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HAMSTER-TO-RAT HEART AND LIVER XENOTRANSPLANTATION WITH FK506 PLUS ANTIPROLIFERATIVE DRUGS[1,2]

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Heterotopic hamster hearts transplanted to unmodified LEW rats underwent humoral rejection in 3 days. Survival was prolonged to a median of 4 days with 2 mg/kg/day FK506. As monotherapy, 15 mg/kg/day cyclophosphamide greatly prolonged graft survival—far more than could be accomplished with RS-61443, brequinar (BQR), mizoribine, methotrexate, or deoxyspergualin. However, when FK506 treatment, which was ineffective alone, was combined with a short induction course (14 or 30 days) of subtherapeutic BQR, RS-61443, or cyclophosphamide, routine survival of heart xenografts was possible for as long as the daily FK506 was continued. In addition, a single large dose of 80 mg/kg cyclophosphamide 10 days preoperatively allowed routine cardiac xenograft survival under FK506. The ability of these antimetabolites to unmask the therapeutic potential of FK506 correlated, although imperfectly, with the prevention of rises of preformed heterospecific cytotoxic antibodies immediately postoperatively. As an adjunct to FK506, azathioprine was of marginal value, whereas mizoribine, methotrexate, and deoxyspergualin (DSPG) were of intermediate efficacy.

After orthotopic hepatic xenotransplantation, the perioperative survival of the liver with its well-known

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resistance to antibodies was less dependent than the heart on the antimetabolite component of the combined drug therapy, but the unsatisfactory results with monotherapy of FK506, BQR, RS-61443, or cyclophosphamide were changed to routine success by combining continuous FK506 with a short course of any of the other drugs. Thus, by breaking down the antibody barrier to xenotransplantation with these so-called antiproliferative drugs, it has been possible with FK506 to transplant heart and liver xenografts with consistent long-term survival of healthy recipients.

FK506, which prevents T cell activation and cytokine secretion by inhibiting the transcription of early genes (*1*), has permitted improvements in the clinical transplantation of a variety of allografts (*2, 3*). However, the drug has a minimal effect on B cells and the antibody response, and does not prevent humoral allograft rejection in the presensitized recipient (*4, 5*) or the rejection of xenografts by heterospecific antibodies (*6, 7*).

In a hamster-to-rat xenograft model, we have combined FK506 with drugs that subvert the action of key enzymes of de novo purine and pyrimidine nucleotide synthesis, thereby inhibiting the DNA synthesis required for expansion of activated T and/or B cell clones. Brequinar (BQR),* an agent the immunosuppressive qualities of which were studied in rats by Cramer et al. (*8*) and RS-61443, a mycophenolic acid derivative showing considerable preclinical and clinical promise (*9–12*), where the new prototype drugs selected. However toward the end of the study, established anti-DNA drugs also were evaluated as adjuvants, including azathioprine, cyclophosphamide, and methotrexate; of these cyclophosphamide, was found to be spectacularly effective. Finally, FK506 was combined with deoxyspergualin (DSPG), a putative antimacrophage/monocyte drug that also has been said to suppress B cell activation and maturation (*13*).

MATERIALS AND METHODS

Animals. Inbred male Lewis rats (LEW, RT1^l) weighing 200–300 g were recipients, and Golden Syrian hamsters weighing 100–150 g were donors (Charles River Lab., Wilmington, MA).

Surgical procedure. The hamster-to-rat xenograft models were those characterized previously by Valdivia and Monden (*14–17*). Operations were under methoxyflurane anesthesia. Heterotopic heart transplantation was performed by anastomosing the donor aorta and pulmonary artery of the xenograft to the recipient infrarenal abdominal aorta and vena cava, respectively. The cardiac grafts were palpated daily for the first month and every other day thereafter. Rejection was diagnosed by the cessation of the heartbeat, and confirmed by direct inspection at reoperation and by histopathology. There was no discard rate of failed experiments.

Liver transplantation after graft cholecystectomy was performed with Kamada's cuff technique for the portal and infrahepatic vena cava anastomoses, revascularizing the portal vein only (*14–17*). Rejection was diagnosed by the death of the recipients, followed by histopathological examination. Animals dying <3 days after surgery were excluded from the study (less than 5% of the total). This small rate of discard was from technical misadventures.

Immunosuppressive agents. FK506: FK506 (a gift of the Fujisawa Pharmaceutical Co., Osaka, Japan) was given i.m. after suspending it in normal saline. The dose for heart recipients was 2.0 mg/kg/day on days 0 to 5 followed by 1.0 mg/kg/day on days 6 to 30. For liver xenotransplantation, 1.0 mg/kg/day was used for the first 30 days. Both liver and heart recipients were given alternate-day injections of 0.5 mg/kg FK506 from days 31 to 100.

Antiproliferative drugs: All of these drugs were prepared daily and administered by gastric instillation. BQR (donated by Du Pont Medical Products, Wilmington, DE) was suspended in distilled water, and adjusted with NaOH to pH 9.0. RS-61443 (donated by Syntex Inc, Palo Alto, CA) was used in a special vehicle that contained 0.5% carboxymethyl-cellulose, 0.4% polysorbate 80, 0.9% benzyl alcohol, and 0.9% sodium chloride in distilled water. Mizoribine (Bredinin, donated by Asahi Chemical Industry Co. Ltd., Tokyo, Japan) was suspended in distilled water. Azathioprine, cyclophosphamide, and methotrexate were bought from commercial pharmacies and suspended in distilled water.

Deoxyspergualine: This gift from Nippon Kayaku Co. Ltd, Tokyo, (later Bristol-Myers Squibb) was suspended in sterile water and administered intramuscularly.

Recipient humoral antibodies. Lymphocytotoxic antibodies: Complement-fixing lymphocytotoxic antibodies were measured in the recipient rat sera by Terasaki's method (*18*), using target lymphocytes prepared from hamster cervical lymph nodes. After washing and isolation, the cells were resuspended at a concentration of 4×10^6/ml. Duplicate samples of 1 μl of various dilutions of serum samples and one μl of lymphocyte suspensions were placed into 72-well tissue-typing trays (Robbins Scientific, Sunnyvale, CA). After incubation for 60 min at room temperature, 5 μl of baby rabbit complement diluted 10 times (Cedarlane Laboratories Limited, Hornby, Ontario) was added to each well with reincubation for another 30 min at room temperature. Then 5 μl of 0.4% trypan blue and 15 μl of barbital buffer were added to each well for staining and fixation. The cell lysis ranged from 0 (undetectable) to 100%. The lymphocytotoxic antibody titer was defined as the highest serum dilution with more than 51% cell lysis. Normal hamster serum served as a negative control.

Indirect immunofluorescence: Snap-frozen normal hamster liver was the target tissue. It was cut into 2-μ sections, incubated with a protein blocking agent (Lipshaw, Pittsburgh, PA), and then incubated again with sera obtained from normal LEW rats (to detect preformed antibodies) or LEW recipients of hamster organs (to detect the antibody response). This was followed by goat antirat IgG or IgM to detect the localization, class, and intensity of the heterospecific antibodies. The location and intensity of the immunoglobulin deposits were determined without knowledge of the treatment regimen.

Statistical analyses. The graft survival was so predictable in unmodified rat recipients (3 days for hearts, 7 days for livers) that all survival extension beyond one day was statistically significant with the Mann-Whitney *U* test. Median survival figures were calculated but survival of individual animals was given in all experimental groups.

RESULTS

Heart xenograft survival. Single drug therapy: When used as a single agent in a dose of 2 mg/kg/day, FK506 increased graft survival by only one day, significantly less prolongation than could be accomplished with BQR, RS-61443, cyclophosphamide, or methotrexate (Table 1). The therapeutic window of BQR was narrow, the drug being almost ineffective at 3 mg/kg/day and clinically toxic if continued at 4.5 mg. The optimal dosing proved to be 4.0 mg/kg/day for 3 days and 3 mg/kg/day thereafter. The dose latitude with RS-61443 appeared to be somewhat greater (Table 1).

Cyclophosphamide was the only drug that permitted consistently successful heart transplantation. All of the animals given 10 or 15 mg/kg/day cyclophosphamide are alive. Although follow-up is short because the experiments were done last, the superiority of cyclophosphamide to any other single agent was evident (Table 1).

Combined therapy: Added to baseline therapy of 2 mg/kg/day FK506 that was ineffectual alone, BQR and RS-61443 both

* Abbreviations: BQR, brequinar; DSPG, deoxyspergualin.

TABLE 1. Hamster heart graft survival in LEW rat recipients treated with FK506, BQR, RS-61443, cyclophosphamide, methotrexate, mizoribine, and deoxyspergualin as single agents[a]

Group	Agent	Dose (mg/kg/day)	Duration (days)	n	Survival (days)	Median survival (days)
1	—	—	—	6	3, 3, 3, 3, 3, 3	3.0
2	FK506	2.0	0 →	6	4, 4, 4, 4, 5, 5	4.0
3	BQR	3.0	−1 →	4	5, 5, 6, 7	5.5
4	BQR	4.0 × 3 → 3.0	−1 →	6	5, 8, 8, 11, 12, >100	9.5
5	BQR	4.5	−1 →	4	7,[b] 9,[b] >75, >75	>42.0
6	RS-61443	20	−1 →	4	4, 5, 5, 6	5.0
7	RS-61443	40	−1 →	4	6, 7, 7, 8	7.0
8	RS-61443	60 × 3 → 40	−1 →	6	6, 8,[b] 9, 9, 20, 23	9.0
9	RS-61443	60	−1 →	4	9, 10, 13, 15	11.5
10	Cyclophosphamide	7.5	−1 →	5	7, 8, 8, 8, 9	8.0
11	Cyclophosphamide	10.0	−1 →	5	14, >15, >15, >15, >15	>15.0
12	Cyclophosphamide	15.0	−1 →	5	>28, >28, >36, >36, >36	>36.0
13	Methotrexate	0.5	−1 →	5	10, 11,[b] 13,[b] 14,[b] >16	13.0
14	Mizoribine	7.5	−1 →	5	4, 4, 4, 4, 5	4.0
15	DSPG	5.0	−1 →	3	4, 4, 4	4.0

[a] BQR, RS-61443, Cyclophosphamide, Methotrexate, Mizoribine and Deoxyspergualin were administered from one day before transplantation and continued until graft rejection.

[b] Animal died with functioning graft.

permitted a spectacular improvement in heart xenograft survival. As when it was used alone, the BQR had a narrow therapeutic window with a single safe dose. The RS-61443 could be used over a wide dose range in combination with FK506 with virtually 100% assurance of long survival (Table 2, groups 7–10), whether it was given for 14 or 30 days.

Duration of follow-up with most of the other antimetabolite drugs has been shorter. However, the early success rate with cyclophosphamide induction 7 to 30 days with doses of 7.5, 10, or 15 mg/kg/day appeared to be at least equivalent to RS-61443 and BQR. Mizoribine was less effective and azathioprine had only a modest therapeutic effect when added in daily high doses to FK506, whereas mizoribine, methotrexate, and DSPG were of intermediate efficacy (Table 2). DSPG was the only drug tested as an adjuvant to FK506 that is not an antimetabolite. The animals given this drug developed alopecia and weight loss during its 30-day administration.

Cyclophosphamide pretreatment: The 5 rats given a single dose of 80 mg/kg cyclophosphamide 10 days preoperatively and then treated with the usual daily regimen of FK506 after transplantation have all accepted their cardiac xenografts, with follow-ups of 2 months (Table 2, group 31).

Liver xenograft survival. Single drug therapy: FK506 alone at 1 mg/kg/day increased survival 5-fold to 34.5 days, with 3 of 10 animals surviving beyond 60 days and one beyond 100 days (Table 3); the results were similar to those reported earlier by Valdivia et al. (*17*). BQR alone doubled median survival but only one of 7 animals lived beyond 19 days (Table 3). Results also were poor with RS-61443 and with the lowest dose of 7.5 mg/kg/day cyclophosphamide.

Combined therapy: When maintenance FK506 was combined with a 14-day course of 3 mg/kg/day BQR, the survival achievable with either drug alone was remarkably enhanced (Table 4) with 4 of 7 animals living for 100 days (group 1). Extending the daily BQR to 30 days (group 2) or giving it every other day from day 14 until 100 days (groups 3) caused a high delayed mortality. The animals appeared to have toxic reactions.

RS-61443 with FK506 also greatly augmented survival when given for 14 days in the 2 relatively low doses of 20 and 30 mg/kg, but the success rate was reduced, apparently because of toxicity, when the RS-61443 was continued for 30 days (Table 4, group 5).

Although the follow-up periods are shorter, the best results were with cyclophosphamide plus FK506 (Table 4, groups 8 and 9). Virtually every animal had long survival—whether cyclophosphamide was given at the daily dose of 7.5 mg/kg, which was ineffective as monotherapy, or at the higher dose of 10 mg.

Splenectomy: The efficacy of FK506 also appeared to be improved with splenectomy (Table 4, group 7), a finding similar to that previously reported with hamster-to-rat heart xenotransplantation under monotherapy with FK506 (*19*) or with liver xenotransplantation under cyclosporine (*16*).

Cyclophosphamide pretreatment: Nine of the 12 rats pretreated 10 days preoperatively with a single dose of 80 mg/kg cyclophosphamide have had long survival posttransplantation under FK506.

Antibody correlation. Under FK506 as single-drug treatment for cardiac xenotransplantation, the heterospecific lymphocytotoxic antibodies that were present pretransplantation had astronomic increases by 3 or 4 postoperative days, coinciding with the humoral rejection of the hearts. Thirty-day induction therapy with BQR and RS-61443 partially prevented this (Fig. 1). However, throughout the course until 100 days, low levels of xenospecific antibodies persisted without apparent harm (Fig. 1).

The antibody titers after liver xenotransplantation under FK506 alone rose 10 times higher than after heart transplantation alone, presumably because the livers survived long enough for the response to be complete (Fig. 2). The addition of BQR for a 14-day induction period blunted the antibody response more than RS-61443, but with both drugs the titer declined toward control levels after the 14-day course of the antiproliferative agents was stopped (Fig. 2).

Humoral antibodies. Indirect immunofluorescence staining of heart and liver xenografts showed that the antibody was principally IgM with light IgG deposition (Table 5). Liver recipients treated with FK506 only had considerable antibody concentration in vessels, but when antiproliferative drug treatment was added the staining was lighter and less blood-vessel-directed.

TABLE 2. Hamster heart graft survival in LEW recipients treated with combination treatment with FK506[a]

Group	Drug combined with FK506	Dose (mg/kg/day)	Duration (days)	n	Survival (days)	Median survival (days)
1	BQR	3.0	−1 → 30	6	4, 5, 5, 5, 7, >100	5.0
2	BQR	4.0 × 3 → 3.0	−1 → 13	5	>100, >100, >100, >100, >100	>100
3	BQR	4.0 × 3 → 3.0	−1 → 30	6	89, >100, >100, >100, >100, >100	>100
4[b]	BQR	4.0 × 3 → 3.0	−1 → 30	6	5, 22, 24, >100, >100, >100	>62.0
5	BQR	4.5	−1 → 30	4	3,[c] 9,[c] >75, >75	>42.0
6	RS-61443	10	−1 → 30	6	4, 5, 5, 5, >100, >100	5.0
7	RS-61443	20	−1 → 13	6	19, >78, >78, >78, >90, >90	>78.0
8	RS-61443	20	−1 → 30	6	>100, >100, >100, >100, >100, >100	>100.0
9	RS-61443	30	−1 → 30	6	>91, >91, >100, >100, >100, >100	>100.0
10	RS-61443	40	−1 → 30	6	>78, >92,[d] >92,[d] >92, >100, >100	>92.0
11	Cyclophosphamide	5	−1 → 30	5	5, 5, 5, 5, 6	5.0
12	Cyclophosphamide	7.5	−1 → 7	5	>35, >35, >35, >35, >35	>35.0
13	Cyclophosphamide	7.5	−1 → 13	5	>49, >49, >49, >50, >50	>49.0
14	Cyclophosphamide	7.5	−1 → 30	5	>64, >64, >64, >64, >64	>64.0
15	Cyclophosphamide	10	−1 → 13	6	>50, >60, >63, >63, >64, >64	>63.0
16	Cyclophosphamide	15	−1 → 9	4	29, >43, >47, >47	>45.0
17	Methotrexate	1.0	−1 → 13	3	7,[c] 7,[c] 9[c]	7.0
18	Methotrexate	1.0	−1 → 5	3	9,[c] 10,[c] >40	10.0
19	Methotrexate	1.0	−1 → 3	7	8, 10, 10, >19, >19, >49, >49	>19.0
20	Methotrexate	1.0	−1 → 1	2	8, 9	8.5
21	Methotrexate	0.5	−1 → 9	4	10,[c] 13,[c] 43, >49	28.0
22	Methotrexate	0.25	−1 → 13	3	4, 4, 6	4.0
23	Mizoribine	5.0	−1 → 30	3	5, 5, 7	5.0
24	Mizoribine	7.5 × 3 → 5.0	−1 → 30	6	3,[c] 5, 5, >77, >77, >100	>41.0
25	Azathioprine	15	−1 → 30	2	4, 5	4.5
26	Azathioprine	45	−1 → 30	3	5, 5, 23[c]	5.0
27	Azathioprine	60	−1 → 30	4	5, 8,[c] 17,[c] 19[c]	12.5
28	DSPG	2.5	−1 → 30	4	4, 5, 5, 5	5.0
29	DSPG	5.0 × 3 → 2.5	−1 → 30	6	23, 38, >70, >70, >70, >76	>70.0
30	DSPG	5.0	−1 → 30	4	9, 24,[c] 32,[c] >76	28.0
31	Cyclophosphamide	80.0 (i.p.)	−10	5	>71, >71, >71, >79, >79	>71.0

[a] FK506 was administered intramuscularly at a dose of 2.0 mg/kg/day for the first 6 days (days 0 to 5), then 1.0 mg/kg/day (days 6 to 30) and continued at 0.5 mg/kg/day every other day (days 31 to 100).

[b] FK506 was used at 1.0 mg/kg/day for the first 30 days (0 to 30) and continued at 0.5 mg/kg/day every other day (days 31 to 100).

[c] Animal died with functioning graft.

[d] RS-61443 treatment was discontinued 25 days after transplantation because of the appearance of diarrhea and body weight loss.

TABLE 3. Hamster liver graft survival in LEW rat recipients treated with FK506, BQR, RS-61443 and cyclophosphamide as single agents

Group	Agent	Dose (mg/kg/day)	Duration (days)	n	Survival (days)	Median survival (days)	1-month survival rate (%)	100-day survival rate (%)
1	—	—	—	8	6, 7, 7, 7, 7, 7, 7, 8	7.0	0	0
2	FK506	1.0	0–30[a]	10	7, 11, 18, 19, 21, 48, 58, 72, 94, >100	34.5	50 (5/10)	10 (1/10)
3	BQR	3.0	0–Death	7	7, 8, 9, 12, 17, 19, 74	12.0	14.3 (1/7)	0
4	RS-61443	20.0	0–Death	5	7, 7, 7, 7, 8	7.0	0	0
5	Cyclophosphamide	7.5	0–Death	5	7, 7, 9, 9, 9	9.0	0	0

[a] FK506 was used at a dose of 1.0 mg/kg/day for the first 30 days (0 to 30) and continued at 0.5 mg/kg/day every other day (days 31 to 100).

DISCUSSION

The moderately difficult hamster-to-rat model of xenotransplantation has been investigated extensively by Valdivia and Monden (*14–17*) and by others, including Knechtle et al. (*20*), who reported the mean survival of heterotopic hearts for >100 days with total-lymphoid irradiation plus cyclosporine. This accomplishment could not be duplicated by later workers (*21, 22*). Marchman et al. (*23*) reported that TLI plus DSPG increased heart xenograft survival to a mean of 26 days. By inhibiting complement fixation with snake venom in cyclosporine-treated rats, Van Den Bogaerde et al. (*24*) were able to increase heart graft survival to a mean of 50 days, with 2 of 10 animals living for 100 days. These last investigators emphasized the duality of the humoral and cellular mechanisms of xenograft rejection.

The special value of the hamster-to-rat xenograft models for the screening of immunosuppressive drug combinations was evident from our experiments. A hamster organ is confronted

TABLE 4. Hamster liver graft survival in LEW rat recipients treated with combination treatment with FK506[a]

Group	Agent	Dose (mg/kg/day)	Duration (days)	n	Survival (days)	Median survival (days)	1-month survival (%)
1	BQR	3.0	0–13	7	21, 42, 81, >100, >100, >100, >100	>100	85.7 (6/7)
2	BQR	3.0	0–30	7	13, 21, 23, 32, 36, 46, >100	32.0	57.1 (4/7)
3	BQR	3.0	0–13[b]	9	15, 15, 19, 30, 38, >100, >100, >100, >100	38.0	66.7 (6/9)
4	RS 61443	20	0–13	10	25, 83, >100, >100, >100, >100, >100, >100, >100, >100	>100.0	90.0 (9/10)
5	RS 61443	20	0–30	9	12, 22, 30, 36, 54, >100, >100, >100, >100	54.0	77.8 (7/9)
6	RS 61443	30	0–13	10	15, 15, 39, 76, >82, >84, >96, >97, >97, >98	>83.0	80.0 (8/10)
7	Splenectomy	—	—	6	9, 11, 18, 61, >100, >100	39.5	50.0 (3/6)
8	Cyclophosphamide	7.5	0 → 9	10	10, 31, >40, >45, >45, >45, >47, >48, >48, >51	>45.0	90.0 (9/10)
9	Cyclophosphamide	10.0	0 → 9	5	>32, >32, >32, >39, >39	>32.0	100.0 (5/5)
10	Cyclophosphamide	80.0 (i.p.)	−10 (once)	12	12, 13, 20, >46, >47, >47, >47, >47, >65, >66, >66, >66	>47.0	75.0 (9/12)

[a] FK506 was used at 1.0 mg/kg/day for the first 30 days (0 to 30) and continued at 0.5 mg/kg/day every other day (days 31 to 100).
[b] BQR was administered for the first 14 days and continued on alternate days from days 14 to 100 at a dose of 3.0 mg/kg/day.

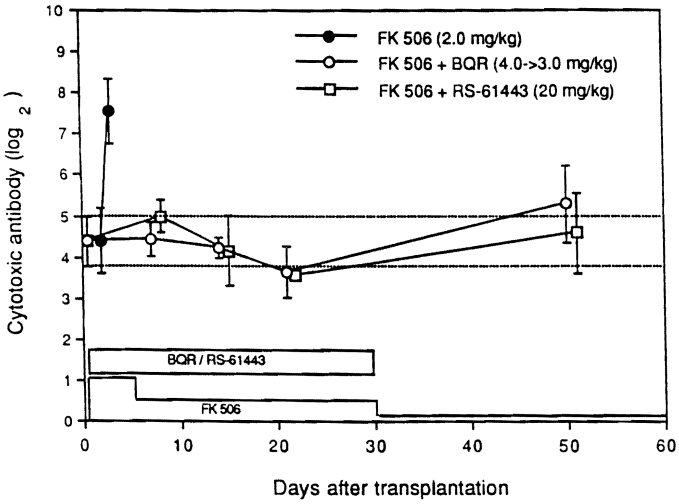

FIGURE 1. Antihamster lymphocytotoxic antibody after heart transplantation.

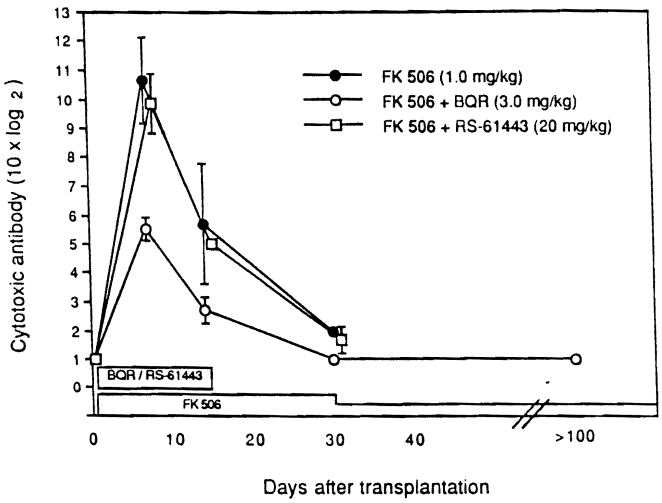

FIGURE 2. Antihamster lymphocytotoxic antibody response after liver transplantation. Note that the log 2 scale is ×10, to accommodate the far greater rise in titer compared with that in heart recipients (see Fig. 1).

in the rat by a moderate titer (1:16–1:32) of preformed heterospecific cytotoxic antibodies, and subsequently by a rapidly gathering antibody storm that destroys cardiac grafts within 3 days in untreated recipients, before there is a trace histopathologically of immunocyte infiltration. The liver, which is more resistant to antibody-mediated injury (*17, 25, 26*), survives this initial onslaught but cellular rejection becomes increasingly evident beyond 3 days until death of the unmodified host after 7 days from combined humoral and cellular rejection. Thus the use of these 2 organs for testing allows at the outset a stratification in untreated animals of the 2 distinctive mechanisms of humoral and cellular graft rejection, and makes it clear that the mitigation or interdiction of either alone will not consistently permit long-term graft survival.

This generalization was verified with testing of all of the individual drugs that contributed to our most effective cocktails. Although FK506 and the antiproliferative drugs used most extensively (RS-61443 and BQR) significantly improved heart and liver xenograft survival when used alone, they did not permit consistent long-term survival. Survival became rou-

TABLE 5. Hepatic specificity of xenoantibodies in sera of liver recipients by indirect immunofluorescence[a]

Treatment	7–8 Days after transplantation		30–40 Days after transplantation	
	Sinusoids	Large vessel endothelium	Sinusoids	Large vessel endothelium
FK506 1.0 mg/kg	+++	+++	±	±
	++	++	±	±
FK506 1.0 mg/kg + RS-61443 20 mg/kg (0–13)	++	++	±	±
	++	++	±	±
FK506 1.0 mg/kg + BQR 3.0 mg/kg (0–13)	+	+	±	±
	+	+	±	±

[a] Immunofluorescent intensity was scored on a qualitative scale from 0 to 3+; values were shown as IgM deposits.

tinely possible when continuous FK506 was combined with a shout course of BQR, which inhibits de novo pyrimidine synthesis by blocking the enzyme dihydroorotate dehydrogenase (*27*), or of RS-61443, which (like mizoribine) inhibits de novo purine synthesis by blocking enzyme inosine monophosphate dehydrogenase (*28*).

Although less-extensive data were acquired with mizoribine and azathioprine, these antiproliferative agents appeared less capable of augmenting FK506 efficiency. In contrast, cyclophosphamide, another purine antimetabolite with considerable B cell specificity (*29*) was unexpectedly effective as monotherapy in prolonging heart xenograft survival and in allowing the routine acceptance of heart xenografts in FK506-treated recipients when the cyclophosphamide was given as a single large dose 10 days before transplantation or in a 9-day course postoperatively in smaller doses. The ability to effectively use cyclophosphamide for daily dosing in the xenograft model has direct clinical implications for clinical xenograft trials because this drug has been shown in extensive clinical trials of renal and liver transplantation to be as safe and effective as azathioprine when used as the baseline drug for chronic therapy (*30–32*). Azathioprine had low efficacy in the hamster-to-rat heart model, and methotrexate and mizoribine were in between.

DSPG, the synthetically hydroxylated fermentation product of *Bacillus lactobacillus*, the mechanism of action of which is not as well understood, appeared capable of filling the same handmaiden role to FK506 as the antimetabolite drugs, but the animals lost weight, developed alopecia, and appeared systemically toxic when effective doses were used. DSPG has been described as ameliorating preformed antibody states, including those in xenotransplantation (*13, 23*).

After orthotopic hepatic xenotransplantation, the perioperative survival of the liver with its well-known resistance to antibodies was less dependent than the heart on the antimetabolite component of the combined drug therapy—and, in contrast to the heart xenograft experiments, cyclophosphamide as monotherapy had little value for hamster-to-rat liver xenotransplantation—seemingly less than BQR or RS-61443. However, when any of these 3 agents was used in a brief induction course for continuous treatment with FK506, the results were converted from unpredictable and unacceptable to almost invariably successful.

Thus, by breaking down the antibody barrier to xenotransplantation with several of these so-called antiproliferative drugs as well as with DSPG, it has been possible with FK506 to transplant hearts and livers in the moderately difficult hamster-to-rat xenotransplantation model as easily as in most allogeneic strain combinations and more easily than in some. The therapeutic benefit of the adjuvant agents correlated with the ability of the adjuvant drug to inhibit the antihamster antibody response perioperatively, although this correlation was imprecise later in the course, the reappearance of the antibodies was not predictably harmful. Once the first 2 weeks were past, treatment with antiproliferative agents was no longer necessary—and, in fact, its continuation may have been a liability in that some of the rats appeared ill unless these agents were stopped. The minor benefit of splenectomy in one of our liver subgroups was thought to be by abridging the humoral immune response. The imperfect correlation between the suppression of generic heterospecific antibodies by these drugs and the clinical results, particularly with cyclophosphamide, has been explained by further studies (manuscript in preparation) showing that xenograft rejection is dependent on an IgM-producing subpopulation of splenic B cells and NK cells.

Thus, we are reporting here the conclusion that interdiction of the B cell proliferative response holds the key to the critical first step of xenotransplantation. This concept should be clinically relevant, provided that the humoral antibody reaction is not so instantaneous that it causes hyperacute rejection in a matter of a few minutes or hours. It is known from experience that baboon-to-human xenotransplantation fulfils this condition (*33, 34*). The prime candidate drug to prevent the heterospecific humoral rejection response is cyclophosphamide, although the experimental drugs RS-61443 and BQR are promising.

Once the antibody barrier is breached, the need for the antiproliferative drugs apparently diminishes, as was shown by the ability to routinely stop cyclophosphamide, RS-61443, and BQR in 2 weeks or less without subsequent humoral rejection in spite of the continued presence or in some animals a delayed rise of the antihamster antibodies. In the hamster-to-rat model, monotherapy with FK506 was all that was required from 2 weeks onward. It was shown previously that with stoppage of FK506 at 100 days, the hamster heart or liver xenograft is rejected, primarily by cellular mechanisms, after several weeks to months (*19*).

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DISCUSSION

DR. AUCHINCLOSS (Boston, Massachusetts): Do you think the two drugs are prolonging survival more effectively than one alone because they're acting synergistically on a single mechanism of rejection, or are they working on two different mechanisms of rejection?

One way of examining that question would be to test the two drugs together in allograft combinations. Do you see the same synergistic effect when using an allograft?

DR. MURASE: In the allograft system we have a different experiment because we use a very low dose of FK506. With antiproliferative drugs at these low doses, an additive or synergistic effect is observed on allograft survival.

DR. AUCHINCLOSS: I'm sorry, you do see synergistic effect?

DR. MURASE: Yes.

DR. SOLLINGER (Madison, Wisconsin): This is the longest graft survival ever reported using drug therapy in this xenograft model.

I have two questions. What did the histology show in your cardiac model? How about FK506 plus Brequinar versus FK506 and RS61443? I am particularly interested in the histology of the coronary arteries.

The second question is if you would have a choice clinically to use either RS61443 or Brequinar in conjunction with FK506, what would you recommend?

DR. MURASE: We have histology for the greater than 100 day survivors. This was reviewed by Dr. Jake Demetris, who found no coronary artery changes.

Perhaps Dr. Starzl should answer the second question.

DR. STARZL (Pittsburgh, Pennsylvania): I think that any of the drugs you mentioned would be fine. My impression is that there is a wider margin of acceptable drug dose with the RS61443 than with Brequinar, which really has to be used right at the 3.5 to 4 mg/kg range. Whereas with RS61443, one can see an effective dose range all the way from just above 10 mg/kg up to 60 mg/kg.

However, I think it's worth pointing out that Cytoxan does the same thing. Although less efficient, methotrexate seems similar. The important point is that Dr. Murase has exposed a generic discovery, not an advocacy of a particular drug. Thus, several of the antimetabolites can be used as an adjuvant to "jump-start" maintenance treatment with FK506. Because of anxiety on the part of the drug companies about using experimental drugs together, it seems likely that a drug like Cytoxan will be used first with FK506 if clinical trials are attempted.

DR. AUCHINCLOSS: Dr. Starzl, you're the only person who has put an animal liver into a human being. With these new drugs now becoming available, are you prepared to do that again?

DR. STARZL: That's a policy decision that I wouldn't care to make myself. It involves the institutional IRBs and it involves the FDA and the NIH. There would have to be a substantial consensus before anyone would want to attempt such a trial. I have the impression, because of the delicate nature of the undertaking, that there will only be one or two opportunities to do it, so it's rather important that it succeed.

However, the basic tools to make it succeed are within our hands. In 1963, we transplanted six baboon-to-human kidney heterografts. None hyperacutely rejected. They all functioned for more than six days, to a maximum of 60 days. All grafts were eventually lost because of the syndrome that Dr. Murase has been able to interdict, that is, the delayed humoral rejection.

I don't know of any undertaking in clinical transplantation that is more thoroughly supported than a potential trial of xenotransplantation. With Dr. Murase's model, which is a very difficult one, she has been consistently able to get better results than most people 6 to 12 months ago could do with a whole variety of fairly difficult allograft models. It is a stunning set of data; except for the FK506, the achievement is not particularly drug specific.

It's like getting to a precious jewel encased in an impermeable shell. She has broken the shell with the antiproliferative drugs. It's quite an amazing story.

DR. AUCHINCLOSS: I agree entirely.

DR. STARZL: Can I just add one thing, because I think Dr. Murase said this in a way that might be misunderstood. At 100 days, the pathology in most of these hearts was absolutely normal. That is, when specimens were presented to Dr. Demetris as unknowns, he had trouble determining which was the xenograft heart and which was the native heart.

DR. BACH (New York, New York): Could you explain what you meant when you say that the heart is simply a vascular form of rejection, whereas the liver is the combination of vascular and cellular? I ask this especially when you show us that at least by the assay you used for natural antibodies, that they go up very much with the combined treatment of FK506, and either Brequinar or RS61443. Yet you get survival despite that.

DR. STARZL: The phenomenon is, of course, one which you have given the name "accommodation." When you and I last discussed this, it was in terms of transfection of human genes into xenograft endothelium. If you can succeed for a while, and it looks as if the magic time is 13 or 14 days, the continuing presence of these preformed antibodies may no do harm. This would not be without precedent, since it has been observed in recipients of allografts who "ride out" preformed cytotoxic antibodies.

But I'm only expressing my own particular brand of wonderment about the fact that enormous delayed rises in titer don't kill the graft.

The differentiation, between humoral and cellular rejection is made possible with 2 different kinds of organ grafts. On one hand, the heart xenograft at three days in untreated rats, or at four days in rats treated with FK506-with no histopathologic evidence of cellular rejection. Also, the liver, known to be relatively resistant to humoral rejection, survives long enough to identify a cell mediated component along with the characteristic changes of humoral rejection in the blood vessels. The cellular component by this time is very aggressive. This combination lesion is not unfamiliar. We have often seen it in allografts.

DR. BACH: That is precisely why I asked the question. It seems that is not induction of accommodation, it is accommodation!

My concern, is that we don't know as much about a combination, such as hamster-to-rat, as we do, for instance about pig-to-nonhuman primate. As such, I'm not quite sure how to think about antibody, complement, or other factors in the vascular rejection.

DR. STARZL: Thank you for putting the question that way. We think the barriers are the same; they differ only quantitatively. At a practical level, what is required to win in a human situation is to pick a species combination where you do not get hyperacute rejection within minutes before you can do something to break through the antibody barrier. With hyperacute rejection, the game is over.

By empirical experience, there are at least 3 animal-human combinations that qualify: the baboon-to-human (the kidney experience of 1963 and the Baby Faye case of 1984), the chimpanzee to human experience of Reemtsma, and the Rhesus monkey experience of Reemtsma. The Rhesus monkey was the least satisfactory because it was fiercely rejected after about three days, but hyperacute rejection did not occur. The best donor, the chimpanzee, cannot be used again because this is an endangered species. This appears to leave us with the baboon.

The work being done at Duke and at your place looking at in vitro antibody reactivities may well be predictive of other interspecies possibilities, but right now it seems to me that the pig-to-human is too tough.

More than 25 years ago, René Kuss of Paris tried a pig-to-human kidney xenotransplant, under Imuran and prednisone, at a time when dialysis was not available. Kuss has described to me in detail how the kidney was hyperacutely rejected in about 15 minutes. Thus the pig cannot qualify unless, or until, we can do something to move that antibody barrier back.