GROWTH INHIBITORY PROPERTIES OF *MYCOPLASMA* ANTIBODY

JACK S. BAILEY, HAROLD W. CLARK, WILLIAM R. FELTS, AND THOMAS McP. BROWN

Arthritis Research Unit, Department of Medicine, The George Washington University, Washington, D.C

Received for publication 3 April 1963

ABSTRACT

BAILEY, JACK S. (The George Washington University, Washington, D.C.), HAROLD W. CLARK, WILLIAM R. FELTS, AND THOMAS McP. BROWN. Growth inhibitory properties of *Mycoplasma* antibody. J. Bacteriol. **86**:147–150. 1963.—A substance in antiserum responsible for growth inhibition of *Mycoplasma* was found to be associated with the water-insoluble globulin fraction. This fraction and the agglutinins removed from agglutinated antigen inhibited the growth of *Mycoplasma* in a similar manner. Sonic oscillation of antiserum broth cultures partially relieved the growth inhibition. The results of this study suggest the existence of neutralized *Mycoplasma* in a latent phase, which could explain their infrequent detection in tissues and fluids. The application of such techniques as sonic oscillation may improve the recovery and identification of *Mycoplasma* from these sources.

The growth of microorganisms of the genus *Mycoplasma*, pleuropneumonia-like organisms (PPLO), has been shown to be inhibited by specific antisera (Edward and Fitzgerald, 1953, 1954; Nicol and Edward, 1953). Although little is known of the inhibition processes, antibody is believed to be the responsible active agent.

The purpose of the present investigation was to determine some of the properties and specific activity of the growth-inhibiting agent or antibody. This study includes an attempt to dissociate the *Mycoplasma* antibody complexes and to recover viable organisms.

MATERIALS AND METHODS

Preparation of antigen and antisera. The organisms used in this study were the *M. hominis* type I strain from tissue cell culture, designated T-5, and a *M. hominis* type II strain, designated CH. The origin of these strains and the methods used to prepare antigen and rabbit antisera have been described previously by Bailey et al. (1961). Commercial pooled rabbit serum was used for the normal control in the agglutination and inhibition tests.

Agglutination and inhibition of growth tests. All agglutination and inhibition-of-growth tests were performed as reported previously by Bailey et al. (1961). Tests for growth inhibition, unless otherwise specified, were made in broth of digested beef heart muscle containing 20% (v/v) human ascitic fluid and 2% rabbit antiserum. A 1-ml amount of antiserum-broth mixtures was inoculated with 0.05 ml of a diluted 24-hr culture containing 100 to 1,500 colony-forming units, as determined by plate count. The agar medium used contained 1.2 g of agar per 100 ml of broth enriched with 20% (v/v) ascitic fluid.

Antibody purification method I. Partial purification of antibody was attained by precipitation of globulins from 5 ml of rabbit antiserum with 2.5 ml of saturated $(NH_4)_2SO_4$, with stirring. This fractionation procedure was repeated, and the precipitated globulin and the supernatant serum fractions were dialyzed against several changes of constantly stirred distilled water at 4 C for 18 hr. After centrifugation of the dialyzed globulins, the water-soluble globulin fraction (SF) was decanted and the insoluble portion (IF) dissolved in 0.85% NaCl. The protein (biuret) concentrations of IF, SF, and the albumin fraction (AF) were adjusted to 1 mg/ml and sterilized with a Swinney filter.

Antibody purification method II. Antibody was purified by adsorption and dissociation of specific antigen-antibody (Ag-Ab) precipitates with concentrated salt solution, according to Heidelberger and Kendall (1936). Samples (2 ml) of antiserum were mixed with 2-ml suspensions of antigen particles (standardized turbidimetrically) under sterile conditions and incubated for 1 hr at 56 C. The antigen-antiserum mixtures were centrifuged for 15 min at 3,000 × *g*. The supernatant fluid was removed and added to the sedimented particles obtained from another 2 ml

TABLE 1. *Growth inhibition of Mycoplasma hominis CH by antiserum fractions (method I)*

Antiserum fractions*	Dilutions in broth cultures†							Control serum (1:10)	Agglutination titer
	1:10	1:20	1:40	1:80	1:160	1:320	1:640		
Soluble (SF)	+	0	0	0	0	0	0	0	1:4
Insoluble (IF)	+	+	+	+	+	+	0	0	1:40
Supernatant fluid (AF)	0	0	0	0	0	0	0	0	0

* Initial protein concentration of fractions: 1.0 mg/ml.

† Symbols: + = inhibition (less than one-half of control colony count); 0 = no inhibiton (similar to control colony count).

TABLE 2. *Properties of Mycoplasma hominis CH antiserum and purified agglutinin (method II)*

Serum	Titer*	Dilution	Growth inhibition†	Protein concn
				mg/ml
Whole antiserum	1,280	1:50	+	70.5
Adsorbed antiserum	160	1:10	±	55.8
Purified agglutinin	128	1:2	+	2.7
Control	0	—	0	68.0

* Reciprocal agglutination titer.

† Symbols: + = inhibition; ± = partial inhibition; 0 = no inhibition.

of standardized antigen. The incubation and centrifugation procedures were repeated. The combined Ag-Ab precipitates were washed four times with cold 0.85% NaCl and suspended in 0.5 ml of 15% NaCl at 37 C for 1 hr to dissociate the antibody. The preparations were centrifuged as before, and the precipitates were extracted two additional times with 15% NaCl. The 1.5 ml of combined extracts were dialyzed for 24 hr in the cold against frequent changes of 0.85% NaCl and refrigerated.

Sonic oscillation. Volumes (1 ml) of broth cultures were transferred to sterile polypropylene centrifuge tubes (13 × 100 mm; Ivan Sorvall, Inc., Norwalk, Conn.). Each tube, held at the top by a rubber stopper, was immersed in the water-filled oscillator cup of a 9-kc Raytheon sonic oscillator for 30 sec. Samples of the cultures were removed before and after sonic oscillation and serial dilutions plated (in duplicate). Colony counts were made after 48 hr of incubation at 37 C; results are expressed as the number of colonies per ml of culture.

RESULTS

Antibody purification method I was used to obtain the IF and SF globulins, and the AF from CH antiserum. Growth of a dilute *Mycoplasma* inoculum was inhibited by 3 μg of the IF globulin at the 1:320 dilution, whereas 100 μg of the SF globulin inhibited growth at the 1:10 dilution (Table 1). The AF did not affect the growth nor agglutinate *Mycoplasma*. Standard suspensions of CH antigen (37 μg of nitrogen per ml) were visibly agglutinated by 25 μg of the active IF globulin at a 1:40 dilution, whereas 250 μg of the less active SF globulin were required for agglutination. It is evident that IF globulin contains the growth-inhibitory activity of the antiserum.

The ability of the IF fraction to agglutinate as well as to inhibit growth at correspondingly higher titers than the SF fraction suggests that the globulins responsible for growth inhibition and agglutination are similar.

Antibody purification method II was used to obtain agglutinins to the CH strain for investigation of their growth-inhibitory activity. As shown in Table 2, the whole antiserum containing 70.5 mg of protein per ml completely inhibited growth when diluted 1:50. The CH-adsorbed antiserum contained 55.8 mg of protein per ml and partially inhibited growth when diluted 1:10. The purified antibody preparation had a protein concentration of 2.7 mg per ml and an agglutination titer of 1:128. Growth of *M. hominis* CH was completely inhibited in 48 hr by the purified antibody when diluted 1:2 in broth culture. These results further indicate that the globulins responsible for growth inhibition and agglutination are similar and perhaps identical.

Recovery of viable Mycoplasma by sonic oscillation. The Ag-Ab aggregates formed in the *Mycoplasma* agglutination tests are noticeably broken up by vigorous agitation. Studies were made to determine whether sonic oscillation could restore the growth of the agglutinin-inhibited *Mycoplasma*. The growth of *Mycoplasma* (CH, T-5) in broth containing homologous antiserum was largely inhibited in 24 hr, with no apparent growth after 144 hr (Table 3). Sonic treatment of antiserum broth cultures of *Mycoplasma* usually resulted in significant increases in colony counts.

Exposure of the 144-hr cultures to sonic oscillation released colony-forming *Mycoplasma*, as evidenced by the colony growth obtained upon subsequent plating.

Discussion

The antiserum fraction responsible for inhibition of *Mycoplasma* growth is apparently a nondialyzable, water-insoluble globulin. Since this antibody fraction can be dissociated from prepared Ag-Ab complexes and remain active in agglutination as well as inhibition tests, the responsible globulins in both instances may be the same.

The reasons for partial and reversible growth inhibition in the presence of excess antibody are not apparent. Contact of *Mycoplasma* with excess homologous antibody appears to result in the establishment of an equilibrium, rather than complete and immediate neutralization of all microorganisms. This observation resembles "The Percentage Law" described by Andrews and Elford (1933) that a given dilution of antiserum neutralized an approximately constant percentage of phage over a wide range of concentration in a given time.

Recently Clark et al. (1963) demonstrated that fluorescent antibody remained fixed to the *Mycoplasma* membrane residue after exposure to sonic oscillation. Our additional observation that viable *Mycoplasma* can be recovered from antibody complex by sonic oscillation appears to exclude lysis as the primary cause of growth inhibition. Therefore, it seems logical to conclude that antibody inhibition of growth is due in some way to the reaction with surface antigens.

Anderson and Doermann (1952) were able to recover 2% of the plaque-forming activity of neutralized T_3 bacteriophage using sonic oscillation. Our recovery of viable *Mycoplasma* from apparently negative antiserum broth cultures after exposure to sonic oscillation suggests a latent phase of the organisms in a neutralized form. Clark et al. (1963) also used sonic oscillation to release viable *Mycoplasma* "bound" in tissue cell cultures. In view of these findings, further investigation of the application of sonic oscillation techniques to body fluids and tissues suspected of containing neutralized *Mycoplasma* appears to be indicated.

Measurement of *Mycoplasma* growth by turbidimetric methods is unreliable and limited. The decreased turbidity resulting from exposure of *Mycoplasma* to surface-active agents and sonic oscillation has been interpreted as lysis by Smith and Sasaki (1958). We have found that decreased turbidity can be caused by fragmentation of the larger aggregates. This was shown by the resultant smaller colony-forming units producing more numerous, smaller, and uniform-sized *Mycoplasma* colonies. Quantitative interpretation of colony counts from broth cultures must be made with caution when the effects of several physical and chemical variables are involved and difficult to control. *Mycoplasma* colony-forming units are known to grow in aggregates of different sizes and shapes, depending upon the media properties. Our observations suggest that fragmentation of neutralized chains or aggregates of *Mycoplasma* with the release of reproductive end groups, the dissociation of antibody, or both are possible mechanisms of inhibition reversal.

TABLE 3. *Effect of sonic oscillation on colony-forming units (CFU) of Mycoplasma in cultures incubated with homologous antiserum and control serum*

Incubation time	CH culture				T-5 culture			
	Antiserum		Control serum		Antiserum		Control serum	
	CFU × 10/ml*		CFU × 10^6/ml		CFU × 10/ml		CFU × 10^6/ml	
	Before†	After†	Before	After	Before	After	Before	After
hr								
24	70	160	6.7	6.6	46	90	10	9
48	6	80	36	22	2	2	400	200
96	16	700	40	18	0	16	150	1.9
144	0	36	30	3.8	0	2	5	0.6

* Average number of CFU per ml.

† Before and after sonic oscillation of culture for 30 sec.

Acknowledgments

This investigation was supported in part by the Arthritis Research Unit, Veterans Administration Hospital, Washington, D.C. Further support was provided by research grant AI 04071 from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service.

LITERATURE CITED

ANDERSON, T. F., AND A. H. DOERMANN. 1952. Sonic reactivation of antiserum-neutralized bacteriophage T3. J. Bacteriol. **63**:291–292.

ANDREWS, C. H., AND W. J. ELFORD. 1933. Observation on anti-phage sera. I. "The percentage law." Brit. J. Exptl. Pathol. **14**:367–383.

BAILEY, J. S., H. W. CLARK, W. R. FELTS, R. C. FOWLER, AND T. MCP. BROWN. 1961. Antigenic properties of pleuropneumonia-like organisms from tissue cell cultures and the human genital area. J. Bacteriol. **82**:542–547.

CLARK, H. W., J. S. BAILEY, R. C. FOWLER, AND T. MCP. BROWN. 1963. Identification of *Mycoplasmataceae* by the fluorescent antibody method. J. Bacteriol. **85**:111–118.

EDWARD, D. G. ff., AND W. A. FITZGERALD. 1953. Inhibition of growth of organisms of the pleuropneumonia group by antibody. J. Gen. Microbiol. **8**:iv.

EDWARD, D. G. ff., AND W. A. FITZGERALD. 1954. Inhibition of growth of pleuropneumonia-like organisms by antibody. J. Pathol. Bacterol. **68**:23–30.

HEIDELBERGER, M., AND F. E. KENDALL. 1936. Quantitative studies on antibody purification. I. The dissociation of precipitates formed by pneumococcus specific polysaccharides and homologous antibodies. J. Exptl. Med. **64**:161–172.

NICOL, E. S., AND D. G. ff. EDWARD. 1953. Role of organisms of the pleuropneumonia group in human genital infections. Brit. J. Venereal Diseases **29**:141–150.

SMITH, P. F., AND S. SASAKI. 1958. Stability of pleuropneumonialike organisms to some physical factors. Appl. Microbiol. **6**:184–189.