204

Clin. exp. Immunol. (1970) **6**, 279–289.

CROSS-REACTIVITY STUDIES OF HORSE, GOAT AND RABBIT ANTI-LYMPHOCYTE GLOBULIN

H. AMEMIYA, N. KASHIWAGI, C. W. PUTNAM AND T. E. STARZL

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

(*Received* 28 *August* 1969)

SUMMARY

In the sera of ten normal humans and twenty-eight candidates for organ transplantation, the passive haemagglutination test detected a 50% incidence of preformed antibodies of low titre directed against horse serum. Such antibodies were also found to cross react with goat or rabbit sera in most instances. Seventeen of the organ recipients were later studied after the institution of treatment with horse antihuman-lymphocyte globulin (ALG). The incidence of anti-horse-serum antibodies rose to 100%. At the same time, an increased activity against goat serum developed; cross-reactions against rabbit serum were also demonstrated but to a less pronounced degree. With immunoelectrophoresis and Ouchterlony diffusion tests, it was shown that the cross-reactivity was to similar although not necessarily identical protein components of the different heterologous sera.

These data suggest that there is an inherent risk in switching from one ALG to another, particularly if horse and goat derivatives are used sequentially. Since rabbit ALG does not cross react so extensively with horse or goat serum it would be predicted to be a relatively safer second-line agent.

These expectations seemed to have been realized in six patients who were given more than one kind of ALG, always beginning with horse globulin. When goat ALG was administered second, anaphylactic reactions tended to appear early, but when the rabbit product was given second or third, it was relatively well tolerated.

INTRODUCTION

In the initial transplantation trials with horse anti-human-lymphocyte globulin (ALG), this agent was administered in combination with azathioprine and prednisone and its use limited to a maximum of four post-operative months (Starzl *et al.*, 1967). It soon became clear, particularly after transplantation between non-related individuals, that an 'escape' from immunosuppressive control often occurred after the discontinuance of the intramuscular

Correspondence: Professor T. E. Starzl, Department of Surgery, University of Colorado School of Medicine, 4200 East Ninth Avenue, Denver, Colorado 80220, U.S.A.

globulin injections (Starzl, 1969; Starzl *et al.*, 1969). Unfortunately, prolongation of the treatment course was often prevented in subsequent cases by the development of toxic reactions to the horse ALG being given. As a consequence, ALG prepared from the serum of immunized rabbits and goats was substituted for the equine globulin on a number of occasions (Starzl, 1969; Starzl *et al.*, 1969).

It is the purpose of this report to comment upon the degree of safety with which a switch may be made from one heterologous serum donor to another, based on observations in a number of cases in which this was actually done. In addition, immunological data will be presented about the cross-reactivity of horse, rabbit and goat ALG. The latter studies were carried out with the passive hemagglutination method, Ouchterlony analysis and immuno-electrophoresis.

METHODS

Clinical cases

Three recipients of livers and three recipients of kidneys were initially treated with horse ALG. Nine to 119 days later, the treatment was stopped, either because of suspected anaphylactic reactions (four examples), because of severe reactions at the intramuscular injection sites (one example), or because the end of an arbitrary treatment period had been reached (one example). Therapy with azathioprine and prednisone was continued without interruption.

Administration of goat ALG was instituted without delay in four cases. In two others, rabbit globulin was started after intervals of 67 and 75 days. Finally, five patients received globulin from all three species, the horse product always being the first. In three cases the goat derivative was used next and in the other two the rabbit ALG was used as the second agent. The data from these cases are summarized in Table 1.

Passive haemagglutination

This examination was the most sensitive one used. In principle, it involves the detection of antibodies in test sera by their ability to cause agglutination of chromic chloride treated 0 negative human red cells that have been coated with heterologous protein to make a complex antigen (Gold & Fudenberg, 1967).

Preparation of antigen. The red cells were coated with the sera of ten different humans, as well as with sera from one horse, one goat and one rabbit. Before mixing with the red cells, the sera were inactivated at 56°C for 30 min, diluted ten-fold with normal saline solution, and added to an equal volume of 0·1% chromic chloride in saline. The solution was then added to an equal volume of packed red cells, incubated at room temperature for 5 min, and the mixture washed four times with saline. The coated cells were separated by centrifugation and resuspended in normal saline solution in a 1% concentration by volume.

Measure of cross-reactivity with specific antisera. The different varieties of coated human red cells were exposed to the following commercial antisera (Hyland Laboratories, Los Angeles, California): (1) rabbit antiserum raised against human serum, (2) rabbit antiserum raised against horse serum, (3) rabbit antiserum raised against goat serum, and (4) goat antiserum raised against rabbit serum. When reacted against normal serum from the donor species, the four commercial antisera defined a minimum of eleven, eight, ten and eleven precipitin bands, respectively. To 0·025 ml of serial two-fold dilutions of the commercial

TABLE 1. Clinical information for six recipients of kidney or liver homografts who were treated with ALG raised in more than one species

Case No.	Type homograft	Horse ALG*			Goat ALG*			Rabbit ALG*			Interval (days) between	
		Order	Duration (days)	Reason for stopping	Order	Duration (days)	Reason for stopping	Order	Duration (days)	Reason for stopping	1 and 2	2 and 3
1	Liver	1	119	End of course	3	83	Local reactions	2	58	Local reactions	75	0
2	Liver	1	18†	Local reactions	3	71	Systemic reaction	2	45	Local reactions	67†	0
3	Liver	1	41	Anaphylactic reaction	2	15	Anaphylactic reaction	3	83	Died	0	0
4	Kidney	1	57	Anaphylactic reaction	2	15	Anaphylactic reaction	3	35	Anaphylactic reaction	0	3
5	Kidney	1	9	Anaphylactic reaction	2	86	Anaphylactic reaction	3	68	End of course	0	4
6	Kidney	1	95	Anaphylactic reaction	2	148	Local reactions				4	

Horse ALG was always used first; the second (and third) species ALG in each case is indicated.

* The leukoagglutinin titres were 1:4000 to 1:8000 for the horse ALG and 1:2000 to 1:4000 for the goat and rabbit products. The protein contents in g/100 ml were: horse ALG, 6·8–7·6; goat ALG, 4·2–8·3; and rabbit ALG, 2·3–5·2. The doses were adjusted according to the titre of the ALG and the patient's weight; most adults received 4–5 ml/injection.

† Desensitization for 12 days was attempted 30 days after stopping horse ALG; the effort was abandoned.

antisera were added equal aliquots of the coated cell suspensions. Following incubation at 4°C for 18 hr, the cells in the micro-plate wells were viewed grossly; the end point was said to be the greatest dilution of antisera causing visible agglutination.

Detection of and cross-reactivity of naturally occurring antibodies. Using exactly the same technique, it was determined if the agglutination described in the above section could be caused by the normal serum of the different species. For example, red cells coated with horse serum were mixed with normal human, goat and rabbit serum; the red cells coated with rabbit serum were exposed to the serum of humans, a goat and a horse, etc.

The search for natural antibodies in humans was a very complete one. The sera examined were obtained from ten healthy donors as well as from twenty-eight patients who ultimately received renal, hepatic, splenic or cardiac homografts. In each case, it was determined if the patient serum was capable of agglutinating red cells coated with horse, rabbit, or goat protein, and if so to what titre.

The measurement of antibodies induced by ALG therapy. Using the same method, antibodies against all three heterologous serum proteins were repeatedly sought before and at varying intervals after the institution of ALG therapy in twenty-one cases. Sixteen of the renal recipients were treated with horse globulin only and one other was administered horse and goat ALG in that order. The four other patients had orthotopic liver transplantation. One had horse ALG only, but the other three were given globulin from all three species. In all recipients, azathioprine and prednisone were begun shortly before or at the time of transplantation and continued indefinitely in varying doses (Starzl, 1969; Starzl *et al.*, 1967).

Immunoelectrophoresis

Although exquisitely sensitive, the passive haemagglutination method did not allow identification of the individual protein components against which antibodies were directed. For this purpose and also to confirm the findings obtained with passive haemagglutination, serum samples were also subjected to immunoelectrophoresis (Scheiddegger, 1955). The 25 × 75 mm microelectrophoretic slide was coated with 2 ml of 0·1 g/100 ml agar gel in pH 8·6 veronal buffer. The centre (antigen) well was charged with 0·001 ml of a 1:1 dilution of normal serum obtained from the human, horse, goat and rabbit. Following electrophoresis for 90 min at 5 V/cm of gel potential difference, the peripheral troughs were filled with 0·07 ml of patient sera or commercial antisera, also in a 1:1 dilution. Immunodiffusion was allowed to continue at 4°C for 2–3 days.

Commercial antisera. The same commercial antisera used for the passive haemagglutination test (see earlier section) were placed in the trough. Thus, the normal serum of the human, horse, goat and rabbit was interacted with antisera raised in the rabbit or goat against these species. Cross reactivity was considered to be present when the different heterologous antisera produced precipitin bands in the same location.

Patient sera. Exactly the same examination was carried out except that the 'antisera' were from the patients who had received ALG prepared from one or more species.

Ouchterlony double diffusion

Sera from seventeen patients receiving horse ALG only were further examined for cross-reactivity with horse, goat and rabbit sera using a standard micro-Ouchterlony method (Ouchterlony, 1958). The patients' serum was placed in the centre well and the heterologous

sera in the three peripheral wells; the volume of serum in each well was 0·01 ml. Lines of fusion between adjacent bands indicated the presence of identically reacting protein fractions.

RESULTS

Clinical observations

Six patients had a change in ALG from a few days to many months after the institution of equine globulin therapy (Table 1). Immediate serious difficulties were not caused by the second ALG but in two cases in which the switch was from horse to goat globulin, reactions after 15 days necessitated a further switch to the rabbit.

Cross-reactivity as studied with specific antisera

Apparent interspecies cross-reactivity was demonstrated with antisera raised against the serum of the human, horse, goat and rabbit (Table 2). Using the passive haemagglutination method, the most powerful cross-species reaction of each of the animal antisera was with human serum. However, there were also cross-reactions to a less pronounced degree within the circle of the three animals. For example, the anti-equine serum antibodies agglutinated red cells coated with horse serum to a titre of 1:128,000; when the red cells were covered with goat protein the titre was 1:256. The same thing was seen with variations in all interspecies combinations tested (Table 2).

TABLE 2. Cross-reactivity of specific commercial antisera with normal human, horse, goat and rabbit serum

Antigen	Specific antisera	Passive haemagglutination titre*	Serum protein components identified by immunoelectrophoresis						
			Albumin	α_1	α_2M	γM	γA	γG	Additional fractions
Human serum	Rabbit anti-human-serum	1:8,000,000	+	+	+	+	+	+	+
	Rabbit anti-horse-serum	1:8,000,000	±		+				
	Rabbit anti-goat-serum	1:4,000,000	+		+			+	
	Goat anti-rabbit-serum	1:4,000,000	+		+	+		+	
Horse serum	Rabbit anti-human-serum	1:256,000	+		±			+	
	Rabbit-anti-horse-serum	1:128,000	+	+	+	+	+	+	+
	Rabbit anti-goat-serum	1:256	+		+		+	+	
	Goat anti-rabbit-serum	1:256			+		+		
Goat serum	Rabbit anti-human-serum	1:4,000	+		+			+	
	Rabbit anti-horse-serum	1:256	+		+			+	
	Rabbit anti-goat-serum	1:1,000,000	+	+	+	+	+	+	+
	Goat anti-rabbit-serum	No reaction							
Rabbit serum	Rabbit anti-human-serum	1:32							
	Rabbit anti-horse-serum	No reaction							
	Rabbit anti-goat-serum	No reaction							
	Goat anti-rabbit-serum	1:256,000	+	+	+	+	+	+	+

The titres given were determined by the passive haemagglutination method. The individual serum protein fractions reacting with the antisera were identified by immunoelectrophoresis.

* The titre is expressed as the reciprocal of the greatest dilution of antisera giving visible agglutination.

With immunoelectrophoresis, it was found that the protein components responsible for the interspecies cross-reactivity were widespread (Table 2). They included the albumin, γG-globulin and especially the macroglobulins.

Naturally occurring antibodies against sera of other species

No naturally occurring antibodies could be detected in the serum of the normal horse, goat or rabbit which were directed against the serum of the other two members of the animal group.

However, there were antibodies in all three animal species which reacted against the serum of some members of the ten man normal human panel. The horse, goat and rabbit sera caused low titre (1:16 to 1:256) agglutination in five out of ten, seven out of ten and one out of ten of the human sera, respectively.

TABLE 3. Frequency of naturally occurring antibodies against normal horse, goat or rabbit serum in twenty-eight pretransplantation patients who had not received ALG and in ten normal donors

	Pre-transplant	Normal donor	Total	Per cent
Patients showing no reactions	7/28	2/10	9/38	23·6
Patients reacting against a single species				
Horse	0	1	1	2·7
Goat	6	3	9	23·7
Rabbit	0	0	0	0
Total	6/28	4/10	10/38	26·4
Patients reacting against two species				
Horse and goat	4	3	7	18·2
Horse and rabbit	1	0	1	2·7
Goat and rabbit	1	0	1	2·7
Total	6/28	3/10	9/38	23·6
Patients reacting against three species	9/28	1/10	10/38	26·4
Total positive reactions	21/28	8/10	29/38	76·4%

A positive reaction was defined as a titre of 1:2 or greater by the passive haemagglutination method.

Testing in the other direction, the sera of the ten normal humans mentioned above as well as sera from twenty-eight transplant candidates very often contained antibodies against normal horse, goat or rabbit serum or some combination of these sera (Table 3). The magnitude of the titres is summarized in Fig. 1. The range of the agglutinating ability was 1:2 to 1:2048; there were eight examples of titres of 1:256 or greater.

Antibodies in the sera of ALG treated patients

Incidence. As already mentioned, there were a number of patients who had antibodies against equine, rabbit or goat serum before receiving treatment with horse ALG; the titres were usually quite low (Fig. 2). From 1 to 4 weeks after the institution of horse ALG there

were sharp increases in the anti-horse-serum antibodies in all of seventeen cases (Fig. 2). In rough parallel with these changes were coincident rises in the activity against both normal goat and rabbit serum, particularly the former (Fig. 2).

Five other patients were first treated with horse ALG and subsequently with goat and rabbit globulin; a sixth patient received secondary therapy only with the goat product. At the outset the changes were as shown in Fig. 2. With the switch to another heterologous globulin, all three titres tended to rise even farther but did not undergo any distinctive subsequent alteration in relation to each other.

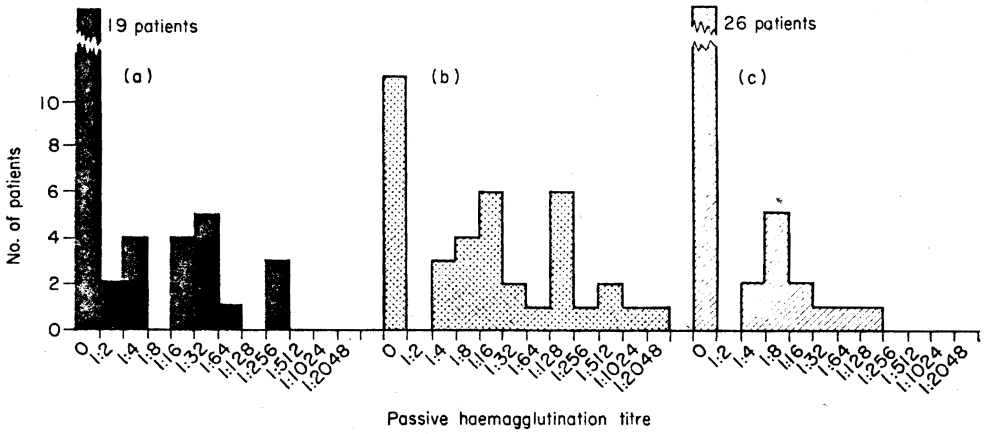

FIG. 1. Distribution curves of naturally occurring anti-horse (a), anti-goat (b) and anti-rabbit (c) antibody titres in the sera of thirty-eight patients who had not received ALG. The titres were quantitated by the passive haemagglutination method and expressed as the reciprocal of the greatest dilution of serum giving visible agglutination.

Cross-reactivity. The foregoing results suggested that the administration of horse ALG to patients caused antibodies to develop not only against equine protein but often against goat and rabbit proteins as well.

With immunoelectrophoresis it was determined to which constituents of the heterologous sera the human antibody response was directed in five patients who had been given only horse ALG. The results are summarized in Fig. 3. The antibodies evoked by the horse globulin had the greatest avidity to the same protein constituents in normal horse, goat and rabbit serum. The first antibodies to appear were usually directed against γA-globulin; later the macroglobulins were a favourite target.

The Ouchterlony analysis was used in sixteen sera from patients treated only with horse ALG to determine further if the cross-reactivity were to identical protein constituents. Fusion of the terminal precipitin lines could be demonstrated in only one instance; the reactions against the horse, rabbit and goat sera were to identical protein components. In the other fifteen cases, identity could not be proved but in seven of these the relative insensitivity of the method resulted in no precipitin bands at all being seen even at the horse serum interface. In several more instances, precipitin bands were absent at the goat and rabbit interfaces, despite the fact that anti-horse, anti-goat and anti-rabbit antibodies had been demonstrated to be present by the other more sensitive techniques.

DISCUSSION

One of the most interesting findings in this study was the presence of preformed anti-animal-serum antibodies in so many patients who had not been exposed to ALG. The explanation for this kind of low titre activity can only be speculated upon. There may have been naturally occurring heterospecific antibodies. It is also conceivable that immunization

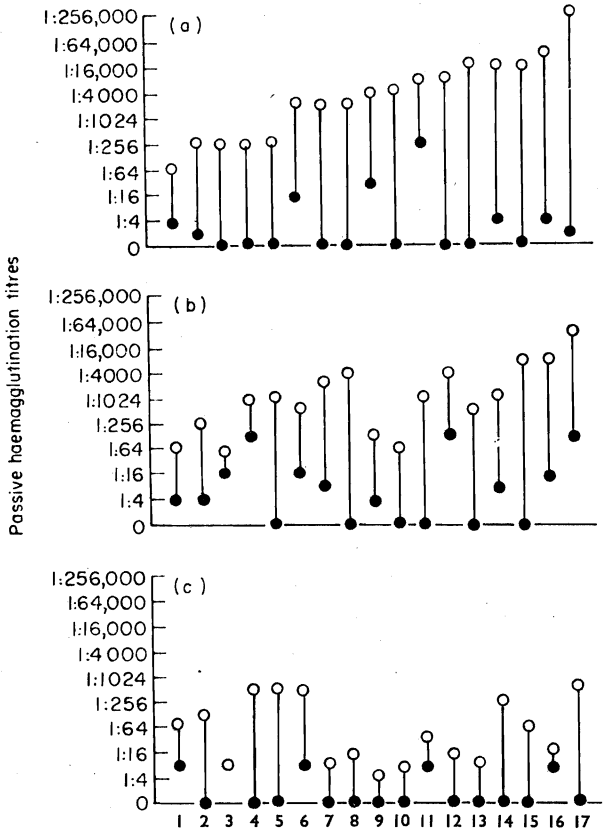

FIG. 2. Elevations in anti-horse- (a), anti-goat- (b) and anti-rabbit-serum (c) antibody titres occurring during the period of horse ALG administration in seventeen recipients of kidney or liver homografts. The antibody levels were quantitated by the passive haemagglutination method. The titres before treatment (●) and the maximum titres (○) attained are given for each patient.

at some time in the distant past with one of the widely employed animal biological products could have been responsible. The latter explanation has been suggested (Arbesman *et al.*, 1960) to account for a very high incidence of horse protein sensitization in a clinic population in the United States.

Whatever the explanation, more than 70% of the individuals amongst our normal volunteers and transplant candidates had antibodies against horse, goat or rabbit proteins. In many of these cases, the pre-existing antibodies reacted against the serum of two of the three

or even all three animal species. After horse ALG had been started the incidence of anti-equine-serum antibodies became 100%. Moreover, the administration of horse ALG proved to be a highly effective way of also inducing antibodies reacting with both goat and rabbit serum, particularly the former. Without exception, the appearance of rising titres of anti-equine activity was accompanied by closely correlated increases in antigoat reactivity. Incidental sensitization to rabbit protein also occurred but not so consistently and to a lesser extent.

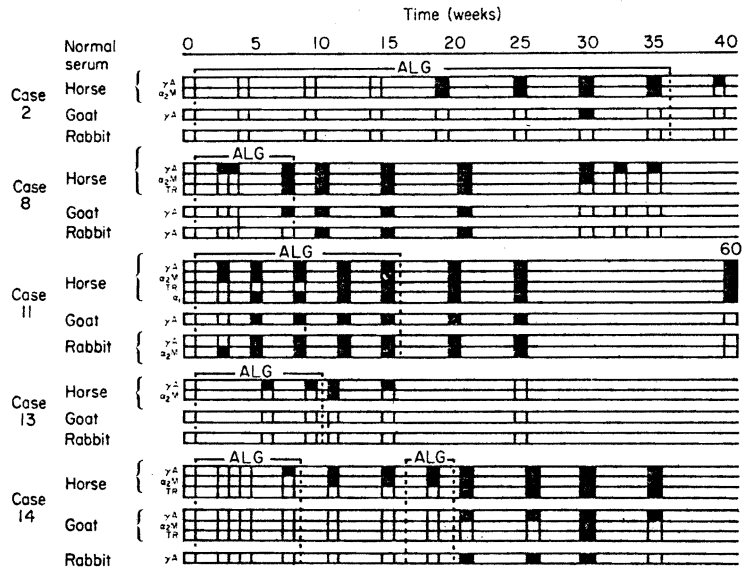

FIG. 3. Immunoelectrophoretic analysis of sequential sera from patients receiving only horse ALG. Each patient serum sample was reacted against normal horse, goat and rabbit sera. Protein components interacting with a given test serum are indicated by a solid square; an open square represents a negative test. The duration of ALG therapy is given for each case. γA, γA-Globulin; α_2M, α_2-macroglobulin; α_1, α_1-globulin; TR, transferrin.

It is impossible to say to what degree the widely reacting heterologous antibodies in the patient sera were caused by varieties of horse protein which are found in essentially identical forms in the goat and rabbit. The data from the Ouchterlony analysis indicated such identity in only one of sixteen experiments; however, this method has the deficiency of insensitivity. At the least, it seems certain that the cross-reactivity was explicable by antigenic similarities in the serum proteins of the three species and that these reacted commonly to the antibodies raised with the serum of one of these species. Furthermore, the magnitude of cross-reactivity seemed greater between the horse and goat than between the horse and rabbit, findings which were consistent with those reported in related kinds of immunological research (Heidelberger & Landsteiner, 1923; James, 1965; Nuttall, 1904).

The development of cross-reacting heterologous antibodies in patients receiving primary treatment with horse ALG should be taken into consideration in planning therapy with a globulin from a second or third species. The data from the present study suggests on immunological grounds that the rabbit would be the preferred secondline heterologous serum donor. This expectation appeared to have been borne out in the clinical observations. Rabbit

ALG given as secondary or tertiary therapy did not cause any early reactions and was continued for 35–83 days.

In contrast, the immunological analyses showed that sensitization to horse serum coincidentally resulted in the almost parallel development of antibodies against goat protein. Consequently, it was not surprising that two of our three patients who were given secondary goat ALG developed anaphylactic reactions within 15 days which were no different than those caused earlier by the horse globulin. The third patient had no difficulty during many ensuing months.

There are other implications to the development of cross-reacting antibodies during ALG therapy. If these are present in significant titres they could undermine the immunosuppressive efficacy of the new globulin after a switch is made from one species source to another. The reports of Lance (1968), Ono *et al.* (1969) and Jooste *et al.* (1968) have all indicated that when immune elimination becomes sufficiently rapid, the ability of ALG to mitigate homograft rejection is at least partly lost.

Both the problems of toxicity and loss of immunosuppressive efficacy should theoretically be minimized by the substitution of rabbit ALG in patients sensitized to horse globulin. The goat product could be reserved for a later time and only if an eventual return to horse globulin proves impossible. If rotation could be restricted to the horse and rabbit, return to equine ALG might ultimately be possible since horse protein sensitization can recede sufficiently under immunosuppression to permit the reinstitution of horse ALG at a later date (Iwasaki *et al.*, 1967; Kashiwagi *et al.*, 1968).

ACKNOWLEDGMENTS

This work was supported by United States Public Health Service Grants AM-06344, AM-07772, FR-00051, AI-04152, FR-00069, AM-12148 and AI-AM-08898.

REFERENCES

ARBESMAN, C.E., KANTOR, S.Z., ROSE, N.R. & WITEBSKY, E. (1960) Serum sickness: Serologic studies following prophylactic tetanus antitoxin. *J. Allergy*, **31**, 257.

GOLD, E.R. & FUDENBERG, H.H. (1967) Chromic chloride: A coupling reagent for passive hemagglutination reactions. *J. Immunol.* **99**, 859.

HEIDELBERGER, M. & LANDSTEINER, K. (1923) On the antigenic properties of hemoglobin. *J. exp. Med.* **38**, 561.

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

JAMES, K. (1965) A study of the α_2-macroglobulin homologues of various species. *Immunology*, **8**, 55.

JOOSTE, S.V., LANCE, E.M., LEVEY, R.H., MEDAWAR, P.B., RUSZKIEWICZ, M., SHARMAN, R. & TAUB, R.N. (1968) Notes on the preparation and assay of anti-lymphocytic serum for use in mice. *Immunology*, **15**, 697.

KASHIWAGI, N., BRANTIGAN, C.O., BRETTSCHNEIDER, L., GROTH, C.G. & STARZL, T.E. (1968) Clinical reactions and serologic changes after the administration of heterologous antilymphocyte globulin to human recipients of renal homografts. *Ann. intern. Med.* **68**, 275.

LANCE, E.M. (1968) The effects of chronic ALS administration in mice. *Advance in Transplantation* (Ed. by J. Dausset, J. Hamburger and G. Mathe), p. 107. Munksgaard, Copenhagen.

NUTTALL, G.H.F. (1904) *Blood Immunity and Blood Relationship*. Cambridge University Press, London.

ONO, K., DEWITT, D.W., WALLACE, J.H. & LINDSEY, E.S. (1969) Immunosuppressive activity of allogeneic antilymphocyte serum in the rat. *Transplantation*, **7**, 122.

OUCHTERLONY, O. (1958) Diffusion-in-gel methods for immunological analysis. *Progr. Allergy*, **5**, 1.

SCHEIDEGGER, J.J. (1955) Une micromethode de l'immuno-electrophorese. *Int. Arch. Allergy*, 7, 103.

STARZL, T.E. (with the assistance of C.W. PUTNAM) (1969) *Experience in Hepatic Transplantation*. Saunders, Philadelphia.

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 antilymphocyte globulin in man. *Transplant. Proc.* **1**, 448.

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