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The Antituberculous *Mycobacterium bovis* BCG Vaccine Is an Attenuated Mycobacterial Producer of Phosphorylated Nonpeptidic Antigens for Human γδ T Cells

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The mycobacterial antigens stimulating human γδ T lymphocytes (R. L. Modlin, C. Permitz, F. M. Hofman, V. Torigian, K. Uemura, T. H. Rea, B. R. Bloom, and M. B. Brenner, Nature (London) 339:544–548, 1989; D. H. Raulet, Annu. Rev. Immunol. 7:175–207, 1989) have been characterized recently in *Mycobacterium tuberculosis* H37Rv as a group of four structurally related nucleotidic or phosphorylated molecules, termed TUBag1 to -4 (tuberculous antigens 1 to 4) (P. Constant, F. Davodeau, M. A. Peyrat, Y. Poquet, G. Puzo, M. Bonneville, and J. J. Fournie, Science 264:267–270, 1994). Here, we analyzed their distribution in different mycobacterial species of the *M. tuberculosis* group, with special emphasis on the human vaccine *Mycobacterium bovis* BCG. We show that the same four TUBag1 to -4 molecules are shared by these mycobacteria. Quantitative comparison reveals, however, that while the pathogen *M. bovis* and *M. tuberculosis* species produce rather high amounts of TUBag, all of the BCG strains have a surprisingly reduced production of TUBag. These observations suggest that among tuberculous mycobacteria, the bacterial TUBag load could, to some extent, constitute an immunological determinant of mycobacterial virulence for humans.

The cellular elements mediating immunity to mycobacteria involve CD4 and CD8 αβ T lymphocytes as well as a minor subset of lymphocytes expressing γδ T-cell receptor (10, 11, 18, 24). In adult humans, up to 95% of the peripheral blood $CD4^-$ $CD8^-$ γδ T lymphocytes express Vγ9Vδ2-encoded T-cell receptors (21), most of which react to mycobacterial ligands (12, 13). Since this subset of lymphocytes is present at low frequencies in the thymus (3, 8, 17), such a peripheral repertoire is probably shaped by a sustained stimulation with few powerful antigens (7). Mycobacteria are omnipresent in the human environment; thus, some shared or cross-reactive mycobacterial antigens could trigger the peripheral γδ T-lymphocyte expansion (9). Furthermore, intradermal injection of viable *Mycobacterium bovis* BCG doses during early infancy constitutes the current antituberculous vaccination in developed countries. Given that BCG bacilli remain viable for years in their hosts, a chronic stimulation of the reactive γδ T cells could operate if their nominal antigens were delivered by this mycobacterium.

It is known (4) that the human pathogen *Mycobacterium tuberculosis* produces a group of four related phosphorylated molecules stimulating human γδ T cells, termed TUBag1 to -4 (for tuberculous antigens), that can be characterized by their collective degradation upon mixed phosphatase treatment (with alkaline phosphatase plus nucleotide pyrophosphatase). The components TUBag1 and TUBag2 are related phosphate monoesters of a hitherto-unidentified low-molecular-weight substituent, whereas the larger TUBag3 and TUBag4 molecules are nucleotidic pyrophosphodiesters of this same unidentified substituent. In TUBag4, the nucleotide is 5′-triphospho-2′-deoxy-β-D-ribofuranosyl-1′-thymine, whereas it has not yet been determined in TUBag3.

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Pathogenicity is rather graded among mycobacterial species, and closely related to the agent of human tuberculosis, *M. bovis* is responsible for bovine tuberculosis and some human tuberculosis. Since this pathogen has been attenuated to yield the variant known today as the BCG vaccine, we were interested in comparing the relative TUBag contents of *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG.

MATERIALS AND METHODS

Mycobacterial cultures. Mycobacteria grown on Sauton medium were collected and extracted overnight (O.N.) with $CHCl_3$-CH_3OH (vol/vol) solvent. The dried extract was partitioned into organic and aqueous phases; the aqueous phase was dried and reconstituted at 100 mg/ml in water as mycobacterial extract (ME) stock solutions stored at −30°C. Clinical *M. tuberculosis* isolates obtained from tuberculosis sputa were expanded twice on Sauton medium before preparation of their MEs.

Biochemistry of TUBag. TUBag were obtained from MEs as already described (4) by, successively, DEAE elution with increasing ammonium acetate concentrations, silica gel elution with increasing percentages of water in isopropanol, and elution by reverse-phase high-performance liquid chromatography (RP-HPLC) on C_{18} columns (Bischoff Chromatography, Leonberg, Germany) with isocratic 0.1 M ammonium acetate. High-pressure anion-exchange chromatography (HPAEC) was performed on a DX 300 apparatus (Dionex, Sunnyvale, Calif.) with an analytical AS11 column (Dionex), a sodium hydroxyde gradient from 0 to 0.05 M associated with a CH_3CN gradient from 0 to 22.5%, and dual conductimetry in a chemical suppression mode plus UV detection. The proliferative response of clone G115 was used to monitor the isolation procedure (see below). Phosphatase treatments involved 3 U of calf intestinal alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.) and 1 U of *Crotalus adamanteus* venom nucleotide pyrophosphatase (Sigma) incubated O.N. at room temperature before stimulation assays.

T-cell lymphoproliferation and FACS analysis. Freshly isolated peripheral blood lymphocytes (PBL) from healthy donors were cultured for 10 days in interleukin-2-supplemented medium in the presence of the specified ME, before live cells were phenotyped by indirect immunofluorescence and fluorescence activated cell sorter (FACS) analysis with monoclonal antibodies 389 (anti-Vδ2) and 360 (anti-Vγ9) (5). The lymphoproliferative response of the $V\gamma9V\delta2^+$ T-cell clone G115 was measured as described previously (4). Amplification ratios were obtained by the following formula: (percentage of $V\gamma9V\delta2^+$ T cells × total number of viable cells obtained at the end of the culture)/(percentage of $V\gamma9V\delta2^+$ T cells at the start of culture × number of seeded cells per well).

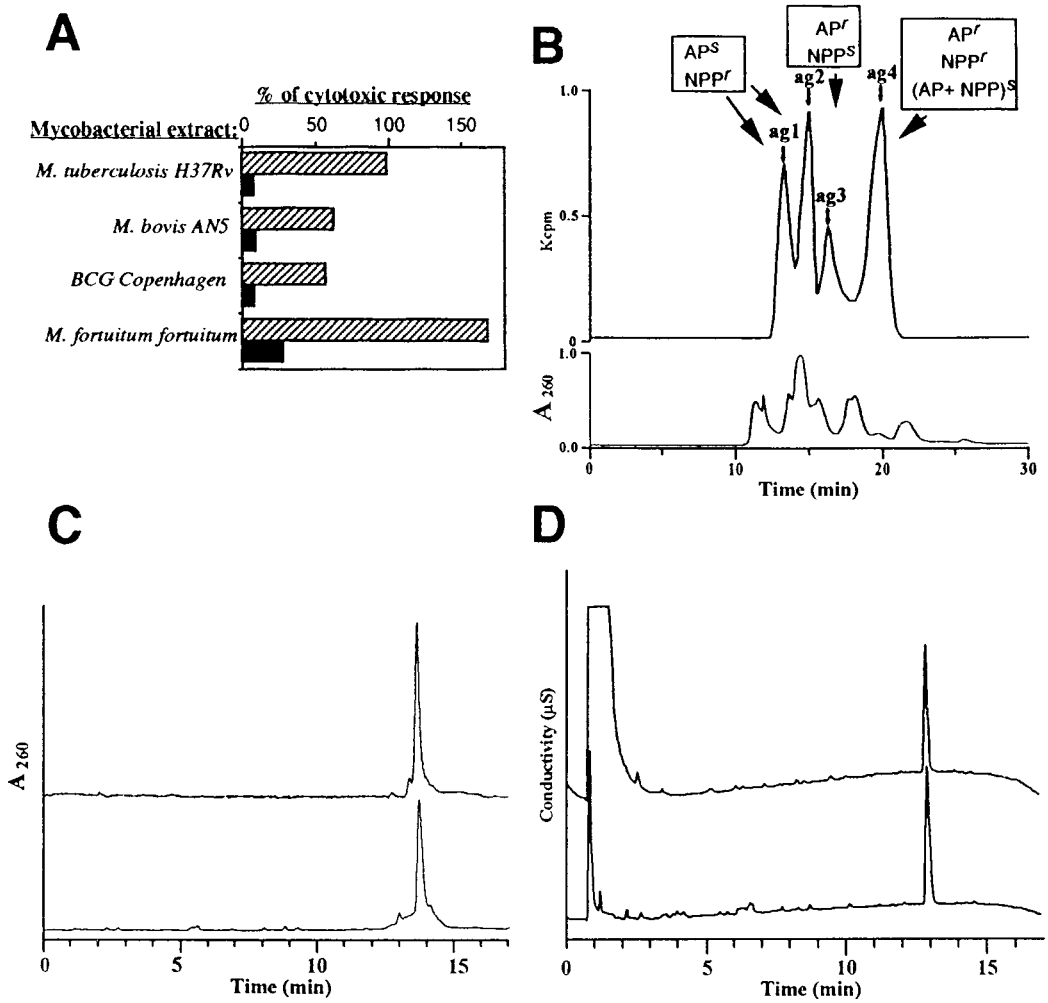

FIG. 1. Similar TUBag1 to -4 are produced by different mycobacteria. (A) ME stimulating the cytotoxicity of a Vγ9Vδ2$^+$ CTL contain TUBag. MEs from the specified species (3 mg/ml) were tested for induction of a short-term cytotoxic response of the G115 Vγ9Vδ2$^+$ T-cell clone prior to (hatched bars) or following (solid bars) mixed phosphatase treatment. The results are expressed relative to those obtained with *M. tuberculosis*, which were set at 100% as an internal reference. (B) Four TUBag are present in BCG (strain Copenhagen). BCG-derived ME separated by C_{18} RP-HPLC was monitored by either proliferation of the G115 Vγ9Vδ2$^+$ T-cell clone (upper profile) or UV absorbance at λ 260 nm (lower profile). Open boxes indicate the sensitivity (*s*) or resistance (*r*) to treatment with either alkaline phosphatase (AP), nucleotide pyrophosphatase (NPP), or both (AP+NPP) of the stimulatory activity defining each TUBag. (C) Comparative HPAEC of purified TUBag3 from *M. tuberculosis* Erdman (upper profile) and *M. fortuitum* fortuitum (lower profile) detected by their UV absorbance at λ 260 nm. (D) Comparative HPAEC of purified TUBag4 from *M. tuberculosis* Erdman (lower profile) and BCG Copenhagen (upper profile) detected by their conductimetry.

Cytotoxicity assays and TUBag titrations. The cytotoxic response assay of the Vγ9Vδ2$^+$ T-cell clone G115 to ME was performed in RPMI medium containing 10% fetal calf serum as described previously (6) with 4.5×10^4 G115 effector cells and 3,000 ^{51}Cr-labelled G115 target cells (effector/target cell ratio, 15:1) incubated for 4 h at 37°C with ME (eight doubling dilutions starting from 3.3 mg/ml) prior to counting the induced ^{51}Cr release. The percentage of specific cytotoxic response is obtained by the following formula: $100 \times$ [(experimental release − spontaneous release)/(maximum release − spontaneous release)]. The cytotoxic responses to ME and phosphatase-treated ME were determined relative to that of untreated *M. tuberculosis*, which was set at 100% as the reference. The comparative quantification in arbitrary stimulation units (ASU) of TUBag loads in each sample was done by comparing the effect-dose titration curve of the sample relative to that of an internal reference of a 100-ASU TUBag batch derived from *M. tuberculosis* as described previously (4). Measurement of specific effector cell autolysis induced by TUBag was done by use of 20,000 Cr-labelled T cells as already described (15).

RESULTS

Presence of the same TUBag in various mycobacterial species. The human γδ T-cell response to TUBag can be monitored conveniently by short-term cytotoxicity assays using relevant Vγ9Vδ2 cytotoxic T lymphocyte (CTL). In this assay (Fig. 1A), phosphatase treatment (alkaline phosphatase plus nucleotide pyrophosphatase) evidenced the presence of TUBag or structurally related metabolites in either the virulent *M. bovis* (strain AN5), the opportunist pathogen *Mycobacterium fortuitum* (strain fortuitum), or BCG (strain Copenhagen). Consequently, the TUBag were separated from each mycobacterial extract by an already described isolation procedure in which both chromatographic characteristics in ion-pair C_{18} RP-HPLC (shown for BCG in Fig. 1B) and sensitivities to alkaline phosphatase and nucleotide pyrophosphatase allowed each TUBag assignment (4). Comparative HPAEC monitored by either UV absorbance at 260 nm (Fig. 1C) or conductimetry (Fig. 1D) confirmed assignment of the nucleotidic compounds TUBag3 and TUBag4 found in the various species. In this way, the four molecular species TUBag1 to -4 (4) were found in *M. tuberculosis* (strain Erdman), *M. bovis* (strain AN5), and BCG (strain Copenhagen), whereas only TUBag1, TUBag2, and TUBag3 could be detected in *M. fortuitum*. Since no other

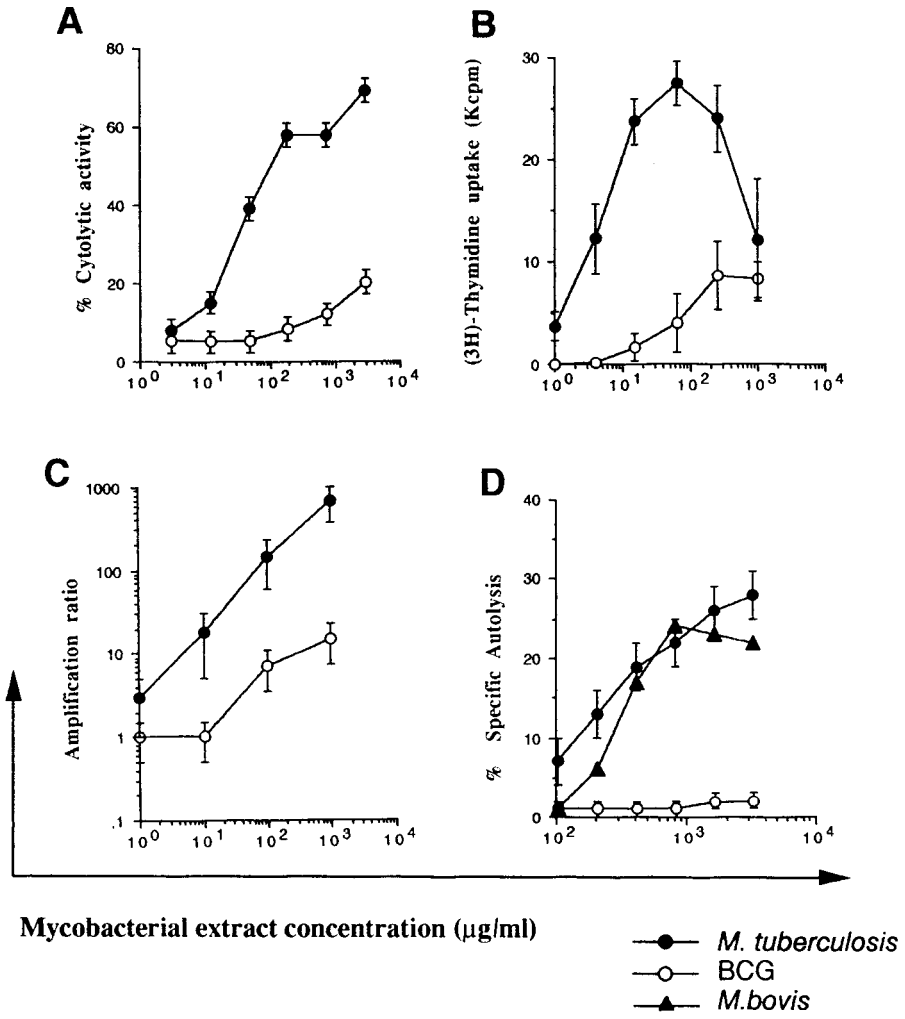

FIG. 2. TUBag loads differ in *M. tuberculosis*, *M. bovis*, and BCG type strains. (A) Vγ9Vδ2$^+$ CTL clone G115-mediated cytotoxicity for BCG ME- versus *M. tuberculosis* ME-pulsed B lymphoblastoid cell line (BLCL) targets with increasing ME doses. (B) ME-stimulated proliferative response of the Vγ9Vδ2$^+$ T-cell clone G115. Results give counts per minute obtained as (cultures with G115 + BLCL + ME) − (G115 + BLCL). (C) ME-stimulated polyclonal amplification of Vγ9Vδ2$^+$ PBL. Fresh PBL cultured 15 days with or without ME were counted and phenotyped for Vγ9Vδ2$^+$ TCR expression, and amplification ratios were estimated by dividing the total number of Vγ9Vδ2$^+$ cells obtained after culture with ME by the total number of Vγ9Vδ2$^+$ cells obtained after culture without ME. (D) ME-induced autocytotoxicity of the Vγ9Vδ2$^+$ CTL clone G115. Data are expressed as percentage of specific lysis of 2×10^4 ^{51}Cr-radiolabelled G115 effector cells incubated with ME. Each dot represents the mean ± standard deviation of three to six independently prepared specific MEs.

stimulating material was detected by these experiments, the results showed that similar TUBag shared by tuberculous and other mycobacteria are the only stimuli for mycobacterium-reactive Vγ9Vδ2$^+$ T cells. In *M. tuberculosis* (all strains investigated), *M. bovis* (strain AN5), and BCG (all strains tested), all of the stimulating TUBag1 to -4 were isolated from bacterial cell extracts, and a total absence of secreted TUBag was demonstrated in all of their corresponding culture fluids, even for those of longer culture durations during which cell wall antigen shedding could be evidenced. Under these conditions (before detectable arabinogalactan cell wall antigen shedding occurred in the culture fluid [data not shown]), conversely, almost 90% of the stimulatory TUBag material found in unrelated fast-growing *M. fortuitum* cultures was secreted in the culture fluids. The age of the culture did not influence the ponderal TUBag yield in the extracts. When probing for qualitative variations in the TUBag yield by age of the mycobacterial cultures (*M. tuberculosis* and BCG), no difference in the ponderal TUBag load titers (ASU per 100 mg of dried extract) of young (1-week) and older (3- to 5-week) cultures was found for either species. Thus, it appears that common TUBag molecules are produced by different mycobacterial species, and whereas intense TUBag secretion occurs in some unrelated species, these mycobacterial compounds appear to be confined to the cell-extractable material in all of the tuberculosis-related mycobacteria.

Type strains of tuberculosis-related mycobacteria have quantitatively different cellular TUBag loads. Titrating the biological responses of a reactive Vγ9Vδ2 T-cell clone to MEs enabled us to compare their respective stimulating antigen loads. These biological responses were lymphoproliferation measured by thymidine uptake and by cell number amplification ratio (4), major histocompatibility complex-unrestricted cytotoxic activity, and induction of specific autolysis (15). When isolating the cellular TUBag from freshly grown BCG cultures, we repeatedly obtained lower TUBag yields from these cultures than from cultures of *M. tuberculosis*. Accordingly, a 50-fold lower amount of TUBag in BCG than in *M.*

TABLE 1. Amplification ratios of Vγ9Vδ2 cell numbers in PBL cultures stimulated with different BCG and *M. tuberculosis* MEs[a]

Culture with:	Amplification ratio at ME dilution of:			
	10^{-2}	10^{-3}	10^{-4}	10^{-5}
BCG				
ME 1	5.8	1.1	1.4	1.0
ME 2	3.1	0.8	0.7	<0.5
ME 3	35.9	19.4	1.4	<0.5
M. tuberculosis H37Rv				
ME 1	935	194	23.8	4.9
ME 2	709	154	10.2	1
ME 3	159	38	1.2	0.6
H37Ra	932	221	36.8	4.7

[a] The results show the amplification ratios of $V\gamma9V\delta2^+$ T-cell numbers from a representative experiment performed in 2-week cultures of PBL from a healthy donor in interleukin-2-supplemented (100 biological response modifier program units per ml) complete medium stimulated with increasing doses of ME prepared from various independent mycobacterial cultures. (For equation to determine amplification ratio, see Materials and Methods.) Subsequent experiments were done with various other PBL cultures from healthy donors, which yielded comparable results.

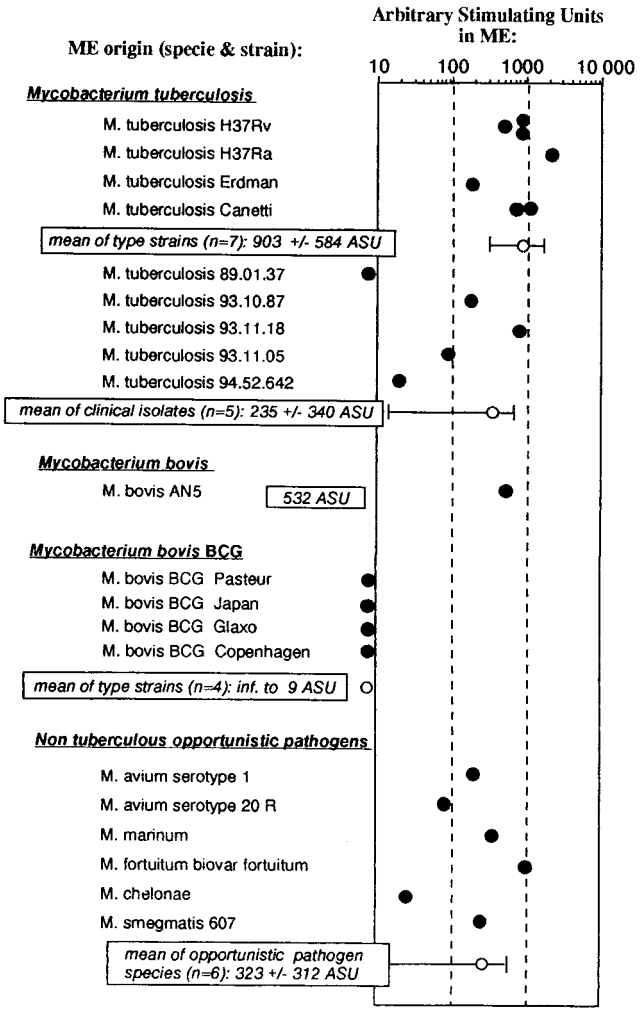

FIG. 3. TUBag loads of various mycobacterial species and strains. Percentage of specific autocytotoxicity in ^{51}Cr-radiolabelled G115 cells incubated with various ME concentrations was expressed as arbitrary stimulation units calculated by comparing the amount of ME yielding 50% of the maximal response with an internal 100-ASU standard TUBag batch. inf., inferior.

tuberculosis was estimated on the basis of the ability to trigger either the cytotoxicity, the proliferation, or the autocytotoxicity of relevant clones or the polyclonal amplification of Vγ9Vδ2 T cells in fresh PBL (Fig. 2A to C). For instance, as shown in Fig. 2A, 10% cytolytic activity was obtained with about 200 μg of BCG ME per ml versus about 4 μg of *M. tuberculosis* ME per ml, whereas, as shown in Fig. 2B, 5 kcpm of proliferation was recorded with either 1 μg of *M. tuberculosis* ME per ml or 62 μg of BCG ME per ml. This difference is not qualitatively attributable to a specific lack of TUBag in BCG, since all four TUBag were present in it (Fig. 1B) as well as in virulent *M. bovis* (data not shown), but rather is attributable to a lower TUBag production by BCG. Interestingly, the virulent *M. bovis* parental species of BCG has a TUBag load almost identical to that of the other pathogen, *M. tuberculosis* (Fig. 2D). Since BCG can undoubtedly be considered nonpathogenic to humans, these data led us to suspect that pathogenic tuberculous mycobacteria produce higher amounts of these TUBag-stimulating human lymphocytes than the vaccinating BCG. This conclusion was not biased by the use of distinct T-cell responses of a single Vγ9Vδ2 T-cell clone, since similar results have been obtained with other mycobacterium-reactive Vγ9Vδ2 T-cell clones. Thus, these different responses to the *M. tuberculosis* and BCG stimuli did not reflect preferential clonal selectivities, as shown by the superior amplification of polyclonal Vγ9Vδ2 T cells in PBL cultures repeatedly induced by *M. tuberculosis* ME compared with induced by BCG ME (Table 1).

Cellular TUBag load level correlates with the pathogenicity of tuberculous mycobacteria for humans. Given that both BCG and *M. tuberculosis* isolates are prone to variations in their cell wall antigen contents and to test whether in diverse isolates of tuberculous-related mycobacteria the bacterial TUBag load correlated with pathogenicity, we simultaneously titrated by short-term cytotoxicity the TUBag-induced responses of a single $V\gamma9V\delta2^+$ T-cell clone, G115 (6), triggered by equivalent extracts from various mycobacterial isolates. This study involved four laboratory type strains of *M. tuberculosis*, five clinical isolates from tuberculosis patients, the AN5 type strain of virulent *M. bovis*, and four different type strains of the nonpathogenic BCG vaccine as well as a panel of nontuberculous potential pathogens (collectively referred to as opportunistic), including *M. avium*, *Mycobacterium marinum*, *M. fortuitum*, *Mycobacterium chelonae*, and the environmental but nevertheless opportunistic human pathogen *Mycobacterium smegmatis* (31). Since considerable TUBag degradation and loss occurs over time in any extract (our unpublished observation), freshly grown mycobacterial cultures were produced from which hydrosoluble extracts (MEs) were simultaneously prepared at the same concentration and titrated with a 100-ASU TUBag reference (Fig. 3).

First, these data showed little variation in the bacterial TUBag amounts estimated in independent cultures of the same strain (*M. tuberculosis* H37Rv), thereby allowing a comparative analysis. Second, like the Erdman and Canetti-derived *M. tuberculosis* laboratory strains, the H37Ra strain (whose classification as avirulent relied upon guinea pig tests [28]) titrates at a high level like strain H37Rv does, that is, at around 900 ASU. Collectively, these laboratory *M. tuberculosis* strains have higher TUBag loads than the average TUBag load of the clinical *M. tuberculosis* isolates (mean, 235 ASU), where the observed variation in TUBag loads possibly reflected growth

differences. Taken together, most of these strains trigger a high cytotoxic response at bacterial extract concentrations of about 10 μg/ml. Third, the bacterial TUBag load of virulent *M. bovis* AN5 (532 ASU) is high, that is, on the same order of magnitude as those of most *M. tuberculosis* strains, whereas the TUBag load of all of the vaccinating BCG strains tested herein was at the lowest level recorded in the study (below 9 ASU, that is, around 10% cytotoxicity with 1,000 μg of BCG extract per ml). In support of the view that BCG is a TUBag hypoproducer variant of *M. bovis* rather than representative of some normal TUBag loads in nontuberculous mycobacteria, the TUBag loads of nontuberculous opportunistic pathogens (*M. avium*, *M. marinum*, *M. chelonae*, *M. fortuitum*, and *M. smegmatis*) were titrated at around 300 ASU, that is, on the same order of magnitude as the TUBag load of *M. bovis* or *M. tuberculosis.*

DISCUSSION

In humans, the peripheral Vγ9Vδ2$^+$ T-cell subset is subjected to an intense postnatal amplification (3, 21) presumably triggered by recurrent antigens (3, 7, 8), some of which derive from mycobacteria (10, 11, 18). On the basis of indirect evidence, it was suggested that such antigens contain carbohydrate (22, 23) and, more recently, phosphate (4, 26, 29). Actually, the *M. tuberculosis* ligand for human γδ T cells is composed of at least four structurally related molecules (TUBag1 to -4) (4). The present report shows that production of such TUBag is not specific to *M. tuberculosis* but common to a variety of different mycobacteria, including environmental species. These data constitute the molecular evidence that these shared TUBag1 to -4 correspond to the predicted few recurrent antigens driving the postnatal expansion of human Vγ9Vδ2 T lymphocytes (7, 9). Besides demonstrating the common production of these phosphorylated TUBag among mycobacteria, this study evidenced a discrepancy in the TUBag load among mycobacteria of the tuberculosis group (*M. tuberculosis*, *M. bovis*, and *M. bovis* BCG). Our data show that BCG produces 50- to 100-fold-lower amounts of these four TUBag substances.

Initially, BCG was deliberately attenuated from a virulent strain of *M. bovis* by serial passage in vitro, but distinct BCG strains derived from the original Pasteur strain developed by Calmette and Guérin, which has been prepared worldwide under differing conditions for vaccine purposes (27). In this study, we found lower TUBag yields in all four vaccinating BCG strains tested than in any virulent *M. bovis* or *M. tuberculosis* strain, as shown by either isolation procedures or comparative titration.

We have recently shown (15) that TUBag stimulation of human Vγ9Vδ2$^+$ T lymphocytes triggers in the T cell an almost immediate, broad, major histocompatibility complex-unrestricted cytotoxic activity for a large spectrum of cell targets, including the effector cells themselves. If these observations remain valid in vivo, then in infectious lesions of mycobacterial origin, such a massive response might participate in autoimmune cell degradation at the site of TUBag contact with reactive Vγ9Vδ2$^+$ T cells. Comparative titrations of cell-bound TUBag indicate that a low TUBag load is produced by the nonpathogenic mycobacterium BCG, which implies that only a nondeleterious level of γδ T-cell response must be triggered by vaccination with BCG. Accordingly, no detectable in vitro cytotoxic response can be recorded in mycobacterium-reactive γδ T cells stimulated with less than 1 mg of BCG-derived extract per ml. Conversely, the highly pathogenic tuberculous mycobacteria mostly harbor a high TUBag load, that is, TUBag loads possibly able to trigger in situ the activation of autoimmune cytotoxicity. So, once *M. tuberculosis* bacteria reach their host tissues, their TUBag charge could induce local autoimmune cytotoxic response and tissular degradation within the mycobacterial ecological niche. In a situation reminiscent of the present observations, a strong stimulation of Vγ9Vδ2$^+$ T cells, presumably driven by TUBag-related stimuli, has been detected with virulent group A but not avirulent group D streptococcal species (2, 20). Together, these observations favor the hypothesis that, in tuberculous mycobacteria, pathogenicity could to some extent be determined by a high level of TUBag and, hence, that a high TUBag load could constitute an immunological determinant of microbial virulence. Since BCG produces the same four TUBag as its virulent *M. bovis* parent does (Fig. 2), it is conceivable that in mycobacteria, TUBag biosynthesis involves a common operon which is down-regulated in the BCG progeny of *M. bovis.*

Studies with the mouse model (14, 16, 19, 25) have led to the hypothesis that γδ T cells act as regulators of the anti-infectious immunity. The present study suggests that in recently infected humans, the high TUBag loads of the *M. tuberculosis* pathogen could drive a massive γδ T-cell activation, whereas a more chronic exposure to lower levels of BCG vaccine-derived TUBag likely results in nondeleterious effects. In agreement with this view, recent infection with *M. tuberculosis* has been found to correlate with the onset of Vγ9Vδ2 T cells in PBL (30). Massive γδ T-cell triggering in multibacillary tuberculosis could then create an imbalance in the regulatory function of the γδ T cells either towards an autoimmune pathogenic cytotoxicity or, conversely, to anergization of potentially reactive γδ T-effector cells as reported for a tuberculosis patient (1). This could reconcile the finding of high TUBag loads in tuberculous mycobacteria and the current view that a nominal γδ T-cell response contributes to immune protection rather than to pathogenesis (1).

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P. Constant and Y. Poquet contributed equally to this study.

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