Peroxide Formation by Mycoplasmas which Infect Man (33362)

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One of the most interesting properties of *M. pneumoniae* is the production of an hemolysin which is active against a number of different types of erythrocytes. When a suspension of red blood cells in agar was applied to an agar culture containing colonies of *M. pneumoniae*, a hemispherical area of hemolysis developed over the site of each colony. This indicated that the hemolysin was small and freely diffusable in agar (1). Subsequently, the hemolysin was found to be small enough to pass through two layers of dialysis membrane and use was made of this property to identify it as a peroxide. Specifically, catalase or peroxidase, enclosed within a dialysis bag which was interposed between mycoplasma colonies and red cells, inactivated the hemolysin (2). Recently the secretion of peroxide has been measured quantitatively. The method employed is based upon a peroxide-dependent inhibition of the enzyme catalase by 3-amino-1,2,4-triazole (3). Other mycoplasmas such as *M. laidlawii*, *M. gallisepticum*, and *M. neurolyticum* have also been shown to secrete a peroxide hemolysin (2, 4).

It has been suggested that peroxide may be one factor in the virulence of *M. pneumoniae* for man (2). It was of interest, therefore, to determine whether other mycoplasmas which infect man also produce peroxide and, if so, whether there are quantiative differences in secretion.

Materials and Methods. Organisms. Mycoplasma strains used in this study included the 8 which infect man and 3 which infect animals. These strains each had a history of multiple passages in mycoplasma media. All of them were cloned 3 × prior to their use in this study. The organisms derived from man were: *M. pneumoniae* (strain FH), *M. hominis* (strain DC-63), *M. orale* type 1 (strain CH-19299), *M. orale* type 2 (strain CH-20247), *M. orale* type 3 (strain DC-1114) (H. Fox, R. H. Purcell, and R. M. Chanock, 1968, to be published); *M. salivarium* (strain PG-20), *M. fermentans* (strain PG-18), and T-strains (T-960, T-354, and Threats-U). The organisms derived from animals were: *M. gallisepticum* (strain PG-31), *M. pulmonis* (strain PG-22), and *M. neurolyticum* (strain PG-28).

Media. Medium used for propagation of *M. hominis*, *M. orale* type 1, *M. orale* type 2, *M. orale* type 3, *M. salivarium*, and *M. neurolyticum* was the agar medium described by Chanock *et al.* (5). For the growth of *M. pneumoniae*, *M. fermentans*, *M. gallisepticum*, and *M. pulmonis* this medium was supplemented with 1% dextrose (final concentration). T-strains were grown on the medium described by Purcell *et al.* (6).

Test for hemolysis. Two ml of agar medium in plastic petri dishes (Falcon Plastics, 35 × 10 mm) was inoculated with 0.05 ml of a dilution of broth culture which contained approximately 100–200 colony forming units (cfu) of organisms. *M. pneumoniae*, *M. hominis*, *M. fermentans*, *M. gallisepticum*, *M. pulmonis*, and *M. neurolyticum* were grown under aerobic conditions while *M. orale* type 1, *M. orale* type 2, *M. orale* type 3, and *M. salivarium* were grown under an atmosphere of 5% CO_2–95% N_2. T-strains were grown under an atmosphere of 20% CO_2–80% N_2.

After 3–5 days of incubation at 37°, the growing colonies were overlayed with 0.5 ml of a guinea pig blood–agar mixture (1 volume of 20%, 3 × washed guinea pig erythrocytes in Alsever's solution mixed with 3 vol of PPLO agar cooled to approximately 45°). Incubation of the overlayed colonies was performed aerobically at 37°, and hemolysis was read at intervals of 24 hr.

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In order to study the nature of the hemolysins produced by various mycoplasmas peroxidase (code HPO-D) or catalase (code CTL) of Worthington Biochemicals, was incorporated into the blood–agar overlay mixture. Catalase inhibition of hemolysis was reversed by means of incorporation of 3-amino-1,2,4-triazole (Baltimore Biological Laboratories), a specific inhibitor of catalase into the blood overlay mixture.

Results. Hemolysin production by different mycoplasma species. Results of these experiments are summarized in Table I. It was found that each of the mycoplasma species tested, except the T-strains, secreted an hemolysin active against guinea pig erythrocytes. However, not all mycoplasmas tested hemolyzed red blood cells to the same extent or at the same rate. Possibly the failure of T-strain mycoplasmas to produce hemolysis may be related to the small size of their colonies.

Hemolytic plaques produced by colonies of *M. pneumoniae, M. gallisepticum, M. pulmonis,* and *M. neurolyticum* were visible as circular zones of 0.5–1 mm in diameter within 24 hr. When incubation was prolonged to 48–72 hr, the diameter of the hemolytic zones reached 1–1.5 mm, while after 96 hr guinea pig red cells often underwent spontaneous lysis. Hemolysis caused by these species was always complete (β-type). In contrast, hemolytic plaques produced by the colonies of *M. hominis, M. orale* type 1, *M. orale* type 2, *M. orale* type 3, *M. salivarium,* and *M. fermentans* started to appear, in most cases, within 48 hr of incubation. Such hemolytic zones did not exceed 1 mm in diameter after 72–96 hr of incubation. The differences in rate and extent of hemolysis observed suggest that there may be quantitative differences in hemolysin production among different mycoplasma species. Among the mycoplasmas of man, other than the T-strains, colony size did not appear to be responsible for the observed differences since *M. pneumoniae,* which produced the smallest colonies, produced the earliest and largest hemolytic plaques.

Hemolysis due to *M. orale* type 2 and *M. salivarium* was complete (β-type) while that caused by *M. hominis, M. orale* type 1, *M. orale* type 3, and *M. fermentans* was only partial (α-type). It was of interest that hemolysis was often found to be of the β-type when the colonies of the latter organisms were overlayed with a thinner layer of red blood cell–agar mixture or a lower concentration of erythrocytes was incorporated in the agar overlay.

Identification of the hemolysins as peroxide. In view of previous findings that the hemolysins of certain mycoplasma species appeared to be peroxide, identification of the hemolysins under study was attempted using the enzymes catalase and peroxidase. These were incorporated into the agar–red blood cell suspension which was used to overlay the mycoplasma colonies.

Results of these experiments are summarized in Table I. It was found that the hemolytic activity of each of the mycoplasmas tested was suppressed by catalase and, with the exception of *M. salivarium,* also by peroxidase. In several instances partial inhibition of hemolysis was observed, i.e., in the presence of either enzyme, small plaques of incomplete hemolysis developed at a delayed rate. The inhibition of hemolysis appeared to be related to the concentration of catalase. Hemolytic activity of *M. hominis, M. orale* type 1, *M. orale* type 2, *M. orale* type 3, and *M. salivarium* was prevented by 2000 U/ml of catalase. This concentration, however, was not sufficient to completely suppress hemolysin formation by *M. pneumoniae, M. fermentans, M. gallisepticum, M. pulmonis,* and *M. neurolyticum,* but greater inhibition was observed when 8000 U/ml of catalase was used.

Peroxidase (1000 U/ml) also inactivated the hemolysins of the various mycoplasmas tested. One thousand U/ml of this enzyme was not effective against the hemolysin of *M. salivarium.*

Reversal of the hemolysin inhibitory effect of catalase and peroxidase. In order to prove the specificity of the inhibitory effect of these enzymes, heated catalase and peroxidase were added to the medium. In addition, the specific inhibitor of catalase, 3-amino-1,2,-

TABLE I. Hemolytic Activity of Various Species of Mycoplasma.

species	Diam (mm) of hemolytic zones at indicated time (hr)[a]				Hemolysis after indicated material added to erythrocyte–agar overlay							
					Catalase (2000 U/ml)		Catalase (8000 U/ml)				Peroxida (1000 U/	
	24	48	72	96	Unheated	Unheated + 3-amino-1,2,4-triazole (3000 μg/ml)	Unheated	Unheated + 3-amino-1,2,4-triazole (μg/ml) 3000	12,000	Heated (100°/30 min)	Unheated	H (30
iae	1	1.5	1.5	L[b]	+	++	±	+	++[c]	++	0	
	0	0.5	1	1	0	++	0	+	++	++	0	
	0	<0.5	0.5	0.5	0	++	0	+	NT[d]	++	0	
	0	0.5	1	1	0	++	0	+	NT	++	0	
	0	0	<0.5	0.5	0	++	0	±	NT	++	0	
m	0	0.5	1	1	0	++	0	±	+	++	++	
ans	0	<0.5	<0.5	0.5	+	++	±	++	++	++	0	
	0	0	0	0	NT	NT	NT	NT	NT	NT	NT	
icum	1	1.5	1.5	1.5	+	++	±	++	++	++	±	
s	1	1.5	1.5	L	+	++	±	++	++	++	±	
icum	0.5	1	1	1	+	++	±	+	++	++	0	

6 expts.

ous lysis of the erythrocytes in agar overlay.

hemolysis and time of development the same as in absence of catalase.

d.

-960, T-354, and Threats-U.

4-triazole (AT), was also used to reverse the inhibitory activity of this enzyme.

Results of these experiments are summarized in Table I. Heat treatment (100°/30 min) of both enzymes resulted in the total loss of their inhibitory effect on hemolysin activity of the mycoplasmas tested. In addition the inhibitory effect of catalase was reversed by AT, a specific inhibitor of catalase which acts only in the presence of peroxide (3). Inhibition produced by 2000 U/ml of catalase was abolished when 3000 μg/ml of AT was incorporated into the red blood cell-agar overlay. However, except for *M. fermentans, M. gallisepticum,* and *M. pulmonis,* this concentration of AT was not sufficient to reverse completely the inhibitory effect of 8000 U/ml of catalase. A complete reversal of the effect of this concentration of catalase on *M. pneumoniae, M. hominis,* and *M. neurolyticum* was achieved when 12000 μg/ml of AT was added to the medium.

It is of interest that the hemolytic zones produced by mycoplasmas in the presence of AT were somewhat larger than zones produced in the absence of this compound. This is probably due to the inhibitory effect of AT on the catalase contained in the horse serum present in the growth medium.

Discussion. Hemolytic activity of *M. pneumoniae, M. laidlawii, M. gallisepticum,* and *M. neurolyticum* was previously shown to be due to peroxide secreted by these organisms (2, 4).

Results of this study have shown that other mycoplasmas which infect man also produce peroxide hemolysins since such hemolycins are inhibited by catalase or peroxidase. However, in comparison with the rapid complete lytic effect of *M. pneumoniae* these mycoplasmas produced smaller hemolytic zones; hemolysis was delayed and was often incomplete. Except for *M. fermentans* hemolysis could also be prevented by a lower concentration of catalase than was required to inhibit hemolysis by *M. pneumoniae.* These findings suggest that the mycoplasmas of man other than *M. pneumoniae* are less potent hemolysin producers, probably due to a lower rate of peroxide secretion.

Recently the rate of peroxide generation by *M. pneumoniae* was measured by a quantitative technique: 10^{10} cfu of this organism produced peroxide at the rate of 0.2–2 μmoles/hr (3). Previously it was also found that this quantity of peroxide generated by the hemolytic drug acetylphenylhydrazine could cause serious oxidative damage to exposed red blood cells (7, 8). On the basis of these findings it was suggested that peroxide secretion by *M. pneumoniae* contributes to the virulence and pathogenicity of this organism.

Since the mycoplasmas which are nonpathogenic for man also secrete peroxide, it would appear that peroxide per se is not a determinant of virulence. More likely, virulence is related to the quantity of peroxide secreted—thus, among the mycoplasmas which infect man, *M. pneumoniae,* which appears to secrete the largest amount of peroxide, is the most pathogenic. Other factors probably also play a role in virulence. The affinity of *M. pneumonia* for respiratory tract epithelium, unique among the mycoplasmas which infect man, may be such a property. This type of attachment provides an unusual opportunity for peroxide secreted by the organism to attack the tissue cell membrane without being rapidly destroyed by catalase or peroxidase present in extracellular body fluids (9).

Summary. The hemolytic activity of 8 human and 3 animal species of mycoplasmas was studied. With the exception of T-strains of human origin, each of the mycoplasmas tested hemolyzed guinea pig red blood cells.

Catalase and peroxidase inhibited the hemolytic activity of the mycoplasmas tested. Heat inactivation reversed the inhibitory effect of these enzymes. In the case of catalase, this effect was also abolished by its specific inhibitor 3-amino-1,2,4-triazole. Thus, hemolysins produced by these mycoplasmas seem to be peroxides. However, when compared to *M. pneumoniae* other mycoplasmas which infect man were found to be less potent producers of peroxide. The relevance of these findings to the virulence of mycoplasmas for man is discussed.

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Kinetics of the Development of Factors Responsible for Interferon-Induced Resistance (33363)

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In interferon (IF)-treated cells the development of antiviral resistance requires the integrity of cellular RNA and protein syntheses (1–4). This finding incidates that IF acts by inducing the cells to produce a peptide or a protein which is directly responsible for the antiviral state. Previous experiments from this laboratory support the view that the production of an antiviral substance (5) in IF-treated cells does not require synthesis of newly induced, intermediary proteins. Therefore, the most satisfactory working model of the induction by IF of the antiviral state would require at least the following sequential events: (a) derepression of the antiviral protein (AVP) cistron; (b) poduction of messenger RNA (mRNA) for AVP; and (c) translation of the mRNA into AVP.

Although this model identifies the main variables, it does not specify many of the kinetics of interaction during establishment of the antiviral state. The object of the present paper is to consider some interactions of the factors which are involved in development and maintenance of the antiviral state in IF-treated mouse embryo (ME) cells. For clarity of presentation the concept of mRNA and AVP will be used to help interpret the findings with the full realization that these concepts are indirectly derived and subject to future reinterpretation.

Materials and Methods. The IF was produced in the serum of NIH strain mice by intravenous injection of Newcastle disease virus (6). Primary ME cell cultures and continuous mouse L cell cultures were prepared as previously described (7). Antiviral resistance was measured by determining the inhibition of yield of vesicular stomatitis virus (VSV) in a single step growth cycle as employed previously (7). Actinomycin D (Merck Sharp and Dohme) was used at a final concentration of 1 μg/ml. Cycloheximide (Sigma Chemical Co.) was used at a final concentration of 10 μg/ml. Previous experiments showed that both drugs exerted 95% inhibitory activity on RNA synthesis and on protein synthesis, respectively, in these cell cultures (5, 7).

Results and Discussion. Estimation of time of production of mRNA for antiviral protein in interferon-treated mouse embryo cells. Figure 1 shows the curve of development of the antiviral state in ME cells treated with different doses of IF. The cells were treated with IF at time 0. At each indicated interval of time, one set of tubes was washed carefully to remove IF and challenged with VSV in

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