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Antigen Provoking Gamma Interferon Production in Response to *Mycobacterium bovis* BCG and Functional Difference in T-Cell Responses to This Antigen between Viable and Killed BCG-Immunized Mice

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It has been shown that gamma interferon (IFN-γ)-producing CD4$^+$ T cells, which are generated only by immunization with viable bacteria, exert a significant role in protective immunity against mycobacteria in mice. In this study, we have tried to determine the antigen recognized by the T cells in search of a possible protective antigen. T cells from viable *Mycobacterium bovis* BCG-immunized mice were stimulated with several antigens, and IFN-γ production was measured. Purified protein derivative and viable and killed BCG lysates caused significant IFN-γ production, and almost the same level of IFN-γ activity was detected in both groups stimulated with viable and killed BCG lysates. However, heat shock protein (HSP) 65 and HSP 70 were not a major antigen for IFN-γ production. The antigen provoking IFN-γ production is localized mainly in the membrane fraction of BCG cells, and the approximate molecular size was 18 kDa. On the other hand, T cells from killed BCG-immunized mice never responded to this antigen for IFN-γ production, whereas they could mount a delayed-type hypersensitivity response. These results showed that the antigen provoking IFN-γ production was present in killed as well as viable BCG. In addition to the antigen presentation by antigen-presenting cells, some kinds of differentiation factor (such as monokines) that are produced only by stimulation with viable cells seemed to be necessary for the development of IFN-γ-producing T cells.

Tuberculosis continues to be a major public health problem. Even now, infection with *Mycobacterium tuberculosis* leads to the deaths of 2 to 3 million people each year (25). It has been reported that the epidemic of human immunodeficiency virus infection has caused the higher case rate of tuberculosis and the high susceptibility to *M. tuberculosis* (2, 18). Significant outbreaks of multidrug-resistant strains of *M. tuberculosis* have further been reported (7). However, our strategy for enhancing the antimycobacterial resistance of the host depends only on vaccinaton with *Mycobacterium bovis* BCG that was developed long time ago, and any improvement has not yet been achieved. The efficacy of BCG vaccination is now coming into question because human trials have failed to demonstrate significant protection (10). It is also indicated that the tuberculin skin test using purified protein derivative (PPD), a widely available mycobacterial antigen for diagnosis, may lead to false-positive reactions because of the antigenic similarities among various mycobacteria. The test cannot specifically identify the infecting mycobacterial species (33). Accordingly, development of better vaccines and diagnostic reagents is required.

It is generally accepted that protective immunity is mediated by T cells which activate macrophages to express enhanced antimycobacterial activity by lymphokine production. CD4$^+$ T cells are considered to be the primary T-cell subset responsible for the immune response to *M. tuberculosis* (22, 28, 30). Thus, one of the approaches for the development of a vaccine and diagnostic reagent is to identify mycobacterial antigens which are recognized by CD4$^+$ T cells regulating protective immunity. Many researchers made an effort to identify the antigens involved in humoral and cell-mediated immunity against mycobacteria, and various antigens have been characterized (37). However, the antigens involved in protective immunity are still not clear. This may be because most of these antigens were clarified by antigen-specific T-cell proliferation, antibody response in mice and humans, and delayed-type hypersensitivity (DTH) response. Recent reports showed that neither DTH, proliferation, nor interleukin-2 (IL-2) production correlated with protective immunity (16, 20, 27, 34). In the study of lymphokines produced specifically by T cells responsible for the expression of protective immunity, we have shown that gamma interferon (IFN-γ) production may be an appropriate marker for CD4$^+$ T cells protective against infection with *M. bovis* BCG (16) and *Listeria monocytogenes* (34). Thus, the antigens which are recognized by IFN-γ-producing CD4$^+$ T cells may be regarded as the reliable protective antigen.

In the present study, we have fractionated PPD and lysates of viable and killed cells of BCG according to the molecular weight by gel electrophoresis and tried to identify the antigen provoking IFN-γ production in search of a possible protective antigen. Our data clearly demonstrated that the antigen exists only in the low-molecular-weight range of both killed BCG lysate and viable BCG lysate as well as in PPD and is not unique to viable cells.

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MATERIALS AND METHODS

Mice. Male C3H/He mice raised and maintained in a specific-pathogen-free condition were used at ages of 8 to 10 weeks.

Reagents. PPD was purchased from Japan BCG Production Inc. (Tokyo, Japan). Recombinant mycobacterial heat shock proteins (HSPs), HSP 65 and HSP 70, were kindly provided from Jan D. A. van Embden with support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

***M. bovis* BCG.** *M. bovis* BCG (Pasteur strain) maintained in our laboratory was grown for 10 to 14 days in Middlebrook 7H9 broth (Difco Laboratories, Detroit, Mich.) supplemented with 5 mg of bovine serum albumin (BSA) per ml, 4 μg of catalase (Sigma Chemical Co., St. Louis, Mo.) per ml, 0.2% glycerol, 2 mg of glucose per ml, 0.85 mg of NaCl per ml, and 0.05 mg of oleic acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan) per ml. Cells were collected by centrifugation at 7,000 × *g* for 15 min, washed with broth without supplements, and dispensed as stock culture at an appropriate concentration. The aliquots were stored at −80°C before use. The concentration of BCG was determined by plating the diluted bacterial suspension onto Ogawa's egg yolk medium plates and counting the colonies after 21 days. Killing of bacteria was achieved by incubation of BCG cells in 1% formaldehyde at 37°C overnight.

Preparation and fractionation of BCG lysates. Viable and killed BCG cells were washed three times with 20 mM Tris-HCl (pH 8.0) containing 0.05% Tween 80. BCG cells resuspended in the buffer were sonicated at 200 W and 1.7 A for 15 min with an Insonator model 200M (Kubota, Tokyo, Japan) and passed three times through a French press (Ohtake, Tokyo, Japan) set at 20,000 lb/in^2. The lysates were separated into subcellular fractions according to the procedure of Orme et al. (29). Briefly, the lysates were centrifuged at 20,000 × *g* for 20 min at 4°C. The resultant pellet contained mostly the cell wall fraction. The supernatant was recentrifuged at 100,000 × *g* for 2 h at 4°C to yield the membrane fraction (pellet) and the cytoplasmic fraction (supernatant). The BCG lysates were also fractionated according to the molecular sizes of the proteins by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using ELFE (Genofit, Geneva, Switzerland). The lysate was treated with the same volume of sample buffer under reducing conditions at 95°C for 5 min and was applied on SDS–12.5% polyacrylamide gel. The electroelution was carried out with a constant 6-mA current, and 1-ml fractions were collected. The fractions were dialyzed against 20 mM Tris-HCl (pH 8.0) to remove SDS, passed through a 0.22-μm-pore-size filter, and then used as fractionated soluble antigens. The protein concentration was determined with a protein assay kit (Bio-Rad Laboratories, Richmond, Calif.). The molecular weight of the proteins in each fraction was estimated with an electrophoresis calibration kit (Pharmacia Fine Chemicals, Uppsala, Sweden).

Immunization of mice and cell cultures. Viable and killed BCG cells were appropriately diluted with phosphate-buffered saline (PBS), mixed with the same volume of incomplete Freund's adjuvant (Difco), and emulsified by stirring vigorously at 15,000 rpm for 10 min on ice with an Ace homogenizer (Nihonseiki Ltd., Tokyo, Japan). We emulsified viable cells of BCG in incomplete Freund's adjuvant in order to immunize mice with two types of vaccines prepared in the same way and in the same route. Mice were immunized by subcutaneous injection with 0.2 ml of the emulsion containing either 10^5 viable or 10^7 killed BCG cells. Killed cells were used at a 100-fold-higher dose to compensate for the number of viable bacteria which grow in vivo. Four weeks after injection, spleens were removed and the T cells were enriched by a passage through a nylon-wool column after lysis of contaminating erythrocytes. Nylon-wool-passed T cells were suspended at a concentration of 5×10^6 cells per ml in culture medium that consisted of RPMI 1640 supplemented with 10% fetal calf serum (Flow Laboratories, Inc., McLean, Va.), 100 U of penicillin G per ml, 100 μg of streptomycin per ml, 5×10^{-5} M 2-mercaptoethanol, 5 g of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) per liter, and 2 g of $NaHCO_3$ per liter. Splenic T cells were stimulated with PPD, HSP 65, HSP 70, BCG lysates, and the fractionated antigens at 37°C in the presence of 2.5×10^4 of peptone-induced peritoneal exudate cells as antigen-presenting cells (APC) per ml. HSPs were provided by J. D. A. van Embden, National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands. The culture supernatants were collected 2 days after stimulation. For measurement of proliferative response, 100 μl of splenic T-cell suspension was distributed in 96-well flat-bottomed culture plates (Costar, Cambridge, Mass.) and the cells were cultured with the antigens and APC for 2 days. Eight hours before harvesting, 18.5 kBq of [^{3}H]thymidine (ICN Radiochemicals, Irvine, Calif.) was added to each well, and the radioactivity incorporated into the cells was measured.

IFN-γ assay. The IFN-γ titer in the supernatants was determined by enzyme-linked immunosorbent assay as previously described (16). Briefly, the supernatants and recombinant mouse IFN-γ (a gift from Central Research Institute, Daiichi Seiyaku Co. Ltd., Tokyo, Japan) were added to wells of enzyme immunoassay plates (Costar) precoated with rat anti-mouse IFN-γ monoclonal antibody (Lee Biomolecular Research Inc., San Diego, Calif.) and 0.5% BSA in 0.05 M carbonate-bicarbonate buffer (pH 9.6). After incubation for 90 min, the plates were washed and incubated with rabbit anti-mouse IFN-γ polyclonal antibody for 90 min. After the plates were washed, peroxidase-conjugated goat anti-rabbit immunoglobulin (Zymed Laboratories, Inc., San Francisco, Calif.) was added and the plates were incubated for 90 min. The plates were washed, and then orthophenylenediamine in phosphate buffer (pH 5.0) with 0.003% H_2O_2 was added as a substrate solution. The reaction was terminated by adding 2.5 M H_2SO_4, and the A_{490} was measured. The IFN-γ titer was expressed as international units per ml.

T-cell Western blot (immunoblot) analysis. Twenty microliters of the fractionated antigen and PPD were subjected to SDS–12.5% PAGE. After electrophoresis, proteins were transferred to a nitrocellulose filter by electroblotting at 90 mA overnight. The filter was washed in PBS containing 0.3% Tween 20 and stained with colloidal gold (AuroDye forte; Amersham International plc, Amersham, United Kingdom). After brief washing with distilled water and air drying of the filter, protein-dotted nitrocellulose areas (20 mm^2) were cut out, dissolved in 0.75 ml of dimethyl sulfoxide for 1 h, and then precipitated by addition of 0.05 M carbonate-bicarbonate buffer (pH 9.6) according to the method of Abou-Zeid et al. (1). The resultant nitrocellulose particles were washed three times, resuspended in 0.5 ml of culture medium or PBS, and kept at −20°C until used as antigens.

DTH assay. Spleen cells obtained from normal mice and from mice immunized with viable and killed BCG were passed through a Sephadex G-10 column (Pharmacia). Nonadherent cells were collected and mixed with PPD (200 μg/ml) or with the fraction (20 μg/ml) containing the antigen provoking IFN-γ production, which was obtained from preparative SDS-PAGE. Fifty microliters of the cell suspension (10^7 cells) was

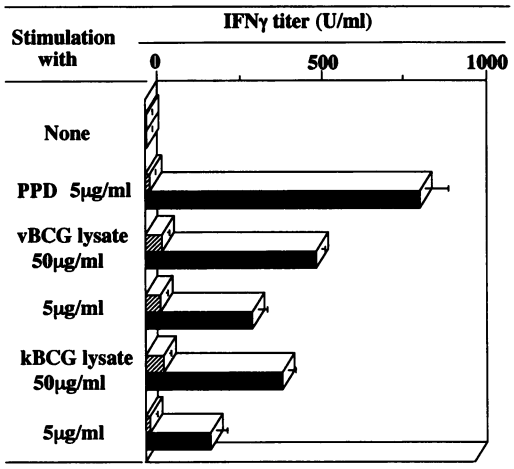

FIG. 1. IFN-γ production by T cells from viable BCG-immunized mice in response to PPD and BCG lysates (v, viable; k, killed). Splenic T cells from viable BCG-immunized mice (closed columns) and from normal mice (hatched columns) were stimulated with PPD and the lysates in the presence of APC for 2 days. Data are expressed as the mean of triplicate values ± standard deviations.

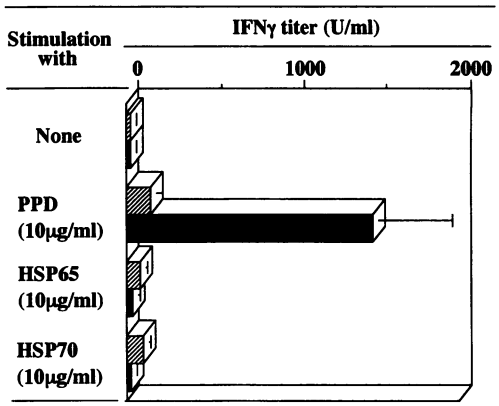

FIG. 2. IFN-γ production by T cells from viable BCG-immunized mice in response to PPD, HSP 65, and HSP 70. Splenic T cells from viable BCG-immunized mice (closed column) and from normal mice (hatched column) were stimulated with PPD and HSPs in the presence of APC for 2 days. Data are expressed as the mean of triplicate values ± standard deviations.

injected into the left hind footpads of normal sex-matched syngeneic mice. The specific swelling was measured 24 h later. DTH was expressed as the difference in footpad swelling between the left and right control footpads.

RESULTS

Determination of the antigen involved in IFN-γ production. We first determined which antigen is recognized by IFN-γ-producing T cells and whether the antigen exists only in viable BCG or is present even in killed BCG. The lysates of viable and killed BCG were prepared by using sonication and a French press, and IFN-γ production by T cells from viable BCG-immunized mice was measured after stimulation with PPD and both lysates. In the present study, splenic T cells, but not purified $CD4^+$ T cells, were used as the producer cells of IFN-γ because the lymphokine production has been shown to be limited to $CD4^+$ T cells that regulate protective immunity (16). PPD usually showed the highest antigenic activity. A significant level of IFN-γ production was also detected after stimulation with both lysates at almost the same level (Fig. 1). These data showed that the antigen recognized by IFN-γ-producing T cells commonly exists in both lysates and was not unique to viable BCG. We also determined the antigenic ability of recombinant HSP 65 and HSP 70. In contrast to PPD and both lysates, HSP 65 and HSP 70 showed poor antigenicity to stimulate IFN-γ production (Fig. 2). Accordingly, HSP 65 and HSP 70 did not seem to be the major antigen recognized by IFN-γ-producing T cells.

In order to determine the antigen involved in IFN-γ production, we carried out T-cell Western blot analysis. PPD (100 μg) was applied to SDS–12.5% PAGE gels and transferred to nitrocellulose filters by electroblotting. The protein-blotted areas (20 mm^2) were cut out, and Western blot antigens were prepared. Splenic T cells from viable BCG-immunized mice were stimulated with 20 μl of the blot antigens, and the cell proliferation and IFN-γ production were measured. The proliferative response was observed in response to a wide spectrum of antigens (Fig. 3b). In contrast, the pattern of IFN-γ production was quite different. A significant level of IFN-γ production was observed only in response to limited fractions containing 14- to 20-kDa antigens (Fig. 3a). Proliferation of T cells from killed BCG-immunized mice was also shown in response to a wide spectrum of the antigens. However, these cells could not produce a significant level of IFN-γ (Fig. 4).

It is shown in Fig. 1 that the antigenic activity for IFN-γ production was almost the same in both viable and killed BCG lysates. If IFN-γ-producing cells recognize only the low-molecular-weight antigen, the antigen must be present in both viable and killed BCG lysates. To confirm this, we prepared fractionated antigens from PPD and viable and killed BCG lysates by preparative SDS-PAGE. PPD and both lysates (3 mg) were applied to SDS-PAGE gels and electroeluted according to molecular weight. Figure 5 shows the representative reelectrophoresis pattern of the fractionated PPD antigens on an SDS–12.5% polyacrylamide slab gel after silver staining. The similar pattern was also seen in the fractions obtained from viable and killed BCG lysates (data not shown). Figure 6 shows IFN-γ production by splenic T cells from viable BCG-immunized mice after stimulation with the fractionated antigens of both lysates and PPD at a concentration of 1 μg/ml. IFN-γ could be produced equally by stimulation with the similar fractions prepared from both viable and killed BCG lysates. Furthermore, the molecular weight of the fractions capable of stimulating IFN-γ production was almost the same as that of PPD. Among the fractions in the low-molecular-weight range, the highest activity was always detected in fraction 5. Though the semipurified fraction still appeared to contain heterogeneous proteins, the molecular mass of major band was approximately 18 kDa. It was shown that the antigen recognized by IFN-γ-producing T cells was present in the 18-kDa region and the amount of antigen in both BCG lysates was almost the same.

DTH mediated by T cells from killed BCG-immunized mice after elicitation with the antigen provoking IFN-γ production. In order to determine whether T cells recognizing the antigen provoking IFN-γ production have developed in mice immunized with killed BCG, DTH response against the antigenic fraction containing the antigen provoking IFN-γ production was examined by an adoptive cell transfer experiment. The antigen could elicit a significant level of DTH equally in both groups of mice receiving cells from killed and viable BCG-

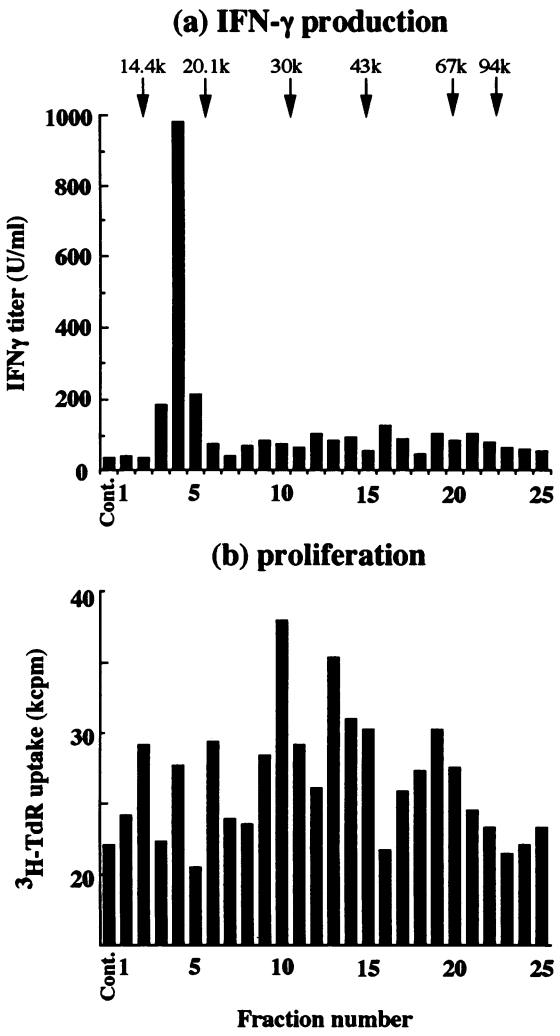

FIG. 3. T-cell Western blot analysis for IFN-γ production and proliferation of T cells from viable BCG-immunized mice. (a) Pattern of IFN-γ activity. Data are expressed as the means of triplicate values. Standard deviations did not exceed 12% of the mean. (b) Pattern of the proliferative response. Data are expressed as the mean [^{3}H]thymidine (^{3}H-TdR) uptake of triplicate cultures. Standard deviations did not exceed 23% of the mean. Cont., response to control nitrocellulose particles. Numbers on the top refer to positions of molecular weight markers.

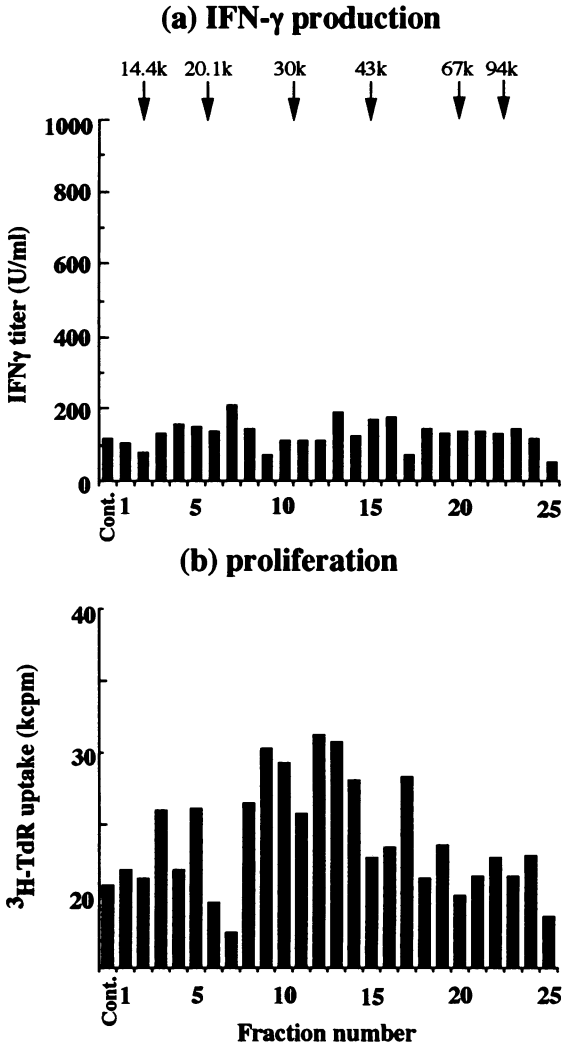

FIG. 4. T-cell Western blot analysis for IFN-γ production and proliferation of T cells from killed BCG-immunized mice. Data are expressed in the same manner as in Fig. 3. Standard deviations of IFN-γ activities and proliferative responses did not exceed 17% of the mean.

immunized mice (Fig. 7). The level of DTH elicited with 10 μg of PPD was almost the same in both groups of mice. The swelling was not a nonspecific reaction, because the level of DTH response in mice receiving normal cells plus the antigen was the same as that in group of mice given the antigen alone. The data suggested that among several T-cell populations which have developed by immunization with killed BCG, T cells responding to the antigen could express DTH but not produce a large amount of IFN-γ.

Location of the antigen in BCG cells. We next examined the location of the antigen in the cells of viable BCG. The lysate of viable BCG was differentially centrifuged and separated into three fractions rich in either cell wall, cytoplasmic membrane, or cytoplasm. An aliquot (100 μg) of each subcellular fraction was applied to SDS–PAGE 12.5% gels and transferred to nitrocellulose. The area containing the 18-kDa region was cut out from each filter paper, and Western blot antigens were prepared. Splenic T cells from viable BCG-immunized mice were stimulated with 20 μl of the blot antigen, and IFN-γ production was determined. All the subcellular fractions obtained from BCG cells showed the antigenicity to induce IFN-γ production. The highest production of IFN-γ was observed in response to the blot antigen obtained from the membrane-rich fraction (Fig. 8). Therefore, the antigen critically important for IFN-γ production appeared to be present mainly in the membrane-rich fraction.

DISCUSSION

IFN-γ is a pleiotropic cytokine with an immunomodulatory effect on a variety of cells. In respect to the effect on macrophages, it has been reported that IFN-γ induces the expression of major histocompatibility complex class II antigen on the surface of macrophages and primes macrophages for the production of nitric oxide and reactive oxygen intermediates,

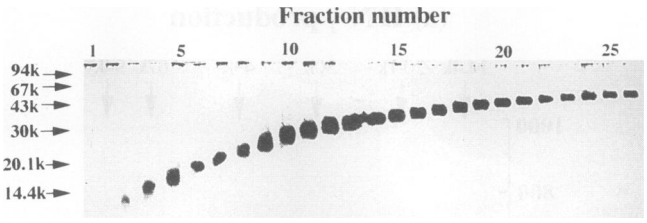

FIG. 5. Pattern of PPD antigens fractionated by preparative SDS-PAGE. PPD (3 mg) was electroeluted from an SDS–12.5% polyacrylamide gel. Ten microliters of the resultant fractions was reelectrophoresed on an SDS–12.5% polyacrylamide slab gel, and silver staining was done. Numbers on the left refer to positions of molecular weight markers.

which may directly cause the intracellular killing of bacteria (6). IFN-γ is widely regarded as a major macrophage-activating factor to enhance antibacterial capacity (4, 24).

IFN-γ is also thought to be the critical factor for the expression of antimycobacterial immunity in mice. It has been shown that lymphocytes from mice immunized with mycobacteria produced a large amount of IFN-γ (32, 35). Our previous study has shown that IFN-γ production, but not IL-2 production or DTH, correlated with the expression of protective immunity against *M. bovis* BCG (16). In an in vitro experiment designed to examine the effect on intracellular growth of mycobacteria, IFN-γ was shown to be capable of stimulating murine macrophages to kill *M. tuberculosis* (11). These results suggested that protective T cells may contribute to antimycobacterial immunity in vivo by the direct activation of macrophages through IFN-γ production.

It has been generally accepted that protective immunity against mycobacteria can be induced by immunization with viable bacteria but is difficult to induce by using killed bacteria for immunization (27). Based on such an observation, there is an idea that only viable bacteria possess the protective antigen, presumably the metabolites of living bacteria rather than constitutive proteins that exist commonly in both dead and viable bacteria (28). In the present study, however, IFN-γ production from T cells, which were obtained from viable BCG-immunized mice and could confer protection against BCG, was observed when the T cells were stimulated with killed BCG lysate as well as viable BCG lysate and PPD. On the basis of the pattern of IFN-γ production after stimulation with the fractionated antigens, it was shown that the antigen which provokes IFN-γ production is present in both lysates at almost the same levels. On the other hand, T cells from mice immunized with killed BCG could induce a DTH response but not produce a large amount of IFN-γ against stimulation with the fractionated antigen. This result showed that T cells responding to the antigen have developed into DTH-mediating cells even after immunization with killed BCG but could not produce IFN-γ in an antigen-specific manner. This finding raises the question of why T cells mediating protective immunity cannot be generated by immunization with killed BCG. In our previous studies using *L. monocytogenes*, a significant difference between viable and killed bacteria was observed in the ability to induce monokine production from macrophages (14, 21). The data showed that production of a large amount of IL-1 was induced by stimulation with viable bacteria but not with killed bacteria. Administration of recombinant IL-1 accelerated the in vivo generation of DTH-mediating T cells in mice immunized with killed *L. monocytogenes*. A similar difference in monokine-producing ability was observed between viable and killed BCG as well (unpublished observation). Therefore, it seems that in addition to the presentation of the protective antigen by macrophages to $CD4^+$ T cells, IL-1 and/or some other monokines are necessary for the development of protective T cells.

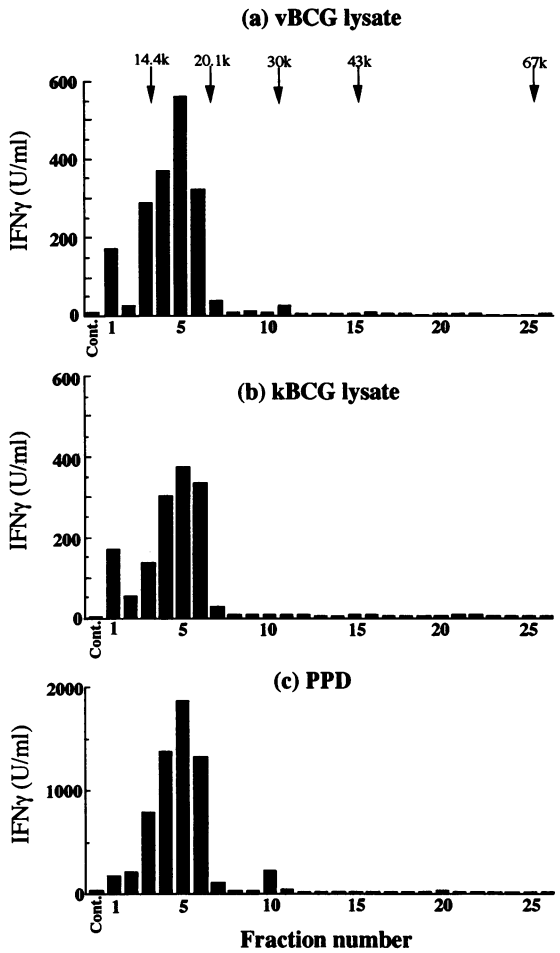

FIG. 6. IFN-γ production of splenic T cells from viable BCG-immunized mice in response to fractions obtained from PPD and BCG lysates by preparative gel electrophoresis. Splenic T cells from viable BCG-immunized mice were stimulated with fractions obtained from viable BCG lysate (a), killed BCG lysate (b), and PPD (c) in the presence of APC for 2 days. Data are expressed as the means of triplicate values. Standard deviations did not exceed 20% of the mean. Numbers on the top refer to positions of molecular weight markers.

Several papers showed that some mycobacterial antigens could stimulate IFN-γ production from T cells sensitized with *M. tuberculosis* (5, 29) and *M. bovis* (13). However, we detected a significant level of IFN-γ production only when T cells were stimulated with the fractions with approximately 18-kDa antigen. The discrepancy between these observations might be due to a difference in culture conditions or strains used for immunization and stimulation as the antigens. In order to determine whether the low-molecular-weight antigen has distinct importance for the expression of protection and is valuable as the primary target in the development of new vaccine, we have preliminarily examined the response of human peripheral blood mononuclear cells obtained from tuberculin-positive healthy donors and patients with pulmonary tuberculosis against fractionated PPD antigens. IFN-γ

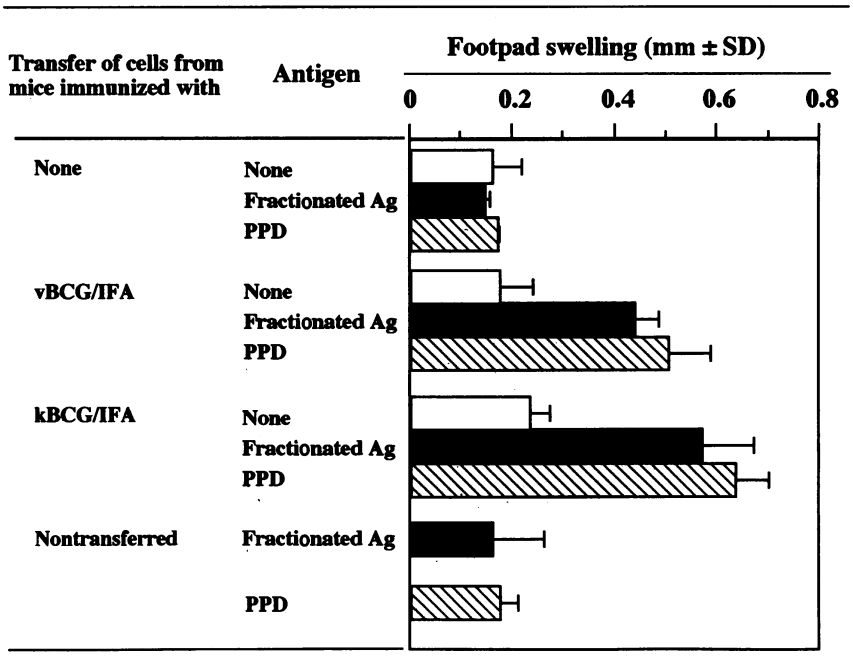

FIG. 7. Adoptive transfer of DTH using T cells responding to the antigen provoking IFN-γ production. Splenic nonadherent cells (10^7) from viable (v) and killed (k) BCG-immunized mice and from normal mice were transferred into footpads of naive mice with the fraction (1 μg) containing the antigen provoking IFN-γ production (Fractionated Ag) or PPD (10 μg) or without antigen (None). Data are expressed as the mean difference in thickness between the left and right footpads of five mice ± standard deviation (SD). IFA, incomplete Freund's adjuvant.

activity was observed in response to the fractions containing 14- to 40-kDa antigens. Among them, the fractions ranging 14 to 20 kDa were the most effective in IFN-γ production (data to be published). Accordingly, it appears that the antigen in a relatively low molecular weight range is critically important for triggering IFN-γ production in humans as well as in mice. The role of IFN-γ in human antimycobacterial resistance is still controversial because there is a report that IFN-γ was not effective by itself in activating human macrophages for killing of *M. tuberculosis* in the in vitro experiment (31). However, it was reported that mycobacterium-reactive $CD4^+$ T-cell clones obtained from tuberculoid leprosy patients produced a large amount of IFN-γ (12). At the site of mycobacterial infection, the IFN-γ concentration was elevated (3). Administration of IFN-γ reduced the bacillary burden in patients with lepromatous leprosy (15). Taken together, IFN-γ may be the lymphokine distinctively required for the expression of protective immunity in humans, while a broad spectrum of cytokines may contribute to antimycobacterial immunity.

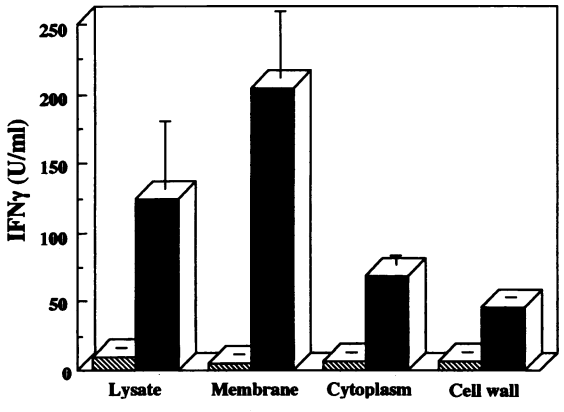

FIG. 8. Location of the antigen provoking IFN-γ production in BCG cells. Splenic T cells from viable BCG-immunized mice (closed column) and from normal mice (hatched column) were stimulated with 20 μl of 18-kDa region-containing Western blot antigen obtained from the indicated subcellular fractions. Data are expressed as the means of triplicate values ± standard deviations.

Several mycobacterial antigens of similar molecular weights have been reported previously (37). Nagai et al. have shown that 18-kDa protein (MPT63) was present in the culture fluid of *M. tuberculosis* and the corresponding antigen also exists in that of *M. bovis* BCG (23). They indicated that MPT63 was secreted mainly in culture fluid and was hardly detected in the lysate of BCG cells. In addition, MPT63 was not capable of inducing a DTH reaction in guinea pigs sensitized with killed *M. tuberculosis*. Thus, it seems that MPT63 is different from the antigen provoking IFN-γ production reported in the present study, in that the antigen existed in the cells of BCG and could induce a significant DTH reaction with an adoptive cell transfer system. Lee et al. purified a 19-kDa membrane protein from *M. tuberculosis* Erdman (19), and a similar protein was demonstrated in *M. bovis* BCG as well. A pI value of the protein was estimated to be 5.9. The deduced amino acid sequence was identical to that of the 16-kDa protein (originally classified as 14 kDa [8]), and there were some similarities in the sequence to that of the *M. leprae* 18-kDa protein. The protein is reported to be capable of inducing a DTH reaction and T-cell proliferation (17). We found that the approximately 18-kDa antigen of the present study was located mainly in the cytoplasmic membrane, and the pI value was preliminarily determined to be about 5.5 to 6.0 from the results of isoelectric

focusing and T-cell Western blot analysis. Thus, the properties of the antigen provoking IFN-γ production seemed to be similar to those of the 19-kDa membrane protein. Another 19-kDa lipoprotein from *M. tuberculosis* was identified by Young et al. as one of the secreted proteins which reacted with TB23 monoclonal antibody (37). The corresponding antigen was also demonstrated in the culture filtrate of *M. bovis* (9, 36). However, it is not yet clear whether the antigen triggering IFN-γ production is among those antigens reported so far or is a novel one. As one candidate for the protective antigen, we are currently trying to purify the antigen and determine its amino acid sequence.

Little is known about the distinctive features of protective T cells. Orme reported that memory immunity against *M. tuberculosis* was mediated by long-lived, cyclophosphamide- and irradiation-resistant $L3T4^+$ $Lyt\text{-}2^-$ T cells (26). We have shown that protective T cells were functionally characterized by the ability to produce IFN-γ. Now, it remains to be determined whether the difference of expression between DTH and protection against BCG is due only to the difference in the ability to produce IFN-γ between protective T cells and DTH-mediating T cells. In this regard, it may be required to look at the qualitative and quantitative differences in production of several lymphokines by these T-cell populations using the antigen reported here. In addition, the ability of the antigen to induce IFN-γ may be useful for the preferential activation and/or expansion of T cells required for the expression of protective immunity in the host that had been immunized or infected with *M. tuberculosis*.

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