Passive Hemagglutination Test for Bovine Chlamydial Abortion

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Purified chlamydial elementary bodies were subjected to sonic lysis to produce soluble antigen for passive hemagglutination (PHA). PHA was more sensitive than complement fixation (CF) in detecting antibody in hyperimmunized rabbits, lambs, and calves. Both CF and PHA tests were used to monitor antibody responses in experimentally infected pregnant cows. All animals responded with rises in titers after experimental infection. Titers declined and then increased a second time near the time of abortion. PHA titers were considerably higher than CF titers.

Many aspects of the immune response to the agent of bovine chlamydial abortion are unknown. Although epidemiological studies indicate that natural infection leads to immunity, experimental stimulation of immunity has been unsuccessful (6). It is unknown whether the protective immune response is humoral or cellular. Humoral response in experimental and natural infections is usually determined by the use of a group-specific complement fixation (CF) test. Group CF serology as employed is of questionable validity, both because it is group rather than species specific and because it is dependent on the bovine immunoglobulin fixation of guinea pig complement when it is known that some bovine immunoglobulin classes will not fix guinea pig complement (3).

An attempt was made to improve serological techniques available for bovine chlamydial abortion in the hope that improved serology would lead to a better understanding of immunity to the disease and perhaps to better serodiagnostic tests. An attempt was made to improve the CF test employed and to apply a passive hemagglutination (PHA) test. Because these serological studies required the production of large yields of purified chlamydiae, it was necessary to develop suitable purification techniques.

MATERIALS AND METHODS

Cultivation and purification of chlamydiae.

The bovine chlamydial abortion isolate EBA59-795 isolated by Storz et al. in 1959 (13) was used in this study. The organism was cultivated in 7-day embryonating chicken eggs.

Embryonating eggs were inoculated with dilutions of chlamydiae which would cause death of egg embryos in 5 to 10 days. Eggs were candled twice daily, and dead eggs were refrigerated prior to harvesting. Infectious yolks were removed aseptically with a needle and syringe, and the yolk sacs were removed with forceps. Yolk and yolk sacs were pooled, and the total volume of the harvest was determined. Sufficient 4 M KCl was added to make the total suspension 1 M KCl. The mixture was homogenized (Omni-mixer, Ivan Sorvall, Inc., Norwalk, Conn.) for 1 min and centrifuged at 800 × g for 20 min. The low-speed centrifugation resulted in an upper lipid layer, a coarse sediment, and an intermediate layer which was harvested and centrifuged at 18,000 × g for 30 min to sediment the elementary bodies. The sediment was rinsed once with 1 M KCl and suspended in a volume of KCl equal to 1/10 the original volume. A 40% sucrose solution was prepared in 1 M KCl and was used to prepare a 0 to 40% sucrose gradient by the method of Ribi and Hoyer (9). Concentrated elementary bodies were applied to the surface of the gradient in a volume of about 3 ml for each 35-ml tube. The tubes were centrifuged for 60 min at 6,000 × g in a refrigerated horizontal rotor (RC2B-Centri-fuge, HB-4 rotor, Ivan Sorvall, Inc., Norwalk, Conn.). A fairly wide band of elementary bodies resulted. The band was harvested with a needle and syringe and blended with an equal volume of fluorocarbon (Freon 114, dichlorotetrafluoroethane, E. I. DuPont De Nemours and Co., Palo Alto, Calif.). The fluorocarbon and extracted material were separated from the elementary bodies by centrifuging for 10 min at 800 × g. Cold saline was added to the fluorocarbon and extracted material and again blended and centrifuged. The two harvests from fluorocarbon were pooled and again sedimented at 18,000 × g for 30 min.

The effectiveness of purification was determined...
by determining egg infectivity and total protein of the starting homogenate and the final product.

Preparation of group-specific CF antigen.

Purified elementary bodies were boiled for 30 min, chilled, and extracted twice with equal volumes of diethyl ether. Extraction was done by mixing in an Erlenmeyer flask on a magnetic stirrer for 15 min. The material was placed in a cold, closed centrifuge tube and centrifuged at 500 × g for 2 min to separate the solvent layers. The ether layer was placed in an open beaker under a chemical hood, and the ether was evaporated by circulating air. The dried ether extract was then dissolved in phosphate-buffered saline (PBS) and stored at 4°C.

Microtiter CF test. Hemolysin and complement were titrated by conventional tube titrations. Two units of complement and two units of hemolysin were used in the tests. Antigen titers were determined by block titration. When serum titrations were done, four complement controls for each microtiter (Cooke Engineering Co., Alexandria, Virginia) plate were prepared in tubes to contain 0.5, 1.0, 1.5, and 2 units of complement and were transferred to the microtiter plates in 0.025-ml amounts. Microtiter plates were incubated for 1 hr at room temperature and for 30 min at 37°C in a circulating-air incubator. Equal volumes of 2.5% sheep erythrocytes and diluted hemolysin were mixed at least 15 min before being added to the wells. The plates with sensitized cells added were incubated at 37°C and monitored by checking the complement controls. When the plates showed complete hemolysis at 2 units and 1.5 units, partial hemolysis at 1 unit, and no hemolysis at 0.5 unit, they were centrifuged to facilitate the interpretation of results.

Soluble antigen preparation. Purified elementary bodies were suspended in sucrose phosphate albumin (14) and mixed with one-half volume of a 50% suspension of 5-μm glass beads. The mixture was sonically treated (model W-140C, Heat Systems-Ultrasonics, Inc., Plainsview, N.Y.) in a stainless-steel cup for three 10-min intervals with 10-min breaks to allow time for cooling. The cup was cooled with crushed ice. The sonically treated material was centrifuged at 20,000 × g for 30 min to remove glass beads, fragments, and undisrupted elementary bodies.

PHA. The procedure for PHA test was that of Stavitsky as modified by Vedros (16). Sheep blood was collected in Alsever’s solution, and the cells were washed three times with saline. One milliliter of packed cells was suspended in 40 ml of PBS, pH 7.2. Tannic acid was prepared daily in a 1:20,000 dilution in saline. One volume of diluted cells was mixed with an equal volume of diluted tannic acid. The mixture was incubated in a water bath at 37°C for 10 min. Cells were sedimented by centrifugation, rinsed once with one volume of PBS, and suspended in one volume of unbuffered saline.

To sensitize cells with antigen a suitable concentration of soluble antigen prepared by sonic treatment was diluted in 0.075 M phosphate-citrate-buffered saline, pH 5.6. One volume of tannic acid-treated cells was mixed with five volumes of diluted antigen and incubated for 10 min at 37°C. Sensitized cells were then sedimented and rinsed once with PBS containing either 1:80 heat-inactivated normal rabbit serum or bovine albumin and resuspended to a final cell concentration of 0.9%. Serum to be tested were heat inactivated in a 1:10 dilution at 56°C for 30 min. One milliliter of inactivated diluted serum was then absorbed with 1.0 ml of packed, rinsed sheep erythrocytes. Controls with each test included untreated erythrocytes with antisera, tannic acid-treated erythrocytes with antisera, tannic acid-treated erythrocytes with buffer, and antigen-sensitized erythrocytes with buffer. All PHA tests were performed by the microtiter test. Disposable trays with V-shaped wells were used. Doubling dilutions of sera in 0.05-ml amounts were performed, and two 0.025-ml drops of sensitized cells were added to each well. Trays were sealed with cellophane tape and incubated overnight at room temperature.

RESULTS

Purification of elementary bodies resulted in a decrease in total infectivity from 10^{-6.3} to 10^{-4.4} 50% chicken embryo lethal doses (ELD_{50}). Protein content was reduced from 11.5 g/100 ml to 0.5 g/100 ml. Specific infectivity expressed as the total infectivity divided by the total grams of protein increased from 5.8 × 10^6 ELD_{50} g to 2.5 × 10^10 ELD_{50} g. The purified product was free from egg-specific antigens as determined by reacting the product against rabbit and anti-yolk sac antibody in CF, PHA, and immunodiffusion tests.

The effect of sonic treatment on purified elementary body suspensions was determined by infectivity titrations and tannic acid-treated cell-sensitizing antigen titrations. Nearly all of the soluble antigen was released from the elementary bodies between 10 and 30 min of ultrasonic treatment. Infectivity of the suspension rapidly decreased during sonic treatment and was less than 10^2 ELD_{50}/ml at the end of 30 min. Soluble antigen could be diluted as much as 1:320 for cell sensitization. At dilutions lower than 1:80, a direct hemagglutination occurred with tannic acid-treated erythrocytes. This reaction is probably not equivalent to the hemagglutination that has been previously reported for various chlamydialae because no hemagglutination occurred with cells that had not been treated with tannic acid. It was found that the soluble antigen could not be preserved by freezing. When stored at 4°C, the antigen titer dropped at least one dilution overnight, and no antigenic activity could be detected after 48 hr.

PHA was compared with CF in monitoring antibody responses in hyperimmunized rabbits.
lambs, and calves. The comparative responses of rabbits immunized with different routes and antigen preparations are shown in Table 1. Antigens were prepared from purified elementary body suspensions containing approximately 10⁶ ELD₅₀/ml. Intraperitoneal injections of 1 ml were given at weekly intervals for 3 weeks and then once every 2 or 3 weeks for an additional 15 weeks, for a total of 11 injections. Subcutaneous injections were made with inocula containing equal volumes of concentrated antigen and adjuvant. A volume of 0.25 ml was injected in each of four sites at weekly intervals for 3 weeks followed by four injections at approximately 1-month intervals. Normal yolk sac antigens contained equal volumes of a 20% (w/v) normal yolk sac suspension and adjuvant. A volume of 0.5 ml was given in each of two sites at weekly intervals for four injections. The highest titers were obtained with elementary bodies injected subcutaneously in incomplete Freund adjuvant. The PHA titers were consistently higher than the CF titers. Hyperimmune antisera to normal yolk sacs did not react with the chlamydial antigens in either the CF or PHA tests. The antibody responses of four heifers are shown in Fig. 1 and 2. The heifers depicted in Fig. 1 were immunized with 5 ml of a 20% suspension of infectious yolk sacs weekly for 5 consecutive weeks. The heifers in Fig. 2 were immunized with 5 ml of a 20% suspension of chlamydiae-infected mouse lung at the same time intervals. In all cases, PHA titers were considerably higher than CF titers. Peak titers were reached after the first or second injections and did not increase after subsequent injections.

The results of injecting four lambs with the same antigens and injection schedules used with the heifers are shown in Fig. 3 and 4. All four lambs had fairly high titers prior to immu-

<table>
<thead>
<tr>
<th>Rabbit no.</th>
<th>Antigen and route</th>
<th>Peak CF titer</th>
<th>Peak PHA titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Intact purified elementary bodies, intraperitoneal</td>
<td>40</td>
<td>640</td>
</tr>
<tr>
<td>2</td>
<td>Intact purified elementary bodies, intraperitoneal</td>
<td>20</td>
<td>1,280</td>
</tr>
<tr>
<td>3</td>
<td>Intact purified elementary bodies, in incomplete Freund adjuvant, subcutaneous</td>
<td>80</td>
<td>2,560</td>
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<td>4</td>
<td>Intact purified elementary bodies, in incomplete Freund adjuvant, subcutaneous</td>
<td>160</td>
<td>10,240</td>
</tr>
<tr>
<td>5</td>
<td>Sonically treated, purified elementary bodies, in incomplete Freund adjuvant, subcutaneous</td>
<td>80</td>
<td>5,120</td>
</tr>
<tr>
<td>6</td>
<td>Sonically treated, purified elementary bodies, in incomplete Freund adjuvant, subcutaneous</td>
<td>80</td>
<td>5,120</td>
</tr>
<tr>
<td>7</td>
<td>Normal yolk sac in incomplete Freund adjuvant</td>
<td>Neg*</td>
<td>Neg</td>
</tr>
<tr>
<td>8</td>
<td>Normal yolk sac in incomplete Freund adjuvant</td>
<td>Neg</td>
<td>Neg</td>
</tr>
</tbody>
</table>

* Neg, negative.

TABLE 1. Serum titers of hyperimmunized rabbits

![Fig. 1. CF and PHA (IHA) titers of heifers immunized with yolk sac-propagated chlamydiae.](image1)

![Fig. 2. CF and PHA (IHA) titers of heifers immunized with mouse lung-propagated chlamydiae.](image2)

The applicability of the PHA test for monitoring antibody responses in cows undergoing experimentally induced chlamydial abortion was then determined. Five cows in the fourth to fifth month of gestation were infected with 20% infectious yolk sac suspensions of the bovine chlamydial abortion isolate. Weekly serum samples were collected for 12 weeks. Three of the five cows aborted between the 7th and 11th week after infection. The serum antibody responses as determined by both the
PHA and CF tests are shown in Fig. 5 through 9. As with the hyperimmunized animals, PHA titers were considerably higher than CF titers. The effect of heat inactivation of serums at 65°C instead of 56°C was determined throughout the course of the response. Titers were lower after 65°C inactivation both during the initial response to challenge and during the secondary rises that occurred with abortion.

Titers, as determined by both tests, were found to rise considerably after initial infection. The titers then dropped and rose again either prior to or at the time of abortion in those animals that aborted. The two cows that calved normally did not show secondary rises in titers.

**DISCUSSION**

A number of methods have been proposed for the purification of chlamydial elementary bodies. Various methods have utilized as starting material yolk sacs (10, 18), yolk (7), and allantoic fluid (11). Most of these methods have included trypsin digestion as part of the purification procedure. Wang et al. (17) demonstrated that digestion with crude trypsin could destroy the protective antigen activity to trachoma in mouse protection tests. This destructive potential suggests that trypsin digestion may be undesirable in the production of purified elementary body suspensions for antigen study.

The purification method described here was developed by adapting and combining methods previously used by others (9, 10, 17) and had the advantage of avoiding trypsin digestion and of utilizing both infected yolk and yolk sacs as starting material. The fact that considerable infectivity was retained in the purified product and the inability to demonstrate egg-specific antigens in the product indicate that this method of purification is satisfactory for the study of chlamydia antigens. The major disadvantage of the method is the use of fluorocarbon in the final purification step. The volatile nature of the fluorocarbon can result in the production of hazardous chlamydial aerosols unless precautions are taken. Aerosols can be avoided by using closed containers when possible and by keeping the material near 0°C.

Benedict and O'Brien (2) reported the use of infected allantoic fluid, centrifuged to remove elementary bodies, as a source of tanned cell-sensitizing antigen. It was observed that the allantoic fluid preparations would agglutinate tanned cells directly at low dilutions. It was
null
with a subsequent decline followed by a secondary response prior to impending abortion. Page et al. (8) reported that on initial exposure to a chlamydial isolate a calf responded only with 19S, 65 C labile antibody and that heat inactivation distinguished between slow- and fast-sedimenting antibody. Storz and McKercher (12) also concluded that removal of antibody activity by inactivation at 65 C indicated only a 19S antibody response. In the present report, none of the cattle responded with only 19S-type antibody production, as indicated by heat sensitivity, which suggests that all animals studied were previously exposed to chlamydiae.

LITERATURE CITED


