Indirect Hemagglutination with *Mycoplasma* Antigens: Effects of pH on Antigen Sensitization of Tanned Fresh and Formalinized Sheep Erythrocytes

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Received for publication 19 July 1971

An investigation of the influence of different factors affecting the sensitivity of the indirect hemagglutination test has been performed with antigens of four mycoplasmas isolated from sheep or goat. Tanned erythrocytes of sheep, fresh and formalinized, were sensitized with the above antigens. It was demonstrated that, with formalinized erythrocytes, the sensitivity was increased by 50 to 100 times when the sensitization was done at a low pH level. The pH level was unimportant for sensitizing fresh erythrocytes. The greatest sensitivity of the indirect hemagglutination test was obtained with fresh rather than formalinized erythrocytes. Three different types of antigens were used, and the most suitable antigen was found to be the supernatant fluid from an ultrasonically treated centrifuged *Mycoplasma* suspension.

The indirect hemagglutination (IHA) test has been increasingly used to demonstrate antibodies in man and animals with *Mycoplasma* infections and in serological comparison of *Mycoplasma* strains. In the most widely used modifications of the test, either fresh tanned or formalinized tanned erythrocytes of sheep are used in the sensitization procedure (1, 5, 7, 9, 11). A buffered suspension of washed *Mycoplasma* cells, a suspension of ultrasonically treated cells, or the supernatant fluid of centrifuged, ultrasonically treated *Mycoplasma* cells is usually used as the antigen.

The pH level recommended for the sensitization procedure varies, but usually a pH from 6.4 to 7.2 is used (3, 5, 7). The test itself is apparently always performed at a pH of 7.2.

The purpose of this work is to elucidate the influence of variations in the pH used for the sensitization procedure as measured by the sensitivity of the test, to evaluate which of the above antigen preparations is the best suited as antigen, and finally to compare the effect of using either formalinized or fresh erythrocytes.

**MATERIALS AND METHODS**

**Strains.** *M. agalactiae* (strain PG 2), *M. mycoides* var. *capri* (strain PG 3), *M. arginiini* (strain 506), and the nonclassified Brack strain 2833 (2), all isolated from sheep or goat, were used as antigens.

**Preparation of antigens.** The mycoplasmas were grown in a medium of heart infusion broth (Difco) supplemented with horse serum (20%), yeast extract (10%), yeast extract (10%), 0.002% deoxyribonucleic acid (DNA; Sigma calf thymus), thallium acetate (0.1%), and penicillin (500 international units/ml).

The pH of the uninoculated medium was 7.4. The cultures were incubated at 37 C for 48 hr. The pH of the medium changed in the acid direction by 1 pH unit when inoculated with *M. mycoides* var. *capri*. With *M. agalactiae* and the Brack strain 2833, the pH did not change significantly, whereas *M. arginiini* changed the pH of the medium slightly in the alkaline direction.

The cultures were harvested in a refrigerated centrifuge (Sorvall R.C. 2B) at 34,000 X g. The sediment was washed twice in phosphate-buffered saline (PBS; pH 7.2), resuspended in buffer corresponding to 1/12 of the original volume, and finally homogenized in a Braun glass homogenizer.

Three different types of antigens were prepared: (i) a suspension of whole cells in PBS (whole antigen); (ii) a suspension of cells ultrasonically treated in a Branson Sonifier (S-75) at 20 kc/min for 10 1-min periods on ice (sonicated treated antigen); (iii) the supernatant fluid of the sonically treated antigen after centrifugation for 1 hr at 34,000 X g (supernatant fluid antigen). All antigens were stored at -70 C in volumes of 1 ml.

**Preparation of antisera.** Antigens for immunization of rabbits were prepared by culturing mycoplasmas in rabbit meat infusion broth supplemented with rabbit serum (10%), yeast extract (10%), 0.002% DNA (Sigma), penicillin, and thallium acetate. The cultures were harvested at the end of the log phase in a refrigerated centrifuge (Sorvall RC-2B) at 12,000 X g for 1 hr. The sediment was washed twice in PBS.
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(\(pH\) 7.2) and resuspended in PBS corresponding to \(\frac{1}{5}\)\(_4\) of the original volume. The antigens were stored at \(-70^\circ C\).

Albino rabbits were used for immunization. Each rabbit was given five intramuscular injections of 1.5 ml of a mixture of antigen and Freund's complete adjuvant (equal parts) at intervals of 2 to 3 days. After a rest of 3 weeks, 1.5 ml of antigen was given intravenously and repeated after a period of 10 days. Ten days after the last injection the rabbits were bled. The sera were inactivated (56 C, 30 min), diluted to 1:10 in PBS, absorbed with washed, packed sheep erythrocytes (1 ml per 1 ml of serum), and stored at \(-20^\circ C\).

**Performance of the IHA test.** In all experiments, erythrocytes (RBC) from the same sheep were used. The blood was collected in Alsever's solution (1:1).

**Preparation of sensitized RBC.** The formalinization was performed according to Adler and Da Massa (1). After this procedure, the RBC were washed five times in PBS and resuspended in PBS to a concentration of 2%. This suspension, which is stable for months, was stored at 4 C. Fresh RBC in Alsever's solution, stored at 4 C, had to be used within 10 days after withdrawal of the blood. The treatment with tannin was done immediately before use as follows. Fresh or formalinized RBC washed three times and resuspended in PBS suspension were mixed with 1 volume of tannin (Merck) in PBS (1:20,000) and incubated on a water bath for 10 min at 37 C, washed twice in PBS, and finally resuspended to the original volume (2%) in a buffer having the desired \(pH\).

The sensitization was performed in the same way for both fresh tanned and formalinized tanned cells with 1 volume of RBC (2%), 0.5 volume of antigen, and 2.5 volumes of the same buffer in which the RBC were suspended. The mixture was incubated in a water bath at 37 C for 20 min and occasionally shaken. After sensitization, the cells were centrifuged for 5 min at 1,500 rev/min, washed in PBS (\(pH\) 7.2), and resuspended to the original volume in PBS with 2% inactivated normal rabbit serum absorbed with RBC as previously described.

Antigens were standardized by checkerboard titrations against homologous hyperimmune sera. The dilution of an antigen giving the highest titer was chosen as 1 antigen unit. In the titrations, 2 units of antigen were used.

The titrations were performed in permanent Lucite microtiter plates with "U"-shaped wells (Cooke Engineering Co.). Serial twofold dilutions of serum were performed in 0.025-ml volumes of PBS with 2% normal rabbit serum. To each serum dilution, 0.025 ml of sensitized RBC was added. The plates were sealed with tape and left overnight at room temperature and evaluated after 16 to 18 hr. As end point, the last dilution giving total agglutination was chosen.

As the control for nonspecific agglutinating factors in sera, RBC were treated as above, with the exception that antigen was replaced with buffer, and RBC-agglutinating ability of the antigens was controlled with sensitized RBC in serum diluent.

**Buffers.** Two buffer systems were used: (i) Michaelis' barbital-sodium acetate buffer covering \(pH\) levels from 2.6 to 9.4; (ii) three different buffers which together covered the same \(pH\) intervals, namely, acetate buffer (\(pH\) 4.0 to 5.5), phosphate buffer (\(pH\) 6.0 to 7.5), and borate buffer (\(pH\) 8.0 to 9.0). All buffers were made according to Geigy Scientific Tables (4). Before use, the buffers were diluted 1:8 in 0.9% NaCl and the \(pH\) was checked (Radiometer, Copenhagen).

**RESULTS**

**Influence of \(pH\) on the potency of the antigen.** The antigen concentrations were determined for each antigen preparation of the four strains held at different \(pH\) values during the sensitization of RBC. Both the serological titers and the titers of antigens varied with different \(pH\) levels. The antigen titer dropped from 16 at \(pH\) 4.5 to 1 at \(pH\) 7.5 with formalinized RBC, whereas the \(pH\) had no influence on the titer with fresh RBC (Fig. 1). Furthermore, the degree of dilution of antigens was critical. The IHA titer was constant at different antigen dilutions to a certain point after which the antibody curve suddenly dropped to zero (Fig. 2). The results illustrated in Fig. 1 and 2 are representative of tests performed repeatedly with the three antigen preparations of the four strains.

**Effect of \(pH\) on the titer of serum.** To determine the influence of \(pH\) variations on the sensitivity of the IHA test and to investigate whether the chemical composition of the buffer had any influence on \(pH\) response, the \(pH\) levels at which the sensitization of the tanned RBC was performed were varied from 4 to 9 at intervals of 0.5 \(pH\) unit with both buffer systems.

The experiments showed that the composition
of the buffer had no significance. The results were identical with both buffer systems.

The influence of the pH on the sensitivity of the IHA test is demonstrated in Fig. 3, which shows the average curves for the serum titers determined at different levels of pH for all three types of antigen preparations. The figure demonstrates that with fresh tanned RBC the level of pH is not significant. The level of pH with formalinized tanned RBC was very significant, as the highest titer was obtained at pH 4.0 to 5.5, the titer at this pH being four to eight times higher than that at neutral pH after which the titer drops suddenly. It is also seen from Fig. 3 that when fresh RBC were used the titer was higher at any pH level than when formalinized cells were used. The curve for the fresh RBC shows only values between pH 5 and 8. Hemolysis of these cells occurred at pH 4 to 5, and spontaneous agglutination was observed at pH levels above 8 to 9.

The influence on the IHA titer of the pH level used for sensitization of tanned RBC was essentially the same with all antigen preparations of the four strains tested, although subject to some variability. For example, with the M. agalactiae antigen and antiserum, a titer of 2,560 was obtained after a sensitization pH of 5.0 compared with a titer of 20 with cells sensitized at pH 8.0, whereas with the M. arginini antigen and antiserum the drop in the IHA titer was less pronounced. But, in all cases, the highest titer was obtained with sensitization at pH 4.5 to 6.0.

Comparison of the three antigen preparations. The unit antigen titer was determined for each type of antigen from the four strains employed when tested at different pH levels. There was no significant difference in the IHA titer with antigens prepared by the three different methods (Fig. 4). Nevertheless, in the author's experience, the supernatant fluid antigen is the most suitable, because this antigen could be diluted further than the two other antigen preparations without loss of activity. Also, the end point of titration was very clear when using the supernatant fluid antigen.

Cross-titration at different pH levels. The four strains of Mycoplasma were cross-titrated with antigens sensitized at different pH levels to determine whether this would give heterologous reactions and to determine whether cross-reactions varied in parallel with homologous reactions. The pH level had no influence on the oc-

![Fig. 2. Antigen titration. Antigen, M. mycoides var. capri (PG 3) supernatant fluid antigen; sensitization pH, 5.5; formalinized RBC.](#)

![Fig. 3. Effect of variation in pH during sensitization on the IHA titer. Antigen, M. mycoides var. capri (PG 3). Average curves for whole antigen, sonically treated antigen, and supernatant fluid antigen. Symbols: •, fresh RBC; ○, formalinized RBC.](#)

![Fig. 4. Effect of variation in pH during RBC sensitization on the IHA titer. Comparison of whole antigen, sonically treated antigen, and supernatant fluid antigen. Antigen, M. agalactiae (PG 2); formalinized RBC. Symbols: •, whole antigen; ○, sonically treated antigen; ×, supernatant fluid antigen.](#)
TABLE 1. Cross-reactions in IHA test between caprine mycoplasmas and their antisera

| Antigen                        | M. agalactiae (PG 2) | M. mycoides var. capri (PG 3) | Brack (strain 2833) | M. arginini (strain 506) |
|--------------------------------|----------------------|--------------------------------|---------------------|-------------------------|
| M. agalactiae (PG 2)           | 2,560                | 0                              | 0                   | 0                       |
| M. mycoides var. capri (PG 3)  | 0                    | 5,120                          | 160                 | 0                       |
| Brack (strain 2833)            | 0                    | 2,560                          | 2,560               | 0                       |
| M. arginini (strain 506)       | 0                    | 0                              | 0                   | 640                     |

* Formalinized erythrocytes were sensitized at pH 5.5; 0 = <20.

currence of heterologous reactions, and the actual cross-reactions varied at the same sensitization pH values to the same extent as the homologous reactions, irrespective of the type of antigen preparation used. The cross-reactivity was the same with fresh and formalinized RBC.

Table 1 shows the results of cross-titrations with supernatant fluid antigen and formalinized RBC sensitized at pH 5.5. It appears from the table that M. mycoides var. capri (strain PG 3) and the Brack strain (2833) isolated from a sheep show cross-reactivity. This relationship will be further examined with other serological tests and biochemical reactions.

**DISCUSSION**

It is concluded from the results obtained that the IHA test is more sensitive with fresh tanned RBC than with formalinized cells when the sensitization of the latter is performed at neutral pH. Disadvantages with fresh RBC are that these cells cannot be stored for more than 10 days and that hemolysis of the fresh tanned cells may occur. Hemolysis is sometimes caused by antigen alone, but more often the hemolysis occurs in the presence of antibodies. The quality of fresh RBC also varies in regard to possible hemolysis and autoagglutination, even when the cells are taken from the same sheep.

Although the formalinized cells can be used after storage for long periods without the occurrence of hemolysis or autoagglutination, they have the disadvantage, on the other hand, of being less sensitive than fresh cells when used in the IHA test. However, this difference can largely be eliminated by sensitizing the formalinized cells at low pH. In contrast to the present results, Herbert (6), using ovalbumin as antigen, found only a small increase in the IHA titer after sensitizing formalinized cells at low pH. His findings, however, are not directly comparable with the present results because of the complex composition of the Mycoplasma antigens.

In this paper, the tests described only involve antigens prepared from ovine and caprine mycoplasmas and antisera prepared in rabbits, but additional tests with human mycoplasmas, classical as well as T mycoplasmas, support the results reported here.

Pollack et al. (8), using the tetrazolium reduction inhibition and gel diffusion tests, found that the antigenicity of M. pneumoniae decreases with decreasing pH in the media during growth of the organisms. In the present work, no correlation between the pH changes in the growth media and the pH dependency of the IHA test could be demonstrated.

It is concluded that the most suitable antigen for the IHA test is obtained by using the supernatant fluid after centrifugation of an ultrasonically treated Mycoplasma cell suspension and that the sensitivity of the test with formalinized cells is increased significantly when the sensitization of the cells is performed at a low pH.

**ACKNOWLEDGMENTS**

The author thanks E. A. Freundt and H. Emr for valuable discussions during the course of this investigation.

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