Virulence of *Mycobacterium tuberculosis* and Susceptibility to Peroxidative Killing Systems

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At sub-bactericidal concentrations of hydrogen peroxide, *Mycobacterium tuberculosis* was killed by hydrogen peroxide/peroxidase/halide microbicidal systems. The halide cofactor could be either iodide or, with much lower efficiency, chloride. Omission of any one of the reactants eliminated the tuberculocidal effect. Differences in susceptibility between different strains of *M. tuberculosis* did not correlate with virulence differences. The observations are discussed in the context of host defence mechanisms against tuberculosis.

INTRODUCTION

How macrophages kill *Mycobacterium tuberculosis* is not yet established. Hydrogen peroxide (H_2O_2) is produced by macrophages (Klebanoff & Hamon, 1975) and resistance to it is a virulence determinant in *M. tuberculosis* (Mitchison *et al.*, 1963). However, studies of strains with high virulence and of strains with low virulence, either on primary isolation from Indian patients (group 1) or after mutation to isoniazid resistance (group 2) or after prolonged maintenance on laboratory media (group 3), showed that possession of peroxide resistance, although necessary, is not sufficient for virulence (Jackett *et al.*, 1978). In particular, the resistance of the laboratory-attenuated strain H37Ra to H_2O_2 is comparable to that of virulent strains. This suggests that resistance to additional bactericidal macrophage products might also be required. However, virulence does not correlate with resistance to low pH or to superoxide (Jackett *et al.*, 1978).

Since Reiter *et al.* (1964) and Klebanoff & Luebke (1965) demonstrated the antibacterial effect of the reaction between H_2O_2 , peroxidase and thiocyanate, leucocytic peroxidase (myeloperoxidase) has come to be regarded as a major component of antimicrobial host defences, acting by greatly enhancing the killing power of H_2O_2 in the presence of an oxidizable cofactor. The latter can be a halide or thyroxine instead of thiocyanate (Klebanoff & Hamon, 1972; Klebanoff, 1975). It is not clear, however, whether such a system operates in macrophages. The peroxidase content of macrophages is low relative to that of polymorphonuclear phagocytes (Klebanoff & Hamon, 1975). If the virulence of *M. tuberculosis* could be shown to correlate with resistance to a peroxidative killing system, then evidence would be forthcoming for such a system, present in macrophages, having a significant function. The strains of *M. tuberculosis* which had been compared previously for their resistance to low pH, superoxide and H_2O_2 were therefore examined for susceptibility to peroxidase-mediated killing systems using concentrations of H_2O_2 which were themselves sub-bactericidal.

METHODS

Organisms. Six strains of *Mycobacterium tuberculosis* and *Escherichia coli* B were used. The origins and properties of the *M. tuberculosis* strains have been described previously (Jackett *et al.*, 1978). Briefly, two strains (I2646 and 79499) were of high virulence and four (H37Ra, B1453, 79112 and its isoniazid-resistant mutant 79112R) were of low virulence in the guinea pig. Both high-virulence strains were catalase-positive and H₂O₂-resistant. Of the low-virulence strains, H37Ra (group 3) and 79112 (group 1) were catalase-positive; B1453 (group 2) and 79112R were catalase-negative. H37Ra was resistant to H₂O₂; the other three were sensitive to H₂O₂.

Growth conditions. *Mycobacterium tuberculosis* was grown at 37 °C in catalase-free liquid medium 7H9 (Jackett *et al.*, 1978). For most tests, late-exponential phase (7 d) cultures containing about 1×10^8 colony-forming units (c.f.u.) ml⁻¹ were used. Mid-exponential phase (4 d) cultures containing about 1×10^7 c.f.u. ml⁻¹ were used where stated. For tests with *E. coli* B, late-exponential phase (3 h) cultures grown at 37 °C in nutrient broth (Oxoid no. 2) to a concentration of 2×10^8 c.f.u. ml⁻¹ were used.

Peroxidative system susceptibility tests. In the basic susceptibility tests, bacterial survival was assessed in the presence of three reactants: (i) lactoperoxidase, (ii) an oxidizable cofactor which was either iodide, chloride, thiocyanate or thyroxine, and (iii) H₂O₂ added as reagent or generated *in situ* by glucose oxidase-catalysed oxidation of glucose. Controls in which individual components of the reaction mixture were omitted were included in each experiment. In the standard peroxidative killing tests of Klebanoff (1970), lactate buffer at the optimum pH 4.5 to 5.0 is used. However, this buffer was found to be toxic to *M. tuberculosis* and so tests were done in a non-toxic buffer composed of 50 mM-citric acid/50 mM-sodium phosphate (pH 5.0). A culture was diluted to 4×10^5 bacteria ml⁻¹ (Thoma counting chamber) in double-strength buffer. Samples of bacterial suspension (500 µl) were dispensed into 75 × 10 mm glass tubes and 200 µl 50 mM-glucose in water was added to each tube. Lactoperoxidase (200 or 400 U), oxidizable cofactor (various amounts) and glucose oxidase (0.005 to 0.28 U) or reagent H₂O₂ (250 nmol) were added as separate 100 µl solutions in water. Where reactants were omitted for control purposes, water was added to bring the final reaction volume to 1 ml. All reaction tubes were prepared in duplicate and glucose oxidase or H₂O₂ was added last. After 1 h incubation in a 37 °C water bath, 100 µl samples were removed, diluted 10- and 100-fold in 0.1% (w/v) bovine serum albumin fraction V (Armour Pharmaceuticals, Eastbourne, Sussex) and c.f.u. ml⁻¹ were assessed on blood agar base (Oxoid) or 7H11 agar (Jackett *et al.*, 1978) for *E. coli* and *M. tuberculosis*, respectively. The number of c.f.u. in a test mixture was expressed as a percentage of the number of c.f.u. in the corresponding control tubes which contained bacteria, glucose and buffer only. The figure so obtained was termed the percentage survival. The statistical significance of differences in percentage survival in different test conditions was assessed by analysis of variance.

Test reagents. Glucose oxidase (type V) containing about 200 U (mg protein)⁻¹ was obtained from Sigma. One unit oxidizes 1 µmol β-D-glucose to D-glucuronic acid and H₂O₂ per min at pH 5.1 and 35 °C. Lactoperoxidase (Boehringer) contained 90 U (µg protein)⁻¹, standardized with *o*-dianisidine as substrate (Klebanoff, 1965). DL-Thyroxine and potassium thiocyanate were obtained from Sigma. All other reagents were of analytical grade (BDH).

RESULTS

In all experiments in which a bactericidal effect was seen with a complete mixture of H₂O₂ (or glucose oxidase), peroxidase and an oxidizable cofactor, omission of any one of the reactants eliminated the killing effect. For brevity, the results with these controls are not presented.

Killing of E. coli

Preliminary experiments with *E. coli* confirmed that each of the oxidizable cofactors, iodide, chloride, thiocyanate or thyroxine, was effective in peroxidative killing (Table 1). Iodide was more effective than the other cofactors. Essentially identical results were obtained when the organism was grown in 7H9 medium or tested in 100 mM-lactate buffer pH 5.0 (not shown).

Killing of M. tuberculosis

Susceptibility with iodide as cofactor. *Mycobacterium tuberculosis* was killed by the interaction of H₂O₂ (enzymically generated or added), lactoperoxidase and 10 µM-iodide (Table 1). Even with 1 µM-iodide the system was effective, the percentage survival in the complete 1 µM-iodide system being significantly different ($P < 0.05$; all strains considered

Table 1. *Susceptibility of five strains of M. tuberculosis and E. coli B to peroxidative enzyme systems*

Bacterial suspensions (2×10^8 bacteria ml^{-1}) were incubated with glucose (10 mM) for 1 h at 37°C , at pH 5.0, in 50 mM-citric acid/50 mM-sodium phosphate. Tests and controls (organisms in glucose buffer only) were incubated in duplicate in two experiments. Survival in test conditions is expressed as a percentage of the survival in controls, i.e.

$$\frac{\text{c.f.u. ml}^{-1} \text{ in test conditions}}{\text{c.f.u. ml}^{-1} \text{ in buffer control}} \times 100; \text{ each value is the geometric mean of four estimates.}$$

Test conditions			Mean percentage survival					
Glucose oxidase (U ml^{-1})	Lacto-peroxidase (U ml^{-1})	Cofactor (μM)	I2646 (H)*	79499 (H)	H37Ra (L)*	B1453 (L)	79112 (L)	<i>E. coli</i> B
0.02	—	—	97	87	116	82	103	95
0.28	—	—	93	80	105	79	68	50
0.02	200	KI (1)	55	48	100	54	90	52
0.02	200	KI (10)	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
0.02	200	KCl (100)	80	107	103	58	56	50
0.28	200	KCl (100)	71	61	94	76	104	35
0.02	200	Thyroxine (5)	80	122	99	74	94	1.5
0.02	200	Thiocyanate (625)	89	91	102	88	97	< 0.1
H ₂ O ₂ (250 μM)	200	KI (10)	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1

* H and L, High and low virulence, respectively, in the guinea pig (root index of virulence > 1 or < 1 ; Mitchison *et al.*, 1961).

Table 2. *Effect of phase of growth and lactoperoxidase concentration on the susceptibility of six strains of M. tuberculosis to chloride-dependent peroxidative killing systems*

Bacteria were grown to either the mid-exponential or late-exponential phase of growth and their survival in each killing system was assessed and expressed as described in Table 1.

Test conditions			Mean percentage survival					
Glucose oxidase (U ml^{-1})	Lacto-peroxidase (U ml^{-1})	KCl (mM)	I2646	79499	H37Ra	B1453	79112	79112R
Mid-exponential phase								
0.28	—	—	72	96	102	90	98	92*
0.28	200	100	55	89	70	91	29	50*
0.28	400	100	46	81	56	67	25	38*
Late-exponential phase								
0.005	—	—	101†	119†	113†	99†	112†	98
0.28	—	—	97	94	84	100	92	0.4†
0.28	200	100	63	80	66	100	97	85*

* Glucose oxidase used at 0.005 U ml^{-1} .

† Results are the mean of duplicate tests in one experiment.

together) from that in the glucose oxidase control. Although some strains were more susceptible than others, susceptibility did not correlate with either low virulence or susceptibility to H₂O₂. Cofactors other than iodide were ineffective. Although there was some indication of an effect with 100 μM -chloride on some strains (Table 1), a series of experiments in which chloride was tested at 1 and 10 mM (in the presence of glucose oxidase at 0.02 or 0.28 U ml^{-1} and peroxidase at 200 U ml^{-1}) failed to substantiate this effect. In contrast,

chloride was consistently effective as cofactor in the peroxidative killing of *E. coli* (results not shown).

Susceptibility with chloride as cofactor. The glucose/glucose oxidase/lactoperoxidase/chloride system was examined further in experiments in which the chloride concentration was increased to 100 mM to more closely reflect physiological concentrations. Because bacteria are often most susceptible to bactericidal agents when they are growing most actively (Winslow & Walker, 1939; Meynell, 1961), the effect of the phase of growth was also investigated. With this higher chloride concentration, a small bactericidal effect of the system against some strains was again seen (Table 2). Although mid-exponential phase cultures were no more susceptible to H_2O_2 (glucose oxidase control) than late-exponential phase cultures, the younger cultures of strains 79112 and 79112R showed greater susceptibility to the complete system than the older cultures, i.e. the difference in percentage survival between the glucose oxidase control and the complete system was greater with the younger than with the older cultures ($P < 0.01$, for both strains). The phase of growth had no effect on the susceptibility of the other strains to the complete system. The bactericidal effect was increased when the peroxidase concentration was increased, i.e. the difference in percentage survival between the glucose oxidase control and the complete system was greater with 400 than with 200 U peroxidase ml^{-1} ($P < 0.01$, over all strains). No correlation between the degree of killing and either virulence or H_2O_2 susceptibility of the strains was apparent.

DISCUSSION

The brief reports that mycobacteria, including *M. tuberculosis*, are susceptible to peroxidase-mediated killing with iodide (Demoulin-Brahy *et al.*, 1975; Delville *et al.*, 1976; Avila *et al.*, 1976) are confirmed and extended here. Although iodide as low as $1 \mu M$ is an effective cofactor for the peroxidase-mediated tuberculocidal reaction, this concentration may not be reached in macrophages. Thyroid hormones are regarded as a natural source of iodide for the killing system in granulocytes (Klebanoff, 1970) but the amounts of thyroid hormones or iodide in macrophages are not known. Hyperthyroidism, induced in rabbits by injection of either thyroxine or triiodothyronine, can enhance resistance to infection with *M. tuberculosis* (Lurie, 1964). Although no beneficial effect of triiodothyronine administration is observed in patients with pulmonary tuberculosis (Nodine *et al.*, 1959), increased resistance is seen in guinea pigs (Bloch, 1963). Furthermore, macrophages from triiodothyronine-treated guinea pigs appear to suppress growth of *M. tuberculosis* to a greater extent than do macrophages from normal rabbits (Hsu & Kapral, 1960). However, in the face of the complexity of the hormonally elicited changes, it is no more than a possibility that increased availability of iodide for a peroxidative killing system is involved. Because the availability of iodide for a tuberculocidal peroxidative reaction *in vivo* is questionable, it is particularly pertinent that in the *in vitro* system iodide can be replaced by chloride. Although the chloride dependent system is much less efficient (the halide concentration required for an equivalent tuberculocidal effect being 10^6 times greater with chloride than with iodide), chloride is likely to be adequately available in macrophages (Klebanoff, 1970). Although the efficiency with which toxic products are generated from the H_2O_2 /peroxidase/chloride system is critically dependent on the ratios of the reactants (Sbarra *et al.*, 1976, 1977), the system, unlike the iodide-dependent system (Klebanoff, 1967), can operate optimally even at neutral pH. The tuberculocidal effect of the chloride-dependent reaction may therefore exceed that of the iodide-dependent reaction in the bacterial environment *in vivo*, particularly if low pH does not prevail.

The peroxidase-mediated systems in effect convert innocuous concentrations of H_2O_2 into tuberculocidal reactants. This raises the question of whether *M. tuberculosis* encounters peroxidase within macrophages. Briefly, peroxidase is present in granules (presumably lysosomes) in bone marrow promonocytes in man, mouse and guinea pig and in lesser

amounts in circulating and exudative monocytes of the same species; it is less abundant than in polymorphonuclear leucocytes and essentially absent from the granules of mature macrophages in these species and in the rat and rabbit; however, peroxidase can perhaps transfer from granulocytes to macrophages during the complex cell interactions in inflammatory exudates (Klebanoff & Hamon, 1975). The small amounts of peroxidase present in macrophage golgi vesicles and in nuclear and endoplasmic reticulum membranes are of doubtful relevance to microbial killing. Peroxidase is absent from granules of rabbit alveolar and mouse peritoneal macrophages (Biggar & Sturgess, 1976; Lepper & Hart, 1976) and alveolar macrophage bactericidal activity against Gram-positive and Gram-negative organisms is not peroxidase-mediated (Biggar *et al.*, 1976). However, peroxidase can transfer from granules to phagocytic vesicles in casein-elicited peritoneal monocytes of the guinea pig (Daems *et al.*, 1975) and mouse (Lepper & Hart, 1976) and is involved in the ability of human monocytes to kill *Candida* (Lehrer, 1970). Thus, peroxidase may be potentially less available for tuberculocidal reactions in macrophages than in monocytes.

Monocytes produce H_2O_2 (Klebanoff & Hamon, 1975) and therefore the peroxidase of monocytes might play an important role in killing *M. tuberculosis* in tuberculous granulomas of high cell-turnover type. These develop with the onset of cell-mediated immunity and immature macrophages or monocytes predominate (Dannenberg *et al.*, 1972; Spector, 1975). Demonstration of a correlation between virulence and resistance to peroxidase-mediated killing systems would have supported this view but absence of correlation, as found here, does not constitute evidence to the contrary. *Mycobacterium tuberculosis* might simply be less prone to vary in its resistance to peroxidative systems than in its resistance to other host defences, e.g. H_2O_2 . It is nevertheless possible that the organism avoids exposure to peroxidase *in vivo*. The organism can inhibit fusion of lysosomes with the phagocytic vacuole in macrophages (Draper & Hart, 1975; Lowrie *et al.*, 1975) and possibly also in monocytes (Lepper & Hart, 1976) when the phagocytes are in monolayer culture. Direct investigation of the extent of *in vivo* peroxidase exposure is clearly needed.

If a peroxidase/ H_2O_2 /halide tuberculocidal system does operate *in vivo*, the present study indicates that organisms with high and low virulence are likely to be equally susceptible to its effects. Low virulence in strain H37Ra (laboratory-attenuated, low-virulence group 3) therefore probably cannot be explained by loss of resistance to the peroxidase system. Previous studies also showed that this strain was not deficient in resistance to H_2O_2 , low pH or superoxide, alone or in combination (Jackett *et al.*, 1978). Hence, although low virulence of *M. tuberculosis* isolated from Indian patients or of isoniazid-resistant mutants (low-virulence groups 1 and 2 respectively) can be attributed to loss of H_2O_2 resistance, the basis of low virulence in group 3 organisms is still uncertain.

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