

Allogeneic Hematolymphoid Microchimerism and Prevention of Autoimmune Disease in the Rat

A Relationship between Allo- and Autoimmunity

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Abstract

Conventional allogeneic bone marrow transplantation after myeloablation can prevent experimental autoimmunity and has been proposed as treatment for humans. However, trace populations of donor hematolymphoid cells persisting in solid organ allograft recipients have been associated in some circumstances with therapeutic effects similar to replacement of the entire bone marrow. We therefore examined whether inducing hematolymphoid microchimerism without myeloablation could confer the ability to resist mercuric chloride (HgCl₂)-induced autoimmunity.

Brown-Norway (BN) rats were pretreated with a syngeneic or allogeneic bone marrow infusion under transient FK506 immunosuppression before receiving HgCl₂. They were compared with BN rats receiving either no pretreatment (naive) or FK506 alone. Administration of HgCl₂ to naive BN rats induced marked autoantibody production, systemic vasculitis and lymphocytic infiltration of the kidneys, liver and skin in all of the animals and a 47% mortality. In contrast, BN rats pretreated with HgCl₂-resistant allogeneic Lewis bone marrow and transient FK506 showed less clinical disease and were completely protected from mortality. More specifically, IgG anti-laminin autoantibody production was decreased by 40% ($P < 0.05$), and there was less histopathological tissue injury ($P < 0.005$), less in vitro autoreactivity ($P < 0.05$), less of an increase in class II MHC expression on B cells ($P < 0.01$), and 22% less weight loss ($P < 0.01$), compared with controls. Protection from the experimental autoimmunity was associated with signs of low grade activation of the BN immune system, which included: increased numbers of circulating B and activated T cells before administration of HgCl₂, and less autoreactivity and spontaneous proliferation in vitro after HgCl₂. (*J. Clin. Invest.* 1996; 97:217-225.) Key words: bone marrow transplantation • microchimerism • immunosuppression • mercuric chloride • vasculitis

Introduction

Autoimmune diseases affect ~ 2.5% of the general population (1-3) and account for 20-25% of the patients on waiting lists

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for liver and kidney transplantation (4, 5). Although T lymphocytes play an important role in these disorders, the exact pathogenesis remains unknown.

Two primary theories have been proposed to explain the generation of pathogenic autoreactivity. While both concede the immune system to be the disease mediator, the first theory attributes autoimmunity to a failure of central tolerance. This reasoning would support the use of immunosuppressive therapy as treatment (6, 7). The other hypothesis implicates immune network abnormalities and dysfunctional peripheral control mechanisms as important contributing factors. This viewpoint would support stimulation of ill-defined "peripheral immune regulatory networks" as a therapeutic approach (8, 9).

Conventional allogeneic bone marrow transplantation after recipient pre-conditioning with lethal irradiation, has already been used to correct immune system abnormalities and cure autoimmune disorders in experimental animals and advocated as therapy in humans (10, 11). Although the concept of endowing the recipient with a normal (donor) immune system is beguilingly simple, evidence has accrued which suggests that such a draconian step is not required. A surprising therapeutic effect can be achieved in some instances with the presence of only a trace population of persistent donor leukocytes (microchimerism), such as that recently observed in successfully treated animal and human whole organ allograft recipients under conventional immunosuppression (12-15). Examples of the profound immunological sequelae of microchimerism include avoidance of graft-versus-host reactions (13-17); tolerance to whole vascularized organ allografts from the original donor (12-15, 18, 19); transfer of delayed-type hypersensitivity responses (20) and switching of ABO blood group types (21).

Indeed, we have postulated that successful whole organ transplantation allows a small component of the donor immune system to disseminate from the allograft, and eventually integrate with, and change, the overall properties of the much larger recipient immune system (22, 23)—similar to the "tolerance by network stimulation" hypothesis of Coutinho (24, 25). The immunologic consequences of whole organ transplantation are thus the same as if bone marrow alone was infused, as we have previously shown in experiments using Lewis (LEW) to Brown-Norway (BN) rat transplantation models (15, 23). In BN rats, injection of HgCl₂ induces the emergence of autoreactive anti-Ia T lymphocytes that stimulate the production of several well-defined autoantibodies by B cells (26). This results in a widespread vasculitis, with lymphocytic infiltration and damage to the skin and visceral organs, and upregulation of class II MHC antigens on affected tissues (26, 27). In animals that survive the acute syndrome, the autoreactive cells di-

1. Abbreviations used in this paper: AI, autoimmune; BN, Brown-Norway rat; LEW, Lewis rat; HgCl₂, mercuric chloride; MLR, mixed leukocyte reaction.

minish and regulatory T cells appear, which render the rats resistant to additional HgCl_2 injections (28). These regulatory T cells can then be used to transfer resistance to naive BN rats (28).

In the present experiments we have tested the hypothesis that the induction of microchimerism (LEW \rightarrow BN) with a bone marrow infusion might confer the normal resistance to mercuric chloride (HgCl_2) autoimmunity of the LEW rat on the normally AI-sensitive BN recipient.

Methods

Animals. Adult 7–8-wk-old inbred male rats weighing 200–250 grams were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN) and maintained in specific pathogen-free animal facilities with access to rat chow and water *ad libitum*. Lewis rats (LEW; RT1^b) were used as bone marrow allograft donors. Brown-Norway (BN; RT1^a) rats were used as recipients and as the susceptible strain for HgCl_2 -induced AI disease.

Induction and monitoring of autoimmune disease, and transplantation procedures. Autoimmune disease was reproducibly induced in naive BN rats (group II, $n = 15$) with five subcutaneous injections of 100 $\mu\text{g}/100$ grams bodyweight of HgCl_2 (Sigma Chemical Co., St. Louis, MO) given over 9 d, from day 0 to day 8, as a 0.1% solution in distilled water. In this model, autoantibody production routinely peaks at 12–14 d, whereas the worst clinical disease occurs around 21 d (29). BN rats (group I, $n = 5$) receiving equivalent injections of carrier only, served as negative controls.

As previously described (23), hematolymphoid microchimerism was produced 100 d before subsequent HgCl_2 therapy by infusing 2.5×10^6 unfractionated bone marrow cells (~ 1 donor/recipient) into the penile vein of BN recipients. The bone marrow was harvested from the femurs, tibias and humeri of LEW donor rats using RPMI 1640 supplemented with 25 mM Hepes buffer, 2 mM L-glutamine, 50 U/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin (all obtained from GIBCO, Life Technologies Inc., Grand Island, NY). The BN recipients were then treated with daily intramuscular FK506 injections (dissolved in HCO-60 and D mannitol; Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan) of 1 mg/kg per day for 14 d and with two extra injections of the same dose on day -80 and day -72, at which stage all immunosuppression was stopped. On day 0 the same HgCl_2 protocol as above was commenced. Alone, this transient FK506 treatment protocol has been shown to have no effect on organ allograft survival by day 0 (23).

BN rats ($n = 13$) rendered microchimeric in this fashion (group V) were compared with two control groups: BN rats ($n = 6$) that received only the FK506 treatment protocol outlined above (group III), and BN rats ($n = 11$) that received an identical amount of syngeneic BN bone marrow plus the same FK506 regimen (group IV) (see Table I).

All animals were examined daily for clinical signs of AI disease (hair loss, erythema affecting the paws, chest, and peri-oral area), and weighed every second day. "Minor" changes were defined as detectable perioral and pedal erythema; "moderate" changes were obvious perioral, labial and pedal erythema with trace ulceration; "severe" changes were defined as extensive erythema of the chest wall, perioral, labial, and footpads with swelling, ulceration, and crusting of the lesions.

Enzyme linked immunosorbent assays. ELISAs were performed using a modification of a previously described technique (30). Polystyrene microtiter plates (Nunc, Kamstrup, Denmark) were coated with 10 $\mu\text{g}/\text{ml}$ laminin (Sigma Chemical Co., St. Louis, MO) in 10 mM Tris-HCl buffer (pH 7.4). After washing with PBS containing 0.05% Tween 20, the laminin-coated plates were blocked with protein blocking agent (Lipshaw Immunon, Pittsburgh, PA), washed, and incubated for 60 min with 100 μl test serum diluted to 1:100. Wells were then washed and incubated with either alkaline phosphatase-conju-

gated goat anti-rat IgG or goat anti-rat IgM (both diluted 1:80; Jackson ImmunoResearch Inc., West Grove, PA). Alkaline phosphatase activity was localized with *p*-nitro-phenyl-phosphate substrate and buffer (Moss Incorporated, Pasadena, MD) and the product was quantitated by measuring extinction at 405 nm using a V-max kinetic microplate reader (Molecular Devices, Menlo Park, CA). Sera were assayed in quadruplicate and results (optical density/1000) are expressed as mean \pm SD for each time point.

White cell counts and flow cytometric analysis. Blood samples were taken from the tail artery and lymphocyte counts were assessed with a hemocytometer after suspension of heparinized blood at a 1:1 dilution in 2.86% glacial acetic acid. Double immunostaining flow cytometric analysis was used to determine lymphocyte lineage profiles. After RBC lysis (Red Cell Lysing Buffer [pH 7.5]; Stem Cell Chemical Co.), 2.5×10^5 cells were incubated for 20 min at 4°C in wells containing biotinylated OX-19 (CD5; peripheral T cells), OX-6 (MHC class II common determinant) in combination with FITC-conjugated OX-19, W3/25 (CD4; T helper subset, macrophages) or OX-8 (CD8; MHC class I restricted T cells, NK cells). To measure B cells, lymphocytes were single stained with unconjugated OX-33 (CD45RA; B-cell common leukocyte antigen). All primary antibodies were of mouse IgG₁ isotype and were obtained from Serotec (Oxford, England). After washing with ice cold PBS containing 1% BSA and 0.1% sodium azide (both from Sigma Chemical Co.), either streptavidin-phycoerythrin (Accurate Chemical Corp., Westbury, NY) or FITC-conjugated goat anti-mouse antibody (for OX-33; Serotec) were added for a further 20 min before washing. Cells were fixed with 1% paraformaldehyde and analyzed on an EPICS Elite flow cytometer (Coulter Corporation, Hialeah, FL) with acquisition of ten thousand lymphocyte-gated events for each sample. FITC-conjugated mouse IgG₁ isotype and goat anti-mouse FITC were used as controls, and unstained cells were incorporated to assess autofluorescence.

Histology and immunohistochemistry. Recognizing that animals with the most severe pathology died early of their disease, those animals that survived for 30 d from each group were killed for histopathological analysis. Tissue samples from the kidney, skin, liver, and lung were fixed in 10% neutral-buffered formalin, paraffin-embedded and routinely stained with haematoxylin and eosin. Additional tissue fragments from the same organs were snap-frozen in optimal cold temperature compound (O.C.T.; Miles Inc., Elkhart, IN) for immunofluorescent studies that were carried out on 4- μm cryostat sections fixed in cold acetone at -20°C and stained with FITC-conjugated goat anti-rat IgG or IgM (Cappel, Organon Teknika Corp., West Chester, PA) at dilutions of 1:100 and 1:50, respectively. Normal BN kidneys and spleen tissues were used as negative and positive controls, respectively. The routine light microscopy and immunofluorescence studies were blindly evaluated by two observers (C. P. Delaney and A. J. Demetris).

The severity of the tissue pathology associated with HgCl_2 -induced autoimmunity was semi-quantitatively evaluated, based on a combination of the severity of inflammation and tissue injury in each organ. Lung pathology was excluded because of spontaneous complicating changes observed in the BN strain. No inflammation or tissue injury was given a score of 0. The combination of moderate, predominantly mononuclear inflammation associated with tissue injury in > one-half of the area of affected organs was given a score of three. The total score for an individual rat was obtained by adding the score from each organ.

Mixed leukocyte reactions (MLR) and proliferative assays. Lymphocytes were obtained from the cervical lymph nodes of subject animals. Stimulator cells were harvested from normal BN animals and irradiated with 2000 rads. Varying numbers of responder cells were mixed with a constant number of stimulators (10^5) in 96-well U-bottomed plates (Falcon, Oxnard, CA) to give final responder:stimulator ratios of 4:1 in a final volume of 200 μl of complete medium (RPMI 1640 with added 10% normal BN serum, 5 $\mu\text{g}/\text{ml}$ of gentamicin, 5.5×10^{-5} M 2-ME, 2 mM L-glutamine, 10 mM Hepes buffer and 0.5 $\mu\text{g}/\text{ml}$

Table 1. Clinical Features, Weight Loss, and Mortality of Normal and Pretreated BN Rats Given HgCl₂

Group	Pretreatment	n	Clinical manifestations after HgCl ₂ *				Weight loss [†] %	Deaths at day 30	
			None	Minor	Moderate	Severe		Number	Percent
I	Neg. control (saline)	5	5	—	—	—	—	0	0
II	Pos. control	15	—	2	6	7	22	7	47
III	FK506	6	2	3	1	—	21	2	33
IV	BN (syngeneic) bone marrow plus FK506	11	2	5	2	2	20	1 [‡]	9
V	LEW (allogeneic) bone marrow plus FK506	13	11 [‡]	2	—	—	16 [§]	0 [‡]	0

Although pretreatment with FK506 with or without syngeneic bone marrow (groups III and IV) conferred some protection from HgCl₂ autoimmunity, animals treated with allogeneic bone marrow infusion plus transient FK506 at day -100 (group V) had the most effective protection from the clinical features and mortality associated with the disease. All significant differences are indicated and statistical analyses used are indicated in footnotes. *Erythema affecting paws, chest and peri-oral area (see Fig. 1, Methods). [†]Maximum mean weight change, as a percentage of initial body-weight. [‡] $P < 0.005$ vs. group II, and IV, Chi-squared test. [§] $P < 0.01$ vs. group II, Chi-squared test. ^{||} $P < 0.005$ vs. groups II, III, and IV, Student's *t* test.

of NMA). Cultures were incubated at 37°C for 96 h in 5% CO₂ in humidified air. Each well was pulsed with 0.2 µCi/ml final concentration of tritiated thymidine (2 Ci/mM; New England Nuclear, Beverly, MA) for the final 7 h of incubation, before automatic harvesting (Skatron, Lier, Norway) and analysis in a liquid scintillation counter (Wallac, Gaithersburg, MD).

For baseline proliferation assays, 10⁵ cells from subject animals at day 0 in each group being tested were placed in quadruplicate in 96-well plates using complete medium, cultured for 24 h, labeled and harvested as for the MLR plates. Proliferation assays and MLR were also repeated at day 30, using cervical lymph node-derived lymphocytes from animals surviving HgCl₂ disease. Results presented throughout the manuscript are from a minimum of three experiments.

Statistical analysis. Results are expressed as mean ± SD or mean ± SEM. Student's *t* test and the Chi-squared test were used as indicated in the text, and a *P* value of < 0.05 was considered to be significant.

Results

Protection from autoimmune disease. Naive BN animals given no treatment other than HgCl₂ (group II) developed a severe AI syndrome associated with significant weight loss and a 47% mortality by day 30 (Table I). The obvious clinical manifestations included peri-oral, labial, anterior chest wall and pedal erythema (Fig. 1 A), with associated hair loss and ulceration in the same distribution.

Histopathological examination of the skin showed a lymphoplasmacytic infiltrate in the upper dermis, which extended into basal epidermal and hair shaft epithelium, where it was associated with prominent epithelial cell apoptosis (Fig. 2). Analysis of the kidneys revealed mild mesangial hypercellularity, with a mild to moderate lymphoplasmacytic and eosinophilic interstitial inflammatory infiltrate, associated with tubu-

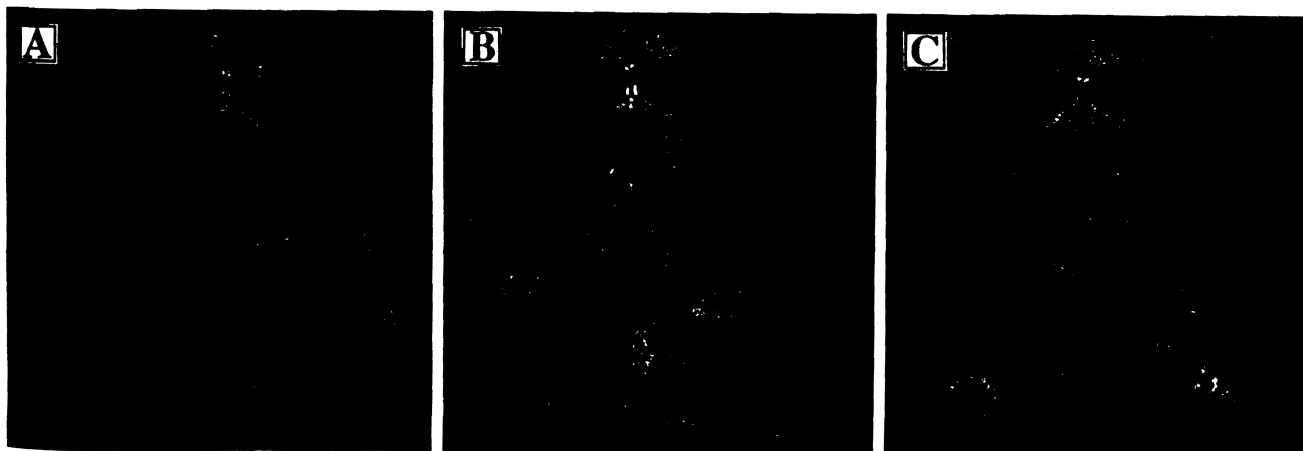


Figure 1. Demonstration of the amount of protection from the clinical manifestations of HgCl₂-induced AI disease that can be achieved with different pretreatment protocols. Normal BN animals given HgCl₂ develop severe clinical changes (peri-oral, chest wall, and foot-pad erythema and ulceration) after 21 d (group II: A). Pretreatment with syngeneic bone marrow and transient FK506 (group IV) affords partial protection from these changes (B), as does FK506 alone (group III). However, an allogeneic bone marrow infusion with transient FK506 (group V) abrogates these clinical features and the animals appear normal at all times (C), significantly better than the protection seen in groups III and IV.

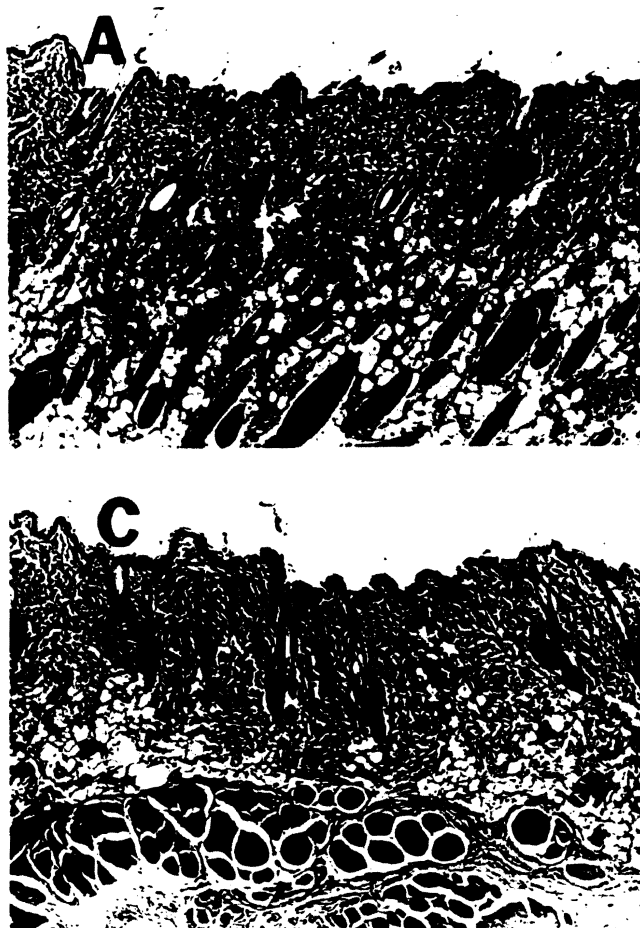


Figure 2. Light microscopic evaluation of hematoxylin and eosin stained sections from the skin of negative control animals (A; group I), positive controls (B; group II) and animals pretreated with allogeneic bone marrow and transient FK506 (C; group V), 30 d after induction AI disease with HgCl_2 . Note the moderate lymphocytic inflammatory infiltrate of the upper dermis which extends into the hair shafts and epidermis and is associated with epithelial cell apoptosis (inset). These changes were partially prevented in groups III and IV, but completely prevented in group V animals (see Table II).

lar epithelial infiltration and damage (Fig. 3). This resulted in tubular dilatation at the corticomedullary junction. Inflammation of a similar composition was present in the portal tracts of the liver, where infiltration and bile duct injury were evident (Table II).

All of the treatment protocols used resulted in at least some protection from the clinical manifestations and mortality

seen after HgCl_2 injections, with significant protection seen in Groups IV and V (Table I, Fig. 1). However, the most comprehensive protection was observed in BN animals pretreated at day -100 with an *allogeneic* bone marrow infusion in combination with the transient course of FK506 immunosuppression (group V) (Table I). The complete absence of mortality, the significantly reduced weight loss, the presence of minimal der-

Table II. Histopathological Evaluation of Inflammation and Damage Occurring in Animals That Survived for 30 d after Starting HgCl_2 Injections

Group	Treatment	n	HgCl_2	Mean score per tissue [†]			Total*	Immunofluorescence (IgM)
				Skin	Kidney	Liver		Kidney [‡]
I	Naive	3	-	0.0	0.0	0.2	0.2	0
II	Naive	5	+	1.6	1.6	1.4	4.6	5
III	FK506 alone	5	+	0.2 ^{††}	1.5 ^{††}	0.8	2.5 ^{††}	2.5
IV	BN (syngeneic) bone marrow plus FK506	10	+	0.8 ^{††}	1.2 ^{**}	1.0	3.0 ^{††}	3.5
V	LEW (allogeneic) bone marrow plus FK506	11	+	0.0 [†]	0.8 [†]	0.6 [†]	1.4 [†]	1.5

Rats dying before 30 d were not included in this analysis. Significant differences are indicated (Student's *t* test). * Total score for each group by adding scores for separate tissues. [†] Mean score for each tissue after blinded histological evaluation of sections. [‡] Representative score on a scale of 0-5, after blind grading of kidney sections stained with goat anti-rat IgM. [†] $P < 0.05$ vs. group II. ^{††} $P < 0.005$ vs. group II. ^{**} $P < 0.05$ vs. group V. ^{†††} $P < 0.005$ vs. group V.

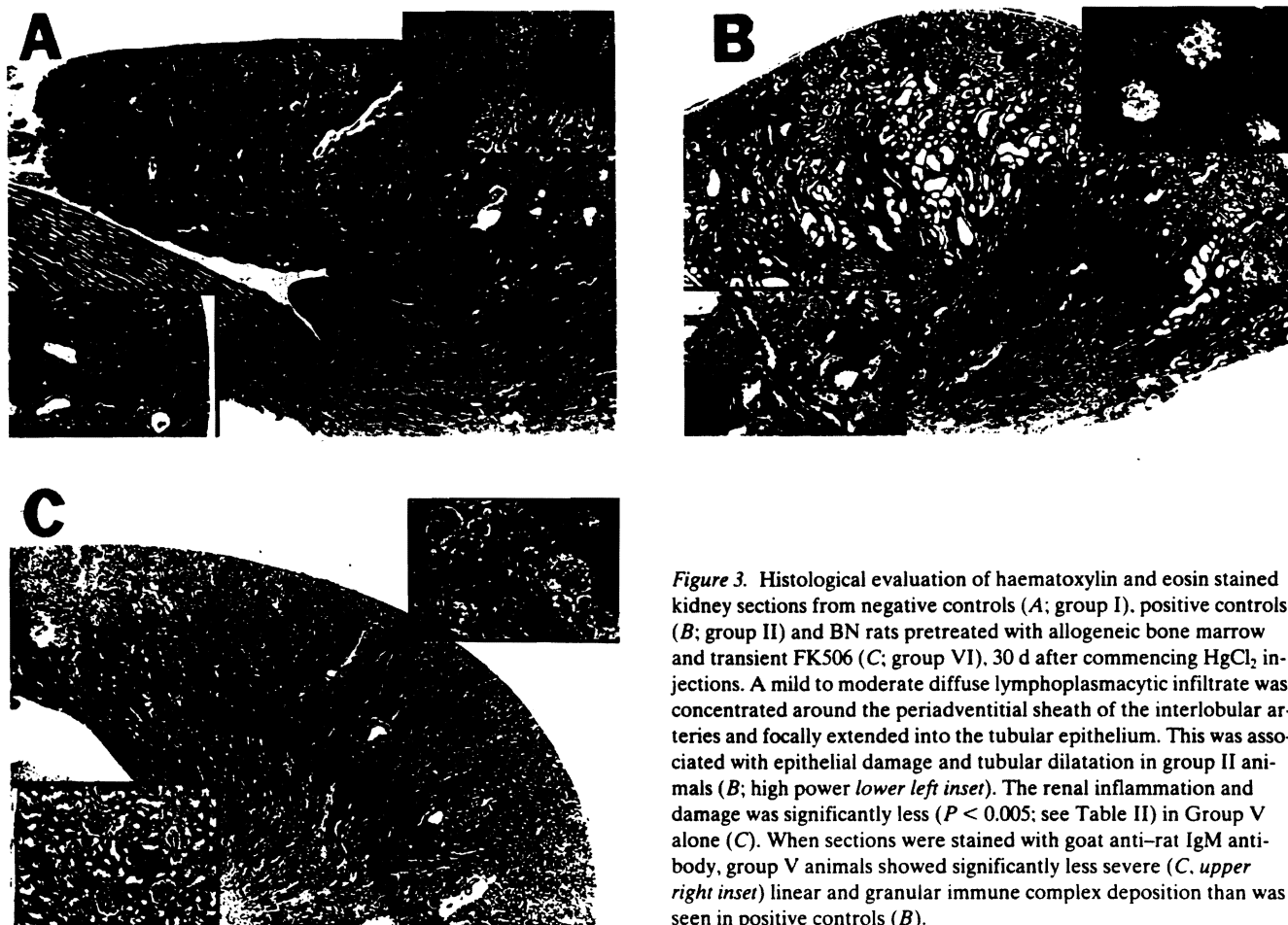


Figure 3. Histological evaluation of haematoxylin and eosin stained kidney sections from negative controls (A; group I), positive controls (B; group II) and BN rats pretreated with allogeneic bone marrow and transient FK506 (C; group VI), 30 d after commencing HgCl_2 injections. A mild to moderate diffuse lymphoplasmacytic infiltrate was concentrated around the periadventitial sheath of the interlobular arteries and focally extended into the tubular epithelium. This was associated with epithelial damage and tubular dilatation in group II animals (B; high power lower left inset). The renal inflammation and damage was significantly less ($P < 0.005$; see Table II) in Group V alone (C). When sections were stained with goat anti-rat IgM antibody, group V animals showed significantly less severe (C, upper right inset) linear and granular immune complex deposition than was seen in positive controls (B).

mal changes (in only two animals), and the less severe histopathologic evidence of tissue injury documented the clinical and pathological amelioration seen in these animals (Table I, Fig. 1).

During the experiments, protection from HgCl_2 autoimmunity was first noticed as less severe skin lesions (Fig. 2) and a reduction in weight loss (Table I). Naive BN rats that received FK506 with or without syngeneic bone marrow (groups III and IV) showed less dermal hair loss and ulceration grossly, and less dermal inflammation microscopically, than untreated naive BN rats. However, the BN rats that received allogeneic bone marrow and FK506 (group V) were essentially completely protected ($P < 0.005$) (Table I). Microscopically, there also was a decrease in renal (Fig. 3) and hepatic inflammation and damage in all groups that received pretreatment (groups III–V) as compared to positive controls (group II), but these parameters also were most significant in group V.

Semi-quantitative grading of immunoglobulin deposits correlated excellently with the routine histopathologic findings (Table II). Naive BN rats given HgCl_2 alone (group II) consistently contained the most intense glomerular capillary loop deposits of IgG and IgM, whereas they were less intense in rats from groups II, IV and, particularly, group V (Fig. 3).

Serological evaluation. Fig. 4 illustrates the significant reduction in mean IgG anti-laminin levels seen at day 14 in BN rats treated with transient FK506, with (group V) or without (group III) allogeneic LEW bone marrow. There was also a trend towards less autoantibody production in rats treated

with FK506 and syngeneic bone marrow, but this did not reach statistical significance. Protection from IgM anti-laminin autoantibodies was more easily achieved in all groups and a rise was only seen in unprotected (group II) animals (data not shown).

Peripheral blood lymphocyte analysis (Table III). As compared to baseline levels on day 0, naive BN rats given HgCl_2 (group II) showed a significantly increased peripheral blood lymphocyte count at day 14 that was largely attributable to a significant increase in B cells (OX-33⁺/CD45RA B cell common leukocyte antigen). An associated reduction of T helper cells (CD5⁺/CD4⁺) and in upregulation of MHC class II expression on T cells (CD5⁺/MHC class II⁺) also occurred in these animals.

In contrast to naive BN animals, rats pre-treated with FK506 with or without a syngeneic or allogeneic bone marrow infusion (groups III–V) showed an increased peripheral lymphocyte count, a decreased percentage of T helper cells and an increased percentage of MHC class II positive T cells at day 0. Furthermore, group V animals had significantly higher baseline B cell levels on day 0 than animals in other groups. These findings suggest the presence of low-grade immune activation in animals preconditioned with allogeneic bone marrow and transient FK506, before the administration of HgCl_2 .

After treatment with HgCl_2 , the changes in B and T helper leukocyte profiles of preconditioned rats were similar to those of naive animals, except for animals protected by allogeneic bone marrow and FK506 (group V). In this group and those

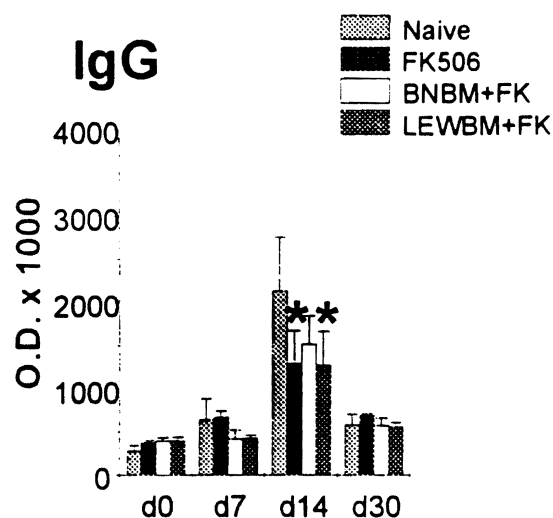


Figure 4. ELISA for IgG anti-laminin autoantibodies in protected and unprotected animals after administration of HgCl_2 . Syngeneic BN bone marrow infusion with FK506 (BNBM + FK; group IV) conferred some protection against elevations of autoantibody titres at day 14, however, a significant reduction in peak Ab titre was only achieved in animals treated with transient FK506 alone (FK506; group IV) or allogeneic LEW bone marrow with transient FK506 (LEWBM + FK; group V) (*; $P < 0.05$, Student's t test). No autoantibody increase was seen in the negative control animals treated with carrier only (data not shown). Eight samples from each group at each time point were examined and assayed in quadruplicate. Results are expressed as mean \pm S.D. for each time point.

given FK506 alone, the expected reduction of Th cells was not seen. More importantly, the significant increase of B cells was completely prevented in group V alone, correlating with the significantly lower auto-antibody levels, less severe disease and prevention of mortality seen in these animals.

Because of an earlier work describing increased MHC class II expression on B cells between days 3 and 15 after treatment with HgCl_2 (31), the protective effect of allogeneic bone marrow infusion on this parameter was also assessed by measuring the mean channel fluorescence (MCF) intensity of OX-6 expression on all peripheral blood lymphocytes (as $> 95\%$ of OX-6 $^+$ peripheral blood leukocytes are B cells; data not

shown). To control for possible slight variations between staining intensity on different days, all samples were stained at the same time for this experiment. On day 0, there was no difference between the MCF of samples (four animals analyzed per group) from groups II (no pretreatment), IV (syngeneic bone marrow), and V (allogeneic bone marrow). In contrast, the increased MCF, which occurred in group II and IV animals at day 7 (to 18.8 and 15.9, respectively), was significantly reduced in group V animals (to 13.9; $P < 0.01$, Student's t test).

Spontaneous proliferation and autoreactivity assays. When placed in wells without external stimulation of any kind, lymphocytes from group V animals at day 0 showed increased rates of thymidine incorporation (Fig. 5A), as compared with naive BN rats (group II at day 0) and transiently FK506 treated recipients (group III) on day 0, although this did not reach statistical significance ($P = 0.28$, Student's t test). The same was true for MLR reactivity at day 0 (Fig. 5C). Although animals from group V responded more vigorously to autologous stimulation, the wide variability between animals made the results statistically insignificant ($P = 0.56$).

Conversely, lymphocytes from group V animals at day 30 showed significantly less baseline proliferation than those from group II and group IV animals (Fig. 5B), and when placed in MLR against normal BN stimulators, a significant reduction in autoreactivity was seen (Fig. 5D).

Discussion

Several of the treatment regimens used in this study afforded some resistance to HgCl_2 -induced autoimmunity, but the best overall serological, histological, and mortality protection was seen in animals pretreated with allogeneic bone marrow and FK506 (group V). This pretreatment protocol is known to induce hematolymphoid microchimerism for at least 100 d, and is associated with donor-specific allogeneic tolerance (15, 23). However, the substantial protection afforded by transient FK506 with or without syngeneic bone marrow (groups III and IV), although clearly not as effective as FK506 plus allogeneic bone marrow (group V), suggests that transient immunosuppression is an important component in the protection protocol.

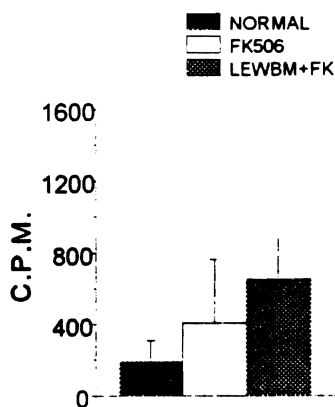
Reference to the work of Hess et al. (32) and others on cyclosporine-induced syngeneic graft-versus-host disease, which HgCl_2 autoimmunity resembles, provides a possible explanation.

Table III. Analysis of Peripheral Blood Lymphocytes of Naive (Group II), FK506 Alone (Group III), and Syngeneic (Group IV: BNBM + FK) and Allogeneic (Group V; LEWBM + FK) Bone Marrow Plus FK506 Animals Given HgCl_2 from Days 0 to 8

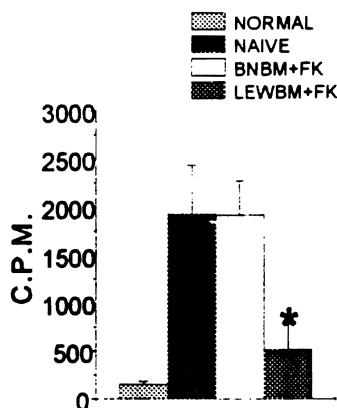
Time	(Group II) Naive (n = 5)			(Group III) FK506 (n = 3)			(Group IV) BNBM+FK (n = 7)			(Group V) LEWBM+FK (n = 6)		
	day 0	day 14	day 30	day 0	day 14	day 30	day 0	day 14	day 30	day 0	day 14	day 30
Lymphocytes ($\times 10^6/\text{ml}$)	7	10*	14*	12 [‡]	7	11	12 [‡]	10	12	10	10	15*
CD5 $^+$ /CD4 $^+$	37	28*	26*	23 [‡]	22	22	29	20**	24	27 [‡]	23	23*
CD5 $^+$ /CD8 $^+$	3.8	4.9	3.8	7.0 [‡]	6.4	4.0	5.8	4.8	4.4	4.5	5.0	4.2
CD5 $^+$ /class II MHC	1.4	3.3*	3.0	3.9 [‡]	3.5	1.7 [‡]	2.7 [‡]	3.7 [‡]	2.5	3.0 [‡]	4.4	3.0
CD45RA:B-cell CLA	39 [§]	49*	41	34 [§]	44*	53*	38 [§]	50**	50*	45	46	46

Mean values are presented throughout and significant differences are indicated (Student's t test throughout). Group II animals at day 0 (naive and before HgCl_2 treatment) are used as baseline controls. Percentage of lymphoid-gated events for double stained populations, except OX-33+. * $P < 0.05$ vs. d0 of same group. [‡] $P < 0.05$ vs. group II (naive BN + HgCl_2) at same time point. [§] $P < 0.05$ vs. group V (allogeneic bone marrow + HgCl_2) at same time point. ^{||} $P = 0.06$ vs. group V at same time point.

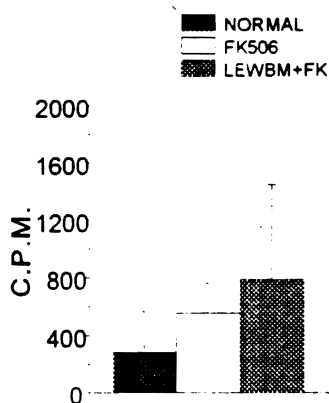
A. Spontaneous proliferation at d0



B. Spontaneous proliferation at d30



C. MLR at d0



D. MLR at d30

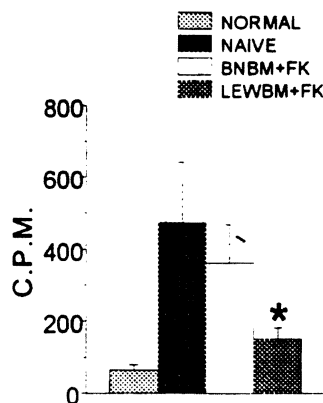


Figure 5. Spontaneous proliferation and MLR assays. On day 0, before starting HgCl_2 administration, lymphocytes from animals given allogeneic LEW bone marrow with transient FK506 at day -100 (LEW + FK; group V) showed greater spontaneous proliferation (A) and an increased ability to respond to syngeneic normal BN lymphocytes in MLR (C) than those from normal BN or FK506 only (FK506) animals, although this did not reach statistical significance ($P = 0.28$ and $P = 0.58$, respectively; Student's t test). In contrast, 30 d after starting HgCl_2 , the spontaneous proliferation (B) and autoreactivity (D) seen in unprotected naive animals (NAIVE; group II) and those protected with syngeneic BN bone marrow and FK506 (BNBM + FK; group IV) was significantly reduced in animals pretreated with allogeneic LEW bone marrow and FK506 ($P < 0.05$, Student's t test). (Data shown are mean \pm SEM of results with at least three animals per group).

tion for the mechanism of action of transient FK506. They have shown that syngeneic GVHD requires: (a) transient cyclosporine, which causes thymic injury, T cell maturational dysregulation and the release of abnormally high numbers of autoreactive cells into the periphery (33); (b) a thymus, to assist in T cell maturation (33); and (c) irradiation or cytoreductive therapy to disable peripheral regulatory networks, including so called "internal image" cells. Since FK506 causes thymic injury similar to cyclosporine (34-36), it is likely that FK506 also releases autoreactive cells into the periphery. These cells share with those generated during HgCl_2 -induced autoimmunity, a functional specificity for self Ia antigens (32, 37). Thus, it is not unreasonable to expect that the transient FK506 regimen, like cyclosporin, releases autoreactive cells, that in turn, produce an autoaggressive response that stimulates regulatory pathways. Thus, rechallenge of these animals with HgCl_2 produces less tissue injury and mortality.

The hypothesis tested in this paper is that by combining the drug effect discussed above with the continued presence of allogeneic cells, even in small numbers, a prolonged stimulus to important regulatory pathways might be provided. Indeed, Rossini et al. have shown the importance of the continued presence of a disease resistant population of cells by using splenocytes from histocompatible, diabetes-resistant, BB/WF donors in diabetes susceptible BB/Wor rats (38). In their ex-

periments, resistance to diabetes was conferred when susceptible rats contained as little as 8.5% of resistant chimeric donor cells in the spleen, and was lost when the chimerism (i.e., donor cells) was not detectable.

There are several possible explanations for the *in vivo* protection from AI seen in this study. The likelihood of each is considered to be in this order of probability: (a) alloactivation of the BN immune system may also stimulate autoreactive T cells and thus mimic the HgCl_2 -induced autoimmune syndrome, making the animals resistant to further activation or change by HgCl_2 ; (b) donor LEW T cells contained in the bone marrow inoculum, which are responsive to Ia determinants on BN cells, may mimic AI disease producing BN cells and stimulate autoantibody production and thus, regulatory networks; and, (c) activation of Th1-type BN T cells by low-grade allostimulation may change the cytokine milieu or immunologic microenvironment of the BN rat, and dampen the Th2-type response normally seen after HgCl_2 administration. We cannot confidently distinguish between these possibilities at this time. However, related studies in our laboratories have shown alloactivation of the BN immune system can trigger production of some of the same autoantibodies as those produced during HgCl_2 -induced autoimmunity (Delaney, manuscript in preparation).

Results such as the increased expression of MHC class II

on T cells, increased numbers of B cells and increased measurements of in vitro baseline proliferative and MLR reactivity in microchimeric animals protected from the autoimmune disease suggest that the processes involve cell activation. Such data support previous suggestions by Coutinho and others that the mechanisms involved in self and allogeneic tolerance are active processes (24, 25), and correlate with the active proliferation known to be associated with microchimerism (39, 40).

Several other lines of investigation also suggest a direct link between allo- and autoreactivity, and draw attention to striking similarities between the mechanisms responsible for self and allogeneic tolerance. Hess et al. have shown that autoreactive CD8 cells emerging during syngeneic graft-versus-host-disease induced by transient cyclosporine therapy, can prolong allograft survival (41). Neonatal allogeneic tolerance achieved through hematolymphoid mixed chimerism correlates with high levels of T and B lymphocyte reactivity (25), a propensity to AI disease (42, 43), and increased production of IL-4. Interestingly, such allogeneic tolerance in neonates can be blocked with anti-IL-4 antibodies (44), whereas administration of exogenous IL-4 has been shown to restore self-tolerance and prevent diabetes in the NOD mouse by reversing a proliferative defect in mature thymocytes (45).

The results presented in this model show effective prevention of the serious features of HgCl₂ autoimmunity, although human AI diseases may be more difficult to treat than experimental models. For example, a single blood transfusion can prevent diabetes in the BB rat, but repeated allogeneic buffy coat transfusions met with little or no success in humans, even in the early stages of diabetes (46). However, the transfused cells in the above study (46) were HLA-DR identical and no significant evidence of lymphoid activation was detected during treatment. In clinical solid organ transplantation, where intense allostimulation and hematolymphoid microchimerism routinely occurs in long term survivors (13), AI disease has a relatively low recurrence rate or is noticeably less severe than before transplantation (47–52). An important exception to this generalization is patients who are HLA identical to the donor, in which case recurrent AI disease is common (53). Although the potent immunosuppression given to prevent rejection may account for these observations, an effect similar to that reported in this manuscript may also be occurring.

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