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IMMUNOLOGY AND SEROLOGY OF *ANAPLASMA MARGINALE*

I. FRACTIONATION OF THE COMPLEMENT-FIXING ANTIGEN

THOMAS E. ROGERS,[1] RICHARD J. HIDALGO, AND GEORGE T. DIMOPOULLOS

Department of Veterinary Science, Agricultural Experiment Station, and Department of Bacteriology, Louisiana State University, Baton Rouge, Louisiana

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ABSTRACT

ROGERS, THOMAS E. (Louisiana State University, Baton Rouge), RICHARD J. HIDALGO, AND GEORGE T. DIMOPOULLOS. Immunology and serology of *Anaplasma marginale*. I. Fractionation of the complement-fixing antigen. J. Bacteriol. **88**:81-86. 1964.—Studies were conducted to fractionate and purify the complement-fixation (CF) antigen of *Anaplasma marginale* in infected erythrocytes of cattle. Initial attempts were made to resolve the antigen from crude stromatal preparations by various chemical and physical methods. Fractionation procedures involving partial and total lipid extraction suggested that the CF antigen was lipoprotein in nature. Fluorocarbon deproteinization of stromatal antigens was also attempted. A method was developed for the preparation of a desirable *Anaplasma* CF antigen which involved disintegration of infected erythrocytes by sonic vibration and separation of the antigen by differential centrifugation. Antigens prepared by this method were highly specific, colorless, did not exhibit anticomplementary activity, and possessed higher titers than standard *Anaplasma* antigens. When density-gradient sedimentation was applied to sonic extracts of infected cells, it was demonstrated that the CF antigen could also be fractionated by this method.

Various methods have been employed in the preparation of antigens for use in the complement-fixation (CF) test for the diagnosis of bovine anaplasmosis (Gates et al., 1954*a*). These CF antigens consist of erythrocytic stromata prepared from animals which show a high percentage of erythrocytes containing *Anaplasma* bodies (AB). Although these antigens are crude and have disadvantages of possessing considerable color, turbidity, and low titer, they are the only ones available for the diagnosis of anaplasmosis by the CF test (Gates et al., 1954*b*). There is a need for an antigen with more desirable properties. This report describes the fractionation of erythrocytes infected with *A. marginale* and the preparation of a more suitable antigen from infected erythrocytic material.

[1] Present address: Department of Microbiology, Medical College of South Carolina, Charleston.

MATERIALS AND METHODS

Preparation of stromata. The methods for infecting and maintaining calves and for observations during the disease were described previously (Dimopoullos and Bedell, 1962). Blood from 12 *Anaplasma*-infected calves was obtained by exsanguination, and was collected in heparin sodium solution (0.3 ml per 50 ml of blood; 1,000 USP units per ml) when microscopic examination of Giemsa-stained blood smears demonstrated at least 72 and as high as 90% of the erythrocytes to contain AB. In addition, blood from six normal calves was similarly obtained. The cells were separated from the plasma by centrifugation, washed three times with 0.85% NaCl solution, and pooled. Stromata were prepared according to previously described methods (Dimopoullos and Bedell, 1960).

Antigenic titer. For the determination of the presence of antigenic activity and antigenic titer, samples of the stromatal pools were diluted 1:10 with Veronal-buffered saline, pH 7.2 (CF buffer). CF antigenic activity was determined according to protocol (U.S. Department of Agriculture, 1958).

Extraction of stromata. Portions of the stromata were diluted 1:1 with distilled water and freeze-dried. The dried stromata were fractionated by use of techniques similar to those which have been employed in the separation of the components of human red blood cell membranes (Calvin et al., 1946; Moskowitz et al., 1950; Moskowitz and Calvin, 1952). The processes involved stepwise lipid extraction of the dried stromata. In preparing fractions for determination of anti-

genic activity, solvents were removed by use of a flash evaporator. Residues were mixed with CF buffer in a ratio of 1:5. All fractions were tested for antigenic activity with standard anti-*Anaplasma* and normal sera.

One-step extraction was also made with lipid solvents. Dried stromata (5 ml, diluted 1:10 with CF buffer) was added to 200 ml of a 3:1 ethanol-ethyl ether solution and mixed overnight at 4 C. The mixtures were centrifuged for 15 min at 400 × *g*, and supernatant fluids and sediments were collected. Ether and alcohol were removed by use of a flash evaporator. Residual solvents were removed from the sediment produced by centrifugation in the same manner. Similar extractions were made with mixtures of chloroform and methanol. All fractions were suspended in 5 ml of CF buffer and titrated for antigenicity.

Extraction with fluorocarbon. For extraction, 180 ml of Genetron 113; 18 ml of fresh, packed, wet stromata; and 72 ml of phosphate buffer (pH 7.2) were mixed and homogenized for 5 min at 16,000 rev/min in an Omnimixer at 4 C (Gessler et al., 1956*a*, *b*; Porter, 1956). After homogenizing, the emulsion was broken by centrifugation for 10 min at 1,000 × *g*. The aqueous layer was removed and tested for CF antigenic activity without further treatment. Double and triple extractions by the same techniques were also made on additional stromata.

A second technique (Halonen et al., 1958) was employed whereby 20 ml of the stromata (suspended in distilled water, 1:1) were exposed to sonic vibration for 10 min. Afterwards, the material was mixed with 10 ml of Genetron 113 and homogenized as before. The homogenate was centrifuged for 10 min at 700 × *g*. A 1-ml amount of the aqueous layer was removed, diluted with 4 ml of CF buffer, and titrated. The procedure was repeated on another 20-ml portion of stromata, but the aqueous layer was centrifuged for 90 min at 105,000 × *g*. A brown supernatant fluid was collected, and the pellet was homogenized in 10 ml of CF buffer. Both of the materials were tested for CF antigenicity.

Preparation of CF antigen from sonic extract. Red blood cells from acutely infected animals were separated and washed according to the procedure previously described. Packed cells were suspended in an equal volume of phosphate-buffered saline (pH 7). The suspensions were subjected to sonic vibration in 25-ml quantities for 10 min at 17 to 20 C by use of a 50-w, 9-kc Raytheon sonic oscillator (Bedell and Dimopoullos, 1963; Rogers and Dimopoullos, 1963). The sonic extract was centrifuged at 700 × *g* for 30 min, and the supernatant fluid was recovered and centrifuged for 1 hr at 105,000 × *g* at 1 C. The resulting sediment was washed in 0.067 M Na_2HPO_4 (pH 8.3) at 105,000 × *g* for 1-hr intervals until the supernatant fluid did not contain red color due to hemoglobin.

The sediment was then resuspended in 10 volumes of phosphate buffer (pH 7) and subjected to sonic vibration until the pellet was dispersed and a homogeneous suspension remained. Dowex 2X-4 ion-exchange resin was added to the suspension at the rate of 0.5 g per 10 ml, and the mixture was stirred for 10 min. After decanting from the resin, the suspension, together with the previously described sonic preparation and the first high-speed supernatant fluid, were titrated according to the U.S. Department of Agriculture (1958) protocol for CF antigenic activity.

Density-gradient sedimentation. Gradients were prepared by layering 4, 7, 7, and 7 ml (Brakke, 1960) of Veronal-buffered saline solution containing 600, 500, 400, and 300 mg of sucrose per ml, respectively, in cellulose nitrate tubes (2.5 by 7.6 cm). Quantities (2 ml) of the sonic extract were layered over the gradient columns, and the tubes were centrifuged at 50,000 × *g* for 1 hr in a swinging bucket rotor (Spinco, SW-25.1). Layers of the gradient column were removed, from top to bottom, by use of a pipette with a curved tip drawn out to a small opening at the end. All layers of fractionated material were titrated for CF activity according to protocol. Smears were prepared of all separated layers and were examined microscopically after staining with Giemsa stain. Attempts were made to further purify the fraction showing the highest CF activity by recycling the material by use of the gradient technique described above. Comparisons were also made of CF antigenic activity in this layer in the presence and absence of sucrose. Sucrose was removed by repeated washing of layers in Veronal-buffered saline at 50,000 × *g* for 30 min.

RESULTS

CF antigenicity of stromatal components. Stromatal antigen, prepared by lysis and precipitation of pooled infected erythrocytes in carbon

dioxide-saturated distilled water, possessed a titer of 1:10 and compared favorably with the standard antigen (*Anaplasma* CF antigen, U.S. Department of Agriculture). The presence of CF antigenic activity in fractionated stromatal components was determined. When fractions were titrated with anti-*Anaplasma* and negative sera, the amount of antigenic activity in the fraction corresponding to stromin was essentially unchanged from the unfractionated stromata. Titrations of suspensions of the fractions derived from the stromin corresponding to elinin resulted in antigenic titers as high as 1:320. However, when a number of positive and negative sera were tested, with the elinin fraction as the CF antigen, considerable anticomplementary activity in the antigen was demonstrated. Agglutination of the sheep red blood cells also occurred in many samples. Upon further fractionation by complete removal of lipids, CF activity was not found in the resulting fractions. Fractions of stromata prepared in a similar manner from normal erythrocytes did not show CF antigenicity.

When untreated stromatal antigens were extracted with mixtures of alcohol and ether, and chloroform and methanol, the resulting residues and lipid extracts did not show any CF antigenic activity when tested with anti-*Anaplasma* serums.

High-speed homogenization of aqueous stromatal suspensions with Genetron 113 resulted in the separation of three distinct layers. Thick stromatal material was present at the interface of the aqueous layer and the lower Genetron layer. The aqueous material was highly anticomplementary. Similar results were obtained after repeated extractions of the aqueous layer. When the sonic extracts of stromata were extracted with Genetron and the resulting aqueous suspensions were tested for CF activity, titers of 1:5 to 1:10 were obtained, and anticomplementary activity was not detected. High-speed centrifugation of these aqueous suspensions and subsequent testing of supernatant fluid and sediment for CF activity showed that the active material was present in the sediment. The antigenic activity of the material never exceeded that of standard stromatal antigen. However, the extraction process removed considerable color and undesirable stromatal material and produced a clearer antigen with no anticomplementary properties.

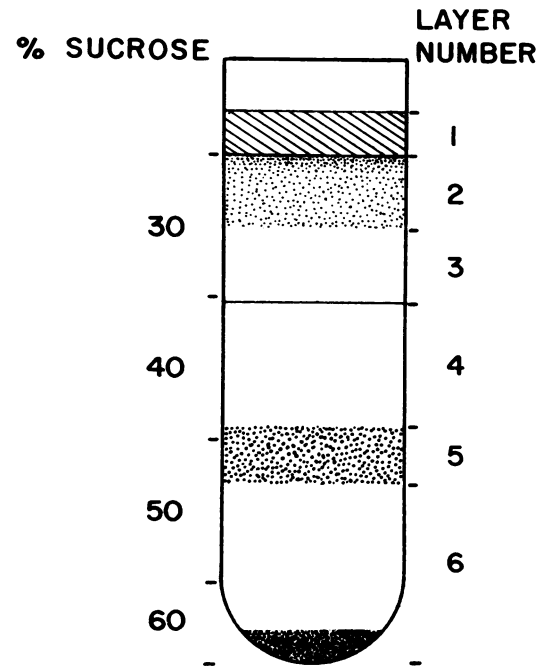

FIG. 1. *Distribution of sonic extract of Anaplasma marginale-infected erythrocytes after density-gradient sedimentation in sucrose.*

Antigen prepared from sonic extract. Suspensions of washed *Anaplasma*-infected red blood cells were disintegrated by sonic vibration for 5 min; then residual intact cells and a majority of the hemoglobin were removed by differential centrifugation. This procedure resulted in a final CF antigen preparation with highly desirable characteristics, i.e., high CF antigenic titer, absence of excessive color due to hemoglobin, and absence of anticomplementary properties in dilutions considerably lower than that of the final titer. Titers of CF antigens prepared in this manner were as much as 25 times higher than those of standard stromatal antigens when tested according to protocol. When tested with anti-*Anaplasma* and normal sera, these antigens were found to be highly specific. Antigenic material was not removed by washing the antigen in buffer to remove hemoglobin, since the supernatant fluid from the high-speed sedimentation procedures did not possess CF activity.

Density-gradient sedimentation. A schematic representation of a typical sedimentation experiment is shown (Fig. 1). Layers were removed in descending order and designated 1 through 6.

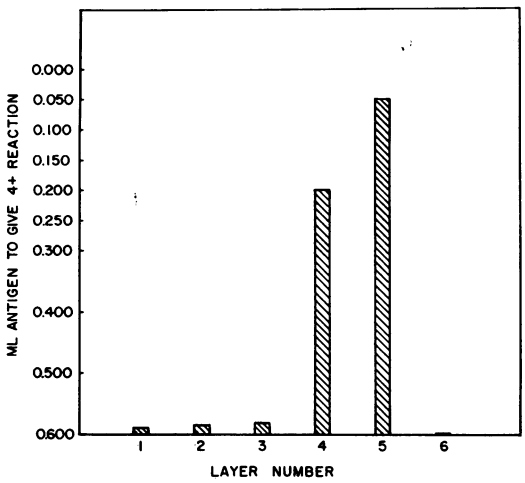

FIG. 2. *Distribution of Anaplasma marginale complement-fixing antigenic activity in fractions of sonic extract of infected erythrocytes separated by density-gradient sedimentation in sucrose.*

Layer 1 contained low-density material which was amorphous in nature as determined by examination of stained preparations. Particulate material, such as AB or cellular debris, was not observed in layers 1, 3, and 4. Layer 5, a very compact layer possessing the highest CF titer, consisted of a light-brown material. When smears of this layer were stained and examined by light microscopy, they were found to contain very high numbers of AB and considerable amorphous acidophilic- and basophilic-staining material. Electron microscopy confirmed the fact that the layer contained large amounts of amorphous material and AB. Layer 6 contained a pellet consisting of acidophilic-staining stromatal material. This layer contained no CF activity. The distribution of CF activity in the gradient layers is indicated (Fig. 2). The fraction showing highest antigenic activity (layer 5) was relatively free from hemoglobin and, when prepared in the proper dilution for the CF test, was only slightly opaque as compared with stromatal antigens ordinarily employed in the CF test for anaplasmosis. Anticomplementary activity was not encountered. Recycling of layer 5 resulted in a preparation containing AB in a higher state of purity, but an increase in CF activity was not observed. Sucrose had no apparent effect on antigenic activity.

The relative CF activity of these preparations when used as *Anaplasma* CF antigen was as follows: standard *Anaplasma* CF antigen (supplied by Technical Services, U.S. Department of Agriculture, Agricultural Research Service, Animal Disease Eradication Division), 1:10; CO_2-water precipitated stromata, 1:10; fraction corresponding to stromin, 1:10; fraction corresponding to elinin, 1:320; CF antigen prepared from sonic extract, 1:250.

DISCUSSION

Fractionation of stromata prepared from *Anaplasma*-infected erythrocytes showed that the CF antigenic substance was present in the fractions corresponding to stromin and elinin. The retention of all CF antigenic activity by the fraction corresponding to stromin is not particularly significant, except that it demonstrates the relative stability of the CF antigen in an alkaline solution. In addition, stromatal CF antigens may be cleared of almost all of the hemoglobin by this treatment so that color no longer interferes with the determination of the degree of hemolysis in the CF test. This has been one of the major problems with stromatal preparations of the type presently used for routine diagnosis. There is an apparent loss of specificity upon extraction of ether-soluble lipids to produce the fractions corresponding to elinin. In addition, the loss of CF activity which results when all lipids are removed by extraction with alcohol-ether or methanol-chloroform indicates that the antigen has an active lipid moiety. Neither the lipid extracts nor the protein residue retained CF antigenicity. These characteristics present evidence that the antigen is lipoprotein in nature. The possibility of denaturation of the antigenic substance by these procedures has been considered. However, purification of blood group antigens by the same methods has been successful (Moskowitz et al., 1950).

Fluorocarbons are used for isolation of virus particles to provide greater control over the specific gravity and viscosity of the isolating medium (Gessler et al., 1956*a*). In virus isolation methods, such solvents allow separation of the nucleoproteins of viruses from nonviral proteins and lipids. According to Gessler et al. (1956*b*), the principle involved is a preferential wetting or attraction of the surface of the viral particle by water, while nonviral protein particles and lipids are preferentially attracted by the fluorocarbon

solvents. This method was applied to homogenates of *Anaplasma*-infected erythrocytic stromata as a possible means of obtaining CF antigenic material of high purity and activity. The factors responsible for failure to obtain fractions which were antigenically active when stromata were homogenized and extracted with Genetron 113 are not clear. However, similar treatment of sonic extracts of the stromata resulted in an aqueous layer which contained CF-active material. This is probably due to inadequate homogenization or lack of disintegration of erythrocytic stromata; as Gessler et al. (1956*b*) pointed out, a very thorough mincing and homogenization of tissue is important. Sonic treatment, therefore, apparently liberated more of the antigenic substance in the form of small particles which remained in the aqueous layer of lower density than the Genetron 113. The absence of anticomplementary activity in the aqueous layer was probably accounted for by the removal of this activity in the dense stromatal material separated at the interface by the centrifugation process. Lipid extraction by the fluorocarbon solvent probably occurred, and antigenic activity may have been decreased by the removal of lipid. For example, Halonen et al. (1958) found that fluorocarbons reduced the specific reaction of ECHO CF antigens. The addition of serum aided in preventing loss of specific activity. Another possible explanation for low titers in the aqueous layer is that CF activity is not completely extracted from the stromatal material even by multiple treatments.

Disintegration of *Anaplasma*-infected erythrocytes by sonic vibration followed by the removal of residual intact cells and hemoglobin by differential centrifugation resulted in a preparation with high CF antigenic activity. Titers ordinarily obtained with standard stromatal antigens range from 1:10 to 1:15, whereas the antigens prepared by this method possess titers as high as 1:250 when tested with identical anti-*Anaplasma* sera. Moreover, the complete absence of red color due to hemoglobin and lack of significant anticomplementary activity are very encouraging. Preliminary testing of numerous known anti-*Anaplasma* and normal sera indicates that the antigen preparation has a high degree of specificity. When sera of known antibody titers were tested with standard and experimental antigens, the titers were found to deviate no more than one dilution, if any. Such an antigen would lend itself to use in a micro-CF test for anaplasmosis.

Density-gradient sedimentation of sonically disintegrated preparations of *A. marginale*-infected erythrocytes was also useful in fractionation of CF antigens. The antigens obtained by this method contained very little color, since most of the hemoglobin did not migrate with the CF-active fractions during the sedimentation process. The fractionation resulted in the preparation of an antigen of higher CF titer than that of standard stromatal antigens but lower than those produced by differential centrifugation of sonic extracts as previously described. The lower titers were encountered in the fractions obtained by the density-gradient method because CF activity was contained in more than one fraction. Much of the inactive cellular debris separated at a different rate than the active portion. This probably accounted for the lack of anticomplementary activity in the antigenically active fractions. These results show that density-gradient sedimentation can be employed to prepare *Anaplasma* antigens from the sonic extract of infected red blood cells.

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