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Absence of Hydrogen Peroxide Production by or Catalase Action in *Rickettsia prowazeki*

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Glutamic acid oxidation by *Rickettsia prowazeki* is not accompanied by hydrogen peroxide generation, nor is added hydrogen peroxide degraded, as measured by a manometric technique.

The production of hydrogen peroxide and the capacity to degrade it or the lack of either or both of these activities is important in the biology of many microorganisms (10). These properties assume special significance in the case of pathogenic microorganisms because of the role of the myeloperoxidase system in the interaction of these organisms with polymorphonuclear leukocytes (8). The mechanism of intracellular killing by macrophages appears to differ from that of polymorphonuclear leukocytes because the former is not coupled to oxidative phosphorylation (9). However, in both cases hydrogen peroxide production appears to be involved. *Rickettsia prowazeki* is an obligate intracellular parasite which has the capacity (i) to penetrate host cells directly through the plasma membrane (C. L. Wisseman, Jr., and A. D. Waddell, manuscript in preparation) into the cytoplasm where it multiplies free, unbounded by a vacuolar membrane, and (ii), in the absence of antibodies, to survive for at least a limited period of time within phagocytic vacuoles of human macrophages and to escape from the vacuole into the cytoplasm where it then multiplies (6). When coated with appropriate antibodies, which are not rickettsiacidal, it is destroyed within the vacuoles of human macrophages even though the rickettsial envelope resists lysozyme action (7). The mechanisms involved in these processes are unknown. However, because hydrogen peroxide has been implicated in both action on cell membranes, as with certain mycoplasma (5), and intracellular killing (8), the capacity of *R. prowazeki* to produce and degrade hydrogen peroxide was studied.

Two somewhat different procedures were employed for the purification of *R. prowazeki*, Breinl strain, from infected yolk sacs. When hydrogen peroxide production was to be determined, infected yolk sacs were blended in sucrose-phosphate-glutamate to form a 25% (wet wt/vol) homogenate. This material was centrifuged (27,000 × *g* for 30 min), and the pellet, after resuspension, was placed on a continuous 5 to 30% sucrose gradient containing 0.01 M KCl and 0.01 M tris(hydroxymethyl)aminomethane-maleate buffer, pH 6.2, and centrifuged again (1,000 × *g* for 45 min). The rickettsial band was suspended in a medium which had been shown previously to maintain rickettsial oxidative activity for 4 h; this medium contained 0.115 M NaCl, 0.02 M KCl, 0.004 M $MgCl_2$, 0.001 M $CaCl_2$, 0.005 M K_2HPO_4, 0.02 M K glutamate, 10 μg of nicotinamide adenine dinucleotide per ml, 1 μg of coenzyme A per ml, 0.05% bovine serum albumin, and 0.01 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer, pH 7.1. *Streptococcus pneumoniae* (ATCC 6301), used as a positive control, was grown in Trypticase soy broth (Baltimore Biological Laboratory) and harvested at 18 h. It was suspended in the same medium as the rickettsiae, except that glucose (0.2%) was substituted for glutamate as an energy source. Hydrogen peroxide production was measured by the technique of Cohen and Somerson (4) whereby the secretion rate of H_2O_2 is measured by an H_2O_2-dependent inhibition of catalase by 3-amino-1,2,4-triazole. The specific procedure employed here has been described previously (11).

The rickettsiae which were used for the determination of catalase activity were first partially purified by a batch sucrose method (3 volumes of yolk sac and 5 volumes of 50% [wt/vol] sucrose [C. L. Wisseman, Jr., and A. D. Waddell, unpublished data]). This suspension was centrifuged at 20,200 × *g* for 30 min, and the pellet was suspended in PBS ($Na_2HPO_4 \cdot KH_2PO_4$, 0.01 M; NaCl, 0.12 M, pH 7.0). The suspension was centrifuged at 120 × *g* for 5 min, and 2 to 3 ml of the supernatant was layered onto continuous Renografin gradients (30 to 45% Renografin in PBS) (12). The gradient was centrifuged at 23,-

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000 rpm (60 min; SW25.1 rotor). The rickettsial band was diluted with 2 parts of PBS and centrifuged at 20,200 × *g* for 30 min. The pellet was resuspended in PBS, and a second Renografin gradient centrifugation was carried out. The final rickettsial suspension was made in 0.05 M potassium phosphate buffer, pH 7.0. *Escherichia coli* strain K-12, used as a positive control, was grown in Trypticase soy broth for 18 h, washed three times in 0.05 M potassium phosphate, pH 7.0, and resuspended in the same phosphate buffer. Catalase activity was determined manometrically by measuring the amount of oxygen liberated from added H_2O_2 (11).

Table 1 shows that a heavy suspension of *R. prowazeki* (optical density at 725 nm [1-cm light path], 1.20) caused negligible inactivation of catalase in the presence of 3-amino-1,2,4-triazole over a 150-min period, regardless of whether glutamate, a substrate for oxidative metabolism (2, 3), was present and, hence, gave no evidence of H_2O_2 production. In contrast, in the same test system, *S. pneumoniae*, even at a turbidity of less than 5% of that of the rickettsial suspension, produced sufficient H_2O_2 to almost completely inactivate the catalase in 30 min.

Preliminary qualitative tests for catalase activity in purified *R. prowazeki*, i.e., observation for bubble formation when H_2O_2 was added to a heavy rickettsial suspension, were negative. A subsequent sensitive (<10 μl of O_2 liberated) quantitative test for catalase activity with a heavy rickettsial suspension (Table 2) again gave no evidence for catalase activity, whereas an *E. coli* suspension of the same turbidity caused essentially complete degradation of H_2O_2 and quantitative evolution of O_2 within 10 to 15 min.

R. prowazeki in extracellular suspension appears to lack both the capacity to produce H_2O_2 during glutamic acid oxidation and to degrade H_2O_2 through catalase-type action. Thus, H_2O_2 production cannot account for the production of the plasma membrane defect associated with rickettsial hemolysis and assumed in rickettsial penetration of host cells. Nor does H_2O_2 of rickettsial origin contribute to intraphagosomal rickettsial destruction, as has been shown to occur under some circumstances with certain bacteria (8). The lack of the H_2O_2-catalase system in *R. prowazeki* may constitute one of the adaptations associated with obligate intracellular parasitism. Because H_2O_2 is not a significant component of the intracytoplasmic environment of the eucaryotic host cells (8), catalase activity would not be required for survival in this niche. Conversely, H_2O_2 production by the rickettsiae might be toxic to the host cell and, thus, limit host cell capacity to support rickettsial growth.

TABLE 1. *H_2O_2 generation by R. prowazeki and S. pneumoniae as measured by the catalase-aminotriazole technique*

Time (min)	% Inhibition of catalase activity				
	Control (No cells)	*R. prowazeki*[a]		*S. pneumoniae*[b]	
		Glutamate	No glutamate	1 × concn	0.1 × concn
0	0	0	0	0	0
30	0	0	0	98	96
60	0	4	4	100	100
150	0	6	4	100	100

[a] Optical density of *R. prowazeki* at 725 nm (1-cm light path), 120.

[b] Optical density of *S. pneumoniae* at 725 nm (1-cm light path), 0.54 (1 × concentration) and 0.05 (0.1 × concentration).

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TABLE 2. *Catalase activity in R. prowazeki and E. coli*

Time (min)	Cumulative oxygen produced (μl)			
	H_2O_2 and no cells	*E. coli* and no H_2O_2	*E. coli*[a] with H_2O_2	*R. prowazeki*[a] with H_2O_2
5	−1,[b] 1	0, 2	74, 92	−1, 1
10	−3, −2	1, 1	218, 229	−4, −3
15	−2, 0	2, 1	243, 257	−2, −2
20	−2, 0	6, 3	257, 267	−2, −2
25	−2, −2	3, 1	255, 267	−4, −4

[a] Both cell suspensions (*E. coli* and *R. prowazeki*) were adjusted to the same optical density (optical density at 725 nm [1-cm light path], 1.00).

[b] Microliters of oxygen produced in the presence of 20 μmol of H_2O_2.

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