THE ROLE OF HUMORAL ANTIBODY IN THE REJECTION OF PRIMARY RENAL ALLOGRAFTS IN SHEEP

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Although there is a significant body of evidence which suggests that humoral antibody plays an important role in the rejection of primary renal allografts (1–20), transplantation reactions are usually held to be cell-mediated immune phenomena (21). It would seem unlikely that uniquely humoral or cell-mediated reactions occur, for the final outcome of any immunological event will be decided by a multiplicity of interactions which occur between enormous numbers of proliferating and differentiating cells and their immediate environment. In primary renal allografts in sheep, the cell-mediated component of the reaction involves the migration of thousands of millions of lymphocytes from the blood stream of the recipient into the graft and out of it by way of the lymph (15, 22). Many of these cells are proliferating and they synthesize and secrete protein actively during their migration. Although much of this protein can be separated into 19S and 7S fractions, it does not appear to be immunoglobulin.[1]

Antibodies are produced by a population of fixed cells in the lymph node, regional to where the allograft is installed, and also in the spleen; these antibodies have been shown to be cytotoxic for lymphocytes from the kidney donor.[1] The present paper reports experiments which we have done to determine the role of these antibodies in the rejection of primary renal allografts in sheep. To this end the characteristics of the antibody found in the circulation and recovered from allograft tissue have been compared. The destructive effect of normally produced and passively infused immune serum and globulin has been monitored by studying the character and tempo of the rejection process as it occurs in the lymph and relating this to pathological changes within the graft itself.

Materials and Methods

Animals.—Merino, Merino-Border Leicester, and Merino-Romney Marsh cross ewes between 6 mo and 6 yr of age were used for the experiments. The sheep were housed indoors in metabolism cages and fed water, lucerne chaff, and grain oats ad libitum.

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[1] Pedersen, N. C., E. P. Adams, and B. Morris. The response of the lymphoid system to renal allografts in sheep. Manuscript in preparation.

Surgical Procedures.—Transplantation of the kidneys and the cannulation of the various lymphatic vessels were carried out as described by Pedersen and Morris (15, 22).

Collection of Lymph and Cell Counting.—Lymph samples were collected continuously from a variety of lymphatics in the renal allograft recipient. The collections were made in polyethylene bottles containing powdered heparin and penicillin which were attached to the sheep. Total white cell counts and red cell counts were done with an electronic cell counter (Coulter model B, Coulter Electronics, Inc., Fine Particle Group, Hialeah, Fla.). Differential cell counts were made on Leishman-stained smears.

Protein Determinations.—Protein concentrations in lymph plasma and blood serum were measured by the biuret reaction.

Antibody Determinations.—Lymphocyte-agglutinating and cytotoxic antibody titers were determined by methods described previously (reference 15 and footnote 1) using lymphocytes collected from a chronic fistula in the efferent popliteal duct of the kidney donor. This duct was cannulated before removing the kidney for grafting. Lymphocyte cytotoxic antibody titers were expressed as the percentage of ^{51}Cr released and agglutinating antibody titers in $\log_2$ dilutions of serum or lymph. Hemagglutinating and hemolytic antibody titers were measured by standard methods.

Separation of Globulins.—Immunoglobulins were separated into their various classes on G-200 Sephadex columns (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). The columns were equilibrated with 0.2 M sodium chloride-0.1 M Tris buffer, pH 8.0. Three protein fractions were separated from the column and these were equivalent to fractions with sedimentation constants of 19S, 7S, and 5.5S. The fractions were tested for their antibody activity against lymphocytes from the kidney donor and the term IgM has been used for the antibody recovered in the first protein peak excluded from the column. This antibody was sensitive to 2-mercaptoethanol. The antibody recovered from the second peak was 2-mercaptoethanol resistant and has been referred to as IgG.

Elution of Antibodies from Renal Allograft Tissue.—The kidney grafts were perfused with 500 ml 0.9% NaCl solution as soon as they were recovered from the recipients to remove as much blood from them as possible. The tissue was then minced and homogenized with 250 ml of phosphate-buffered saline (PBS). The homogenate was centrifuged at 4°C, the upper layer decanted, and the tissue pellet washed in many changes of cold PBS to remove unbound protein and antibody globulin. The tissue homogenate was then mixed with 250 ml of ice-cold glycine-HCl buffer (pH 2.2) and stirred slowly for 15 min at 4°C to elute the antibody from the tissue homogenate. The mixture was adjusted to pH 7.4 with 10 N NaOH, centrifuged further to remove any denatured proteins, and then dialyzed overnight at 4°C against several changes of 0.9% NaCl solution. After dialysis the fluid was again centrifuged to remove any insoluble protein material and then reduced to a vol of 5–15 ml by positive pressure dialysis.

RESULTS

The Characteristics of the Humoral Antibody Produced in Response to a Primary Renal Allograft.—Antibody, as measured by its activity against donor lymphocytes, appeared in the blood serum of recipient ewes between 93 and 193 h after the grafts were installed. In most cases this was about 48 h before lymph flow from the grafts ceased (Table I). The antibody titers in the blood rose rapidly, and continued to increase after the flow of lymph from the graft had stopped (Fig. 1). Although both lymphocyte-agglutinating and cytotoxic antibodies were detected in most of the recipients there were two sheep which had only cytotoxic antibodies (Table I). Antibody present in the blood serum at the time of rejection usually possessed agglutinating and cytotoxic activity in both the IgM and IgG fractions (Fig. 2).

TABLE I

The Characteristics of the Serum Antibody Produced in Sheep in Response to Primary Renal Allografts, the Time of its Appearance in the Blood, and the time at which Lymph Flow from the Grafts Ceased

Sheep no.	Time antibody first appeared	Time lymph flow ceased	Type of antibody	
			Agglutinating	Cytotoxic
	h	*h*		
1	135	183.5	+*	NT§
2	95	150	+	NT
3	160	205	+	NT
4	132	150.5	+	NT
5	120	147.5	+	NT
6	123	165	+	NT
7	93	166	+	NT
8	157	172	+	+
9	169	238	+	+
10	100	187	−‡	+
11	122	188	+	+
12	116	172.5	+	+
13	158	182	−	+
14	193	193	+	+
15	178	187	+	+

*+, present.
‡−, absent.
§NT, not tested.

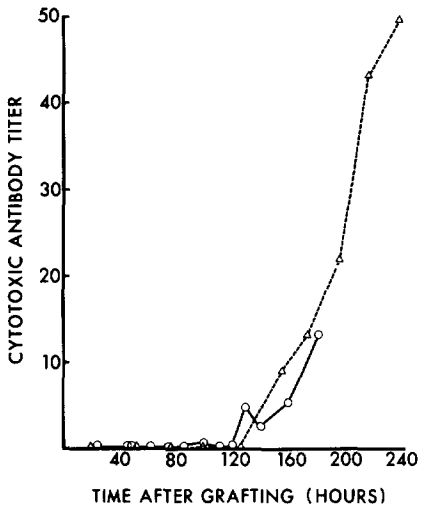

FIG. 1. The antibody titers in the circulating blood of the recipient (△---△), and in the lymph from a primary renal allograft (○—○) during its lifetime. Lymph flow from the graft ceased at 190 h and this was taken as the time of rejection.

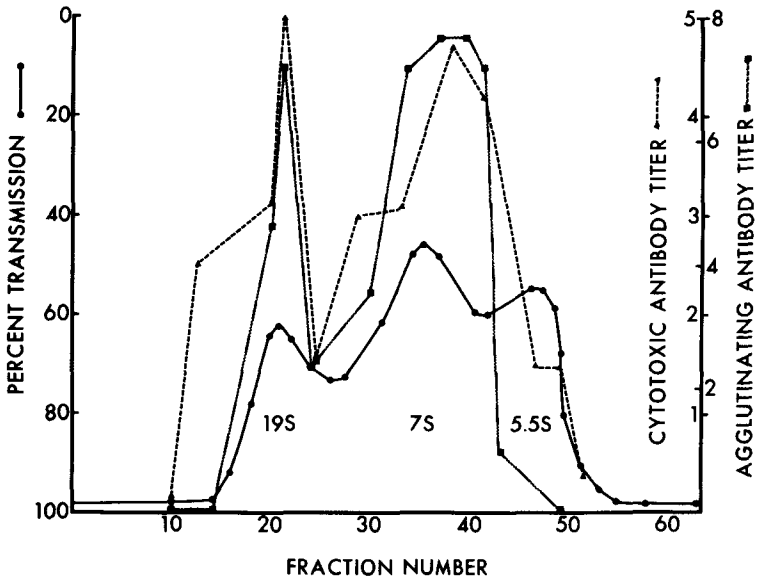

FIG. 2. The characteristics of the antibody recovered from the blood of a recipient sheep carrying a primary renal allograft. The antibody was separated by Sephadex G-200 chromatography and the cytotoxic and agglutinating activities of the various fractions measured.

Serum obtained from sheep that had rejected a previous primary renal allograft, often could be shown by cross-absorption studies to have activity against a number of different antigenic determinants present on lymphocytes. There was considerable cross-reactivity between the sera of sheep that had rejected a primary allograft and lymphocytes obtained from sheep other than the kidney donors (Table II). This cross-reactivity was more extensive with high titered sera. Two of these sera had $\log_2$ agglutinating and cytotoxic titers between 4 and 13 when tested against lymphocytes from a range of 16 unrelated sheep.

Hemolytic or hemagglutinating antibodies against the red cells of the kidney donors were not detected in the present series of experiments. No tests were made to determine whether there were antibodies reactive against antigenic determinants other than those present on lymphocytes and red blood cells.

The Binding of Antibodies Reactive against Donor Lymphocytes to Primary Renal Allografts.—To determine the extent to which antidonor lymphocyte antibody was being bound to the allograft, kidneys in the process of being rejected were removed from three sheep 188–193 h after transplantation. Antibodies present on the homogenized graft tissue were eluted, separated into IgM and IgG classes, and the separate fractions assayed for cytotoxic and agglutinating activity against lymphocytes from the respective kidney donors.

The titers of antibody in the final concentrate of the elution fluid are given in Table III. In the first sheep it was equal to the titer in the blood serum at

TABLE II

The Cross-Reactivity of Antisera from Renal Allograft Recipients Tested against Lymphocytes from the Kidney Donor and from Eight Unrelated Sheep

Recipient sheep sera	Donor sheep lymphocytes	Lymphocyte agglutinating antibody titer of sera from renal allograft recipients tested against lymphocytes from the kidney donor and from other unrelated sheep: Unrelated sheep lymphocytes							
		1	2	3	4	5	6	7	8
8B	1:128	−*	+++	−	+++	+++	−	+	+++
12B	1:128	+++‡	++	−	+++	+++	−	+++	+++
13B	1:32	+§	−	−	−	−	−	−	−
14B	1:256	++‖	+++	++	+++	−	++	−	+++
15B	1:512	++	+++	+++	+++	−	++	+++	+++
16B	1:32	−	−	−	−	−	−	−	−
18B	1:64	+++	++	−	−	+++	−	++	++
19B	1:256	+++	+++	+++	+++	+++	+++	+++	−
20B	1:128	+++	+++	−	++	+++	−	+++	+++
21B	1:256	+++	+++	++	+++	++	++	−	+++
26B	1:4,000	+++	−	+	−	+++	+	+++	+++
27B	1:1,000	++	+++	+++	−	+++	+++	−	−
28B	1:64	+++	+++	−	+++	+++	+++	−	+++

*−, negative
‡++, 1:10.
§+, 1:5.
‖+++, 1:20 or greater.

TABLE III

The Titer of Antidonor Lymphocyte Antibody in the Final Concentrate of the Elution Fluid which Contained the Antibody Recovered from Primary Renal Allograft Tissue

Time of removal of graft	Final volume of the concentrated eluate	Log_2 serum antibody titers at the time graft was removed		Log_2 antibody titers in the concentrated eluate	
		Agglutinating	Cytotoxic	Agglutination	Cytotoxic
h	*ml*				
188	5	12	6	12	6
190	7	0	3	0	7
193	15	1	0	6	5

the time the graft was removed while in the other two sheep it was significantly higher. As there was no detectable antibody in the final washings of the allograft homogenates before the antibody elution procedure was begun, the antibody detected was considered to have been complexed previously with allograft antigens and to have been fixed to the graft tissue.

The Effect of Graft Removal on the Time of Appearance and the Levels of Antibody in the Blood Serum.—In a previous paper (15) we reported that a close temporal relationship existed between the appearance of cytotoxic and agglutinating antibodies in the blood and an increase in the protein and red cell content of the lymph coming from the allograft. This finding suggested that the appearance of antibody in the blood was related to the destruction of the endothelium of the graft which led in turn to severe alterations in the permeability of

the graft vasculature. It was possible however that the binding of antibody by the allograft tissues reduced the titers of circulating antibody in the blood below detectable levels until relatively late in the life of the graft, thereby giving the impression that the synthesis of antibody was a late event in the rejection process. To establish how long the graft had to be in place to initiate antibody production and to see what effect the graft had on the level of circulating antibody in the blood, kidneys were transplanted into sheep and then removed at varying times before rejection occurred normally. The titers of antibody in the blood serum were followed daily up until the graft was removed and subsequently for a further 10–14 days. If antibody was being absorbed by the grafted kidney at a rate sufficient to affect its level in the circulation it was argued that the removal of the graft would result in an increase in the serum antibody titers, provided this antibody was being produced outside the graft itself.

Fig. 3 shows the serum antibody response in recipient sheep from which renal allografts were removed at various times after grafting. When the grafts were removed before they had been in place for 120 h, no detectable antibody subsequently appeared in the recipient's serum. If the grafts were removed after 120 h the kinetics of appearance of antibody in the blood and the final titers reached were similar to those in recipients in which the grafts were left in place until rejection was completed normally. The sheep which had grafts removed before 120 h and which failed to produce detectable levels of serum antibody were, nevertheless, sensitized, for when they were given the second

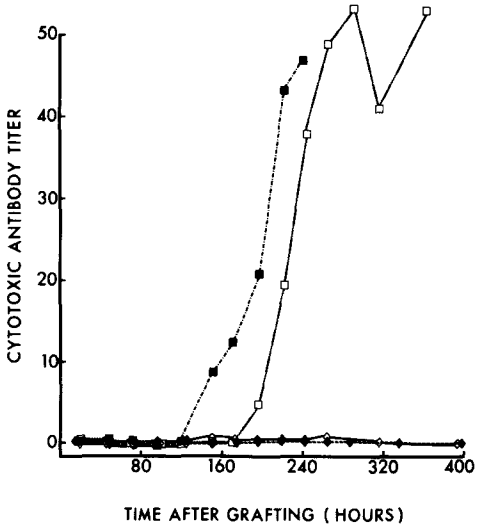

FIG. 3. The effect of removing renal allografts at various times after transplantation on the levels of cytotoxic antibody produced in the blood of the recipient. ◇, graft removed after 121 h; ◆, graft removed after 73.5 h; □, graft removed after 148 h; and ■, graft allowed to remain until rejected.

kidney from the original donor, it was rejected in an accelerated fashion and the antibody produced was typical of a secondary response (Fig. 4).

These experiments showed that the graft had either to be in place for a critical period of around 120 h to stimulate a measurable humoral antibody response in the recipient, or less likely, that the graft itself was the site of antibody production up until 120 h. The results also showed that the amount of antibody absorbed by the graft from the blood after 120 h was not sufficient to alter the kinetics of the immune response to any extent and that antibody production occurred principally outside the allograft. There was also a clear difference in the time at which sensitization of the recipient occurred and the time at which the maximum humoral immune response was initiated.

The Effect of Passively Transferred Alloantibody on Renal Allografts.—A cause and effect relationship was sought between the appearance of antibody in the circulation and the destruction of the renal allografts. Experiments were done with antisera or globulin fractions obtained from sheep that had rejected primary renal grafts. This material was infused into a third party sheep carrying the second kidney from the original donor against whose first kidney the antiserum or globulin fraction had been raised. To prevent any of the infused antibody from being absorbed out by cross-reacting determinants on the tissues of the third party recipient, tests were made on large numbers of potential recipients to select animals whose lymphocytes showed no reactivity with the particular antiserum or globulin fraction being used. The antiserum or globulin was administered 24 h after the allograft was installed. This experiment was carried out on three sheep using various protocols to administer the antiserum. In the first sheep 155 ml of immune serum was given intravenously and 300 ml

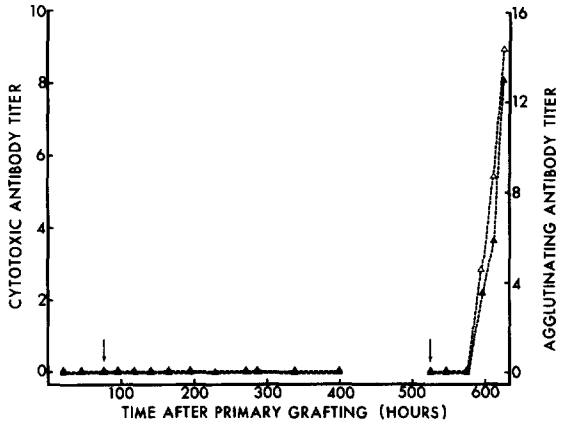

FIG. 4. The effect of a previous renal allograft, removed 72 h after installation, on the rejection of a second allograft from the same donor. The first arrow indicates the time of removal of the first graft; the second arrow indicates the time the second graft was installed. △—△, lymphocyte agglutinating antibody titer; and ▲---▲, lymphocyte cytotoxic antibody titer.

subcutaneously at the same time; the second sheep was given the globulin fraction prepared from 432 ml of immune serum intravenously, at the rate of 12 ml/h for 24 hr; while the third sheep was given the globulin from 564 ml of immune serum at 3.0 ml/h into the renal artery for 18 h and 15.0 ml/h for a further 8 h. The infused material had $\log_2$ agglutinating titers of between 15 and 16 when tested against donor lymphocytes. Two of the renal allografts were rejected within 24 h of beginning the infusion of the antiserum while the third graft survived for 65 h. The total time each of these three grafts survived was significantly shorter than for normal primary allografts in sheep. The changes monitored in the graft lymph were essentially similar in the three experiments. Shortly after giving the antiserum or the globulin fraction, the numbers of red cells and the protein content of the lymph began to increase. The rate of lymph flow fell shortly after this and then some hours later ceased abruptly as the graft was finally destroyed (Fig. 5).

The histology of the renal allografts destroyed by the administration of antiserum was characteristic. The cortex and medulla showed extensive destruction of the blood capillary endothelium with interstitial hemorrhage and poly-

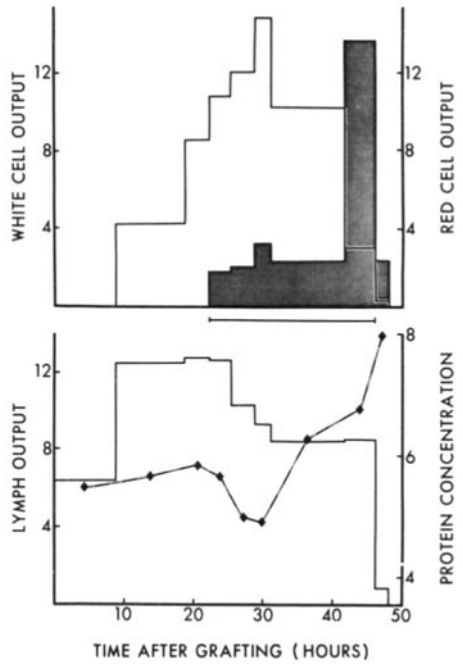

FIG. 5. The effect of an intravenous infusion of specific immune globulin on the formation and composition of lymph from a renal allograft. The upper graph shows the output of white cells per hour $\times 10^7$ and the output of red cells per hour $\times 10^8$ (hatched area). The lower graph shows the output of lymph flow, ml/h (—) and the lymph protein concentration, g/100 ml (◆). Globulin was infused during the period indicated by the horizontal line below the upper graph.

morphonuclear infiltration. There were hemorrhages in the glomeruli and proteinaceous material in the Bowman's capsule and in the renal tubules. Only a small number of lymphocytes had infiltrated into the two allografts that were destroyed within 24 h of giving the antiserum but more lymphocytes were present in the graft that survived for 65 h. This finding was similar to that reported by Pedersen and Morris (15) for allografts rejected by sensitized recipients. These histological changes are shown in Fig. 6.

DISCUSSION

Our experimental results support the view that humoral antibody plays a significant role in the rejection of primary renal allografts in sheep. The role of cell-mediated mechanisms in the rejection of renal allografts in sheep has not yet been determined. However, it would seem likely that if cell-mediated mechanisms operate they would do so synergistically with humoral antibody. In addition to a direct cytotoxic effect, humoral antibody may also confer some specific cytolytic potential on lymphoid cells in the manner described by Perlmann and Holm (23) and Van Boxel et al. (24).

While sensitization to a secondary graft occurred within 72 h after primary grafting, it appeared that the initial graft had to be left in place for a period of 120 h before the primary humoral antibody response was fully expressed. Removing the graft before 120 h may have restricted the humoral antibody response by limiting the release of antigens from the graft or by limiting the adjuvant or enhancing effects of mediators produced by cells in the graft not concerned in specific antibody production. A similar relationship between the time grafts are left in place and the magnitude of the host's immunological response has been described previously. Dempster et al. (25) removed skin grafts from mice and rats immediately before the time when rejection was anticipated and they removed renal allografts from dogs as soon as the grafts ceased to make urine. They found that a second graft from the same donor placed in the recipients 10–14 days later was rejected in a primary fashion and they interpreted these findings to mean that the allograft tissue only became antigenic after it was disrupted by the rejection process. Sparks et al. (26) also showed a significant reduction in the humoral antibody response to skin grafts in mice when the grafts were removed before their being rejected. They concluded that the continued presence of alloantigens was important for maximal antibody production.

The alloantibodies produced in the host during the primary rejection were specific for antigens on the donor sheep's lymphocytes and kidney tissue. These antibodies did not react to antigens of the donor's red cells, indicating that none of the multiplicity of antigenic determinants present on sheep red cells (27) are histocompatibility antigens. The histocompatibility antigens of sheep resemble in their tissue distribution and complexity the HL-A histocompatibility antigens of man.

SUMMARY

Antibody which had cytotoxic and agglutinating activity against donor lymphocytes appeared in the blood stream of primary renal allograft recipients usually within 48 h of the graft being finally rejected. Appearance of the anti-

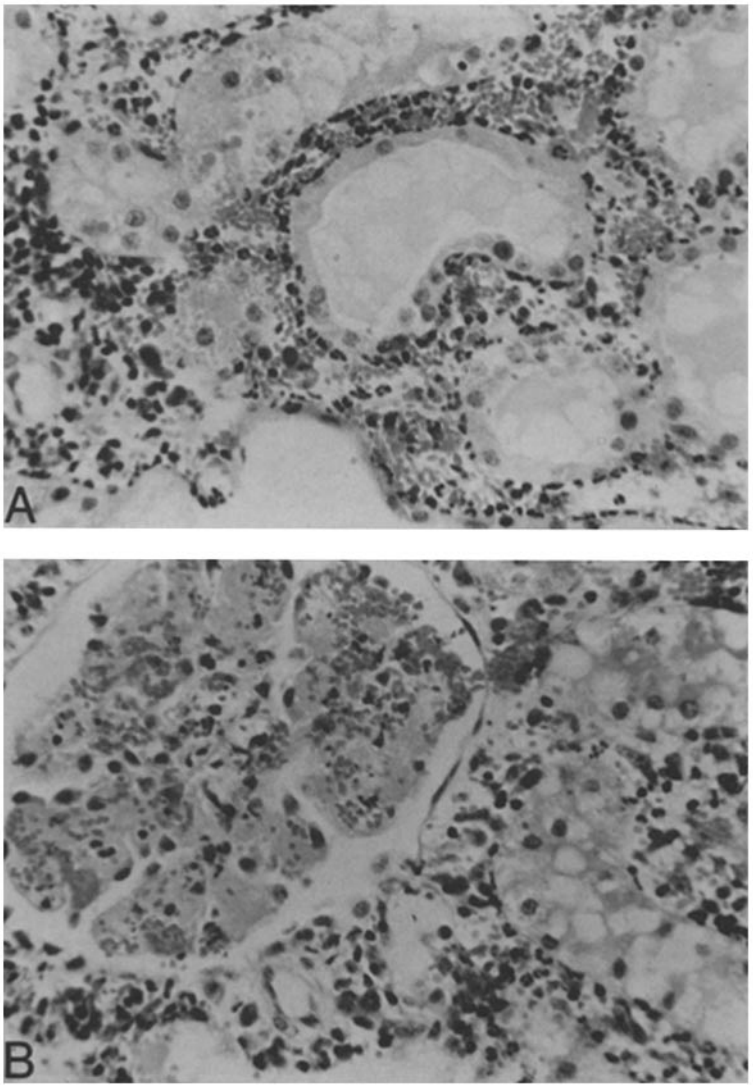

FIG. 6. The histological appearance of tissue from a renal allograft after intravenous infusion of specific immune globulin into the recipient. (A) interstitial hemorrhage and polymorphonuclear infiltration into the intertubular spaces (B) hemorrhagic destruction of a glomerulus and adjacent tubules. Hemotoxin and eosin × 450.

body in the blood was associated with severe alterations in vascular permeability and this led to increases in the numbers of red cells and in the protein content of the lymph coming from the allograft. It was possible to elute cytotoxic and agglutinating antibody from renal allograft tissue, showing that this type of antibody was bound to graft antigens during the rejection process. The transfusion of whole serum or serum globulins obtained from sheep that had previously rejected allografts led to the destruction of recently installed renal grafts and the histological changes produced in these grafts and the alterations in the composition of the lymph coming from them were similar to those seen in the terminal stages of primary rejection. These findings have led us to the conclusion that in the sheep, at least the terminal stage of primary renal allograft rejection is mediated by humoral antibody.

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REFERENCES

1. Almgard, L. E., and S. E. Svehag. 1968. Humoral antibodies in canine renal transplantation. *Acta Pathol. Microbiol. Scand.* **73**:605.
2. Altman, B. 1963. Tissue transplantation: circulating antibody in the homotransplantation of kidney and skin. *Ann. R. Coll. Surg. Engl.* **33**:79.
3. Bussy, J. G., F. Sabiston, and W. W. Pfaff. 1967. Humoral cytotoxic antibodies in allograft rejection. *Surg. Forum.* **18**:233.
4. Cochrum, K. C., W. C. Davis, S. L. Kountz, and H. H. Fudenberg. 1969. Renal autograft rejection initiated by passive transfer of immune plasma. *Transplant. Proc.* **1**:301.
5. Dubernard, J. M., C. B. Carpenter, G. J. Busch, A. G. Diethelm, and J. E. Murray. 1968. Rejection of canine renal allografts by passive transfer of sensitized serum. *Surgery (St. Louis).* **64**:752.
6. French, M. E., and R. J. Batchelor. 1969. Immunological enhancement of rat kidney grafts. *Lancet.* **2**:1103.
7. Guttmann, R. D., R. R. Lindquist, R. M. Parker, C. B. Carpenter, and J. P. Merrill. 1967. Renal transplantation in the inbred rat. I. Morphologic, immunologic and functional alterations during acute rejection. *Transplantation (Baltimore).* **5**:668.
8. Hager, E. B., M. P. DuPuy, and D. F. Wallach. 1964. Immunologic suicide and studies on the role of antibody and complement in canine kidney homograft rejection. *Ann. N. Y. Acad. Sci.* **120**:447.
9. Hampers, C. L., P. Kolker, and E. B. Hager. 1967. Isolation and characterization of antibodies and other immunologically reactive substances from rejecting renal allografts. *J. Immunol.* **99**:514.
10. Holter, A. R., T. J. McKearn, M. R. Neu, F. W. Fitch, and F. P. Stuart. 1972. Renal transplantation in the rabbit. I. Development of a model for study of hyperacute rejection and immunological enhancement. *Transplantation (Baltimore).* **13**:244.

11. Horowitz, R. E., L. Burrows, F. Paronetto, D. Dreiling, and A. E. Kark. 1965. Immunologic observations on homografts. II. The canine kidney. *Transplantation (Baltimore)*. **3**:318.
12. Klassen, J., and F. Milgrom. 1971. Studies on cortical necrosis in renal grafts. *Transplant. Proc.* **3**:598.
13. McKenzie, I. F. C., and S. Whittingham, 1968. Deposits of immunoglobulin and fibrin in human allografted kidneys. *Lancet.* **2**:1313.
14. Patel, R., and P. I. Terasaki. 1969. Significance of the positive crossmatch test in kidney transplantation. *N. Engl. J. Med.* **280**:735.
15. Pedersen, N. C., and B. Morris. 1974. The rate of formation and the composition of lymph from primary and secondary renal allografts. *Transplantation (Baltimore)* **17**:48.
16. Spong, F. W., J. D. Feldman, and S. Lee. 1968. Transplantation antibody associated with first-set renal homografts. *J. Immunol.* **101**:418.
17. Straus, A., F. Straus, and D. Roseman. 1971. Allogeneic "rejection" of *in situ* rat kidney using humoral antibody. *Transplant. Proc.* **3**:570.
18. Terasaki, P. I., T. Akiyama, J. D. McCleland, and J. A. Cannon. 1962. Renal damage produced *in vivo* by homologous mouse antisera. *Ann. N. Y. Acad. Sci.* **99**:645.
19. Williams, G. M., D. M. Hume, R. P. Hudson, Jr., P. J. Morris, K. Kano, and F. Milgrom. 1968. "Hyperacute" renal-homograft rejection in man. *N. Engl. J. Med.* **279**:611.
20. Yamada, T., and J. H. Kay. 1968. Kidney homotransplantation with special reference to cytotoxic antibody response. *Surgery (St. Louis)*. **63**:637.
21. Brent, L. 1971. Pathogenic role of delayed hypersensitivity and antibody in allograft reactions. Cellular Interactions in the Immune Response. 2nd International Convocation on Immunology. S. Karger AG., Basel, Switzerland. 250.
22. Pedersen, N. C., and B. Morris. 1970. The role of the lymphatic system in the rejection of homografts: a study of lymph from renal transplants. *J. Exp. Med.* **131**:936.
23. Perlmann, P., and G. Holm. 1969. Cytotoxic effects of lymphoid cells *in vitro*. *Adv. Immunol.* **11**:117.
24. Van Boxel, J. A., J. D. Stobo, W. E. Paul, and I. Green. 1972. Antibody-dependent lymphoid cell-mediated cytotoxicity: no requirement for thymus-derived lymphocytes. *Science (Wash. D. C.)*. **175**:194.
25. Dempster, W. J., J. S. Calnan, and A. E. Kulatilake. 1963. Time of antigen release from homografts. *Br. Med. J.* **1**:23.
26. Sparks, F. C., T. G. Canty, C. C. Ting, W. G. Hammond, and R. B. Heberman. 1970. Antibody response to skin allografts in mice. *Proc. Soc. Exp. Biol. Med.* **133**:1392.
27. Rasmusen, B. A. 1962. Blood groups in sheep. *Ann. N. Y. Acad. Sci.* **97**:306.