

Antibody localization in horse, rabbit, and goat antilymphocyte sera

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During the last 3 years, there has been an increasing clinical use of heterologous antilymphocyte sera (ALS) or various globulin fractions (ALG) of these antisera.^{4, 15, 17-19, 21, 22} The most commonly used serum donor has been the horse. Numerous efforts have been made to refine the globulin removed from the raw serum for eventual injection so that the ratio of desired equine antibodies to irrelevant protein would be increased. These attempts at purification were based upon evidence from many sources that the immunosuppressive activity of ALS is heavily concentrated in IgG or 7S gamma globulin.^{6, 8, 9, 23}

In our own institution, a highly purified horse IgG (ALGG) was given a clinical trial in early 1968 with extremely disappointing results. The ALGG, which was administered in combination with azathioprine and prednisone, appeared to have a reduced ability to mitigate the rejection of renal homografts.^{11, 19} A similar loss of efficacy of refined horse ALGG was noted by Clunie and associates³ in canine experiments.

As a consequence of these findings, the present study was undertaken to re-evaluate the location of various antibodies as well as the immunosuppressive effect in horse ALS. In addition, the same information about antibody localization was sought for ALS raised in rabbits and goats.

METHODS

Immunization. The immune serum obtained from the three different animals was initially treated in the same way. It was sterilized by millipore filtration, inactivated by heating at 56° C. for 30 minutes, and then stored at 4° C. until testing. However, there were some differences in the details of the prior immunization.

Horse. One horse received subcutaneous injections of 2.5 to 5 × 10¹⁰ canine splenic lymphocytes approximately once a week for 9 weeks before bleeding. Two other animals were immunized with human spleen lymphocytes for 6 and 9 weeks, respectively, following the same schedule.

Rabbit. Six New Zealand white rabbits were injected intraperitoneally once a week for 4 weeks with an average of 5 × 10⁹ human spleen lymphocytes per injection. One week after the last immunizing dose,

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they were bled by heart puncture. The sera from the six rabbits were pooled for analysis.

Goat. Before being bled, one goat was immunized for 12 weeks by the same technique as that described for the horse except that the individual cell doses were 1×10^{10} .

Analysis of proteins. The proteins were separated by DEAE-cellulose column chromatography. Five to 6 ml. of raw ALS were brought to pH 8.0 by dialyzing against 0.01M sodium phosphate; the sera were then applied to a 2 by 20 cm. column containing 20 Gm. of activated DEAE-cellulose* that had been prebuffered to pH 8.0 with 0.01M sodium phosphate and packed under 10 pounds of pressure. The samples were followed through the column by gradient elution with a solution of sodium phosphate that began at pH 8.0 (0.01M) and finished after 900 ml. with pH 4.5 (0.3M). The flow rate through the column was about 1 ml. per minute; 5 ml. were collected in each test tube. The optical densities of the eluates were measured spectrophotometrically at 280 $m\mu$. The collecting tubes containing protein "peaks" were pooled and designated fractions I to VII (horse and rabbit) and I to VI (goat); for subsequent analyses these fractions were lyophilized and reconstituted at 50 times their original concentration.

The total protein concentrations of the raw sera and the various fractions were determined by the biuret method of Henry and co-workers.⁷ The presence and quantity of IgG in any of the fractions was detected by the agar immunodiffusion technique† of Fahey and McKelvey.⁵ In addition, protein constituents were identified with cellulose acetate membrane electrophoresis‡ and by microimmunoelectrophoresis§ with commercial antisera.||

*Whatman DE32, H. Reede Angel Inc., London, England.

†The antirabbit IgG and antigoaat IgG were obtained from Hyland Laboratories, Los Angeles, Calif. The anti-horse IgG was raised in rabbits with pure horse IgG.

‡Beckman Instruments, Inc., Fullerton, Calif.

§LKB-Producter AB, Stockholm, Sweden.

||Hyland Laboratories, Los Angeles, Calif.

Lymphocytotoxicity, leukoagglutination, hemagglutination, and thromboagglutination activities of the sera and the protein fractions were determined as described previously,¹⁰ except for one modification in the thromboagglutinin titration. The incubation period for the thromboagglutination was for 6 hours without agitation, instead of for 20 minutes with agitation.

Ammonium sulfate fractionation.

Rabbit and goat ALS. Twenty milliliters of each of the two raw antisera were precipitated with 33, 36, and 40 percent saturated ammonium sulfate (SAS). After reconstituting the sediment to 20 ml. with normal saline, a second precipitation was carried out with the same concentration of SAS as before. The final sediment was then reconstituted to 10 ml. and dialyzed against normal saline before being submitted to analysis by the techniques described in the preceding section.

Horse ALS. The differential ammonium sulfate fractionation of Allen and associates¹ was used to obtain two kinds of horse protein that were rich, respectively, in gamma globulin and T-equine (also known as gamma₁ or beta₂) globulin. Sixty milliliters of horse ALS were added to the same volume of normal saline and precipitated with 33 percent SAS. The sediment was reconstituted to 60 ml. with normal saline and again precipitated with 33 percent SAS. The final precipitate was reconstituted with normal saline to one third the original serum volume and dialyzed against normal saline. The product was designated as Fraction G.

The supernatant from the first 33 percent SAS precipitation step was precipitated with 50 percent SAS. The precipitate obtained was reconstituted to 60 ml. with normal saline, and a second 33 percent SAS precipitation was performed. This sediment was discarded, and the supernatant was again precipitated with 50 percent SAS. The last precipitate was reconstituted to one third the volume of the original serum and dialyzed against normal saline. The end product was designated as Fraction T.

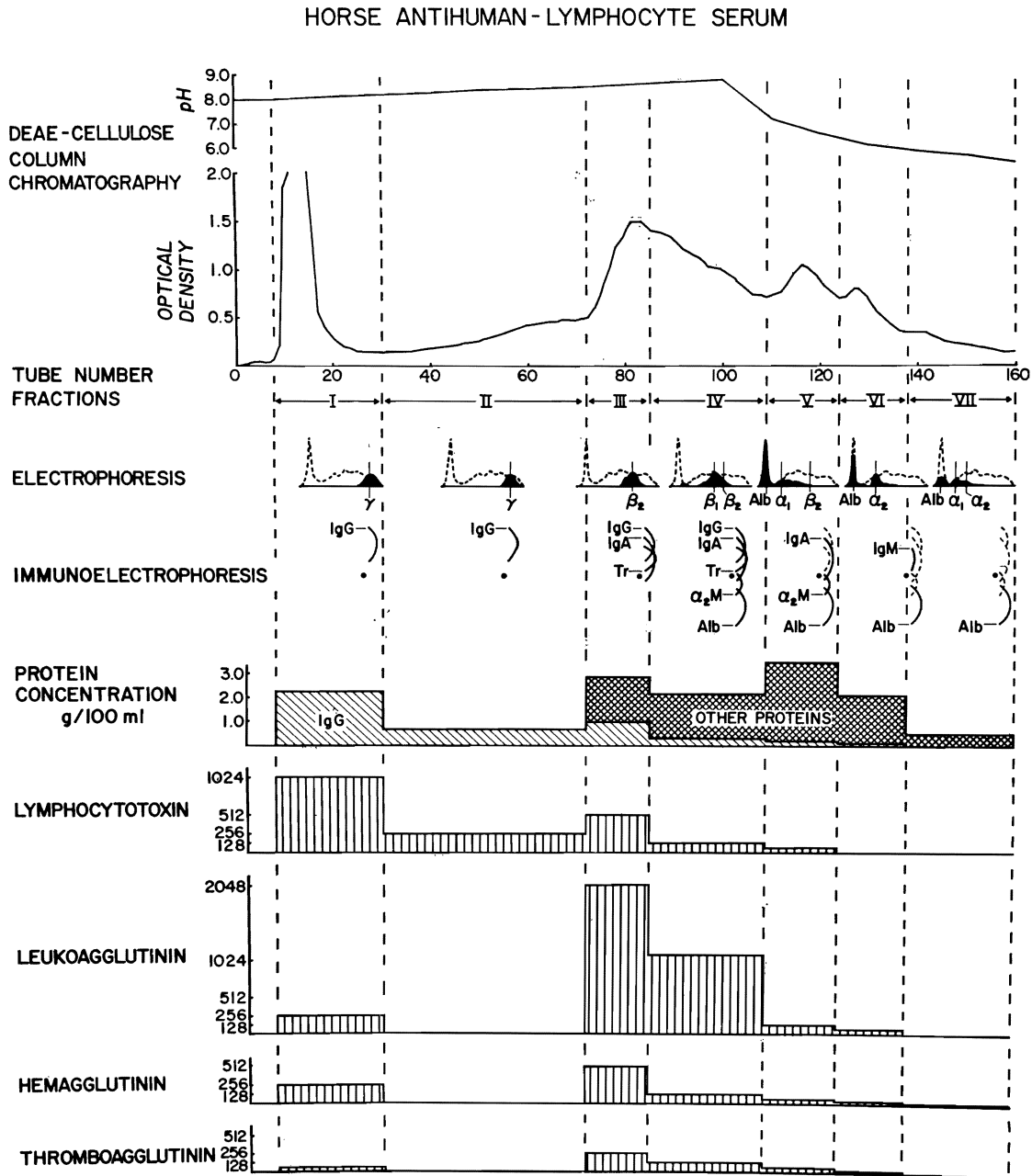


Fig. 1. Analysis of the location of antibodies in the serum of a horse that had been immunized for 6 weeks with weekly subcutaneous injections of human splenic cells. Adjuvant was not used in this or any of the other heterologous serum donors. The multiple analytic techniques shown were used to determine the varieties, concentrations, and activities of the proteins in the different fractions. *Alb* = albumin; α_1 = alpha₁ globulin; α_2 = alpha₂ globulin; α_2M = alpha₂ macroglobulin; β_1 = beta₁ globulin; β_2 = beta₂ globulin or T-equine; γ = gamma globulin; *IgA* = immunoglobulin A; *IgG* = immunoglobulin G; *IgM* = immunoglobulin M; *Tr* = Transferrin.

In vivo testing. Serum was obtained from the horse that had been immunized for 9 weeks against canine splenic lymphocytes; preliminary absorption was carried out with dog erythrocytes, thrombocytes, and plasma. The above described Fractions G and T were

then prepared and either diluted or concentrated so that the gamma globulin and T-equine concentrations, respectively, were equal to those in the original absorbed serum. They were then tested for their ability, when given subcutaneously, to induce

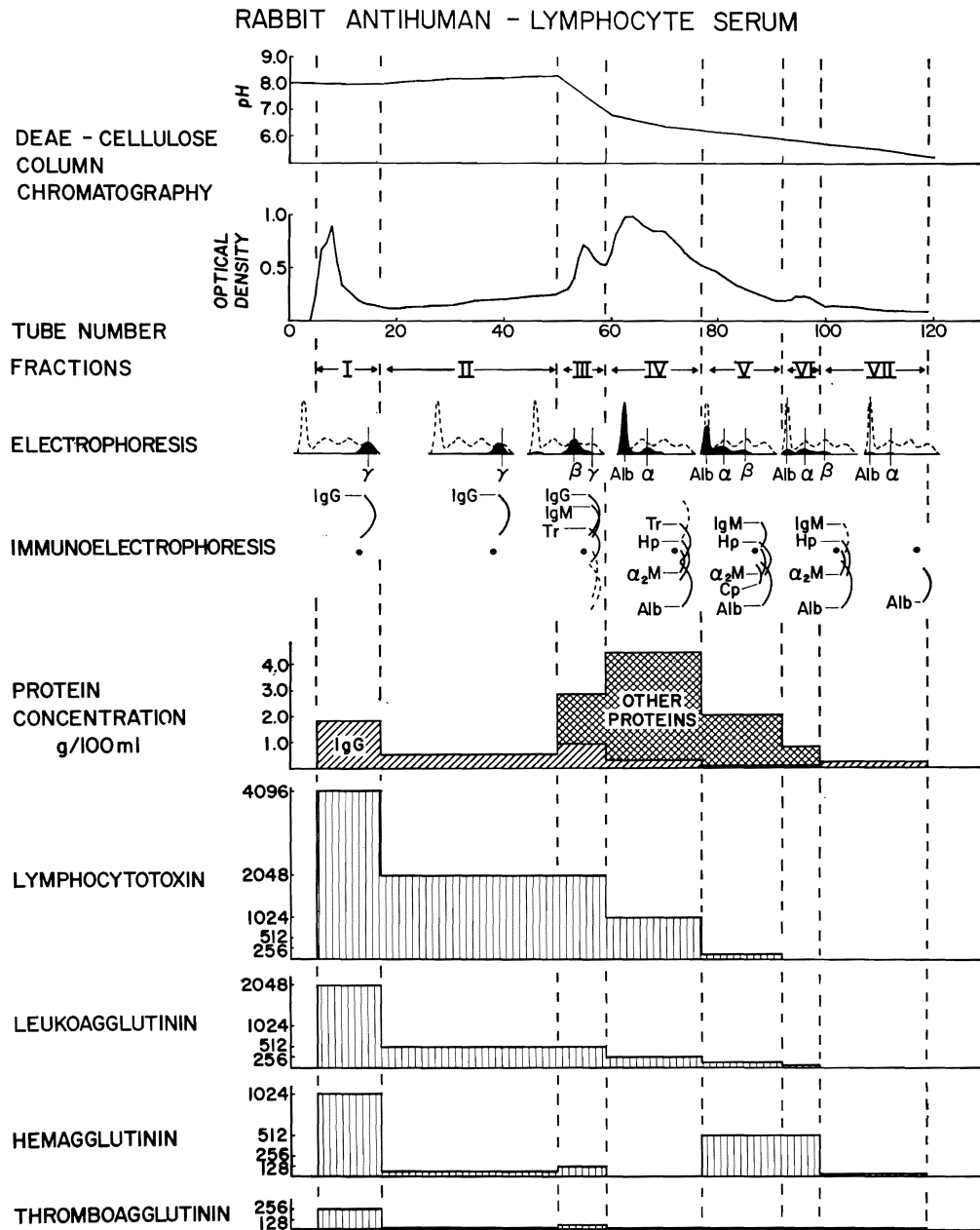


Fig. 2. The localization of antibodies in the pooled sera of six rabbits that had been immunized weekly for 4 weeks with human spleen cells subcutaneously. The protein constituents for which abbreviations were not given in Fig. 1 are *Cp* = ceruloplasmin and *Hp* = haptoglobin.

lymphopenia and to mitigate rejection of renal homografts. The results were compared with those obtained with the use of the absorbed ALS from which the fractions were obtained. Another group of control ani-

mals was untreated. All recipient dogs in the three test series were injected daily with 0.5 ml. per kilogram of the appropriate material, beginning 1 day before transplantation and continuing until the day of death.

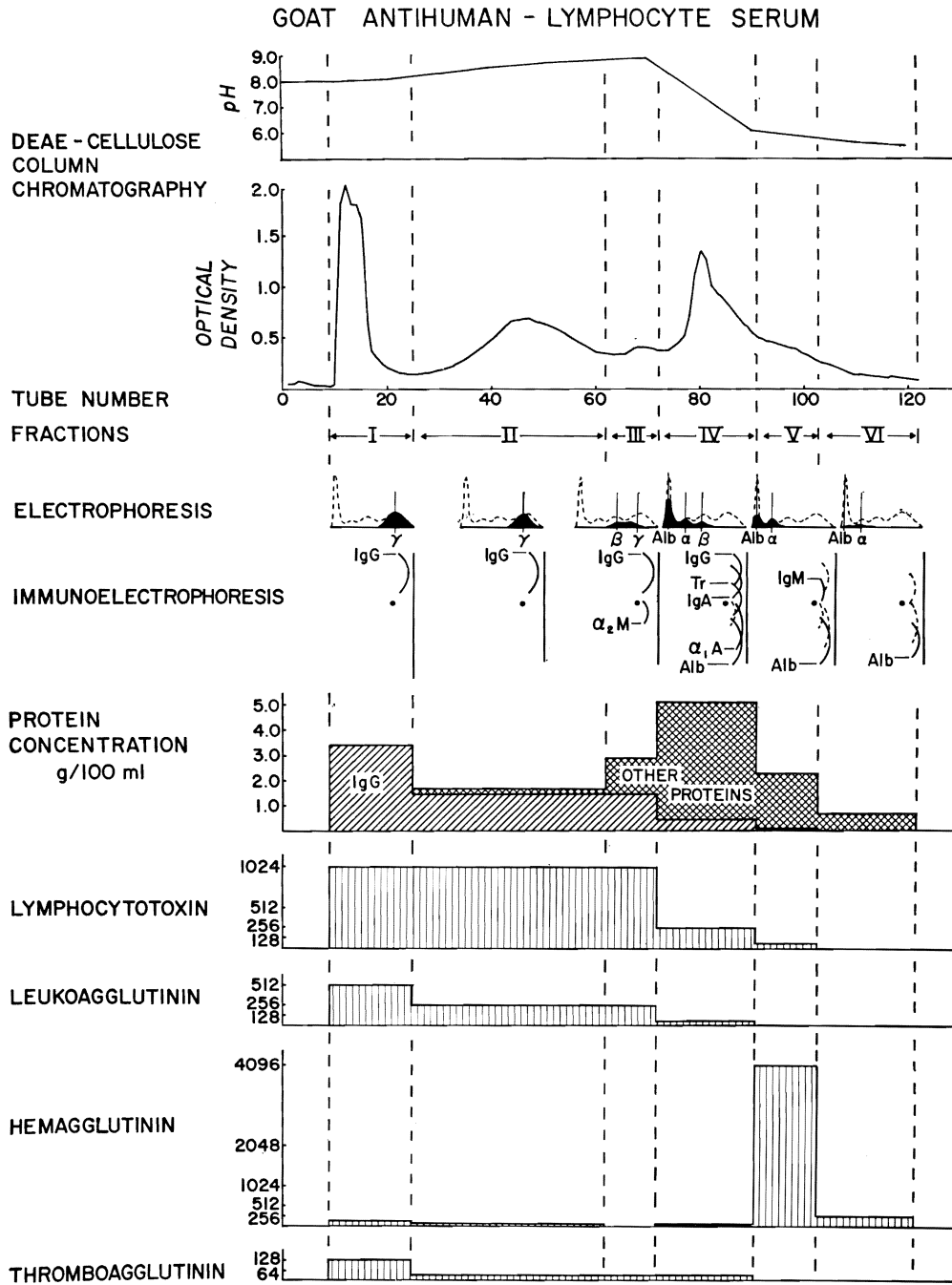


Fig. 3. The antibodies in goat ALS raised with human splenic cells over a period of 12 weeks. The only abbreviation not used in Figs. 1 and 2 is α_1A = alpha₁A globulin.

Table I. *The protein concentrations and titers of crude globulin obtained by double precipitation with different proportions of saturated ammonium sulfate (SAS)**

	Volume (ml.)	Protein concentration (Gm.%)		Antibodies	
		Total	Gamma	Leukoagglutinin	Lympho- cytotoxin
<i>Rabbit ALS</i>	20.0	7.3	3.2	1:1,024	1:4,096
33% SAS	10.5	2.2	1.9	1:1,024	1:2,048
36% SAS	11.8	2.5	2.0	1:1,024	1:4,096
40% SAS	11.7	2.6	1.9	1:1,024	1:4,096
<i>Goat ALS</i>	20.0	7.7	3.6	1:512	1:2,048
33% SAS	11.5	4.9	4.4	1:512	1:1,024
36% SAS	12.5	5.4	4.7	1:512	1:1,024
40% SAS	13.2	5.4	4.5	1:512	1:2,048

*The gamma contribution to the total protein was calculated from electrophoresis scans.

The peripheral lymphocytes were frequently counted.

RESULTS

Chromatography and antibody localization.

Horse ALS. The results were essentially the same in the two horses that were given human splenic tissue as well as in the horse immunized with canine spleen. There were seven protein peaks. The lymphocytotoxic activity was very heavily, if not exclusively, concentrated in the IgG (Fig. 1). This was particularly evident in the pooled Fractions I and II in which only IgG was represented. The lymphocytotoxins in the Fractions III and IV were probably also contained in the IgG which contributed to these heterogeneous collections.

In contrast, more than 80 percent of the leukoagglutinins were in the Fractions III and IV which consisted predominantly of IgA (also known as "fast" gamma G, or IgG(T)). The hemagglutinins and thromboagglutinins were mostly in the same location, although these antibodies were also detectable in the IgG (Fig. 1).

Rabbit ALS. This antiserum was also separable into seven fractions. However, almost all the measured antibodies were in the IgG (Fig. 2). The only significant spread was of the hemagglutinins which were found in the IgM as well (Fig. 2).

Goat ALS. This goat ALS was separable into six fractions (Fig. 3). The distribution

was very similar to that in the rabbit except that the hemagglutinins were almost exclusively in the IgM. The other antibodies were in the IgG.

Ammonium sulfate fractions.

Rabbit and goat ALS. The properties of the crude globulin obtained in both species by double precipitation with 33, 36, or 40 percent SAS are shown in Table I and Fig. 4. With 40 percent SAS, the antiwhite cell titers were fully retained. At 33 percent SAS, there was some loss of lymphocytotoxicity, but the precipitated protein was much less heterogeneous.

Horse ALS. The properties of the Fractions G and T removed by differential ammonium sulfate precipitation were essentially the same with the use of the serum of the two horses immunized with human splenic lymphocytes and with the use of the serum of the third animal which was given canine spleen. Fraction G had a very high proportion of IgG compared with the T-equine content. The Fraction T also contained IgG (Fig. 5), but the predominant immunoglobulin was IgA. Before testing Fraction G for its immunosuppressive effect (see below), it was diluted to give the same gamma globulin content as in the ALS. A similar adjustment was made for the T-equine content of Fraction T. The protein concentrations and the antibody activities of the adjusted fractions are shown in Table II.

In vivo testing. Both the absorbed horse

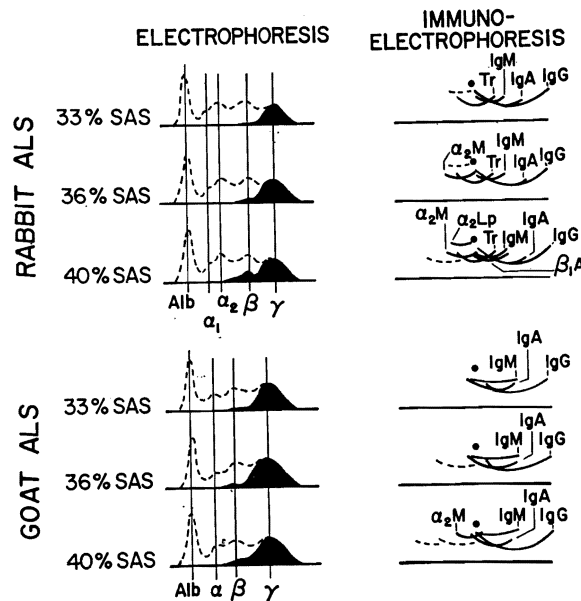


Fig. 4. The electrophoretic and immunoelectrophoretic features of the crude globulin precipitated from rabbit and goat ALS with different percentages of saturated ammonium sulfate (SAS). The only abbreviations not used in Figs. 1 to 3 are α_2Lp = alpha₂ lipoprotein and β_1A = beta₁ A globulin.

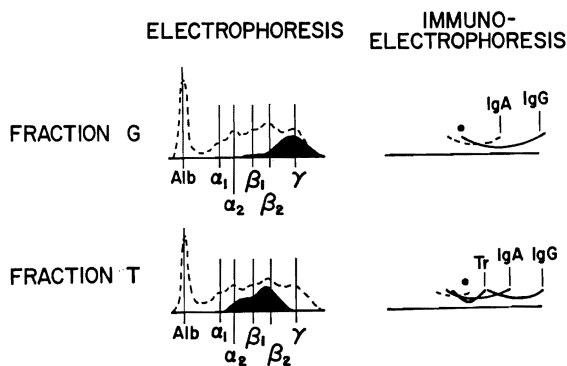


Fig. 5. Electrophoresis and immunoelectrophoresis of the globulin fractions removed by differential ammonium sulfate precipitation from horse anti-dog-lymphocyte serum. Note the marked heterogeneity of the fraction T. The two fractions were tested for their ability to mitigate renal homograft rejection.

antidog-lymphocyte serum and the Fraction T removed from it caused prompt lymphopenia with declines of the peripheral lymphocyte counts in dogs to 1,500 mm.³ or less. There was a delayed lymphopenia with the administration of Fraction G but to a

lesser degree and only after 10 days of continuous treatment.

The results of canine renal homotransplantation between nonrelated mongrel donors and recipients are shown in Table II. Of the 43 transplantations performed, six were eliminated from the analysis because of technical accidents which spoiled the experiments.

The dogs treated with ALS and the Fractions G and T had a significantly longer survival than the animals given no therapy. There seemed to be no difference when ALS and Fraction T ($p > 0.1$) were used, whereas the animals injected with Fraction G lived for somewhat shorter periods ($p < 0.01$).

DISCUSSION

The two animals that have been most commonly used as sources for ALS have been the rabbit and the horse. In the immunized rabbit, there has been little doubt that the antiwhite cell antibodies and the immunosuppressive effect reside in the gamma-G globulin.^{9, 12-14, 20} The findings in the pres-

Table II. Results of canine renal transplantation with whole horse ALS or globulin fractions being

	Protein concentration (Gm. %)				Antibodies	
	Gamma	T-equine	Beta, alpha, and albumin	Total	Leukoagglutinin	Lymphocytotoxin
Absorbed ALS	1.7	1.6	3.8	7.1	1:2,048	1:512
Fraction G	1.9	0.7	0.4	3.0	1:512	1:512
Fraction T	0.7	1.6	2.6	4.9	1:2,048	1:256
No treatment						

*By concentration or dilution, it was attempted to give the Fraction G injections the same gamma globulin content as that in the Fraction T with extraneous protein.

ent study were in agreement with these earlier investigations in that both the leucoagglutinins and lymphocytotoxins of the rabbit serum were in the IgG. Moreover, essentially all the undesirable thromboagglutinins and the preponderance of the hemagglutinins were in the same location; some hemagglutinin activity also appeared to be in the IgM. The analysis of goat ALS provided almost identical results except that the hemagglutinins were exclusively found in the IgM.

In the horse, a greater spread of the various antibodies has been reported by several authors during the first few months after beginning immunization.^{2, 8, 9, 16, 19} Most of these publications contained data indicating that leucoagglutinins or cytotoxins or both were present not only in the IgG ("slow" gamma G) but also in the T-equine globulin of which the principal constituent is IgA or "fast" gamma G; the skin graft experiments of James and Medawar⁹ suggested that a Sephadex-separated 7S globulin that may have contained IgA was immunosuppressive. At a later time, after 3 to 11 months of immunization, Pichlmayer and associates¹⁶ showed a shift of the antiwhite cell antibodies to the IgM fraction.

The three horses in the present study which had received splenic cells for 6 to 9 weeks were considered to be early serum donors. Essentially no leucoagglutinins or lymphocytotoxins were in the IgM. However, abundant antibodies were present in the T-equine globulin and, in fact, most of the leucoagglutinins were in this location whereas the lymphocytotoxins were primar-

ily in the IgG. The patterns of localization and the separability of these two kinds of activity were similar to the findings described by Carraz and co-workers.²

If, as appears to be the case, there are two distinct kinds of antiwhite cell antibodies in horse ALS, both apparently have immunosuppressive qualities as judged by the canine kidney transplantation experiments that were done using the ALG prepared by differential ammonium sulfate precipitation. The IgA-rich Fraction T which contained high leucoagglutinin but relatively low lymphocytotoxin titers was at least as effective as the IgG-rich Fraction G which had a greater lymphocytotoxicity relative to its leucoagglutinating properties.

The results of these studies have not weakened the generally held proposition that most of the active portion of equine ALS in the first months after the beginning of immunization is 7S gamma globulin. However, they do provide a possible explanation for the loss of potency seen with highly refined horse IgG since this fraction ("slow" gamma G) represents only part of the total activity. The other portion consists of the "fast" gamma G (IgA), so called because of its slightly greater electrophoretic mobility. The IgA, which is buried in the T-equine globulin, is discarded in the process of refinement of IgG. Separation of the IgA from the other components of the heterogenous T-equine globulin and retention of it with the more easily removable IgG is a practical problem which has not yet been satisfactorily solved for mass production.

Such difficulties can be avoided in a num-

used*

Hemagglutinin	Thromboagglutinin	No. dogs	Survival			P value	
			Mean	Range	S.E.(I)	To control	To ALS
1:128	1:32	6	20.6	10-50	7.5	<0.01	—
1:128	1:16	10	16.3	9-34	3.3	<0.01	<0.01
1:128	1:32	10	19.4	11-35	3.3	<0.01	>0.1
		11	9.9	5-21	3.5	—	—

ALS doses. A similar adjustment was made for the T-equine content of the Fraction T. Note the high degree of contamination of

ber of other species. For example, the desired immunoglobulins in both the rabbit and goat are in the IgG and in a condensed and relatively isolated segment of the serum, thereby greatly simplifying the methodology for their nearly complete removal in an unadulterated form. The main disadvantage is that large amounts of ALS cannot be so easily raised because of the limiting factor of low blood volume in small serum donors.

SUMMARY

The localization of antibodies was studied in rabbit, goat, and horse ALS raised by weekly immunization with canine or human spleen cells for 4 to 12 weeks. A combination of analytic techniques was used including column chromatography, electrophoresis, immunoelectrophoresis, determination of protein concentration, and measurement of antibody titers.

In the rabbit and goat ALS, virtually all of the leukoagglutinins and lymphocytotoxins were in the easily separable IgG; accidentally induced thromboagglutinins were in the same location. In the rabbit hemagglutinins were found in both the IgG and IgM, whereas in the goat these were almost exclusively in the IgM.

The antiwhite cell antibodies were most widely distributed in the horse. The cytotoxins were primarily in the IgG, but the leukoagglutinins were most heavily concentrated in the T-equine globulin which consists mostly of IgA. By differential ammonium sulfate precipitation of a horse antidog lymphocyte serum, fractions were prepared that were rich in IgG and IgA. Both were

able to delay the rejection of canine renal homografts, the IgA-rich preparation to a somewhat greater degree. The findings in this study have been discussed in relation to the refining techniques that have been used for the production of globulin from heterologous ALS.

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