuously without the addition of feeder cells and therefore represented a pure T cell population. The DBS43 transfectant specifically released IL-2 when exposed either to synthetic MEP (Figure 3, right) or to mycobacterial extracts (data not shown). The addition of the autologous EBV-transformed B cell line as a possible APC resulted in a minor enhancement (approximately 2-fold) in the DBS43 IL-2 response. The parent T cell clone, DG.SF13, from which the DBS43 TCR cDNAs were cloned, also responded either to MEP (Figure 3, left) or to mycobacteria (data not shown), although the addition of the B cells augmented the amount of IL-2 released in response to MEP by 50-fold. Thus, Vγ2Vδ2$^+$ T cells could respond both to natural mycobacterial antigens and to synthetic monoalkyl phosphates in the absence of other cells. Although accessory cells or APCs were not required by most Vγ2Vδ2$^+$ T cells, the presence of accessory cells or APCs augmented the magnitude of the responses. Interestingly, the group of γδ clones that did require accessory cells for prenyl pyrophosphate recognition (clones HF.2, JN.23, and JN.24) are from the distinct functional subset of CD4$^+$ γδ T cells (Morita et al., 1991; Spits et al., 1991) and thus, this subset may have presentation requirements distinct from most γδ T cells.

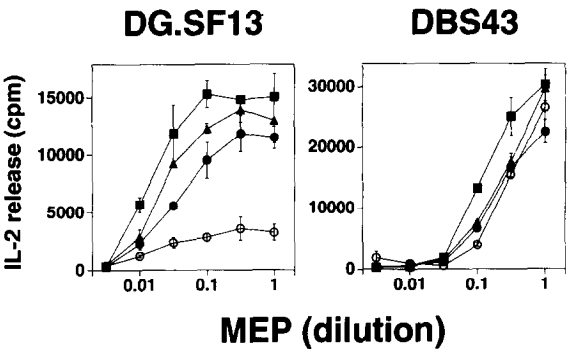


Figure 3. IL-2 Release to Monoethyl Phosphate by the Vγ2Vδ2 TCR Transfectant, DBS43, in the Absence of Accessory Cells

The DBS43 transfectant or the TCR-parent DG.SF13 T cell clone was incubated with MEP for 24 hr in the absence (open circle) or presence of varying numbers of DG.EBV cells (5 × 10^4 [closed circle], 1 × 10^5 [closed triangle], and 2 × 10^5 [closed square] per well). The supernatants then were harvested and IL-2 levels were assessed by proliferation of the IL-2-dependent CTLL20 cell line. In the absence of MEP, the DBS43 transfectant did not release detectable IL-2. Note that the DBS43 transfectant did not require DG.EBV cells for maximal IL-2 release, whereas the TCR parent DG.SF13 clone required DG. EBV cells for maximal IL-2 release.

T Cell-T Cell Contact Is Required for the Recognition of Prenyl Pyrophosphate Antigens in the Absence of APCs

As most Vγ2Vδ2$^+$ T cells could respond to prenyl pyrophosphate antigens in the absence of other cell types, it was possible that γδ T cells recognized these antigens alone in solution. Alternatively, presentation could have occurred through the ability of γδ T cells to associate with the prenyl pyrophosphate antigens and to present them to neighboring T cells. Such T cell presentation of antigen has been demonstrated for MHC class I- and II-restricted αβ T cell clones in the presence of preprocessed peptides (LaSalle et al., 1993; Pichler and Wyss-Coray, 1994; Su et al., 1993; Suhrbier et al., 1993).

To determine the mechanism of recognition employed by γδ T cells, calcium flux by a γδ T cell clone was assessed following exposure to prenyl pyrophosphate antigens. For these studies, the DG.SF68 γδ T cell clone was loaded with the calcium indicator, indo-1, and the intracellular calcium levels of the T cell clone (expressed as the mean ratio of indo-1 fluorescence at 410/480 nm) were determined by flow cytometry. Following the addition of MEP or mycobacterial extract (Figure 4A, upper left, first arrow) to a single cell suspension of DG.SF68 cells, intracellular calcium levels were determined for 6 min. No calcium flux was detected for such a suspension of single cells (Figure 4A, upper left). However, when an identical aliquot of DG.SF68 cells in suspension with MEP was centrifuged for 0.5 min to effect cell–cell contact (Figure 4A, upper right, second arrow), resuspended, and analyzed by flow cytometry, a clear peak of calcium flux (3- to 5-fold increase over baseline levels) occurred, indicating TCR signaling (Figure 4A, upper right). No such calcium flux was noted in control αβ T cells (Figure 4A, bottom).

To examine directly whether solitary T cells were capable of responding to prenyl pyrophosphate antigens, γδ T cells were exposed to the synthetic analog MEP, and cellular calcium flux was assessed by microscope-based laser cytometry. Indo-1-loaded T cells were observed individually under a microscope and intracellular calcium levels were determined by the ratio of indo-1 fluorescence at 400 nm to that at 480 nm. As seen in representative pseudocolor images (Figure 4B, top), solitary γδ T cells exposed to MEP exhibited no change in intracellular calcium levels (as represented by the blue color), although exposure to a calcium ionophore caused large increases in intracellular calcium levels (as represented by the green–red color). In contrast, T cells exposed to MEP and placed in contact with other T cells by centrifugation exhibited increases in intracellular calcium levels (Figure 4B, bottom middle, see clusters of green–yellow cells). No such increases in calcium levels were seen after centrifugation in the absence of MEP (Figure 4B, bottom left). Thus, unlike compounds such as ionomycin, neurotransmitters, and hormones, which activate individual cells without a cell–cell contact requirement, the activation of γδ T cells by prenyl pyrophosphate antigens required cell–cell contact.

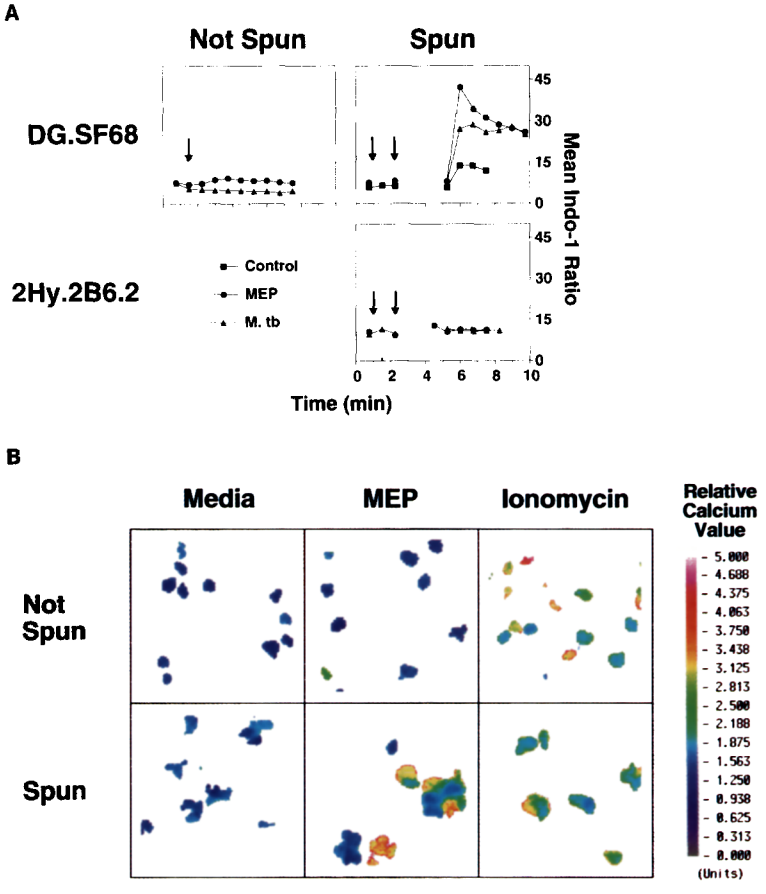


Figure 4. T Cell–T Cell Contact Is Required for Calcium Flux to Prenyl Pyrophosphate Antigens in the Absence of Accessory Cells

(A) Determination of calcium flux by flow cytometry. The Vγ2Vδ2$^+$ T cell clone, DG.SF68 (top), or the MBP-specific αβ clone, 2Hy.2B6.2 (bottom), were loaded with indo-1 and assessed for calcium flux by flow cytometry. Media (closed square), MEP (closed circle), or M. tuberculosis extract (closed triangle) was added at the first arrow and, at the second arrow, the cells were centrifuged for 1 min (right) and reapplied to the cytometer after 2–3 min, or were not centrifuged (left). The mean indo-1 ratio is plotted. Note that calcium flux by the γδ T cell was seen with both MEP and the mycobacterial extract only after centrifugation resulted in T cell–T cell contact. No such cell–cell contact requirement was noted for calcium flux induction by mitogenic TCR signaling with a polyvalent IgM anti-CD3 MAb or with the calcium ionophore, ionomycin (data not shown).

(B) Determination of relative intracellular calcium ion concentrations in individual T cells by interactive laser cytometry. The γδ T cell clone, CP.1.15, was loaded with indo-1 and analyzed for $[Ca^{2+}]_i$ response at 4–6 min to media (left), MEP (1/1000 dilution, middle), or ionomycin (2 μg/ml, right) without (top) or with (bottom) centrifugation to effect cell–cell contact. The colorimetric scale represents relative units of $[Ca^{2+}]_i$ based on the ratio of indo-1 fluorescence intensities at 400 nm and 480 nm. Representative micrographs are shown. The percentage of responsive T cells (indo-1 ratio > 2.0) under each condition was 0% (0 of 14 cells) for not spun-media; 8% (1 of 12 cells) for not spun-MEP; 96% (25 of 26 cells) for not spun-ionomycin; 7% (5 of 77 cells) for spun-media; 63% (47 of 73 cells) for spun-MEP; and 92% (24 of 26 cells) for spun-ionomycin. Note the increased calcium ratio in the clusters of T cells upon centrifugation in the presence of MEP (middle panel, bottom).

Recognition of Prenyl Pyrophosphate Antigens Does Not Require MHC Class II, Classical MHC Class I, β_2m, CD1, TAP1/TAP2, or DMA/DMB Expression

Since cell–cell contact was necessary for the recognition of the prenyl pyrophosphate antigens by γδ T cells, we examined the requirement for the expression of known antigen-presenting molecules for this recognition using several approaches. First, MAbs against MHC class I, MHC class II, β_2m, or CD1 molecules were used to attempt to block the recognition of nonpeptide antigens in cytolytic or proliferative assays. Second, the Vγ2Vδ2 TCR transfectant, DBS43, was examined for MHC class II expression. Lastly, mutant cell lines lacking MHC class I, MHC class II, or CD1 molecules were used either as targets for lysis by $V\gamma2V\delta2^+$ T cells in the presence of prenyl pyrophosphate antigens or as accessory cells to augment the proliferation of $V\gamma2V\delta2^+$ T cells in response to such antigens. The $CD4^+$ γδ T cell clone, HF.2, was used as the responder cell for the proliferation assays, because this clone required accessory cells to respond to prenyl pyrophosphate antigens (see Figure 2A). Because similar accessory cell-dependent antigen-specific $CD4^+$ αβ T cell clones proliferate to preprocessed peptide antigens only in the presence of accessory cells expressing the restricting MHC molecule (Sinigaglia et al., 1988), studies were performed to determine whether expression of classical antigen-presenting molecules on the accessory cells was required for the recognition of prenyl pyrophosphate antigens by this clone.

The addition of MAbs specific for MHC class I, MHC class II, β_2m, or CD1a, CD1b, or CD1c molecules did not significantly block the recognition of mycobacterial antigens or MEP by $CD4^-CD8^-$ $V\gamma2V\delta2^+$ T cell clones in both cytolytic and proliferative assays (data not shown). This suggested that known presentation elements may not present the nonpeptide prenyl pyrophosphate antigens.

To assess further the requirement for MHC class II expression, we examined MHC class II expression on the γδ TCR transfectant DBS43. The DBS43 transfectant totally lacked expression of class II molecules (Figure 5A), yet was able to recognize prenyl pyrophosphate antigens in the absence of other cells (see Figure 3). To confirm further that MHC class II does not present the nonpeptide antigens, the mutant MHC class II–negative cell line RJ-2.2.5, and its MHC class II–positive parent cell line Raji, were used as targets for lysis by the $V\gamma2V\delta2^+$ T cell clone DG.SF8 in the presence of prenyl pyrophosphate antigens. Staphylococcal enterotoxin A (SEA) was used as a control stimulus because it is recognized by Vγ2-bearing γδ T cells when presented by MHC class II molecules (Rust et al., 1990). As expected, in the presence of SEA the DG.SF8 T cell clone lysed the class II–positive parent cell line, Raji, but failed to lyse the class II–negative mutant cell line, RJ-2.2.5 (Figure 5B). In contrast, in the presence of a mycobacterial extract the DG.SF8 clone lysed both the Raji and the mutant RJ-2.2.5 line (Figure 5B), indicating that MHC class II expression is not required for the recognition of mycobacterial antigen-sensitized target cells. Similarly, a variety of class II–deficient EBV-transformed B cell lines functioned as target cells for a second $V\gamma2V\delta2^+$ T cell clone LG.C6, in the presence but not in the absence of a mycobacterial extract (Figure 5C).

To assess further the requirement for classical class I molecules, we used the mutant APC 721.221, which lacks HLA-A, HLA-B, and HLA-C expression, and the mutant APC FO-1, which lacks β_2m expression (D'Urso et al., 1991; Rajagopalan and Brenner, 1994) as accessory cells. Both mutant APCs presented the mycobacterial antigens to the APC-dependent γδ clone HF.2 (Figure 5D, experiment 1). Expression of CD1a, CD1b, and CD1c molecules also was not required, since EBV-transformed B cell lines used in these experiments do not express these molecules (data not shown) yet presented prenyl pyrophosphate antigens. Taken together, these data utilizing MAb blocking, TCR transfectants, and mutant-presenting cell lines suggested that the recognition of prenyl pyrophosphate antigens by γδ T cells did not require classical MHC class I, MHC class II, or CD1 expression.

Although MHC class I and II molecules were not required for the presentation of prenyl pyrophosphate antigens, it remained possible that the TAP1 and TAP2 or DMA and DMB molecules might play a role. To determine the requirement for these molecules, the mutant cell line 721.174 was used as an APC. This cell line has deletions in the MHC locus resulting in a loss of class II molecules, the TAP1 and TAP2 peptide transporters that are critical for the peptide loading of class I molecules (Spies et al., 1990), and the DMA and DMB molecules that play roles in the peptide loading of class II molecules (Denzin et al., 1994). Despite these defects, the 721.174 cell line efficiently presented prenyl pyrophosphate antigens in a mycobacterial extract to the HF.2 γδ T cell clone (Figure 5D, experiment 2). Thus, the requirements for the presentation of monoalkyl phosphate antigens to $V\gamma2V\delta2^+$ T cell clones such as HF.2 were clearly distinct from the requirements for the presentation of protein antigens using MHC class I or class II antigen-presenting pathways.

Prenyl Pyrophosphate Antigens Do Not Require Antigen Uptake or Processing

Although the MHC class I and class II peptide loading pathways were not required for prenyl pyrophosphate antigen presentation, there may be other intracellular pathway(s) required for prenyl pyrophosphate antigen presentation. To assess this possibility, EBV-transformed B cells were fixed to prevent the uptake and processing of the prenyl pyrophosphate antigens. Then, the fixed cells were used as APCs for the APC-dependent γδ clone HF.2. Both the untreated B cells and the fixed B cells were capable of presenting the antigens in a mycobacterial extract to the HF.2 clone (Figure 6, top, experiment 1; mean proliferation of 17,290 cpm for the untreated cells versus 10,647 cpm for the fixed cells). Similarly, both untreated and fixed B cells were capable of efficiently presenting synthetic isopentenyl pyrophosphate and the prenyl pyrophosphate antigens in a mycobacterial supernatant (Figure 6, bottom, experiment 2). In contrast, fixed PBMC failed to present antigen to the M. tuberculosis–specific $CD4^+$ αβ T cell

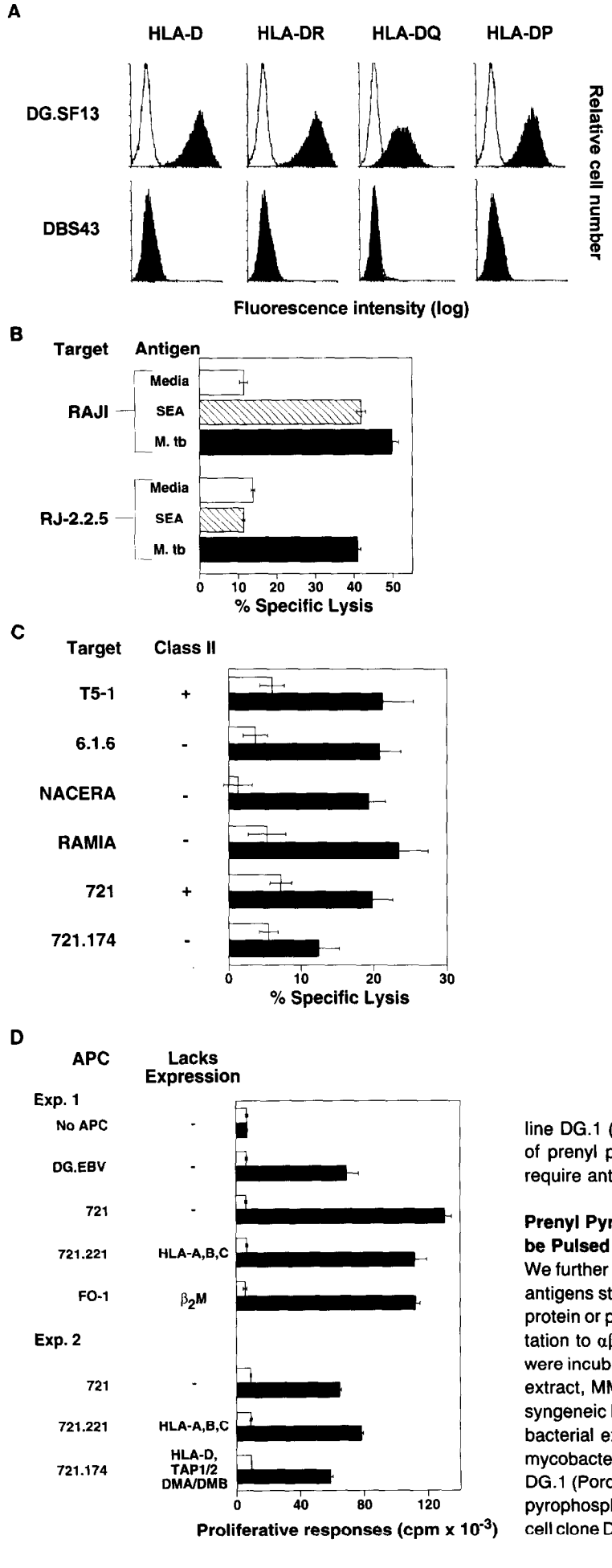


Figure 5. Vγ2Vδ2+ T Cells Respond to Prenyl Pyrophosphate Antigens in the Absence of MHC Class II Molecules (HLA-DR, HLA-DQ, HLA-DP, HLA-DMA, or HLA-DMB), Classical MHC Class I Molecules (HLA-A, HLA-B, or HLA-C), β_2m-Dependent Molecules, and TAP1/TAP2 Transporters

(A) The Vγ2Vδ2 TCR transfectant, DBS43, is MHC class II negative. MHC class II expression by the γδ T cell clone, DG.SF13, and the Vγ2Vδ2 TCR transfectant, DBS43, was assessed by staining with class II–specific MAbs and analyzing by one-color flow cytometry. The MAbs used were anti-pan class II (IVA12), anti-HLA-DR (LB3.1), anti-HLA-DQ (Leu10), and anti-HLA-DP (B7/21). Control MAb (P3) staining is shown as open profiles, whereas class II staining is shown as closed profiles.

(B) MHC class II expression is not required for the killing of target cells by γδ T cells upon exposure to a mycobacterial extract. The Burkitt's lymphoma, Raji, and its class II–deficient mutant, RJ-2.2.5, were used as chromium labeled targets in a standard 4 hr cytotoxicity assay with the γδ clone, DG.SF8, in the absence (open bars) or the presence of the superantigen, SEA (striped bars) or a M. tuberculosis extract (closed bars). E:T was 100:1. Note that the recognition of the SEA superantigen by γδ T cells required class II expression, whereas the recognition of mycobacterial extract did not.

(C) Parent and mutant EBV lines lacking class II expression due to induced mutations (6.1.6 and 721.174) or inherited mutations (Nacera and Ramia) were used as targets in a 4 hr cytotoxicity assay as described above, except clone LG.C6 was used as the effector in the absence (open bars) or the presence (closed bars) of M. tuberculosis extract. E:T was 31.6:1.

(D) Expression of classical MHC class I, β_2m-dependent molecules, MHC class II, TAP1/2, and DMA/DMB is not required for the recognition of prenyl pyrophosphate antigens by the Vγ2Vδ2+ HF.2 clone. The HF.2 T cell clone (APC dependent) was cultured with (experiment 1) the syngeneic EBV line DG.EBV, the parent EBV line 721, the mutant EBV line 721.221 (lacking HLA-A, HLA-B and HLA-C), and the melanoma cell line FO-1, (lacking β_2m), or with (experiment 2) 721, 721.221, and 721.174 (lacking class II, TAP1/TAP2, and DMA/DMB) in the absence (open bars) or presence (closed bars) of M. tuberculosis extract.

line DG.1 (data not shown). Therefore, the presentation of prenyl pyrophosphate antigens to γδ T cells did not require antigen uptake or processing.

Prenyl Pyrophosphate Antigens Cannot be Pulsed on APCs

We further determined whether the prenyl pyrophosphate antigens stably associated with APCs as do conventional protein or peptide antigens and superantigens for presentation to αβ T cells. Syngeneic EBV-transformed B cells were incubated for 4 hr with either a crude M. tuberculosis extract, MMP, MEP, or PPD and then washed. Whereas syngeneic EBV-transformed B cells pulsed with the mycobacterial extract, or the PPD preparation stimulated the mycobacteria-specific DR7-restricted CD4+ αβ T cell line DG.1 (Porcelli et al., 1992), B cells pulsed with the prenyl pyrophosphate antigens did not stimulate the Vγ2Vδ2+ T cell clone DG.SF68 (Figure 7). Since γδ T cells themselves

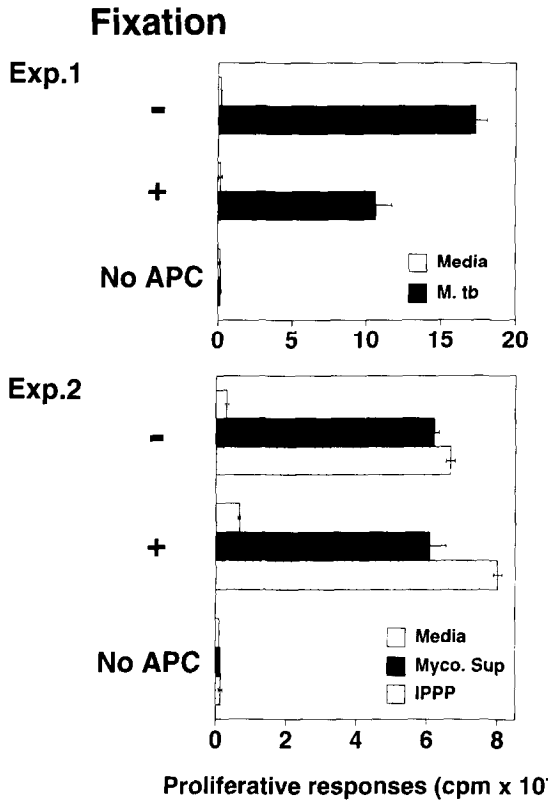


Figure 6. Lack of Antigen Uptake and Processing Requirement for Prenyl Pyrophosphate Antigen Recognition

The APC-dependent Vγ2Vδ2+ T cell clone, HF.2, was cultured with unfixed or fixed EBV-transformed B cells (experiment 1, top) in the absence (open bars) or presence (closed bars) of M. tuberculosis extract or (experiment 2, bottom) in the absence (open bars) or presence of mycobacterial supernatant (closed bars) or synthetic isopentenyl pyrophosphate (31.6 μM) (striped bars). APC were fixed by a 15 s exposure to 0.05% glutaraldehyde.

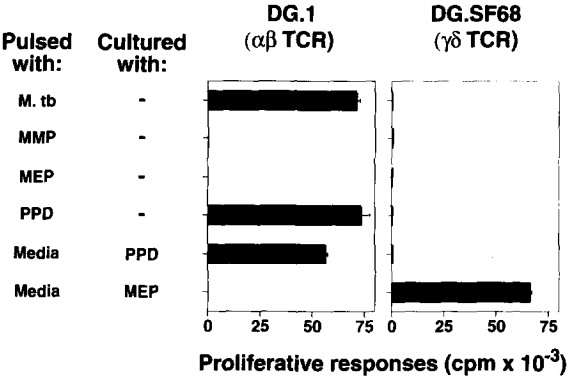


Figure 7. Prenyl Pyrophosphate Antigens Cannot Be "Pulsed" onto APCs

The syngeneic EBV-transformed B cell line, DG.EBV, was treated with mitomycin C, pulsed with either crude mycobacterial extract, monomethyl phosphate (MMP), monoethyl phosphate (MEP), or PPD by incubation for 4 hr at 37°C, and then washed three times. The γδ T cell clone, DG.SF68, or the mycobacteria-specific CD4+ αβ T cell line, DG.1, then were cultured with the pulsed APC in the presence of 0.03 nM rIL-2 for 48 hr. MEP and PPD were added as positive controls for each APC group (only the unpulsed control APC group is shown as the other groups gave similar levels of proliferation).

may express a presenting element, the γδ T cell clone DG.SF68 was pulsed with the prenyl pyrophosphate antigens, treated with mitomycin C, and used as presenting cells for untreated DG.SF68 T cells. Again, no stimulation of the untreated DG.SF68 T cells was noted (data not shown). Further attempts to pulse prenyl pyrophosphate antigens onto syngeneic and allogeneic PBMC using phosphate-containing or phosphate-free wash buffers also were unsuccessful (data not shown). Thus, pulsing APC with synthetic and partially purified natural prenyl pyrophosphate antigens did not result in APC stimulatory for Vγ2Vδ2+ T cells, suggesting that these antigens could not stably associate with the APC cell surface in an immunogenic form. Rather, the continual presence of these antigens was required for T cell activation.

Discussion

We have examined the presentation of nonpeptide prenyl pyrophosphate antigens to human γδ T cells. We find that the recognition of prenyl pyrophosphate antigens by Vγ2Vδ2+ T cells can occur through an extracellular pathway involving the secretion of prenyl pyrophosphate antigens by mycobacteria. Whereas cell–cell contact between γδ T cells (or between γδ T cells and other cell types) is required for prenyl pyrophosphate recognition, γδ T cells do not require specialized accessory cells for recognition to occur. Moreover, antigen uptake and processing is not required for recognition by Vγ2Vδ2+ cells, as evidenced by the ability of mutant APCs lacking peptide loading pathways and fixed APCs to present the prenyl pyrophosphate antigens. Finally, the expression of classical MHC class I, MHC class II, and CD1a, CD1b, or CD1c molecules is not required for prenyl pyrophosphate antigen recognition by γδ T cells, confirming some earlier studies (Fisch et al., 1990; Holoshitz et al., 1989, 1993). Clearly, Vγ2Vδ2+ T cells recognize prenyl pyrophosphate antigens in a manner distinct from that of αβ T cell recognition of conventional protein antigens, CD1-restricted lipid antigens, and superantigens.

In some aspects, nonpeptide prenyl pyrophosphate recognition by γδ T cells resembles peptide antigen recognition by αβ T cells (Wyss-Coray et al., 1992; Yewdell and Bennink, 1992). Like the recognition of nonpeptide antigens, the recognition of peptide antigens by MHC class I– or MHC class II–restricted human T cells does not require professional APCs due to the ability of T cells to present to other T cells through cell–cell contact (LaSalle et al., 1993). Moreover, like nonpeptide antigen recognition, peptide antigen recognition does not require antigen uptake or processing, because peptides can directly bind to cell surface MHC class I or class II molecules (Hosken et al., 1989). There are, however, important differences between peptide recognition and prenyl pyrophosphate recognition. Whereas peptide antigens are presented by either MHC class I or class II molecules, prenyl pyrophosphate antigens do not appear to use these molecules or

CD1 molecules in the recognition process. Moreover, whereas peptide antigens form stable complexes with MHC molecules on the surface of APCs, prenyl pyrophosphate antigens do not. Finally, whereas exogenous peptide antigens must be generated from proteins by proteolysis (Moore et al., 1988; Sherman et al., 1992), the prenyl pyrophosphate antigens are secreted in an immunogenic form by several mycobacterial species.

There is strong evidence that the recognition of prenyl pyrophosphate molecules is TCR mediated. First, this recognition can be blocked by MAbs to the γδ TCR (Munk et al., 1990; Tanaka et al., 1994). Second, the γδ T cell expansions noted in response to these molecules are restricted to γδ cells expressing the Vγ2 (also termed Vγ9) and Vδ2 gene segments (De Libero et al., 1991; Kabelitz et al., 1991; Panchamoorthy et al., 1991; Tanaka et al., 1994), and only γδ T cell clones expressing Vγ2Vδ2 TCR are reported to respond to the prenyl pyrophosphate antigens (Davodeau et al., 1993; De Libero et al., 1991). Finally, transfection of cDNAs encoding a Vγ2Vδ2 TCR into the TCR loss mutant of the αβ T cell tumor, Jurkat, confers responsiveness to the nonpeptide prenyl pyrophosphate antigens, whereas transfer of cDNAs encoding a Vγ1Vδ1 TCR do not (Bukowski et al., 1995). Thus, since recognition involves the γδ TCR, the prenyl pyrophosphate molecules are likely to function as antigens for γδ T cells rather than as mitogens.

Models of Prenyl Pyrophosphate Antigen Recognition

Although our data are most consistent with TCR-mediated recognition of prenyl pyrophosphate molecules, it remains possible that these molecules function to enhance the recognition of another ligand rather than acting as classical antigens. However, we favor models in which there is direct recognition of either the prenyl pyrophosphate molecules or the complex of the prenyl pyrophosphate molecules and a presenting element (Figure 8). In these models, the Vγ2Vδ2 TCR binds to prenyl pyrophosphate antigens either in solution or at the cell surface with or without a presenting molecule (Figure 8). If presenting molecules exist for prenyl pyrophosphate antigens, they are not conventional MHC class I, MHC class II, or CD1 proteins. The hypothetical presenting molecules would be unable to form stable complexes with prenyl pyrophosphate antigens and would bind these antigens rapidly (in minutes), as evidenced by the rapid induction of calcium flux in spun T cells after antigen addition, but would also have high antigen dissociation rates.

In the proposed models, we hypothesize that the γδ TCR binds directly to prenyl pyrophosphate antigens. Evidence for the direct recognition of foreign antigens by γδ T cells has been presented for the herpes simplex virus glycoprotein gI (Sciammas et al., 1994), for allogeneic MHC class II and TL molecules (Schild et al., 1994), and for native tetanus toxin (H. B. et al., unpublished data). Moreover, the length of the δ chain CDR3 region most resembles that of immunoglobulin heavy chains, leading some authors to suggest immunoglobulin-like recognition properties for

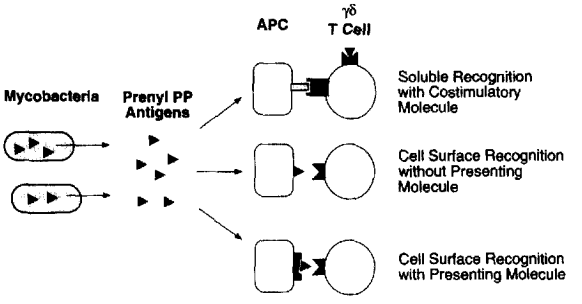


Figure 8. Possible Models of Prenyl Pyrophosphate Antigen Recognition

Prenyl PP is prenyl pyrophosphate. Note that although TCR recognition of the prenyl pyrophosphate antigens is diagrammed as involving cognate interactions with the antigen binding region of the TCR, recognition through the framework regions of the TCR variable domain cannot be excluded.

the γδ TCR (Rock et al., 1994). Thus, the Vγ2Vδ2 TCR could recognize prenyl pyrophosphate antigens in a fashion similar to that of antibodies that bind to soluble haptens such as dinitrophenol or azobezenearsonate, or to hapten-specific T cells (Morita et al., 1986; Rao et al., 1984a, 1984b; Siliciano et al., 1986). However, the recognition of soluble prenyl pyrophosphate antigens, as with the recognition of soluble haptens by B or T cells, would not be expected to cross-link the TCR. This would make it necessary for the prenyl pyrophosphate antigens to associate with the cell surface if T cell activation is to occur. If recognition of soluble prenyl pyrophosphate antigens without a presenting molecule does occur, the cell–cell contact requirement for γδ T cell activation could be explained by the need for an additional interaction with a costimulatory or accessory molecule on a cell surface.

Our data do not support models where prenyl pyrophosphate antigens induce a change in the presenting cell, such as the induction of a heat shock protein or the chemical modification of the cell surface that is recognized by the γδ T cell. Such changes would have to be rapidly induced on fixed cells and rapidly lost, given our inability to effect stable changes in the APCs that can be detected by γδ T cells. Similarly, our data do not support models where prenyl pyrophosphate molecules stimulate cytokine secretion, which in turn specifically stimulates Vγ2Vδ2$^+$ T cell proliferation and activation. Such a model would be inconsistent with the IL-2 release observed in response to prenyl pyrophosphate molecules by the Vγ2Vδ2 TCR transfectant but not the Vγ1Vδ1 TCR transfectant. Furthermore, the mutant Jurkat T cell line used here was derived from an αβ T cell tumor unlikely to express unique γδ T cell–specific cytokine receptors.

Potential In Vivo Relevance of Extracellular Presentation of Prenyl Pyrophosphate Antigens

Our data demonstrate that prenyl pyrophosphate antigens can use a novel extracellular pathway for antigen presentation distinct from the intracellular and extracellular pathways used for antigens and superantigens presented by

MHC class I, MHC class II, and CD1 molecules. We hypothesize that the secretion of prenyl pyrophosphate antigens by mycobacteria and other bacteria allows γδ T cells in vivo to recognize these antigens directly without requiring antigen uptake and processing. In fact, the intracellular passage of prenyl pyrophosphate antigens through the acidic environment of endosomes and lysosomes would be expected to inactivate these compounds by the hydrolysis of the pyrophosphate moiety from the prenyl chain (Logan, 1972).

The rapid recognition of prenyl pyrophosphate antigens secreted by a number of microorganisms and presented by an extracellular pathway may allow Vγ2Vδ2$^+$ T cells to complement the recognition of bacterial and parasitic protein antigens presented by intracellular pathways to αβ T cells. Supporting this possibility, elevated levels of Vγ2Vδ2$^+$ T cells are seen at least as early as 7 days after salmonella infection and 9 days after tularemia infection (Sumida et al., 1992). Moreover, systemic salmonella infections in humans (where bacteria can be cultured from the blood) are associated with the highest elevations of Vγ2Vδ2$^+$ T cells (Hara et al., 1992). After activation by prenyl pyrophosphate antigens, these Vγ2Vδ2$^+$ T cells can secrete a number of cytokines, including IFNγ (Barnes et al., 1992; Follows et al., 1992). Therefore, besides providing critical cytokines required for the elimination of bacteria, γδ T cells could also influence the differentiation of naive microbe-specific αβ T cells into cells secreting T_H1 cytokines. Evidence for such a role for γδ T cells has recently been reported for listeria infections in the mouse (Ferrick et al., 1995). Clearly, the unique chemical structure of the prenyl pyrophosphate antigens and the differences in their requirements for recognition underscores the distinct nature of this antigen presentation system for γδ T cells. The concepts of T cell recognition of conventional protein antigens and protein superantigens should be expanded to include the recognition of nonpeptide prenyl pyrophosphate antigens.

Experimental Procedures

Derivation of and Culture Conditions for T Cell Clones

T cell lines and clones were maintained by periodic restimulation with phytohemagglutinin as previously described (Morita et al., 1994). The derivation of the CD4$^-$CD8$^-$ and CD8$^+$ γδ T cell clones and the weakly cytotoxic CD4$^+$ γδ T cell clones, HF.2, JN.23, and JN.24, have been described (Morita et al., 1991; Spits et al., 1991; Tanaka et al., 1994). The DG.1 line is a CD4$^+$ PPD-specific αβ T cell line (Porcelli et al., 1992) and the 2Hy.2B6.2 clone is a CD4$^+$ αβ T cell clone that recognizes myelin basic protein (residues 84–102) (Ota et al., 1990). SP-F3 is a CD4$^+$ αβ T cell clone that recognizes tetanus toxoid C fragment (residues 947–961) (Roncarolo et al., 1988; data not shown).

Antigens

Sonicated mycobacterial extract was prepared from a selected lot of M. tuberculosis strain H37Ra (Lot 77645990, Difco, Detroit, Michigan) or from cultured M. smegmatis as described (Morita et al., 1994). The prenyl pyrophosphate antigens from mycobacterial supernatant was partially purified from M. fortuitum as described (Tanaka et al., 1994). MEP was prepared and purified by anion exchange (Tanaka et al., 1994). Monomethyl phosphate, isopentenyl pyrophosphate, and dimethylallyl pyrophosphate were obtained from Sigma. Staphylococcal enterotoxin B was obtained from Toxin Technology, Incorporated (Sarasota, Florida).

Mycobacterial Culture and the Secretion of the Nonpeptide Antigen

Separate cultures of M. fortuitum (a rapid growing mycobacterial species) were set up in parallel for the indicated period in Middlebrook's media. At each timepoint, the optical density of the bacteria was measured at 600 nm and the bacteria from 50 ml of media were harvested by centrifugation and then probe sonicated. The total bioactivity was determined for the spent media and the bacterial lysates by bioassay. We defined 1 U of bioactivity as the concentration of antigen necessary to achieve half-maximal proliferation of the DG.SF68 T cell clone. Assays for this experiment were performed at the same time and a standard preparation of mycobacteria with known biological activity was assayed to standardize the biological units.

Mycobacterial secretion of antigen and its recognition by γδ T cells was simultaneously evaluated in a transwell system (Costar, Cambridge, Massachusetts). Live M. smegmatis in logarithmic phase growth (OD_{600} was 0.7) were harvested from 5 ml of culture broth, washed three times in phosphate-buffered saline (PBS), and resuspended in 0.5 ml PBS. Serial dilutions of live mycobacteria, mycobacterial supernatant, synthetic isopentenyl pyrophosphate, MEP, and tetanus toxin were added to the inner wells of a transwell in 0.1 ml. The inner well was separated from the outer well by a 0.4 μm membrane. Soluble antigens also were added directly to the outer wells of control cultures. Equal numbers (6 × 10^6 cells/well) of the γδ T cell clone CP.1.15 and the mitomycin C-treated EBV-transformed B cell line DG.EBV were added to each outer well in 0.9 ml of RPMI 1640 media, and cultured for 24 hr. Then, the cells in the outer well were resuspended, 200 μl of the cell suspension were added to three wells of a 96-well plate, pulsed with [^{3}H]thymidine, and harvested 24 hr later. Soluble antigen preparation induced equal levels of specific proliferation whether added to the inner wells or added directly to the outer wells.

Proliferation Assays

T cell proliferation assays were performed as described (Morita et al., 1994). In brief, T cells were plated in triplicate in round-bottomed 96-well plates at 5–10 × 10^4 T cells per well with 1 × 10^5 irradiated syngeneic or allogeneic PBMC. Alternatively, PBMCs were replaced with mitomycin C-treated EBV-transformed lymphoblastoid B cells. Fixation of APC was by a 15 s exposure to 0.05% glutaraldehyde (Sigma, Missouri) followed by washing three times. Because the γδ T cell response to mycobacteria is not MHC restricted (Kabelitz et al., 1990b), allogeneic cells were suitable APCs. The cultures were pulsed with 1 μCi of [^{3}H]thymidine (2 Ci/mmol) on day 1 and harvested 16–18 hr later. In some cases, low levels of rIL-2 (0.03 nM) were added to the culture media. Mean proliferation and SEM are shown.

For T cells lacking APC, resting γδ T cell clones were used 2–16 weeks following restimulation. By this time, irradiated APCs had died and the cells were 98%–99$^+$% T cells as determined by ungated one-color flow cytometry with the anti-TCRδ1 MAb (data not shown). For further purification, resting T cells were stained with MAbs against a macrophage surface marker (CD14, MAb 3C10) and a B cell surface marker (complement receptor 2 or CD21, MAb HB5) and then negatively selected with magnetic beads (Morita et al., 1991). A portion of the resulting purified T cell population was stained with FITC-labeled control or anti-TCRδ1 MAbs and analyzed by flow cytometry.

Stimulation of γδ T Cell Clone and γδ TCR Transfectant with Antigen for IL-2 Release

The γδ T cell clone DG.SF13 and the γδ TCR transfectant DBS43 have been described (Bukowski et al., 1995). In brief, the γδ TCR transfectant DBS43 was derived by transfection of the γ and δ TCR cDNAs of the DG.SF13 clone into the TCR–Jurkat mutant J.RT3-T3.5 (Saito et al., 1987). For IL-2 release, T cells or TCR transfectants (1 × 10^5 cells) were cultured either alone or with varying numbers of mitomycin C-treated DG.EBV tumor cells (as syngeneic APCs), in wells of a 96-well flat-bottomed plate (ICN Biochemicals, Costa Mesa, California) in 200 μl of media. The prenyl pyrophosphate analog, MEP, was added as partially purified MEP (1/20 starting dilution). Phorbol myristate acetate, a required costimulator (Saito et al., 1987), was present at 10 ng/ml. After 24 hr at 37°C, the supernatants were harvested and diluted to 1/8. IL-2 release was determined by the prolifera-

tion of the IL-2-dependent T cell line CTLL-20 as described (Morita et al., 1991). IL-2 release was not noted in control wells lacking MEP.

Measurement of Ca Flux by Flow Cytometry

T cell clones were incubated in 5 μM indo-1 (Molecular Probes, Incorporated, Eugene, Oregon) at 1 × 10^7 cells/ml for 30 min at 37°C. Indo-1-loaded T cells were diluted to 1 × 10^6 cells/ml and kept at 4°C until use. T cells (without APC) then were incubated at 37°C for 2 min, analyzed for 30 s to establish baseline levels, and antigen was added. For "not spun" samples, the cells were analyzed for an additional 7 min. For "spun" samples, the cells were analyzed for an additional 1 min to establish baseline calcium flux after antigen addition. The T cells then were removed, centrifuged for 20 s in a microcentrifuge to effect cell–cell contact, and incubated for a further 1–2 min at 37°C. The cells were resuspended, applied to the cytometer, and analyzed for an additional 5–6 min. As a positive control, TCR was cross-linked by the IgM anti-CD3 MAb, 2Ad-2A2 (a gift from Dr. Reinherz, Dana Farber Cancer Institute, Boston, Massachusetts). Maximum and minimum $[Ca^{2+}]_i$ levels were determined by the addition of ionomycin (2 μg/ml from Sigma) and EGTA (80 mM), respectively. The mean ratio of indo-1 fluorescence at 410/480 nm is shown.

Measurement of Ca Flux in Individual Cells by Interactive Laser Cytometry

T cell clones were loaded with indo-1 (2 μM) in the absence of APCs as above, washed twice, and analyzed using an interactive laser cytometer (Meridian Instruments ACAS 570, Okemos, Michigan). For "not spun" samples, indo-1-loaded T cells (1 × 10^6 cells/ml) were incubated at 37°C for 4 min, antigen was added, and the T cells plated on a glass cover slip. For "spun" samples, indo-1-loaded T cells (1 × 10^6 cells/ml) were incubated at 37°C for 4 min, antigen was added, and the T cells were centrifuged in a microcentrifuge to effect cell–cell contact. After 1 min at 37°C, the cells were gently resuspended and plated on a glass cover slip. Plated T cells were visualized on an Olympus IMT-2 inverted fluorescence microscope and illuminated with 355 nm UV light from an argon laser. The ratio of fluorescence emission at 400 nm (bound Ca^{2+}) to that at 480 nm (unbound Ca^{2+}) was taken as a relative measure of $[Ca^{2+}]_i$. The ratios of indo-1 fluorescence were represented by pseudocolor hues. A computer-interfaced xy scanning stage was used to generate quantitative two-dimensional images of $[Ca^{2+}]_i$ for individual T cells. Ionomycin (2 μg/ml) was added as a positive control for maximal $[Ca^{2+}]_i$ levels. Representative microscope fields at 3–5 min after additions are shown.

MAb Blocking Studies

MAbs to MHC class I, MHC class II, β_2m, and CD1 were; W6/32 (anti-pan class I), BB7.7 (anti-class I), PA2.6 (anti-class I), IVA12 (anti-HLA-D), LB3.1 (anti-HLA-DR), L243 (anti-HLA-DR), B7/21.1 (anti-HLA-DP), IVD12 (anti-HLA-DQ), BBM.1 (anti-β_2m), L368 (anti-β_2m), OKT6 (anti-CD1a), 4A7.6.2 (anti-CD1b), BCDIb2.1 (anti-CD1b), 10C3 (anti-CD1c), and 3C11 (anti-CD1d). Prior to T cell addition, MAbs were added as either dialyzed ascites fluid (1/200 dilution), overgrown culture supernatant (1/4 dilution), or purified antibody (12.5 μg/ml) to cytotoxicity or proliferation assays with suboptimal concentration of prenyl pyrophosphate antigens. All MAbs were shown to either block control T cell responses or to stain on FACS analysis.

Immunofluorescence Analysis

For determination of MHC class II expression, the γδ T cells clone, DG.SF13, the Vγ2Vδ2 TCR transfectant, DBS43, the EBV-transformed B cell line, 721, and the class II–negative deletion mutant of the 721 cell line, 721.174, were stained with anti-class II MAbs. The MAbs used were anti-pan class II (IVA12), anti-HLA-DR (LB3.1), anti-HLA-DQ (Leu10), anti-HLA-DP (B7/21), or the control MAb (P3). The anti-HLA-DQ MAbs, SPV-L3 and IVD12, also were used and did not stain DBS43 (data not shown). Stained cells were analyzed by one-color flow cytometry performed with a FACsort flow cytometer using Lysis II software (Becton Dickenson and Company, Palo Alto, California) (Morita et al., 1994).

Cytotoxicity and Proliferation Assays with MHC Class I, MHC Class II, and TAP1/TAP2-Deficient Tumor APC

Cytotoxicity was measured using a standard 4 hr ^{51}Cr-release assay (Morita et al., 1991). Chromium-labeled targets and the γδ T cell clones, DG.SF8 or LG.C6, were mixed at an E:T ratio of 100:1 in the absence or continual presence of mycobacterial extract (1/35 dilution) or the staphylococcal superantigen SEA (1 μg/ml). Targets cells were the Burkitt's lymphoma Raji and its MHC class II–negative mutant, RJ-2.2.5 (Accolla, 1983); T5-1, and its class II–deficient mutant, 6.1.6 (Gladstone and Pious, 1978); 721 and its class II deletion mutant, 721.174 (DeMars et al., 1984); and the natural class II–deficient cell lines, Nacera and Ramia, derived from two patients with bare lymphocyte syndrome (De Préval et al., 1985). The RJ-2.2.5, 6.1.6, Nacera, and Ramia mutants lack all class II molecules due to the absence of different transactivating factors for class II transcription (Bénichou and Strominger, 1991). The 721.174 mutant lacks class II genes, TAP1 and TAP2 peptide transporter genes, and the DMA and DMB class II genes due to deletions in the MHC locus (DeMars et al., 1984; Denzin et al., 1994; Spies et al., 1990). The 721.221 mutant lacks cell surface expression of HLA-A, HLA-B, and HLA-C (Shimizu and DeMars, 1989). The mutant melanoma cell line, FO-1, is β_2m deficient due to a defect in β_2m gene expression (D'Urso et al., 1991) and lacks detectable assembled class I molecules as determined by reactivity with the W6/32 MAb (Rajagopalan and Brenner, 1994).

Pulsing of APCs

APCs (as DG.EBV, a syngeneic EBV-transformed B cell line) were mitomycin C treated, and incubated with either a M. tuberculosis extract (1/2 dilution), monomethyl phosphate (2.5 mM), monoethyl phosphate (1/100 dilution), or PPD (50 μg/ml) for 4 hr at 37°C. The cells then were washed three times at 4°C with Puck's saline (phosphate-free buffer) or with PBS. The γδ T cell clone, DG.SF68, or the mycobacteria-specific $CD4^+$ αβ T cell line, DG.1, were incubated with the pulsed APC in the presence of 0.03 nM rIL-2. [^{3}H]thymidine (1 γCi) was added to the cultures on day 1 and the cells were harvested at day 2. Each pulsed APC group also was cultured with T cells in the continued presence of M. tuberculosis extract, MMP, MEP, and PPD as a positive control for each APC group (only the unpulsed control APC group is shown). No proliferation was noted in the absence of T cells. In other experiments, different types of APC were used, including irradiated syngeneic and allogeneic PBMC, mitomycin C-treated allogeneic EBV cells, and mitomycin C-treated γδ T cells (DG.SF68), and the length of pulsing was varied.

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