Purification of Equine Infectious Anemia Virus Antigen by Affinity Chromatography

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Affinity chromatography was performed to obtain highly purified antigen from equine infectious anemia (EIA) virus. After crude antigen was concentrated by polyethylene glycol precipitation of culture fluids from equine dermal cells persistently infected with EIA virus, and after the virus was disrupted with ether, it was added to a column of cyanogen bromide-activated Sepharose 4B to which EIA-specific antibody had been conjugated. The antigen was effectively released from the column with 5 M MgCl₂ and proved to be highly purified. Passive hemagglutination tests on sera from EIA infections were carried out, using the purified antigen. Results indicated that the passive hemagglutination test with the antigen was a specific laboratory test with high sensitivity for EIA infection.

Group-specific antigen derived from equine infectious anemia (EIA) virus has been widely used for the diagnosis of EIA, especially in immunodiffusion tests (1, 6, 9, 10). However, the antigen generally contains contaminants other than the viral components, such as bovine serum or cell debris, and this occasionally causes nonspecific reactions. Therefore, purified antigen is desirable, not only to avoid nonspecific reactions, but also for developing more sensitive serological tests than immunodiffusion.

The present paper describes purification of the antigen by affinity chromatography and preliminary trials of passive hemagglutination (PHA) in EIA, using the purified antigen.

MATERIALS AND METHODS

Preparation of crude antigen. Culture fluids were obtained from equine dermal cells that were persistently infected with EIA virus and kindly provided by W. A. Malmquist, National Animal Disease Laboratory, Ames, Iowa (6). The cells were grown and maintained in Eagle minimal essential medium, containing 10 and 5% bovine serum, respectively. The culture fluid was harvested at 5- to 7-day intervals, and the virus was concentrated with polyethylene glycol no. 20,000 at a concentration of 4%. The concentrated virus was then suspended to $\frac{1}{30}$ of the original volume in phosphate-buffered saline (pH 7.4) containing 0.1% Tween 80 and treated with 2 volumes of ethyl ether, as previously described (10).

Affinity chromatography. The immunoglobulin G-rich fraction was prepared from infected horse serum by salting out with 1.22 M ammonium sulfate. Approximately 20 mg of the protein was bound to 1 g of dried cyanogen bromide-activated Sepha-

rose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) by mixing for 4 h at room temperature in 0.1 M carbonate buffer (pH 9.0). The gel conjugated with EIA-specific antibody was put into a column 5.0 cm in length and 2.5 cm in diameter, and the crude virus antigen, dialyzed against 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.0) containing 0.5 M NaCl, was added to the column. After incubation, the column was washed with the same buffer until the optical density of the effluent decreased less than 0.01 at 280 nm. The antigen bound to antibody was then eluted from the column. The released antigen was concentrated and dialyzed successively in a collodion bag (Sartorius-membrane filter GmbH; Göttingen, West Germany) against 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.0) and phosphate-buffered saline.

Titration of antigen and determination of its specific activity. Antigenic titer was determined by the complement fixation test using the microtiter technique (14). The titer was expressed as the reciprocal of the highest dilution showing a positive reaction. Specific activity was expressed as the antigenic titer per milligram of protein in the complement fixation test.

Immunodiffusion reaction. The procedure for the immunodiffusion reaction has been previously described (9).

Protein measurement. Protein concentration was determined by the method of Lowry et al. (5). Bovine serum albumin (Cohn V fraction) was used as a standard.

RESULTS

Standardization of procedures for affinity chromatography. Preliminary studies indicated that the specific antibody titer of the immunoglobulin G fraction, the temperature and

time of incubation of antibody and antigen on the column, and the solution used for dissociation of antigen from the antibody were especially important in affinity chromatography. For the antibody against EIA virus, the immunoglobulin G fraction was prepared from infected horse serums that had a precipitating antibody level of more than 1:32 (8) and was conjugated to cyanogen bromide-activated Sepharose 4B as described in Materials and Methods. Incubation periods and temperatures for producing antibody-antigen complexes in the column and reagents used for dissociation of antigen were tested under various conditions and combinations. As a result, the antigen was shown to conjugate completely with antibody for 18 h at 4°C. MgCl_2 , 5 M, was found to be the best solution for releasing antigen from the column, as compared with glycine hydrochloride buffer (2, 7), 1.5 M NaCl (13), and 5 M NaI (3).

Based on these results, 5-ml amounts of crude antigen with titers of 1:32 to 1:512 and protein concentrations between 4 and 10 mg/ml were added to columns in which approximately 10 ml of gel conjugated with antibody had been packed. After incubation for 18 h at 4°C, contaminating proteins were removed by washing with 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.0). Then the antigen was eluted with 5 M MgCl_2 at room temperature.

One example of the antigen elution profile is presented in Fig. 1. The Sepharose coupled with antibody could be used at least five times without loss of efficiency.

Purity of the antigen. By the procedure described above, purity of the antigen obtained was increased 50 or more times over that of the crude antigen (Table 1). Recovery of the antigen was almost complete. To confirm the purity of the antigen, it was reacted with anti-bovine serum produced in horses, since bovine serum was considered to be the major contaminant in the starting crude antigen. No reaction was observed between the antigen and the horse anti-bovine serum in immunodiffusion reactions (Fig. 2). These results indicate that the antigen

obtained by affinity chromatography is free of bovine serum contaminants.

PHA test using the purified antigen. PHA reactions were performed, using the purified antigen. Sheep erythrocytes fixed with Formalin and treated with tannic acid were sensitized with the purified antigen in phosphate-buffered saline (pH 6.4) for 30 min at 37°C. Then, serum samples from horses experimentally infected with EIA virus were reacted against the erythrocytes. Agglutination occurred only with EIA-infected horse serums.

FIG. 1. Elution profile of antigen from the column in affinity chromatography. A 5-ml amount of crude antigen was added to a column with dimensions of 5.0 by 2.5 cm. Chromatography was carried out at room temperature at a flow rate of 30 ml/h. The eluate was collected into 3-ml fractions with a fraction collector. First, the contaminating proteins were removed from the column with 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.0). Then, purified antigen was eluted with 5 M MgCl_2 solution. The elution pattern and concentration of MgCl_2 in the eluate are magnified in the figure shown in the upper right. The antigen was eluted in fractions 49 to 52, with a peak at fraction 50. Optical density (OD) was derived from measurements on 10-fold dilutions of fractions.

TABLE 1. Purification of virus antigen by affinity chromatography

Expt no.	Antigen								Recovery (%)
	Crude				Purified				
	Vol (ml)	CF ^a titer	Protein (mg/ml)	Sp act	Vol (ml)	CF titer	Protein (mg/ml)	Sp act	
1	5	1:64	4.8	13.3	5	1:64	0.092	695.6	100
2	5	1:64	5.3	12.1	5	1:64	0.078	810.0	100

^a CF, Complement fixation.

One example is shown in Fig. 3. The horse (no. 521) was experimentally inoculated with the P337 strain of EIA virus and had severe fever 13 and 35 days after inoculation. As can be seen in the figure, hemagglutinating antibody was demonstrated in the serum samples obtained

26 days or later after inoculation. Antibody levels by PHA were 40 to 80 times higher than those determined by immunodiffusion tests. Immune responses of a horse (no. 580) to EIA virus infection were measured sequentially by PHA and precipitating antibody titers (Fig. 4).

FIG. 2. Immunodiffusion reaction between purified antigen and horse anti-bovine serum. Antigens before purification (CAG) and purified by affinity chromatography (PAG) were placed in the central wells of (A) and (B), respectively. Horse serum infected with EIA was used as reference positive serum (PS). Well 1 was filled with a serum sample collected from a horse with EIA. Well 2 was filled with serum collected from an uninfected horse. These two horses were inoculated repeatedly with rhinopneumonitis vaccine containing bovine serum and cultured cell debris. Nonspecific reactions observed in (A) (between CAG and samples 1 and 2) were demonstrated beforehand to be due to bovine serum and its antibody.

FIG. 3. Demonstration of PHA with infected horse sera. Positive reactions were recognized in sera obtained 26 days or later after inoculation.

FIG. 4. Development of antibodies in a horse experimentally inoculated with equine infectious anemia virus. Antibodies measured by PHA (○) and by agar gel immunodiffusion (AGI) (△) were determined. Development of both types of antibodies coincided well, and both remained positive until at least 120 days after infection.

Appearance of PHA and precipitating antibodies coincided well, and both types of antibody were detectable 25 days after inoculation. Antibody levels in this horse ranged from 40 to 160 by PHA tests and from 2 to 4 by immunodiffusion tests.

DISCUSSION

There have been several reports on purification of EIA virus antigen by Sephadex G-100 column chromatography (16), free-flow electrophoresis, density gradient centrifugation, or gel filtration with Sephadex G-200 (12). We have also attempted some of the methods described above, but the results obtained were not satisfactory from the standpoints of recovery and purity of antigen.

Affinity chromatography has been used for purification or isolation of a variety of proteins, including enzymes. We have, however, seen only a few reports on purification of virus or virus components (3, 4, 11, 15). In the present paper, the group-specific antigen of EIA virus (9) was successfully purified by affinity chromatography. The most important factor for effective recovery of the antigen was the use of the specific precipitating antibody-rich immuno-

globulin G fraction. The antigen was bound to the antibody at 4°C overnight and released completely with 5 M MgCl₂. We are now using the antigen for PHA tests in EIA to diagnose cases that cannot be definitively diagnosed by immunodiffusion tests. Standardization of the hemagglutination test is still under investigation; however, preliminary results showed the test to be very sensitive, as compared with the immunodiffusion test.

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