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Improvements in the preparation of heterologous antilymphocyte globulin with special reference to absorption and diethylaminoethyl cellulose batch production

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For almost two years, horse antihuman-lymphocyte globulin (ALG) has been administered intramuscularly to patients in our institutions as an adjuvant immunosuppressive agent after homotransplantation of kidneys and livers. For most of this period, the ALG was prepared by precipitation with ammonium sulfate according to the methods described by Iwasaki and co-workers.[4] The evidence is strong[9-12] that there was a resulting improvement in patient care. However, a number of annoying or alarming toxic manifestations were observed.[5, 12] These included intense pain at the injection site, fever, thrombocytopenia, skin rashes, the development of antibodies against the equine protein, and anaphylactic reactions.

From the beginning, there was reason to believe that at least some of the side reactions could be minimized by improving the ALG. For example, one possibility was that the local pain was an Arthus reaction, since the ALG contained antibodies against several human plasma proteins, particularly fibrinogen. The variable presence of antiplatelet antibodies could explain the thrombocytopenia. The precipitating antibodies developed by the patients after weeks or months of therapy were directed principally against the small amounts of alpha and beta globulins contained in the ALG[5]; rarely were there detectable precipitins against the gamma-G globulin which is thought to be the active constituent of ALG.

This report will describe efforts to improve the quality of antilymphocyte globulin. These first involved changing the techniques for preliminary absorption of the raw serum. In addition, a batch technique was developed for the extraction on a mass-production basis of pure antilymphocyte gamma-G globulin (ALGG). The resulting product was tested in canine recipients of renal homografts to be sure that it had not lost its immunosuppressive efficacy in the process of refinement. Finally, a comparable antihuman ALGG was used clinically.

METHODS

Modifications of absorption. Certain details of absorption were changed from the technique originally described by Iwasaki

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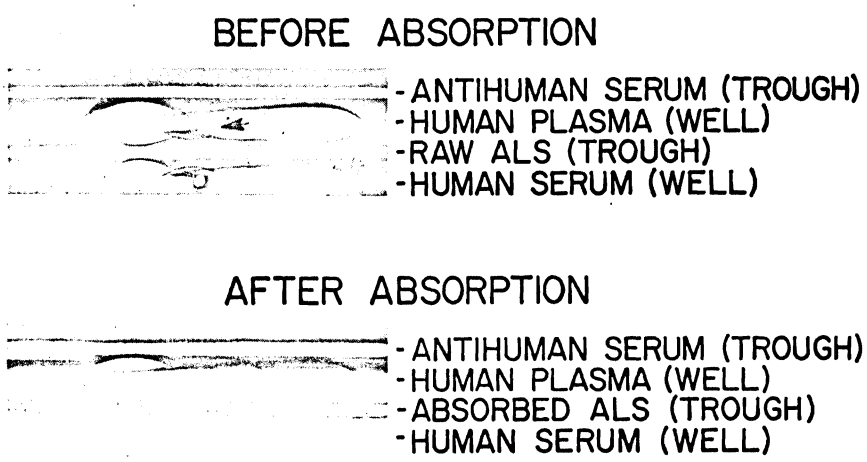

Fig. 1. Immunoelectrophoresis of immune horse serum (ALS) before and after absorption with human plasma (1 part to 10 parts horse serum), red cells, and thrombocytes. Above: Before absorption. Note the precipitin lines against most of the human plasma protein fractions including fibrinogen (arrow). Below: After absorption. All precipitin lines have disappeared except for a faint one in the alpha$_2$ region.

and associates.[4] Previously, the raw horse serum was first decomplemented by heating to 56° C., then absorbed against human red cells, and finally absorbed against pooled human serum. The decomplementation was done first, in order to avoid hemolysis of the added human red cells. The procedure had two flaws: (1) The use of serum for absorption precluded the removal of antifibrinogen (Fig. 1) and antithrombocyte antibodies. (2) The precipitation of equine antibodies directed against other human plasma proteins (Fig. 1) was made inefficient by the absence of complement at this stage.

These objections were eliminated by the following procedure. Whole blood was collected from multiple donors. EDTA was used as an anticoagulant to prevent platelet aggregation. The red cells, plasma, and platelets were then separated with differential centrifugation. The human plasma was added to the unaltered horse serum. Various volume ratios were tested after incubation for 2 hours at 37° C. and 18 hours at 4° C. With the addition of one part of human plasma to two parts of horse serum, all of the equine precipitating antibodies were eliminated. At a ratio of 1:10 the removal was not quite complete (Fig. 1). However, the 1:10 proportion was routinely used in order to prevent excess dilution of the ALS.

Before incubation, the human platelets isolated earlier were added to the mixture in quantities determined by the premeasured level of the antiplatelet activity in the ALS. In a given laboratory, an estimate of the number of platelets needed can be obtained by establishing standards such as shown in Table I. The addition of even very large volumes of platelet packs did not materially reduce the antileukocyte titer in the eventual supernatant fluid (Table I).

After centrifugation for 15 minutes at 10,000 r.p.m., the supernatant fluid was heated at 56° C. for 30 minutes. Absorption as previously described[4] was now carried out with the human red cells. Hemolysis did not occur. At the completion of the various absorption procedures, the total protein content of the horse serum had decreased by 15 to 20 percent.

Preparation of gamma-G globulin. The optimum conditions for efficient batch mixing were determined by trial and error, with the use of crude ALS. DEAE-cellulose* was added to the horse serum in amounts far in

*Cellex-D, exchange capacity 0.8 mEq. per gram, Bio-Rad Laboratories, Richmond, Calif.

Table I. *Absorption of thromboagglutinins in raw antihuman ALS with human platelets*

No. of platelets added to 2 ml. of ALS	*Thromboagglutinin titer*	*Leukoagglutinin titer*	*Lymphocytotoxin titer*
Control	1:48	1:2,000	1:36,000
1.9×10^7	1:48		
3.8×10^7	1:48		
7.5×10^7	1:24	1:2,000	1:18,000
1.5×10^8	1:12		
3.0×10^8	1:6	1:2,000	1:18,000
6.0×10^8	1:6		
1.2×10^9	1:3	1:1,000	1:18,000
2.4×10^9	1:3		
4.8×10^9	0	1:2,000	1:18,000

excess of those calculated to be necessary according to the protein content of the serum and the manufacturer's extraction assay of the DEAE; 1 Gm. of dry diethylaminoethyl (DEAE)-cellulose (wet weight 8 Gm.) was used per milliliter of ALS. The pH and molar concentration were controlled by the addition of 20 ml. of various phosphate buffers per milliliter of serum. The mixture was stirred for 3 hours at 4° C. and centrifuged at 10,000 r.p.m. for 10 minutes. The supernatant fluid was removed and concentrated to the original serum volume by ultrafiltration. It was now tested for total protein content, protein fractions, and leukoagglutinin titers. The potency per weight of protein was related to that of the original ALS by the formula:

$$\text{Potency coefficient} = \frac{\dfrac{\text{Titer ALG}}{\text{Protein concentration ALG}}}{\dfrac{\text{Titer ALS}}{\text{Protein concentration ALS}}}$$

The most efficient extraction of pure ALG was with a pH of 7.0 and a molarity of 0.01 (Table II).

The large quantities of DEAE-cellulose used per unit of serum in the foregoing process made it an impractical means for mass production. Changes were made to rectify this deficiency. First, crude globulin was removed from the absorbed horse serum as a preliminary step, with the use of a single precipitation with 0.4 saturation ammonium sulfate.[4] The precipitate was dissolved in normal saline to half the volume of the absorbed serum and dialyzed with tap water to remove excess ammonium sulfate. The resulting product, which was now ready for the additional batch extraction, is shown in Fig. 2. It contained alpha, beta, and gamma globulin and a trace of albumin.

Next, each milliliter of raw globulin solution was mixed with 0.4 Gm. of DEAE-cellulose instead of 1 Gm. This adjustment necessitated another change, since it could be shown by column chromatography that the large volumes of sodium phosphate buffer now eventually permitted the washing through of significant quantities of beta globulin. When the quantity of buffer added to the batch was reduced to 2.4 ml. per milliliter of globulin solution, the supernatant fluid again contained pure gamma-G globulin.

This supernatant fluid was dialyzed against normal saline to insure isotonicity, concentrated with ultrafiltration to the original volume of the raw globulin solution, sterilized by passage through a Millipore filter, and stored in sterile bottles. It had a pH of 7.01 to 7.08.

Immunologic testing. The protein concentration was determined of the crude serum, intermediate products, and the final globulin. The constituents were identified with cellulose acetate electrophoresis and immunoelectrophoresis. A profile of the specific activity of the ALS or its by-products was obtained by measuring the titers of leukoagglutinins,[2] lymphocytotoxins,[13] and antiplatelet antibodies.[3] Hemagglutinins and hemolysins were determined with twofold antibody dilution. The precipitins were first studied with a standard ring test with the use of twofold antigen dilution. The specific canine or human plasma proteins against which the precipitins reacted were then identified by immunoelectrophoresis.

The final antihuman ALGG was compared with ammonium sulfate–precipitated ALG prepared from the same absorbed serum in an additional way. Both were sub-

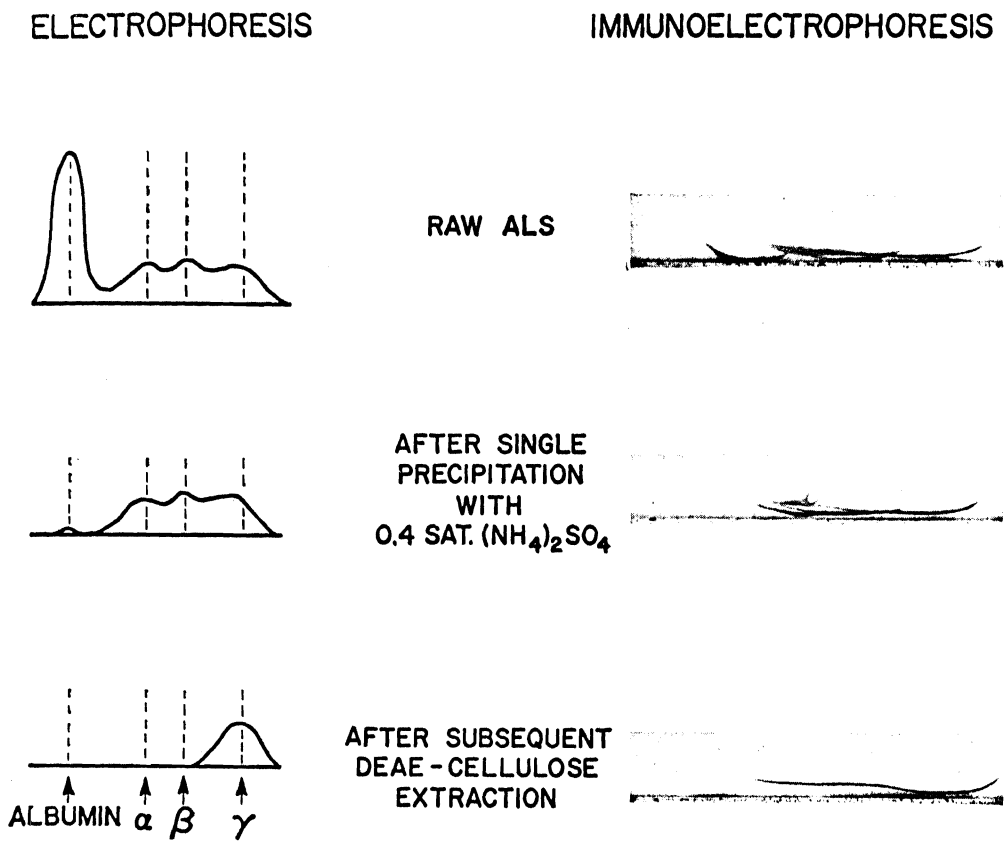

Fig. 2. Electrophoretic and immunoelectrophoretic patterns of unabsorbed antihuman ALS, the raw globulin obtained with a single ammonium sulfate precipitation of absorbed ALS, and the final product (pure gamma-G globulin) after subsequent DEAE-cellulose batch extraction.

jected to immunoelectrophoresis against the serum from a patient who had been sensitized against horse protein. By so doing, it was possible to determine which antigens in these two products would react with this particular patient's serum.

Testing in animals. Several batches of ALGG were tested for pyrogenicity by intravenously injecting rabbits with 50 mg. per kilogram. Fever was not observed. LD_{50} experiments in mice were not pursued when it was found that no deaths occurred even when intraperitoneal doses as great as 1 mg. per gram did not cause death.

The immunosuppressive efficacy of the canine ALGG was assessed after pelvic renal homotransplantation to 11 recipients subjected to concomitant bilateral nephrectomy. Nonrelated mongrel donors were used. The ALGG was started two days before operation and continued daily thereafter for at least one month postoperatively in animals that lived this long. The individual subcutaneous doses were 0.125 ml. per kilogram of an injectate that had a protein concentration of 3.3 to 3.9 Gm. percent and a leukoagglutinin titer of 1:4,000. The survival was compared to that of 10 unaltered control animals. At autopsy the homografts were fixed in 10 percent formalin for studies with light microscopy.

Clinical trials. After the appropriate animal experimentation had been completed, human ALGG was given to patients in a regimen comparable to that described in previous reports on ALG.[11, 12] It was used as an adjuvant to therapy with azathioprine and prednisone in five recipients of kidney

Table II. *The effect of pH and molar concentration of sodium phosphate buffer in the DEAE-cellulose mix on the quantity and characteristics of the protein in the supernatant. The highest yield of pure gamma-G globulin was with pH 7.0 and molarity 0.01*

pH	*Molarity*	*Protein content (Gm. %)*	*Leukoagglutinin titer*	*Potency coefficient**	*Globulin fractions found with immunoelectrophoresis*
Raw ALS		8.20	1:8,000	1.00	
Supernatant fluid after DEAE-cellulose extraction					
7.5	0.005	0.88	1:2,000	2.36	γG
7.5	0.01	1.29	1:4,000	3.18	γG
7.0	0.005	1.43	1:4,000	2.87	γG
7.0	0.01	1.96	1:8,000	4.12	γG
6.3	0.005	2.20	1:8,000	3.78	γG, γA, γM, β
6.3	0.01	2.70	1:8,000	3.04	γG, γA, γM, β

*Defined in the text.

or liver homografts. The brief follow-up precludes a meaningful evaluation of the immunosuppressive effect. However, the toxicity was studied by the same clinical and serologic criteria which were applied to the previously used ALG.[5] Principal emphasis was upon the clinical findings of pain, fever, and anaphylactic reactions and upon the immunologic data provided by skin testing and serologic measurements of the recipient's serum.

RESULTS

In vitro features of the human ALGG. The final product was pure gamma-G globulin (Fig. 2) with a protein concentration of 3.2 to 3.5 Gm. percent, a leukoagglutinin titer of 1:2,000 to 1:4,000, and a lymphocytotoxicity titer of 1:18,000 to 1:36,000 (Table III).* The loss of gamma-G globulin in the total process was approximately one half. Absorption and ammonium sulfate precipitation accounted for most of this loss since 70 percent and 55 percent, respectively, remained after the two initial steps. After the final batch treatment, approximately 53 percent could still be recovered.

*The disparity between the leukoagglutinin and lymphocytotoxin titers reflects principally differences in the way in which the tests are performed.

The potency coefficient of the ALGG as defined earlier was much greater than that of ALG produced from the same serum with previously employed techniques,[4] since an equivalent anti–white-cell activity was present in the ALGG in spite of a much-reduced protein concentration (Table III). In both the ALG and ALGG, the effectiveness of the improved absorption techniques was reflected in the low titers of antired cell, antithrombocyte, and precipitating antibodies (Table III). The lack of specific activity against human plasma proteins was particularly well demonstrated with immunoelectrophoresis (Fig. 1).

When ALGG was tested with immunoelectrophoresis against the serum of a patient sensitized against horse protein, there were no precipitin lines. In contrast, the sensitized patient's serum reacted with the beta globulin in the ammonium sulfate–prepared ALG.

Immunosuppressive effect of ALGG in dogs. With survival credit for individual dogs limited to 50 days, the 11 recipients treated with ALGG had a mean survival of 23.2 ± 15.7 (S.D.) days as compared with 13.4 ± 7.8 in 10 untreated controls ($p<0.05$). Of the treated dogs, 5, 4, 3, and 2 lived for more than 15, 20, 30, and 50 days, respec-

Table III. *Comparative chemical and immunologic data on ALGG produced with DEAE-cellulose batch technique and ALG prepared with double ammonium sulfate precipitations from the same raw ALS*

	ALS	*ALG*	*ALGG*
Total protein (Gm. %)	9.6	7.4	3.6
Anti-WBC activity			
Leukoagglutinin titer	1:4,000	1:4,000	1:4,000
Potency coefficient*	1.00	1.30	2.67
Lymphocytotoxin titer	1:18,000	1:36,000	1:36,000
Potency coefficient*	1.00	2.59	5.34
Purity			
Immunoelectrophoresis		α, β, γ	γG
Electrophoresis		α, β, γ	γ
Anti-RBC activity			
Hemoagglutinin titer	1:2,000	1:64	1:64
Hemolysin titer	1:16	0	0
Antiplatelet activity			
Thromboagglutinin titer	1:48	1:3	1:3
Antiplasma protein activity			
Precipitin titer	1:2,560	1:40	1:40

*Defined in text.

tively. Of the control dogs, all but one died in less than 15 days, the exceptional animal living for 35 days. Postmortem examination of the homografts in both the treated and control animals showed typical features of rejection, although this was less advanced in the former series.

Clinical trials. The 5 patients had pain at the intramuscular injection sites. This was similar to that caused by the previously used crude ALG. Occasional low fevers also seemed related to the injections of ALGG, but with a much-reduced frequency compared to the old ALG. Although the globulin had a low antiplatelet titer, transient thrombocytopenia was seen in 2 cases.

During the 6 weeks of therapy, no precipitin antibody response has occurred. During the same time, more than 90 percent of patients treated in the past with ammonium sulfate–precipitated ALG exhibited increases in precipitins.[5] However, all 5 patients have had increases in hemagglutinin titers, to a maximum of 1:8 to 1:32. No anaphylactic reactions or generalized skin rashes have thus far been seen.

DISCUSSION

The results in this study have shown that a high-quality gamma-G globulin (ALGG) can be produced with a batch technique from the serum of immunized horses. The loss rate of the desired antibody was no greater and it was probably less than when multiple ammonium sulfate precipitations were used as the sole means of extracting the globulin. When tested as an immunosuppressive agent in dogs, the ALGG had approximately the same ability to prevent or delay homograft rejection as was previously reported from this laboratory[11] for unrefined ALS or ammonium sulfate–precipitated ALG.

The primary theoretical advantage of batch techniques is that large quantities of a pure protein fraction should be producible with less extensive equipment than is needed for column extraction. Both methods are based upon the same chemical principle. The feasibility of batch methods was demonstrated by Stanworth[8] in 1960, also using DEAE-cellulose, and later by Sgouris and co-workers.[7] DEAE-Sephadex has been used

for the same purpose by Baumstark and co-workers,[1] and tested for the preparation of ALG by Perper and co-workers.[6] In all of these reports, except that of Perper and his associates, the amounts of gamma-G globulin produced per volume of mix were very small. With the technique described in the present study, the objection was overcome by a first-step ammonium sulfate precipitation and by the appropriate manipulation of the amounts of DEAE-cellulose and phosphate buffers.

In dogs, the ALGG produced by this method had an immunosuppressive effect which was neither better nor worse than that of less pure ALS or ALG preparations; the same is probably true for the globulin produced for clinical use. Whether human ALGG proves to have striking advantages over the previously used ALG remains to be seen. In spite of the improved absorption procedures, the high degree of purification, and the reduced protein content, the injection of ALGG was still painful and in some cases seemed to cause thrombocytopenia. After several weeks of administration, there were rises in hemagglutinin titers which indicated a response to Forssman antigen which is present in horse but not human protein. However, the precipitin response seen in past cases and directed primarily against alpha and beta globulins, in ammonium sulfate precipitated ALG, was eliminated with the use of ALGG. Thus, it is possible that the chief benefit of ALGG will be in a reduction in the incidence of delayed complications of foreign protein therapy such as anaphylaxis. To date, anaphylactic reactions have not been seen with the re-

SUMMARY

Antilymphocyte gamma-G globulin [illegible] was produced from the serum of [illegible] horses. Modifications of the pre- [illegible] absorption techniques permitted [illegible] of undesirable, extraneous anti- [illegible] the use of a batch technique, [illegible] globulin could then be re- [illegible] quantities. The resulting product was first confirmed to have immunosuppressive qualities in dogs and then given a clinical trial. In patients, its administration occasionally caused low-grade fever and thrombocytopenia. Pain at the injection site was not eliminated. Precipitin antibody responses have apparently been prevented in the patients but not a host response to Forssman antigens.

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ADDENDUM

ALGG was given to a total of 13 recipients of intrafamilial renal homografts. The results obtained in these patients, sandwiched in between the more extensive trials with the ammonium sulfate–precipitated ALG, were distinctly disappointing. Although there were no anaphylactic reactions and few other side effects, the immunosuppressive potency appeared to have been greatly reduced by the process of refinement. There were a greater number of severe rejections in the 13 ALGG-treated patients than in the 72 cases in which the crude globulin had been used (Table IV).

Table IV. *Postoperative morbidity and mortality rate in 13 patients treated with ALGG prepared by the DEAE-cellulose batch technique and in 72 patients receiving ammonium sulfate–precipitated ALG*

Series	*No. of cases*	*BUN (150 mg. %)*	*Post-operative dialysis*	*Deaths*
ALGG	13	5	4	6
ALG	72*	3	1	3

*Fifty-eight of these cases were before the ALGG trial; 14 were after.

A probable explanation for this finding has recently been published.[8a] In horses subjected to long-term immunization, there is apparently a variable shift of the desired antibodies into the T-equine fraction, which is largely eliminated by the purification technique described in the present communication. This apparently had not yet occurred in horses used for the canine test system, but had evidently done so in the chronically immunized horses which provided the antihuman ALS. Consequently, we have returned to the use of the less pure ammonium sulfate–precipitated ALG in subsequent clinical cases.

In the rabbit and goat, there appears to be less tendency for migration of the active immunoglobulin portion. Thus, the batch technique may prove to be more valuable and reliable in these 2 species.

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