259

THE NATURE OF ANTIPLATELET ACTIVITY IN ANTILYMPHOBLAST ALG

WITH SPECIAL REFERENCE TO CROSS-REACTING ANTIBODY, IMMUNOCHEMICAL CHARACTERIZATION, AND COOMBS' POSITIVE THROMBOCYTOPENIA IN ALG-TREATED RENAL RECIPIENTS

H. AMEMIYA, T. YOKOYAMA, C. W. PUTNAM
M. TORISU AND T. E. STARZL

THE NATURE OF ANTIPLATELET ACTIVITY IN ANTILYMPHOBLAST ALG

WITH SPECIAL REFERENCE TO CROSS-REACTING ANTIBODY, IMMUNOCHEMICAL CHARACTERIZATION, AND COOMBS' POSITIVE THROMBOCYTOPENIA IN ALG-TREATED RENAL RECIPIENTS

H. AMEMIYA, T. YOKOYAMA, C. W. PUTNAM, M. TORISU AND T. E. STARZL

Department of Surgery, University of Colorado School of Medicine, and Denver Veterans Administration Hospital, Denver, Colorado

SUMMARY

ALG raised against lymphoblasts grown in pure culture for many generations contained antiplatelet activity. The thromboagglutinins could be completely removed by absorption with lymphoblasts, indicating that they had been raised to antigens shared by lymphoblasts and platelets. Anti-spleen ALG possessed levels of such anti-platelet antibodies but in substantially higher titres, because an additional contribution was made by contaminating thrombocytes in the immunizing injectate. By chromatographic separation and immunoelectrophoretic analysis of the eluate from platelet–antibody complexes generated during the absorption of anti-spleen ALG with thrombocytes, the thromboagglutinins were shown to reside almost exclusively (97·7%) in the area of the IgA fraction. The direct Coombs' test, reacting platelets from patients receiving equine anti-spleen ALG with guinea-pig antisera against normal horse serum, showed a highly significant, but not invariable correlation with clinical platelet depression, which was not, however, clearly related to the thromboagglutinin titre of the ALG being administered.

INTRODUCTION

Thrombocytopenia often has been observed in recipients of organ transplants under treatment with differing immunosuppressive regimens. More than one factor has been indicted, including simple bone marrow depression (Starzl, 1964). In addition, previous reports from our department showed a relationship between thrombocytopenia and graft platelet entrapment during acute (Groth *et al.*, 1968) and hyperacute rejection (Giles *et al.*, 1970; Simpson *et al.*, 1970; Starzl *et al.*, 1970). Sequestration of thrombocytes in grafts can also occur by a non-immunologic pathway, particularly in livers (Hutchison *et al.*, 1968).

Correspondence: Dr T. E. Starzl, Veterans Administration Hospital, 1055 Clermont Street, Denver, Colorado 80220, U.S.A.

417

It is equally well known that horse antilymphocyte serum (ALS) can cause thrombo-cytopenia (Starzl *et al.*, 1967; Ono *et al.*, 1969; Starzl, 1969). Work in our laboratory (Starzl, 1969; Starzl *et al.*, 1969) and by Pichlmayer *et al.* (1968) and James *et al.* (1970) suggested that some platelet killing could be expected even with ALS raised with pure collections of lymphocytes because of antigens shared between platelets and lymphocytes. Nevertheless, Najarian *et al.* (1969, 1970) have claimed that ALS raised in horses against cultured human lymphoblasts (grown by Dr G. Moore, Roswell Park Memorial Institute, Buffalo, New York) did not produce thrombocytopenia in any of their patients.

In the present investigation, the antiplatelet activity of horse ALS raised by splenic lymphocytes was compared to that in equine ALS raised against cultured lymphoblasts (grown by Dr G. Moore). Antiplatelet activity was found in both products and charac-terized for its immunochemical nature, specificity, and cross-reactivity. In addition, studies were performed in thirty-six kidney recipients treated with horse antispleen ALS in order to show the mechanism of the induced thrombocytopenia. The degree of platelet depression was shown to correlate with the coating of patients' platelets with horse IgA (positive direct Coombs' test.)

METHODS

Preparation and testing of ALS

The antigen. The cell suspensions were injected in horses subcutaneously over 3–6 months. Four horses received fourteen to thirty inoculations with splenic lymphocytes (Iwasaki *et al.*, 1967); the other two were given seventeen to twenty injections over 2–3 months of cultured lymphoblasts that had been growing for more than 1000 generations in the laboratory of Dr G. Moore, Buffalo, New York.

Titres. Antibodies raised in the two different kinds of horses were measured in the raw horse serum at various phases of processing and in the refined antilymphocyte globulin (ALG). Haemagglutinins, leukoagglutinins and lymphocytotoxins were measured by techniques previously reported from our laboratory (Iwasaki *et al.*, 1967; Kashiwagi *et al.*, 1970). The thromboagglutinins were determined by the method described by Kashiwagi *et al.* (1970), incubating for 6 hr without agitation.

Absorption procedures. The crude antisplenic (and the antilymphoblast) ALS for clinical use were absorbed (Iwasaki *et al.*, 1967; Kashiwagi *et al.*, 1969) with pooled fresh human red blood cells and with human platelets to remove excessive haemagglutinins and thrombo-agglutinins respectively. The ALG was then prepared by ammonium sulphate precipitation (Iwasaki *et al.*, 1967).

Other absorption studies

Antilymphoblast ALS from two different horses was diluted five times and then absorbed four to six times (37°C for 2 hr and 4°C for 12 hr) with an equal volume of human red cells that were purified eight times by differential centrifugation. In addition, diluted ALS was absorbed two or three times under the same conditions with three times the volume of packed lymphoblasts. Haemagglutinin, leukoagglutinin, thromboagglutinin and lymphocytotoxin titres were measured before and after these procedures.

The nature of the antiplatelet antibody

After the platelet absorption mentioned previously, the agglutinated platelets were

assumed to have complexed with specific antibody. The platelets were suspended in normal saline and submitted to differential centrifugation six to ten times to remove contaminating red and white blood cells.

Elution of antibody. The thromboagglutinins were eluted by the method of Stelos (1967). After suspending 3 g of agglutinated platelets in 20 ml glycine-HCl buffer (pH 2·3, 0·15 M) at 4°C for 20 min while stirring, the supernatant containing antiplatelet antibody was removed by centrifugation at 7000 *g* for 30 min and the pH adjusted to 8·0 with saturated NaOH solution. Precipitation was then carried out with ammonium sulphate (50% sat.). The precipitate was dissolved in distilled water and dialysed against tap water and normal saline solution for chromatography as described below.

DEAE-cellulose column chromatography. Gradient chromatography was carried out in a 28 × 4 cm column containing activated Whatman DE-32 cellulose (H. Reeve Angel & Co. Ltd, London, England) (Sober & Peterson, 1958). The samples and cellulose were equilibrated with the initial sodium phosphate buffer (pH 8·0, 0·005 M). Elution was at a rate of 20 ml/hr. After the first peak was completely eluted, the acidity and molality of the buffer were gradually increased to pH 5·0, 0·2 M. The protein concentration of each tube was measured with a Hitachi-Perkins spectrophotometer at a wave length of 280 mμ. The thromboagglutinin and haemagglutinin titres of each tube were then determined.

Immunoelectrophoresis. A representative tube from each of the three major protein peaks defined by chromatography was analysed by Scheidegger's (1955) method. The sample was placed in the centre well of a 0·1% agar gel plate and electrophoresed at a potential difference of 5 V/cm for 90 min. The two troughs were then filled with guinea-pig anti-horse IgG and IgA antisera (raised in our laboratory by immunization with a purified IgA fraction; Oriol, Binaghi & Boussac-Aron., 1968), which are said by Oriol *et al.* (1968) to react only with the heavy chains of equine immunoglobulin molecules. Incubation was for 48 hr at 20°C (Amemiya *et al.*, 1970).

Coombs' test of patients' platelets

The platelets of thirty-six kidney recipients were studied for coating antibodies after treatment postoperatively with antisplenic ALS for 11–93 (mean 30·0 ± 12 SD) days. Five ml of blood were mixed with 0·5 ml of 5% EDTA in saline and centrifuged at 1200 *g* for 60 sec to separate the platelet-rich plasma. The latter was washed once in 10 volumes of Eagle's basal medium containing one-tenth volume EDTA–saline solution, and recentrifuged (1000 *g* for 5 min). The thrombocyte plug was resuspended in EDTA-Eagle's solution to a cell concentration of about 500,000/mm^3. Red and white blood cell contamination was 0·10 ± 0·06% and 0·98 ± 1·15%, respectively.

Preparation of Coombs' reagent. Rabbit antiserum to normal horse serum (Hyland Corporation, Los Angeles, California) was completely absorbed with human O, A and B red blood cells. After absorption, the antiserum was sufficiently diluted with buffered saline solution to eliminate all positive reactions with normal human thrombocytes. Usually the dilution ratio was sixteen times the original antiserum concentration. Finally, the antiserum was further diluted with the same solution so that it gave a precipitation titre of 1 : 500 when reacted by Ouchterlony's antigen dilution method with normal horse serum, the initial protein concentration of which was standardized at 1 g%.

The direct Coombs' test. Using a microtitre plate, the Coombs' reagent was serially diluted from 1:1 to 1:64 with phosphate buffered saline solution (pH 7·1, 0·15 M). An

equal volume (0·025 ml) of the platelet suspension was added to each well and the tray incubated for 6 hr at 20°C following which the configuration in each well was read with the magnifying reflector.

RESULTS

Antibodies in the ALS

Antispleen ALS. During immunization, extremely high titres of lymphocytotoxins and leukoagglutinins were raised. Coincidentally, haemagglutinin titres increased to 1 : 4096 and thromboagglutinins to 1 : 512 (Fig. 1). As mentioned earlier, these last two antibodies were absorbed out before globulin extraction.
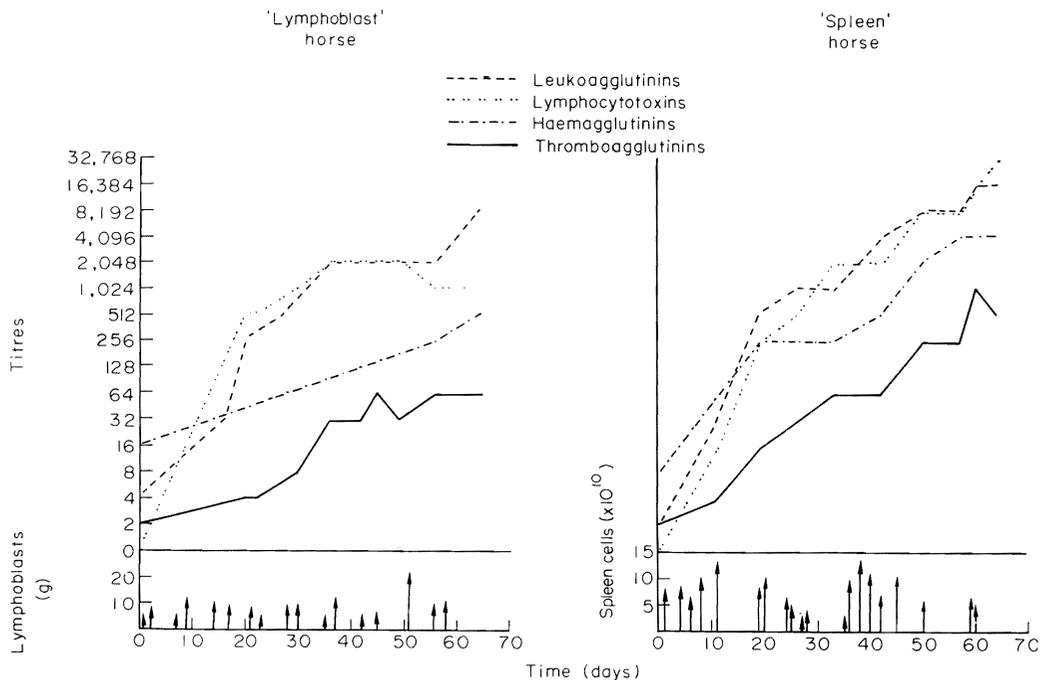

Fig. 1. Immunizing schedules and the resulting lymphocytotoxin, leukoagglutinin, haemag-glutinin, and thromboagglutinin titres for two horses inoculated with human lymphoblasts and spleen cells, respectively. The lymphoblast dose is given as the weight of the cell suspension in grams (1 g equals approximately 10^9 lymphoblasts, of which usually less than 30% were viable by the Erythrosin-B dye exclusion test). Only the lymphocytes in the spleen cell sus-pension were counted; of these, 30–70% were thought to be viable. The titres are expressed as the reciprocal of the greatest dilution of serum giving a positive reaction by that test.

Antilymphoblast ALS. The immunizing dose schedule in terms of cell number and the timing of injections was comparable to that with the 'spleen' horse. The animal responded with the highest rises in the leukoagglutinins and lymphocytotoxins. However, there were also significant increases in the haemagglutinins and thromboagglutinins to 1 : 512 and to 1 : 64, respectively (Fig. 1).

The effect of absorption upon antilymphoblast ALS

The raw antilymphoblast ALS obtained from the two different horses was diluted with buffered saline solution to the titres and protein contents shown in Table 1. Multiple absorptions with red blood cells completely removed the haemagglutinins but had no significant effect upon leukoagglutinins, lymphocytotoxins or thromboagglutinins.

In contrast (Table 1), absorption two or three times with packed lymphoblasts removed not only the lymphocytotoxins and leukoagglutinins but most or all of the thrombo-agglutinins and haemagglutinins as well. The absorption of the anti-platelet and anti-red cell antibodies could in no sense be ascribed to accidental contamination by platelets and/or red cells since the lymphoblasts had been in a blood free cell culture for many generations.
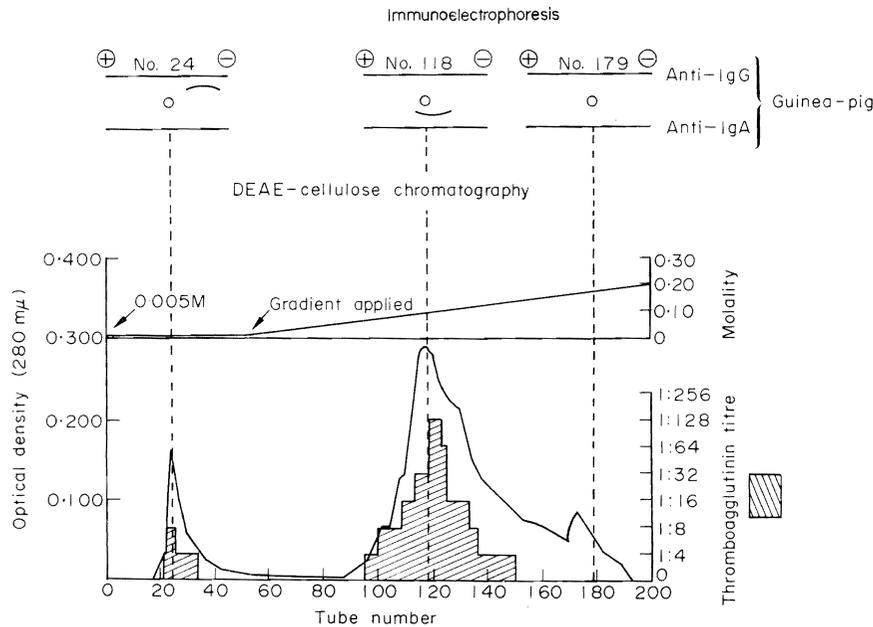

FIG. 2. Chromatographic and immunoelectrophoretic analyses of thromboagglutinins eluted from platelet–antibody complexes formed during the absorption of horse antispleen ALS by human thrombocytes. Two protein peaks contained antiplatelet activity. The larger was identified by immunoelectrophoresis with guinea-pig antisera as equine IgA; the smaller was IgG.

The nature of the antiplatelet antibody

After chromatography of the eluate from the platelet–antibody complexes, antiplatelet activity was seen in two of the three protein peaks; the largest, which was estimated to contain 97·7% of the total, was IgA (Fig. 2) insofar as could be determined with guinea-pig antisera. The smaller antibody peak was IgG. The third protein peak which did not contain antibody was highly acid and probably represented breakdown derivatives of the platelets.

Clinical studies and Coombs' tests

The results with chromatography prompted the use of a rabbit serum raised against whole horse serum as the Coombs' reagent since the common practice of using an anti-IgG

C

TABLE 1. Absorption of antilymphoblast ALS with human red blood cells and lymphoblasts

ALS from:	Procedure	Titres of:				Protein Concentration* (g%)
		Lymphocytotoxins	Leukoagglutinins	Thromboagglutinins	Haemagglutinins	
Horse 1	Not absorbed	128	512	32	64	1·12
	Absorbed ×6 with RBCs	128	256	32	0	1·12
	Absorbed ×3 with lymphoblasts	0	0	0	0	1·12
Horse 2	Not absorbed	256	1024	512	128	1·06
	Absorbed ×4 with RBCs	256	1024	512	0	1·06
	Absorbed ×2 with lymphoblasts	4	4	8	32	1·06

*The protein concentration was determined by the biuret method.

serum would have detected only a tiny fraction (less than 3%) of the total antiplatelet activity.

Thrombocytopenia versus a positive Coombs' test. There was a highly significant correlation ($P<$ 0·01) between the development of a positive Coombs' test and thrombocytopenia (Table 2). However (Fig. 3), it was often noticed that a Coombs' test became positive some days or weeks before the maximum depression of the platelet count. Conversely, recovery of platelets was often delayed for some time after discontinuance of ALG therapy and even sometimes for several days after the Coombs' test became negative (Fig. 3).

Thrombocytopenia versus antiplatelet titre of ALG. There was no significant difference in platelet depression between those patients receiving ALG with a very low antithrombocyte titre and those patients whose ALG had titres greater than 1:256. Similarly, these titres did not correlate with the presence of a positive Coombs' test (Table 3).

TABLE 2. Chi-square analysis of the incidence of platelet depression versus the results of the Coombs' test in thirty-six recipients of renal homografts treated with antispleen ALG

Platelet depression†	Coombs' test*		Total
	Positive	Negative	
Present	8	3	11
Absent	2	23	25
Total	10	26	36

$$\chi^2 = 12{\cdot}89 > 6{\cdot}63\ (P < 0{\cdot}01)$$

* The criteria for inclusion in the Coombs' positive group were positive reactions on two successive occasions or frequent (30% or more) positive Coombs' tests during the period of ALG administration.

† Platelet depression was defined as a thrombocyte count on two consecutive days of less than 150,000/mm³ (or less than the pre-ALG control if the latter were below 150,000/mm³).

DISCUSSION

Antiplatelet activity in ALS and ALG has been recognized to be a significant hazard since the earliest clinical trials with these agents (Starzl *et al.*, 1967; Starzl, 1969). From the beginning, two distinct aetiologic factors seemed likely. One which was emphasized from our laboratory (Starzl, 1969; Ono *et al.*, 1969) and by Woiwod *et al.* (1970) was that an antibody could accidentally be raised against platelets as well as against erythrocytes by the inadvertent inclusion of thrombocytes and red cells in the cell suspensions used for immunization. Ways of reducing this kind of contamination in preparing spleens and other tissues for injection have been described by Townsend *et al.* (1969) but Woiwod *et al.* (1970) have underscored the difficulties of getting rid of all platelets.

The second possible factor which was supported by several authors (Pichlmayer *et al.*, 1968; James *et al.*, 1970; Starzl ,1969; Starzl *et al.*, 1969) but not by others (Najarian *et al.*, 1969, 1970) was that platelets and red cells had certain antigens in common with lymphoid
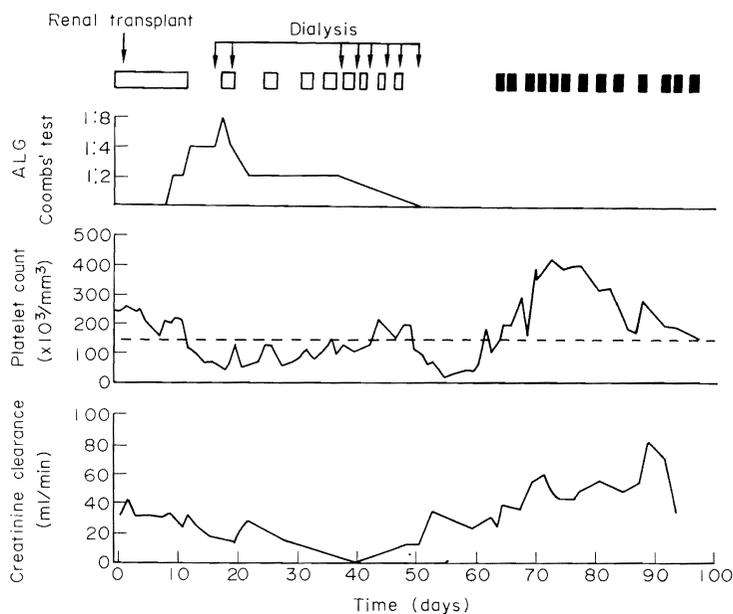
FIG. 3. The course of a recipient of a cadaveric renal homograft whose thrombocytopenia was associated with Coombs' positive reactivity. Note that the Coombs' test turned positive before depression of the platelet count could be documented. After stopping the antispleen ALG, the Coombs' tests rapidly became negative. The platelet count, however, did not return to normal for about 3 weeks, but then remained above 150,000/mm³ even though treatment with a new batch (shaded blocks) of ALG was instituted.

TABLE 3. Incidence of thrombocyte depression and positive Coombs' tests in fifteen patients receiving ALG with low thromboagglutinin titres and in seven recipients given high thromboagglutinin titre ALG

Thromboagglutin in titre of ALG	No. of patients	Thrombocyte depression*	Positive Coombs' reaction*
≤ 1 : 32	15	4 (26·6%)	5 (33·3%)
≥ 1 : 256	7	2 (28·6%)	1 (14·3%)

* See Table 2 for criteria.

cells and that, as a consequence, purely antilymphocyte antibodies cross-reacted with the other formed elements. Incontrovertible proof for this later hypothesis awaited the use of cultured lymphoblasts for immunizing antigen, since this approach ruled out for the first time all possibility of extraneous red cells or platelets in the injectates. Under these conditions, in the present study antilymphoblast ALS was shown to contain haemagglutinins and thromboagglutinins.

These findings were hardly surprising in view of the evidence that platelets share antigens with a variety of other cells (Gilboa-Garber & Nelken, 1961; Shulman, Moor-Jankowsky & Hiller, 1965; Hanna & Nelken, 1968). However, the 'sharing' is undoubtedly variable in different cell membranes. For example, in the present study lymphoblasts injected in horses elicited unwanted haemagglutinins and thromboagglutinins in addition to the

desired leukoagglutinins and lymphocytotoxins. All of these antibodies were absorbed with lymphoblasts. In contrast, red cells were effective absorbers solely against haemagglutinins. Past work has showed that absorption with platelets can be carried out without reducing lymphocytotoxin titres (Starzl, 1969; Starzl *et al.*, 1969). Thus, complete representation of all the antigens in the system being tested could be found only in the lymphoblasts.

At the moment, there seems no reason to suspect that thromboagglutinins raised with platelets are inherently either more or less thrombocytopenic than those resulting from shared lymphocyte antigens. It seems more likely that any *in vivo* effect would represent roughly the sum of both these factors. Immunochemically, two distinct kinds of thrombo-agglutinins could not be identified in antispleen ALS. Almost all antiplatelet identity seemed to be in IgA, as was previously claimed by Kashiwagi *et al.* (1970).

With the use of the direct Coombs' test, an idea was obtained of the regularity with which IgA attached to circulating platelets and contributed to thrombocytopenia. There was a highly significant correlation between positive Coombs' tests and platelet depression. However, the association was not invariable and in addition there were some patients with thrombocytopenia who had negative Coombs' tests. Moreover, the strength of the anti-platelet titre in the ALG did not correlate either with the incidence of positive Coombs' tests or the severity of thrombocytopenia, as might have been predicted by the data of Kamoun & Hamburger (1970). Consequently, it seems likely that the other factors mentioned in the introduction of this article played an important role in many of the post-transplantation thrombocytopenias. These could include the pre-existing state of the recipient haematopoietic system, bone marrow depression post-transplantation by immuno-suppressants, platelet sequestration in the graft because of rejection or mechanical trauma, and other less well defined processes that could contribute either to accelerated platelet destruction or to retarded platelet production.

REFERENCES

AMEMIYA, H., KASHIWAGI, N., PUTNAM, C.W. & STARZL, T.E. (1970) Cross reactivity studies of horse, goat and rabbit anti-lymphocyte globulin. *Clin. exp. Immunol.* **6**, 279.

GILBOA-GARBER, N. & NELKEN, D. (1961) Specific antigens of human leukocytes and thrombocytes. *Nature (Lond.),* **192**, 466.

GILES, G.R., BOEHMIG, H.J., AMEMIYA, H., HALGRIMSON, C.G. & STARZL, T.E. (1970) Clinical hetero-transplantation of the liver. *Transplant. Proc.* **2**, 506.

GROTH, C.G., PORTER, K.A., OTTE, J.B., DALOZE, P.M., MARCHIORO, T.L., BRETTSCHNEIDER, L. & STARZL, T.E. (1968) Studies of blood flow and ultrastructural changes in rejecting and non-rejecting canine orthotopic liver homografts. *Surgery,* **63**, 247.

HANNA, N. & NELKEN, D. (1968) Thrombocyte specific antigens. *Vox Sang.* **14**, 57.

HUTCHISON, D.E., GENTON, E., PORTER, K.A., DALOZE, P.M., HUGUET, C., BRETTSCHNEIDER, L., GROTH, C.G. & STARZL, T.E. (1968) Platelet changes following clinical and experimental hepatic homo-transplantation. *Arch. Surg.* **97**, 27.

IWASAKI, Y., PORTER, K.A., AMEND, J.R., MARCHIORO, T.L., ZUHLKE, V. & STARZL, T.E. (1967) The preparation and testing of horse anti-dog and anti-human antilymphoid plasma or serum and its protein fractions. *Surg. Gynec. Obstet.* **124**, 1.

JAMES, K., PULLAR, D.M., JAMES, V.S., WOOD, A., EPPS, H.B.G. & RAHR, L. (1970) The development and distribution of antilymphocytic and other antibodies in horses immunized with human lymphoid antigens. *Transplantation*, **10**, 208.

KAMOUN, P.P. & HAMBURGER, J. (1970) Measurement of antiplatelet activity of antilymphocyte sera based on the inhibition of 5-hydroxytryptamine uptake by blood platelets. *Transplantation*, **10**, 53.

KASHIWAGI, N., GROTH, C.G., AMEND, J.R., GECELTER, L., BLANCHARD, H. & STARZL, T.E. (1969) Improvements in the preparation of heterologous antilymphocyte globulin with special reference to absorption and diethylaminoethyl cellulose batch production. *Surgery*, **65**, 477.

KASHIWAGI, N., SHERER, D., TOWNSEND, C.M., JACOBS, R., ONO, K., KAPUR, B. & STARZL, T.E. (1970) Antibody localization in horse, rabbit, and goat antilymphocyte sera. *Surgery*, **67**, 789.

NAJARIAN, J.S., SIMMONS, R.L., GEWURZ, H., MOBERG, A., MERKEL, F. & MOORE, G.E. (1969) Anti-serum to cultured human lymphoblasts: preparation, purification and immunosuppressive properties in man. *Ann. Surg.* **170**, 617.

NAJARIAN, J.S., SIMMONS, R.L., GEWURZ, H., MOBERG, A., MERKEL, F. & MOORE, G.E. (1970) Antihuman lymphoblast globulin. *Fed. Proc.* **29**, 197.

ONO, K., BELL, P.R.F., KASHIWAGI, N. & STARZL, T.E. (1969) The comparative *in vitro* and *in vivo* activity of ALS raised by immunization with thymic, splenic and lymph node lymphocytes. *Surgery*, **66**, 698.

ORIOL, R., BINAGHI, R. & BOUSSAC-ARON, Y. (1968) Preparation d'anticorps monospecifiques anti-immuno-globulines. *Ann. Inst. Pasteur*, **114**, 52.

PICHLMAYER, R., BRENDEL, W., TSIRIMBAS, A., BOCK, E., THIERFELDER, S., FATEH-MOGHADAM, A., HORNUNG, B. & PFISTERER, H. (1968) Use of heterologous anti-lymphocyte sera in man. *J. cardiovasc. Surg. (VIII Congress of the International Cardiovascular Society)*, p. 57.

SCHEIDEGGER, J.J. (1955) Une micro-methode de l'immune-electrophorese. *Int. Arch. Allergy*, **7**, 103.

SHULMAN, N.R., MOOR-JANKOWSKY, J. & HILLER, M.C. (1965) Platelet and leukocyte isoantigens common to man and other animals. *Ser. Haemat.* **11**, 113.

SIMPSON, K.M., BUNCH, D.L., AMEMIYA, H., BOEHMIG, H.J., WILSON, C.B., DIXON, F.J., COBURG, A.J., HATHAWAY, W.E., GILES, G.R. & STARZL, T.E. (1970) Humoral antibodies and coagulation mechanisms in the accelerated or hyperacute rejection of renal homografts in sensitized canine recipients. *Surgery* **68**, 77.

SOBER, H.A. & PETERSON, E.A. (1958) Protein chromatography on ion exchange cellulose. *Fed. Proc.* **17**, 1116.

STARZL, T.E. (1964) *Experience in Renal Transplantation.* Saunders, Philadelphia.

STARZL, T.E. (1969) *Experience in Hepatic Transplantation.* Saunders, Philadelphia.

STARZL, T.E., BOEHMIG, H.J., AMEMIYA, H., WILSON, C.B,, DIXON, F.J., GILES, G.R., SIMPSON, K.M. & HALGRIMSON, C.G. (1970) Clotting changes, including disseminated intravascular coagulation, during rapid renal homograft rejection. *New Engl. J. Med.* **283**, 383.

STARZL, T.E., BRETTSCHNEIDER, L., PENN, I., SCHMIDT, R.W., BELL, P., KASHIWAGI, N., TOWNSEND, C.M. & PUTNAM, C.W. (1969) A trial with heterologous anti-lymphocyte globulin in man. *Transplant. Proc.* **1**, 448.

STARZL, T.E., PORTER, K.A., IWASAKI, Y., MARCHIORO, T.L. & KASHIWAGI, N. (1967) The use of anti-lymphocyte globulin in human renal homotransplantation. *Antilymphocytic Serum* (Ed. by G. E. W. Wolstenholme and M. O'Connor), pp. 4–34. Churchill, London.

STELOS, P. (1967) Isolation of specific antibody from complexes of antigen and antibody. *Handbook of Experimental Immunology* (Ed. by D. M. Weir), pp. 112–119. Blackwell Scientific Publications, Oxford.

TOWNSEND, C.M., JR, VYVIAL, T.M., WOOD, A.J., REMMERS, A.R., JR, SARLES, H.E., CANALES, C.O., BEATHARD, G.A., FISH, J.C., MUNIZ, F., LEVIN, W. C. & RITZMANN, S.E. (1969) Antihuman lymphocyte globulin. Simplified production procedure for human use. Preliminary report, *Texas Rep. Biol. Med.* **27**, 2.

WOIWOD, A.J., COURTENAY, J.S., EDWARDS, D.C., EPPS, H.B.G., KNIGHT, R.R., MOSENDALE, B., PHILLIPS, A.W., RAHR, L., THOMAS, D., WOODROOFFE, J.G. & ZOLA, H. (1970) The preparation and properties of horse antihuman lymphocyte serum and globulin. *Transplantation*, **10**, 173.