A major T-cell-inducing cytosolic 23 kDa protein antigen of the vaccine candidate *Mycobacterium habana* is superoxide dismutase

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This study describes the purification and immunochemical characterization of a major 23 kDa cytosolic protein antigen of the vaccine candidate *Mycobacterium habana* (TMC 5135). The 23 kDa protein alone was salted out from the cytosol at an ammonium sulfate saturation of 80–95%. It represented about 1.5% of the total cytosolic protein, appeared glycosylated by staining with periodic acid/Schiff’s reagent, and showed a pI of approximately 5.3. Its native molecular mass was determined as approximately 48 kDa, suggesting a homodimeric configuration. Immunoblotting with the WHO-IMMLEP/IMMTUB mAbs mc5041 and IT61 and activity staining after native PAGE established its identity as a mycobacterial superoxide dismutase (SOD) of the Fe/Mn type. The sequence of the 18 N-terminal amino acids, which also contained the binding site for mc5041, showed a close resemblance, not only with the reported deduced sequences of *Mycobacterium leprae* and *Mycobacterium tuberculosis* Fe/MnSODs, but also with human MnSOD. In order to study its immunopathological relevance, the protein was subjected to *in vivo* and *in vitro* assays for T cell activation. It induced, in a dose-related manner, skin delayed hypersensitivity in guinea-pigs and lymphocyte proliferation in BALB/c mice primed with *M. habana*. Most significantly, it also induced lymphocyte proliferative responses, in a manner analogous to *M. leprae*, in human subjects comprising tuberculoid leprosy patients and healthy contacts.

**Keywords:** vaccine, *Mycobacterium habana*, 23 kDa protein, superoxide dismutase, T cell response

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**INTRODUCTION**

A formidable worldwide resurgence of tuberculosis is being witnessed in the wake of AIDS (Weiss, 1992), and leprosy, although on the decline, persists as a major public health problem in developing countries (WHO, 1988). Dismal performances of BCG in some populations, as well as of other candidate integral vaccines undergoing human trials (Fine & Rodriguez, 1990; Convit et al., 1992), have underlined the need for molecular characterization of immunopathologically important mycobacterial constituents in the quest for better drugs, diagnostic techniques and vaccines. Attention has primarily been focused on proteins, with a growing realization that the T-cell-mediated immune responses generated by them (Germain, 1994) could be of a diagnostic or prophylactic value. In addition, proteins working as vital enzymes or virulence factors for the microbe could serve as suitable targets for new drug development. Thus, an array of antigenic and/or enzymic proteins has been isolated and characterized from various compartments of a mycobacterial cell, viz. the cytosol, cell membrane and cell wall, and from the growth medium (Young et al., 1992). A good number of these proteins, particularly the cytosolic ones, have also been genetically cloned, and studies addressing the vaccine or diagnostic potential of

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**Abbreviations:** DH, delayed hypersensitivity; IEF, isoelectric focusing; PAS reagent, periodic acid/Schiff’s reagent; PHA, phytohaemagglutinin; SOD, superoxide dismutase.

The EMBL accession number for the sequence reported in this paper is P80582.
some of them are well underway (Young & Cole, 1993; Gelber et al., 1994).

*Mycobacterium habana* (TMC 5135, *Mycobacterium simiae* serovar 1) has shown promising results as a candidate vaccine against leprosy and tuberculosis in the mouse model in studies conducted at our laboratory (Gupta et al., 1979) and elsewhere (Singh et al., 1989). It could also prime monkeys for the lepromin skin test (Singh et al., 1992). Further, this cultivable mycobacterial strain was found to share some of the antigens with *Mycobacterium leprae*, including a specific determinant on an 18 kDa heat-shock protein (Lamb et al., 1990). We report here the isolation and immunochemical characterization of a prominent 23 kDa cytosolic protein of *M. habana*, whose real identity, in the course of the study, was established as a superoxide dismutase (SOD) bearing structural and antigenic homologies with Fe/MnSODs of *Mycobacterium tuberculosis* and *M. leprae*. The immunological importance of the purified protein was assessed by the parameters for T cell activation, viz. skin delayed hypersensitivity (DH) in guinea-pigs and lymphocyte proliferation assays in mice as well as in human subjects comprising leprosy patients and healthy contacts.

**METHODS**

**Human subjects.** Fourteen individuals were selected for this study. Eleven of them were patients of tuberculoid or borderline tuberculous leprosy, classified according to the clinico-bacteriological and immunohistological criteria of Ridley & Jopling (1966). They were admitted to the wards of the Skin Centre, Base Hospital, Lucknow, India, and were receiving multidrug treatment (WHO, 1988) for periods ranging from 1 to 10 months. The remaining three subjects were healthy laboratory workers environmentally exposed to mycobacterial antigens (healthy contacts). Informed consent was obtained from all subjects prior to drawing a 10 ml sample of venous blood.

**Protein purification.** *M. habana* (TMC 5135) was cultured in Sauton’s liquid medium at 37 °C on an orbital shaker. Cells were harvested in late-exponential phase (3 weeks) and subjected to the recommended protocol for subcellular fractionation (Brodie et al., 1979). Briefly, batches of bacterial cells (5 g wet wt per batch) were washed and suspended (0-2 g ml⁻¹) in sonication buffer (50 mM Tris/HCl, pH 7.4, with 10 mM MgCl₂, 1 mM PMSF and 1 mM EGTA) and sonicated for a total of 10 min using 50% output (100% = 475 W) and 50% duty cycle (on/off) of a sonicator (Heat Systems Ultrasonics). The sonicate was centrifuged at 23000 g for 30 min to remove the broken cell walls and the supernatant was re-centrifuged at 150000 g for 90 min to remove the membrane fragments and recover the cytosol. Protein estimations were done by a modified Lowry method (Markwell et al., 1978).

The major 23 kDa cytosolic protein of *M. habana*, as visualized by SDS-PAGE (described below), could be purified to homogeneity by a temperature-controlled fractional salt precipitation procedure (Scopes, 1987). Initially, cytosol (about 3 mg protein ml⁻¹, 30 ml per batch) was subjected to serial precipitations at 20%, 40%, 60%, 80% and 95% ammonium sulfate (enzyme grade, Sigma) saturation. The precipitates were collected by centrifugation, dissolved and dialysed against PBS (10 mM sodium phosphate, 150 mM NaCl; pH 7.4), and subjected to SDS-PAGE. After observing that the 23 kDa protein was almost exclusively precipitated between 80% and 95% salt saturation, the procedure was simplified as follows. All other cytosolic proteins were precipitated in the first step by applying 80% salt saturation, then, in the second step, saturation of the supernatant was raised to 95% to precipitate the 23 kDa protein. In the majority of the batches (six out of eight), the isolated protein exhibited single band purity by both Coomassie blue and silver staining after SDS-PAGE. Minor contaminants in occasional batches could easily be removed by applying a second cycle of salt precipitation to the sample after making it free from ammonium sulfate by extensive dialysis against PBS. Homogeneity of various batches of isolated protein was also confirmed by isoelectric focusing (IEF), using the method described below.

**SDS-PAGE and specific staining.** SDS-PAGE under reducing conditions was done by the method of Laemmli (1970), using a 12.5% (w/v) resolving gel in a mini slab-gel apparatus (Brotviga). Molecular mass markers were obtained from Sigma. After electrophoresis, gels were stained for proteins with either Coomassie blue or a silver stain (Wray et al., 1981). Staining was also done with periodic acid/Schiff’s (PAS) reagent in order to visualize glycosylation (Leach et al., 1980).

**IEF.** This was done according to the procedure described by Dunbar et al. (1990) in cylindrical gels (4 mm diameter) using ampholytes purchased from Pharmacia. The upper, cathode buffer was 0.02 M NaOH and 0.085% phosphoric acid was used as the lower, anode buffer. After prefocusing the ampholytes (250 V for 2 h), IEF of a 5–10 μg protein sample was carried out at 1000 V for 6 h. The gels were fixed and stained with Coomassie blue. pl values of standard markers (Sigma) were plotted against the respective migration distances and the pl of purified protein was extrapolated from this curve.

**Determination of native molecular mass.** The native protein molecular mass marker kit of Sigma was used and supplier’s instructions (Technical Bulletin no. MKR-137) were followed. Briefly, purified protein and markers were electrophoresed on a set of cylindrical gels with different polyacrylamide concentrations (7%, 9%, 10% and 12%, w/v). For each protein, 100 log(Rₑ × 100) was plotted against the percentage gel concentration and individual slopes (= retardation coefficient) were determined. Afterwards, log (negative) slope was plotted against log molecular mass for each marker and from this linear plot, the molecular mass of purified protein was extrapolated.

**N-terminal amino acid sequence analysis.** The protocol of Matsudaira (1987) was followed. The purified protein was electroblotted on a PVDF membrane (Immobilon-P, 0.45 μm; Sigma) using CAPS buffer. The blotted protein was visualized by Coomassie blue staining and the corresponding membrane area was excised, washed, dried and used for N-terminal sequencing. The sequencing was done using the model 470A Gas Phase Sequenator (Applied Biosystems) at the DBT facility, Indian Institute of Science, Bangalore, India (courtesy of Professor N. Appaji Rao).

**Anti-SOD antibodies and immunoblotting.** A panel of mAbs, including those that react with two distinct but shared epitopes of mycobacterial SOD, mc5041 (SA1D2D) and IT61 (F116-5) (Khanolkar-Young et al., 1992), was provided by IMMLEP/IMMTUB Monoclonal Antibody Bank of WHO (courtesy of Dr T. M. Shinnick). A monospecific polyclonal antibody against purified the 23 kDa protein of *M. habana* was raised by immunizing (intradermal, multiple sites) a rabbit with the protein emulsified in Freund’s incomplete adjuvant. Optimal titres were attained after the third biweekly booster.
For immunoblotting, antigens resolved by SDS-PAGE were electrophoretically blotted (Towbin et al., 1979) on nitrocellulose paper (0.45 μm pore size; Sigma) using a Trans-Blot apparatus (Bio-Rad). Later, individual strips cut out from paper were probed with anti-SOD antibodies. Briefly, strips were blocked (2 h at room temperature) with 3% (w/v) skimmed milk powder (Aniskspray, Lipton) and incubated (2 h, room temperature) with appropriate dilutions of anti-SOD antibodies (1:50 for IT6, 1:500 for mc5041 and 1:500 for rabbit antiserum) prepared in 1% milk. The washed strips were reincubated (2 h, room temperature) with 1:1000 dilutions (in 1% milk) of affinity-purified, peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulins (Sigma). After final washings, the colour was developed with 4-chloronaphthol (Sigma) as substrate.

**Activity staining in a gel.** The purified protein as well as the cytosol of *M. habana* were subjected to non-denaturing (native) slab-gel PAGE in a 12.5% gel using the buffer system of Laemmli (1970), omitting SDS and reducing agent. SOD activity was visualized by negative staining with nitro blue tetrazolium (NBT) (Beauchamp & Fridovich, 1971). Briefly, gels were soaked in a solution of 0.2% NBT, 0.025 M N,N,N',N'-tetramethylethylenediamine and 2.8 × 10^{-6} M riboflavin in potassium phosphate buffer (50 mM, pH 7.8) for 30 min at room temperature and later illuminated with fluorescent light until colourless zones indicating SOD activity were visible against a uniformly blue background. For the determination of the metal cofactor by the enzyme inhibition method (Takao et al., 1991), gel strips, after native PAGE, were incubated at room temperature for 30 min with 10 mM potassium cyanide or 10 mM sodium azide or for 1 h with 0.5 mM hydrogen peroxide. The control strip was incubated with phosphate buffer alone. SOD activity was stained by the NBT method.

**DH tests in guinea-pigs.** A previously described protocol (Sinha et al., 1987) was followed. Guinea-pigs were primed with an autoclaved saline suspension of *M. habana* by administering two intradermal injections (0.1 ml each) in the groin region (3 mg wet bacterial wt per animal). After 4 weeks, the animals were tested (in groups of three) for skin DH with indicated doses of antigens (in 0.1 ml saline). The mean diameter of the erythematous reaction was recorded at specified time intervals. A group of unimmunized animals served as a negative control.

**T cell proliferation assays in mice.** BALB/c mice were primed by intradermal injections (on both flanks) of an autoclaved saline suspension of *M. habana* (1.5 mg wet bacterial wt per mouse). After 3–4 weeks, the animals were sacrificed by cervical dislocation. The spleen was removed and mashed gently in culture medium (RPMI 1640, Sigma) over a fine stainless-steel wire mesh using the glass piston of a syringe. The single-cell culture medium (RPMI 1640, Sigma) over a fine stainless-steel wire mesh using the glass piston of a syringe. The single-cell culture suspension was then passed through a column of nylon wool (BioTest AG) for the enrichment of T cells (Julius et al., 1973). Cells in the eluate were washed, adjusted to 2 × 10^6 cells ml^{-1}, and dispensed (0.1 ml per well) into 96-well flat-bottomed culture plates (Corning). Cultures were set in triplicate, with or without the indicated concentrations of antigen or mitogen, in culture medium supplemented with 10% heat-inactivated FCS and 10^{-3} M 2-mercaptoethanol (total culture volume = 0.2 ml per well). Incubations were done for 4 d in a CO_2 incubator, and 18 h prior to termination, the cultures were pulsed with 1 μCi (37 kBq) [^3H]thymidine [methyl-T, specific activity approx. 20 Ci mmol^{-1} (740 GBq); from BARC, Bombay, India] per well. Cells were harvested on a cell harvester and the radioactivity was measured in a liquid scintillation counter.

**Human T cell proliferation assays.** Mononuclear cells were isolated from heparinized blood by density gradient centrifugation over Ficoll-isopaque (Boyum, 1968). The washed cells were counted, adjusted to 2 × 10^8 cells ml^{-1} in culture medium (RPMI 1640), and dispensed (0.1 ml per well) into 96-well flat-bottomed culture plates. Cultures were set in triplicate, with or without the indicated concentrations of antigen or mitogen, in culture medium supplemented with 10% heat-inactivated pooled normal human serum (total volume = 0.2 ml per well) and incubated for 6 d in a CO_2 incubator. The cultures were pulsed with [^3H]thymidine (1 μCi (37 kBq) per well) 18 h before harvesting, and the radioactivity of the harvested cells was measured in a liquid scintillation counter.

**RESULTS**

**Homogeneity and yield of purified protein**

SDS-PAGE of the *M. habana* cytosol under reducing conditions revealed the presence of a prominent 23 kDa protein. Its abundance is evident from Fig. 1(a), which shows protein profiles of serially diluted cytosol. By PAS staining, the 23 kDa protein appeared to be glycosylated, like the majority of the other proteins (Fig. 1b). All

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**Fig. 1.** (a) SDS-PAGE of serial twofold dilutions of *M. habana* cytosol showing the abundance of the 23 kDa protein. Lanes: 1, 30 μg cytosolic protein; 2, 15 μg protein; 3, 7.5 μg protein; 4, 3.75 μg protein. (b) SDS-PAGE and PAS staining of cytosolic proteins showing glycosylation of the 23 kDa protein (arrow) and other proteins.
cytosolic proteins, except the 23 kDa protein, were precipitated at 80% saturation with ammonium sulfate, whereas the 23 kDa protein alone was precipitated between 80 and 95% saturation (Fig. 2a). Precision in salt saturation as a function of temperature was found to be a critical requirement for this purification process. SDSPAGE and silver staining served as the first criterion for homogeneity of the purified protein (Fig. 2b). The second criterion was IEF, in which the protein was focused as a single band at a PI of about 5.3. Its amenability to N-terminal sequence analysis (described below) was also evidence of homogeneity. The native molecular mass of purified protein was determined to be approximately 48 kDa, indicating its natural presence as a homodimer.

One gram wet bacterial weight yielded about 20 mg cytosolic protein, from which about 0.3 mg 23 kDa protein could be isolated (mean of five batches). Thus the isolated protein constituted approximately 1.5% of the total cytosolic protein.

**Characterization of the protein as SOD**

Initially, in view of the similarity in molecular masses, immunoblotting of purified protein was attempted with IMMLEP/IMMTUB mAb IT61 (F116-5). This antibody is known to react with a common epitope of the 23 kDa mycobacterial antigen, identified later as SOD. After observing a positive reaction, another mAb, mc5041 (SAID2D), against *M. leprae* SOD, described earlier as a 28 kDa antigen (Young et al., 1985), was used which also produced a positive reaction. Results of immunoblotting with both the mAbs and a rabbit antiserum raised against the purified 23 kDa *M. habana* protein are shown in Fig. 3(b).

Further characterization of the enzyme was done by activity staining in a native gel in the presence or absence of cofactor-specific inhibitors. Sensitivity to cyanide denotes Cu,ZnSOD whereas Fe/MnSODs are only partially inhibited by azide or hydrogen peroxide and not at all by cyanide. As seen in Fig. 3(a), the presence of SOD activity was evident in the cytosol as well as in purified protein. None of the inhibitors abrogated this activity, indicating the probability of the enzyme being of the Fe/Mn type.

**Structural identity with related SODs**

The N-terminal sequence of the purified protein, up to 18 amino acid residues, was determined as follows: Ala-Glu-Tyr-Thr-Leu-Pro-Asp-Leu-Gly-Trp-Asp-Tyr-Ala-Ala-Ser-Gly-Pro-Gly18. A comparison of this partial sequence was made with that of other SODs reported in the literature. A striking resemblance of *M. habana* SOD was observed with Fe/MnSODs of *M. tuberculosis* (Zhang et al., 1991), *M. leprae* (Thangaraj et al., 1989) and man (Barra et al., 1984), whereas there was no such homology.
**Fig. 4.** A comparison of the N-terminal sequence of *M. habana* SOD with that of *M. leprae* MnSOD (Thangaraj et al., 1989), *M. tuberculosis* Fe/MnSOD (Zhang et al., 1991), human MnSOD (Barra et al., 1984) and human Cu,ZnSOD (Jabusch et al., 1980). Blanks (—) indicate amino acid residues identical to *M. habana* SOD at the corresponding positions; + + + indicates the mapped epitope for anti-SOD mAb mc5041 (SA1D2D) in the case of *M. leprae* (Thangaraj et al., 1990).

![Diagram](image)

**Fig. 5.** DH responses to *M. habana* cytosol and purified protein (SOD) in guinea-pigs. All reactions peaked at 24 h and those of < 5 mm (shaded area) were considered negative. (a) Dose response with cytosol tested at 2.5 (○), 1.25 (●), 0.625 (□) and 0.312 (■) µg protein. (b) Dose response with SOD tested at 25 µg protein (▼) and its twofold dilutions, which did not produce any reaction (values correspond to baseline). (c) DH with 5 (□) and 2.5 (○) µg cytosolic protein. Equivalent reactions were produced by 10 (△) and 5 (▲) µg SOD (d).

**DISCUSSION**

The family of SODs (EC 1.15.1.1) normally functions to protect cells from toxic effects of superoxide anions \( \left( O_2^- \right) \), generated during various metabolic processes, by catalysing their dismutation to hydrogen peroxide and...
Table 1. Murine lymphocyte proliferative responses

The optimal doses of PHA, *M. habana* cytosol and SOD per 10^6 cells were worked out separately. The values are the mean Ac.p.m. [= c.p.m.(antigen/mitogen – medium)]. The SEM of triplicate cultures was < 15% of the mean.

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>Controls</th>
<th>PHA (10 µg ml⁻¹)</th>
<th><em>M. habana</em> cytosol (25 µg ml⁻¹)</th>
<th>SOD (12.5 µg ml⁻¹)</th>
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<tbody>
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60862 ± 5286* 12417 ± 2285* 3027 ± 570*

*Mean ± SEM.

Table 2. Human lymphocyte proliferative responses

The optimal doses of PHA, *M. leprae* cytosol, *M. habana* cytosol and SOD per 10^6 cells were worked out separately. The values are the mean Ac.p.m. [= c.p.m.(antigen/mitogen – medium)]. The SEM of triplicate cultures was < 15% of the mean.

<table>
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<th>Patient no./type*</th>
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<th><em>M. leprae</em> cytosol (25 µg ml⁻¹)</th>
<th><em>M. habana</em> cytosol (25 µg ml⁻¹)</th>
<th>SOD (12.5 µg ml⁻¹)</th>
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*TT, Tuberculoid leprosy; BT, borderline tuberculoid leprosy; HC, healthy contact.

Molecular oxygen (Fridovich, 1986). The importance of \( \text{O}_2^- \) has been accentuated by the fact that it may combine with nitric oxide (NO), another potent cytotoxic molecule, to transiently produce peroxynitrite (ONOO\(^{-}\)), which could be more lethal than either of the reactants (Radi et al., 1991). Since \( \text{O}_2^- \) and NO are also primary reaction products of a 'respiratory burst', the major microbicidal mechanism of an activated phagocyte, SODs of various pathogens have been viewed as virulence factors (Beaman & Beaman, 1984). This understanding has triggered a series of studies pertaining to structure–function analysis of SODs from various parasites as well as their hosts. Some biochemical properties of *M. leprae* and *M. tuberculosis* SODs were initially reported by Kusunose et al. (1976, 1981) and Wheeler & Gregory (1980). The genes for both SODs have been cloned and sequenced (Thangaraj et al., 1989, 1990; Zhang et al., 1991) and some B cell epitopes on these proteins have been identified with the help of mAbs (Young et al., 1985; Khanolkar-Young et al., 1992). Very recently, crystal-
lization and three-dimensional structure determination of the recombinant *M. tuberculosis* (H₃₇Ra) SOD has been reported (Cooper et al., 1995).

It was not immediately obvious that the prominent 23 kDa cytosolic protein of *M. habana* was, in fact, SOD. Activity staining in denaturing gels was not attempted since it was highly unlikely for an enzyme to retain its activity under such conditions. Secondly, considerable uncertainty existed about the molecular mass of a mycobacterial SOD. Its subunit size has been reported as 28 kDa for *M. leprae* (Young et al., 1985) and 21 kDa for *M. tuberculosis* (Kusunose et al., 1976). On the other hand, the subunit size of both the SODs as deduced from respective DNA sequences has been found to be 23 kDa. Uncertainty also prevails regarding the native molecular configuration of SODs, which in the case of *M. habana* appeared to be homodimeric. Native SODs from various microbial sources have been found to exist in configurations ranging from monomeric (Spiegelhalder et al., 1993) to tetrameric. *M. tuberculosis* SOD exists as a homotetramer of 88 kDa (Kusunose et al., 1976) and the native molecular mass of *M. leprae* SOD has been reported as 40 kDa (? dimer) (Kusunose et al., 1981). Thirdly, despite the lack of signal peptide sequence (Zhang et al., 1991), mycobacterial and other SODs have been regarded as predominantly secretory molecules (Kusunose et al., 1976; Cooper et al., 1995), whereas in the case of *M. habana*, its secretory level was below the detection limits (data not shown). Nonetheless, in some avirulent mycobacteria, the enzyme has been found to remain intracellular (Kusunose et al., 1976).

The *M. habana* protein could be isolated from the cytosol solely by means of a ‘salting out’ procedure. It is well documented that SODs from various sources get precipitated at a relatively higher ammonium sulfate saturation, between 62.5 and 95% (Kusunose et al., 1976; Beaman et al., 1982; Takao et al., 1991). However, purification to homogeneity has invariably involved additional steps; typically, a passage through anion-exchange and gel-filtration columns. In the case of *M. habana*, SOD happened to be the only protein precipitable at >80% salt saturation. This unusually high solubility of the protein should be viewed in the context of reported differences in solubility of SODs across the mycobacterial species. For example, precipitation of *M. tuberculosis* SOD required an ammonium sulfate concentration in the range 62.5-82.5%, whereas in the case of *Mycobacterium smegmatis* it was 70-85% (Kusunose et al., 1976). As for yield, *M. habana* SOD represented as much as 1-5% of all cytosolic proteins. Identically high levels of cytosolic SOD (about 2%) have also been reported in *Nocardia* (Beaman et al., 1982). However, the corresponding value for *M. tuberculosis* was only about 0.4% (Kusunose et al., 1976). The difference in cytosolic levels of SOD in various species could reflect the variability in its predisposition to behave as a secretory molecule, or merely the difference in growth conditions.

The N-terminal sequence of *M. habana* SOD closely resembled the corresponding (deduced) sequences of *M. leprae* and *M. tuberculosis*. It also showed a structural relationship with human MnSOD (mitochondrial) but not with human Cu,ZnSOD (cytosolic). Its metal cofactor was determined as Fe/Mn, which agreed with these observations. The reported cofactor is Mn for the *M. leprae* enzyme and Fe for *M. tuberculosis*. However, the deduced sequence of the *M. tuberculosis* enzyme is similar to MnSODs (Zhang et al., 1991). These discrepancies may perhaps be reconciled with a recent observation that the choice between Fe or Mn as a cofactor could largely depend on the culture conditions (Meier et al., 1982). Another important structural feature of the *M. habana* enzyme was its apparent glycosylation. Such ‘post-translational’ modifications are known to distinguish native somatic proteins from their recombinant counterparts. Indeed, a good number of mycobacterial proteins have shown evidence of glycosylation (Espitia et al., 1995; Dobos et al., 1995; and other references cited within these papers). Nevertheless, it is desirable to confirm the glycosylation in SOD by more stringent methods, considering the limitations of PAS staining (Leach et al., 1980).

The most significant aspect of this study was recognition of *M. habana* SOD by T cells of not only guinea-pigs and mice primed with *M. habana*, but also of human subjects infected with *M. leprae* or exposed to environmental mycobacteria. These observations underline the relevance of ‘shared’ T cell epitopes of mycobacterial SOD. Interestingly, in patients with tuberculoid leprosy, the patterns of T cell recognition of SOD and *M. leprae* extract were similar. More information on structural and antigenic aspects of *M. habana* SOD is needed to understand this phenomenon. Meanwhile, the cross-reactivity between SOD of *M. habana* and *M. leprae* may be added to the list of known antigenic resemblances between the species (Lamb et al., 1990). Further, glycosylation as it may occur in the native enzyme could play an important immunological role, e.g. by modifying the process of antigen presentation to the T cells (Ishioka et al., 1992). Whatever the reason, these results indicate the possible immunopathological importance of mycobacterial SODs. Such an indication was provided earlier by Khanolkar et al. (1989), who observed that about 55% of leprosy cases had deposition of *M. leprae* SOD in the skin. Immune recognition of the mycobacterial SOD may be seen in the larger perspective of immunodominance of those antigens which are synthesized in copious amounts under stressful situations, e.g. heat-shock proteins (Young et al., 1988). Enhanced synthesis of SOD has also been reported under conditions of stress (Amano et al., 1994) and, like heat-shock proteins, SODs from parasites bear considerable structural homologies with the host enzyme. As high as 67% homology has been noted between *M. leprae* and human MnSODs (Thangaraj et al., 1990). Nevertheless, serological differences (Kusunose et al., 1976), as well as genus and species-specific DNA sequences (Zolg & Philippi-Schulz, 1994) of mycobacterial SODs, have been reported. Finally, in a recent report on comparative evaluation of vaccine potentials of recombinant or somatic antigens, *M. leprae* SOD was
found to offer significant protection in the mouse footpad
model of leprosy (Gelber et al., 1994). In the same
experiment, the 65 kDa heat-shock protein, a better
known homologous antigen, also provided good pro-
tection.

In conclusion, our study focuses attention on the structure
and antigenicity of mycobacterial SOD, visualized initially
as a major 23 kDa cytosolic protein of the vaccine
candidate M. banana, and isolated in the native state. In the
light of its immunopathological importance, it was
particularly interesting to note that the enzyme was
recognized by T cells of leprosy patients in a manner
analogous to M. leprae. These observations put myco-
bacterial SOD in the same class as mycobacterial heat-
shock proteins, which are highly antigenic, even immuno-
protective, despite bearing a prominent structural hom-
ology with the corresponding host proteins.

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