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Preparation and purification of horse antihuman lymphocyte globulin (ALG)

Kohki Konomi, M.D., Ph.D.*
Division of Research

Sharad D. Deodhar, M.D., Ph.D.
Division of Pathology

In recent years great interest has been aroused in the immunosuppressive activity of antilymphocyte serum (ALS) or antilymphocyte globulin (ALG). The immunosuppressive activity of ALG has been demonstrated in various experimental models, and it has been claimed that ALG may be the most potent immunosuppressive agent yet known. ALG has also proved to be helpful as an adjunct in the immunosuppressive regimen for human renal transplantation. Starzl and associates[1] reported that with use of ALG prepared by the ammonium sulfate fractionation method there was 95 percent one-year survival among patients who underwent renal transplantation. The major complications of ALG therapy noted by these authors included severe pain at the site of ALG injection, and a low but significant incidence of anaphylactic reactions.

Recently we investigated the problems of the preparation, purification, and clinical use of horse antihuman lymphocyte globulin as related to the Cleveland Clinic renal transplantation program. By using a DEAE-Sephadex batch fractionation method for the purification of ALG we have been able to obtain the IgG component of horse serum with a more than 95 percent purity. We have used this material in the treatment of 41 patients during the last year; this report summarizes our experience with respect to the methods of preparation and purification of ALG.

Materials and Preparation of ALG

Spleen cell antigen. The antigenic preparation used for the immunization of horses consisted of a spleen cell suspension prepared in the following manner under sterile conditions. Excised human spleens, of patients with terminal renal failure who were undergoing bilateral nephrectomy and splenectomy in preparation for the renal homotransplants, were perfused with large amounts (4 to 5 liters) of cold Ringer's lactate solution until the perfusate emerging from the splenic vein was relatively clear and free of erythrocytes. The spleens were then put through a meat grinder and the minced splenic tissue was put through con-

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* *Fellow, Division of Research (Immunology).*

secutive stainless steel sieves of various meshes from 20 to 40 to 80. The cell suspension collected in sieves from meshes 40 and 80 were used in the final preparation. The cell suspensions were diluted with saline to a cell concentration of from 50 to 100 million cells per milliliter.

Animals. Horses and ponies have been used for the purposes of immunization and both have proved to be equally satisfactory.

DEAE-Sephadex A-50, capacity 3.5 ± 0.5 meq per gram, particle size from 40 to 120 μ, was obtained from Pharmacia Fine Chemicals, Inc.

Immunization. About 100 ml of spleen cell suspension, prepared as just described, was injected subcutaneously in the appropriate horse at different sites. An injection was administered once a week for the first four weeks, then once every other week for the next four weeks, and finally once a month thereafter. In each pony, about 60 ml of the spleen cell suspension was injected at any one time. In certain cases, the spleens were perfused, kept frozen, and then the cell suspension was prepared after the frozen spleens had been thawed. The horse sera were titrated for leukoagglutinin titers from time to time, and when the titer had reached a level in the range of 1:4000 to 1:8000, which usually took about three to four months, from 2 to 3 liters of blood was drawn from each horse once per month; the sera were isolated, heated at 56 C for 30 minutes, and finally stored at −20 C.

PURIFICATION OF ALG

Absorption with human erythrocytes and plasma. To remove the human erythrocyte and plasma protein antibodies from the horse crude ALS, the following procedure was used. To 100 ml of crude ALS was added 5 ml of human plasma that had been heated to 56 C for 30 min. The mixture was stored overnight at 4 C. It was then centrifuged at 5000 rpm for 30 min at 4 C. To the supernatant, 4 ml of packed, human AB-positive erythrocytes was added, and the mixture was stirred gently for 4 hr at 4 C. The packed erythrocyte preparation was obtained from the same person whose plasma was used in the initial absorption. The erythrocytes were washed with saline four or five times to remove the buffy coat and serum proteins as completely as possible. After absorption, the packed erythrocytes were removed by centrifugation at 2000 rpm at 4 C for 10 min. This procedure was repeated by another absorption with fresh packed erythrocytes, also for 4 hr. The supernatant obtained after the second absorption was then purified by the DEAE-Sephadex batch fractionation procedure.

Preparation of DEAE-Sephadex. The DEAE-Sephadex was prepared essentially like that described by Perper and associates.[2] DEAE-Sephadex A-50 was first washed extensively with distilled water and de-fined. It was then washed with 0.5 N sodium hydroxide, then with distilled water, then with 0.5 N hydrochloric acid, and with distilled water again, and finally with 0.05 M phosphate buffer (pH 7.85). The gel was allowed to dry by suction on the Buchner funnel;

the final product weighed from 1200 to 1300 g, starting with 80 g of DEAE-Sephadex.

Purification procedure. To 1000 ml of the absorbed crude serum, 400 g (wet weight) of DEAE-Sephadex was added, and the mixture stirred at 4 C for 1 hr and then filtered through a Buchner funnel. To the filtrate, an additional, fresh 400 g of DEAE-Sephadex was added, and the same process was repeated three successive times. Treatment in this manner for at least four times is essential for obtaining a highly purified IgG fraction of γ-globulin. The final filtrate was dialyzed against 4 liters of 0.03 M phosphate buffer (pH 7.3) overnight, and the dialysate was then lyophilized.

The dry powder was dissolved in distilled water and the protein concentration adjusted to 4 g per 100 ml, the final molarity of the phosphate buffer was between 0.12 and 0.15 M. Merthiolate was added in the ratio of 1 mg per milliliter (0.1 percent), and the final preparation was stored in small aliquots at −20 C. The purity of the final preparation was evaluated by determination of paper electrophoretic pattern, immunoelectrophoretic pattern, protein content, pH, leukoagglutinin titer, erythrocyte agglutinin titer, and by ultracentrifugation. Sterility was assessed on the basis of aerobic (blood agar plate) and anaerobic (thio-tube) cultures of the final preparation. We have found, recently, that citrated plasma can be used instead of serum in the purification procedure. The overall procedure for the preparation and purification of ALG is summarized in *Figure 1.*

Leukoagglutinin and erythrocyte agglutinin titers. Leukoagglutinin titer against human buffy coat cells was measured by the method of Payne[3] and Dausset[4] with slight modifications. Serial twofold dilutions of the test serum were made in buffered saline, and to each solution 0.1 ml of the leukocyte suspension was added. The tubes were then read for agglutination after incubation for 1 hr at 37 C. The maximum dilution of the ALG preparation which gave a positive agglutination (more than 50 percent of the cells agglutinated) was considered

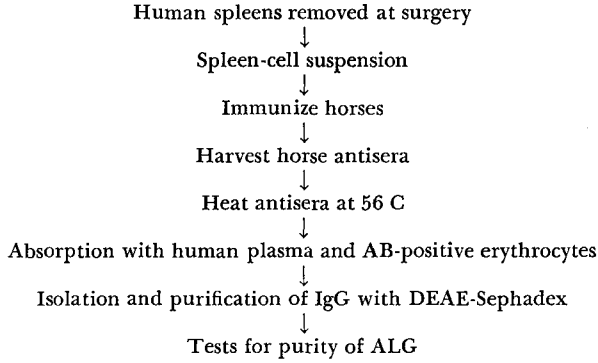

Fig. 1. Outline of preparation and purification of ALG.

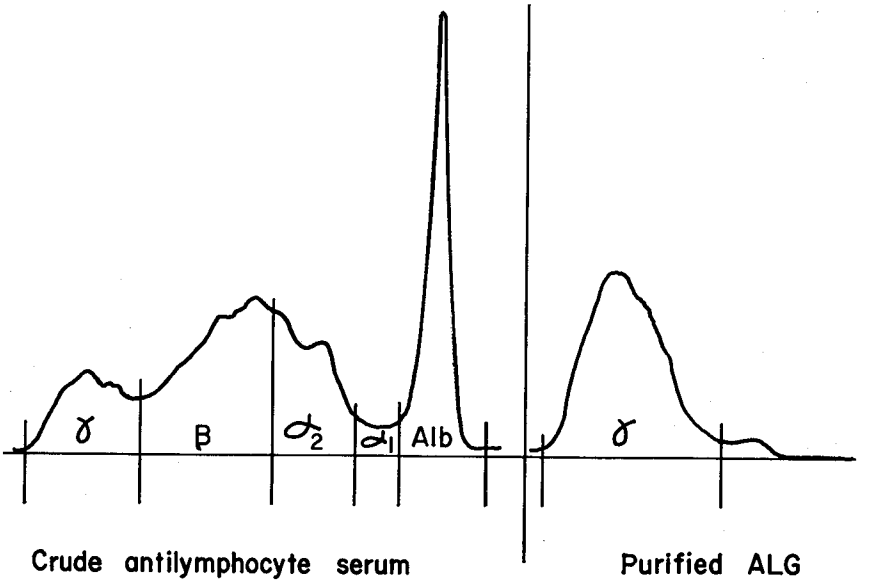

Fig. 2. Paper electrophoretic pattern of crude and of purified ALG. Electrophoresis was carried out on the Beckman paper electrophoretic system with barbital buffer (B_2) pH 8.6.

the titer of that preparation. Erythrocyte agglutinin titers against human erythrocytes were determined by adding two drops of a 4 percent erythrocyte suspension to a mixture of 0.1 ml of saline and 0.1 ml of the test material prepared by serial twofold dilutions of the ALG preparation. The tubes were then read for agglutination after incubation for 30 min at 37 C and centrifugation at 2000 rpm for 5 min.

Detection of transferrin. The final preparation was examined for transferrin content by means of the antibody agar diffusion method, with plates obtained from Hyland Laboratories, Los Angeles, California.

RESULTANT PRODUCT

The final preparation was found to be 95 percent or more γ-globulin, according to paper electrophoresis (*Fig. 2*), and also by the demonstration of a single line on immunoelectrophoresis corresponding to IgG (*Fig. 3*). Ultracentrifugal analysis also showed a single moving boundary corresponding to a protein component of Svedberg constant of 7 (*Fig. 4*). The leukoagglutinin titer of the final preparation was usually in the range of 1:2000 to 1:4000, and the erythrocyte agglutinin titer was in the range of 1:128 to 1:256. The overall recovery of IgG was in the range of 50 percent. The final pH and phosphate molarity were 7.3 to 7.4, and 0.12 to 0.15 M, respectively, and these values were considered adequate for clinical use. The various properties of the final product

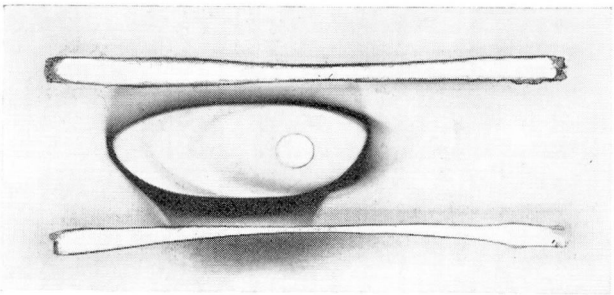

Fig. 3. Immunoelectrophoretic pattern of purified ALG. Pattern developed with ALG in the center well, and rabbit antiserum to horse serum proteins in the peripheral troughs.

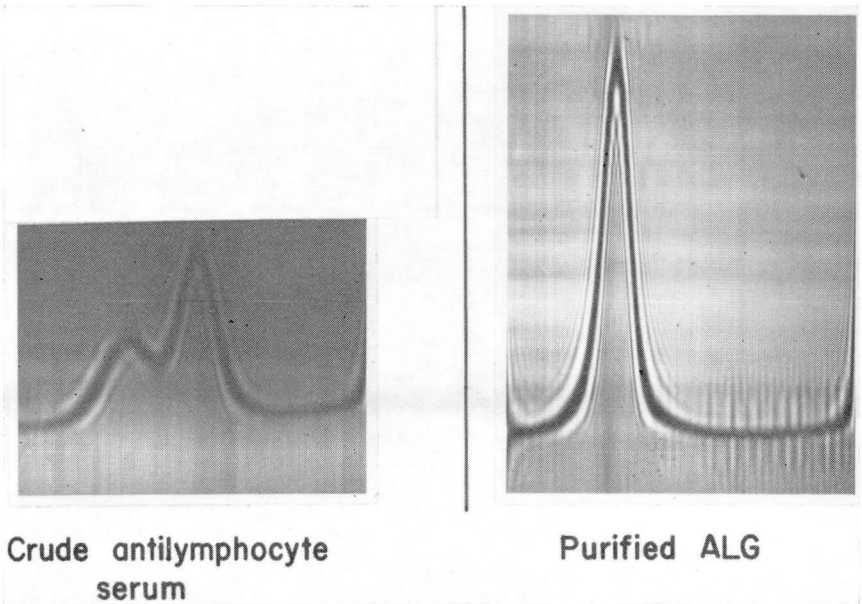

Fig. 4. Ultracentrifugal patterns of crude and of purified ALG. Pattern developed with speed, 52,640 rpm; temperature, 20 C; exposure intervals, 16 minutes; concentration used in cells, 1 percent. The single moving boundary of ALG corresponded to protein of Svedberg constant 7.

are summarized in *Table 1*. With essentially a clean technic and the addition of merthiolate to the final preparation, we have found that sterility was adequately maintained. The final product contained little or no β-globulins and transferrin, which are known to be highly immunogenic,[5] and hence their removal was a great advantage in the present purification procedure. The high purity of this material has also rendered it relatively safe for human use. Our clinical use of ALG will be presented in another report.

Table 1.—*Properties of purified ALG*

Property	Unit
Protein content	4 g per 100 ml
pH	7.3–7.4
Phosphate molarity	0.12–0.15 M
Paper electrophoretic pattern	>95% IgG
Immunoelectrophoretic pattern	Single line of IgG
Ultracentrifugal pattern	Single 7-S—fraction
Leukoagglutinin titer	1:2000–1:4000
Erythrocyte agglutinin titer	1:128–1:256

DISCUSSION

A number of procedures have been developed for the purification of γ-globulin from crude serum. Recently, Iwasaki and associates[6] reported the purification of human ALG from horse crude serum by an ammonium sulfate fractionation method and also by a DEAE-cellulose column fractionation method. The ammonium sulfate method failed to give us satisfactory results, and the final preparations were always contaminated with significant amounts of α- and β-globulins. Also, in clinical use, the ammonium sulfate preparations usually caused more pain and other local reactions than those now observed with the product obtained by the DEAE-Sephadex method. Column fractionation with DEAE-cellulose is extremely time consuming, and it does not lend itself to preparation of satisfactory amounts for clinical use on a large scale.

Recently Perper and associates[2] reported purification of the IgC component of horse antigoat ALS by the DEAE-Sephadex batch fractionation method. We have extended this method, with some modifications, to the purification of anti-human ALS, and our results have been comparable to those obtained by Perper and associates in the case of antigoat ALS. The almost complete removal of β-globulins and transferrin by the present method also offers a distinct advantage; the ammonium sulfate fractionation method, by contrast, did not remove those components effectively. Another advantage of the method we used is that large quantitites of sera can be processed rapidly and easily with a fairly high recovery (50 percent) of the IgG γ-globulin.

It should be pointed out that in the purification procedure we used, no attempt was made to purify the active, specific antibody representing antilymphocyte activity. Future efforts in this regard should be directed toward (1) isolation of the specific antibodies with the immunosuppressive effect of ALS, and (2) preparation of the active fragment of the antibody molecule that contains the immunosuppressive activity. With such purified materials it may be possible some day almost completely to eliminate the dangers of hypersensitivity reactions associated with the administration of foreign proteins such as those present in ALS.

Summary

The purified IgG fraction containing human antilymphocyte globulin has been isolated from immunized horse sera by a DEAE-Sephadex batch fractionation method. The recovery of IgG was about 50 percent, and more than 95 percent of the final product was found to be IgG, according to paper electrophoretic, immunoelectrophoretic, and ultracentrifugal analyses. The leukoagglutinin activity of the crude serum was present to a large extent in the isolated IgG fraction. The DEAE-Sephadex batch fractionation method offered the distinct advantages of the simplicity and rapidity of the procedure, the feasibility of handling large amounts, the purity of the final product, and the low incidence of local reactions at the site of injection in its clinical test use.

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