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FK 506 reverses acute graft-versus-host disease after allogeneic bone marrow transplantation in rats

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Severe graft-versus-host disease was induced by transplantation of ACI rat bone marrow and spleen cells into irradiated Lewis rat recipients. Treatment with FK 506 or cyclosporine A (CsA) was started after clinical and histologic evidence of acute GVHD was present. A 14-day course of FK 506 at 1.0 mg/kg/day could rescue 100% of the animals suffering from GVHD. In contrast only one half of the animals treated with CsA at a high dose of 25 mg/kg/day recovered. After cessation of immunosuppressive therapy, FK 506–treated animals displayed a marked prolonged disease-free interval as compared to CsA-treated bone marrow recipients. Recurrence of the disease in these animals could be prevented when FK 506 treatment was continued after the induction period with a low maintenance dose of 0.1 mg/kg/day every other day. (SURGERY 1991;110:357-64.)

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TRANSPLANTATION OF ALLOGENEIC BONE marrow (BM) has become an accepted treatment for a variety of malignant and nonmalignant disorders.[1] However, acute graft-versus-host disease (GVHD) develops in about 40% to 80% of these patients despite prophylactic immunosuppression.[2] Treatment of acute GVHD, once established, has been attempted with a variety of regimens including cyclosporine A (CsA),[3] methylprednisolone,[4] antithymocyte globulin,[5] monoclonal antibodies against T cells,[6] and combinations of these agents. Despite these advances in immunosuppression, GVHD remains the major cause of morbidity and death during the first months after allogeneic bone marrow transplantation (BMTx).[7]

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Because of the limited success of current therapy, more potent immunosuppressive agents are required to reverse established GVHD. The new immunosuppressive agent FK 506 has been shown to suppress immune responses[8] and to prolong graft survival in rats,[9,10] dogs,[11] baboons,[12] and more recently in humans.[13-15] Although its mode of action is similar to CsA, in vitro and in vivo assay experiments have shown FK 506 to be far more potent than CsA.[8-16] Therefore we evaluated the use of FK 506 for the treatment of established acute GVHD in a rat model.

MATERIAL AND METHODS

Animals. Male Lewis ($RT1^l$) and male ACI ($RT1^a$) rats weighing 200 to 225 grams were purchased from Harlan Sprague-Dawley, Indianapolis, Ind., and maintained in a laminar flow caging system (Thoren Caging Systems, Hazelton, Pa.).

BM and spleen cell transplantation. BM and spleens from ACI donors were harvested, and a total of 60×10^6 BM cells and 30×10^6 spleen cells were transplanted to Lewis recipients through the penile vein 2 hours after 1000 rad total-body irradiation, delivered from ^{137}Cs source. Animals received penicillin/streptomycin/gentamicin on day 0 followed by gentamicin injections on days 1 and 2.

Immunosuppression. FK 506 was provided by Fu-

Table I. Grading of GVHD

*Clinical assessment**	*Histopathologic assessment*	
	Severity	*Skin*
Erythematous ear		
Weight loss	+	Vacuolar degeneration and necrosis of the basal layer of epidermis and
Hyperkeratosis of the footpad		mild lymphocyte infiltration of the epidermis
Unkempt appearance	++	Eosinophilic necrosis, spongiosis, dyskeratosis, and above
Dermatitis	+++	Massive lymphocyte infiltration of the epidermis and
Diarrhea		focal microscopic epidermal-dermal separation

*Three or more signs considered positive for GVHD.

Table II. Treatment groups

Group	*n*	*Reconstitution*	*Immunosuppression*	*Dose (mg/kg)*	*Days of therapy*
1	6	None	None	—	—
2	10	ACI	None	—	—
3	12	ACI	CsA	15	12-25
4	9	ACI	CsA	25	12-25
5	8	LEW	CsA	25	12-25
6	8	ACI	FK-506	1	12-25
7	6	ACI	FK-506	1.5	12-25
8	8	ACI	FK-506	1.0 + 0.1	12-25 + 27-40

jisawa Pharmaceutical Company, Osaka, Japan. The powder with carrier solvent, HCO-60 and D-mannitol, was diluted in normal saline. CsA obtained from Sandoz Pharmaceuticals, Hanover, N.J., was dissolved at 15 mg or 25 mg in 1 ml of intralipid for intramuscular injections. Both agents were prepared shortly before daily intramuscular administration.

Assessment of GVHD. Rats were weighed every 3 days and assessed daily for clinical signs of GVHD. Histologic and clinical criteria of GVHD are described in Table I.[17-19] Weekly ear biopsies were taken from three to four animals of each group. The grading of histopathologic findings was assessed in a blind manner.

Assessment of chimerism. Four weeks after BMTx, four rats from each group were randomly chosen and assessed for the presence of ACI donor-type lymphocytes in the peripheral blood with three different monoclonal antibodies for detection of lymphocyte major histocompatibility complex class I antigens[20] (Fig. 1). Monoclonal antibody (MAb) 163 (IgG2b) is specific for the RT1.A^1 antigen on Lewis cells, although MAb 211 (IgG2b) is specific for the RT1.A^a antigen on ACI cells. MAb 42-RT1.A^n was used as an irrelevant control. MAb 163, 211, and 42 were provided by H.W. Kunz, PhD, University of Pittsburgh, Department of Pathology, Pittsburgh, Pa.

The MAb 163, 211, and 42 were added as primary biotinylated antibodies at a 1:50 dilution and were incubated for 60 minutes at 4° C. Cells were resuspended in fluorescein isothiocynate-conjugated avidin as a secondary marker and were analyzed on a flow cytometer (Epics Profile; Coulter Electronics, Hialeah, Fla.).

Statistical analysis. Survival was calculated by the life-table method, with the mean survival time being derived from that time at which 50% of the animals were surviving. The follow-up in this study was 60 days. Comparisons of survival were analyzed with the Wilcoxon's signed rank test with unequal variances. Two-tailed p values <0.05 were considered statistically significant.

RESULTS

Assessment of chimerism. Flow-cytometric analysis of peripheral blood lymphocytes showed reconstitution of the allogeneic BM recipients with more than 95% ACI cells in all tested animals (Fig. 1).

Recovery from GVHD. Treatment groups are presented in Table II. Control animals not rescued with BM infusion after 1000 rad lethal total body irradiation died within 16 days (group 1). Animals receiving allogeneic BM + spleen cells after total body irradiation developed acute GVHD at a median day of 11, with a

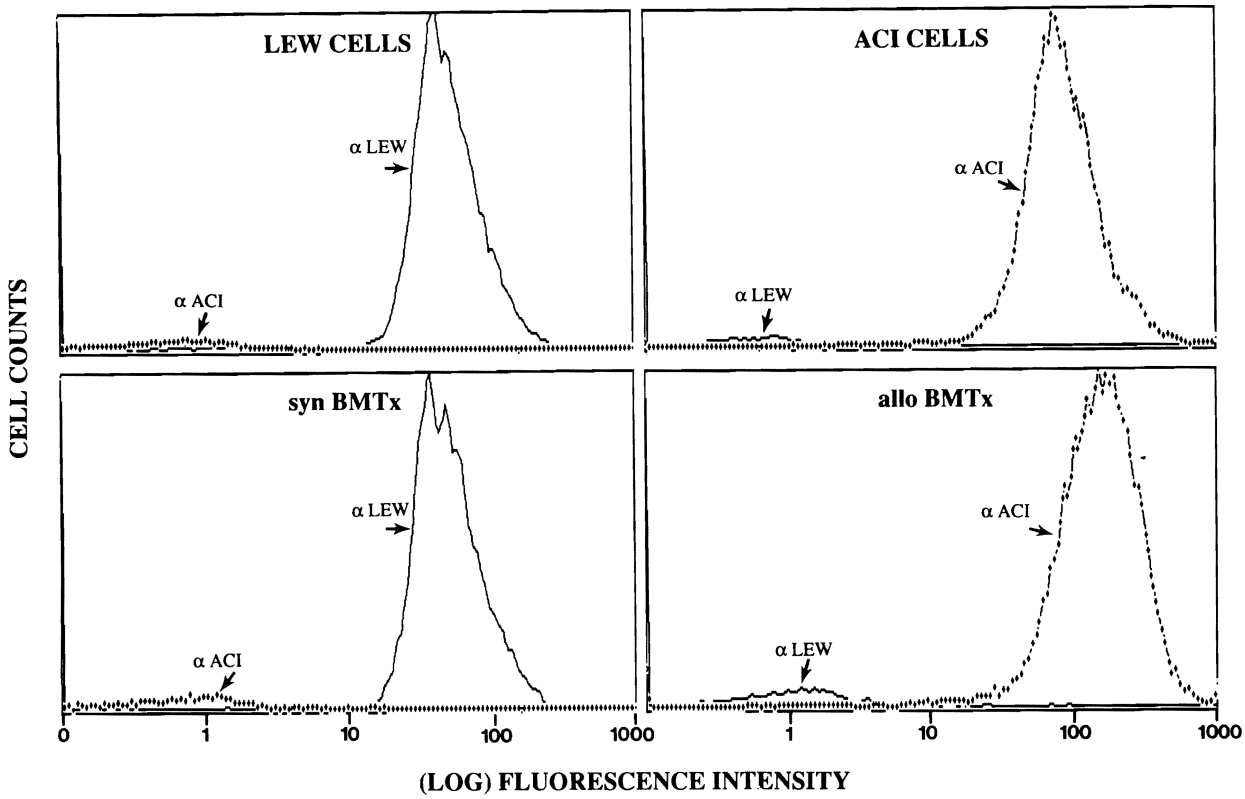

Fig. 1. Single-color flow-cytometric assessment of cell phenotypes from Lewis *(LEW)* and ACI controls, a syngeneic reconstituted and a representative allogeneic BM recipient. Four weeks after BMTx, peripheral blood lymphocytes were tested for the presence of donor type ACI lymphoid cells with MAb 163 (anti-LEW) and MAb 211 (anti-ACI). Animals receiving allogeneic BM repopulated with 95% donor type ACI cells.

range of 8 to 12 days. All animals that did not receive immunosuppressive therapy (group 2) died within 26 days with severe symptoms of GVHD.

The 14-day course of immunosuppressive therapy was initiated on day 12 after BMTx after clinical and histologic GVHD were present. CsA given at 15 or 25 mg/kg per day on days 12 to 25 (groups 3 and 4) was able to reverse GVHD in 42% and 55%, respectively (Table III). The remaining allogeneic BM recipients died either during therapy or failed to recover from GVHD.

All animals receiving FK 506 at 1.0 or 1.5 mg/kg/day, for the same 14-day period (groups 6 and 7), were rescued, with no death caused by GVHD (Table III). The animals treated with FK 506 recovered slightly earlier at 19 to 21 days (groups 6 and 7), compared to 23 and 24 days in the CsA-treated animals (groups 3 and 4).

Recurrence of GVHD after cessation of immunosuppressive therapy. In all CsA-treated animals and in 75% and 50% of animals (groups 6 and 7) receiving a 14-day course of 1.0 and 1.5 mg/kg/day FK 506, respectively, GVHD eventually recurred after immunosuppressive therapy was stopped (Table IV). Recurrence of GVHD in FK 506–treated animals was substantially delayed, when compared to the CsA-treated groups. Recurrence of GVHD was prevented in animals in group 8, which received low-dose treatment with FK 506 at 0.1 mg/kg/day every other day up to 40 days, after the same 14-day induction treatment with 1.0 mg/kg/day used in group 6. With this regimen, GVHD recurrence was always prevented during the study period of 60 days. In all animals spared for 60 days, no matter in which group, GVHD recurred, except for three animals, which were treated with FK 506 (3/27) and survived without displaying GVHD for more than 100 days.

Treatment death in CsA-treated animals was higher than in animals receiving FK 506 (Fig. 2). Six of 12 animals given CsA at 15 mg/kg/day and four of nine animals receiving CsA at 25 mg/kg/day died during therapy. No death was seen in the FK 506–treated

Table III. Recovery from GVHD

Group	Treatment	Dose (mg/kg)	Days	n	Recovery from GVHD			Survival (days)	p value†
					%	Yes/no	Day*		
2	Untr BMTx	—	—	10	0	0/10	—	16, 18, 19, 19, 20, 25, 26, 26, 26, 26	—
3	CsA	15	12-25	12	42	5/12	23	14, 14, 14, 17, 22, 22, 42, 42, 42, 42, 42, 42	NS‡
4	CsA	25	12-25	9	55	5/9	24	14, 16, 17, 18, 36, 40, 40, 41, 41	NS‡
5	CsA	25	12-25	8	—	—	—	All >60	<0.009
6	FK-506	1	12-25	8	100	8/8	21	48, 54, 54, 57, 57, >60, >60, >60	<0.01
7	FK-506	1.5	12-25	6	100	6/6	19	53, 53, >60, >60, >60, >60	<0.03
8	FK-506	1 + 0.1 qod	12-25 + 27-40	8	100	8/8	21	All >60	<0.009

qod, Every other day.
†Wilcoxon signed rank test for survival of treatment (groups 3 through 8) versus untreated BMTx (group 2).
‡Not significant, $p > 0.05$

Table IV. Recurrence of GVHD after cessation of immunosuppresive therapy

Group	Treatment	Dose (mg/kg)	Days	n	Recurrence of GVHD			MST
					%	Yes/no	Day*	
2	Untr BMTx	—	—	10	—	—	—	—
3	CsA	15	12-25	12	100	5/5	28	32
4	CsA	25	12-25	9	100	5/5	35	38
6	FK-506	1	12-25	8	75	6/8	52	57
7	FK-506	1.5	12-25	6	50	3/6	56	>60
8	FK-506	1 + 0.1 qod	12-25 + 27-40	8	0	0/8	—	>60

MST, Median survival time in days; *qod*, every other day.
*Median day of recurrence.

Table V. Histopathologic assessment of GVHD

Group	Treatment	Dose (mg/kg)	Days	n	GVHD at days after BMTx		
					12	25	45
2	Untr BMTx	—	—	3	2+	2+	—*
3	CsA	15	12-25	4	1-2+	1+	—*
4	CsA	25	12-25	4	1-2+	0-1+	—*
6	FK-506	1	12-25	4	1-2+	0	0-1+
7	FK-506	1.5	12-25	4	1-2+	0	0-1+
8	FK-506	1.0 + 0.1 qod	12-25 + 27-40	4	1-2+	0	0

**qod*, Every other day. Not available because all animals died because of GVHD.

groups during the same time period. Prolongation of survival time was statistically significant for all FK 506–treated groups, compared to untreated BM recipients (Table III) and compared to both groups given CsA (versus group 4 [CsA 25 mg]; groups 6, $p > 0.01$; group 7, $p > 0.03$; group 8, $p > 0.0001$). The prolongation of survival in the CsA-treated animals versus untreated controls was not significant (group 3, $p > 0.41$; group 4, $p > 0.15$).

To assess if toxicity of CsA therapy was a reason for the high mortality rate, one group of animals (group 5) was given syngeneic BM and treated with a dose of 25 mg/kg/day on days 12 to 25. In this group no death was seen up to 60 days after treatment, and all animals were

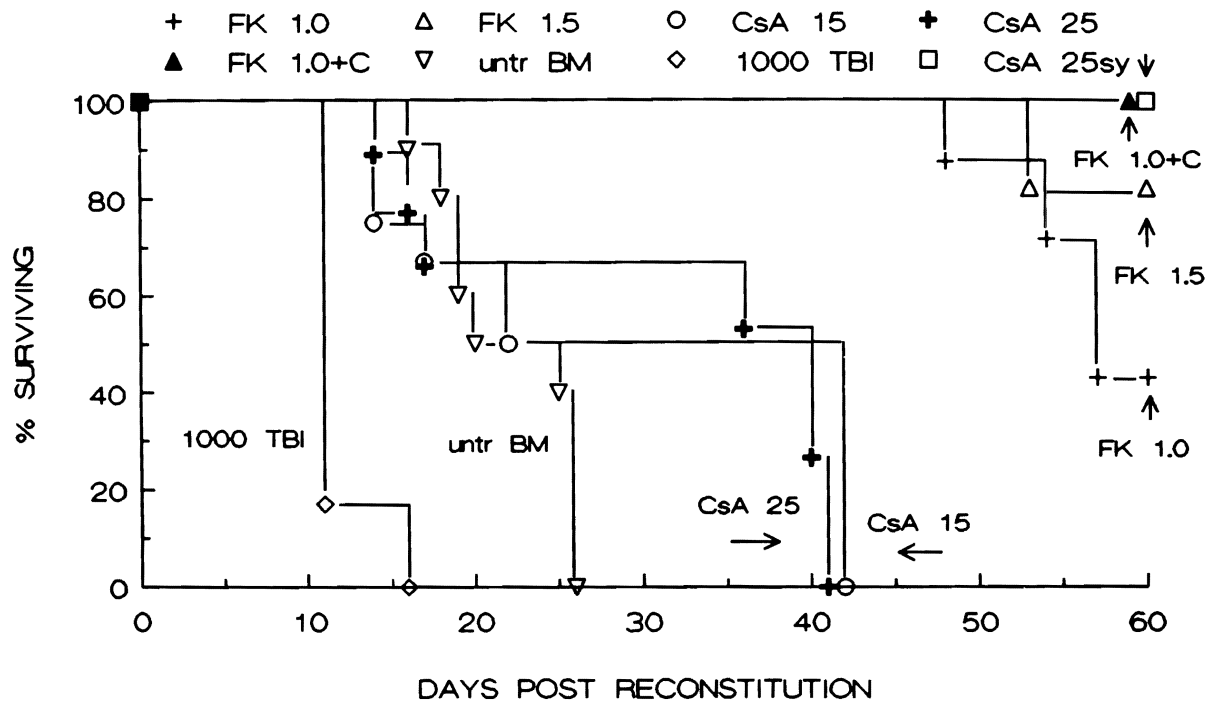

Fig. 2. Survival from GVHD of allogeneic BM recipients treated with CsA or FK 506 at various doses. Animals received either CsA at 25 mg *(CsA 25)* or 15 mg *(CsA 15)* /kg/day or FK 506 at 1.0 mg *(FK 1.0)* or 1.5 mg *(FK 1.5)* /kg/day on day 12 to 25 after BMTx. In one group, FK 506 treatment was continued after the induction period of 1.0 mg/kg/day on days 12 to 25 with 0.1 mg/kg/every other day to day 40 after BMTx *(FK 1.0 + C)*. One group that served as a CsA-toxicity control received a syngeneic BMTx and CsA at 25 mg/kg/day on day 12 to 25. Also included are untreated BM recipients *(untr BM)* and irradiation controls *(1000 TBI)*.

in excellent health without any indication of drug toxicity.

Histologic evidence. All animals displayed histologic evidence of GVHD on day 12 when therapy was started (Table V). After 2 weeks of treatment, histologic signs of GVHD persisted in CsA-treated animals with vacuolar degeneration and lymphocytic infiltration of the epidermis (Fig. 3). In contrast the FK 506–treated animals had no histologic signs of GVHD in biopsies taken on day 25. At 45 days after BMTx no animals from the untreated group or the CsA-treated group were alive. At this time point 20 days after cessation of the 14-day course of immunosuppression, both FK 506–treated groups (groups 6 and 7) displayed evidence of GVHD. Recurrence of GVHD was not seen in the skin of animals receiving continuous low-dose treatment of FK 506 every other day (Table V).

DISCUSSION

We have shown that a 14-day course of FK 506 can reliably reverse established GVHD, something not achieved with CsA. The high mortality rate during CsA treatment could be attributed to death from GVHD, drug toxicity, or a combination of both. CsA toxicity as the primary factor was ruled out in syngeneic BM recipients (group 5), which had no deaths when treated with 25 mg/kg/day. Thus the better results with FK 506 were attributable to the superior immunosuppression with this agent. In vitro studies have shown FK 506 to be about 50 to 100 times more potent than CsA.[9] To achieve an effectiveness of CsA comparable to that of FK 506, doses in the toxic range would be required.[21] However, in the dose used of 25 mg/kg/day, a CsA effect could be seen, a finding consistent with clinical observations that CsA is effective for the treatment of human GVHD.[3] Our experimental results are in contrast to an earlier negative report in which the onset and severity of GVHD in rats was not altered when oral CsA was started 7 days after BMTx.[22] The difference in these results may have been due to the dosage of CsA and the oral route chosen in the earlier study. Absorption of the orally administered drug could be unreliable in such experiments because of damage of the small bowel from total body irradiation and GVHD. This damage was

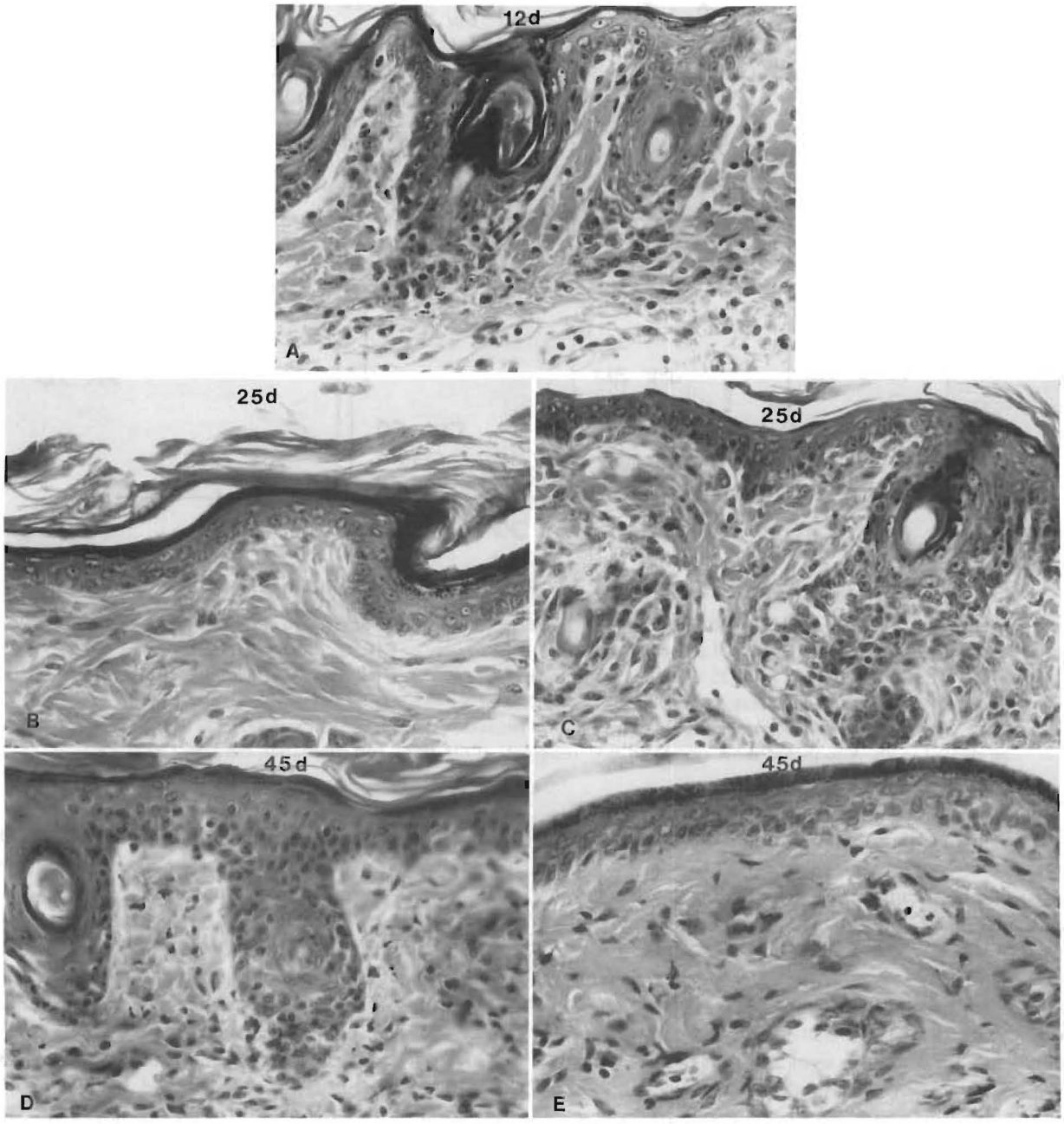

Fig. 3. Ear biopsies from Lewis recipients of allogeneic BM at 12, 25, and 45 days after BMTx. GVHD was present on day 12 (**A**) before treatment was started. Note disruption of the basal membrane, lymphocytic infiltration of the epidermis, and vacuolar degeneration and necrosis. No GVHD was found at the end of the treatment period (**B**) in animals receiving FK 506 at 1.0 mg/kg/day. In contrast, CsA at 25 mg/kg/day was not able to reverse histologic signs of GVHD (**C**). At 45 days after BMTx, GVHD recurred histologically in most rats treated with FK 506 at 1.0 mg/kg/day (**D**). In contrast continuous low-dose treatment with FK 506 at 0.1 mg/kg/day on days 27 to 40 after the induction period (**E**) prevented the recurrence of GVHD. (Hematoxylin-eosin; original magnification ×500.)

seen histopathologically in all of our control animals. In all of our experiments with either CsA or FK 506, the drugs were given intramuscularly.

The mechanism of both immunosuppressive agents has been studied extensively in in vitro experiments. Although CsA and FK 506 bind to different cytosolic

receptors,[23, 24] both have been shown to suppress the expression of the interleukin-2 receptor and the release of interleukin-2[8, 16] and to interfere with the activation of T cells.[16] Why the drugs are effective when given late is speculative. Conceivably the appearance of suppressor cells under delayed therapy could be responsible for reversing signs of GVHD. Spleen cells in CsA-treated BM recipients have been reported to suppress mixed lymphocyte reactions between donor and recipient-type cells.[19] Alternatively they might interfere with the activation and expansion of T cells. Whatever the explanation, clearly both FK 506 and CsA can be therapeutically useful long after immunologic activation starts. One of the striking qualities of FK 506 is its ability to reverse episodes of graft rejection in humans. This was reported in the first trials with FK 506 for the rescue of patients experiencing acute and chronic rejection of solid organ grafts, despite previous treatment with CsA, corticosteroids, and OKT3.[13, 15]

Although the ability to treat GVHD in recipients of BM is an important achievement, prevention of this process may have an even higher priority. We have described elsewhere that GVHD can be avoided with FK 506 in the same rat model herein reported, with greater ease and reliability than with CsA.[25] In addition, these findings have application beyond BMTx. The ability to prevent or control GVHD should change the approach and expectations with the transplantation of solid organs, such as the intestine, which are rich in lymphoid tissue and therefore capable of mounting GVHD.

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DISCUSSION

Dr. Andreas G. Tzakis (Pittsburgh, Pa.). We have followed Dr. Markus' work with great interest because we have

clinical evidence that FK 506 is effective in the treatment of GVHD. We have treated three children at the Children's Hospital in Pittsburgh with FK 506. All did not respond to first-line and second-line conventional treatment. Two of these children had been treated for 8 months, and the third child had been treated for 1½ months. They all had gastrointestinal, skin, and liver involvement; the third patient had alveolitis obliterans as well. The gastrointestinal, skin, and hepatic involvement all resolved within 3 weeks to 3 months after initiation of the treatment. The lung involvement did not improve.

Dr. Roberta E. Sonnino (Cleveland, Ohio). You showed us the result of skin biopsies. Did you look at the other organs that are usually affected by GVHD, and if so, what was the extent of involvement? Were you able to reverse it in all organs?

Also, you obviously continued to use FK 506 at a maintenance low dose. Did you carry the studies out any further after FK 506 was discontinued to see whether you could induce tolerance off immunosuppression as we have found you can do at times with cyclosporine? With bowel transplantations, for instance, rats will develop tolerance to cyclosporine. Did you find a similar situation with FK 506 or did you have to leave them on a low dose permanently?

Dr. Markus (closing). Responding to the last question, we are looking at the prevention of GVHD by FK 506 in a separate study in which we examine all organs. We know that the GVHD will induce effects in other organs. We did reverse the GVHD also in other organs. The liver is a poor organ to follow GVHD in rats because it is not the primary target as it is in humans. A better organ to follow in rats probably is the small intestine.

Concerning the second question, we continued the low-dose treatment further in a couple of animals for up to 60 or 80 days. The rate of tolerance induction was not higher than what we saw in our shorter term treatments. Overall, we have had about 5% to 10% of animals that were free of GVHD and that displayed some sort of tolerance. This incidence was not obviously influenced by the duration of FK 506 treatment.

Dr. Tzakis, this is of course acute GVHD in our rats. The patients who have been treated had chronic GVHD. We are just developing a model to treat the chronic GVHD in rats. We are delighted to hear the positive reports in clinical bone marrow transplantation.