Variation of Superoxide Dismutase Levels in Extracts of *Mycobacterium leprae* from Armadillo Liver[1]

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Superoxide dismutase (SOD) was detected in cell-free extracts of *Mycobacterium leprae* by two research groups working independently ([9, 10, 17]). Using bacteria isolated from armadillo liver, it was shown that the activity was due to a single enzyme ([9, 10, 17]) which was manganese dependent ([10, 17]). Evidence for the bacterial nature of the enzyme was obtained by showing that SOD from *M. leprae* was serologically very similar to *M. lepraemurium* SOD ([9, 10]), present in extracts of *M. leprae* after NaOH-treatment of the bacteria ([17]) which abolishes host-derived activities ([15, 17]), and electrophoretically distinct from the host SODs ([17]). However, there was a hundred-fold discrepancy in the levels of SOD in *M. leprae* measured by the two groups ([9, 17]). In this report, SOD has been measured in 18 extracts of *M. leprae* and correlation with known factors has been looked at, in an attempt to explain the discrepancies in the reported levels of this enzyme in *M. leprae*. No attempt has been made to investigate possible differences in the use of the enzyme assay for SOD, since the two laboratories agreed on levels of the enzyme in *M. phlei* grown in similar conditions ([3, 9, 17]).

MATERIALS AND METHODS

Preparation of cell-free extracts of *M. Leprae*. The source of *M. leprae* was experimentally infected armadillo livers, some of which had been γ-irradiated with 2.5 M Rad ([15]). Leprosy bacilli were isolated by the method of Draper ([18]) which was used by both Kusunose, *et al.* ([9, 10]) and Wheeler and Gregory ([17]). Extracts 1–12 and 14–19 were prepared from bacteria sonicated in low ionic buffer ([17]). Extract 13 was prepared by the method of Kusunose, *et al.* ([9]), modified only in the manufacture of sonicator: bacteria were suspended in 20 mM potassium phosphate (pH 7.9) and disrupted at 100 W for 7 min with two intervals, using a Dawe Soniprobe type 7532A, cooled on wet ice. The sonicate was centrifuged at 75,000 × *g* for 60 min, and the supernatant was used as the cell-free extract.

Assay for SOD. The luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) based assay devised by Bensinger and Johnson ([2]) was used. In order to facilitate comparisons with the literature, the units of SOD were calculated (in this work) as units in the cytochrome c-based assay used in earlier assays of SOD with *M. leprae*. This was carried out by comparing the units of SOD obtained in two extracts of *M. leprae* (extracts 1 and 10), using both the cytochrome c-based and the luminol-based assays. In both assays, one unit of SOD inhibits 50% superoxide-generated chemical reactions (cytochrome c reduction and luminol chemoluminescence) in 3 ml assays. On this basis, the luminol-based assay was found to be 270 ± 10 (mean ± standard error, 4 determinations) times more sensitive than the cytochrome c-based assay for *M. leprae* SOD, thus luminol-based units were divided by 270. In practice, this means that 90 times less *M. leprae* extract was required for the luminol-based assay, since it was scaled down to 1.5 ml; whereas the cytochrome c-based assay was scaled down to 0.5 ml ([17]).

Polyacrylamide gel electrophoresis. Crude extracts of *M. leprae* were applied to gels, electrophoresed, and stained for SOD as described previously ([17]).

Statistical methods. Correlation coefficients (r) were worked out for SOD levels with the factors listed below, and values of p were obtained from t-distribution tables; actual values of t were obtained by analysis of variance of the observations ([4]). Since it is quite likely to find some weak correlations when looking at a number of factors, only correlations with a value of p of ≤ 0.01 were

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TABLE 1. *Superoxide dismutase in extracts of* M. leprae.

Extract	Source[a]	Armadillo	Period of storage at −80°C[a] (weeks)	Period of infection[a] (weeks)	MI[a]	Specific activity of SOD[b]	Notes
1	A1	ar 1	35	61	12	1.15	
2	A1	ar 2	52	56	10	1.44	
3	A1	ar 2	130	56	10	1.55	
4	A1	ar 2	78 + 1[c]	56	ND	0.90	
5	A1	ar 2	78 + 1[c]	56	ND	0.98	γ-irradiated
6	A1	ar 2	79	56	ND	0.97	γ-irradiated
7	A2	ar 3	27	61	12	1.38	
8	A2	ar 3	69	61	12	1.22	γ-irradiated
9	A2	ar 3	69	61	12	1.37	
10	A3	ar 4	63	61	28	2.35	
11	A4	ar 5	9[d]	43	ND	3.84	Died[d]
12	A5	w 39	160	108	12	0.75	γ-irradiated
13	A5	w 39	160	108	ND	0.79	γ-irradiated
14	H1	ar 6	20	78	10	1.30	
15	H1	ar 7	1	69	ND	1.73	
16	H2	ar 8	162	82	33	0.24	
17	H2	ar 9	147	82	26	0.15	
18	H3	ar 10	100	61	ND	0.44	
19	H3	ar 11	108[d]	52	ND	0.78	Died[d]
Kusunose, *et al.*	A5	w 39	40	108	ND	16	Data from reference 9

[a] Factors are defined in materials and methods section.
[b] U SOD/mg protein in a cytochrome c-based assay. Values calculated from the luminol-based assay.
[c] One week at 8°C.
[d] Less than 12 hr at 20°C after death.

accepted. The region of p, between 0.01 and 0.05, is one where correlation is increasingly likely to be observed by chance. "Genstat," ([1]) a statistical package, was used for these manipulations.

Known factors tested for correction with SOD levels

Source. Armadillos were infected with *M. leprae* from five armadillo tissues (A1–5) or three human biopsies (H1–3).

Period of infection. Armadillos were sacrificed when they had a bacterial load of $>5 \times 10^9$. Thus periods of infection are generally similar (Table 1) and the period of the most rapid growth of *M. leprae* (log phase) would have passed. Two armadillos (ar 5 and ar 11) used in this study died before they could be sacrificed.

Period of storage at −80°C. Armadillo tissues were stored separately at −80°C for varying periods. It was possible to study a range of storage periods.

Morphological Index (MI). Since bacterial load was the main consideration in choosing tissues from which to obtain *M. leprae,* it was not possible to "select" suspensions for a range of MIs. Dr. R. J. W. Rees kindly estimated the MIs for all of the suspensions. Values were allocated where no estimates of the MI were made by the statistical package used to work out the analysis of variance.

RESULTS AND DISCUSSION

Confirmation of previous results. Using a factor to convert luminol-based units to cytochrome c-based units (determined with extracts 1 and 10), extracts 16 and 17, which had been used in the work of Wheeler and Gregory ([17]) were assayed, confirming the results obtained in that work (Table 1). Gamma-irradiated "w 39 tissue" (5 g), which had been the source of *M. leprae* used by Kusunose, *et al.* ([9]) was available to me; the leprosy bacilli were harvested and then cell-free extracts were prepared for extract 12 by the method of Wheeler and Gregory ([17]) and for extract 13, by the method of Kusonose, *et al.* ([9]). There was no substantial difference of SOD levels between extracts 12 and 13 (Table 1). However, when Kusunose, *et al.* harvested *M. leprae* from this tissue, it had not been γ-irradiated and so

it was necessary to find out the effect of such irradiation on the SOD in leprosy bacilli.

Effect of γ-irradiation. A portion of armadillo ar 3 tissue stored for 69 weeks at −80°C was γ-irradiated and *M. leprae* were harvested from the irradiated and non-irradiated ar 3 tissue. SOD from the irradiated *M. leprae* (in extract 8) was at 89% of the level in extract 9, from the live *M. leprae*. The slightly lower value after irradiation does not explain the difference in SOD in *M. leprae* harvested from w 39 by Kusunose, *et al.* (9) and reported here.

Effect of storage at 8°C. Extract 11, from leprosy bacilli from an armadillo which had died before it could be sacrificed, had a very high level of SOD (Table 1). A possible explanation for the high SOD was that if the infected tissue was not kept frozen (and stored, at −80°C) and degradation of the tissue by lysosomal enzymes occurred, the level of SOD in the leprosy bacilli might increase as an adaptation to this changed environment. Had the w 39 tissue sent to Japan thawed out in transit, this might explain the high level of SOD reported by Kusunose, *et al.* (9). The results for extracts 4–6 represent an experiment to test this hypothesis. Infected tissue from armadillo ar 2 was either stored at 8°C for seven days, or γ-irradiated, after which some was stored at 8°C for seven days and the rest remained stored at −80°C. Then the leprosy bacilli were isolated and extracts 4, 5, and 6, respectively, were made. Allowing for the effect of irradiation (extracts 5 and 6 would have 1.12 and 1.10 U SOD/mg protein, respectively), the effect of storage at 8°C on live leprosy bacilli in tissue was, if anything, to decrease the level of SOD (in extract 4, Table 1), contradicting the above hypothesis. Subsequently, another suspension of *M. leprae* was isolated from an armadillo (ar 11) that had died, and the level of SOD was only (in extract 19) 0.78 U/mg protein, below the mean value for SOD in *M. leprae* but above the level of SOD in *M. leprae* (in extract 18) from ar 10, which had been inoculated with the *M. leprae* from the same source (Table 1).

Effects of other factors. The correlation between SOD level and factors (Table 1) which, for practical reasons, could not be controlled, was also investigated. This was done by taking the extracts which had not been specially prepared for experiments, thus avoiding any duplicate values which are not applicable (4) when working out values of r (correlation coefficient). In the first place, this definition included extracts (of live *M. leprae* from tissues stored at −80°C) 1–3, 7, 9, 10 and 14–18. Then the irradiated extracts 6 and 12 were added to the analysis, after correction of the SOD value for the effect of irradiation (dividing by 0.89, see above). Finally, extracts 11 and 19, of *M. leprae* from tissue of armadillos which had died, were added. The results of the statistical analysis (Table 2) were inconclusive. The strongest correlation with SOD levels in extracts of *M. leprae* was with the period of storage of infected tissue at −80°C (Table 2); from a line of best fit it was deduced that *M. leprae* from fresh liver would have 1.77 U SOD/mg protein. This corresponds well with the value from extract 17 of *M. leprae* from infected tissue stored at −80°C for only one week. Other factors investigated showed little or no relationship with SOD levels (Table 2).

Contamination with SOD from other organisms. Finally, the possibility of contamination was considered. The number of fast-growing organisms was insignificant (<1 in 10^7 *M. leprae*) and there were no opportunities for them to increase in numbers in these experiments. Difficult-to-grow mycobacterial contaminants (ADMs) have been detected recently in armadillo tissues infected with *M. leprae* (13, 14). Although ADMs were not looked for in this work, gel electrophoresis of extracts 10, 11, 12, 16, and 17 revealed only one band of SOD activity; R_f was 0.67, as observed previously (17). This shows that there was no contamination with host-derived activity and suggests no contamination with SOD from other bacteria in the extracts.

Effect of catalase and peroxidase. The possibility that SOD activity might be overestimated as a result of high levels of catalase was ruled out, since extract 1 (from NaOH treated *M. leprae* in which host-derived catalase activity would be abolished[17]) had full SOD activity, and if 0.1 mM KCN was added (to inhibit peroxidases) to the assay—with extracts 1, 2, and 10—inhibition of luminescence was not altered.

Conclusions. None of the factors in this study, with the exception of time of storage

TABLE 2. *Correlation of superoxide dismutase levels in* M. leprae *with known factors.*

Extracts[a]	Factors[b]								Specific activity Mean ± S.D.
	Source		Period of storage at −80°C		Period of infection		MI		
	r	p[c]	r	p	r	p	r	p	
1–3, 7, 9, 10, 14–18	−0.54	0.025 < 0.05	−0.64	0.01 < 0.025	−0.58	0.025 < 0.05	−0.32	>0.05	1.19 ± 0.64
1–3, 6, 7, 9, 10, 12, 14–18	ND	ND	−0.63	~0.01	−0.46	>0.05	−0.27	>0.05	1.16 ± 0.59
1–3, 6, 7, 9–12, 14–19	−0.45	>0.05	−0.65	<0.01	−0.52	0.025	−0.27	>0.05	1.31 ± 0.88

[a] The selection of extracts was done to avoid duplicate values in the regression analysis (see text "Effects of other factors" for further explanation).
[b] Factors are defined in materials and methods section.
[c] p = probability of getting this correlation by chance. Where $p \leq 0.01$, a correlation is accepted: p should not change, or decrease slightly, with increase in the number of extracts.

of infected tissue at −80°C, influences significantly SOD levels in *M. leprae* (Table 2). This work does not explain the occurrence of high levels of SOD in some suspensions of *M. leprae*; both the SOD level in extract 11 and that reported by Kusunose, *et al.* (9) are beyond 2.58 standard deviations (using the value of 0.88 for S.D.) of the mean ($p < 0.01$). However, a revised value of 1.31 U SOD/mg protein from assaying 15 cell-free extracts from *M. leprae* is presented. It is possible that *M. leprae* SOD is inducible, but it would be very difficult to test this without being able to control the conditions of growth of the bacterium. Inducible SODs are rare among microorganisms (5), and there is no evidence for inducible SOD among the mycobacteria.

Although no catalase of *M. leprae* has been detected yet (8, 17), the possession of very high levels of SOD may compensate for lack of catalase, since the highly toxic hydroxyl radical is produced by the interaction of superoxide and peroxide (6). Speculation upon the significance of these two enzymes for *M. leprae* has been rather contradictory (9, 17). It is therefore suggested that experiments in which leprosy bacilli are exposed to superoxide and peroxide *in vitro* should be devised to obtain further information on the susceptibility of the organism to oxygen-free radicals. Such an approach has been very useful in similar studies on intracellular parasites such as *M. tuberculosis* (7), *Leishmania* spp. (11), and *Toxoplasma gondii* (12).

SUMMARY

Recent improvements in the sensitivity of assay methods for superoxide dismutase (SOD) have enabled the detection of this enzyme in 18 cell-free extracts of purified *Mycobacterium leprae*. By converting back to units of SOD obtained in the cytochrome c-based method previously used in work on this enzyme in mycobacteria, it was shown that extracts of *M. leprae* had 0.15–3.84 U SOD/mg protein (this study). A mean value of 1.31 U/mg protein was calculated. It was not possible to find any factors which could explain the very high levels in some extracts, although correlation with the period of tissue storage at −80°C suggested that *M. leprae* in freshly killed tissue would have 1.77 U SOD/mg protein. The possibility of contamination by SODs from host and oth-

er organisms was unlikely since on gel electrophoresis extracts of *M. leprae* with high levels of SOD showed only a single band of activity characteristic of manganese-dependent SOD previously demonstrated.

RESUMEN

Las recientes mejoras en la sensibilidad de los métodos para cuantificar la superoxido dismutasa (SOD) han hecho posible la detección de esta enzima en 18 extractos libres de células de *Mycobacterium leprae* purificados. Utilizando el método de reducción del citocromo-c, se demostró que los extractos de *M. leprae* tuvieron de 0.15 a 3.84 (media = 1.31) unidades de SOD por mg de proteína. No fue posible encontrar algún factor que pudiera explicar los muy altos niveles de la enzima encontrados en algunos extractos, aunque la correlación con el periodo de almacenamiento del tejido a −80°C sugirió que el *M. leprae* en tejido fresco (recién preparado) debía contener 1.77 U SOD/mg proteína. La posibilidad de contaminación por SODs del huésped o de otros microorganismos es poco probable puesto que los electroferogramas en gel de los extractos de *M. leprae* con niveles elevados de SOD mostraron una sola banda de actividad, característica de la SOD dependiente de manganeso demostrada previamente.

RÉSUMÉ

Des améliorations récentes obtenues dans la sensibilité des méthodes d'évaluation de la superoxyde dismutase (SOD) ont permis la détection de cette enzyme dans dix-huit extraits acellulaires de *Mycobacterium leprae* purifié. En retournant aux unités de SOD obtenues dans la méthode basée sur le cytochrome-c, telle qu'on l'utilisait auparavant dans les travaux sur cette enzyme chez les mycobactéries, on a montré dans cette étude que les extraits de *M. leprae* contenaient 0.15–3.84 unités de SOD par mg de protéine. On a calculé une valeur moyenne de 1.31 U/mg de protéine. Il n'a pas été possible de mettre en évidence des facteurs qui pourraient expliquer les niveaux très élevés observés dans certains extraits, encore que la corrélation notée avec la période d'entreposage à −80°C suggèrent que *M. leprae* dans des tissus fraîchement tués contiendraient 1.77 U SOD/mg de protéine. La possibilité de contamination par le SOD de l'hôte, ou d'autres organismes, est peu vraisemblable car les extraits de *M. leprae* obtenus par électrophorèse sur gel, et présentant des niveaux élevés de SOD, n'ont montré qu'une seule bande d'activité caractéristique du SOD manganèse-dépendant précédemment mis en évidence.

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