PRELIMINARY COMMUNICATIONS

Nature of Hyperacute (Accelerated Second Set) Rejection in Dog Renal Allografts and Effects of Heparin on Rejection Process

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Summary

Renal allografts were exchanged between unrelated mongrel dogs after previous sensitization with skin and kidney grafts from the same donors. Rapid rejection of the renal allografts was associated with the accumulation of platelets and leucocytes in the peritubular and glomerular capillaries but fibrin deposition was not demonstrated.

Heparin infusion delayed but did not prevent the rejection process.

Introduction

Hyperacute rejection of renal allotransplants is becoming an increasingly important problem in major transplantation units due to presensitization of recipients with blood transfusions or, more especially, with previous transplants (Starzl *et al.*, 1968). It has been suggested that intravascular coagulation within the kidney is an important mechanism in this process (Busch *et al.*, 1969; Myburgh *et al.*, 1969) and has been shown in the accelerated rejection of renal xenografts (Rosenberg *et al.*, 1969).

Colman *et al.* (1969) found extensive renal cortical vascular thrombosis in a study of human renal allotransplants undergoing hyperacute, acute, or chronic rejection, although they did not find evidence of a reduction in coagulation factors or circulating fibrinolysins. Heparin has been used to prevent intravascular coagulation within the kidney (Starzl *et al.*, 1968). Although this agent prolongs survival of renal allografts in sensitized recipients, it does not prevent rejection taking place (Macdonald *et al.*, 1970). The object of the present study was to investigate and correlate the early haematological and morphological changes occurring in the hyperacute rejection process in dogs rendered hypersensitive by previous kidney and skin allografts.

Materials and Methods

Three series of dogs were studied. All the dogs chosen were unrelated mongrel dogs weighing about 20-30 kg. In the first series of six, dogs 2, 3, 5, 6, 8, and 9 received skin and, later, kidney grafts from three donors. For each recipient the same donors were used on each occasion. Skin allografts were placed subcutaneously into the thoracolumbar region on days 0, 21, and 35. On day 42 each recipient received a kidney from the donor dog. These kidneys were transplanted to the iliac fossae using siliconized cannulae with a two-way tap. This enabled blood in the renal artery and vein to be sampled immediately a flow was established, and then at 5, 10, 15, 30, and 60 minutes and subsequently at hourly intervals thereafter up to an arbitrary time of six hours if the kidney survived. The use of these cannulae prevented blood loss which normally accompanies vascular anastomosis and they also allowed sampling of blood without contamination from the iliac circulation.

This procedure was then modified to obtain a more rapid rejection in an attempt to simulate the clinical condition. In this second series of dogs (12, 13, 14, 15, 30, 31, and 32) unrelated mongrel dogs were paired and skin grafts were exchanged on day 0 and day 21. On day 35 the first kidneys were exchanged and then removed the next day. One week later (day 42) the second kidneys were exchanged and the haematological changes occurring were monitored as in the first series.

A third series of dogs (16, 17, 26, 27, 28, 29, and 38) were sensitized in exactly the same way as the second series but they received heparin by continuous infusion on restoring the circulation to the second transplanted kidneys.

In order to exclude the possibility that any of the changes observed were due to the surgical procedure alone, renal autografts were performed using exactly the same technique as with the allografts, and identical blood samples were taken.

Kidney tissue was removed for histopathological examination at the time of rejection in the sensitized dogs. Renal biopsies were obtained after 30 minutes from the dogs treated with heparin. Biopsy specimens were taken at one hour in the autografted kidneys. Each donor kidney was perfused immediately after removal and before transplantation with heparinized Hartmann's solution (10 mg of heparin per litre) at 4°C until there was a clear effluent from the renal veins. The kidneys were then stored for a brief period of less than one hour surrounded by ice before transplantation.

Sensitization of the dogs was detected by measuring the lymphocytotoxic titre at each stage of sensitization.

HAEMATOLOGICAL INVESTIGATIONS

Haemoglobin, packed cell volume, total and differential white blood cells counts, platelet counts, and fibrinogen estimations were performed by using standard methods (Dacie and Lewis, 1968) on each blood sample taken from the renal artery and vein.

INTRAVENOUS FLUIDS

Each dog received between 1 and 1½ litres of Hartmann's solution beginning after the induction of anaesthesia at each renal transplantation. The blood pressure was recorded by an indwelling intra-arterial cannula placed in the right foreleg.

LYMPHOCYTOTOXICITY

The lymphocytotoxicity of the sera was estimated before exchange of each skin graft and each renal allograft. Lymphocytes were obtained either from peripheral blood before exchanging each set of skin grafts or from abdominal lymph nodes before exchanging the kidneys.

Ninety-per-cent. pure lymphocyte preparations were obtained

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TABLE I—*Haematological Data and Rejection Times in Dogs Studied*

Recipient Dog. No.	Lymphocytotoxic Antibody Titre	Maximum Arteriovenous Fall Platelets (% Arterial Base)	Final Arterial Platelets (per mm³)	Initial Arterial Platelets (per mm³)	Maximum Arteriovenous Fall Neutrophils (% Arterial Base)	Final Arterial Neutrophils (per mm³)	Initial Arterial Neutrophils (per mm³)	Rejection Time in Minutes
				First Series				
2	1/32	41% (8)	176,000	217,000	10% (8)	16,146	12,465	20
3	1/2,048	61% (4)	170,000	225,000	11% (2)	10,205	5,125	65
5	1/256	29% (5)	232,000	276,000	54% (11)	10,120	9,460	1,560
6	1/1,024	22% (60)	206,000	255,000	20% (31)	8,900	12,876	570
8	1/256	17% (12)	179,000	218,000	38% (31)	7,520	8,218	300
9	1/256	25% (1)	199,000	228,000	66% (1)	13,320	11,790	600
				Second Series				
12	1/2,048	87% (8)	137,000	—	61% (8)	—	21,565	55
13	1/512	88% (15)	289,000	—	80% (6)	—	12,410	20
14	1/512	69% (6)	301,000	218,000	56% (6)	10,556	8,924	25
15	1/256	96% (5)	196,000	445,000	70% (5)	10,736	9,660	15
30	1/512	74% (11)	164,000	223,000	74% (11)	6,030	9,771	70
31	1/1,024	97% (5)	77,000	265,000	91% (6)	2,436	11,500	75
32	1/256	99% (5)	180,000	240,000	88% (5)	11,431	6,300	65
				Third Series (Heparin Treated)				
16	1/1,024	34% (31)	221,000	241,000	51% (1)	14,220	16,744	360
17	1/2,048	32% (6)	407,000	420,000	36% (6)	23,460	12,880	210
26	1/64	12% (6)	246,000	251,000	24% (16)	10,323	5,800	259
27	1/512	17% (11)	485,000	470,000	No change	8,829	7,676	85
28	1/256	1% (10)	133,000	96,000	No change	44,135	25,760	293
29	1/256	43% (6)	423,000	206,000	4% (12)	22,770	11,220	270
38	1/64	51% (6)	305,000	375,000	20% (30)	24,510	17,316	360
33	—	No change	290,000	122,000	11% (5)	32,538	15,721	—
				Autografts				
A34 R.I.F.	—	10% (5)	304,000	253,000	6% (1)	14,694	13,182	—
A34 L.I.F.	—	No change	295,000	251,000	14% (1)	16,598	14,616	—

The figures in parentheses in columns 3 and 6 represent the times of venous sampling in minutes after restoring the circulation to the kidney.

by sedimenting defibrinated blood with plasma gel. The polymorphonuclear leucocytes were removed by using a column of glass beads, and then red blood cells by using an albumin gradient.

The lymphocytes were finally reconstituted in Eagle's medium to a cell count of about 8-15,000/mm³. Serum to be tested was obtained from a clotted blood sample.

Serial dilutions of the serum to be tested were prepared on microtitre plates. Lymphocytes from the donor dog were placed with each dilution, and diluted rabbit complement was added. Controls of lymphocytes in their own serum with complement and in Eagle's medium were tested on each occasion.

After incubation for 30 minutes at 37°C, eosin was used to detect the number of non-viable cells remaining in each well. Counts in excess of 10% over the control values were regarded as positive and the highest dilution in which this could be detected was regarded as the antibody titre.

HAEMAGGLUTININS

Triple dilutions of serum from each sensitized dog were set up against the washed red cells of the opposite member of the pair on a microtitre plate. The serum and red cells were then incubated for two hours and samples were removed from each well and examined for agglutination.

EVIDENCE OF REJECTION

Rejection was thought to have occurred when no further urine appeared and the kidney became soft, flabby, and cyanosed. In all second set kidneys no clots were found in the cannulae at rejection or in the renal arteries or veins.

HISTOLOGICAL PREPARATION

Immediately after rejection the kidneys were removed, wedge-shaped portions about 0·5 cm in thickness, which included both cortex and medulla, were taken and fixed in 10% buffered formalin (pH 7·0) for at least 48 hours. This was followed by secondary fixation in Zenker-formal for six hours. Blocks of tissue from each kidney were embedded in paraffin wax and sections of 3-5 μm thickness were made. These were stained routinely by Mayer's haemalum and eosin, Martius scarlet blue, periodic-acid Schiff, and by Picro-Mallory. In the dogs receiving heparin wedge biopsy specimens of the renal allografts were taken after 30 minutes and processed in the same manner.

Results

REJECTION TIMES

In the first series the rejection time varied from 20 minutes to 26 hours (mean 8 hr 40 min) (table I). In the second series the rejection time varied between 15 and 75 minutes (mean 46 min). Five of the dogs treated with heparin in series 3 (17, 26, 27, 28, and 29) rejected within six hours. In two dogs (16 and 38) the kidneys survived for six hours, which was the arbitrary time limit of perfusion.

LYMPHOCYTOTOXIC TITRE AND HAEMAGGLUTININ TITRE

All but one of the dogs had a raised lymphocytotoxic titre at each stage during sensitization. In the one exception (dog 33) no lymphocytotoxic antibody was detected at any time. There did not appear to be any correlation between the titre and the speed of rejection although the renal allograft in dog 33 continued to function to the end of the experiment.

No haemagglutinins were present in any sera taken before the second kidney allografts.

HAEMATOLOGICAL FINDINGS

Platelets and Leucocytes.—The most striking change was a rapid fall in the platelet count occurring in renal vein samples over the first 15 minutes. This was most striking in the second series of dogs sensitized with skin and kidney allografts. In these the mean level fell by 87% (range 69-99%) compared with the count in the basal arterial sample. There was a similar fall noted in the first series of dogs but this was much less pronounced with a mean of 32% (range 17-61%). The reduction in the neutrophil count over the same period was similarly more noticeable in the second series of dogs with a mean fall of 74% compared to a

TABLE II—*Main Histological Abnormalities Found in Transplants in the Dogs Under Study*

Recipient Dog. No.	Glomeruli			Tubles (Degenerative Changes)		Interstitial Tissues						Intertubular Capillaries					Large Arteries and Veins				Rejection Time in Minutes
	Structural Abnormalities	Increase of W.B.C.	Platelet Aggregation	Vascular Engorgment	Fibrinous Thrombi	Nuclear Pyknosis	Desquamation	Vacuolation	Casts	Oedema	Cell Infil	Engorgment	Increase of W.B.C.	Platelet Aggregation	Thrombi	Structural Abnormalities	Engorgment	Increase of W.B.C.	Platelet Aggregation	Thrombi	
Second Series																					
12	0	++	++	0	0	0	0	+	0	0	0	+	++	+	0	0	0	+−	+	0	55
13	0	0	+++	+	0	0	0	0	0	0	0	+	+−	+	0	0	0	+−	++	0	20
14	0	0	+++	+	0	0	0	0	0	0	0	+	0	++	0	0	0	+−	+++	0	25
15	0	0	+++	+	0	0	0	+	0	0	0	+	+−	++	0	0	+	++	++	0	15
30	0	++	0	0	0	0	0	+	0	0	0	++	++	++	0	0	0	++	++	0	70
31	0	+−	+−	+	0	0	0	+−	0	0	0	+	+−	+	0	0	0	+	++	0	75
32	0	+−	+−	+	0	0	0	+	0	+−	++	+	+	+−	0	0	0	+−	+−	0	65
Third Series (Heparin Treated)																					
16	0	0	++	0	0	0	0	+	+	0	0	+	+−	+	0	0	0	0	+	0	360
17	0	+	++	+	0	0	0	0	++	0	0	+	+	+	0	0	0	0	++	0	210
26 (30 min)	0	0	+−	++	0	0	0	+−	+−	0	+	+	0	+−	0	0	++	0	0	0	
26	0	+	0	+−	0	0	0	+	+−	0	0	+−	+	0	0	0	0	0	0	0	259
27 (30 min)	0	+−	+	++	0	0	0	0	0	+−	0	+	+−	+	0	0	+	0	0	0	
27	0	+	++	++	0	0	0	0	0	0	0	+−	+−	0	0	0	0	+	++	0	85
28 (30 min)	0	+	0	+	0	Mitosis	0	0	+	0	0	+	+	0	0	0	0	0	0	0	
28	0	+	0	0	0	Mitosis	0	0	+	0	0	0	+	0	0	0	0	0	0	0	293
29 (30 min)	0	+	++	0	0	0	0	+	0	0	0	+	0	++	0	0	+−	+−	+	0	
29	0	0	+	+	0	0	0	+	0	0	0	+	0	++	0	0	+	0	+	0	270
33*	0	+	0	+	0	0	0	+	0	0	0	+	+	0	0	0	0	+	0	0	360
38	0	+	++	+	0	0	+	+	0	0	0	+	++	+	0	0	+	++	++	0	360
Autografts																					
A34 L.I.F.	0	0	0	+	0	0	0	0	+	0	0	+	0	0	0	0	0	0	0	0	
A34 R.I.F.	0	0	0	+	0	0	0	0	+	0	0	+	0	0	0	0	0	0	0	0	

* = Non responder. This dog at no time after exchanging skin grafts or the first kidney had a demonstrable lymphocytotoxic titre.
0 = No abnormality seen.
+− = Equivocal abnormalities only.
+ = Mild but definite abnormalities seen.
++ = Severe abnormalities present.

mean fall of 33% (range 10-66%) in the first series. There did not appear to be any correlation between the rejection time and the extent of the fall in the counts. In the heparin-treated group (series 3) the platelet counts also fell but to a lesser degree with a mean of 27% (range 1-51%). In the same group the neutrophil counts dropped in five of the seven dogs studied, but there was no change in two (dogs 27 and 28). There was no significant deterioration in the platelet or leycocyte counts comparing the venous and arterial blood samples in two renal autografts. The non-responder (dog 33) maintained a platelet and leucocyte count in a similar manner. In the dogs of series 1 and 2 arterial platelet counts at the sampling time nearest to rejection were still significantly below the initial arterial levels, again more pronounced in series 2. Four dogs in the heparin-treated group showed slight reduction in the final arterial platelet count, but in three others (dogs 27, 28, and 29) the level was increased. This may have been due to contraction of the spleen, which was noted in most of the dogs at the end of each experiment.

Fibrinogen.—No reduction of fibrinogen was detected in the blood samples removed after transplantation of the renal allografts in any series when using a standard thrombin test or by chemical estimation of the fibrinogen levels.

HISTOPATHOLOGICAL CHANGES

The main abnormalities in all the renal allografts examined were aggregation of platelets and a variable increase in the number of white blood cells in the glomerular and peritubular capillaries (table II). These features were not found in the autografted kidneys. No fibrin thrombi were found in the glomerular or intertubular capillaries or in the larger arteries and veins in any of the kidneys examined.

Discussion

Hyperacute rejection of a human renal allograft may follow sensitization by blood transfusion or a previous kidney transplant (Starzl *et al.*, 1968; Joycey *et al.*, 1972). As the number of patients requiring a second allograft increases this is likely to become more frequent. Attempts have been made to produce experimental models of accelerated graft rejection in animals in order to study this problem. Dempster (1953) showed that rejection of renal allografts occurs within 24 hours of transplantation in dogs presensitized with skin and kidney graft.

Pathological studies of kidneys which have undergone hyperacute rejection have shown the presence of thrombi in the renal vessels, and it has been suggested that intravascular coagulation within the kidney is an important factor in the rejection process. Colman *et al.* (1969) and Starzl *et al.* (1968) reported extensive renal cortical vascular thrombosis in human kidneys which had undergone hyperacute rejection; while in dogs glomerular thrombosis and fibrin platelet thrombi have been found in the rejecting kidneys by Macdonald *et al.* (1970) and Pineo *et al.* (1970). Although Pineo *et al.* described glomerular thrombosis and tubular necrosis, they thought that these changes in themselves were probably not responsible for hyperacute rejection. No fibrin thrombi were found by conventional histological staining techniques in any of the rejecting dog kidneys we have studied. Furthermore, Sharma *et al.* (1972) in a similar study failed to find fibrin deposition in rejected kidneys by electron microscopy. It seems more likely that thrombus formation is a secondary phenomenon occurring after the rejection phase, since in our experiments, where the kidneys were examined immediately on rejection, no fibrin thrombi were found.

Pineo *et al.* (1970) recorded a slight fall in the platelet count in samples of peripheral and renal vein blood taken at hourly intervals after transplantation. We found a much greater fall in the platelet count during the first 15 minutes after grafting, which was followed by a steady rise. The disappearance of platelets during circulation through the kidney correlates with the presence of large numbers of platelets in the glomerular and peritubular capillaries seen histologically. Macdonald *et al.* (1972) also reported a fall in platelets during passage through allografted kidneys in hypersensitized dog recipients. The evidence obtained from studies using xenograft models (Land *et al.*, 1971; Linn *et al.*, 1971; Moberg *et al.*, 1971; Merkel *et al.*,

1971; Slapak *et al.*, 1971) and our own findings make it possible to construct a probable sequence of events in the hyperacute rejection process. The first change appears to be damage to the capillary endothelium presumably by an antibody antigen complex with subsequent binding of complement. This is followed by platelets adhering to the damaged endothelium and subsequent aggregation in the glomerular and peritubular capillaries. Substances released from these damaged platelets could result in the intense vasoconstriction, which together with the platelet plugs themselves could cause acute haemostasis and immediate rejection. Thrombosis is probably a later and secondary change.

The action of heparin in prolonging graft survival is probably due to its direct effect on the platelets. This is consistent with our findings of a reduced fall in the platelet count in the heparin-treated dogs. Linn *et al.* (1971) suggested that substances released by the breakdown of complement contribute to the vasoconstriction during acute rejection. For this reason we established that our heparin preparation was free from anti-complementary activity. Modification of the rejection process with aspirin or cyproheptadine hydrochloride (Periactin) could also be explained by their antagonism to platelet aggregation (O'Brien, 1968; Burrows *et al.*, 1970).

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Amodiaquine-induced Agranulocytosis: Toxic Effect of Amodiaquine in Bone Marrow Cultures in Vitro

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Summary

A case of agranulocytosis is reported in which amodiaquine, to which the patient had been exposed, was found to be toxic to the patient's bone marrow cells when these were cultured in an agar colony system in vitro. This technique should be investigated in other patients with agranulocytosis as a possible means of detecting toxic agents.

Introduction

In most cases of drug-induced blood dyscrasias identification of a causative agent can be made only from the patient's history. This is relatively easy in cases of single drug exposure but more difficult when several drugs are involved. Re-exposure of the patient to the drug cannot be justified as a diagnostic procedure, and firm proof of the harmful nature of a drug has until now been difficult to obtain. This paper reports a case of amodiaquine-induced agranulocytosis in which the toxic effect of amodiaquine was shown by its inhibition of colony growth of the patient's bone marrow cells in vitro. Although the technique of bone marrow culture is well established in the study of granulopoiesis, we are not aware of any previous reports of its use in the investigation of a patient with agranulocytosis.

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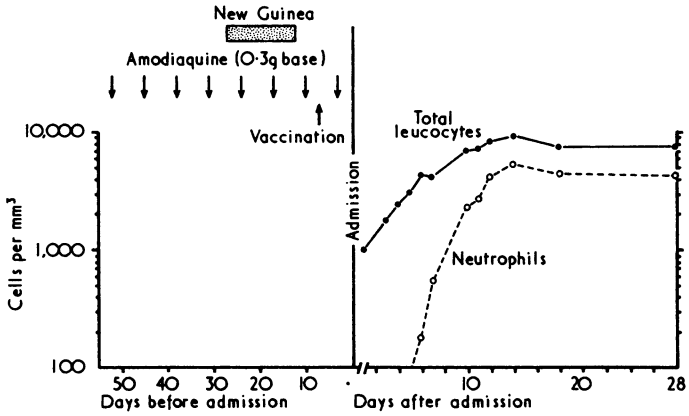
Case Report

A 26-year-old woman visited New Guinea for a two-week holiday in July 1972. Antimalarial prophylaxis consisting of amodiaquine (Camoquin) 300 mg once a week was started four weeks before her departure and continued during and after her holiday (see chart). She took a total of eight doses (2·4 g), the last one three days before her admission. She was febrile and unwell for two days after the second dose, and had diarrhoea for one day on her return, but had otherwise experienced no untoward symptoms. One week before admission she was vaccinated, and immunized against typhoid and cholera. Two days before admission she became febrile, with myalgia and night sweats. Her local doctor prescribed tetracycline and Actifed (triprolidine hydrochloride and pseudephedrine hydrocloride). When a blood count showed agranulocytosis, she was admitted to hospital.

She gave a history of five episodes of respiratory tract infection in the 18 months preceding admission, and she had noted increas-



Clinical course before and after admission. Vaccination against smallpox, typhoid, and cholera, and the duration of patient's stay in New Guinea are shown. Note the expanded time scale after admission.