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Hemolysin of *Mycoplasma pneumoniae*

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ABSTRACT

SOMERSON, NORMAN L. (National Institute of Allergy and Infectious Diseases, Bethesda, Md.), ROBERT H. PURCELL, DAVID TAYLOR-ROBINSON, AND ROBERT M. CHANOCK. Hemolysin of *Mycoplasma pneumoniae*. J. Bacteriol. **89**:813-818. 1965.—Discrete colonies of *Mycoplasma pneumoniae* or *M. laidlawii* on agar produced complete (β) lysis of guinea pig erythrocytes. Crowding of colonies on agar plates, omission of yeast extract from the medium, and incubation under reduced oxygen tension inhibited hemolysin production. Colonies which were not viable after ultraviolet radiation or heating at 56 C did not produce hemolysin. In addition, hemolysis was suppressed in old cultures in which viability was diminished, and was eliminated when colonies were removed from the agar surface by micromanipulation. The hemolysin passed through a viscose dialysis membrane. These findings suggest that the hemolysin is labile, continuously released by the colonies, of low molecular weight, and probably nonprotein in nature.

Hemolysin production is a property exhibited by many mycoplasmas (Edward, 1950; Warren, 1942). Among the human species, only *Mycoplasma pneumoniae* produces rapid, complete (β) lysis of certain mammalian red cells; other human mycoplasmas produce incomplete (α) lysis within a similar time interval. This distinction has proved to be useful for the presumptive identification of *M. pneumoniae* (Somerson, Taylor-Robinson, and Chanock, 1963; Clyde, 1963).

In earlier studies, it was demonstrated that each colony of *M. pneumoniae* releases sufficient hemolysin to produce a distinct plaque, which suggests that hemolysin production is a stable genetic trait. We have speculated that the cold agglutinin response which occurs in primary atypical pneumonia may be related to the action of the β-hemolysin (Chanock et al., 1963). The present study is concerned with the nature and action of the hemolysin and with factors which influence its production in vitro.

MATERIALS AND METHODS

Organisms. The *M. pneumoniae* culture was the FH strain originally isolated by Liu (1957) and subcultured more than 20 times on artificial medium in this laboratory. For studies in which a potent inoculum was required, a higher passage level (170th to 200th) of the strain was used. In addition, the FH strain was adapted to grow on medium without yeast extract by multiple subcultures, and was then employed in several experiments. Although most of the experiments were performed with *M. pneumoniae*, the hemolysin produced by *M. laidlawii* B was studied for comparative purposes. *M. laidlawii* B was obtained from D. G. ff. Edward. Both *M. pneumoniae* and *M. laidlawii* were stored as broth-agar mixtures or broth suspensions at −70 C.

Media. The "standard" agar medium used to grow *M. pneumoniae* was described in detail previously (Taylor-Robinson et al., 1963). It contained 7 parts of PPLO agar (Difco), 2 parts of unheated horse serum, and 1 part of 25% fresh yeast extract. This was supplemented with penicillin, at a final concentration of 1,000 units per ml, and thallium acetate and amphotericin B, both at a final concentration of 2 mg/ml. In later experiments, amphotericin B was omitted, because it was found that this antibiotic is capable of inhibiting other mycoplasma species (Lampen et al., 1963; Chanock, *unpublished data*). Broth medium was similarly prepared except that PPLO broth (Difco) replaced PPLO agar.

In some experiments, yeast extract was omitted from the medium. For studies of nutritional factors, a yeast extract dialysate was substituted for yeast extract. This dialysate was prepared either by (i) dialysis for 48 hr against 10 volumes of water, with reduction to the original volume in a flash evaporator (Buchler Universal) at a temperature not exceeding 60 C, or by (ii) dialysis with a collodion membrane in a VirTis Ultrabac filter.

In experiments with *M. laidlawii*, the standard PPLO medium was used. In addition, the organism was grown in a partially synthetic medium described by Razin and Cohen (1963). In some cases Agar no. 3 (Oxo) was added to this medium.

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Agar plate and broth cultures were incubated aerobically or in anaerobic jars flushed three times with a mixture of 95% nitrogen and 5% carbon dioxide. The viability of mycoplasma colonies grown on agar was tested by push-block subculturing, or by ejecting the agar contents of a plate through a syringe into a measured amount of PPLO broth and plating serial decimal dilutions of this suspension. Colonies overlaid with a guinea pig red blood cell (RBC)-agar mixture were tested for viability in a similar manner.

Tests for hemolysin. The presence of hemolysin active against guinea pig RBC was demonstrated by overlaying mycoplasma colonies with an RBC-agar mixture (Somerson et al., 1963). Guinea pig RBC were washed three times in Alsever's solution, diluted, and added to Difco PPLO agar medium (55 to 60 C) to give a final concentration of 3% blood cells. About 3 ml of the mixture were poured as overlay onto agar plates (5 cm in diameter) which contained surface colonies of mycoplasmas. Plates were inspected daily for plaques resulting from hemolyzed cells.

Isolation of colonies. To obtain discrete mycoplasma colonies, broth suspensions were diluted in serial 10-fold steps, and 0.1 ml of each dilution was inoculated onto agar plates. At various times, plates were examined for colonies and overlaid with the RBC-agar mixture. Plates were usually overlaid 9 to 10 days after inoculation; however, the interval was varied in the nutritional experiments because the time of appearance of colonies varied.

Mycoplasma colonies were removed from the agar surfaces by micromanipulation with a modified Pasteur pipette attached to a suction apparatus. The manipulated area was observed under a dissection microscope to assure that the colonies had been completely removed. The agar removed from the area was only slightly larger in diameter than the colony itself.

Viability and hemolysin production. To determine the effect of ultraviolet radiation on hemolysin production, colonies on agar were placed 10 cm from a General Electric G30T8 lamp for 30 to 45 min.

To determine the effect of heat, plates containing *M. pneumoniae* colonies were sealed with cellophane tape and immersed in a water bath (56 C) for 45 min; another set of replicate plates was similarly incubated in a water bath at 37 C. The RBC-agar overlay was added to the plates immediately after treatment.

To demonstrate the effect of colony age and viability on hemolysin production, agar plates containing *M. pneumoniae* colonies were incubated under aerobic conditions for periods of 5 to 20 days. At 5-day intervals, one plate was overlaid with guinea pig RBC, and the agar from another was syringed into 10 ml of PPLO broth. The broth was diluted in serial 10-fold steps, and 0.1 ml of each dilution was inoculated onto agar plates to determine the number of colony-forming units.

Dialysis experiments. To determine whether the hemolysin was dialyzable, *M. pneumoniae* was grown on standard agar medium for 7 to 10 days. A viscose membrane (Visking brand of cellulose dialysis tubing), cut to the shape of the agar plate, was moistened in saline and placed over the colony-containing agar surface. Such plates were then overlaid with an RBC-agar mixture and incubated aerobically at 37 C. The possibility that *M. pneumoniae* altered the viscose membrane was investigated by testing for alterations in membrane permeability. A segment of the tubing was placed over the colonies and incubated for 24 hr at 37 C. A blood-agar overlay was then added, and the plates were incubated for an additional 48 hr. After plaques of hemolyzed cells had developed, the blood-agar layer was washed from the dialysis tubing. One end of the tubing was closed, bovine albumin was added, and the tubing was completely sealed. The bag so formed was washed in two changes of saline, and dialyzed for 48 hr against an equal quantity of saline. The protein concentration of the dialysate was determined by the method of Lowry et al. (1951).

RESULTS

Effect of colony density on hemolysin production. Discrete colonies of *M. pneumoniae* or *M. laidlawii* grown on agar produced complete (β) lysis of guinea pig RBC. In many experiments, plaques resulting from hemolyzed cells could be seen within 12 to 15 hr. The first change was a green zone, interpreted as incomplete or α-hemolysis. Subsequently, all red cells in the affected area lysed, and the plaque increased in size with time. Each plaque had a mycoplasma colony at its center, and the periphery of the plaque was free from detectable mycoplasma colonies. Hemolysis did not occur or was incomplete when the number of colonies exceeded 10^4 per plate. This type of inhibition occurred commonly on plates with a high colony density resulting from inoculation by the push-block technique. The phenomenon of inhibition is illustrated in the following experiment. A broth suspension of *M. pneumoniae* was diluted in serial 10-fold steps, and 0.1 ml of each dilution was plated onto standard PPLO agar medium. The plates were overlaid after 9 days and observed for hemolysis. No plaques were seen on plates inoculated with the undiluted suspension or the 10^{-1} dilution (Fig. 1). A few small, incomplete plaques developed at the 10^{-2} dilution. Numerous large, clear plaques developed on plates inoculated with the 10^{-3} and 10^{-4} dilutions.

Effect of nutrition on hemolysin production. The components of the growth medium also influenced the occurrence of hemolysis. Typical colonies of *M. laidlawii* developed on a semisynthetic agar medium, but the colonies failed to produce plaques of lysed erythrocytes. When the organ-

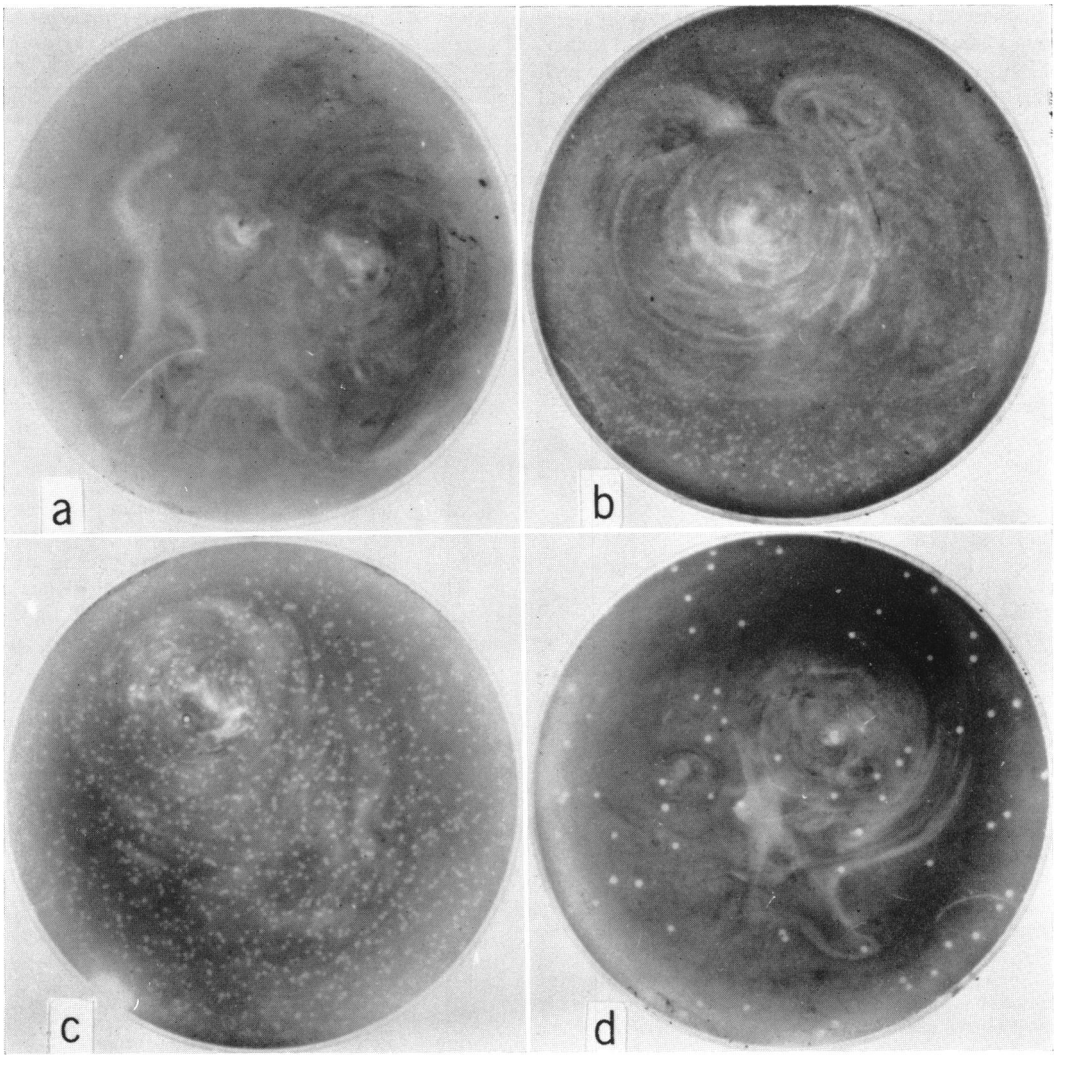

FIG. 1. *Effect of colony density on plaques resulting from lysis of guinea pig RBC by Mycoplasma pneumoniae. PPLO agar plates were inoculated with serial 10-fold dilutions of a broth suspension of M. pneumoniae. Note the absence of plaques at the 10^{-1} dilution (a) and the large discrete plaques at the 10^{-4} dilution (d).*

isms were grown on an enriched medium containing 20% horse serum and 2.5% yeast extract, a clear (β) plaque of lysed erythrocytes was produced by each colony.

Similar observations were made with a strain of *M. pneumoniae* which had been selected for its capacity to grow in the absence of yeast extract. Colonies of this strain, grown on a yeast extract-free PPLO agar medium, failed to produce β-hemolysis, whereas hemolysis developed when yeast extract or a dialysate of yeast extract was added to the medium. This finding suggests that the essential nutrients supplied by yeast extract are of low molecular weight. In some instances, it was possible to overcome the effect of a nutritionally deficient medium by adding yeast and horse serum to the RBC-agar overlay.

Effect of atmosphere on hemolysin production. The gaseous composition of the atmosphere, both before and after RBC overlay, also affected plaque formation (Fig. 2). *M. pneumoniae* could be grown equally well aerobically or under reduced oxygen tension, but production of hemolysin was suppressed under the latter condition. Plaques resulting from hemolyzed cells developed when cultures which were originally

incubated under reduced oxygen tension were overlaid and subsequently incubated aerobically. Less hemolysis was evident with colonies which were originally grown aerobically, overlaid, and then further incubated under reduced oxygen tension. These results suggest that an aerobic atmosphere favors the production or demonstration, or both, of hemolysin.

Correlation of viability with hemolysin production. Heating at 56 C for 45 min or ultraviolet radiation inactivated *M. pneumoniae*. Little or no hemolysis occurred when cultures were subjected to either procedure and then overlaid with an RBC-agar mixture (Table 1). In addition, the extent of hemolysis was directly related to the number of viable organisms present in aging cultures of *M. pneumoniae*. Young colonies on plates incubated for 5 days, and colonies on 20-day-old plates, produced less hemolysis than colonies on 10- or 15-day plates (Table 2). Finally, hemolysis could not be demonstrated on plates from which the colonies had been removed immediately prior to overlay with the RBC-agar mixture. These findings suggest that the hemolysin is quite labile and that the presence of actively growing organisms is required for its demonstration.

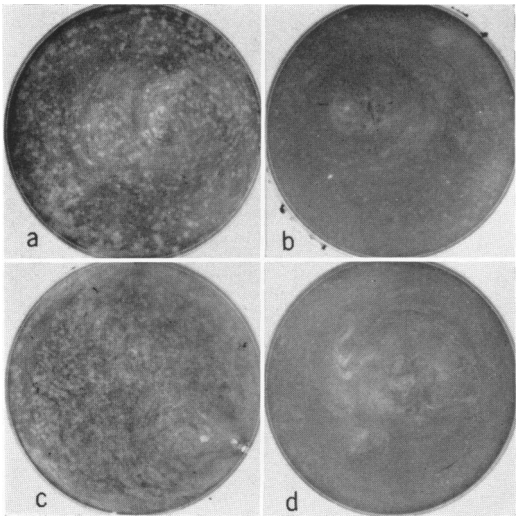

FIG. 2. *Effect of atmosphere on plaques resulting from lysis of guinea pig RBC by Mycoplasma pneumoniae. The incubation atmosphere for colony growth and before addition of the RBC overlay and the incubation atmosphere after overlay were as follows: (a) aerobic before and after overlay; (b) anaerobic before and after overlay; (c) anaerobic before and aerobic after overlay; (d) aerobic before and anaerobic after overlay.*

TABLE 1. *Effect of heat and ultraviolet radiation on viability and hemolysin production of Mycoplasma pneumoniae*

Treatment (45 min) of agar plates*	Size of plaques of lysed erythrocytes when colonies were overlaid with guinea pig RBC	CFU per 0.1 ml of agar suspension
56 C	0†	8
37 C	4	10^3
Ultraviolet radiation	1	0
Untreated	4	$10^{3.3}$

* Each plate was inoculated with 10^2 colony-forming units (CFU).

† Arbitrary grading of plaque size on 0 to 4 scale; 0 = no hemolysis.

TABLE 2. *Correlation of hemolysis produced by Mycoplasma pneumoniae with viability (reproductive capacity) of the organism*

Days of incubation*	Size of plaques of lysed erythrocytes after guinea pig RBC overlay	CFU per 0.1 ml of agar-broth suspension†
5	2–3‡	60
10	3–4	3,000
15	3–4	2,000
20	1	1

* Plates inoculated with approximately 10^3 colony-forming units (CFU) of *M. pneumoniae*.

† Obtained by syringing agar contents of one plate into 10 ml of standard PPLO broth.

‡ Arbitrary grading of plaque size on 1 to 4 scale; 0 = no hemolysis.

Size of hemolysin. The interposition of a viscose dialysis membrane between mycoplasma colonies and a blood-agar overlay did not prevent the appearance of plaques of hemolyzed cells (Fig. 3). Plaques which formed in the blood-agar mixture placed over the membrane were similar to those which formed without an interposed membrane. The permeability of the viscose sheet was not altered by *M. pneumoniae*, because the membrane did not permit passage of albumin into the dialysate. Thus, the hemolysin is dialyzable, and, therefore, presumably nonprotein and of low molecular weight.

DISCUSSION

Information about the synthesis and chemical nature of hemolysins produced by mycoplasmas is limited because (i) the assay procedure requires actively growing colonies on a solid agar medium, (ii) the hemolysin is quite labile, and (iii) hemolysin production requires a complex medium, the constituents of which are at this time poorly defined. In addition, quantitation of hemolysin

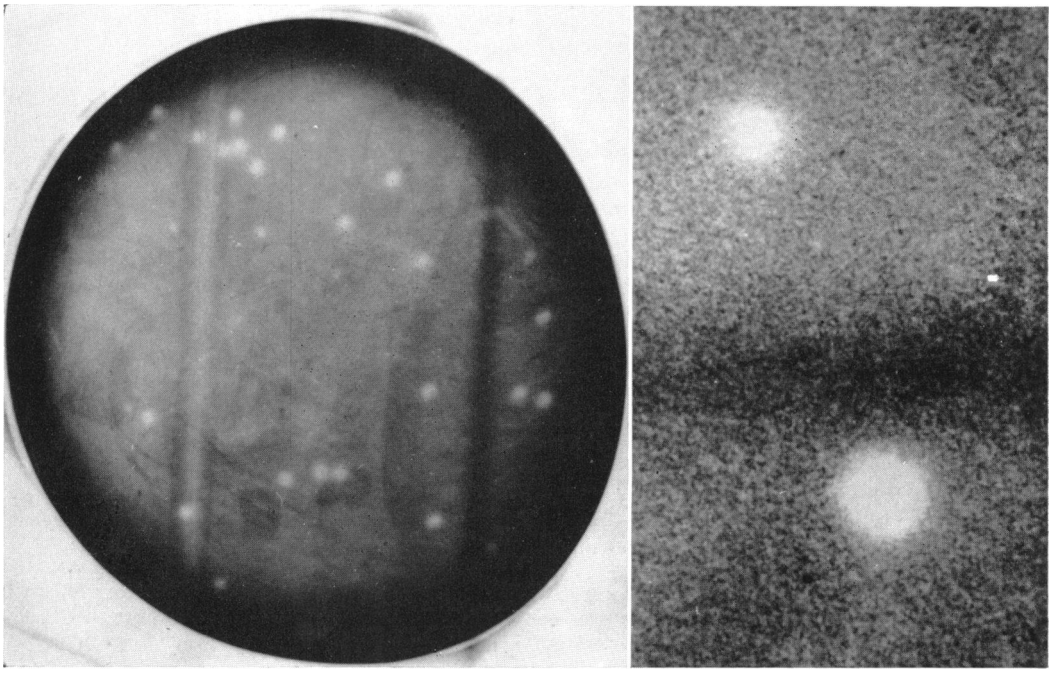

FIG. 3. *Plaques resulting from lysis of erythrocytes in a guinea pig RBC-agar overlay which was placed over a viscose dialysis membrane. The membrane was interposed between Mycoplasma pneumoniae colonies and the overlay. On the left, the entire membrane and overlay were removed from the agar surface before photography. On the right, fivefold magnification of plaques of hemolyzed cells.*

production is hampered by such unknown factors as rates of diffusion, adsorption, and destruction of hemolysin in agar. Hemolysin production in broth cultures cannot be demonstrated consistently, and oxygen tension does not appear to be the only factor concerned. Other investigations have shown that the production of α-hemolysin by staphylococci in broth is enhanced by the addition of a small amount of agar to the broth (Parish and Clark, 1932; Tasman and van der Slot, 1953). The role of agar in the elaboration of hemolysin by mycoplasmas may be related to nutrients in the agar or to the removal of inhibitory substances from the medium.

Hemolysin production appeared to be correlated with viability of the organism. Thus, ultraviolet radiation or exposure to a temperature of 56 C abolished hemolysis. Hemolysis was also suppressed in old cultures in which viability had diminished. In addition, no trace of hemolysin could be found in the surrounding agar medium after the colonies had been removed. These findings suggest that the hemolysin is extremely labile and is constantly being released by the mycoplasma colony.

The inhibition of hemolysin production during high-density growth of *M. pneumoniae* and *M. laidlawii* might be due to metabolic inhibition, but it is more likely to be caused by depletion of essential nutrients from the medium. The latter explanation is suggested by the failure of colonies to form hemolysin when yeast extract is omitted from the medium. If the nutrient supplements are limited, only partial or α-hemolysis occurs. The nutritional requirements do not appear to be protein in nature, because a dialysate prepared from yeast extract permits hemolysis to occur. Nutritional deficiency is not the only condition which inhibits hemolysin production; anaerobiosis has a similar suppressive effect. This oxygen requirement may be indicative of an enzyme-catalyzed oxidation of lipid and subsequent hemolytic activity. The lability of the hemolysin is consistent with the unstable nature of intermediates of lipid oxidation.

The hemolysins of *M. pneumoniae* and *M. laidlawii* differ from many bacterial hemolysins. The α-hemolysin of staphylococci and the β-hemolysin of *Listeria monocytogenes* appear to be protein in nature (Elek, 1959; Girard, Sbarra, and Bardawil, 1963; Jenkins, Njoku-Obi, and Adams, 1964). The α toxin of *Clostridium perfringens* is hemolytic and has been identified as lecithinase C (MacFarlane and Knight, 1941;

Zamecnik, Brewster, and Lipmann, 1947). The hemolysin of *Pseudomonas aeruginosa* is unusually stable to heat, acid, and alkali, characteristics which strongly suggest that this hemolysin is not an enzyme; the possibility that it is a protein has been advanced (Berk, 1962). The exact nature of the hemolysin of *M. pneumoniae* is also unknown. Our findings indicate that it is of low molecular weight, freely diffusible, and probably nonprotein in nature, although the possibility that it is a small protein or polypeptide cannot be eliminated.

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