3-Formyl-1-butyl Pyrophosphate A Novel Mycobacterial Metabolite-activating Human γδ T Cells*

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Most human blood γδ T cells react without major histocompatibility complex restriction to small phospho- rlated nonpeptide antigens (phosphoantigens) that are abundantly produced by mycobacteria and several other microbial pathogens. Although isopentenyl pyrophosphate has been identified as a mycobacterial antigen for γδ T cells, the structure of several other stimulating compounds with bioactivities around 1000-fold higher than isopentenyl pyrophosphate remains to be elucidated. This paper describes the structural identification of 3-formyl-1-butyl-pyrophosphate as the core of several non-prenyl mycobacterial phosphoantigens bioactive at the nM range. Recognition of this molecule by γδ T cells is very selective and relies on its aldehyde and pyrophosphate groups. This novel pyrophosphorylated aldehyde most probably corresponds to a metabolic intermediate of the non-mevalonate pathway of prenyl phosphate biosynthesis in eubacteria and algae. The reactivity to 3-formyl-1-butyl-pyrophosphate supports the view that human γδ T cells are physiologically devoted to antimicrobial surveillance.

Although the vast majority of T lymphocytes recognize via their αβ TCR1 antigenic peptides associated to major histocompatibility complex molecules, the so-called unconventional T cells that often express γδ TCR recognize their ligands in a different way. The prominent γδ T cell subset in human blood expresses the Vγ9/Vδ2 TCR and responds to nonpeptide antigens produced by various microbial pathogens, such as mycobacteria. The mycobacterial stimuli for these T cells have been characterized independently by two groups as nonpeptide phosphoantigens; V, variable; Rt, retention time.

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EXPERIMENTAL PROCEDURES

Structural Analysis of Phosphoantigens—TUBag1 and TUBag2 were purified from 20 liters of Mycobacterium fortuitum culture medium using HPLC and HPAEC as described (5). Ion Trap electrospray ionization mass spectrometry (LCQ, Finnigan Thermoquest, Les Ulis, France) was done in negative mode with scanning over a mass range of 100–2000 mass units, and TUBag samples were diluted 1 µg/ml in isopropanol/water (v/v) to which 1% triethylamine had been added and were introduced by continuous infusion at 3 µl/min. MS* was done on the specified main ions with a window of 5 mass units using He collision with 25% total beam energy. Structural combinations were calculated with Molgen (Molgen Softwares, Bayreuth, Germany). HPAEC and HPLC were coupled to a photodiode array detector (Millenium, Waters) scanning at 1 spectrum/s between λ 200 and 800 nm with a 0.5 nm window. Chemical synthesis of pyrophosphate esters and of UTP-γesters was achieved by nucleophilic displacement of tosylated alcohols with pyrophosphate or reaction of the corresponding alcohols with carbodiimide-activated UTP as described (9–11). The structures of the compounds were checked by MS*, 1H NMR and HPAEC. Chemical treatments of isopentenyl-PP, 2-butanone-1-yl-PP, and 3-formyl-1-bu- tyl-PP (TUBag1) were done as follows. Samples were added to 5 mM NaIO4 before cell assays was done by the addition of a few drops of glycerol to the sample. Reduction was performed by adding 100 µl of 10 mM NaBH4 (Aldrich) containing 4 mM NH2OH for 40 min at room temperature, followed by neutralization with 5 µl of methanol. Similar

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‡ The abbreviations used are: TCR, T cell receptor; DXP, 1-deoxy-3xylose-5-phosphate; MS*, multiple stages mass spectrometry; HPLC, high performance liquid chromatography; HPAEC, high pH anion exchange chromatography; PP, pyrophosphate; IPP, isopentenyl-PP; PPi, inorganic PP; m, multiplet; TUBag, Mycobacterium tuberculosis antigens; V, variable; Rt, retention time.
Mycobacterial 3-Formyl-1-butyl Pyrophosphate Antigen

FIG. 1. Ion Trap electrospray ionization mass spectrometry of mycobacterial phosphoantigens. The postulated structures for TUBag3 and its MS fragments are shown above. A, negative MS1 of TUBag3, infusion of 3 μg/min in isopropanol/water/triethylamine (50/50/5); B, negative MS2 of the TUBag3 pseudomolecular ion m/z 567 selected from A and shown by an arrow; C, negative MS3 of ion m/z 261 selected from B and shown by an arrow.

Bioassays of Human γδ T Cell Activation—Bioactivities of the specified molecules were drawn from the titration of the autotocytotoxic response of a γδ V9/V2 T cell clone stimulated by serial dilutions of the phosphoantigens (12). The ability of the molecules to trigger selective expansion of γδ V9/V2 peripheral blood lymphocytes in short term culture assays was assessed as described previously (12). Briefly, peripheral blood lymphocytes from healthy donors were cultured for 8 days with the specified molecules in culture medium supplemented with recombinant human interleukin 2, and the frequency of CD3-positive T cells (monoclonal antibodies from Coulter-Immunotech, Marseille, France) was estimated by flow cytometry using a FACScan apparatus (Becton Dickinson, Mountain View, CA). Polyclonal V9/V2 T cells stimulated by untreated or NaBH4-treated phosphoantigens were analyzed using a microphysiometer (Cytosensor, Molecular Devices, Crawley, UK), with 8 × 105 cells/experiment, a flow (100 μl/min) of low-buffered RPMI (Molecular Devices, Crawley, UK) alone (Fig. 4, control lanes) or containing the specified stimulus (added at a time shown by arrow), and data collection rate of 90 s.

RESULTS

Molecular Masses of TUBag1 and -3—Thirty μg of pure TUBag3 (UTP-γ-X) molecule obtained as described (5) was analyzed by Ion Trap electrospray ionization-MS in negative mode (Fig. 1A). The spectrum showed abundant ions at m/z 567 and m/z 589, corresponding to (M-H)- and (M+Na-2H)-, respectively. This result unambiguously indicates a molecular mass of 568 atomic mass units for TUBag3, i.e. 84 atomic mass units above that of UTP. Subsequent negative MS2 from m/z 567 (Fig. 1B) yielded the fragments ([UTP-H]-, [UDP-H]-, and [UMP-H]-, demonstrating the UTP-γ-X structure formerly assigned to TUBag3 (5). Most importantly, this negative MS2 spectrum showed fragments at m/z 261 and m/z 243, corresponding to the (XPP-H)- fragment and its anhydro derivative (XPP-H2O-H)-. Thus, X-OH has a molecular mass of 102, and the PP monoester which corresponds to TUBag1 (6), has a molecular mass of 262 atomic mass units, confirming a recent observation (13). This conclusion was supported by negative MS3 from m/z 261, which yielded an anhydro fragment (m/z 243) and two diagnostic fragments corresponding to a ketene (CH2=CO) loss (m/z 219) and a PP, fragment (m/z 159, Fig. 1C). Moreover, these data indicate the presence of a carbonyl group in XPP and establish the structure of the PP monoester. This negative MS3 spectrum from TUBag3 was identical to the negative MS2 spectrum from ions at m/z 261 observed in the negative MS1 spectrum from TUBag1, obtained in similar conditions as in Fig. 1A (data not shown). No anion was generated from X-OH in these experiments, so this molecule presumably contains few oxygen atoms and no carboxylic group. Furthermore, its even mass indicates either an absence of the N atom or the presence of an even number of N atoms. With a general formula C1–7, H10–4, O1–4, N2 or 4, 21,533 isomeric structures correspond to a molecular mass of 102 atomic mass units, among which only 1065 are noncarboxylic with at least one primary alcohol as the phosphorylation site.

The X Motiey Contains a Carboxyl Group—To screen the 1065 structural options, we synthesized organic PP esters of 262 atomic mass units (in acidic form) bearing either linear saturated alkanes, polyols, rings, ketones, or lactones, and we searched for compounds with Rt on HPAEC matching that of the mycobacterial XPP TUBag1 (5). Only carbonyl-containing phosphoesters did so (Fig. 2) and presented phosphoantigenic bioactivity for γδ T cell clones, although in the micromolar range, as already found with prenyl-PP (1). Since similar results were obtained with the UTP-γ-ester analogues when compared with the mycobacterial X-UTP (TUBag3); these data suggest the presence of a carbonyl group in X. This assumption was confirmed by spectroscopy. First, a Fourier transform infrared spectrum of TUBag1 (5 μg) in water presented a C=O stretching band at 1670 cm⁻¹ (versus 1700 cm⁻¹ for a synthetic ketone-PP reference; not shown). Second, based on the weak ϵ of C=O at λ 260–300 nm in UV (εmax < 30 m⁻¹ cm⁻¹), a photodiode array comparison of HPLC-purified TUBag1 to relevant standards (Fig. 3) evidenced a weak absorption at λmax between 265 and 268.6 nm. Similar data were obtained with synthetic hydroxyketones standards, whereas conversely, PP, IPP, or pentanol do not absorb in this range. Thus, in agreement with the ketene loss from X-PP observed in the negative MS2 fragmentation (Fig. 1C), X contains a noncarboxylic C=O group whose local charge (−0.3) accounts for its peculiar HPAEC Rt.

TUBag1 Is a 3-Formyl-1-butyl-PP—When their bioactivities for γδ T cells were titrated by serial dilutions, mycobacterial phosphoantigens were stimulatory at nanomolar concentrations (5). However, synthetic keto-pyrophaosphoesters or UTP-γ-esters such as 2-butanone-1-yl-PP, 3-pentanone-1-yl-PP, 4-pentanone-1-yl-PP, or 2-methyl-3-butanone-1-yl-(or 2-yl)-γ-UTP had much weaker (5–80 μM) phosphoantigenic bioactivities. Therefore, we compared the functional group of mycobacterial X-PP to that of a synthetic keto-pyrophaosphoester reference by titrating their bioactivities after selective chemical treatments (Table I). The bioactivities of untreated TUBag1 (5 nM), 2-butanone-1-yl-PP (30 μM), and isopentenyl-PP (3 μM) were unaffected by treatment with 5 mM NaOH, pH 7. On the contrary, TUBag1 (or whole mycobacterial extract), but not IPP (nor any prenyl phosphate), was totally inactivated by reduction with 10 mM NaBH4, as expected for carbonyls (see 2-bu-
Fig. 2. Comparative HPAEC analysis of various alkyl-PP to TUBag1 and TUBag3. The specified molecules were analyzed on a DX500 HPAEC apparatus as already described (7) and compared with inorganic phosphate, PP, TUBag1, and TUBag3 references. For clarity, the eluting positions of the above-specified compounds have been indicated by arrows. Note that butanone-1-yl-PP and butanone-1-yl-UTP present the same Rt as TUBag1 and TUBag3, respectively. µS, microsiemens.

Fig. 3. Spectroscopic analysis of 3-formyl-1-butyl-PP. Top, comparative photodiode array absorption in the λ 200–300-nm region showing absorption (Abs) due to the carbonyl of HPLC-purified TUBag1 (6) in water solvent and spectra recorded in the same conditions of the following references: 1, 3-methyl-1-pentanol; 2, PP, 3, isopentenyl-PP; 4, 1-hydroxy-2-methyl-3-butanone; 5, 1-hydroxy-4-pentanone. The observed λmax from the 250–300-nm region, with 0.5-nm precision are given above. Bottom, structure of 3-formyl-1-butyl-PP and its NMR attributions collected from previous TUBag3 and TUBag4 spectral data (5, 6, 15).

Table I

Phosphoantigen bioactivity after chemical treatment

Bioactivity was measured by induction of an autocytotoxic response of a V9/V962 T cell clone stimulated by serial dilutions of samples in three to five independent experiments. Untreated bioactive sample concentrations are: IPP, 3 µM 2-butanone-1-yl-PP, 80 µM. Mycobacterial TUBag1 bioactivity was tested at up to a 1:1000 dilution, corresponding to 5–10 µM TUBag1 (7). †, bioactive molecule; ‡, abrogation of bioactivity; ††, not tested.

|          | NaO4 | NaBH4 | NaBH3CN | KmNO4 | Br2H3O6 |
|----------|------|-------|---------|-------|---------|
| IPP      | +    | +     | +       | +     | +       |
| 2-butanone-1-yl-PP | +    | +     | -       | +     | +       |
| Mycobacterial TUBag1 | +    | +     | -       | +     | ††      |

Tanone-1-yl-PP in Table I). Furthermore, bioactivity of TUBag1 (or that of whole mycobacterial extract) was also totally abrogated by oxidation with 1 mM KMnO4, a treatment that did not affect bioactivity of IPP, prenyl phosphates, or 2-butanone-1-yl-PP (Table I). These data clearly demonstrate that mycobacterial phosphoantigens are different from prenyl phosphates. IPP oxidation by KMnO4 produced the diol-PP, CH2OH-C(CH3)OH-CH2-C(Me)-OP, with a molecular mass of 280 atomic mass units, an HPAEC Rt identical to that of TUBag1, and a phosphoantigenic bioactivity similar to that of IPP (3–5 μM in autacytotoxic response of a V9/V962 T cell clone, not shown). The bioactivity of KMnO4-oxidized IPP is due to the C3–C4 diol moiety, as this latter was chemically converted by 5 mM NaIO4 into 3-butanone-1-yl-PP (same HPAEC Rt as TUBag1, bioactivity 50–80 µM), an unstable compound prone to β elimination into 1-buten-3-one (HPAEC Rt = 0 min, biologically inactive) and PPi. The low bioactivity of C3–C4 diol suggests that dihydroxylated phosphoantigens are unlikely to be the natural antigens. Thus, TUBag1 contains a primary alcohol (phosphorylation site) and a carbonyl (reduced by NaBH4 and oxidized by KMnO4) TUBag1 does not contain any imine group (C=N), as it was resistant to treatment with 10 mM NaBH4-CN at neutral pH (14). To check for the presence of HC=O or of enol, TUBag1 and IPP were treated by cold bromine water (0.5 mM, pH 6), which adds double bonds or readily oxidizes aldehydes with α-H. Br2-treated IPP remained bioactive, indicating that this treatment did not degrade the phosphate bonds and that unsaturation was not mandatory for bioactivity. Conversely, the bioactivity of Br2-treated TUBag1 was completely abolished (Table I), implying the presence of an aldehyde that was either oxidized by aqueous Br2 and KMnO4 or reduced by NaBH4 into distinct inactive molecules. In agreement with the above spectroscopic data and MS fragmentation pattern of TUBag1, this conclusion was further supported by a weak positive Schiff staining of concentrated HPLC fractions of TUBag1. Previous data from NMR analysis of TUBag3 (5) and TUBag4 (6) completed this identification. In both 1H spectra from TUBag4 or TUBag3, two primary alcohol protons (CH2O--P--) were detected at 4.10 ppm (m) and 4.20 ppm (m), respectively. These protons coupled together (J = 15 Hz) and to two adjacent methylenic (CH2--CH2--O--P-) protons at 3.47 ppm and 3.57 ppm in TUBag3 and at 3.49 ppm and 3.58 ppm in TUBag4, indicating the presence of a dimethylene group in both antigens. In addition, a well defined 1H-1H coupling was observed in TUBag4 between the protons at 1.26 ppm (3H, J = 7.6 Hz, Me) and 4.10 ppm (1H), which implies a H2C-CH group, which was confirmed by 1H,13C homonuclear multiple quantum and homonuclear multiple bond correlation spectroscopy experiments (CN: 22 ppm; C6: 65 or 75 ppm (15)). Together with a primary alcohol and an aldehyde group, these results establish that the free X alcohol of 102 atomic mass units phosphorylated in the mycobacterial metabolites
TUBag1, 3, and 4 is 3-formyl-1-butanol: H$_2$C-HC(CHO)-CH$_2$-CH$_2$-OH. Consequently, based on former structural evidence (5, 6) and the present findings, the complete structures of mycobacterial phosphoantigens TUBag1, 3, and 4 can be established as, respectively, 3-formyl-1-butyl-PP, 3-formyl-1-butyl-γ-UTP, and 3-formyl-1-butyl-γ-TTP. No fully convincing MS of TUBag2 could be obtained. Nevertheless, its slightly higher Rt on C$_{18}$ HPLC (6) and on HPAEC (7) and the preliminary report of a mycobacterial aldehydic$^2$ phosphoantigen of mass 276 (8) suggest that TUBag2 corresponds to a closely related longer chain homolog of TUBag1 such as 3-formyl-1-pentyl-PP.

Structural Determinants of 3-Formyl-1-butyl-PP, Which Affect γδ T Cell Reactivity—Reactivity of human γδ T cells to phosphoantigens is not strictly selective, because various phosphoesters comprising a linear C$_2$ core as ethyl-PP are stimulatory, although at nearly 10$^3$ times higher concentrations (EC$_{50}$% = 10–30 μM). Since isoamyl-PP (the saturated analogue of IPP) does not activate γδ T cells, the relatively higher bioactivity of isopentenyl-PP (EC$_{50}$% = 1–3 μM) has been attributed to its prenyl unsaturation (1). This olefin is better recognized than its related C$_3$-C$_4$ diol-PP (see KMnO$_4$-treated IPP in Table I, EC$_{50}$% = 10 μM) and than the subsequent C$_3$-carbonyl derivative (NaIO$_4$ oxidized C$_3$-C$_4$ diol-PP: EC$_{50}$% = 50–80 μM, data not shown). Similarly, the potent activity of 3-formyl-1-butyl-PP on human γδ T cells (5–10 nM range (5)) is clearly conferred by its aldehyde group. Although NaBH$_4$ reduction of 3-formyl-1-butyl-PP changed slightly, its topology (increasing the antigen volume from 179 Å$^3$ to 183 Å$^3$ and lowering the local dipolarity at C$_6$ from 2.2 to 1.4 Debye), this subtle structural alteration totally abrogated the bioactivity of this compound (Table I and Fig. 4). Activation of human γδ T cells by mycobacterial ligands such as the purified 3-formyl-1-butyl-PP induced a powerful in vitro expansion of Vγ9Vδ2 T cells from peripheral blood lymphocytes of healthy donors, whereas the topologically resembling NaBH$_4$-reduced 3-formyl-1-butyl PP did not (Fig. 4, upper panel). T cell proliferation is, however, a late event, usually detected after several days of in vitro cultures stimulated from the beginning of the assay. Hence, the γδ cell unresponsiveness to NaBH$_4$-reduced 3-formyl-1-butyl-PP could possibly reflect the capacity of this compound to inhibit late steps of cell proliferation without actually interfering with its initial recognition by T cells. We assayed whether early steps of γδ T cell activation by TUBag1 were also crucially determined by its aldehyde function.

The early responses of polyclonal γδ T cell lines to 3-formyl-1-butyl-PP and to its NaBH$_4$-reduced derivative were compared using a microphysiometer monitoring the acidification rate of culture media (16). We recorded a rapid cell response to mycobacterial 3-formyl-1-butyl-PP and found that its aldehyde-to-alcohol reduction abrogated its bioactivity (Fig. 4, lower panel). These experiments confirmed that the γδ T lymphocyte unresponsiveness to reduced antigen is due to abrogation of early events leading to cell activation such as antigen recognition.

**DISCUSSION**

Human Vγ9/Vδ2 T cells predominate in adult blood but not in thymus (20–23). This age-related peripheral bias most likely reflects a chronic exposure to highly recurrent ligands (24). Accordingly, the broad reactivity of Vγ9/Vδ2 T cells to various microbial, viral, and protozoan pathogens arises from their unique ability to specifically recognize a peculiar set of natural nonpeptide-phosphorylated ligands called phosphoantigens (2, 13, 25). Several structurally different phosphoantigens occur in nature (26–28), and in particular, microbial pathogens that infect humans and primates show a marked tendency to synthesize such metabolites (25). Here we found that several mycobacterial phosphoantigens comprise a 3-formyl-1-butyl-PP moiety, either alone, like the formerly described TUBag1 antigen, or as a nucleotide conjugate like the α-phosphodiester of 5’-UMP and 5’-TMP in TUBag3 and TUBag4, respectively. Furthermore, we found that the γδ cell-mediated recognition of such mycobacterial 3-formyl-1-butyl-PP ligand involves determinants defined by the PP, as described previously (5–7, 15), but also by the aldehyde group elucidated in this study (Fig. 4). Interestingly, this single antigen specificity is recovered in a polyclonal γδ T cell population drawn from distinct healthy individuals, which confirms several earlier reports about the lack of major histocompatibility complex restriction and limited bias of TCR repertoire of such a T cell recognition pattern (3, 4, 30, 31). The polyclonal specificity for the aldehyde motif is, however, very selective, as NaBH$_4$ reduction induces a slight change to the topology of the antigen while abrogating its recognition, a conclusion fully consistent with the recent finding that the TCR Jγ region determines subtle changes in phosphoantigen selectivity (8). Thus, the physiological recognition of mycobacterial phosphoantigens (i.e. at nanomolar concentrations) is actually a highly selective process for detecting a defined, although widespread, set of target cells that secrete these ligands.

- 3-Formyl-1-butyl-PP is a five-carbon molecule of linear structure with a unique ramification distal from the diphosphorylated hydroxyl. This type of phosphoester does not correspond to any formerly described natural compound and, consequently, cannot be strictly assigned to any known metabolic route. However, two structures resembling five-carbon phosphodiesters,
isopentenyl- and dimethylallyl-PP, are ubiquitous precursors for steroids and isoprenoids. Although in most cells, such compounds come from acetyl coenzyme A and mevalonate, in algae and in several eubacterium including *Corynebacterium* and *Mycobacterium* (17), their biosynthesis proceeds by a separate route that has been discovered recently (18, 19). This latter, referred to as the Rohmer’s or DXP (1-deoxy-D-xylulose-5-phosphate) pathway, arises from the decarboxylate condensation of glyceraldehyde 3-phosphate and pyruvate (summarized in Fig. 5) by a transketolase enzyme called DXP synthase, into deoxyxylulose 5-phosphate, which is in turn converted by a DXP reductoisomerase into 2-C-methyl-D-erythritol-4-phosphate, a linear trihydroxylated five-carbon molecule with a unique CC ramification distal from the diphosphorylated hydroxyl. This triol monophosphate has been found in several bacteria and is assumed to lead to isopentenyl-PP biosynthesis through several yet-unidentified reducing and phosphorylating steps. Its highly relevant structure, harboring both an oxidation level intermediate between 2-C-methyl-D-erythritol and isopentenyl and a lower phosphorylation suggests that the mycobacterial 3-formyl-1-butyl-PP clearly constitutes one of such late C5-PP intermediates (17–19). In this sense, although natural 3-formyl-1-butyl-PP had not been described yet, it appears an ideal candidate precursor of mycobacterial prenyl phosphates biosynthesis by Rohmer’s pathway.

By targeting their reactivity toward the mevalonate-independent pathway of isoprenoid biosynthesis proper to mycobacteria and to some other eubacteria, human γδ T lymphocytes are enabled to detect very low amounts of proliferating pathogens that secrete such compounds in their environment (5). This antigen targeting brings a double advantage. First, it allows the immune system to avoid a permanent activation by prenyl phosphates, since these latter are ubiquitous metabolites found in all living cells, including the γδ T lymphocytes themselves. Second, the non-mevalonate pathway has been reported so far only in eubacteria, fungi, and algae, representing, therefore, a highly selective target for a suitably focused immune surveillance by T cells that do not use major histocompatibility complex presentation to initiate their function. It is likely that related γδ T lymphocyte-stimulating metabolites will be discovered in other microbial pathogens and plants.

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**Fig. 5.** Structural similarities of the metabolic intermediates from the non-mevalonate pathway of isopentenyl-PP biosynthesis with 3-formyl-1-butyl-PP (*inset*). The non-mevalonate pathway metabolites and enzymes are drawn according to Refs. 29, 32, 33. TPP, thiamine pyrophosphate.
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