# Guinea Pig Cellular Immune Responses to Proteins Secreted by *Mycobacterium tuberculosis*

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To study the immunological activity of proteins secreted by *Mycobacterium tuberculosis*, we carried out comparative studies in guinea pigs infected intravenously with $2.5 \times 10^3$ CFU of this organism or with $2.5 \times 10^4$ CFU of *Mycobacterium bovis* BCG. Groups of infected guinea pigs were skin tested with fractions of secreted proteins covering well-defined narrow-molecular-mass regions, or such fractions were used for lymphocyte stimulation experiments. The lymphocyte stimulation experiments showed that the fraction containing proteins with molecular masses below 10 kDa had a superior stimulating capacity in tuberculous guinea pigs whereas the 24- to 30-kDa fraction gave significantly higher skin reactions in this group compared with BCG-vaccinated guinea pigs. A precise mapping within the region from 23 to 35 kDa by using a combination of narrow overlapping fractions and purified proteins enabled the identification of the 24-kDa antigen MPT64 as a molecule specific for tuberculous infection. Thus, MPT64 is a promising candidate for a specific diagnostic skin test reagent for human tuberculosis.

The fact that the incidence of tuberculosis (TB) is increasing in developing as well as industrialized countries has led to renewed efforts to improve existing methods in the control of the disease. Two major products presently used to control the disease, the *Mycobacterium bovis* BCG vaccine and the diagnostic reagent tuberculin purified protein derivative (PPD), both have shortcomings: the efficacy of BCG remains a matter of controversy (24), and the diagnostic value of tuberculin is weakened by the fact that sensitivity is induced by a broad range of mycobacteria. In particular, the fact that successful BCG vaccination as well as infection with *Mycobacterium tuberculosis* lead to high-level tuberculin reactivity complicates the interpretation of tuberculin test results. An important approach to improvement of the existing BCG vaccine has been the study of proteins secreted by *M. tuberculosis* during growth, a group of potentially protective antigens (4, 6). The rationale behind this line of work has been discussed recently (21). The biological activities of secreted proteins have this far primarily been studied in the mouse model of TB, and this group of antigens has been demonstrated to contain important target molecules, which are responded to strongly at the height of infection as well as in mice in a state of memory immunity (4, 6). The present series of experiments was initiated with two main objectives: first, to study the biological activity of secreted proteins from *M. tuberculosis* in guinea pigs, a very relevant animal model of human TB (13), and second, to study this activity with particular emphasis on the identification of candidates for improved diagnostic skin test reagents for the disease. The results obtained suggest that guinea pig T cells respond strongly to secreted antigens during tuberculous infection and that a secreted 24-kDa protein termed MPT64 is a promising candidate for a specific diagnostic reagent.

## MATERIALS AND METHODS

**Guinea pigs.** Outbred guinea pigs from strain Ssc:AL bred at Statens Seruminstitut were used throughout the study. Female guinea pigs weighing 300 to 400 g at sensitization were used.

**Bacteria.** *M. tuberculosis* H37Rv or R1609 (isolate from a Danish TB patient) was grown in suspension in modified Sauton medium (10). *M. bovis* BCG Copenhagen (Danish isolate 1331) and *M. bovis* BCG Tokyo were obtained as freeze-dried preparations (Statens Seruminstitut).

**Infection and immunization of guinea pigs.** Guinea pigs were infected intravenously (i.v.) in an ear vein with $2.5 \times 10^3$ CFU of *M. tuberculosis* given in a volume of 0.1 ml. Infection by the same route with BCG was routinely done with $2.5 \times 10^4$ CFU. Vaccinations with BCG were done with four intradermal (i.d.) injections on the abdomen with 0.1 ml of BCG vaccine reconstituted according to the manufacturer's instructions. BCG Copenhagen contained approximately $4 \times 10^6$ CFU/ml of the reconstituted preparation. Immunizations with killed bacteria were done similarly with four 0.1-ml injections i.d. on the abdomen of a suspension of glutaraldehyde-killed bacteria at 0.4 mg (semidry weight)/ml of paraffin oil (Marcol 52). Guinea pigs were skin tested or lymphoid cells isolated for stimulation experiments 3 weeks following infection or immunization.

**Aerosol infection.** A frozen aliquot of *M. tuberculosis* Erdman containing $2.7 \times 10^7$ CFU/ml was thawed and diluted in phosphate-buffered saline (PBS) to a concentration of $10^4$ CFU/ml. This suspension was injected into the nebulizer-venturi unit of a Glas-Col inhalation exposure system, which is a whole-body exposure chamber. The guinea pigs were infected in two groups of 10 animals each. The airflow through the nebulizer and the nebulizing time were adjusted to make the infection give rise to the formation of 2 to 10 (mean of 5) primary lesions in the lungs of the animals.

**Mycobacterial antigens.** Tuberculin PPD RT23, 1 and 10 tuberculin units (TU) (Statens Seruminstitut), was used for skin testing. Tuberculin PPD RT42 (Statens Seruminstitut) for in vitro tests was used for the lymphocyte stimulation experiments.

Short-term culture filtrate (ST-CF) was produced as described previously (5). In brief, *M. tuberculosis* H37Rv bacteria ($8 \times 10^6$ CFU/ml) were grown in modified Sauton medium without Tween 80 on an orbital shaker for 4 to 7 days. The culture supernatants were sterile filtered and concentrated on an Amicon YM3 membrane (Amicon, Danvers, Mass.). Bacteria were killed by incubation overnight in 2% glutaraldehyde in PBS. The suspension was washed three times in PBS, and a rough estimate of bacterial numbers based on the semidry mass was obtained.

The MPT59 and MPT64 antigens were purified by physical-chemical methods as described previously (19). The 38-kDa antigen was prepared in our laboratory by affinity chromatography using monoclonal antibody (MAb) HBT12 (25).

**Preparative SDS-PAGE.** ST-CF was divided into 10 fractions (F1 to F10) by preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described previously (4). Briefly, ST-CF was separated simultaneously in four gels, the gels were cut into fractions, and the protein was eluted overnight with 0.1% (wt/vol) SDS in PBS. Corresponding fractions were pooled and passed twice through Extractigel columns (Pierce Europe B. V., Oud-Bei-

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jerland, The Netherlands) to remove the SDS. After this treatment, no toxicity in cell cultures was present (1a). The protein concentration of the fractions was estimated by the Coomassie brilliant blue method (8) with bovine serum albumin as the standard, and all fractions were adjusted and used in the same concentration. The fractions were analyzed by nonreducing SDS-PAGE by using a 10 to 20% acrylamide gel. The gel was fixed and silver stained. All fractions were aliquoted and kept frozen at $-20°C$.

**Preparative column SDS-PAGE.** Forty milligrams of ST-CF was applied to a Prep-cell column (Bio-Rad, Richmond, Calif.) containing a 16% homogeneous gel matrix. The separation was performed at 200 V overnight, and fractions were collected. Fractions holding molecules within the 23- to 35-kDa range were buffer exchanged into PBS and passed through Extractigel columns. The preparations were adjusted to 20 to 30 μg/ml and kept at $-80°C$ until use as skin test antigens.

**MAbs.** MAbs were prepared as described previously (3, 17, 23).

**Skin tests.** Guinea pigs were shaved on the back and given i.d. injections of 0.1 ml of physiological PBS (pH 7.4) containing the quantity of antigen indicated in the figure legends. Guinea pigs were depilated on the back 24 h later, and reactions were read by two independent readers, each measuring two transverse diameters of the erythemas. Reaction diameters are given as means corresponding to a single diameter.

**Lymphocyte stimulation tests.** Peripheral blood lymphocytes (PBL) were isolated from blood drawn by cardiac puncture, using EDTA as an anticoagulant. Erythrocytes were removed by Ficoll density gradient (density = 1.09) centrifugation. Lymphocytes were washed twice and counted, and the cell concentration was adjusted to $2 \times 10^6$ cells per ml in RPMI 1640 with supplements including 5% fetal calf serum. Spleen peripheral lymphocytes (SPL) were isolated by pressing spleens through a wire mesh. Erythrocytes were lysed by treatment with 0.84% $NH_4Cl$. The lymphocytes were washed twice, and the cell concentration was adjusted to $2 \times 10^6$ cells per ml of RPMI with supplements.

One-tenth milliliter of cells was cultured with 0.1 ml of antigen or mitogen in triplicate for 6 days, the last 22 h in the presence of 1 μCi of [³H]thymidine. Antigens were used in suboptimal concentrations to avoid high-dose inhibition and to allow better discrimination between immunogens. Cultures were harvested, and incorporated [³H]thymidine was counted in a scintillation counter. Results were expressed as counts per minute, using geometric means of triplicate cultures, or as stimulation indices (SIs) calculated as the ratio between antigen-stimulated and nonstimulated cultures.

**Statistical methods.** Two-way analyses of variance with the factor sensitization group and the fraction and antigen numbers were carried out on diameters of skin reactions or log-transformed counts-per-minute values. The variations between guinea pigs were thus included in the residual variations. Least significant differences between skin test means in two sensitization groups were estimated and are indicated in the figure legends. Similarly, differences between geometric mean counts-per-minute responses were estimated and indicated in the figure legends as least significant ratio or SI.

## RESULTS

**Delayed-type hypersensitivity (DTH)-inducing capacity of secreted protein fractions in tuberculous and BCG-infected guinea pigs.** Secreted proteins contained in ST-CF have previously been demonstrated to hold key antigen target molecules giving immune responses in animals infected with *M. tuberculosis* or vaccinated with BCG (4, 6, 15). To investigate possible differences in the specificity of responses induced by these mycobacteria, we established a model suitable for comparative studies. The sensitizing effects of $2.5 \times 10^3$ CFU of *M. tuberculosis* and of $2.5 \times 10^3$, $2.5 \times 10^4$, and $2.5 \times 10^5$ CFU of BCG Copenhagen (Danish isolate 1331) given i.v. were compared by tuberculin skin tests 21 days after infection. This period of time was found sufficient to give a fully mature primary tuberculin response (results not shown). Such a rather short time span has previously been demonstrated to be optimal for responses to secreted antigens (6). The intermediate dose of $2.5 \times 10^4$ CFU of BCG was chosen for the studies described below because it induced a tuberculin reactivity similar to that following infection of guinea pigs with our standard inoculum of $2.5 \times 10^3$ CFU of *M. tuberculosis* (results not shown).

To study the specificity of the response to secreted antigens, ST-CF was separated into 10 fractions covering the molecular mass span from 0 to 200 kDa by preparative SDS-PAGE. The fractions each held from two to five protein bands, and there was only a minimal overlap between neighboring fractions, as
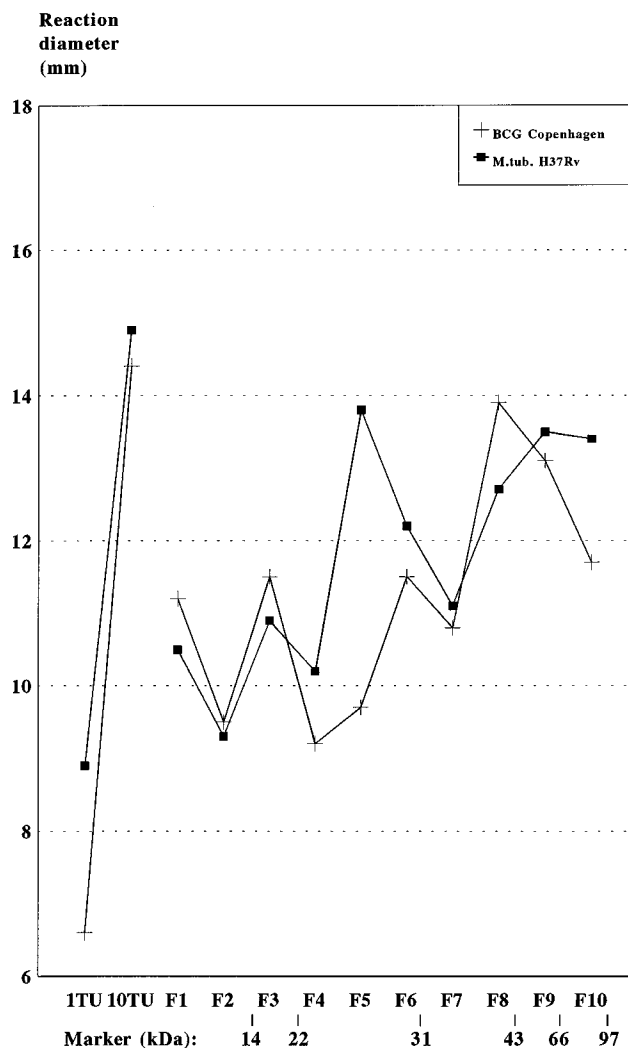


FIG. 1. Skin reactions to 1 and 10 TU of tuberculin and to 1 μg of the indicated fractions in guinea pigs infected i.v. with *M. tuberculosis* (M.tub.) H37Rv ($n = 26$) or BCG Copenhagen ($n = 16$). Mean reactions from three experiments with each infecting agent are shown. The least significant difference was 1.63 mm.

demonstrated previously (6). The ability of these fractions to induce DTH was studied in a series of guinea pig experiments carried out in groups of 8 to 10 animals. Groups were sensitized by i.v. infection with *M. tuberculosis* H37Rv or BCG Copenhagen (Danish isolate 1331). All guinea pigs were skin tested with tuberculin (1 and 10 TU) and with 1 μg of each of fractions F1 to F10, which cover the molecular mass spans indicated in Fig. 1. The results show similar tuberculin reactivities in both groups, emphasized by identical reactions to both 1 and 10 TU (Fig. 1). All fractions studied induced intermediate to high-level DTH reactions in sensitized guinea pigs but gave no reactions in unsensitized animals (not shown). The patterns of reactivity to most of the fractions were similar in the sensitized groups, and only fraction 5, which spans from 24 to 30 kDa, and the high-molecular-weight fraction F10 induced significantly higher reactions in guinea pigs infected with *M. tuberculosis* than in animals given BCG Copenhagen (Fig. 1).

**Lymphocyte-stimulating capacity of secreted protein fractions.** In a parallel series of experiments, we studied the lym-
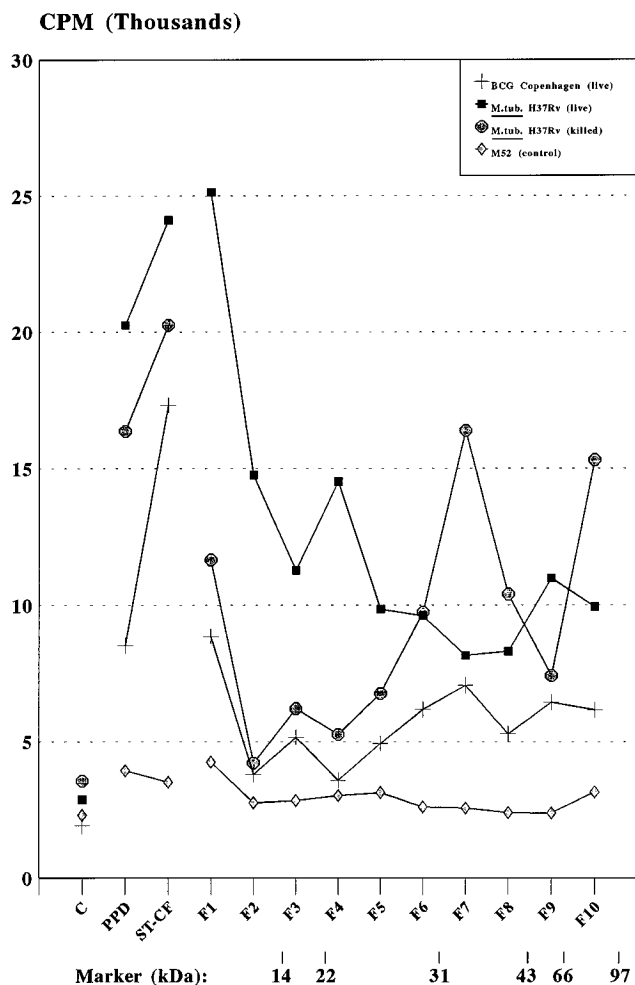
FIG. 2. Lymphocyte stimulation results with SPL from groups of guinea pigs not immunized (M52) (*n* = 7), infected i.v. with BCG Copenhagen (*n* = 6) or *M. tuberculosis* (*M.tub.*) (*n* = 8), or immunized with killed *M. tuberculosis* in oil (*n* = 5). Antigen stimulation was performed with a concentration of 1 μg/ml. C, control culture; ST-CF, unfractionated secreted proteins. Geometric mean reactions are shown. Ratios larger than 2.3 were statistically significant.



FIG. 3. Mean skin reactions to 10 TU of tuberculin and to 2 to 3 μg of Prep-cell fractions A to M in groups of 10 guinea pigs infected i.v. with BCG Copenhagen or *M. tuberculosis* (*M.tub.*). The least significant difference was 1.27 mm.

phocyte-stimulating capacity of the same ST-CF fractions used in the skin test experiments summarized in Fig. 1. The stimulating capacity was studied in SPL and PBL isolated from guinea pigs infected i.v. with *M. tuberculosis* H37Rv or BCG Copenhagen. Other groups in this experiment were immunized with a preparation of killed *M. tuberculosis* in oil or were given oil alone (control group). Because the two cell types gave quite similar results, only responses to SPL are shown (Fig. 2). None of the fractions stimulated cells from the control group. Cells from all sensitized groups reacted well to 1-μg/ml concentrations of both of the multiantigen preparations PPD and ST-CF, the latter giving the highest reactions in all sensitized groups. This experiment also demonstrate that in contrast to the very similar DTH-inducing capacity of *M. tuberculosis* and BCG on our model, *M. tuberculosis* is by far the most efficient inducer of in vitro reactivity to the preparations tested. It is obvious from the figures that fraction F1, which contains molecules smaller than 10 kDa, possesses a stimulating capacity superior to that of the other fractions. Interestingly, the superior DTH-inducing capacity of F5 was not seen in these in vitro experiments. A comparison of the killed and live sensitization shows that the

killed *M. tuberculosis*-in-oil preparation induced a peak proliferation at F7 (35 to 40 kDa), which is absent in infected guinea pigs.

**DTH-inducing capacity of intermediate (23- to 35-kDa)-molecular-mass ST-CF fractions.** To explore the difference in DTH reactivity to F5 between *M. tuberculosis* and BCG Copenhagen sensitization (Fig. 1), a set of skin test experiments was carried out with high-resolution ST-CF fractions in the 23- to 35-kDa molecular mass region. The 13 fractions were obtained from column SDS-PAGE. In contrast to the fractions prepared by elution, this panel of fractions was overlapping (not shown), thereby allowing a precise identification of molecules with peak activity in the molecular mass range under study. Two groups of guinea pigs were infected with *M. tuberculosis* or BCG Copenhagen and skin tested with 10 TU of tuberculin and with 2 to 3 μg of the fractions designated A to M (Fig. 3). The results confirmed and extended the finding obtained with F5 (Fig. 1) by precisely mapping an area around fraction C (~24 kDa), which gives highly significant larger reactions in the *M. tuberculosis* than in the BCG Copenhagen group (Fig. 3). The fractions did not give reactions in nonsensitized guinea pigs (results not shown).
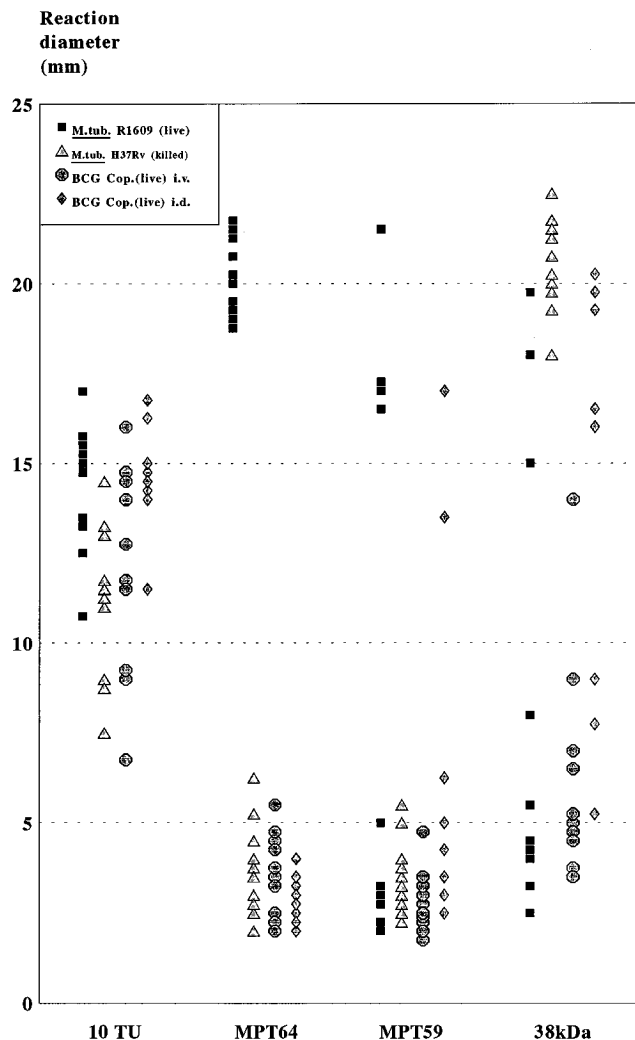
**Reaction
diameter
(mm)**



FIG. 4. Distribution of skin reactions to 10 TU of tuberculin and to 1 μg of the indicated antigens in three groups of 10 guinea pigs infected i.v. with *M. tuberculosis* (*M.tub.*) or BCG Copenhagen (Cop.), vaccinated i.d. with BCG Copenhagen, or immunized with killed *M. tuberculosis* in oil. Each symbol corresponds to a reaction in one guinea pig.

**Biological activities of three antigens purified from *M. tuberculosis*.** In the experiments described above, we observed two major qualitative differences caused by the choice of method of sensitization. One was the higher reactions in tuberculous guinea pigs to the fraction containing a 24-kDa antigen (Fig. 1), and the other was the pronounced lymphocyte stimulation to the 35- to 40-kDa region seen in animals sensitized with killed tubercle bacilli (Fig. 2). In an attempt to

identify the antigens responsible for these differences, the presence of three previously characterized proteins with molecular masses of 24 kDa (MPT64) (12), 31 to 32 kDa (the antigen 85 complex) (9, 11, 19), and 38 kDa (PstS) (2) in the high-resolution fractions was investigated by SDS-PAGE analysis of the fractions followed by immunoblotting with the relevant MAbs (Table 1). The results show that the region inducing peak skin test activity in tuberculous guinea pigs corresponds to the peak reactivity with MAb C24b2, indicative of maximum concentrations of the antigen MPT64. The 38-kDa antigen identified by MAb HBT12 could by comparison be detected only in fraction M covering the molecular mass range from 35 to 38 kDa, which was found to induce peak stimulation in animals sensitized with killed bacilli (Fig. 2). We therefore continued by carrying out experiments with the MPT59 (part of the antigen 85 complex), MPT64, and 38-kDa molecules. Groups of guinea pigs were infected i.v. with *M. tuberculosis* or BCG Copenhagen, vaccinated i.d. with BCG Copenhagen or immunized with killed *M. tuberculosis* in oil, and 3 weeks later skin tested with 10 TU of tuberculin and 1 μg of the purified antigens. The results show similar tuberculin reactivities in the groups but distinct patterns of reactivity to the purified antigens (Fig. 4). Only guinea pigs infected with *M. tuberculosis* gave skin reactions to MPT64 and MPT59, although a large proportion of guinea pigs were nonresponders to the latter antigen. These results have been confirmed by using three different strains of *M. tuberculosis*. Aerosol infection with *M. tuberculosis* likewise induces a strong MPT64 reactivity (Fig. 5). Figure 5 shows an increase in reactivity to MPT64 from 3 to 6 weeks following infection. Similar experiments at 8 and 11 weeks postinfection did not show a further increase in reactivity to this antigen (not shown).

Repeated experiments with MPT64 have shown that approximately 10% of the outbred guinea pig population are nonresponders to MPT64. Guinea pigs vaccinated with BCG Tokyo react similarly to MPT64 as when infected with *M. tuberculosis* (results not shown). Reactions to the 38-kDa antigens were consistently very high in guinea pigs sensitized with killed *M. tuberculosis*. The infected groups, in contrast, gave low and variable responses to this antigen.

Quite similar results were obtained with the lymphocyte stimulation technique using PBL (Fig. 6). With the exception of guinea pigs given the control preparation M52, all groups responded very well to PPD. In contrast, only the group infected with *M. tuberculosis* gave a mean net stimulation with MPT64. None of the groups gave significant mean responses to MPT59. All groups sensitized with mycobacteria responded to the 38-kDa antigen, with the highest reactions seen in the group given killed *M. tuberculosis* in oil.

These results indicate that the MPT64 and the 38-kDa antigens are likely to be responsible for the qualitative differences seen with the ST-CF fractions.

TABLE 1. Presence of defined mycobacterial antigens in narrow-molecular-mass fractions of ST-CF within the 23- to 35-kDa range

| Antigen | Reactive MAb | Presence in fraction[a]: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 2 | 3 | 4 | 5 | 7 | 8 | 9 | 10 | 13 |
| MPT64 (24 kDa) | C24b2 | ++ | +++ | ++ | + | | | | | |
| Antigen 85 (31–32 kDa) | HYT27 | | | | | ++ | +++ | +++ | ++ | |
| PstS (38 kDa) | HBT12 | | | | | | | | | + |

[a] Fractions were separated by SDS-PAGE and immunoblotted. Estimates of relative concentrations are based on intensities of the reactions with the MAbs: +, small amounts; ++, intermediate amounts; +++, large amounts. No antigen was detected on fractions 1, 6, 11, and 12.
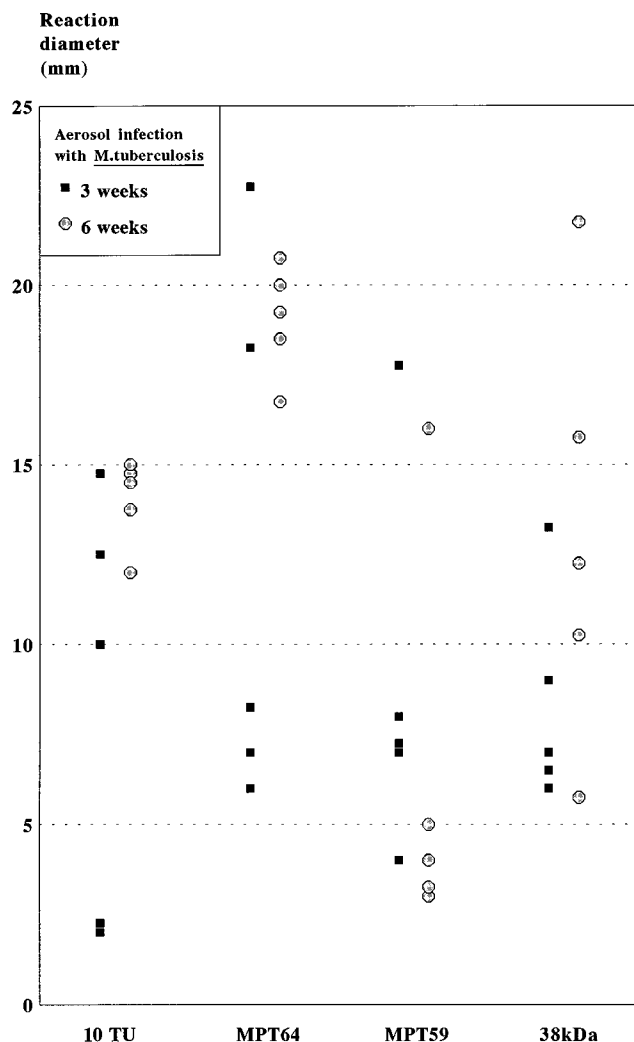
**Reaction
diameter
(mm)**



FIG. 5. Distribution of skin reactions to 10 TU of tuberculin and to 1 μg of the indicated antigens in two groups of five guinea pigs infected with *M. tuberculosis* Erdman by the aerosol route 3 or 6 weeks previously. Each symbol corresponds to a reaction in one guinea pig.

## DISCUSSION

It has been generally recognized for many years that a live sensitization with for example, BCG vaccine is imperative for the induction of specific protective immunity against TB. Given this dogma, much interest has in recent years focused on the group of proteins actively secreted from *M. tuberculosis* as a source of potentially protective antigens (1, 6, 21). Studies from our laboratory using a mouse model of TB have demonstrated secreted antigens to be key antigenic targets during the natural infection and have pinpointed potentially protective proteins in the molecular mass regions below 10 kDa and in the 25- to 33-kDa interval (6).

In the present study, the immunological activity of secreted protein fractions was studied by skin tests and lymphocyte stimulation tests in the guinea pig model of TB infection. Results from skin tests in infected guinea pigs showed intermediate to high-level reactions to all fractions. In contrast, the stimulation experiments with PBL as well as SPL supported previous findings in the mouse model by showing superior stimulation by antigens with a molecular mass below 10 kDa

(6). The qualitative differences between the skin tests and lymphocyte stimulation in the patterns of reactivity to the fractions support the contention that populations of T lymphocytes, which may differ in specificity, mediate these responses. The superior stimulating capacity of the lowest-molecular-weight fraction was seen when guinea pigs were sensitized with live BCG or in particular *M. tuberculosis*, but also after sensitization with killed *M. tuberculosis* in oil. These findings extend the results obtained in the mouse model and indicate that this region may contain promiscuous epitopes recognized by genetically heterogeneous donors during the first phase of infection. Only few low-molecular-mass secreted protein antigens have to date been purified and characterized. The 10-kDa GroES proteins have been demonstrated to be highly immunoreactive T-cell antigens recognized by TB and leprosy patients (7, 18). Recent results from our laboratory have pinpointed a previously uncharacterized 6-kDa protein antigen recognized strongly by murine memory effector cells (1b). Studies are in progress in our laboratory to define the precise identity of the target in the guinea pig model. In contrast to live sensitization, immunization with killed bacteria induced an additional peak sensitivity of both cell types studied to the 35- to 40-kDa fraction 7 (Fig. 2). This region contains detectable amounts of the 38-kDa antigen (Table 1) (4). We identified this molecule as responsible for the peak reactivity by comparing the activities of affinity-purified 38-kDa antigen from *M. tuberculosis* in guinea pigs sensitized with live or killed mycobacteria. These skin test and lymphocyte stimulation experiments showed a superior activity of the 38-kDa antigen in guinea pigs sensitized with killed *M. tuberculosis* compared with infected animals (Fig. 4 and 6). This result confirms previous observations that the 38-kDa antigen is immunodominant in guinea pigs sensitized in this manner (14). Because the killed preparation used is highly enriched in cell wall-derived proteins, these observations also support the contention that the antigen, which is a lipoprotein, may be anchored in the cell membrane (26).

The fact that tuberculin sensitivity is induced by a wide range of mycobacterial infections, including vaccination with BCG, limits the diagnostic benefit of the reagent. A more specific skin test diagnostic is clearly desirable. We observed that skin tests with the 24- to 30-kDa fraction of secreted proteins gave significantly higher reactions in tuberculous guinea pigs than in animals vaccinated with BCG Copenhagen (Fig. 1). A fine analysis of this molecular mass area with a high resolution set of fractions showed that the specific component responsible for these skin reactions was centered around 24 kDa (Fig. 3). The fraction in question was demonstrated by immunoblotting experiments to contain the MPT64 molecule (Table 1). Subsequent skin test experiments with purified MPT64 identified this molecule as a potentially useful specific diagnostic reagent: the majority (~90%) of guinea pigs infected i.v. or by the aerosol route with *M. tuberculosis* react strongly to the antigen, and no reactions were seen in animals vaccinated with BCG or immunized with killed *M. tuberculosis*. A parallel set of lymphocyte stimulation experiments gave quite similar results: stimulation with MPT64 was seen only in guinea pigs infected with *M. tuberculosis*. We have shown MPT64 reactivity by skin test experiments in guinea pigs infected with one of three different strains of *M. tuberculosis*, i.e., H37Rv, Erdman, and R1609, an isolate from a Danish TB patient. Furthermore, the gene has been identified in all of 56 isolates from Tanzanian and Danish patients. No isolate has thus far been devoid of the gene (19a), indicating its universal presence in *M. tuberculosis*.

The positive reactions to MPT64- in BCG Tokyo-vaccinated guinea pigs confirm previous observations by Harboe et al. (12), who used the analogous preparation from BCG, MPB64.
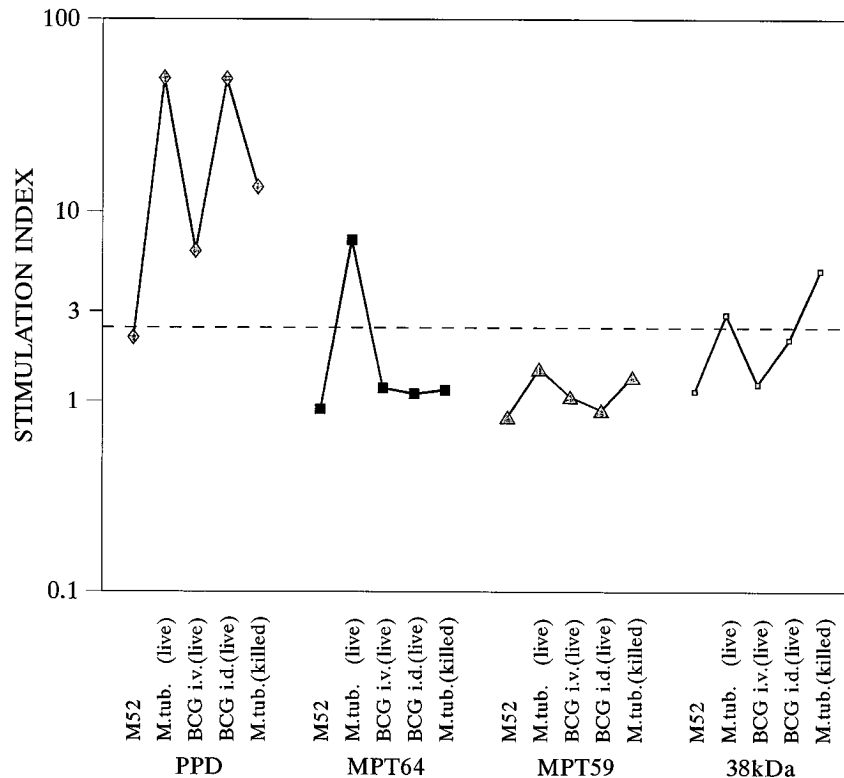
FIG. 6. Lymphocyte stimulation results with PBL from groups of guinea pigs not immunized (M52), infected i.v. with *M. tuberculosis* (M.tub.) H37Rv or BCG Copenhagen, vaccinated i.d. with BCG Copenhagen, or immunized with killed *M. tuberculosis* in oil. Cells were stimulated with the indicated antigens at 1 μg/ml. Geometric mean SI values from six to eight guinea pigs are shown. Mean SI values higher than 2.5 (indicated by the dotted line) are statistically significant.

The differences between the BCG Tokyo and Copenhagen (Danish isolate 1331) strains in the ability to induce sensitivity to MPT64 is in agreement with the recent demonstration that the gene encoding the antigen is present in the Tokyo strain and absent in the Danish strain (16, 20) and with the previous demonstration that the strains belong to distinct BCG families (12). In contrast to our findings, Harboe et al. (12) saw skin reactions in guinea pigs sensitized with killed *M. tuberculosis*. This apparent discrepancy may be explained by our previous observation (unpublished) that insufficient washing of the killed bacteria before preparation of the oil immunogen leads to MPT64 sensitization of immunized guinea pigs.

In summary, these studies of the immunological activity of secreted proteins from *M. tuberculosis* in guinea pigs have confirmed previous findings in the mouse model of strongly T-cell-stimulating molecules of low molecular weight and have led to the identification of MPT64 as a potentially useful diagnostic reagent for TB. These two lines of research are being further pursued in our laboratory.

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