Characterization of an Equine Infectious Anemia Antigen Extracted from Infected Horse Spleen Tissue

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The spleens of horses infected with equine infectious anemia contain an antigen that is useful for a diagnostic immunodiffusion test. This antigen was extracted from the spleen by homogenization of the tissue, centrifugation, and precipitation from the supernatant fluid at 50% saturation with (NH₄)₂SO₄. The antigen was purified by subjecting it to two cycles of electrophoresis in a continuous free-flow electrophoresis cell and finally filtering through a column of Sephadex G-200 gel. The antigen was found to be a small protein with a molecular weight of 27,500 and sedimentation coefficient of 2.1S. Its density was about 1.18 and its isoelectric point 5.8. At 45 to 50 C, it coagulated, losing its antigenicity. The antigen was useful for assaying antibody in the serum from infected horses by using the complement fixation test or the immunodiffusion test. Complement-fixing antibodies were found to be more transient than the precipitating antibodies.

Previous work from this laboratory (5) reported the development of an immunodiffusion reaction that has been established as useful for the diagnosis of equine infectious anemia (EIA). Essentially, the procedure consists of an antigen extracted from the spleen of horses in the early acute phase of EIA infection reacting with antibodies in the serum of horses in somewhat later stages of the disease. The antigen used in this reaction must be extracted from the spleen of horses exhibiting the acute symptoms of EIA; spleens taken at other stages of the infection do not yield adequate antigen. The specificity of this test for EIA has been widely confirmed by several diagnostic laboratories. Three other reports of immunodiffusion reactions for EIA have been published, the most recent of which is that of Nakajima and Ushimi (10) utilizing a purified virus preparation as antigen. An earlier paper by Livingston et al. (9) reported the measurement of a circulating antigen in the serum of infected horses by using an antibody reagent produced by inoculating sheep with cell-free culture fluids from EIA-infected horse leukocytes. There is no apparent relationship between the spleen antigen and this latter test of Livingston et al., and the relationship with the Nakajima et al. purified virus antigen has not been established. The third report of an immunodiffusion reaction in EIA was described by Saxer and Fuentes in several papers (11–13). They reported using an antigen in the pancreas of infected horses. It has not been determined whether this is the same reaction as we report here. In our hands, the reaction between pancreas antigen and serum from infected horses appeared as a diffuse, cloudy line with serum from both normal and infected horses. This report contains a description of methods of purification and characterization of the infected spleen antigen and a discussion of its usefulness in the complement fixation reaction.

MATERIALS AND METHODS

Antigen preparation. Spleens were taken from horses infected with the Wyoming isolate of EIA virus 8 to 11 days after inoculation and after 3 to 5 days of high fever. The pulp was minced, frozen and thawed, mixed with an equal volume (w/v) of phosphate-buffered saline (pH 7.2), and homogenized in a VirTis homogenizer. The homogenate was centrifuged at 27,000 × g for 30 min, and the precipitate was discarded. The supernatant fluid was brought to 50% saturation with an equal volume of saturated (NH₄)₂SO₄ at 5 C. The mixture was centrifuged again at 27,000 × g for 1 hr, and the supernatant fluid was discarded. The precipitate was dissolved in a minimum amount of phosphate-buffered saline and centrifuged at 105,400 × g for 30 min. The supernatant fluid contained the concentrated crude antigen.

The crude antigen was considerably purified by cycling it twice through a free-flow electrophoresis apparatus (model FF4, Brinkmann Instruments, Inc., Westbury, N.Y.) at a density of 1.18. After the final cycle, the antigen was precipitated with (NH₄)₂SO₄ at 50% saturation and redissolved in distilled water.
Inc., Westbury, N.Y.) in which the antigen was continuously fed into a chamber at a rate of 1.7 ml per hour. Under these conditions, the antigen was subjected to an electrical field of 200 mV (900 kV) for about 10 min. The separation chamber buffer was 0.08 M tris(hydroxymethyl)aminomethane (Tris)-citrate (pH 8.6), and the temperature was regulated at 10 C. The fractions containing antigen were combined, dialyzed, lyophilized to concentrate, and then recycled in the same manner. The antigen was concentrated by lyophilization a second time and passed through a column (2.5 by 100 cm) filled with Sephadex G-200. The antigen (0.5 ml) was applied by ascending flow, and filtration was carried out with Tris-0.5 M NaCl buffer at pH 7.2. The flow rate was 0.5 ml/min, and the effluent fractions were continuously monitored at 280 nm with an LKB Uvicord with recorder. The samples containing antigenic activity, as determined in the immunodiffusion test, were dialyzed, concentrated by lyophilization, and used in the following experiments.

Density gradient. A cesium chloride gradient was prepared in 5-ml tubes for use in density gradient experiments which used a swinging bucket rotor (SW 50, Beckman Instruments, Inc., Fullerton, Calif.). The linear gradient ranged in density from 1.05 to 1.26 g/ml. EIA antigen (0.5 ml) was layered on top of the gradient and centrifuged at 114,000 Xg for 16 hr. Fractions of 0.5 ml were taken by puncturing the bottom of the cellulose tube, and the presence of antigen was determined by immunodiffusion and complement fixation techniques after dialysis against phosphate-buffered saline to remove the CsCl.

Ultracentrifugation measurements. Sedimentation velocity method of measuring the sedimentation coefficient under standard conditions was followed. A standard double-sector cell was used with the antigen concentration adjusted to an optical density reading at 280 nm of about 0.500. The velocity was set at 52,000 rev/min, and sedimentation was followed by the ultraviolet scanner. Vp was assumed to be 0.740, and the sedimentation coefficient was calculated and corrected for standard conditions. The conventional sedimentation equilibrium method was used to measure the molecular weight of the antigen. The speed was set at 35,000 rev/min until equilibrium was reached as determined by ultraviolet scanning. Calculation of the molecular weight was by the Svedberg equation (4).

Isoelectric point. The isoelectric point was determined with the use of ampholine electrofocusing equipment (ampholine electrofocusing, LKB Instruments, Inc., Rockville, Md.). A pH gradient was prepared of carrier ampholytes with a range of pH 4 to 7. A 0.5-ml portion of the sample was distributed throughout the gradient and 500 v was applied across the column for 72 hr. Fractions containing 0.5 ml were drawn off the bottom of the column, and the pH and presence of antigenic activity of each were determined.

Heat stability. Samples (0.5 ml) of the purified antigen were heated in a water bath at the following temperatures: 37, 45, 50, and 60 C for 30 min. The samples were cooled immediately and assayed in the plate immunodiffusion test for antigenic activity.

Membrane filtration. The purified antigen was passed through a 10-nm membrane filter (Millipore Corp., Bedford, Mass.), checked for antigenicity by immunodiffusion, and inoculated into a pony as a test for separation of infectivity from antigenicity.

Complement fixation. The five 50% hemolytic unit complement fixation procedure as described in Kabat and Mayer (8) was followed. The serum from horses experimentally infected with the same strain of virus as the antigen was assayed for antibodies. In addition, sera from field cases of EIA were included to determine its usefulness for diagnosis.

Immunodiffusion. Microscopic slides were overlaid with a thin layer of 1.0% Noble special agar in borate buffer (0.5 M, pH 8.6, + 0.15 M NaCl), and a plastic template containing the wells was placed over the agar. The wells in the template were 3 mm in diameter, 5 mm deep, and 5 mm apart. The precipitin line usually appeared within 24 to 48 hr at room temperature.

RESULTS

Electrophoresis. The optical density at 280 nm of each sample is represented in Fig. 1. Notice that most of the ultraviolet-absorbing material was deposited around fraction 10. The EIA antigen, on the other hand, was found by immunodiffusion and complement fixation to be deposited in samples 55 through 65, under the second peak. The point of application of the antigen was almost directly above the antigen peak, indicating that it is only weakly negatively charged at pH 8.6. A recycling of fractions 55 through 65, under identical conditions, gave the results shown in Fig. 2. The antigen was found in nearly the same position as the previous electrophoresis run.

Gel filtration. The last step of purification was gel filtration. The antigen from electrophoretically

![Fig. 1. Electrophoretic migration of spleen antigen in free-flowing electrophoresis chamber. Antigen was applied continuously at a port above tube no. 60.](http://iai.asm.org/Downloadedfrom)
recycled fractions 60 to 66 was applied at the bottom of a 100-cm Sephadex G-200 column separated by ascending elution. There was one heavy, early peak that contained no antigenic activity and a much smaller peak containing all of the antigenic activity. This antigen, when set up opposite antisera from EIA-infected horses, resulted in a single line of microimmunodiffusion plates. Figure 3 shows the precipitin line of several antisera with the antigen in the middle well.

**Density gradient.** Six cesium chloride gradients were run and the fractions were obtained by puncturing the tubes. Density was measured by a refractometer (Bausch & Lomb, Abbe-3L model), and antigenicity was determined, after dialysis, in the immunodiffusion and complement fixation tests. The results are shown in Table 1.

**Sedimentation rate and molecular weight.** Several determinations gave values of corrected sedimentation coefficient, $S_{20w}$ of 2.1 and a molecular weight of 27,500.

**Isoelectric point.** Analysis of fractions ranging in pH from 4 through 7.8 indicated that the antigen was located in a band at the pH 5.8 level.

**Heat inactivation and membrane filtration.** The results of heat inactivation and membrane filtration studies indicated that antigenic activity was lost at 60 and 50°C and was considerably reduced at 45°C. The antigen passed through the 10-nm filter and was noninfectious for a pony. Unfiltered antigen was infectious for a pony.

**Complement fixation.** Assays were made on a

### Table 1. Cesium chloride density gradient centrifugation of spleen-derived equine infectious anemia antigen

<table>
<thead>
<tr>
<th>Fraction no.</th>
<th>Density</th>
<th>Antigenic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.22</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.21</td>
<td>++</td>
</tr>
<tr>
<td>3</td>
<td>1.19</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.18</td>
<td>++</td>
</tr>
<tr>
<td>5</td>
<td>1.16</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>1.14</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1.11</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1.08</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1.06</td>
<td></td>
</tr>
</tbody>
</table>

- Fractions (0.5 ml) from bottom to top of tube.
- Given as $S_{20w}$.
- Antigenic activity determined by immunodiffusion reaction.

### Table 2. Complement fixation assay of antibodies in serum of an equine infectious anemia-infected horse

<table>
<thead>
<tr>
<th>DPI</th>
<th>Titer</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>1:10</td>
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<tr>
<td>31</td>
<td>1:80</td>
</tr>
<tr>
<td>61</td>
<td>1:20</td>
</tr>
<tr>
<td>90</td>
<td>1:10</td>
</tr>
</tbody>
</table>

- The five 50% hemolytic unit complement fixation test of Kabat and Mayer (8). A 0.4-ml antiserum dilution, 0.5-ml guinea pig complement, and 0.4-ml antigen dilution in an overnight fixation at 4°C were used.
- Days postinoculation.
- Degree of lysis was estimated visually, and titer was taken as the highest dilution of serum that, when mixed with two units of antigen, resulted in 50% lysis.
large number of sera from EIA-infected horses. In many of the sera that exhibited high precipitating antibody in the immunodiffusion test, the complement fixation titers were low or even negative. Table 2 contains the complement fixation titers of an experimentally infected horse. Sera taken later than 90 days, in other horses, often were negative.

DISCUSSION

It is evident that there is a small antigen, specific for EIA, in the spleen of infected horses. Precipitating antibodies against this antigen have been detected in the serum of infected horses starting at about 14 days postinoculation, and their presence has been detected continually after all clinical signs of the disease have disappeared. In addition, complement-fixing antibodies have been detected early after infection is initiated, reaching peak titers around a month after infection, and dropping to a low level or disappearing. Apparently a transient stimulation of antibodies of an immunoglobulin class that fixes complement occurs slightly before or concurrently with a more lasting antibody of a class of immunoglobulin that precipitates with antigen but does not fix complement. This common phenomenon with virus infections was reported by Nakajima and Ushimi (10) with their EIA virus antigen and antisera from infected horses.

Earlier work (5) has established that extracts from normal spleens do not show antigenic activity with antibodies in EIA-infected horses nor have antibodies against the antigens been found in the serum of normal horses. Likewise, normal spleen tissue extractions, subjected to electrophoresis and gel filtration as described above, have no antigenic activity nor a protein of the characteristics described here.

Although this study has been useful in characterizing the spleen antigen, the significance and origin of this nonviral antigen are unknown. Several workers have already reported the use of antigen from the spleen of infected horses (1, 11) in serological tests, and others have found that such reactions were nonspecific (2, 3, 7). The reason for the nonspecificity is unclear in view of the clearly specific nature of this antigen in our hands. On occasion, with crude spleen antigen, a second non-EIA antigen has been obtained that gives a reaction which is nonspecific and possibly a horse tissue antigen-antibody reaction. These workers may have been using infected spleens taken at a time when this antigen was predominant. The line of identity in the immunodiffusion test with purified spleen antigen has been useful in demonstrating nonspecific reactions, whereas the complement fixation test could not make this distinction and may have led to positive results that were obtained with normal horse sera.

The antigen we have described has exhibited some similarities with the "virus infection-associated antigen" Cowan and Graves (6) describe for foot-and-mouth disease. They speculate the possibilities that such antigens may be normal cell components that are altered as a consequence of infection or possibly an enzyme involved in virus replication.

This antigen has been demonstrated to be noninfective by inoculation into susceptible horses with a 10-nm filtered preparation. Ultraviolet spectrophotometric scanning gives only one extinction peak (at 280 nm). In addition, its small size of 2.1S and 27,500 molecular weight rule out the virus antigen that has been reported to fall between 110 and 120S (10).

LITERATURE CITED