

# HAMSTER TO RAT KIDNEY XENOTRANSPLANTATION

## EFFECTS OF FK 506, CYCLOPHOSPHAMIDE, ORGAN PERFUSION, AND COMPLEMENT INHIBITION

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Hamster to rat renal xenotransplantation was performed with recipient nephrectomies. Recipients were treated beginning on day 0 with continuous FK 506 monotherapy, a 7-day or open-ended monotherapeutic course of cyclophosphamide (CP),\* and the two drug regimens combined. CP alone (10 mg/kg/day) prevented a xenospecific antibody response and tripled median survival of the kidney (defined as recipient death) from 6 (control) to 18.5 days whereas FK 506 alone had no effect. The drugs in combination were no better than CP alone (15 days) unless the 5-day course of CP was given at a higher dose (15 mg/kg) and started 3 days preoperatively (79 days). In further experiments, adjuvant measures were added to the minimally effective FK 506/7-day CP regimen which gave a median survival of only 15 days. In the most successful modification, intraoperative antibody depletion by the temporary transplantation of third party hamster liver or en bloc kidneys increased median survival from 15 to 34 and 48 days, respectively. An intraoperative i.v. dose administration of the anticomplement drug K76 instead of antibody depletion increased survival to 26 days. Although the events of kidney rejection were similar to those of heart xenografts and partially forestalled by the antibody inhibiting CP treatment, or by antibody depletion, survival for >100 days was accomplished in only 5 of 86 treated animals. The poorer survival previously reported with cardiac xenotransplantation is largely explained by the life support requirement of the kidneys. Renal failure was responsible for almost all deaths before 60 days, and subnormal renal failure was a pervasive adverse factor thereafter, frequently caused by pyelonephritis which is suspected to have had an immunologic etiology.

Hamster to rat heart and liver transplantations have been widely used to study xenograft rejection (1-9), but there has been no recorded experience with the kidney except for our preliminary technical description of a model (10). We report here the effects on the hamster renal xenograft of a variety of familiar or new therapeutic protocols, most of which already have been evaluated previously with other organs. These included FK 506, cyclophosphamide (CP), the anticomplement drug K76, and antibody depletion by prior or contemporaneous transplantation of donor species kidneys or livers. Long survival of the kidney xenograft proved more difficult to

achieve than previously observed with either the heart or liver.

### MATERIALS AND METHODS

#### *Animals*

Inbred male Lewis rats (LEW, RT1<sup>1</sup> Harlan Sprague Dawley Inc., Indianapolis, IN) weighing 200-300 g were recipients, and Golden Syrian hamsters (Charles River Laboratory, Wilmington, MA) weighing 100-150 g were donors.

#### *Surgical Procedure and Animal Care*

*Orthotopic kidney transplantation.* Methoxyflurane inhalation anesthesia was given. The hamster left kidney was mobilized and removed, leaving the left renal artery in continuity with a segment of the aorta, and the left renal vein with a cuff of the inferior vena cava (10). The full ureter was retained in continuity with a piece of bladder. After donor heparinization (200 U), the xenograft was excised and infused via the aorta with 1-3 ml cold lactated Ringer's solution. Using a 10-0 Novafil suture, the end of the graft aorta was anastomosed to the side of the recipient infrarenal aorta and the graft left renal vein cuff to the side of the adjacent inferior vena cava. The donor bladder patch was sewn to the recipient bladder, followed by recipient nephrectomies. Graft rejection was defined as the time of death. Animals that died within 7 days with obvious surgical complications (15%) were eliminated from analysis. The most common causes of exclusion were leak or hemorrhage at the bladder anastomosis.

*Antibody depletion.* The "expendable" organs were from third party hamster donors. The portal vein of the donor liver or the aorta in continuity with en bloc kidneys were arterialized by cuff anastomosis to the carotid artery, with venous drainage into the recipient jugular vein. After ligating the unused open vessels of the grafts, these "antibody trap" organs were perfused for 1.5-2 hr, beginning before and continuing throughout transplantation of the definitive kidneys, and then disconnected.

*Animal care and sampling.* Postoperative intramuscular cefamandole nafate was given for 3 days. Body weight and activity were recorded daily. Blood (0.5 ml) was taken from the tail vein 1 day after grafting and weekly thereafter postoperatively; serum creatinine and anti-hamster lymphocytotoxic antibodies were measured in the samples.

#### *Immunosuppressive Agents*

FK 506 (Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan) was dissolved in normal saline and given intramuscularly in doses of 1 or 2 mg/kg/day. CP, prepared daily in distilled water, was given by gastric instillation in doses of 7.5-15 mg/kg/day. The anticomplement agent, K76 monocarboxylic acid (K76) (11, 12) (Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan), was dissolved in normal saline, and 200 mg/kg was injected intravenously 30 min before graft revascularization.

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\* Abbreviation: CP, cyclophosphamide.

### Experimental Design

The course of unmodified animals (group 1) was compared with that under FK 506 or CP alone (groups 2-4 and 10), and in combination (groups 5 and 6). The group 5 regimen of FK 506 and CP was selected for the addition of further therapeutic variables: antibody depletion with third party liver and kidney in groups 11 and 12, respectively, an increased dose of CP which was advanced into the preoperative period in group 14, and a prevascularization intravenous dose of the complement inhibiting drug, K76 in group 13. The treatment schedules for experimental and control groups are summarized in Table 1.

### Antibody Studies

*Of serum.* Lymphocytotoxic antibodies were measured with a complement-fixing assay, using donor hamster lymphocytes as targets (3, 13). The recipient sera were heat-inactivated (56°C for 30 min) and serially diluted with RPMI 1640. One microliter of various dilutions of serum samples and 1  $\mu$ l of hamster lymphocyte suspensions ( $4 \times 10^6$ /ml) were incubated for 30 min at room temperature. After addition of 5  $\mu$ l baby rabbit complement (1:5 dilution, Cedarlane Laboratories Limited, Hornby, Ontario), the mixture was reincubated for 60 min at room temperature. After trypan blue staining and fixation, cell lysis was scored, and the lymphocytotoxic antibody titer was defined as the most dilute serum sample with greater than 50% cell lysis. Samples were run in duplicate.

*Of tissue.* Direct immunofluorescence studies were performed in separate animals. Untreated animals were killed 1 hr (n=3), 1 day (n=3), or 3 days (n=2) after transplantation. Animals which were antibody-depleted with kidney perfusion and treated with FK 506 and CP (as in group 12) were killed 1 hr (n=2), 1 day (n=3), or 6 days (n=3) after transplantation. K76-treated animals (treated as in group 13) were also killed 1 hr after grafting (n=3). The xenografts were embedded in optimal cold temperature compound at -76°C, cut into 4- $\mu$ m sections, and incubated with FITC-conjugated goat anti-rat IgG or IgM (Accurate Chemical & Scientific Corp., Westbury, NY) or anti-rat complement C3 (Organon Teknika Corporation, Cappel Research Products, Durham, NC). The location and intensity of Ig and complement deposits were examined blindly without knowledge of treatment protocols or the time of sampling.

### Statistical Analysis

Mann-Whitney *U* test was used to compare statistical differences in animal survival between groups. A *P* value less than 0.05 was considered significant.

## RESULTS

### Animal Survival and Graft Function

*Animal survival.* The 6-day survival of untreated controls was not increased with FK 506 monotherapy (group 2, Table 1) but rose to 8 days and 18.5 days with daily 7.5 and 10 mg/kg CP monotherapy, respectively (groups 3 and 4). The CP benefit was not augmented with the addition of FK 506 (groups 5 and 6). The combination of 1 mg/kg/day FK 506 and a 7-day postoperative course of 10 mg/kg CP (group 5) which gave a median survival of 15 days was chosen as baseline for the evaluation of added therapeutic variables that were ineffectual when used alone (groups 7-9).

The 15-day median survival of group 5 was prolonged to 34 days (vs. group 5, *P*=0.06, NS) by perioperative antibody depletion with hamster livers (group 11, Table 1), to 48 days (*P*<0.05) when paired kidneys were used for the depletion (group 12), and to 26 days with a single prevascularization dose of i.v. K76 (*P*=0.1, NS) (group 13). However, the longest survival of 79 days was in animals given continuous FK 506 plus the higher daily dose of 15 mg/kg CP for 5 days divided equally between the pre- and postoperative periods (group 14); animals given the same regimen of CP alone died within 10 days (group 10).

Only 5 animals of the 96 in the study lived for more than 100 days (Table 1)—2/8 in group 12 and 3/9 in group 14.

*Graft function and body weight.* The animals of groups 1-6 were ill almost from the time of xenotransplantation—losing weight and with rapid increases in serum creatinine with a few exceptions that were multiple only in group 4 (Fig.

TABLE 1. LEW rat recipient survival after hamster kidney transplantation: effect of FK 506, CP, K76, and organ perfusion

Group	Treatment			Organ perfusion	n	Survival (days)	Median (days)	<i>P</i>	
	FK 506 <sup>a</sup> (mg/kg/day)	CP (mg/kg/day)	Duration (days)					(vs. group 1)	(vs. group 5)
1	—	—	—	—	10	5, 5, 5, 5, 6, 6, 6, 6, 6, 6	6.0	—	—
2	2.0	—	—	—	11	5, 5, 5, 5, 6, 6, 6, 6, 6, 7, 7	6.0	NS <sup>c</sup>	—
3	—	7.5	0→death	—	7	6, 7, 8, 8, 8, 9, 20	8.0	<0.005	—
4	—	10.0	0→death	—	6	6, 11, 13, 24, 28, 30	18.5	<0.005	—
5	1.0	10.0	0→6	—	7	5, 5, 6, 15, 18, 27, 30	15.0	<0.005	—
6	2.0×6→1.0	10.0	0→6	—	6	8, 10, 11, 12, 33, 57	11.5	<0.005	<0.05
7	—	—	—	Liver	3	5, 5, 6	5.0	NS	—
8	—	—	—	Kidney	3	5, 6, 6	6.0	NS	—
9 <sup>b</sup>	—	—	—	—	5	7, 7, 7, 7, 7	7.0	<0.005	—
10	—	15.0	-3→1	—	6	9, 9, 9, 9, 10, 10	9.0	<0.005	—
11	1.0	10.0	0→6	Liver	9	12, 13, 14, 22, 34, 37, 38, 79, 86	34.0	<0.001	NS
12	1.0	10.0	0→6	Kidney	8	9, 12, 26, 33, 63, 92, 130, 133	48.0	<0.001	<0.05
13 <sup>b</sup>	1.0	10.0	0→6	—	6	9, 19, 25, 27, 43, 46	26.0	<0.005	NS
14	1.0	15.0	-3→1	—	9	7, 11, 13, 16, 79, 79, 102, 118, 126	79.0	<0.001	NS

<sup>a</sup> FK 506 was intramuscularly injected at indicated doses for 30 days and continued at 0.5 mg/kg/day every other day thereafter.

<sup>b</sup> K76 (200 mg/kg i.v.) was given before transplantation as a bolus injection.

<sup>c</sup> NS, not significant (Mann-Whitney *U* test).

1). The rats in group 4 which had open-ended treatment with CP appeared to die of drug toxicity.

In contrast, the majority of animals in groups 11, 12, and 14 reversed the early postoperative weight loss and had periods of stability for days or weeks before secondary deterioration that most commonly was associated with sharp rises in serum creatinine (Fig. 1). Eleven rats which survived for more than 60 days, including the five that went beyond 100 days, had final serum creatinine values that were at or below 3 mg%, but none was normal.

*Antibody Studies*

*Of serum.* Anti-hamster lymphocytotoxic antibodies promptly rose in the untreated animals of group 1 and in those treated only with FK 506, reaching  $2^8$  to  $2^{13}$  by the time of death (Fig. 2). CP in doses of 10 mg/kg/day prevented this increase in all animals whether or not the CP was open-ended or limited to 6 days. The CP benefit was independent of FK 506 and was not obviously enhanced by antibody depletion. In rats that survived for more than 1 month, titers drifted up late, but never to high levels (Fig. 2).

*Of tissue.* The most remarkable change seen by direct immunofluorescence in the untreated kidney xenograft 1 hr after reperfusion was intense deposition of IgM>>IgG in the main vessels (Fig. 3). These increased with time. By 1-3 days the glomerular capillary loops and interstitium became IgM positive (Fig. 4); however, IgG deposits are not prominent.

When preformed antibodies were depleted either by kidney or liver perfusion, or pretreatment with CP, IgM deposits in main vessels 1 hr after grafting were significantly decreased (Fig. 5).

*Histopathology*

Mild spotty hyalinization was observed in the untreated kidney grafts obtained 1 hr after grafting. Hyalinoid degeneration increased with time and involved whole vessel walls in 24 hr. Three to 5 days after transplantation, the vessels showed classical features of humoral rejection. Microthrombi were present in small arteries, arterioles, and glomerular capillaries, resulting in extensive tubular necrosis. There

were diffuse interstitial hemorrhages and perivascular and glomerular neutrophil infiltration, but no mononuclear infiltrates (Fig. 6).

In long-surviving animals, mild to moderate cellular infiltrates were seen in a few sections. Vasculitis, including intimal proliferation and obliterative arteriopathy, was occasionally observed, suggesting that antibody-mediated graft damage was not completely abolished. Evidence of pyelonephritis caused by urinary tract obstruction was a common finding in long-surviving animals.

*Complement Studies*

One hour after transplantation untreated hamster kidney showed diffuse rat C3 deposits along the main vessels. Preoperative bolus K76 injection (200 mg/kg) completely prevented C3 deposition (Fig. 7).

DISCUSSION

The events and mechanisms of hamster heart and liver rejection in rat recipients are well-known. The existence of low level hamster specific xenoantibodies in normal rats has been demonstrated with in vitro cytotoxic assays using hamster lymphocytes or endothelial cells as targets (2-9, 14, 15) and with an indirect immunofluorescence assay showing xenospecific Ig binding to tissues (3, 6, 14). Despite these ominous findings, hyperacute rejection of the heart and liver does not occur in this species combination. However, rapid B cell activation and xenospecific antibody production causes pure humoral rejection of the heart in 3 days (3-9, 14). The liver with its well-known resistance to antibody rejection (16) is destroyed in 7 days by a combination of humoral and cellular rejection (3, 4, 14).

The exclusively humoral kidney xenograft rejection observed in the present study, occurring over a 3-day period rather than hyperacutely, was comparable histopathologically to that of the hamster heart. Within less than 1 hr, deposition of rat IgM>>IgG on the endothelium of the main intraparenchymal renal vessels was striking, but less than in the hyperacutely rejecting pig kidney transplanted to the dog (17), Rhesus monkey (18, 19), or baboon (20). As with ham-

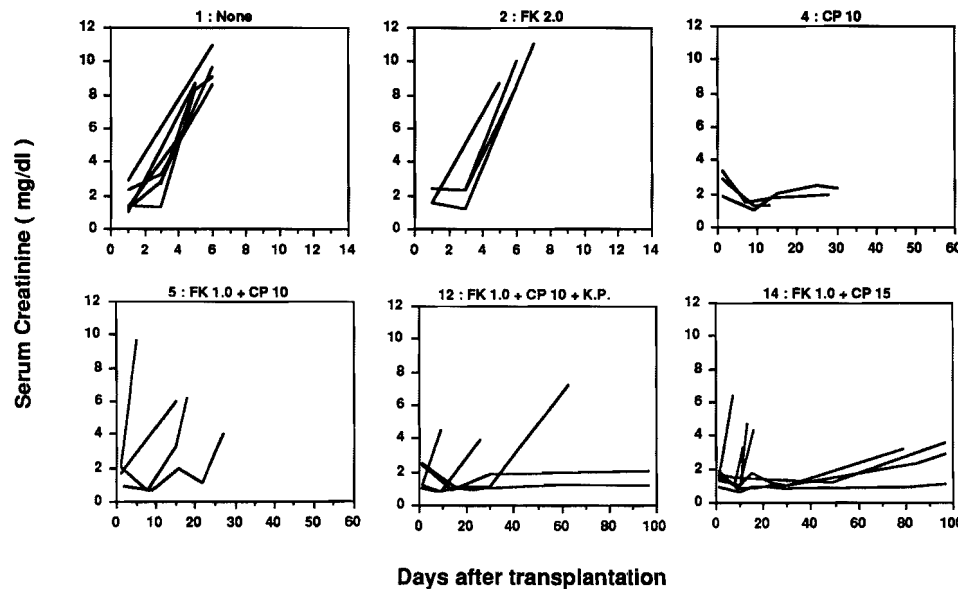


FIGURE 1. Changes of serum creatinine levels after hamster kidney transplantation in animals of groups 1, 2, 4, 5, 12, and 14. The treatment protocol for each group is identical to that described in the text and Table 1.

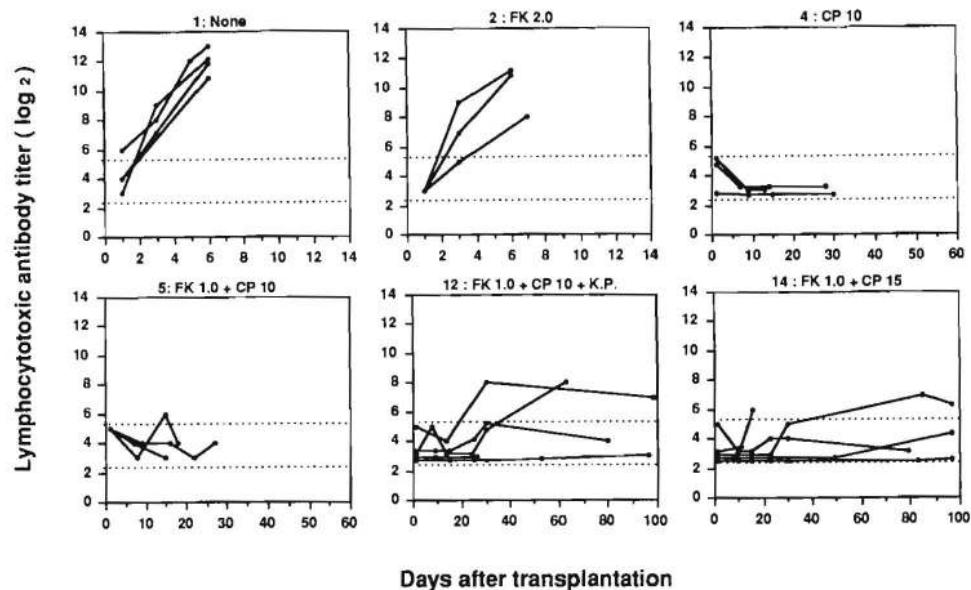


FIGURE 2. Changes of anti-hamster lymphocytotoxic antibody titers after hamster kidney transplantation. Groups are identical to those in Figure 1 and in the text.

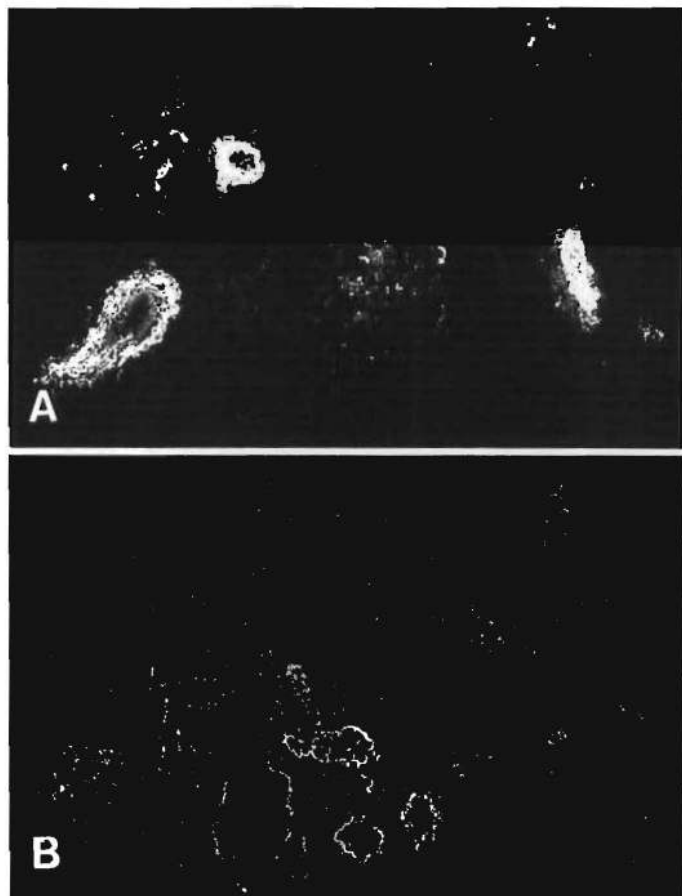


FIGURE 3. Hamster kidney graft 1 hr after grafting in untreated Lewis rat recipient. Intense IgM deposition on the vessels (A) and weak deposits of IgG (B). (Direct immunofluorescence, FITC-conjugated goat anti rat IgG or IgM.)

ster hearts, the humoral rejection was not altered by treatment with the T cell-directed FK 506, but was markedly attenuated by suppressing B cell antibody production with CP. The efficacy of CP in the *kidney* experiments, as in previous reports with the

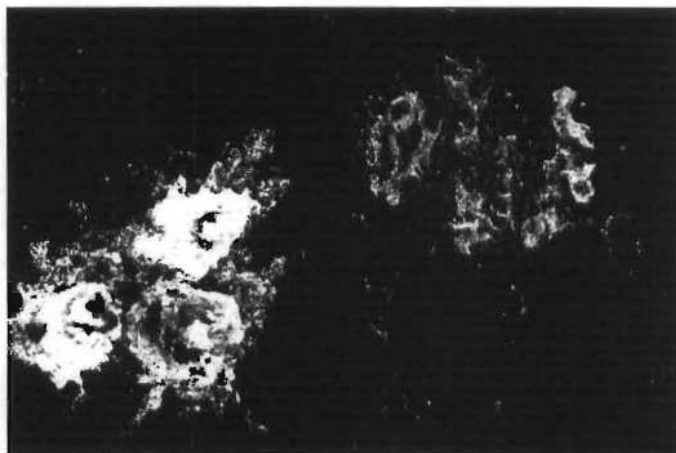


FIGURE 4. Untreated hamster kidney graft 5 days after transplantation in the rat. Intense IgM deposits are seen in the glomerular loops and the interstitium. (Direct immunofluorescence, FITC-conjugated goat anti rat IgM.)

other organs, was attributable to its prevention of the astronomical increases in lymphocyte toxicity titers that otherwise occur. The humoral rejection also was mitigated by depleting the antibodies with "forerunner" donor organs as has been described in hypersensitized allograft recipients (21), and with various species combinations to protect kidney, heart, or liver xenografts (17, 22-24). As we reported long ago in a direct comparison of the liver, kidney, and spleen, none of these organs was strikingly superior for antibody depletion (17). We obtained better results in the present study using en bloc kidneys than we did with the liver.

The critical role of complement in humoral rejection has been well-demonstrated with transplantation across widely diverse species barriers as well as with transplantation to hyperimmunized allograft recipients. In most such experiments, the antibody-initiated classical pathway of complement activation has been incriminated (25). However, hyperacute allograft or xenograft rejection also can be caused by activation of the alternative complement pathway not involving antibody collabora-



FIGURE 5. Hamster kidney graft 1 hr after grafting in recipient pretreated with kidney perfusion (right) or high dose CP (left). Intense rat IgM deposits were significantly reduced, compared with those of untreated recipient in Figure 3. (Direct immunofluorescence, FITC-conjugated goat anti rat IgM.)

tion (26, 27). Anticomplement agents which interfere with both pathways at different levels include cobra venom factor (28) and recombinant soluble complement receptor type I (29, 30). Prolonged heart graft survival in the concordant hamster-rat model has been accomplished by combining cobra venom factor and CsA (5). These agents mitigate hyperacute rejection in pig-to-dog (28), pig-to-primate (18–20), and guinea pig-to-rat (20, 29, 30) xenografts, but the effect is temporary in the discordant species.

K76, which inhibits C5 convertase and thus disables both pathways proximal to the membrane attack complex, has been used in the guinea pig-to-rat heart model with minimal effect when given intraperitoneally (31), but with a much greater prolongation of survival when the drug was administered intravenously at the same dose (32). Intravenous K76 in the present study also appeared to prolong hamster-to-rat kidney xenograft survival, but with the small number of experiments ( $n=6$ ) and the short survival of one of the K76-treated animals, the therapeutic effect was short of statistical significance ( $P=0.1$ ) when compared with FK 506-CP alone (median 15 vs. 26 days).

Although the events of kidney xenograft rejection and the degree of their amelioration with antibody control strategies were generally comparable to those reported earlier with the heart, animal survival was inferior. Rather than connoting an immunologic disadvantage of the kidney, this can be explained by the life support function of the kidney xenograft compared with the physiologically superfluous role of the heterotopic heart. Deaths before 60 days almost invariably were preceded by unremitting weight loss, and in most animals, except those of group 4 treated with presumably toxic open-ended CP, this was associated with striking increases in serum creatinine despite suppression of the antibody response which outlasted CP treatment in more successful cases. After 60 days, maintenance or gain in weight was associated with better, but still abnormally elevated, creatinine concentrations, consistent with a pervasive mortality factor of renal failure. These animals produced a large volume of unconcentrated urine, which sometimes reached three to six times more volume than that of normal rat. This

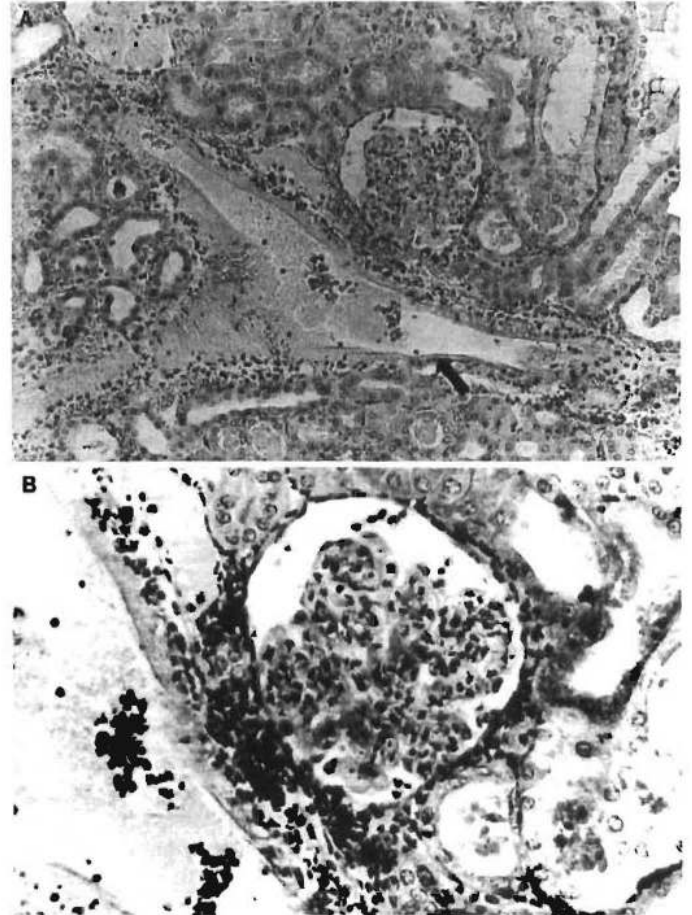


FIGURE 6. (A) Untreated hamster kidney graft 5 days after grafting. Platelet and neutrophilic sludging can be seen in this interlobular artery (arrow) and the glomerulus immediately above the center of the photomicrograph (H&E, 100 $\times$ ). (B) A high magnification of the glomerulus shows capillary loops thrombosis, neutrophilic sludging and collapse of the loops, all of which are indicative of humoral rejection (H&E, 300 $\times$ ).

impaired renal function might be explained by the chronic immunological damages. However, there might be a functional disparity between the rat and hamster kidney. This possibility is currently under investigation.

Histopathologically, vascular damage and signs of pyelonephritis reminiscent of the vasculopathic biliary tract lesions previously reported in liver xenografts were the most common abnormalities in the long surviving kidneys. As with the biliary tree, obstruction of the graft urinary tract for technical reasons often could not be ruled out. Several methods for urinary tract reconstruction have been described to decrease the incidence of pyelonephritis and stone formation in the tiny rat drainage conduits (33). The ureter-ureter and ureter-bladder anastomoses, which have been used for the rat kidney transplantation with more than 85% success, always resulted in anastomosis breakdown in the hamster-to-rat transplantation, because of the short and thin hamster ureter. We chose the technique of bladder-to-bladder reconstruction; however, the poor vascularization of the distal graft ureter and bladder could defeat the purpose intended. The alternative possibility that obstruction of xenograft collecting systems had an immunologic etiology is supported

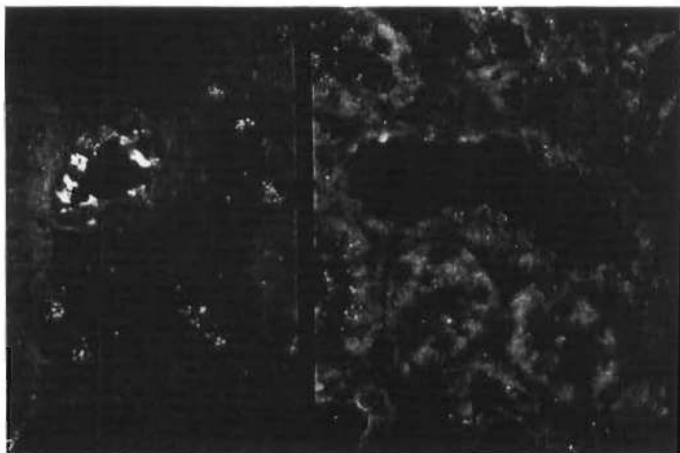


FIGURE 7. Intense rat complement C3 deposits were seen in the main vessels of untreated hamster kidney graft (left: 1 hr after grafting). In K76-treated animal (200 mg/kg i.v.), C3 deposition was dramatically reduced (right). (Direct immunofluorescence, FITC-conjugated goat anti rat C3.)

by experience in liver transplantation in which biliary obstruction was dramatically reduced by using better immunosuppressive protocols (3).

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