A Rapid Method for Purification of Large Quantities of Anti-Lymphocytic Serum.* (32150)

R. J. PERPER, J. T. OKIMOTO, K. C. COCHRUM, N. RAMSEY, AND J. S. NAJARIAN
University of California School of Medicine, San Francisco

Anti-lymphocytic serum has potent immunosuppressive properties(1-4). Recent clinical and experimental studies with this agent have led to increasing interest in purification of the antibody activity(5). Toxic reactions have occurred in recipients of large quantities of unmodified serum(1,3,4). These reactions have been attributed to the presence of red cell antibodies and to the antigenic properties of heterologous proteins(4-6). After absorption of red cell antibodies, immediate acute toxicity has been reduced(4,5) but renal lesions later developed(5), possibly because of complex disease(7).

The horse has often been used as a source of anti-lymphocytic serum (HALS). Although this species has some advantages as a source of large quantities of ALS, its serum proteins are markedly antigenic when administered to most species. The present study describes a rapid method of purifying large quantities of IgG from HALS by absorption of the red cell antibodies and by the removal of nonantibody proteins. A DEAE batch technique, devised for human IgG purification (8,9) was modified and used for the purification of horse IgG. Continuous intravenous administration of the purified gamma globulin resulted in a leukopenia equal to that achieved with a dosage of whole serum which contained 7 times the total proteins and twice the gamma globulin.

Materials and methods. Immunization. A horse weighing 390 kg was given an intravenous injection of 1×10^9 lymphocytes obtained from the thoracic duct of goats followed by 8 biweekly intradermal injections of the same number of cells. By a modification of the technique of Ellis(10) permanent recirculating thoracic duct fistulae were established in the goats, which remained patent for 4-6 weeks.

Humoral antibody determinations. Cytotoxic antibody titer of the HALS was determined by Walford's technique(11). The number of target lymphocytes was adjusted to 1×10^5 to eliminate prozoning. Before the samples were tested for cytotoxicity they were dialyzed against phosphate buffered saline to insure uniformity of ion concentrations.

Red cell antibody determinations were performed by Hildermann's(12) method and guinea pig serum (1:10 dilution) was used as the source of complement for hemolytic tests.

Preparation of serum. Absorption of red cell antibodies. After the HALS was inactivated at 56°C for 30 minutes it was absorbed twice at 4°C on equal volumes of buffy coat-free, washed, pooled and packed goat erythrocytes. Red cell hemagglutinins and hemolysins as well as cytotoxic antibody titers present in the HALS and which were directed against goat red cells and lymphocytes were determined before and after each absorption. Similar antibody measurements were performed before and after absorptions with serum erythrocyte ratios of 2:1, 5:1, 20:1, and 40:1.

Gamma globulin purification. A modification of the DEAE-Sephadex batch technique described by Baumstark *et al*(8) was used to fractionate the IgG from HALS.

Preparation of DEAE-Sephadex (quantity needed for 1000 cc serum). One hundred g of DEAE-Sephadex A-50 (coarse) were suspended in 8 liters of distilled water, which was mechanically agitated for 30 minutes. After the Sephadex settled for 10 minutes the supernatant which contained the "fines" was decanted by suction. The Sephadex was resuspended in the same volume of water and the "defining" process was repeated until the supernatant was clear (approximately 8 times). The final suspension was passed through a Buchner funnel (#6) and the filtrate was discarded. The gel remaining in the funnel was washed with 4 liters of 0.5 N NaOH followed by 4 washes with distilled

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water. This was then repeated using 4 liters of 0.5 N HCl followed by another four 3-liter washes of distilled water. The gel removed from the funnel was resuspended in 8 liters of distilled water and the residual "fines" were removed 4 times. The final suspension was passed through the funnel, and the gel was removed and resuspended in 4 liters .05 M phosphate buffer pH 7.85, where it was allowed to equilibrate with agitation for 15 minutes. The gel was poured through the funnel again and it was washed once with 4 liters of the same buffer. The gel was allowed to dry by suction on the funnel and it was removed and weighed (1200-1350 g).

The Sephadex was regenerated by the method of Baumstark *et al*(8). The saturated gel was sequentially washed in 1.5 M NaCl, distilled water, 0.5 M NaOH, 95% ethanol and finally reconstituted to the chloride form by the addition of 0.5 M HCl.

Experimental procedure. One thousand ml of absorbed whole HALS was added to 400 g of the equilibrated Sephadex and placed on a magnetic stirrer at 4°C for one hour. The gel-serum mixture was then separated by passage through a Buchner funnel and the supernatant which contained the partially purified gamma globulin was saved. The supernatant was further purified by 2 additional exposures to fresh 400 g aliquots of Sephadex by the same protocol. After each exposure to the Sephadex, pH, total proteins, albumin globulin ratios, Cl—, Na+ and K+ determinations were made of the supernatant and the total volume was recorded.

After each passage, an aliquot was tested for cytotoxic antibody titers, and immunoelectrophoretic analysis of the proteins was performed by using anti-horse gamma globulin and anti-horse whole serum. After 3 exposures to the Sephadex an aliquot of the supernatant was analyzed on the ultracentrifuge and the sedimentation constant of the single peak was calculated(13).

Effect of pretreatment with NH_4SO_4. Whole serum was precipitated once with 33% NH_4SO_4 by standard techniques(14). Cytotoxic antibody titers were determined and immunoelectrophoretic analysis was performed on the resuspended precipitate. The serum was then further purified by the DEAE-Sephadex batch technique described above.

Effect of differing DEAE-Sephadex serum ratios. Experiments were performed in which Sephadex serum ratios of 0.2:1, 0.4:1.0, and 0.6:1.0 were used. The Sephadex was weighed in its hydrated form as it appeared on the Buchner funnel after the supernatant was removed. Aliquots of serum were treated with each concentration of Sephadex. The total recovery and degree of purification were determined as above.

Biological activity. Before they were injected the preparations were sterilized by passage through a Seitz filter (pore size 1 μ). Two preparations were used for injections. In one series of experiments HALS was adjusted to 0.15 M by addition of NaCl before injection and in the other series it was injected without adjustment of molarity. Five goats were given intravenous injections of raw HALS at a dose of 2 cc/kg for 5 weeks. The raw HALS contained 63 mg protein/cc (15 mg IgG). Five other animals received the purified gamma globulin from HALS in the same volume, but it contained only 8.5 mg protein (gamma globulin only). Complete blood counts were performed on samples obtained daily.

One mg aliquots of HALS and normal horse gamma globulin were radio-iodinated with ^{125}I and ^{131}I, respectively, by the method previously described(15). The specific activity of the labeled proteins varied from 200-500 μC/mg. Fifty to 100 μg of ^{125}I labeled HALS and 50-100 μg ^{131}I labeled normal horse gamma globulin were simultaneously injected into each of 3 separate goats and the biological half-life was determined by daily measurement of radioactivity in aliquots of blood.

Results. The goat red cell hemolytic titer of different HALS preparations varied from 1:16-1:32 and the hemagglutinin titer varied from 1:32-1:64. In 15 minutes at 0°C 1 ml of packed goat red cells completely absorbed measurable red cell antibodies from 40 ml of HALS. The absorption of the red cell antibodies did not decrease the cytotoxic antibody titer, which remained at 1:2048.

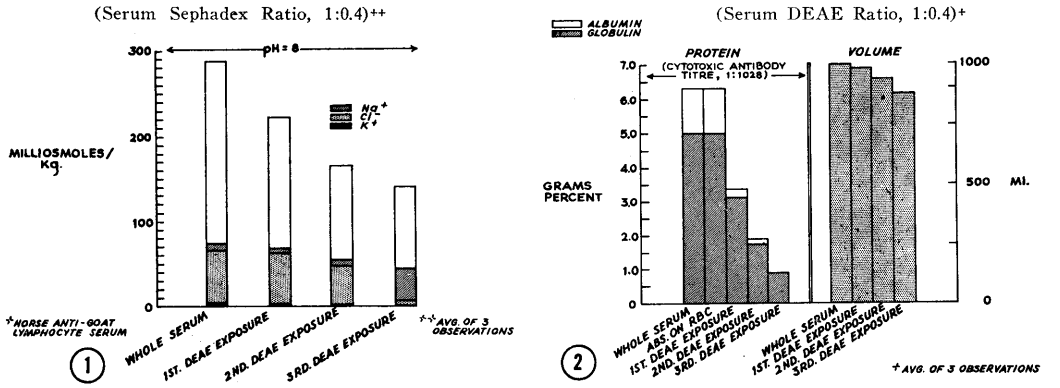

FIG. 1. Soluble ion concentrations following successive treatment of whole undialysed horse anti-lymphocytic serum (HALS) with DEAE-Sephadex.

FIG. 2. Alteration of volume, total protein, albumin and globulin content of whole undialysed HALS following successive treatments with DEAE-Sephadex.

Gamma globulin purification. Fig. 1 and 2 summarize the modifications of whole undialyzed serum by 3 exposures to DEAE-Sephadex. The pH remained unchanged but the univalent and divalent ions were reduced by at least 40% after 3 exposures (Fig. 1). The cytotoxic antibody titer remained constant, and the volume of the serum was reduced by only 2% following each passage through the gel. Total protein concentration was reduced by 88% and at the third passage all the protein remaining was pure gamma globulin (Fig. 2). Fig. 3 illustrates the immunoelectrophoretic pattern of the supernatant following each exposure to the Sephadex.

The final preparation was electrophoretically pure and demonstrated the 3 antigenic components of horse gamma globulin described by Rockey *et al*(16). Fig. 4 illustrates the single protein peak present when the final preparation was analyzed in the ultracentrifuge. This peak had a S_{20} value of 6.76.

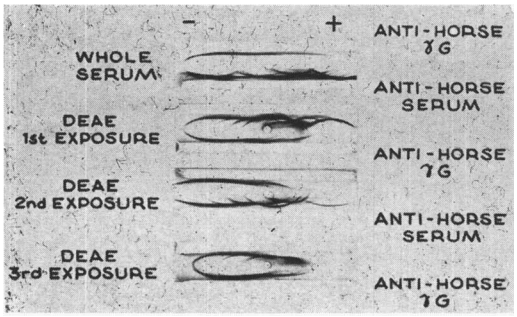

FIG. 3. Immunoelectrophoretic analysis of HALS following successive treatment with DEAE-Sephadex.

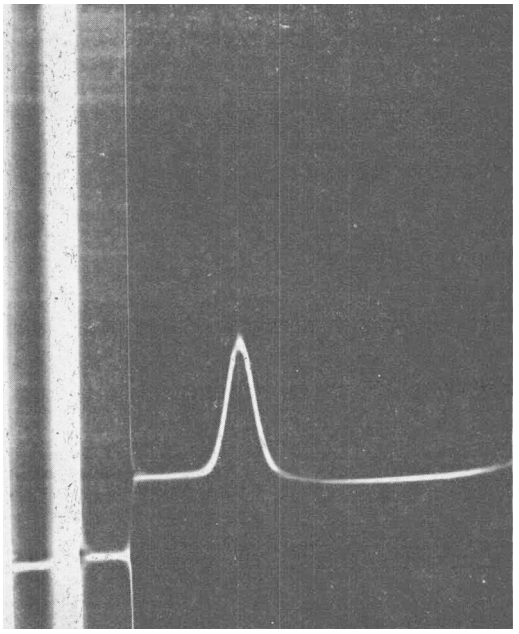

FIG. 4. Ultracentrifugal pattern of IgG obtained from HALS exposed 3 times to DEAE-Sephadex using batch procedures. Protein concentration 10 mg per milliliter .05 M phosphate buffer pH 7.85. Temperature 20°C, second exposure taken 16 min after reaching 59,780 rpm. Diaphram angle 40°.

Each successive passage through the gel removed progressively more serum proteins as can be seen from Fig. 1 and 3. Transferrin was the protein that persisten longest. When the original serum was contaminated with hemoglobin, four exposures to Sephadex were occasionally required to remove all residual transferrin. Albumin was still present after the

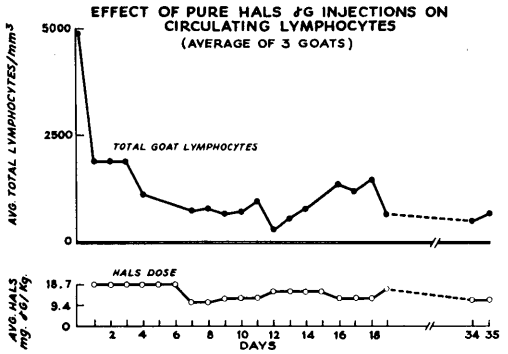

FIG. 5. *In vivo* leukopenia produced by injections of pure IgG obtained from HALS.

second passage (Fig. 3) but in very low concentrations (1.3 mg/cc).

A single precipitation of undialyzed serum at 33% NH_4SO_4 saturation did not completely remove the albumin which was demonstrated immunoelectrophoretically. The cytotoxic titer was unaltered during the precipitation, centrifugation, dialyses, and pervaporation procedures.

The most efficient ratio of serum to DEAE-Sephadex was found to be 1:0.4. When the Sephadex concentration was increased, the degree of purity following each exposure remained unchanged. When the serum Sephadex ratio was decreased to 1:0.2, four exposures to the gel were required to eliminate all contaminating proteins.

Biologic activity. The lymphopenia induced by daily injections of purified gamma globulin from HALS is summarized in Fig. 5. The total lymphocyte count was immediately depressed (3-6 hours) from 4900/mm^3 to 1900/mm^3 and was maintained at approximately this level for up to 5 weeks by daily intravenous injections of 8.5-21 mg/kg of pure absorbed gamma globulin (cytotoxic titer 1:2048). The gamma globulin was stored at 0°C without loss of activity. No toxic reactions, either acute or chronic, were observed during or after daily intravenous administration of the IgG for up to 5 weeks. The animals had no signs of anorexia or depression, and BUN and hemoglobulin concentrations remained normal. In contrast, in order to achieve the same degree of leukopenia 2-3 cc/kg whole HALS was required (15-45 mg/kg of gamma globulin and 63-189 mg/kg protein). When absorbed whole HALS was given, immediate acute toxicity, which has been attributed to red cell antibodies, did not occur; however, chronic toxicity did. The manifestations of acute and chronic reactions following injection of whole HALS have been described by Abaza *et al*(1). The immediate acute reaction is characterized by salivation and prostration whereas the chronic reactions are characterized by dypsnea and depression following injection with gradual development of depression and weight loss. When the daily dosage of HALS was lowered sufficiently to reverse these symptoms the lymphocyte count returned to normal values. Thus in this species daily intravenous administration of raw HALS for longer than 3 weeks was found to be unmanageable. After sterilization, local effects were not observed when the pure preparation was given unmodified intravenously for periods of up to 5 weeks, but when given subcutaneously marked local tissue reaction occurred. This localized effect was prevented by adding sufficient sodium chloride to restore the original ionic strength. When the purified HALS IgG was radio-iodinated with ^{125}I and nonimmune gamma globulin with ^{131}I the biological half-life of the HALS was 11-1/2 days and the normal horse gamma globulin was 8-1/2 days.

Discussion. The DEAE-Sephadex batch method is a rapid, economical and relatively simple method for obtaining large quantities of pure horse gamma globulin. When a large quantity of DEAE-Sephadex was prepared in advance, 8.5 g of electrophoretically pure gamma globulin was obtained in 4 hours from 1000 cc of whole serum. This represents a 56% recovery, assuming the gamma globulin content of normal horse serum to be 24% (17). We have been able to safely draw 3 titers of blood every 2 weeks from a 400 kg horse, therefore 25 g of gamma globulin can be obtained from a single horse per month. The gamma globulin has been stored at 0°C or lyophilized without loss of antibody activity. The final gamma globulin preparation was contained in approximately the same volume as the original serum. Attempts were made to concentrate the gamma globulin by pervaporation and lyophilization, but only a

2-fold increase *in vitro* cytotoxic activity resulted.

The molarity and the pH of the buffer used to equilibrate the DEAE-Sephadex were critical. If the pH or the osmolarity of the buffer varied or if the gel was not adequately equilibrated with buffer, other serum proteins, especially transferrin, were washed through the gel and appeared with the IgG. Klinman *et al*(18) have described the purification of horse gamma globulin with DEAE cellulose and its elution with the same buffer used in this study. They also described the 3 antigenic constituents of horse gamma globulin which we observed.

It was possible to absorb the red cell antibodies present in the HALS with very small amounts of red cells, which made is unnecessary to maintain a large number of animals as red cell donors. This illustrated the advantages of maintaining low red cell antibody titers in the serum donor by immunization with pure lymphocyte preparations. We observed no loss of cytotoxic titer of the HALS when the red cell antibodies were removed by absorption.

Ammonium sulfate precipitation of the globulins from large volumes of serum was found to be impractical. Adequate preparative purification was possible with small volumes but with large volumes centrifugation, dialysis and concentration were cumbersome and time consuming. Furthermore, more than one precipitation was necessary to remove the albumin completely and even trace amounts of contaminating proteins when injected daily can be antigenic.

Iwasaki *et al*(5), recently reported the purification of HALS by multiple ammonium sulfate precipitations at 40% saturation. They also described a low yield of antibody globulin following precipitation at 33% saturation, which we have also observed. When large volumes of serum are to be processed, the multiple steps which Iwasaki describes seem to be much less practical than the batch DEAE methods described here. These authors also performed column chromatography with DEAE cellulose and suggested the use of a .05 M buffer pH 6 to elute antibody protein from large volumes of dialyzed serum. When this buffer was used in our experiments and those of Iwasaki, although antibody activity was present in the eluate, it was contained in beta globulins, T equine fractions as well as in gamma globulin. Furthermore, purification of large quantities of proteins by column chromatography has not previously been shown to be practical.

The purification procedures we have described differed from the batch methods used by others for the purification of gamma globulin from other species(8,9). Other investigators have obtained yields as high as 97% using undialyzed serum, but to achieve this yield of gamma globulin it was necessary to wash the DEAE repeatedly with buffer. Therefore, the final preparation was diluted 3 to 4 times. We were willing to accept a lower yield to eliminate the necessity of concentrating the final solution. We have obtained yields compatible with that described by others when the adherent gamma globulin was eluted from DEAE by additional washings.

The whole HALS contained 88% more foreign proteins and approximately 50% more gamma globulin that was present in the purified preparation. Therefore, when purified gamma globulin was used it was possible to obtain the same degree of leukopenia with 1/2 the gamma globulin contained in the whole serum. An explanation for this paradox may be found in previous studies in which antibodies were shown to develop against the gamma globulin of heterologous ALS, although in lower titer than against nonimmune heterologous gamma globulin(19). It is likely that the more foreign protein injected the greater will be the recipient's antibody response even when the antigen is contained in heterologous ALS. Experiments are now in progress to establish that purified IgG from HALS is more effective in lower doses than the IgG contained in raw HALS because of a reduction of its immune elimination.

Summary. A rapid simple method of obtaining large amounts of antibody gamma globulin from horse anti-lymphocytic serum (HALS) is described. Undialyzed serum was used and DEAE-Sephadex was employed as the anionic exchanger using a "batch" method. Cytotoxic antibody titers, pH and serum

volume were unchanged during absorption of erythrocyte antibodies and purification. The final preparation was electrophoretically pure, gave a single 6.76 peak on the ultracentrifuge, and was structurally unaltered as evidenced by *in vivo* traces studies, and immunoelectrophoretic analysis. *In vivo* toxicity did not occur during long term intravenous administration of the purified preparation. The resultant lymphopenia was equal to that achieved with whole serum containing two times the gamma globulin and 7 times the total proteins.

1. Abaza, H. M., Nolan, B., Watt, J. G., Woodruff, F. A., Transplantation, 1966, v4, 618.

2. Monaco, A. P., Wood, M. L., Gray, J. G., Russell, P. S., J. Immunol., 1966, v96, 229.

3. Sacks, J. H., Filippone, D. R., Hume, D. M., Transplantation, 1964, v2, 60.

4. Huntley, R. T., Iwasaki, Y., Marchioro, T. L., Jeejeebhoy, H., Porter, K. A., Starzl, T. E., Surg., Forum, 1966, v17, 230.

5. Iwasaki, Y., Porter, K. A., Amend, J. R., Marchioro, T. L., Zühlke, V., Starzl, T. E., Surg. Gynec. & Obstet., 1967, v124, 1.

6. Monaco, A. P., Abbott, W. M., Othersen, H. B., Simmons, R. L., Wood, M. L., Flax, M. H., Science, 1966, v153, 1264.

7. Dixon, F. J., Harvey Lectures, 1962-1963, p21, Series 58.

8. Baumstark, J. S., Laffin, R. J., Bardawil, W. A., Arch. Biochem. & Biophys., 1964, v108, 514.

9. James, K., Stanworth, D. R., J. Chromat., 1964, v15, 324.

10. Ellis, F. G., Surgery, 1966, v60, 1251.

11. Walford, R. L., Gallagher, R., Troup, G. M., Transplantation, 1965, v3, 387.

12. Hildemann, W. H., Transpl. Bull., 1957, v4, 148.

13. Perper, R. J., Najarian, J. S., Transplantation, in press.

14. Kabat E. A., Mayer, M. M., Experimental Immunochemistry, 2 ed., C. C Thomas, Springfield, Ill., 1966, p762.

15. Najarian, J. S., Perper, R. J., Surgery, in press.

16. Rockey, J. H., Klinman, N. R., Karush, F., J. Exp. Med., 1964, v120, 589.

17. Cohn, E. J., Chem. Rev., 1941, v28, 395.

18. Klinman, N. R., Rockey, J. H., Frauengerger, G., Karush, F., J. Immunol., 1966, v96, 587.

19. Gray, J. G., Monaco, A. P., Wood, M. L., Russell, P. S., ibid., 1966, v96, 217.

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Antagonism of Intravenous Digitoxigenin Lethality by Reserpine Pretreatment in the Mouse.* (32151)

G. L. LAGE AND J. L. SPRATT† (Introduced by G. L. Plaa)

Department of Pharmacology, College of Medicine, University of Iowa, Iowa City

Dick *et al*(1) reported increased arrhythmias from digitalis in patients receiving reserpine therapy. Roberts *et al*(2) reported that reserpine treatment increased the amount of acetylstrophanthin required to produce ventricular arrhythmias in the dog. On the other hand, Nash *et al*(3) reported that ouabain lethality was decreased with reserpine pretreatment in rats. Takagi *et al*(4) also reported decreased lethality of digoxin with reserpine pretreatment in dogs. From these various reports, it would appear that although reserpine may increase the possibility of arrhythmias induced by digitalis glycosides it also decreases lethal potential of the glycosides.

Considering the known central and peripheral effects of reserpine, the current study was therefore undertaken to investigate possible effects of reserpine on the lethality of digitoxigenin. The latter compound was selected since it is a potent convulsant and was the most toxic of various structurally-related compounds when intravenously administered to the mouse(5).

Methods. Crystalline digitoxigenin was obtained from Lachat Chemicals, Inc., Chicago, Ill., and was dissolved in a final solution of 47.5% ethanol in 0.9% saline. Solubilized

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