The Susceptibility of Strains of *Mycobacterium tuberculosis* to Catalase-mediated Peroxidative Killing

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At low pH and with continuous low concentrations of hydrogen peroxide generated *in situ*, catalase was able to replace peroxidase in the peroxidase/hydrogen peroxide/iodide microbicidal system. The system was effective against *Escherichia coli* and *Mycobacterium tuberculosis*. Iodide could not be replaced by chloride. The system was effective in lactate buffer, but not in citrate/phosphate buffer. Strains of *M. tuberculosis* with high and low virulence were equally susceptible. The observations are discussed in the context of an involvement of host-cell catalase in a possible intracellular killing mechanism against *M. tuberculosis*.

INTRODUCTION

Histopathological studies indicate that the major cells involved in host resistance to *Mycobacterium tuberculosis* are macrophages (Lurie, 1964). Although it is probable that immunologically activated macrophages in particular can kill even virulent tubercle bacilli *in vivo*, this is as yet unproven. Nevertheless, normal mouse peritoneal macrophages can show transient ability to inhibit growth of virulent *M. tuberculosis* strain H37Rv and kill attenuated strain H37Ra *in vitro* (Hart & Armstrong, 1974). Thus, the isolated macrophage appears to have some tuberculocidal potential that might find more powerful and more sustained expression *in vivo*. The nature of the killing mechanisms involved is unknown. However, there are pointers to the involvement of H_2O_2 . H_2O_2 is produced by macrophages (Klebanoff & Hamon, 1975; Rister & Baehner, 1977; Nathan & Root, 1977; Boxer *et al.*, 1979), and resistance to H_2O_2 is a virulence determinant in *M. tuberculosis* (Mitchison *et al.*, 1963; Narayanan-Nair *et al.*, 1964). Although susceptibility to H_2O_2 correlates with low virulence in many strains, laboratory-attenuated strains retain resistance to H_2O_2 (Jackett *et al.*, 1978*a*). This implies that different antimicrobial mechanisms within the macrophage other than the direct action of H_2O_2 kill these strains. However, the possibility remains that an H_2O_2 -mediated mechanism may be involved. Since their first discovery (Reiter *et al.*, 1964; Klebanoff & Luebke, 1965) peroxidase-mediated systems have been shown to contribute to microbial killing in polymorphonuclear leucocytes, by enhancing greatly the bactericidal power of H_2O_2 (Klebanoff & Clark, 1978). In previous studies, strains of *M. tuberculosis* of diverse virulence in the guinea-pig were tested for their susceptibility to peroxidase/ H_2O_2 /halide systems (Jackett *et al.*, 1978*b*). All strains were susceptible to such a system with iodide and, to a much lesser extent, chloride as cofactor, and no correlation with virulence was observed.

The peroxidase content of macrophages is very low relative to that of polymorphonuclear leucocytes (Biggar & Sturgess, 1976; Lepper & Hart, 1976; Ogawa *et al.*, 1978). However, Klebanoff (1969, 1970) showed that catalase can replace peroxidase in the killing system

under certain conditions *in vitro*. Activity against a wide variety of micro-organisms was shown but the studies did not include *M. tuberculosis*.

Normally, catalase is found in the peroxisomes and cytosol (Lowrie *et al.*, 1977; Davies *et al.*, 1979), but transfer of macrophage catalase into the phagocytic vacuole during phagocytosis has been reported (Stossel *et al.*, 1972; Eguchi *et al.*, 1979).

If strains of *M. tuberculosis* could be shown to be susceptible to catalase-mediated systems *in vitro*, and if high susceptibility was correlated with low virulence, this might imply the involvement of the systems *in vivo*. The strains of *M. tuberculosis* previously studied (Jackett *et al.*, 1978*a, b*) were therefore examined for their susceptibility to catalase-mediated systems.

METHODS

Organisms. A strain of *Escherichia coli* B was used for preliminary experiments. Six strains of *Mycobacterium tuberculosis* were used. The properties of these strains have been reported previously (Jackett *et al.*, 1978*a*). Two strains (I2646 and 79499) were of high virulence and four (H37Ra, B1453, 79112 and the isoniazid-resistant variant 79112R) of low virulence in the guinea-pig. Both strains of high virulence were resistant to H_2O_2 ; of the strains of low virulence, the laboratory-attenuated strain H37Ra was resistant to H_2O_2 and the other three were sensitive to H_2O_2 .

Growth conditions. *Escherichia coli* B was grown at 37 °C in nutrient broth (Oxoid no. 2) and used after 3 h incubation, i.e. at the late-exponential phase of growth. *Mycobacterium tuberculosis* was grown at 37 °C in catalase-free liquid medium 7H9 (Jackett *et al.*, 1978*a*) and used for tests after 4 or 7 d, i.e. at the mid- and late-exponential phases of growth, respectively (Jackett *et al.*, 1978*b*).

Catalase-mediated killing tests. The test system consisted of three components: (i) H_2O_2 generated *in situ* by glucose oxidase-catalysed oxidation of glucose, (ii) catalase, and (iii) a halide which was either iodide or chloride. Each experiment included comprehensive controls where single and multiple components were omitted. Two buffers were compared: a buffer composed of 50 mM-citric acid and 50 mM-sodium phosphate (citrate/phosphate), and 25 mM-lactate buffer, both at pH 5.0. Klebanoff (1970) measured the peroxidative killing of *E. coli* in 50 mM-lactate at pH 4.5 to 5.0, but this proved to be toxic for *M. tuberculosis*. The details of the test method have been described previously (Jackett *et al.*, 1978*b*) with the exception that catalase (1000 or 3000 U) was now substituted for peroxidase. Briefly, bacteria suspended in test mixtures to a final volume of 1 ml were incubated for 1 h in a 37 °C water-bath. At the end of the incubation period 100 μ l samples were removed, diluted in 0.01 % (w/v) bovine serum albumin fraction V (Armour Pharmaceuticals, Eastbourne, Sussex) and colony-forming units (c.f.u.) were assessed on blood-agar base (Oxoid) or 7H11 agar (Jackett *et al.*, 1978*a*) 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 control tubes containing glucose and buffer only. 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 200 U (mg protein)⁻¹ was obtained from Sigma. Catalase was obtained from Boehringer [beef liver, 50 U (mg protein)⁻¹: 1 unit decomposes 1 μ mol H_2O_2 min⁻¹ at pH 7.0 and 25 °C]. All other reagents were analytical grade (BDH).

RESULTS

In experiments where bacterial killing was demonstrated with the combination of glucose, glucose oxidase, catalase and halide, omission of one or more of the components resulted in loss of the bactericidal effect. In most instances, for brevity, these controls have been omitted from the tables.

Killing of E. coli

Experiments with *E. coli* confirmed that a catalase-mediated peroxidative killing system was effective (Table 1). However, the bactericidal effect was only evident in lactate buffer. In addition, the system was bactericidal only in the presence of iodide. No killing was observed when iodide was replaced by chloride.

Table 1. *Susceptibility of E. coli B to catalase-mediated killing*

Bacterial suspensions (2×10^5 bacteria ml^{-1}) were incubated with the various additives and glucose (10 mM) for 1 h at 37 °C, at pH 5.0. Tests and controls (organisms in glucose buffer only) were incubated in duplicate in two experiments for each buffer. Survival in test conditions is expressed as a percentage of the survival in controls, i.e. $100 \times (\text{c.f.u. ml}^{-1} \text{ in test conditions})/(\text{c.f.u. ml}^{-1} \text{ in buffer control})$; each value is the geometric mean of four observations.

Test conditions			Mean percentage survival	
Glucose oxidase* (U ml^{-1})	Catalase (U ml^{-1})	Cofactor (mM)	Citrate/phosphate buffer (50 mM)	Lactate buffer (25 mM)
0.02/0.005	—	—	113	38
0.02/0.005	1000	—	92	100
0.02/0.005	—	KI (0.1)	100	42
0.02/0.005	—	KCl (100)	107	40
0.02/0.005	1000	KI (0.1)	86	< 0.1
0.02/0.005	1000	KCl (100)	92	113
0.005/0.005	LP, 200†	KI (0.01)	< 0.1	< 0.1

* Concentrations in citrate/phosphate buffer and lactate buffer, respectively.

† Containing lactoperoxidase (LP) instead of catalase.

Table 2. *Susceptibility of M. tuberculosis to catalase-mediated killing in lactate buffer*

Experimental conditions and methods of estimation of survival are as in Table 1.

Test conditions			Mean percentage survival					
Glucose oxidase (U ml^{-1})	Catalase (U ml^{-1})	Cofactor (mM)	Strains of <i>M. tuberculosis</i>					
			I2646 (H)*	79499 (H)	H37Ra (L)	B1453 (L)	79112 (L)	79112R (L)
0.02	—	—	96	88	96	88	84	81
0.28	—	—	35	38	94	70	56	44
—	—	KCl (100)	88	83	65	78	39	55
0.02	3000	KCl (100)	69	63	57	84	42	36
0.28	3000	KCl (100)	67	36	72	87	50	43
0.02	3000	KI (0.01)	63	18	28	68	58	25
0.28	3000	KI (0.01)	38	15	59	73	55	37
0.02	3000	KI (0.1)			No survival			
0.28	3000	KI (0.1)	8	4	5	18	5	2
0.02	LP, 200†	KI (0.01)			No survival			

* H and L, high and low virulence in the guinea-pig, respectively.

† Containing lactoperoxidase (LP) instead of catalase.

Killing of *M. tuberculosis*

Phase of culture. Overall, there was no difference in the susceptibilities of the strains in mid-exponential (4 d) or late-exponential (7 d) phases of growth. Therefore, the results for the 4 d cultures (mid-exponential phase) only are presented.

Susceptibility with iodide as cofactor. The results closely paralleled those with *E. coli*, but increased concentrations of enzymes were needed for killing (Table 2). There was no killing in citrate/phosphate buffer (results not shown). In lactate buffer, 100 μM -iodide was more effective than 10 μM -iodide. At the lower concentration of iodide, there was no significant difference between the effects of glucose oxidase at 0.02 or 0.28 U ml^{-1} . However, at 100 μM -iodide, the considerable bactericidal effect was greater with the lower concentration of glucose oxidase. Other results (not shown) indicated that under any of the conditions tested 1000 U catalase ml^{-1} was less effective than 3000 U ml^{-1} . Between *M. tuberculosis* strains minor differences in susceptibility did not correlate with differences in virulence.

Susceptibility with chloride as cofactor. No killing was seen with chloride. Although there was some reduction in numbers of c.f.u. with the complete catalase/H₂O₂/chloride system, this effect was no greater than that of glucose oxidase and chloride alone. Indeed, with the 7 d cultures, addition of catalase appeared antagonistic to killing, and protected the organisms.

DISCUSSION

The reports that catalase can substitute for peroxidase in microbicidal reactions *in vitro* against a variety of micro-organisms (Klebanoff, 1969, 1970) have now been confirmed with *E. coli* and extended to *M. tuberculosis*.

Although all of the strains of *M. tuberculosis* studied here were killed by a catalase-mediated system and there was some difference in survival between the strains ($P < 0.01$), there was no correlation between low virulence and high susceptibility to catalase-mediated killing systems.

The catalase-mediated antibacterial activity was only observed in lactate buffer. The mechanism by which lactate supported the killing of bacteria by catalase, whereas citrate/phosphate did not, is unknown. However, it has been known for a long time that citrate ions can inhibit the activity of some enzymes and that lactic acid is toxic to a variety of bacteria at low pH (Dubos, 1955*a*). Although the concentration of lactate used here was selected as being the highest at which no lactate toxicity was detectable with any of the strains of *M. tuberculosis* during 90 min at 37 °C, it is possible that this concentration nevertheless produced sub-lethal damage that facilitated susceptibility to the catalase system. Lactate also enhanced the bactericidal power of the *in situ*-generated H₂O₂ in the absence of halide and catalase. Both lactate and H₂O₂ are produced in increased amounts during phagocytosis (Oren *et al.*, 1963; Ouchi *et al.*, 1965; Gee *et al.*, 1970, 1971; Stossel *et al.*, 1972; Drath *et al.*, 1978).

That catalase can function peroxidatively has been known for many years. The essential condition for such action is the presence of a low steady state concentration of H₂O₂ relative to the catalase concentration and peroxidative activity is further favoured by low pH (Klebanoff, 1969). Preliminary results (which have not been given here) showing that peroxide added directly was ineffective, together with the observations that decreasing the rate of H₂O₂ generation by lowering the glucose oxidase concentration enhanced killing of tubercle bacilli (Table 2), are consistent with the involvement of a peroxidative activity of catalase. The *M. tuberculosis* strains tested were evidently more resistant to the toxic products of the reaction than was *E. coli* since higher concentrations of reactants were needed to kill *M. tuberculosis*. Suitable conditions of low pH and continuous H₂O₂ evolution may be found within the phagocytic vacuoles of certain types of macrophages (Klebanoff & Hamon, 1975).

Abundant catalase has been found in pulmonary alveolar macrophages of rabbit (Gee *et al.*, 1970; Stossel *et al.*, 1972; Lowrie *et al.*, 1977) and rat (Davies *et al.*, 1979) and peritoneal exudate macrophages of guinea-pig (Simmons & Karnovsky, 1973). The enzyme is about equally distributed between non-lysosomal granules (presumptive peroxisomes) and cytosol in the rabbit and rat cells (Stossel *et al.*, 1972; Davies *et al.*, 1979). Mouse peritoneal macrophages contain less than one-tenth as much activity (Simmons & Karnovsky, 1973) and this is virtually confined to non-lysosomal granules (D. B. Lowrie, unpublished results). Peroxidative activity, which was probably due to catalase rather than myeloperoxidase, has been demonstrated in granule fractions of the pulmonary macrophage of the rabbit. The activity was diminished by the catalase inhibitor 3-amino-1,2,4-triazole, and the granule fractions showed bactericidal activity against *E. coli* in the presence of a H₂O₂-generating system and iodide in lactate buffer at pH 4.5 (Paul *et al.*, 1973). If a catalase-dependent system contributes to killing in the phagocytic vacuoles of intact cells then catalase translocation from either cytosol or granules into vacuoles must occur. There is evidence that

catalase enters phagocytic vacuoles which contain oil droplets in rabbit pulmonary macrophages (Stossel *et al.*, 1972) or which contain latex particles in rat peritoneal macrophages (Eguchi *et al.*, 1979), but no evidence of such transfer was found in rat Kupffer cells containing latex (Fahimi *et al.*, 1976). Evidence for transfer into vacuoles containing tubercle bacilli is clearly needed, particularly as live tubercle bacilli can inhibit fusion of their phagocytic vacuoles with lysosomes (Hart *et al.*, 1972; Lowrie *et al.*, 1979).

There is abundant chloride in macrophages (Klebanoff, 1975; Castranova *et al.*, 1979), and presumably also in phagocytic vacuoles, but we have confirmed that catalase-dependent killing does not occur with chloride as the cofactor in place of iodide. Although iodide is effective at concentrations as low as 1 μM in peroxidase-mediated killing of *M. tuberculosis* (Jackett *et al.*, 1978*b*), this iodide concentration was ineffective with catalase. The concentration required was at least tenfold higher, with some effect seen at 10 μM . It is not known if this concentration of iodide is reached within the macrophage, but treatment with iodine-containing hormones can enhance host resistance to tuberculosis. This has been seen in rabbits (Lurie, 1964), mice (Dubos, 1955*b*) and guinea-pigs (Bloch, 1963), and macrophages from triiodothyronine-treated guinea-pigs had enhanced ability to suppress growth of *M. tuberculosis in vitro* (Hsu & Kapral, 1960). However, administration of thyroid hormones in amounts sufficient to inhibit weight-gain had adverse effects on host resistance in mice (Dubos, 1955*b*) and no beneficial effects in man (Nodine *et al.*, 1959). Whether the beneficial effects seen at lower doses in animals are a consequence of increased iodide availability for catalase-dependent killing in infected macrophages has not been established.

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