Stable Mycoplasma Antigen Preparations for Indirect Hemagglutination Tests

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In immunological studies of mycoplasmas, the use of glutaraldehyde for the fixative makes it possible to use erythrocytes from commercially available defibrinated sheep blood. It eliminates the necessity of having to screen blood from individual sheep to obtain a suitable source of erythrocytes, as when employing tannic acid for fixation and sensitization. The chemical bonding of soluble mycoplasma proteins to glutaraldehyde-fixed sheep erythrocytes by bis-diazotized 3,3’dimethoxy derivative, benzidine, yields preparations that are satisfactory antigens for performing the indirect hemagglutination test by the microtiter technique. The antigenic preparations are satisfactory for use after storage at 4 or −10°C for many months. Incorporation of 5% glycerine in the final suspending milieu makes it possible to obtain uniform suspensions of the fixed and sensitized sheep erythrocytes after freezing and after repeated freezing and thawing. Proteins from Mycoplasma arthritidis and M. hominis have been coupled to glutaraldehyde-fixed erythrocytes by diazotization. The last mentioned preparation detected the presence of antibodies in titers greater than 1:10 in 37% of 237 pregnant women whose ages ranged between 20 and 30 years. There was no correlation between the presence of specific antibodies in the blood and the isolation of M. hominis from the cervical canal.

The agglutination reaction is one of the more sensitive and practical methods used for the in vitro detection of specific antibodies. Antigen binding procedures are very sensitive methods for detecting antibodies and have been applied to the detection of antibodies to Mycoplasma pneumoniae by Brunner et al. (3). However, the use of radioactive materials places such procedures beyond the ordinary diagnostic laboratory. It is the purpose of this report to describe a simple and practical test for the detection of mycoplasma antibodies that can be performed in a clinical laboratory with the usual equipment and personnel.

Mycoplasma cells are so small and many species are so difficult to grow that in most cases it is too impractical to obtain adequate amounts of suitable suspensions for direct agglutination tests. It is almost a necessity to use carrier particles for the mycoplasma antigens if an agglutination test is to be performed. Latex particles are satisfactory carriers, but the sensitized particles remain satisfactory as antigens for only a month or two when stored at 4°C (6), and the latex suspensions cannot be frozen. Blood from individual sheep is often unsatisfactory when tannic acid is employed for fixing and sensitizing the cells (11). Sheep erythrocytes fixed with glutaraldehyde and sensitized with proteins by chemical binding are satisfactory antigens in the indirect hemagglutination test, but the suspensions have been reported by Bing et al. (1) to clump after freezing and thawing.

Described in this communication are procedures for fixing sheep erythrocytes from commercially available defibrinated sheep blood (as used in the laboratory for the preparation of various culture media) with glutaraldehyde, and bonding mycoplasma proteins to the cells with bis-diazotized dianisidine. Suspensions can be prepared that are stable for many months or even years when stored at 4 or −10°C and give uniform suspensions after being frozen. The results of testing human serum specimens are included.

MATERIALS AND METHODS

Culture strains. M. hominis PG-21 and M. arthritidis PG-27 (previously designated M. hominis type 2 (6)) were used.

Preparation of glutaraldehyde-fixed sheep erythrocytes. The glutaraldehyde-fixed sheep eryth-
erythrocytes were prepared for the most part by the method of Bing et al. (1). The erythrocytes were prepared from defibrinated sheep blood obtained commercially and washed three times with 0.15 M NaCl. After the packed cells were chilled in an ice bath, they were fixed in a cold 1% (vol/vol) glutaraldehyde (25% solution, technical grade) solution containing 1 volume of 0.15 M sodium phosphate buffer, pH 8.2, 9 volumes of 0.15 M NaCl, and 5 volumes of deionized water. The cells were added slowly drop by drop from a pipette to the chilled fixing solution in the ice bath with vigorous stirring (this procedure is very important). The volume of glutaraldehyde solution used to dilute the packed cells should be of sufficient quantity to make a 1 to 2% cell suspension. The cell suspension was kept in the ice bath for 30 min and then placed at 4°C for another 30 min with occasional mixing throughout the fixation period. The glutaraldehyde-fixed cells were centrifuged and washed with 10 changes of 0.15 M NaCl and finally suspended in a sufficient volume of 0.11 M sodium phosphate buffer, pH 7.3, to give a 30% cell suspension. Methionolate was added to a final concentration of 1:10,000 (wt/vol) as a preservative, and the suspension was stored at 4°C until it was used.

Sensitization of glutaraldehyde-fixed sheep erythrocytes with mycoplasma proteins by bis-diazo 3,3′-dimethoxy derivative, benzidine (BDB). Portions of freeze-thaw cell-free extract precipitated with (NH₄)₂SO₄ between 50 and 75% saturation were conjugated to the glutaraldehyde-fixed erythrocytes with BDB by means of a modified method of Borek (2). One milliliter of a 30% glutaraldehyde-fixed sheep erythrocyte suspension was washed twice with saline, and the cells were then suspended in 0.1 M citrate buffer, pH 5.0, to give a 2% cell suspension. To this was added 10 mg or more of mycoplasma protein; this was then thoroughly mixed and chilled in an ice bath. To this mixture were then added 3-ml samples of cold-solutions BDB consisting of 13.4 mg of 3,3′dimethoxybenzidine and 7.6 mg of NaNO₂ in 10 ml of 0.034 N HCl. The reaction mixture was kept in an ice bath for 30 min with frequent stirring and was then transferred to a 4°C refrigerator for an additional 1.5 h with occasional mixing. The reaction was terminated by adjusting the pH to 7.4 with 1 N NaOH and then by centrifugation; the supernatant was discarded, and the cells were washed twice with buffered saline, pH 7.4, once with M/15 sodium phosphate buffer, pH 7.4, and were finally washed once with M/15 sodium phosphate-buffered saline, pH 6.4, containing 0.25% normal rabbit serum.

The protein-sensitized cells were resuspended in the same final washing buffer solution to a concentration of 2% cell suspension; methionolate was added to a final concentration of 1:10,000. Glycerin (5% concentration) was added to the final suspending milieu if the cells were to be stored at −10°C.

The 10-mg portions of mycoplasma proteins gave satisfactory preparations with the quantities of cells and reagents employed, so other amounts were not tried.

**Mycoplasma protein extracts.** The mycoplasmal suspensions were grown in 16-liter batches (8), and the cells were collected from 48-h cultures by centrifugation at 35,000 × g, washed once with 0.85% NaCl, and suspended in deionized water in the ratio of the sediment from 1 liter of culture in 2 ml of water. The cells were disrupted by repeated cycles of freezing and thawing. The cell residue was collected by centrifugation at 35,000 × g, resuspended in fresh deionized water, subjected to additional cycles of freezing and thawing, and centrifuged. The supernatants were pooled and held overnight at −10°C. The particulate material that appeared when the frozen aqueous extract melted was removed by centrifugation at 32,000 × g for 20 min at 4°C. The clear supernatant containing soluble mycoplasmal proteins was fractioned with (NH₄)₂SO₄ as the first step in separating proteins with specific activities (7). All solutions were maintained and all procedures were carried out at 4°C. A portion of the clear supernatant was mixed with an equal volume of saturated (NH₄)₂SO₄. After 24 h the mixture was centrifuged, and to the supernate was added sufficient (NH₄)₂SO₄ to make a 75% saturated solution. After 24 h the mixture was centrifuged, and the sediment was dissolved in a small amount of deionized water, transferred to dialyzing cellulose tubing, and dialyzed against several changes of saline during a period of 2 days. The dialyzed protein solution was concentrated by pervaporation and centrifuged to remove any particulate material. This report pertains to work with the 75% (NH₄)₂SO₄ precipitated fraction.

**Indirect hemagglutination technique.** The microtiter technique of Sever (10) was used. Serial twofold dilutions of immune sera were prepared in 0.025-ml amounts in M/15 sodium phosphate-buffered saline, pH 6.4, containing 0.25% normal rabbit serum. The mycoplasma proteins conjugated to glutaraldehyde-fixed sheep erythrocytes, 2% suspension, were employed as antigens and added in 0.025-ml amounts per well. Sensitized cells that had been frozen in the presence of 5% glycerine were used as such or centrifuged, the supernatant was discarded, and the cells were resuspended in the M/15 sodium phosphate-buffered saline, pH 6.4, containing 0.25% normal rabbit serum. Pre-immunization rabbit serum and diluent controls were included. The contents of the microtiter plates were thoroughly mixed by tapping, held at room temperature, and examined for agglutination after 1 to 4 h and overnight.

**RESULTS**

The presence of 5% glycerine in the final suspending milieu enabled the sensitized cells to be stored at −10°C and give uniform suspensions upon thawing. This procedure overcame the difficulties reported by Bing et al. (1) and Neimark (9). The glycerinated suspensions gave uniform suspensions even after repeated cycles of freezing and thawing. The thawed suspensions of cells can be used in the indirect hemagglutination test or they can be centrifuged and the cells resuspended in the buffered saline-normal rabbit serum solution. The
glycerine does not appear to interfere with the agglutination reaction.

The stability of preparations of soluble mycoplasma proteins chemically bonded to glutaraldehyde-fixed sheep erythrocytes was demonstrated by the retention of their antigenic activity over periods of several months or years when stored at 4 or \(-10\) C. The titer, taken as a 1+ reaction after overnight at room temperature, did not fluctuate from an end point by more than one dilution over a period of up to 2 years when all of the material had been used for the tests. A 1+ reaction was recorded as the highest serum dilution that showed a definite agglutination at the periphery of the microtiter well with 75% of the sensitized erythrocytes aggregated at the center and forming a coarse granular agglutinated mass.

The sensitivity of preparations of soluble mycoplasma proteins chemically bonded to glutaraldehyde-fixed sheep erythrocytes to detect specific antibodies was demonstrated by their ability to react in high dilutions of specific immune serum. The sera from rabbits immunized with suspensions of whole mycoplasma cells have shown a maximum titer of 1:40,960 when tested with homologous antigen in the indirect hemagglutination test. Very slight cross-reaction occurred with rabbit anti-\(M.\ hominis\) hyperimmune serum and antigens prepared from \(M.\ arthritidis\). Weak reactions (1+) occurred in the 1:4 and 1:8 dilutions.

The ability to detect specific antibodies in human sera was determined by performing the indirect hemagglutination tests on 259 samples of sera submitted to a diagnostic serological laboratory for syphilis serology tests. The antigen was glutaraldehyde-fixed sheep erythrocytes sensitized with \(M.\ hominis\) proteins prepared from a freeze-thaw extract after precipitation with 75% saturation \(\left(\text{NH}_4\right)_2\text{SO}_4\). The results are summarized in Table 1. The controls with each serum and fixed but unsensitized cells were negative, so the results are not included in the table. Individuals of both sexes were included in the younger and older age groups, but only healthy pregnant females were included in the 20- to 30-year group. Cervical cultures were performed on individuals in this group, and there was no significant difference in antibody titers between those individuals from whom \(M.\ hominis\) was cultured and those from whom the organism was not cultured.

### DISCUSSION

Mycoplasmas are more apt to be present in the cervixes of women during the ages of greatest sexual activity. The failure to find a correlation with a positive cervical culture and a high titer of specific antibody is not necessarily incongruous. It could indicate that a representative sample of the cervical secretion was not obtained, sufficient numbers of organisms were not being shed at the time the specimen was collected to be detected in a culture, the individual was not harboring the organism at the particular time, the organisms are not sufficiently invasive to produce a good antibody response, or the organisms may not be good antigens for producing antibodies. Our finding that 37% of individuals in the 20- to 30-year age group had a titer of 1:10 or greater is comparable to the 33% reported by Millian and Spigland (5) for normal individuals in the 20- to 39-year age group and to the 38% reported by Taylor-Robinson et al. (11) for individuals in the 20- to 29-year age group without respiratory illness. Only 3.7% of 109 female blood donors reported by Lemcke and Csonka (4) had titers of 1:10 or greater when their sera were tested by the complement-fixation test. This could indicate the greater sensitivity of the indirect hemagglutination test compared to the complement-fixation test for detecting specific antibodies against \(M.\ hominis\), or it could indicate that the two tests are detecting different globulins in the sera.

Being able to use sheep cells obtained from commercially available defibrinated sheep blood is of great practical value; as Taylor-Robinson et al. (11) reported, the blood from only two of seven sheep was satisfactory for the indirect hemagglutination test when the erythrocytes were treated with tannic acid. We also encountered great difficulties with erythrocytes obtained from various sheep when employing tannic acid treatment, and even the blood from the same sheep varied with different bleedings. Glutaraldehyde fixation and commercially available defibrinated sheep blood appear to obviate these difficulties.

The stability of glutaraldehyde-fixed sheep erythrocytes sensitized with mycoplasma proteins and stored at temperatures of 4 and \(-10\) C

| Table 1. Reactivity of glutaraldehyde-fixed sheep erythrocytes sensitized with \(M.\ hominis\) proteins and sera from individuals in various age groups |
|-----------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| No.             | Ages              | Reciprocal of final dilution of serum | <2    | <10   | 10-16 | 32    | 64    | 128   |
| 14              | <1 wk             |                                 | 10    | 2     | 1     | 0     | 0     |
| 237             | 20-30 yr          |                                 | 100   | 50    | 56    | 22    | 7     |
| 8               | >50 yr            |                                 | 4     | 3     | 0     | 1     | 0     |
for periods of months and years makes it possible for the preparations to be produced in quantities to make large-scale testing possible and to place the same material in the hands of various workers.

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