

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

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 tuberculoid 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 multi-drug 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 23 000 g for 30 min to remove the broken cell walls and the supernatant was recentrifuged at 150 000 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 sub-

jected 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 (Brovinga). 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. pI values of standard markers (Sigma) were plotted against the respective migration distances and the pI 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(R_F × 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 (Anikspray, Lipton) and incubated (2 h, room temperature) with appropriate dilutions of anti-SOD antibodies (1:50 for IT61, 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 2.8×10^5 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 suspension thus obtained was sedimented by centrifugation (400 g for 10 min) and treated with lysis buffer (0.14 M NH₄Cl, 0.017 M Tris/HCl, pH 7.4) to lyse the erythrocytes. The remaining cells were washed and suspended in medium containing 5% (v/v) foetal calf serum (FCS, Biological Industries), and 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 tissue culture plates (Corning). Cultures were set in triplicate, with or without the indicated doses (in 10 µl) of antigen or mitogen, in medium supplemented with 10% heat-inactivated FCS and 10^{-5} M 2-mercaptoethanol (total culture volume = 0.2 ml per well). Incubations were done for 4 d in a CO₂ 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 2×10^6 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 CO₂ 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

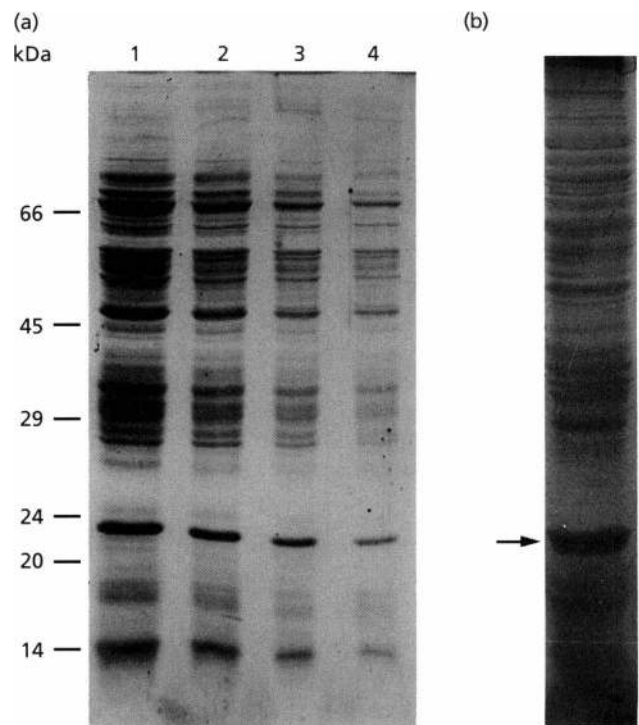


Fig. 1. (a) SDS-PAGE of serial twofold dilutions of *M. habana* cytosol showing the abundance of the 23 kDa protein. Lanes: 1, 30 µg cytosolic protein; 2, 15 µg protein; 3, 7.5 µg protein; 4, 3.75 µg protein. (b) SDS-PAGE and PAS staining of cytosolic proteins showing glycosylation of the 23 kDa protein (arrow) and other proteins.

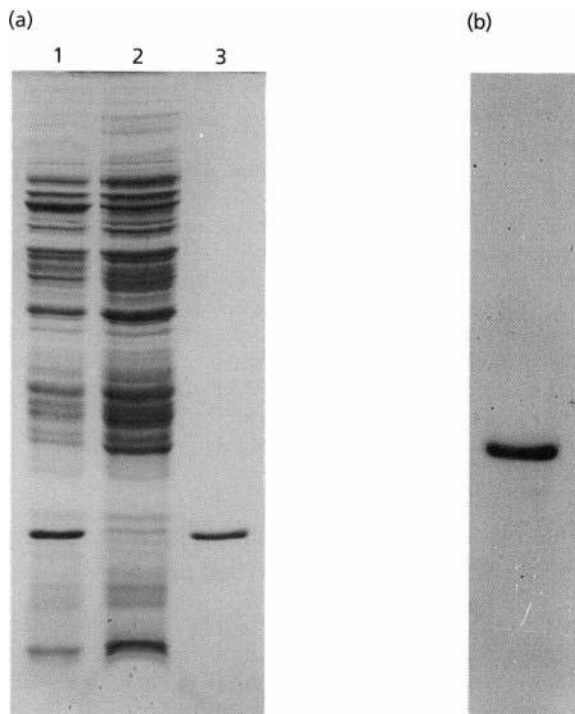


Fig. 2. (a) Purification of the 23 kDa protein monitored by SDS-PAGE and Coomassie blue staining. Lanes: 1, cytosol; 2, cytosolic protein precipitated at 80% salt saturation; 3, 23 kDa protein precipitated at 80–95% salt saturation. (b) Purity of the isolated protein indicated by silver staining.

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. SDS-PAGE 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

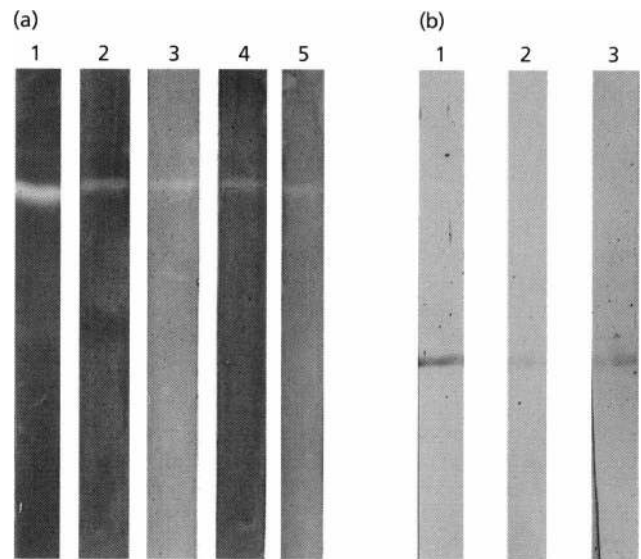


Fig. 3. (a) Characterization of the isolated protein as SOD by activity staining after native gel electrophoresis. Lanes: 1, cytosol; 2, isolated protein. Enzyme activity was not inhibited by KCN (lane 3), NaN₃ (lane 4) or H₂O₂ (lane 5), indicating the presence of Fe/Mn as the metal cofactor. (b) Sharing of B cell epitopes with other mycobacterial SODs seen by immunoblotting using rabbit anti-*M. habana* SOD antiserum (lane 1) and mAbs IT61 (lane 2) and mc5041 (lane 3).

observing a positive reaction, another mAb, mc5041 (SA1D2D), 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: ¹Ala-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

<i>M. habana</i>	1	18
Fe/Mn, native:	A E Y T L P D L G W D Y A A S G P G	
<i>M. leprae</i>		
Mn, deduced:	V - - - - - D - - - - - L E - H	
<i>M. tuberculosis</i> , H ₃₇ Ra		
Fe/Mn, deduced:	- - - - - D - - - G - L E - H	
Human		
Mn, native:	K H S - - - - P Y - - G - L E - H	
Human		
Cu,Zn, native:	- T K A V C V - K G - G P V Q - S I	

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).

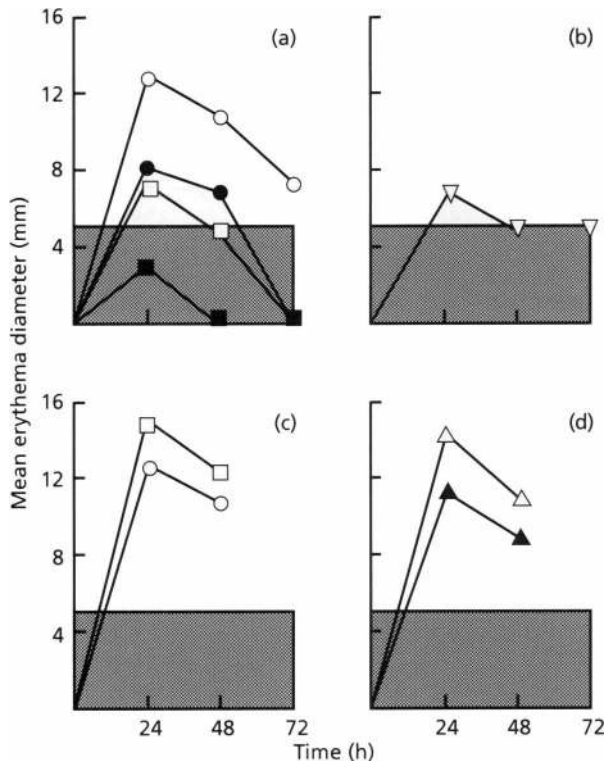


Fig. 5. DH responses to *M. habana* cytosol and purified protein (SOD) in guinea-pigs. All reactions peaked at 24 h and those of < 5 mm (shaded area) were considered negative. (a) Dose response with cytosol tested at 2.5 (○), 1.25 (●), 0.625 (□) and 0.312 (■) µg protein. (b) Dose response with SOD tested at 2.5 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).

with human Cu,ZnSOD (Jabusch *et al.*, 1980) (Fig. 4). This confirmed the findings about the metal cofactor of *M. habana* SOD based on enzyme inhibition studies. This comparison also explained the reactivity of mAb SA1D2D

with *M. habana* SOD, since the corresponding epitope has been mapped to the N-terminus of *M. leprae* SOD (Thangaraj *et al.*, 1990), as indicated in Fig. 4.

DH response in guinea-pigs

Initially, a dose response was determined using serial twofold dilutions of cytosol or purified protein, beginning at 2.5 µg (Fig. 5). The mean diameter of erythema was recorded at the specified time intervals. The positive reactions peaked at 24 h. At least 0.625 µg cytosolic protein was needed to elicit a positive reaction (Fig. 5a), whereas the corresponding value for purified protein was 2.5 µg (Fig. 5b). In the second experiment, guinea-pig DH responses to the selected doses of cytosol and SOD were evaluated. Comparable reactions were seen when the tested dose of SOD was twice that of the cytosol (Fig. 5c, d). Unimmunized guinea-pigs did not react with any of the antigen doses.

Murine T cell proliferation

Lymphocyte proliferative responses of BALB/c mice to phytohaemagglutinin (PHA, a T cell mitogen), *M. habana* cytosol and 23 kDa protein (SOD) are shown in Table 1. Optimal doses, as indicated in the Table, were determined on the basis of pilot experiments. The mean response to SOD alone was about 25% of that obtained with whole cytosol.

Human T cell proliferation

In order to evaluate the human-T-cell-activating potential of the purified protein, the T cell donors were selected from those individuals who are generally regarded as 'responders' for mycobacterial antigens, viz. tubercloid leprosy patients and healthy contacts (Ridley & Jopling, 1966; Young *et al.*, 1992).

T cell proliferative responses (Table 2) were recorded with predetermined optimal doses of PHA (serving as positive control), *M. leprae* cytosol, *M. habana* cytosol and the 23 kDa protein (SOD). On the basis of their response to *M. leprae* cytosol, the donors were arbitrarily categorized as 'high responders' (Δ c.p.m. > 10 000) or 'low responders' (Δ c.p.m. < 2000). Accordingly, six out of eight high responders for *M. leprae* cytosol also turned out to be high responders for SOD (Δ c.p.m. = 16 332–46 820) and all six low responders showed low responses to SOD as well (Δ c.p.m. = 14–888). The proliferative responses to *M. habana* cytosol, on the other hand, did not follow this pattern so closely. Four out of six low responders for *M. leprae* cytosol showed a medium to high response for *M. habana* cytosol (Δ c.p.m. = 8967–51 509).

DISCUSSION

The family of SODs (EC 1.15.1.1) normally functions to protect cells from toxic effects of superoxide anions (O_2^-), 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 Δ c.p.m. [= c.p.m.(antigen/mitogen - medium)]. The SEM of triplicate cultures was < 15% of the mean.

Animal no.	Controls		Antigens	
	Medium only	PHA (10 μ g ml ⁻¹)	<i>M. habana</i> cytosol (25 μ g ml ⁻¹)	SOD (12.5 μ g ml ⁻¹)
1	4805	66289	11460	4420
2	2419	77364	10060	1755
3	1716	60317	6569	3264
4	4950	46425	20297	4037
5	3222	53915	13699	1659
		60862 \pm 5286*	12417 \pm 2285*	3027 \pm 570*

*Mean \pm 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 Δ c.p.m. [= c.p.m.(antigen/mitogen - medium)]. The SEM of triplicate cultures was < 15% of the mean.

Patient no./type*	Controls		Antigens		
	Medium only	PHA (2 μ g ml ⁻¹)	<i>M. leprae</i> cytosol (25 μ g ml ⁻¹)	<i>M. habana</i> cytosol (25 μ g ml ⁻¹)	SOD (12.5 μ g ml ⁻¹)
High responders					
1/TT	670	72068	16408	22874	16332
2/BT	2338	28638	14564	54090	18647
3/TT	625	58711	66707	162146	46820
4/BT	3937	22819	63686	143771	26979
5/BT	2002	106590	18427	15766	19598
6/HC	521	115615	37086	50514	23633
7/BT	443	133405	12115	45662	862
8/HC	590	165251	12499	112740	297
Low responders					
9/HC	754	116745	1784	21247	14
10/BT	1846	94022	287	51509	180
11/TT	321	98026	408	11161	122
12/TT	497	125744	888	8967	888
13/BT	500	123532	34	2100	617
14/TT	310	250530	228	1457	173

* TT, Tuberculoid leprosy; BT, borderline tuberculoid leprosy; HC, healthy contact.

molecular oxygen (Fridovich, 1986). The importance of 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 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 (Spiegelhalter *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 protection.

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. habana*, 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 mycobacterial SOD in the same class as mycobacterial heat-shock proteins, which are highly antigenic, even immunoprotective, despite bearing a prominent structural homology with the corresponding host proteins.

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