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Received 20 February 1996.
Accepted 16 April 1996.

0041-1337/96/6205-681$03.00/0
TRANSPLANTATION

1885

Vol. 62, 681–688, No. 5, September 15, 1996
Printed in U.S.A.

EFFECT OF ANTICOMPLEMENT AGENT K76 COOH ON HAMSTER-TO-RAT AND GUINEA PIG-TO-RAT HEART XENOTRANSPLANTATION[1]

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In normal rats, the xenobiotic K76 inhibited the C5 and probably the C2 and C3 steps of complement and effectively depressed classical complement pathway activity, alternative complement pathway activity, and the C3 complement component during and well beyond the drug's 3-hr half-life. It was tested alone and with intramuscular tacrolimus (TAC) and/or intragastric cyclophosphamide (CP) in rat recipients of heterotopic hearts from guinea pig (discordant) and hamster (concordant) donors. Single prevascularization doses of 100 and 200 mg/kg increased the median survival time of guinea pig hearts from 0.17 hr in untreated controls to 1.7 hr and 10.2 hr, respectively; with repeated injections of the 200-mg dose every 9–12 hr, graft survival time was increased to 18.1 hr. Pretreatment of guinea pig heart recipients for 10 days with TAC and CP, with or without perioperative splenectomy or infusion of donor bone marrow, further increased median graft survival time to 24 hr. Among the guinea pig recipients, the majority of treated animals died with a beating heart from respiratory failure that was ascribed to anaphylatoxins. Hamster heart survival also was increased with monotherapy using 200 mg/kg b.i.d. i.v. K76 (limited by protocol to 6 days), but only from 3 to 4 days. Survival was prolonged to 7 days with the addition to K76 of intragastric CP at 5 mg/kg per day begun 1 day before operation (to a limit of 9 days); it was prolonged to 4.5 days with the addition of intramuscular TAC at 2 mg/kg per day beginning on the day of transplantation and continued indefinitely. In contrast to the limited efficacy of the single drugs, or any two drugs in combination, the three drugs together (K76, CP, and TAC) in the same dose schedules increased median graft survival time to 61 days. Antihamster antibodies rapidly increased during the first 5 days after transplantation, and plateaued at an abnormal level in animals with long graft survival times without immediate humoral rejection. However, rejection could not be reliably prevented, and was present even in most of the xenografts recovered from most of the animals dying (usually from infection) with a beating heart. Thus, although effective complement inhibition with K76 was achieved in both guinea pig- and hamster-to-rat heart transplant models, the results suggest that effective interruption of the complement cascade will have a limited role, if any, in the induction of xenograft acceptance.

[1] This study was supported by research grants from the Veterans Administration and project grant no. DK 29961 from the National Institute of Health, Bethesda, MD.

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K76 monocarboxylic acid is a xenobiotic purified from the culture of the fungus *Stachybotrys complementi* that inhibits the C5 step of the complement cascade (*1*) by fusion of the molecule with the C5 complement component (*2*). Reported in vivo effects of K76 include protection of guinea pigs and mice from Forssman shock (*3*), mitigation of experimental glomerulonephritis in rats (*3, 4*), and amelioration of experimental (*5*) and clinical ulcerative colitis (*6*). In 1993, Miyagawa et al. (*7*) showed that intraperitoneally administered K76 modestly prolonged the survival of heterotopic guinea pig hearts after their transplantation to rats. With intravenous administration, a far more dramatic therapeutic effect was demonstrated (*8, 9*). K76 also substantially prolonged kidney xenograft survival after pig→dog transplantation (*8*).

We further document herein the remarkable ability of K76 to ameliorate the hyperacute rejection (HAR*) that follows guinea pig→rat xenotransplantation. However, this accomplishment, as with other complement-inactivating or -depleting procedures, has no immediate clinical application because the characteristic HAR that defines discordant xenograft rejection was merely delayed while recipient mortality increased in proportion to the efficacy of xenograft protection. Consequently, we systematically added K76 to a double-drug cocktail of subtherapeutic doses of tacrolimus (TAC) and cyclophosphamide (CP) and tested different drug combinations and dose schedules with hamster→rat heart xenotransplantation. Our conclusion from the experiments was that agents that efficiently inhibit complement impose thereby inherent risks and consequently are not likely to have a maintenance role in cross-species transplantation, even between concordant species.

MATERIALS AND METHODS

Experimental Models and Drugs

Animals. Inbred male Lewis rats weighing 200–250 g (Harlan Sprague Dawley Inc., Indianapolis, IN) were used as recipients. Outbred male Syrian hamsters weighing 100–150 g and outbred Hartley guinea pigs weighing 150–300 g (Charles River Laboratories, Wilmington, MA) were used as donors.

Transplant operations. The animals were anesthetized with methoxyflurane. Heart grafts were transplanted into the abdomen of recipients using the technique of Ono and Lindsey (*10*). Rejection was diagnosed by the cessation of a palpable beat of the graft, followed by direct inspection and histopathological examination.

Immunosuppressive drugs. K76 (donated by Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan) was suspended in normal saline, adjusted to pH 7.8 with NaOH, and administered as a bolus intravenous injection via the penile vein over 1–2 min. Plasma concentrations were measured using a high-performance liquid chromatography method (*11*). TAC (Fujisawa Pharmaceutical Co., Osaka, Japan) was injected intramuscularly after suspension in normal saline. CP was prepared daily in distilled water and administered orally by gastric instillation.

Experimental Design (Survival)

Guinea pig→rat transplantation. Monotherapy with intravenous K76 was given in different doses 30 min before revascularization, with repeat doses 9–12 hr later in rats whose graft survival exceeded 9 hr (group 6). The results were compared with those for untreated controls (group 1). The best survival time with monotherapy was obtained with two doses of 200 mg/kg (group 6). This therapy was then systematically combined with other kinds of 10-day pretreatment with TAC, CP, or both together in doses shown in Table 1. In two additional groups given the triple- or double-drug therapy, 250×10^6 unaltered guinea pig bone marrow cells were given on day −10 (groups 11 and 12). Finally, adjuvant splenectomy was combined with K76/TAC/CP therapy in group 13.

Hamster→rat transplantation. K76 doses of 200 mg/kg every 12 hr were begun from 30 min before revascularization (in most experiments) to as late as the third postoperative day (groups 8, 9, and 10) and stopped after day 4, 6, or 8 (Table 2). The drug was tested alone, with TAC at 2 mg/kg per day, with CP at 2.5–10 mg/kg per day, or in combination with both TAC and CP (Table 2). TAC was begun on the day of operation, with the dose reductions on postoperative days 6, 14, and 30 in the different groups shown in Table 2. CP was begun 1 day before the operation and stopped on the days shown in Table 2.

Experimental Design (Nonsurvival)

K76 pharmacokinetic and immunologic studies were obtained in naive rats and with both experimental models using the therapy that provided the best survival time without either adjuvant bone marrow or splenectomy. The latter animals were killed while their xenograft hearts were still beating for histopathologic studies.

Guinea pig→rat. Animals pretreated for 10 days with TAC and CP plus repeat doses of K76 (Table 1, group 8) were killed after 0.5, 1.5, 3, 6, 16, 20, and 25 hr (n=1-3 each).

Hamster→rat. Animals treated with K76, TAC, and CP (Table 2, group 7) were killed on postoperative days 1, 3, 5, 7, 10, 14, and 28 (n=3 each).

Immunologic and Coagulation Assays

Antihamster antibodies. Rat serum samples were heat inactivated (56°C for 30 min), serially diluted with RPMI 1640 medium, and studied with a complement-dependent lymphocytotoxic assay. Hamster lymphocyte targets were prepared from cervical lymph nodes and suspended at a concentration of 2×10^6 cells/ml in medium supplemented with 20% heat-inactivated normal hamster serum. One microliter of diluted serum sample and of hamster lymphocyte suspension were incubated for 30 min at room temperature. Five microliters of baby rabbit complement (1/5 dilution, Cedar Lane Laboratories Ltd, Hornby, Ontario, Canada) were added to each well and reincubated for 60 min at room temperature. After staining with 0.4% trypan blue, the cytotoxic antibody titer was defined as the highest serum dilution with more than 51% of cell lysis.

Clotting factors. Prothrombin time (PT), activated partial thromboplastin time (APTT), and plasma fibrogen concentrations were measured by standard laboratory procedures and were expressed as percentages of the mean value in normal Lewis rats.

Recipient complement. Classical complement pathway activity (CH_{50}) was assayed using the method of Kabat and Mayer (*12*). Serially diluted serum (0.5 ml) and 0.5 ml of sensitized sheep erythrocytes (1×10^8 cell/ml) were incubated in 1.5 ml of GVB (isotonic Vernal-buffered saline with 0.1% gelatine) containing 0.15 mM $CaCl_2$ and 1.0 mM $MgCl_2$, for 60 min at 37°C, and analyzed by spectrophotometer at 412 nm. Alternative complement pathway activity (ACH_{50}) was assayed using rabbit erythrocytes suspended in GVB containing 2 mM Mg^{++} and 8 mM EDTA (5×10^7 cell/ml). Fifty microliters of serum sample and 0.1 ml of rabbit erythrocytes were incubated for 60 min at 37°C and analyzed by spectrophotometer at 412 nm. C3 level was measured with the single radial immunodiffusion method using goat antirat C3 serum (Organon Teknika Corp., Durham, NC). Results were expressed as percentages of the mean value in normal Lewis rats.

* Abbreviations: ACH_{50}, alternative complement pathway activity; APTT, activated partial thromboplastin time; CH_{50}, classical complement pathway activity; CP, cyclophosphamide; CVF, cobra venom fraction; HAR, hyperacute rejection; PT, prothrombin time; TAC, tacrolimus.

TABLE 1. Heterotopic heart and recipient survival after guinea pig → rat transplantation under different therapies

Group	Treatment (duration)				n	Survival (hr)	Median (hr)	Mortality (%)	P (vs. group 1)	P (vs. group 6)
	K76 (mg/kg/dose)	TAC (mg/kg/day)	CP (mg/kg/day)	BM/SN[a]						
1	—	—	—	—	10	0.11, 0.12, 0.13, 0.13, 0.17, 0.18, 0.18, 0.18, 0.27, 0.27	0.17	0	—	<0.005
2	100×1	—	—	—	5	0.33, 0.8, 1.70, 5.5, 5.5[b]	1.70	20	<0.005	<0.01
3	200×1	—	—	—	10	4.66[b], 6.50, 8.40, 8.67, 8.80, 11.5, 18.70, 20.0, 24.3, 27.05	10.15	10	<0.0005	0.16
4	300×1	—	—	—	5	2.0[b], 3.13[b], 4.0[b], 13.47, 27.58	4.00	60	<0.005	0.1
5	400×1	—	—	—	3	1.50[b], 5.90[b], 15.3[b]	5.90	100	<0.005	0.07
6	200×2[c]	—	—	—	6	10.0[b], 13.5[b], 16.0[b], 20.17, 20.55[b], 28.9[b]	18.09	83	<0.005	—
7	—	1.0 (d −10~)	10 (d −10~)	—	4	0.18, 0.19, 0.29, 0.68	0.24	0	0.04	0.01
8	200×2[c]	1.0 (d −10~)	7.5 (d −10~)	—	6	21.5[b], 23.0, 24.0, 24.0[b], 25.33[b], 35.5[b]	24.00	67	<0.005	0.04
9	200×2[c]	1.0 (d −10~)	—	—	6	3.33[b], 8.58[b], 9.92[b], 16.5[b], 23.5, 31.0	13.21	67	<0.005	0.52
10	200×2[c]	—	10 (d −10~)	—	4	8.42[b], 13.17, 31.33, 33.57	22.25	25	<0.005	0.47
11	200×2[c]	1.0 (d −10~)	10 (d −10~)	BM (d-10)	4	21.25, 23.25, 26.00[b], 26.25[b]	24.63	50	<0.005	0.08
12	200×2[c]	1.0 (d −10~)	—	BM (d-10)	4	4.0[b], 4.58[b], 11.0, 12.08	7.79	50	<0.005	0.03
13	200×2[c]	1.0 (d −10~)	10 (d −10~)	SN (d-10)	7	11.75[b], 12.38[b], 23.17[b], 24.25, 26.75, 28.33, 30.25[b]	24.25	57	<0.001	0.32

[a] BM, guinea pig bone marrow transfusion; SN, splenectomy.
[b] Animal died with beating heart graft.
[c] K76 was given 30 min before graft revascularization and every 9–12 hr after transplantation.

Statistical Analysis

The results of complement studies, K76 plasma level, PT, APTT, and fibrinogen levels were expressed as mean ± SD. The Mann-Whitney *U* test was used for statistical analysis of xenograft survival and $P<0.01$ was considered significant.

RESULTS

Survival

Guinea pig→rat transplantation. Median heart survival time was increased 10-fold (from 0.17 to 1.7 hr) with a single prerevascularization dose of K76, 60 times (to 10.15 hr) with one dose of 200 mg, and 106-fold (to 18.1 hr) when a repeat dose of the drug was given 9–12 hr after transplantation (Table 1, group 6). Single doses of 300 mg/kg and 400 mg/kg were also highly protective (up to 27.6 hr). However, most of the rats with long xenograft survival times after treatment with a single high or double 200-mg/kg doses of K76 had respiratory arrest, which terminated 11 of the 14 experiments in groups 4–6 by death while the heart was still beating (Table 1). The dose of 200 mg/kg every 9–12 hr (group 6) was selected as the standard for combination with other therapeutic modalities.

K76 effectiveness was the principal determinant of graft survival, no matter what therapeutic variable—TAC, CP, the two drugs together, donor species bone marrow, or recipient splenectomy—was added (Table 1, groups 8–13). Although a 10-day preoperative course of TAC plus CP (group 7, Table 1) increased median heart survival time from 0.17 to 0.24 hr ($P=0.04$), the addition of optimal-dose K76 to this regimen (group 8) resulted in only a marginal prolongation of graft survival compared with that with K76 alone (24 hr vs. 18 hr, $P=0.04$). The results were essentially the same when the rat recipients were also splenectomized ($P=0.32$) or primed with guinea pig bone marrow on day −10 ($P=0.08$).

None of the guinea pig xenografts were normal (see below). The high mortality (>50%) of dose-effective K76, whether used alone or with other treatment, was almost always due to respiratory failure. The lungs of these animals were architecturally intact, except for evidence of smooth muscle contraction and a characteristic neutrophil infiltration beneath bronchial veins. However, mast cell degranulation was not prominent in the infiltrate.

Hamster→rat transplantation. K76 in doses of 200 mg/kg beginning 30 min before revascularization and every 12 hr thereafter increased median heart survival from 3 to 4 days (Table 2, group 2). The effect was similar to monotherapy with TAC (group 3) or CP (group 4). As reported previously (*13*), combining TAC with subtherapeutic doses of CP increased survival to 5 days (group 5, Table 2). The effect of additional K76 was assessed by comparison with group 5.

Significant prolongation of the survival beyond that ob-

TABLE 2. Heterotopic heart and recipient survival after hamster → rat xenotransplantation with K76, tacrolimus, and cyclophosphamide

Group	Treatment[a] (duration)			n	Survival (days)	Median (days)	Mortality (%)	P (vs. group 5)
	K76 (mg/kg/day)	Induction TAC[b] (mg/kg/day)	CP (mg/kg/day)					
1	—	—	—	6	3, 3, 3, 3, 3, 3	3.0	0	<0.005
2	200×2 (d 0 to 6)	—	—	6	4, 4, 4, 4, 5, 5	4.0	0	0.02
3	—	2.0 (d 0 to 13)	—	6	4, 4, 4, 4, 5, 5	4.0	0	0.02
4	—	—	5 (d-1 to 13)	3	4, 4, 5	4.0	0	0.06
5	—	2.0 (d 0 to 13)	5 (d-1 to 13)	9	5, 5, 5, 5, 5, 5, 5, 6, 6	5.0	0	—
6	200×2 (d 0 to 6)	2.0 (d 0 to 13)	5 (d-1 to 13)	6	6, 24[c], 26[c], 27[c], 32[c], 41[c]	26.5	83	<0.005
7	200×2 (d 0 to 6)	2.0 (d 0 to 5)	5 (d-1 to 7)	7	19, 28, 60, 61[c], 68, 89[c], >100	61.0	28	<0.001
8	200×2 (d 3 to 8)	2.0 (d 0 to 13)	5 (d-1 to 13)	6	18[c], 20[c], 20[c], 23[c], 30[c], 32[c]	21.5	100	<0.005
9	200×2 (d 3 to 8)	2.0 (d 0 to 5)	5 (d-1 to 7)	8	9[c], 20, 27, 34, 46[c], 54, 61[c], >100	40.0	37	<0.001
10	200×2 (d 3 to 6)	2.0 (d 0 to 13)	5 (d-1 to 13)	4	27[c], 30, 30, 31[c]	30.0	50	<0.01
11	200×2 (d 0 to 4)	2.0 (d 0 to 13)	5 (d-1 to 13)	5	7, 7, 7, 7, 31	7.0	0	<0.005
12	100×2 (d 0 to 6)	2.0 (d 0 to 13)	5 (d-1 to 13)	4	5, 5, 6, 9	5.5	0	0.35
13	200×2 (d 0 to 6)	2.0 (d 0 to 13)	—	4	4, 4, 5, 5	4.5	0	0.09
14	200×2 (d 0 to 6)	—	5 (d-1 to 7)	3	7, 7, 7	7.0	0	0.01
15	200×2 (d 0 to 6)	2.0 (d 0 to 13)	2.5 (d-1 to 13)	6	4, 5, 5, 5, 6, 26	5.0	0	0.94
16	200×2 (d 0 to 6)	2.0 (d 0 to 5)	10 (d-1 to 7)	5	100[c], >100×4	>100	20	<0.005

[a] K76 (200 mg/kg) was injected intravenously 30 min before revascularization and continued every 12 hr for days indicated. Cyclophosphamide was given only on days indicated.

[b] Tacrolimus was continued at daily doses of 1.0 mg/kg (day 6 or 14 to 30) and 0.5 mg/kg (day 31 to 100, every other day).

[c] Animal died with beating heart graft.

tained in group 5 was observed with the addition to TAC/CP of a 6-day course of 200 mg of K76 every 12 hr beginning on day 0 (groups 6 and 7) or postoperative day 3 (groups 8 and 9) and by giving a 4-day K76 course of the same daily doses beginning on postoperative day 3 (group 10). The effect of a short course of K76 was largely lost when this was stopped at day 4 (group 11), and the effect of a full-duration course was lost when this was given at half doses daily (group 12). The best median survival time of 61 days (group 7, Table 2) was with a 6-day full-dose course of K76, a reduced dose of TAC during induction, and an abbreviated course of CP. The highest mortality was associated with the most effective regimens for the prevention of rejection; 11 of the 12 rats in groups 6 and 8 died with beating grafts after 18–41 days (mean, 27 days), with infection as the usual postmortem diagnosis. Although a lower mortality (0–50%) was achieved in the experiments of groups 7, 9, 10, and 12 by reducing the cumulative exposure to TAC (groups 7 and 9), CP (groups 7 and 9), or K76 (groups 10 and 12), the tradeoff was rejection within 7–68 days of 14 of the 16 xenografts borne by animals who survived to the end of the experiment.

The dependence of a K76 effect upon the baseline TAC/CP treatment was demonstrated in further experiments. Omission of either CP (group 13, Table 2) or TAC (group 14) eliminated most or all, respectively, of the K76 efficacy. Similarly, reducing CP dosage by half in combination with full TAC plus K76 dosing reduced heart graft survival time almost to that of K76 monotherapy (group 15). In contrast, doubling the CP doses (to 10 mg/kg per day) allowed consistent animal and graft survival time >100 days (group 16, Table 2). These results in group 16 were the same as reported before the same TAC/CP regimen without K76 was used (*13*).

Nonsurvival Studies

Normal Lewis rats. One hour after an intravenous bolus injection of 200 mg/kg, K76 plasma levels were 650 μg/ml. Levels decreased rapidly during the next 4 hr, but trace concentrations remained at 12.5 hr (Fig. 1A). During the first 3.5 hr when plasma K76 levels were maintained >160 μg/ml, C3 level, ACH_{50}, and CH_{50} were suppressed to less than 44%, 18%, and 76%, respectively, of initial values (Fig. 1B). Although CH_{50} returned to normal by 6 hr, ACH_{50} and C3 activity remained depressed to 60% of control at 12 hr. PT and APTT were nearly doubled 2 hr after injection, but fibrinogen levels were not significantly changed (Fig. 1C).

After guinea pig→rat transplantation. Animals pretreated for 10 days with TAC and CP (as in group 8, Table 1) were given 200 mg/kg K76 30 min before and 9.5 and 22.5 hr after heart revascularization. The average K76 plasma concentration was 890 μg/ml when the xenograft blood supply was restored and was maintained between 100 and 300 μg/ml throughout the next day (Fig. 2A), during which time CH_{50}, ACH_{50}, and C3 activities were suppressed to <50% of pretransplant values (Fig. 2B).

However, histopathological examination of the still-functioning grafts 6 hr after transplantation showed evidence of focal hemorrhagic myocardial necrosis and varying degrees of periarterial hemorrhage (Fig. 3). These changes were minimal for the first 6 hr but slowly increased with time. Heart grafts that were still beating 20 hr after transplantation had massive hemorrhagic necrosis with coronary thrombosis (Fig. 3).

After hamster→rat transplantation. Animals pretreated with CP for 1 day and started on TAC perioperatively were given 200 mg/kg K76 30 min before xenograft revascularize-

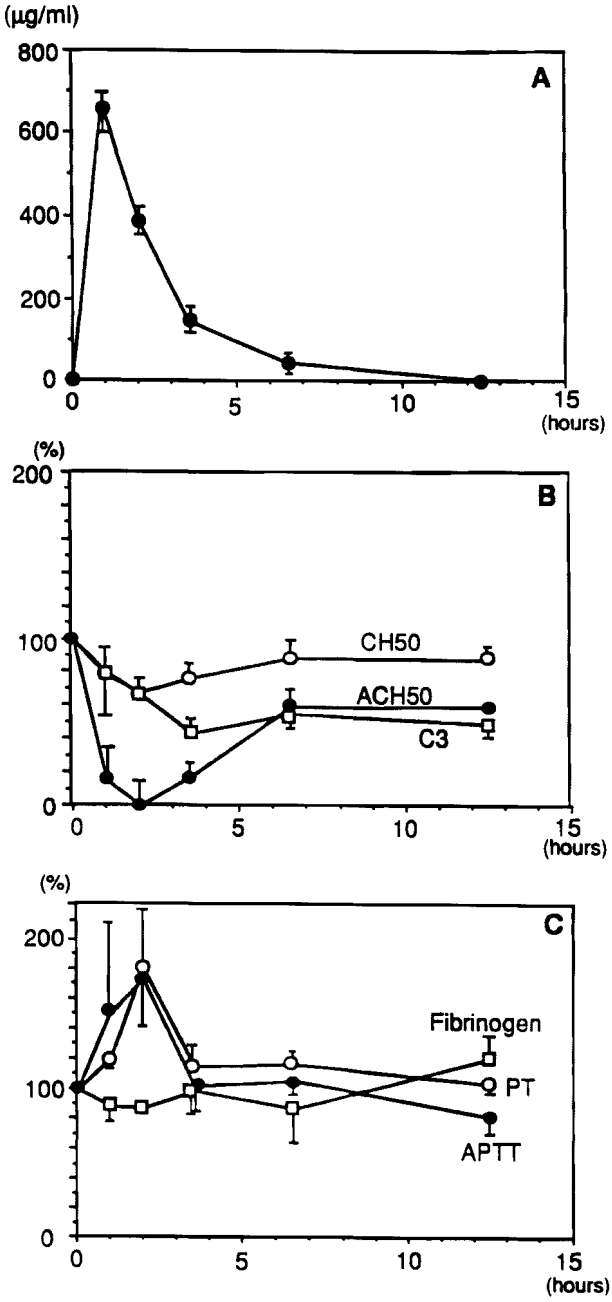

FIGURE 1. Effect of bolus K76 injection (200 mg/kg) in normal Lewis rats (n=2). The values in panels B and C are expressed as percentages of initial value in normal Lewis rats. (A) Plasma K-76 level; (B) complement activity; (C) PT, APTT, and fibrinogen levels.

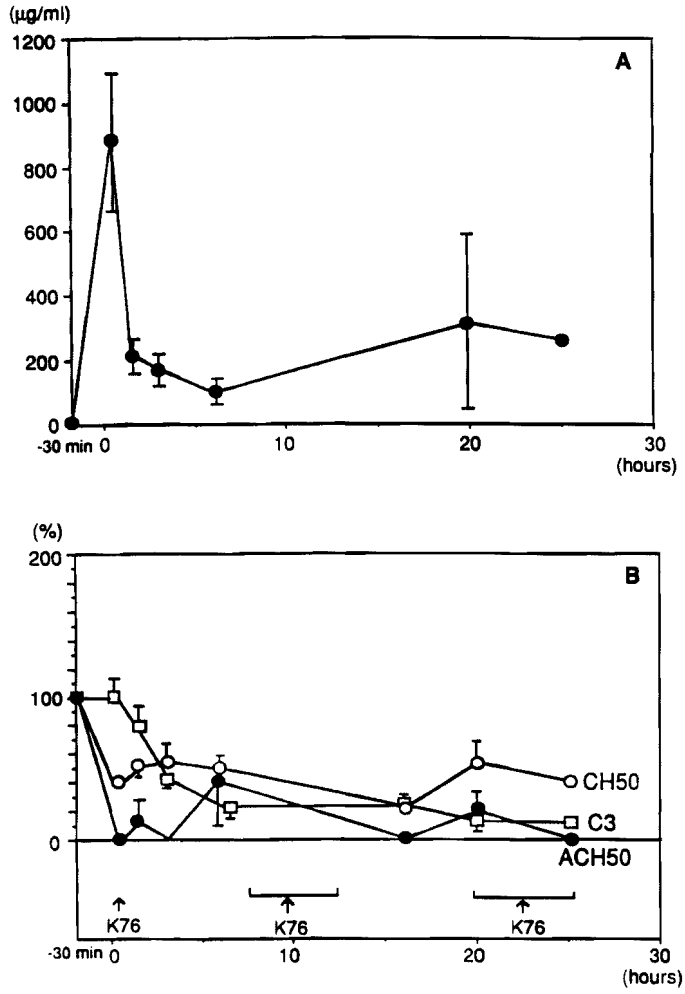

FIGURE 2. Effect of K76 in recipients of guinea pig hearts treated with K76, TAC, and CP as in group 8 (Table 1, n=1-3). K76 (200 mg/kg) was given 30 min before revascularization and repeated every 9–12 hr after grafting. TAC (1.0 mg/kg per day) and CP (7.5 mg/kg per day) were given for 10 days before operation. The values in panel B are expressed as percentages of normal Lewis rats. (A) Plasma K76 level after transplantation; (B) CH_{50}, ACH_{50}, and C3 levels.

tion and every 12 hr thereafter (as in group 7, Table 2). K76 plasma levels were maintained between 50 and 400 μg/ml. For 5 days after transplantation, antihamster lymphocytotoxic antibody increased from the initial level of 2^5 to 2^8, an elevation similar to that in untreated animals (data not shown) and in animals of group 5 (Table 2) treated with only CP and TAC (Fig. 4A). The titers in the K76-treated animals whose heart grafts still beat receded subsequently and plateaued at a higher level than before operation throughout the rest of the month (Fig. 4A). CH_{50}, ACH_{50}, and C3 levels were markedly suppressed during the 6 days of K76 treatment (Fig. 4B). The hearts had histopathologic findings of rejection similar to those reported previously (*14*).

DISCUSSION

The therapeutic implication of inhibiting or depleting complement has been a recurring theme of transplantation research since the HAR syndromes were recognized in allograft recipients who had preformed antigraft antibodies (*15–17*), the same events were described in discordant xenotransplant models (*18*), and Gewurz et al. (*19*) demonstrated that the HAR of organ allografts and xenografts was a complement activation syndrome. Sporadic clinical reports in which antigraft antibodies could not be detected (*20*) showed that the complement activation of HAR could occur by the alternative as opposed to the classical antibody-initiated pathway. The analogy to the Shwartzman reaction (*21, 22*) and recognition that vulnerability of the graft microvasculature is the critical pathogenetic feature were also well-known features of HAR more than 25 years ago (*22*).

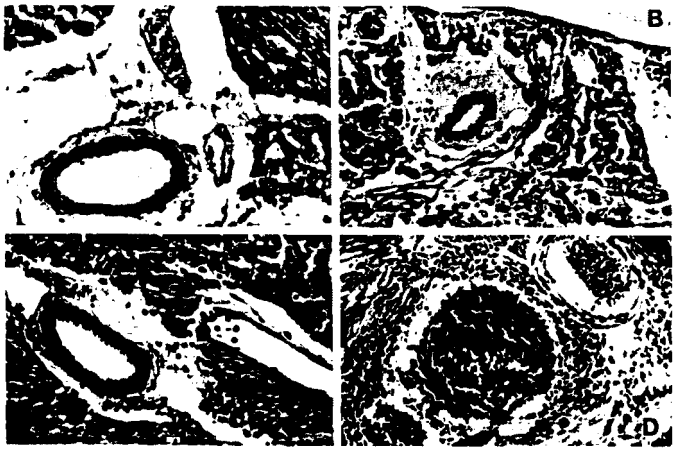

FIGURE 3. Guinea pig heart grafts in recipients treated with K76, TAC, and CP as group 8 (Table 1). (A) At 1.5 hr after revascularization, slight endothelial degeneration and neutrophil aggregation with perivascular edema were seen without any remarkable changes in cardiac parenchyma. (B) Three hours after revascularization, similar changes were seen 1.5 hr after transplantation, with evident neutrophil infiltration in the perivascular area. Parenchyma remained relatively normal. (C) By 6 hr after revascularization, moderate endothelial edema and mild vascular smooth muscle degeneration had occurred. A subpopulation of cardiac muscle showed mild to moderate degeneration with slight congestion. (D) At 20 hr after transplantation, arteries and veins had severe vascular damage, with fibrin thrombus and congestion. Marked degeneration and necrosis with congestion and hemorrhage were seen in parenchyma without inflammatory cell infiltration (hematoxylin and eosin, ×100).

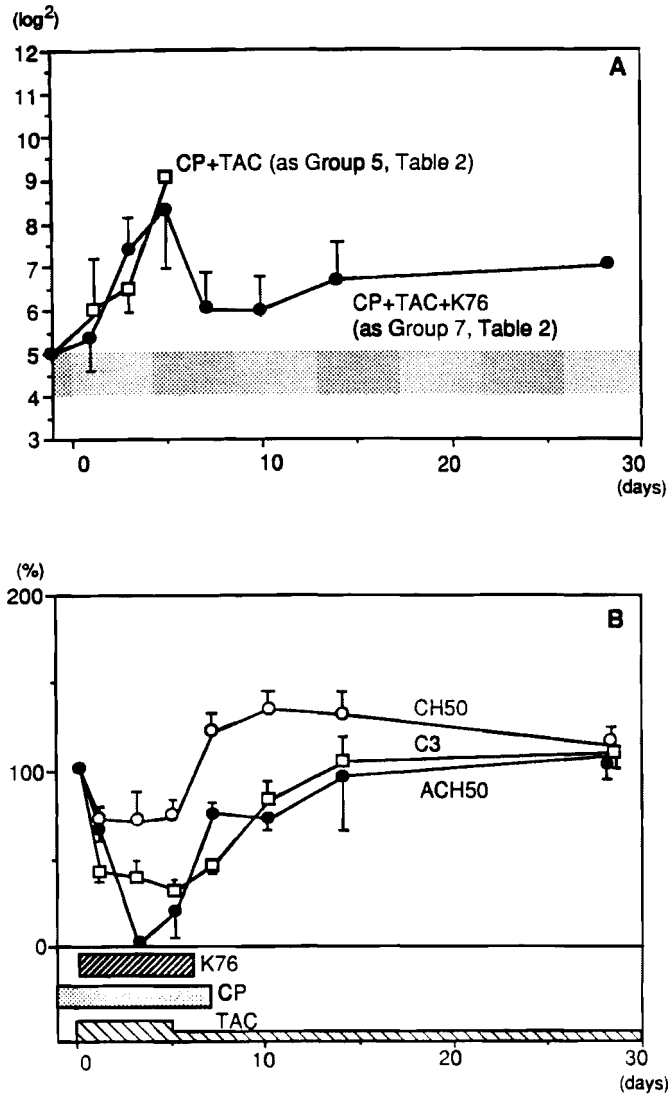

FIGURE 4. Effect of K76 in recipients of hamster hearts treated with K76, TAC, and CP as group 7 (Table 2, n=3). K76 (200 mg/kg) was injected intravenously 30 min before revascularization and continued every 12 hr for 6 days (days 0–6). TAC was injected intramuscularly at 2.0 mg/kg per day on days 0–5, 1.0 mg/kg per day on days 6–30, and 0.5 mg/kg per day every other day thereafter. CP was given orally at a daily dose of 5.0 mg/kg for 9 days (days −1 to 7). The values are expressed as percentages of normal Lewis rats. (A) Anti-hamster lymphocytotoxic antibody titer using CDCC assay (□, antibody levels in normal LEW rats); (B) CH_{50}, ACH_{50}, and C3 levels.

Complement inhibition to control HAR was first attempted with crude cobra venom (*19*) only slightly less successfully than with the more controllable and less toxic cobra venom fraction (CVF), which causes profound depletion of C3, factor B, and terminal components of the membrane attack complex (*23*, *24*). Two other relatively nontoxic drugs allow interdiction of the complement cascade at levels that block the alternative as well as the classical pathway: soluble complement receptor type 1 (*25–27*), which acts as C3 and C5 convertase, and the sesquiterpene compound K76 of the present study. Although these three "modern" agents dramatically extend whole organ survival in so-called discordant xenotransplant models, HAR promptly supervenes when treatment is stopped or develops slowly even when it is continued.

Evidence that K76 inhibits the C5 step of complement activation was obtained by exposing sensitized sheep red blood cells incubated with K76 to guinea pig complement at various stages of the complement activation cascade (*1*). However, a 15–25% step inhibition of C2 and C3 also occurs (*2*), but not enough to explain the lack of tissue C3 deposition in K76-treated rat recipients of hamster kidneys (*28*) and in a rabbit model of ulcerative colitis (*5*), or to explain the profound C3 depression reported herein. K76 may cause inappropriate C3-target cell binding, which could be the ultimate explanation for the failure of C5 activation (W. Miyazaki, unpublished data). Whatever the principal site of action, it is far enough along the complement cascade to suppress both the classical and alternative pathways, an important notation because both are thought to participate in the hyperacute xenograft rejection characteristic of the guinea pig→rat model (*29*, *30*). Failure of anti-μ antibodies to have a strong therapeutic effect in this difficult model (*30*) has been construed as evidence of the importance of alternative pathway activation.

The ability of K76 to delay guinea pig heart HAR by the rat was comparable to that of CVF (*31*, *32*) and soluble complement receptor type 1 (*25*, *26*). However, the price was a high mortality, roughly proportional to the duration of heart xenograft survival. In contrast to this "toxicity" in the guinea pig xenograft rat recipients, K76 was well tolerated in unaltered rats and caused no mortality when used twice daily for 6 days as monotherapy for rat recipients of hamster hearts. The greater than 50% mortality of rats bearing guinea pig

xenografts for long periods was usually due to respiratory arrest, which suggests that the continued perfusion of the xenografted heart was the lethal factor, possibly because of accumulation of anaphylatoxins.

Anaphylatoxins are associated with degranulation of mast cells and basophils, and release of mediators that induce neutrophil aggregation, smooth muscle contraction, and increased vascular permeability. The neutrophil infiltration beneath bronchial veins and the evidence of smooth muscle contractions in K76-treated recipients of guinea pig hearts were consistent with this pathogenesis of respiratory failure. Respiratory arrest has also been seen after guinea pig→rat heart transplantation when the xenograft survival was prolonged with the normally nontoxic anti-inflammatory drug lisofylline (unpublished observations).

The high death rate of rat recipients whose guinea pig hearts were still beating made it impossible to establish with certainty whether adjuvant therapy with TAC and/or CP augmented the dramatic effect of K76 on xenograft survival. With the easier hamster heart→rat model, this was feasible, but only under restrictive experimental conditions. When K76 was given every 12 hr as the sole treatment for as long as the transplanted heart beat, median hamster xenograft survival time was increased from 3 to 4 days ($P=0.02$), the same prolongation as with monotherapy using daily therapeutic doses of TAC or suboptimal doses of CP. When used together, the three agents were synergistic, with loss of effectiveness if any one of the three agents was omitted, if the already suboptimal CP dose was reduced further, or if K76 administration was stopped short of the posttransplant days 5 and 6, during which time the development of xenogeneic antibodies characteristic of this model reaches a peak. The median survival time in the best combined treatment group was 61 days, only slightly better than the 49 days reported by Van den Bogaerde et al. (*33*) using cyclosporine in combination with CVF. Because the half-life of an intravenous bolus of K76 is less than 6 hr compared with the 30 hr of CVF (*24*), maintenance of stable blood levels was undoubtedly less even.

However, the results with the best K76-containing triple-agent regimen were distinctly inferior to what has been reported with optimal dosing of TAC or cyclosporine combined with CP (*13*, *34*) or other antimetabolite drugs (*13*) with which 100% hamster heart survival for 100 days in rat recipients can be routinely obtained. The high mortality of the K76-containing regimens stemmed largely from infection, including the frequent presence of microabscesses in the liver. However, the infections were not attributable per se to the K76, but rather to inadequate control of rejection, which was almost universal. The role of inadequate control of rejection in causing infection was demonstrated by negative example in animals of group 16 (Table 2) given optimal TAC/CP treatment to which K76 was added. All of these rats survived with beating hearts for 100 days, as described previously using the same doses of TAC/CP alone.

In conclusion, K76 is a powerful inhibitor of complement activation and appears to interrupt both the classical and alternative activation cascades. It dramatically delays the hyperacute rejection of discordant xenografts and can also augment minimally effective TAC/CP regimens in concordant models. However, efficient complement inhibition appeared to be a relatively fruitless strategy for both the discordant and concordant xenotransplantation models used in our experiments. After guinea pig→rat transplantation, the prolongation of xenograft survival correlated with a high mortality that was associated more with the continued presence of the xenograft in the circulation than with use of the anticomplement agent. With hamster→rat transplantation, the results when K76 was combined with TAC and low-dose CP were no better than with appropriate doses of TAC and CP only.

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Received 24 January 1996.
Accepted 16 April 1996.