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Mycoplasma-Latex Agglutination Reaction

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ABSTRACT

MORTON, HARRY E. (University of Pennsylvania, Philadelphia). *Mycoplasma*-latex agglutination reaction. J. Bacteriol. **92**:1196–1205. 1966.—The building up of *Mycoplasma* cell mass through adsorption to carrier particles as a method for enhancing the agglutination reaction to identify *Mycoplasma* is described. *Mycoplasma* cells of human, avian, swine, goat, sewage, and tissue-culture origin were adsorbed to latex particles (0.81 μ) and then were agglutinated by immune sera. The adsorption was demonstrated by electron microscopy. Either the cells or their antibodies, depending on which came into contact with the latex particles first, were adsorbed. The test, completed in less than 2 hr, consisted of serially diluting immune sera with buffered saline, adding the antigen, incubating in a water bath, centrifuging, and reading the reaction under 50× microscope magnification. The antigen in each reaction tube, representing the growth from about 1.6 ml of culture, was estimated to contain 23 μg of protein (approximately one-tenth the amount of *Mycoplasma* cells needed for a direct agglutination reaction). In the sera from rabbits undergoing immunization with *Mycoplasma* antigens, the presence of anti-*Mycoplasma* antibodies was detected much sooner in the *Mycoplasma*-latex agglutination reaction test than in the agar-gel diffusion reaction and the growth inhibition tests. Four different lots of latex particles showed excellent uniformity of behavior and stability during storage and testing.

Mycoplasmas (pleuropneumonia-like organisms, PPLO) are recognized generally by their characteristic colony morphology, nutritional requirements, and lack of a typical bacterial form. However, definitive identification of species or strains usually has to be made serologically. This is true especially with most strains isolated from man. The detection of anti-*Mycoplasma* antibodies is essential in determining the role *Mycoplasma* plays in diseases of man.

The serological methods used for detecting specific antibodies are limited, and some may or may not be applicable to mycoplasmas because of the unusual nature of these organisms. Until more is known about the mycoplasmas, particularly their chemical nature and their way of producing disease in man, the mycoplasma surface antigens and their specific antibodies will require serious consideration. These antigens are important factors in the invasion of the body by microorganisms, and their antibodies are usually concerned in the host's immune mechanisms for eliminating the invader.

Although it is possible to perform hemagglutination and hemagglutination-inhibition tests with intact cells of *Mycoplasma* isolated from avian sources, the strains isolated from man do not attach to either untreated or tanned erythrocytes. Moreover, the erythrocytes are selective in their adsorption; untreated erythrocytes adsorb carbohydrates, and tanned erythrocytes adsorb proteins. Sonic extracts of mycoplasmas isolated from man sensitize tanned erythrocytes (6) and react in the indirect hemagglutination test, but a great variation in titer of the same serum, depending upon the source of the sheep erythrocytes, has been reported (32). However, the reactions, reported to be more specific than those of the complement-fixation test (31), indicate that the adsorption of mycoplasma antigens to carrier particles may possess advantages for detecting anti-mycoplasma antibodies.

The precipitation test, as carried out by the agar-gel diffusion technique, is not practical, because it is difficult to put the entire mycoplasma cells into solution, and because the reaction is less sensitive than that of other serological procedures.

Inhibition of the growth of mycoplasmas by their specific immune sera is a useful key in

identifying strains, but it has certain accompanying disadvantages. An actively growing culture is needed. Inhibition of growth is much more difficult with eugonic strains than with dysgonic strains. Inhibiting immune sera are difficult to produce. The reactions are sometimes poorly defined; for instance, a well-defined zone of inhibition may appear by gross inspection, whereas PPLO colonies are observed microscopically in the area of even the highest concentration of immune serum. Immune sera may have high titers, when tested by the agglutination reaction, and at the same time fail to inhibit growth. Conversely, in the test described, differences in agglutination were detected between strains which are inhibited by the same immune sera.

The complement-fixation reaction has been used for detecting anti-*Mycoplasma* antibodies and identifying strains. However, investigators have pointed out the difficulties of the test in regard to anticomplementary action of either the antigens or antisera, or both, and to the frequency of cross-reactions. Furthermore, the cumbersome procedure leaves room for a better method for detecting antibodies and antigens.

Agglutination is the most sensitive in vitro method for detecting the reaction between particulate antigens and their antibodies. The smallness of the *Mycoplasma* cells, however, requires large numbers of cells to form visible aggregates or aggregates which will settle readily by gravity. Adsorbing *Mycoplasma* cells to carrier particles offers a means of increasing the mass of the cells and, at the same time, of maintaining the specificity of the *Mycoplasma* surface antigens. A variety of substances are available to serve as carrier particles.

A procedure for adsorbing *Mycoplasma* cells to latex particles to enhance their mass is described in the present work. The *Mycoplasma*-latex suspensions are employed in an agglutination reaction to detect anti-*Mycoplasma* antibodies by a practical procedure which can be completed in a relatively short time. Anti-*Mycoplasma* antibodies can also be adsorbed to latex particles and used for detecting specific antigens.

MATERIALS AND METHODS

Carrier particles. Latex (Difco), 0.81 μ, was diluted 1:5 in deionized water. This stock dilution of latex particles (LP) was stored at 4 to 10 C and was stable for several weeks under these conditions.

Buffer solution. Glycine-buffered saline (GBS), *p*H 8.2, was prepared by dissolving 7.505 g of glycine and 5.85 g of NaCl in sufficient deionized water to make 1 liter. The reaction was adjusted to *p*H 8.2 by the addition of 1 N NaOH. Usually, about 2.5 ml of the alkali were required to adjust 1 liter of solution.

A stock solution of bovine serum albumin, fraction V (Nutritional Biochemicals Corp., Cleveland, Ohio) was prepared by adding 1 g of the dried bovine serum albumin (BSA) to 5 ml of deionized water and storing at 4 to 10 C. Prior to setting up the PPLO-latex agglutination tests, 1 ml of the 1:5 stock solution of BSA was added to each 100 ml of GBS. Normal rabbit serum in a final dilution of 1:1,000 in the GBS had an action similar to that of 1:500 BSA in stabilizing the suspensions of some strains of PPLO which agglutinated spontaneously in 0.85% NaCl or in the GBS.

Antibody source. Rabbit immune sera were usually used for the source of antibodies. The rabbits were immunized by the intravenous injection of saline suspensions of PPLO cells, by intramuscular or intramuscular and intradermal injections of PPLO cells or fractions of cells mixed with sodium alginate-calcium gluconate, Freund's incomplete, or Freund's complete adjuvant. (Detailed methods of immunization will be published elsewhere.) The rabbits were bled at intervals; the sera were inactivated once at 56 C for 20 min and were stored at −10 C or in the freeze-dried state. Preservatives were not added to the sera or suspensions of cells. For normal rabbit sera, either the sera obtained from some of the rabbits before immunization or pooled sterile normal rabbit serum (Difco), as employed in tissue culture work, was used. In some instances, sera from hospital patients were used in the PPLO-latex agglutination test.

Culture strains. Representative strains of PPLO employed were obtained from the following people. *M. hominis* type 1 PG-21 was received from D. G. ff. Edward, Beckenham, Kent, England, and PG-25, from J. S. Bailey, George Washington University Hospital, Washington, D.C. *M. hominis* type 2 PG-27 (designated as Campo-W), acquired originally from Louis Dienes, was sent to D. G. ff. Edward who designated it as PG-27. It was given to E. Klieneberger-Nobel, who gave it to Ruth G. Wittler, Walter Reed Army Institute of Research, Washington, D.C. She brought it back to the United States and deposited it in the American Type Culture Collection. It was sent to our laboratory as culture No. 14152. Type 2 strain Campo-SS, given to Shogo Sasaki, Keio University, Tokyo, Japan, was received from him. Type 2 strain Campo-A (a cloned strain) was also used. Strains 07-S and 39-S were acquired from Paul F. Smith, University of South Dakota, Vermillion; CH, from Jack S. Bailey. Jack S. Bailey and Paul F. Smith both supplied tissue culture strain T-5; tissue culture strain C-3 was also used. *M. arthritidis* strain PG-6 was received from D. G. ff. Edward. Laidlaw A and Laidlaw B were used. Avian strains A-5969 were sent by Harold Morowitz, Yale University, New Haven, Conn. Avian strain T came from Jack S Bailey. *M. pneumoniae* and strains isolated from swine (SW-1), goats (KHS), and patients (09, 029, and 0143) were also used.

Techniques. The cultures of PPLO were grown in broth, usually with shaking, at 37 C. The cells were harvested, after approximately 48 or 72 hr, by running

the cultures through a Servall SS-3 centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) with a continuous-flow attachment at a flow rate of about 4 liters per hr and at a speed of 15,000 rev/min, estimated as ca. 27,000 $\times$ *g*. The cultures were usually transferred every 2nd day to fresh broth, with inocula of about 10%; the final quantity was approximately 4 liters of broth contained in a 6-liter flask. After the third transfer, most cultures were growing vigorously. By centrifuging the most active, such as the saprophytic strains, after 24 or 48 hr, and the slower growing parasitic strains after 48 or 72 hr, it was possible to harvest cells of the strains studied at about the height of the logarithmic phase of the growth curve or at the beginning of the decline phase.

For the strains studied, the culture media described by Morton, Smith, and Leberman (19), with slight modifications, were employed (18). All of the parasitic strains of PPLO required the addition of an enrichment to the culture media; however, the saprophytic strains showed better growth in the presence of some enrichment. Therefore, 1% of PPLO Serum Fraction (Difco; 29) was usually added to both agar and broth media. When the organisms were used to immunize rabbits, 10% normal rabbit serum was used instead of the PPLO Serum Fraction. Since the broth was free from sediment, it was not necessary to wash the centrifuged cells.

The centrifuged PPLO cells, when suspended in 0.85% NaCl so that 1 ml of suspension contained the growth from 500 to 1,000 ml of culture, usually assayed 3 to 9 mg of N per ml. The cells were satisfactorily preserved by storage at -10 C, since little antigenic material appeared to be released into the saline by the occasional thawing and refreezing of the suspensions.

For use in the latex agglutination test, the PPLO suspensions were diluted with 0.85% NaCl to a turbidity giving a reading of 200 in the Klett-Summerson colorimeter with a 420-mμ filter. These suspensions contained ca. 75 μg of N per ml as estimated by the micro-Kjeldahl method. Micro-Kjeldahl determinations on six different suspensions of five strains of *Mycoplasma* with Klett readings of 195 to 204 gave the following values of milligrams of N per milliliter: Laidlaw A, 0.070; Campo W, 0.0725, 0.0750; PG-21, 0.085; and patient's strains 09 and 029 gave 0.065 and 0.0825, respectively. It was helpful to subject the suspensions to high-frequency sound waves with the Bronson Sonifier, model S-75 (Heat Systems Co., Great Neck, Long Island, N.Y.), for 10 sec or more to insure a uniform suspension, because some suspensions, being granular after the freezing, were unsuitable for agglutination work without redispersing the cells.

The amount of antigen-latex suspension necessary for performing the PPLO-latex agglutination test was prepared in the following manner. To 0.4 ml of 1:5 latex suspension were added 1 ml of PPLO suspension or other antigen preparation and, after 10 min at room temperature, 8.6 ml of GBS (*p*H 8.2) containing 1:500 BSA. After another 10 min at room temperature, the antigen preparation was ready to use; 0.5 ml was added to each of several tubes (13 $\times$ 100 mm) containing 0.5-ml amounts of serial twofold dilutions of serum in the GBS containing 1:500 BSA. Suitable controls were included. The contents of the tubes were thoroughly mixed by shaking. The tests were incubated at 42 C in a water bath and were centrifuged in an International no. 2 centrifuge for 10 min at 2,000 rev/min, estimated as 950 $\times$ *g*. A no. 945 head, capable of holding up to 100 tubes, was used for centrifuging.

Electron microscopy. Three materials were examined with an electron microscope. The first was from the control tube in a PPLO-latex agglutination test, consisting of the LP sensitized with PPLO cells and diluted with the GBS (*p*H 8.2), containing BSA 1:500. The second was from a tube in the PPLO-latex agglutination test showing a +2 reaction. Reactions stronger than +2 gave aggregates too large to be examined in the electron microscope. The material consisted of the LP sensitized with PPLO cells and specific rabbit immune serum diluted with the GBS and BSA to a final dilution of 1:160. The third was LP in the anti-PPLO immune serum which had been diluted with the GBS and BSA diluent to a final concentration of 1:160.

The materials to be examined were mixed in an equal volume of 2% phosphotungstic acid, adjusted to *p*H 7.0 with 0.1 N KOH. A drop of the mixture was then air-dried on carbon-stabilized pyroxylin films attached to 3.01-mm copper grids and was examined in the Siemens Elmiskop I electron microscope, with double condenser illumination.

RESULTS

The concentration of LP has to permit easy readings of the reactions under the conditions of the test. The concentration finally selected was 1:250 in the reaction tubes, the same as employed by Florman and Scoma (10), and nearly that employed by other investigators (7, 9, 13, 16, 17) in procedures with other antigens.

The concentration of PPLO cells which required the minimal amount of antigen while giving the maximal sensitivity and an easily read reaction was selected. Suspensions of PPLO cells giving a reading of 200 in the Klett-Summerson colorimeter best satisfied these conditions (Table 1). Variations in the concentration of the suspensions of PPLO cells did not appreciably alter the end point of the reaction; the intensity of the reactions was more likely to be altered.

Only 0.032 ml of concentrated suspension of PPLO cells (Campo-W strain) was needed to prepare a 9-ml suspension with the Klett reading of 200, used for the test cited in Table 1. Thus, cells from about 1.6 ml of culture were contained in the antigen added to each reaction tube. Since a suspension of PPLO cells giving a Klett reading of 200 has been estimated to contain about 468 μg of protein per ml, approximately 23 μg of protein was contained in the

TABLE 1. *Effects of varied concentrations of Mycoplasma hominis (Campo-W strain) cells on intensity of latex agglutination reaction*

Klett reading of suspension	Time of reading[a]	Antigen[b] control	Reciprocal of final dilution of serum									
			20	40	80	160	320	640	1,280	2,560	5,120	10,240
300	Initial	—[c]	3	3	4	4	3	1	1	1	—	—
	Final	—	4	3	4	4	3	1	1	—	—	—
200	Initial	—	3	3	4	4	4	3	1	1	—	—
	Final	—	3	3	3	4	4	3	1	1	1	—
100	Initial	—	3	3	3	3	3	3	2	—	—	—
	Final	1	3	2	2	3	3	3	3	1	1	1

[a] Final readings were made after holding the tests at 4 C overnight and recentrifuging.

[b] Antigen control: antigen-latex suspension in buffer diluent. At the Klett reading of 100, a serum control (1:20 dilution of serum and latex, no antigen) was also done with negative results.

[c] The numbers 4, 3, 2, 1 represent ++++, +++, ++, and + reactions, respectively; — represents no reaction.

reaction mixture in each tube. Suspensions with a Klett reading of 200 were about the lightest that could be employed in direct agglutination tests. Therefore, only one-tenth as much PPLO suspension was needed for a latex-agglutination test as for a direct agglutination test.

Since washing the PPLO-latex suspension with GBS did not increase the sensitivity of the reaction, it was not used as a routine procedure.

Incubation of latex preparations described by several authors varied between 1.5 hr at 56 C and 18 hr at 37 C. Little difference appeared in the results when the latex and PPLO cells were incubated for 1 hr at 37 and 42 C. The incubation temperature of 42 C for 1 hr was arbitrarily selected because the reactions appeared to be a little stronger than at the lower temperature. In the technique employed, the duration of incubation perhaps could be shortened. A 30-min incubation has given satisfactory results.

Centrifuging the test preparations was essential in determining whether aggregation of the particles had taken place after the reagents had had an opportunity to react. A positive reaction, remaining undetected without this step, could be seen easily after centrifugation at 2,000 rev/min for 10 min. Tubes in which a strong reaction had taken place showed the LP as a small button on the bottom of the tube, with the supernatant fluid clear. In tubes where no reaction had taken place, the LP appeared as a thin layer over the curved bottom of the tube, somewhat resembling a positive reaction in hemagglutination tests, and the supernatant fluid was not clear. Titers could be determined by gross inspection, but they were usually at least two dilutions higher when the contents of the tubes were examined microscopically.

The nature of the reaction in each tube was determined by gently resuspending the sediment: the tube was tapped or swirled lightly, and then was examined under the microscope at a magnification of 50×. The tubes were placed as nearly horizontal as possible on the microscope stage and were viewed with a 10× eyepiece and a 5× objective. Massive clumps in a clear supernatant fluid were interpreted as a maximal reaction and were recorded as 4 or 4+. A barely detectable positive reaction, recorded as 1, showed particles definitely larger than in the control tube. Clumps intermediate in size were recorded as 3 or 2 (Fig. 1).

Before being centrifuged, the contents of some of the tubes were drawn up into capillary tubes and were allowed to stand, as described by Philp et al. (23), but positive reactions could not be detected.

Since a method for preparing a satisfactory antigen by sensitizing LP with PPLO cells was found, it was determined how long such antigen would be satisfactory if stored at temperatures of 4 to 10 C. A large quantity of antigen was prepared, stored in a refrigerator, and tested at intervals with the same lot of immune serum (Table 2). Antigen stored up to 70 days gave essentially the same end point with the same serum; the variations in results at the different test periods were within the limits of experimental error and illustrated the reproducibility of the test. However, after storage for 127 days, the antigen agglutinated spontaneously in the control tube and was unsatisfactory.

Since much less PPLO antigen was employed in the latex reaction in comparison with that employed in the direct agglutination reaction, even though the smaller amount of specific antigen was carried on inert particles of much larger size, it was important to compare the

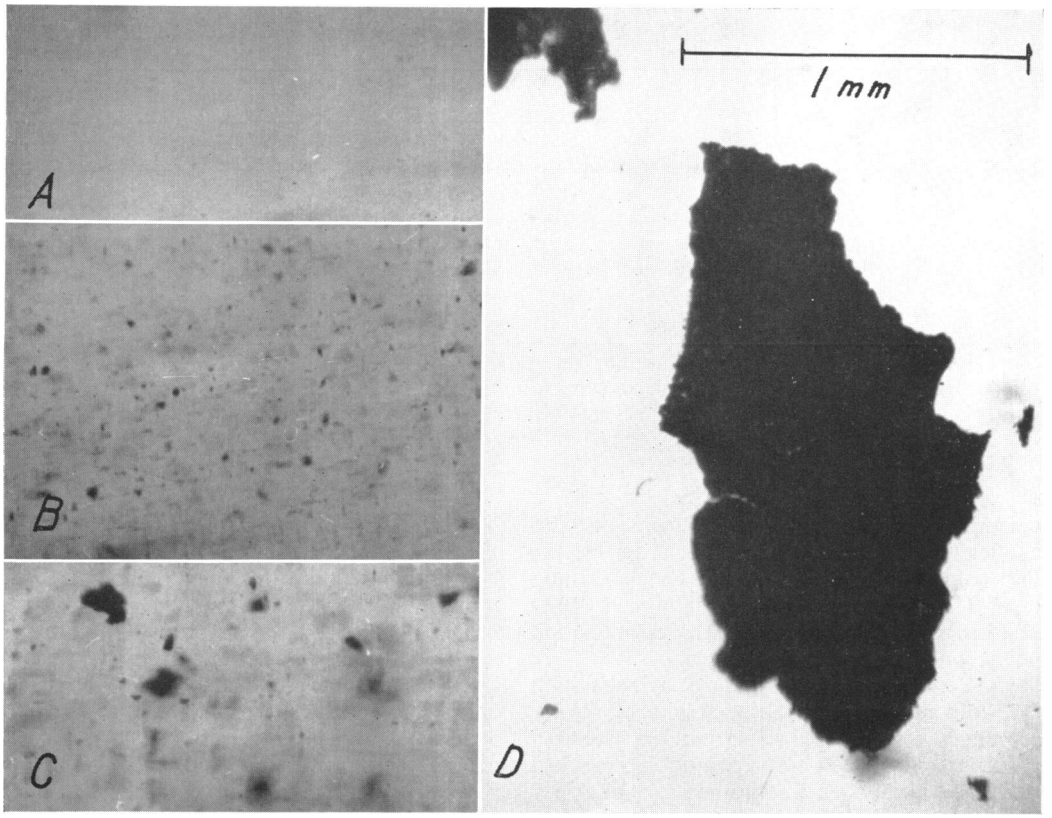

FIG. 1. *Reactions of PPLO-adsorbed latex particles in presence of immune serum. Photomicrographs of resuspended sediments after centrifugation. (A) Appearance of a negative reaction; (B) and (C) ++ and +++ reactions, respectively. Many particles not agglutinated. (D) A ++++ reaction, all particles aggregated into large masses and suspending fluid clear.*

sensitivities of the two types of tests. It was necessary to centrifuge the tubes in the direct agglutination test, as was done in the latex agglutination test, to make accurate readings. This may be due to the small size of the PPLO cells. In general, the sensitivity of the latex agglutination test was comparable to that of the direct agglutination reaction, as noted by the reactions listed in lines 1 to 10, Table 3. The latex reaction seemed more sensitive in detecting cross-reactions, as indicated in lines 11 and 12, Table 3. In other instances, cross-reactions of some *M. hominis* type 2 strains were shown to be specific antigen-antibody reactions. After immune serum to strain C-3, a type 2 strain, was adsorbed with Campo-W cells, the activity of the serum was eliminated for strains C-3, Campo-W, and 39-S, and practically eliminated for strains 07-S and CH.

In addition to the anti-Campo-W serum agglutinating its homologous strain, Campo-SS, and other representative strains of *M. hominis* type 2, such as 07-S, 39-S, C-3, and CH, it also agglutinated *M. arthritidis*. The similarity of *M. arthritidis* and *M. hominis* type 2 strains has been mentioned recently by various investigators. The specificity of the reactions was verified by growth inhibition tests.

The same type of supporting evidence for specificity was obtained with strains of *M. hominis* type 1. Anti-PG-25 serum agglutinated patients' strains no. 029 and 0,143 to partial titer and inhibited their growth. After portions of the immune serum were adsorbed with strains 029 and 0,143, the adsorbed sera no longer agglutinated or inhibited the growth of the strains used for the adsorption.

A commercially available suspension of the Mac strain of *M. pneumoniae* was adsorbed to LP and was agglutinated by a diagnostic serum to a dilution comparable to the stated complement-fixation titer of the serum.

The tissue culture strain of *Mycoplasma*, T-5, was agglutinated, and its growth was inhibited

by only its own immune serum. Thus, it represented a serological type distinct from the strains isolated from human sources.

The *Mycoplasma*-latex reaction differentiated between Laidlaw A and B strains, whereas the growth inhibition test did not. The growth of Laidlaw A and B strains was inhibited by the homologous and heterologous sera; whereas both strains were agglutinated to equal titer by anti-Laidlaw A serum, anti-Laidlaw B serum agglutinated Laidlaw A weakly and to a small fraction of the titer of the serum.

The cross-reactions between serological types suggested some common minor antigens. For example, an anti-Campo-W serum (titer 1:5,120) agglutinated weakly PG-25 at 1:80, Laidlaw A weakly at 1:40, and did not agglutinate Laidlaw B. An anti-PG-25 serum (titer 1:1,280) agglutinated Campo-W weakly at 1:80 and did not agglutinate the Laidlaw strains. An anti-Laidlaw A serum, which agglutinated Laidlaw A and B strains at dilutions of 1:5,120, agglutinated Campo-W at 1:320 and PG-25 weakly at 1:80. On the other hand, an anti-Laidlaw B serum (titer > 1:10,240) agglutinated Laidlaw A at 1:1,280 and weakly agglutinated Campo-W and PG-25 at dilutions of 1:80 and 1:160, respectively.

Strains of PPLO originally isolated from poultry (A-5969 and T) and goats (KHS) adsorbed to LP and worked as well in the agglutination test as did the strains from human sources.

TABLE 2. *Effect of duration of storage at 2 to 10 C of latex particles sensitized with PPLO cells (Campo W strain) for reliable use in the agglutination reaction*[a]

Age of antigen-latex suspension (days)	Antigen control	Reciprocal of final dilution of serum							
		20	40	80	160	320	640	1,280	2,560
0	—	3	4	4	4	4	4	1	—
3	—	3	3	3	4	4	4	3	—
7	—	2	3	4	4	4	3	—	—
14	—	3	3	3	4	4	4	3	—
28	—	2	1	1	3	4	4	1	—
37	—	2	2	2	3	4	4	3	—
49	—	2	3	3	4	4	3	—	—
70	—	2	2	3	3	3	4	3	1
127	2	3	3	3	3	3	3	3	3

[a] See footnote to Table 1 for explanation of symbols.

TABLE 3. *Typical reactions in the PPLO-latex agglutination test compared with those of direct agglutination test*[a]

Test no.	Serum against[b]	Antigen	Type of test	Reciprocal of final dilution of serum								
				20	40	80	160	320	640	1,280	2,560	5,120
1	HS	Campo-A	Latex		4	3	3	2	1	—		
2	HS	Campo-A	Agg.		4	4	3	2	1	—		
3	HS	Campo-W	Latex	2	2	3	3	3	4	4	—	
4	HS	Campo-W	Agg.	4	4	4	4	4	4	2	—	
5	HS	4330-Y	Latex	3	3	4	4	4	4	2	Tr	
6	HS	4330-Y	Agg.	4	4	4	4	4	3	3	1	
7	HS	Campo-A	Latex	3	2	1	—	—	—			
8	HS	Campo-A	Agg.	1	?	—	—	—	—			
9	HS	4330-S	Latex	1	1	2	3	3	3	4	2	TR
10	HS	4330-S	Agg.	4	4	4	4	4	4	4	2	—
11	4330-Y	Campo-W	Latex	2	2	2	1	TR	TR			
12	4330-Y	Campo-W	Agg.	—	—	—	—	—	—			
13	HS	Campo-W[c]	Latex	3	3	3	3	3	4	4	—	
14	HS	Campo-W[d]	Latex	2	2	3	3	4	4	2	—	
15	HS	Campo-W[e]	Latex	3	3	4	2	1	—	—		
16	HS	Campo-W	Latex	1	2	2	3	3	4	2	1	
17	HS	C-3	Latex	1	3	4	4	4	2	1	—	
18	HS	Campo-W	Latex	3	4	4	4	4	4	—		
19	RS	Campo-W	Latex	?	1	1	1	?	—	—		

[a] TR and question mark represent trace and doubtful reactions, respectively. See footnote to Table 1 for explanation of other symbols. Control serum gave no reaction in all tests.

[b] HS, homologous strain; RS, pooled normal rabbit serum.

[c] Fresh Campo-W.

[d] Lyophilized Campo-W.

[e] Acetone-extracted Campo-W.

The presence of anti-*Mycoplasma* antibodies was detected much sooner in the sera from rabbits, after the animals had been started on a course of immunization with PPLO antigens, by employing the PPLO-latex agglutination reaction than by using the agar gel diffusion reaction and the growth inhibition test.

A prozone was commonly observed in the latex agglutination reaction, as is evident in many of the typical reactions listed in Table 3, especially in lines 16 and 17. The optimal proportions of antigen and antibody appeared significant, since, in a series of tubes giving positive reactions, one or two tubes commonly showed a much stronger reaction than the rest. A strong reaction in every tube, illustrated in line 18, Table 3, was the unusual type of reaction.

A positive reaction was usually not obtained above a 1:20 dilution with sera obtained from individual normal rabbits prior to an immunization procedure. However, pooled normal rabbit serum (Difco) gave a slight reaction at higher dilutions (line 19, Table 3).

Lyophilized suspensions of *Mycoplasma* cells were satisfactory for sensitizing LP, as were saline suspensions of cells, which had been stored in the frozen state. Since the limiting membrane of *Mycoplasma* cells is said to contain lipoproteins, the lipids were extracted from a freeze-dried suspension of PPLO cells with acetone. When these defatted cells were used to sensitize LP, the antigen was found much less active than fresh and lyophilized cells when tested in parallel (lines 13–15, Table 3).

The globulins in immune rabbit serum were precipitated with ammonium sulfate, were dialyzed against 0.85% NaCl, and were used to sensitize LP in a manner similar to that employed by Bloomfield et al. (1). Treating a mixture of the antibody-sensitized LP and PPLO cells by the regular technique resulted in typical agglutination of the LP.

Examination of a mixture of LP and of suspension of PPLO cells, as employed in a control tube in the PPLO-latex agglutination reaction, with an electron microscope showed the PPLO cells to be adsorbed to the LP (Fig. 2B). Homologous antibodies for the PPLO cells agglutinated the sensitized LP, as seen in Fig. 2C.

Most investigators, with the exception of

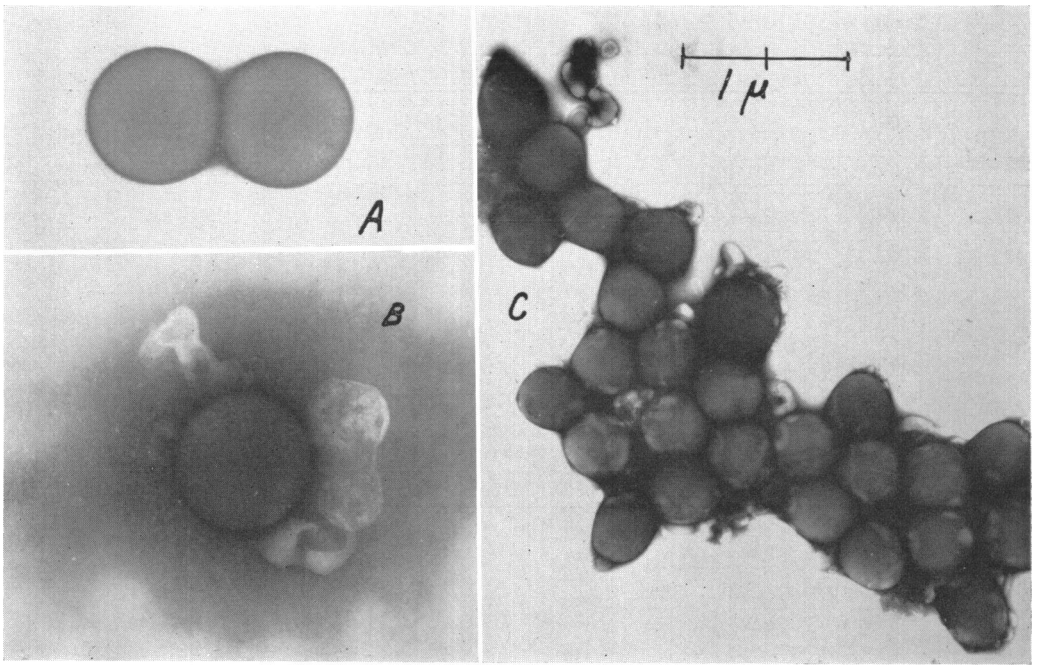

FIG. 2. *Electron micrographs: (A) latex particles suspended in GBS with BSA and 1:160 dilution of rabbit immune serum, the same concentration which produced the reaction pictured in (C); (B) latex particles with adsorbed PPLO cells suspended in the GBS with BSA; (C) small mass of PPLO-adsorbed latex particles agglutinated by 1:160 dilution of rabbit immune serum. The magnification in A and B is twice that in C. The magnification in C is approximately 10,000 × as indicated by the bar.*

Szyfres and Kagan (30), who used LP 0.328 μ in diameter, have used LP of approximately 0.81 μ in diameter. These have appeared to be satisfactory for working with PPLO. One comparison was made with LP 1.305 μ in diameter (provided by V. W. Vanderhoff, The Dow Chemical Co., Midland, Mich.), and readings were more difficult to interpret than when 0.81-μ particles were used.

DISCUSSION

To circumvent the many difficulties encountered when employing erythrocytes in the agglutination reaction for diagnosing rheumatoid arthritis, Singer and Plotz (27) discovered that the biologically inert and uniform polystyrene LP offered many advantages. They determined the concentration of LP, the optimal *p*H of the reacting system, the satisfactory types of buffers, the temperature of incubation, and the amount of centrifugation necessary for an easy differentiation of positive reactions. The statement that antipneumococcus type 7 rabbit serum and pneumococcus type 7 polysaccharide had a great affinity for LP has led to the use of LP with a variety of antigens and has prompted the investigation of their use with *Mycoplasma* antigens.

As observed with diphtheroids which agglutinated spontaneously (10), strains of PPLO that agglutinated spontaneously and could not be used in a conventional agglutination test were employed in the latex agglutination reaction. Suspensions of PPLO cells that tended to agglutinate spontaneously were well dispersed by sonic treatment for a few seconds just prior to mixing with the LP. The sensitized LP were then diluted with the buffer containing a protective colloid, BSA, or normal serum, as suggested by Singer et al. (26).

The stability of the PPLO-latex antigen at 4 C for at least 70 days was slightly better than reported for brucella-latex antigens (9). If more concentrated suspensions of PPLO-LP were stored and diluted just prior to use, as Magwood and Annau (16) did with salmonella-LP suspensions, it might be possible to extend the storage period. It is possible for some sensitized LP to be sufficiently stable to be made available commercially (11). Storage of the PPLO as saline suspensions at -10 C might have accounted for the lack of necessity to wash the PPLO-LP suspensions prior to use in the agglutination test. It has been shown that small amounts of brucella antigen can specifically inhibit the agglutination of sensitized LP (9), so it is necessary that there be no unattached antigen in the suspension of sensitized LP.

The repeated freezing and thawing of the saline suspensions of PPLO cells liberated very little soluble protein material. However, the repeated freezing and thawing of aqueous suspensions of PPLO cells released a considerable amount of soluble protein material. The antigens in these aqueous extracts sensitized LP and then reacted with their specific antibodies, as determined with extracts of other infectious agents which are useful in the diagnosis of such infections as histoplasmosis (2, 25), trichinosis (13, 21), and hydatid disease (7). In these instances, the reactions are as sensitive and specific as colloidion-particle agglutination or the complement-fixation reactions.

Without special treatment of the LP, antibodies may be adsorbed to them. LP, thus sensitized, have been useful in detecting antigens in patients with inflammatory conditions (the C-reactive protein; 28), in infections, such as cryptococcosis (1) and lupus erythematosus (3), and in allergic diseases (4, 12). By means of sensitized LP, it is possible to detect human chorionic gonadotropin hormone (24) and growth hormone (14). In the latter case, it is possible to detect as little as 0.3 μg of antigen. Possibly by isolating the important antigens from PPLO cells, the LP agglutination reaction might be even more sensitive than found by employing whole cells.

A prozone was observed frequently in the mycoplasma-LP reaction, as observed by other investigators working with other antigen-antibody systems. Removal of the albumin portion of immune sera may eliminate the prozone effect (24). Although the LP are considered to be biologically inert, the plain LP have been reported to be agglutinated by sera of patients with rheumatoid arthritis, multiple myeloma, chronic renal disease, and systemic lupus erythematosus (3, 23). These observations point out the necessity of including a plain LP control, in addition to a control of LP treated with antigen, when working with human sera.

Antigens in the supernatant fluids from sonic extracts of PPLO cells will sensitize LP; for this reason, cell suspensions were sonically treated for only a few seconds. This short treatment was sufficient to disperse the cells and not rupture the cell membrane.

The agglutination of LP sensitized with the M protein of *Streptococcus pyogenes* was a sensitive and specific reaction (17), which indicates that specific fractions of bacterial cells, protein in nature, may be used for the sensitizing substances, if desired. As mentioned above, aqueous extracts, representing the contents of the PPLO cells, sensitize LP, and, in preliminary studies,

alkaline extracts of PPLO cells, which gave a strong Millon and negative Molisch reactions, have been found to sensitize LP. It is possible simultaneously to sensitize LP with more than one antigen as, for example, with salmonella (16).

Saline suspensions of PPLO cells, kept frozen for periods ranging from a few days to 2 to 3 years, have been satisfactory for sensitizing LP, which is comparable to the findings with leptospiral suspensions (20). Although it was possible to determine the agglutination reaction with LP coated with leptospiral extracts by examining a drop of the antigen-antibody mixture under the microscope without prior centrifugation (15), it has been necessary to centrifuge the PPLO-sensitized LP-antibody mixtures to detect positive reactions.

Hydatid cyst fluid, most reactive in the latex reaction, was not necessarily the most reactive in the complement-fixation reaction, and vice versa (8). This indicates that the two systems may be reacting with different components, or one system is more sensitive in detecting certain components within an antigenic mixture. Because the suspensions of PPLO cells used for the latex reaction were anticomplementary, comparisons of the sensitivity of the two types of reactions with PPLO cells could not be made.

Bentonite is another example of carrier particles. In a comparative study with hydatid antigens (7), it was found to be slightly more sensitive than the complement-fixation test, as was the latex agglutination test. Bentonite was tried with PPLO cells, but the antigen preparations agglutinated spontaneously.

Taylor-Robinson et al. (32) reported that the erythrocytes from only one of seven sheep tested were suitable for the indirect hemagglutination test, and, even when the erythrocytes from the same sheep were used, reproducibility of results was difficult to obtain. Four different lots of LP were used with no detectable difference noted in their behavior. The small quantity of LP needed and the stability during storage are also desirable attributes of the LP.

Little is known about the mechanism by which material is adsorbed to LP. The earliest work in this area was done with human γ globulin by Oreskes and Singer (22), who estimated that, in the case of soluble materials, one LP approximately 0.8 μ in diameter can adsorb a maximum of about 75,000 molecules of human γ globulin With the aid of the electron microscope, it was demonstrated (16) that crude aqueous extracts of *Salmonella* were attached to portions of the surface of the LP. It was much easier to demonstrate adsorption of PPLO cells to LP. Although it may be at variance with the conclusions of Donaldson and Pennington (5), it seems that the adsorption of antigen or antibodies to LP depends upon the material which first comes in contact with the LP.

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