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EXPRESSION OF AN ALLOGENEIC MHC DRB TRANSGENE, THROUGH RETROVIRAL TRANSDUCTION OF BONE MARROW, INDUCES SPECIFIC REDUCTION OF ALLOREACTIVITY[1]

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***Background.*: Transfer of MHC class II genes, through allogeneic bone marrow (BM) transplantation, induced long-lasting acceptance of renal allografts in miniature swine. To adapt this approach to the clinic, we have now examined whether somatic transfer of allogeneic class II DR genes, into otherwise autologous bone marrow cells (BMC), can provide the matching required for inducing immune tolerance.**

***Methods.* Autologous BMC were transduced ex vivo with recombinant retroviruses for allogeneic DRB followed by BM transplantation. The recipients were then challenged with kidney allografts solely matched to the DRB transgene.**

***Results.* Five miniature swine received autologous BMC conditioned with growth factors and transduced with recombinant retrovirus vectors containing allogeneic (n=4) or syngeneic (n=1) class II DRB genes and a drug-resistance marker. Expression of retrovirus-derived products in BM-derived cells was demonstrated by the detection of drug-resistant colony-forming progenitors and the presence of DRB retrovirus transcripts in peripheral cells. Analysis of selective mixed lymphocyte reaction responses to DR or DQ antigens indicated decreased reactivity toward the transduced DR gene product. Among all of the animals receiving fully mismatched kidney allografts, but with DRB matched to the transduced DRB, the one with the highest gene transduction rate showed stable allograft function and essentially normal renal histology for 2.5 years. A control animal, which received a syngeneic DRB gene, rejected its kidney allograft in 120 days after an earlier rejection crisis.**

***Conclusions.* These studies demonstrate that allogeneic MHC gene transfer into BM provides a new strategy for inducing tolerance across MHC barriers.**

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TABLE 1. Summary of experimental animals

Animal	DRB genotype		Cytokines	Age (wk)	Weight (kg)	Cells/kg[a] ($\times 10^7$)	Engraftment[b] (days)	Time from BMT to KTx	Haplotype kidney donor[c]
	Host	Vector							
10660	cc	d	no	14	9	4	9	152	dd (Id, IId)
10680	dd	c	no	17	14	13	12	162	jj (Ia, IIc)
10697	dd	c	no	17	18	9	13	160	jj (Ia, IIc)
10807	cc	c	no	12	9	8	14	158	dd (Id, IId)
10736	cc	d	yes	17	11	10	14	158	dd (Id, IId)
Avg ± SD				15±2	12±4	9±3	12±2	157±3	

[a] Number of cells from transduction culture infused per kilogram body weight.
[b] Days after BMT to white blood cell count of 1000/mm^3.
[c] SLA haplotypes are given with alleles at the class I (I) and class II (II) loci.

Because of clinical complications associated with chronic immunosuppression and chronic rejection, induction of specific tolerance in adults remains an important goal of transplantation research. We have developed a preclinical model, using MHC-inbred miniature swine (*1*), which exhibit many physiologic and immunologic similarities to human beings (*2*). Selective allogeneic bone marrow transplantation (BMT*) between MHC homozygotes and intra-MHC recombinant donor/recipient pairs has demonstrated the overwhelming importance of class II matching for induction of tolerance to allogeneic kidneys (reviewed in *3*). Thus, a short course of perioperative cyclosporine (CsA) permitted long-term survival of 100% of the class II-matched, class I-mismatched, renal allografts (*4*). With the ultimate goal of adapting this strategy to the clinic, we have begun studies aimed at determining whether transfer of allogeneic class II genes into autologous bone marrow cells (BMC) may substitute for allogeneic BMT and ultimately may permit tolerance induction (*5–8*). For this purpose, pig class II DRB recombinant retroviral vectors have been developed and the expression of the introduced class II β chain has been documented in fibroblasts as well as in murine and porcine bone marrow (BM) progenitors (*5*, *9*).

In the present study, five animals have been reconstituted with autologous BMC transduced with a recombinant retrovirus vector containing the class II DRB transgene from the SLA^d haplotype in the presence of c-Kit ligand (KL) and the fusion molecule PIXY321 (human interleukin [IL]-3 and granulocyte/macrophage-colony-stimulating factor [GM-CSF]). Long-term expression of the transduced DRB^d in BM-derived cells is documented. In addition, we describe the effects of allogeneic DRB expression in vivo on assays of peripheral immunity as well as on induction of unresponsiveness to a subsequent kidney graft expressing the same DR antigens. Together, these results demonstrate the potential efficacy of this approach for the induction of specific unresponsiveness toward transplanted tissues and define this large animal model for the subsequent testing of alternative vectors and transduction protocols.

* Abbreviations: BM, bone marrow; BMC, bone marrow cells; BMT, bone marrow transplantation; CFC, colony-forming cells; CsA, cyclosporine; FBS, fetal bovine serum; GM-CSF, granulocyte/macrophage-colony-stimulating factor; HSC, hematopoietic stem cell; IL, interleukin; KL, c-Kit ligand; KTx, kidney transplantation; LCM, lymphocyte-conditioned medium; LTR, long-terminal repeat; mAb, monoclonal antibody; MLR, mixed lymphocyte reaction; PBL, peripheral blood lymphocytes; PHA, phytohemagglutinin; POD, postoperative day; UT, untranslated; WBC, whole blood cells.

MATERIALS AND METHODS

Animals. Transplant donors and recipients were from our herd of partially inbred miniature swine (reviewed in *3*). The SLA genotypes of the animals included in these studies are shown in Table 1. Genotyping was controlled by strict pedigreed breeding and confirmed by microcytoxicity testing using allospecific antisera (*10*). At the time of BMT, swine were 13–17 weeks old and weighed from 8 to 16 kg. At the time of kidney transplantation, recipients were 8–9 months of age, weighing 20–35 kg. All procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals*, and approved by the Institutional Animal Care and Use Committees of the Massachusetts General Hospital.

Vector construction and virus production. Construction of the retrovirus vector GS4.5 and derivation of high titer producer clones have been described elsewhere (*9*) (see Fig. 1). The retrovirus vector GS7.3 was constructed in a similar fashion using an SLA-DRB^c cDNA (*11*). The GS4.5 construct was transferred into the ampho-

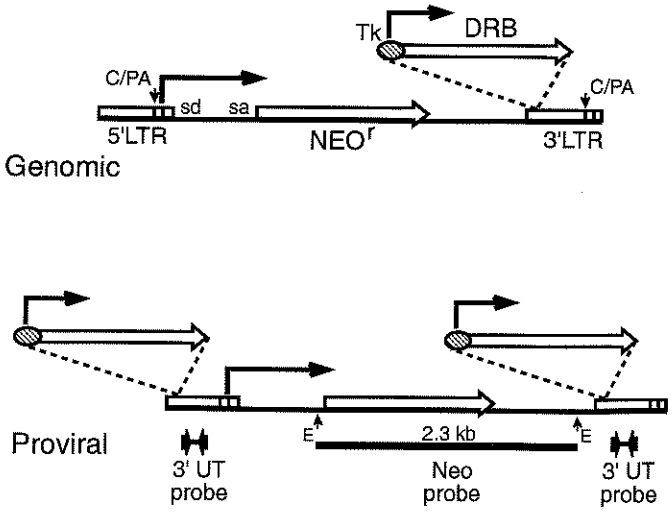

FIGURE 1. Retrovirus vectors. The top diagram depicts the structure of the recombinant DRB plasmid. cDNA for the β chain of SLA class II DR from either the *d* (vector GS4.5 (*9*)) or the *c* (vector GS7.3) allele, together with a promoter from the herpes simplex virus thymidine kinase gene (*51*) (Tk, hatched oval), were inserted into the cloning site of the 3′ LTR of the N2A double-copy vector (*52*). Transcription units (arrows): the neomycin phosphotransferase gene (NEO^R); the splice donor (sd) and cryptic splice acceptor (sa) sites as well as the cleavage/polyadenylation (C/PA) sites are indicated. The bottom diagram illustrates the configuration of the proviral form of the DRB-retroviral genome. Large blunt arrows bracket the polymerase chain reaction-amplified 3′ UT probe used for Northern blot analysis (see Fig. 3 for details).

tropic packaging line PA317 (*12*), whereas the GS7.3 construct was packaged into the GP+env Am12 line (*13*). Cell clones were derived that produced virus at approximately 10^6 colony-forming units/ml, based on drug-resistance titering on NIH 3T3 cells (*9*). These producer clones were also found to be free of competent helper virus as evaluated by a marker-rescue assay (*14, 15*). Virus producer cells were cultured at 37°C in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY) supplemented with 16% heat-inactivated fetal bovine serum (FBS; Sigma Chemical Co., St. Louis, MO), 2 mM L-glutamine (Life Technologies), 1 mM sodium pyruvate (Whittaker Bioproducts, Walkersville, MD), 0.1 mM nonessential amino acids (Whittaker), and penicillin/streptomycin antibiotics (Life Technologies). Virus-containing supernatant was collected from subconfluent to confluent plates after 24 hr with fresh media, passed through a 0.45 μM low protein-binding filter (Sterifil D-HV; Millipore, Bedford, MA), and stored at −70°C before use.

Clonogenic progenitor assay. As described elsewhere (*5, 16*), swine BMC were suspended in plating medium consisting of Iscove's modified Dulbecco's media (Life Technologies), 30% heat-inactivated defined FBS (HyClone, Logan, UT), 2 mM L-glutamine, 10^{-4} M β-mercaptoethanol (Sigma), antibiotics, 0.8% methylcellulose (by adding 36% v/v of a commercially prepared solution; Terry Fox Laboratory Media Preparation Service, Vancouver, BC), and growth factors (described below). For drug selection, the neomycin analog G418 (Geneticin, Life Technologies) was added at a final active component concentration of 1.3 mg/ml. This preparation was then dispensed into 35-mm suspension culture plates in duplicate at 1.1 ml per plate, and incubated at 37°C. Colonies of ≥100 cells were enumerated microscopically after 12–16 days, and consisted predominantly of granulocytes and macrophages (*16*). Phytohemagglutinin (PHA)-stimulated swine lymphocyte-conditioned medium (LCM) was prepared by incubating peripheral blood lymphocytes (PBL) isolated from a Ficoll gradient (LSM; Organon Teknika Corp., Durham, NC) at 10^6 cells/ml in Iscove's modified Dulbecco's media, 20% defined FBS, 2 mM L-glutamine, 10^{-4} M β-mercaptoethanol, antibiotics, and 1% v/v PHA (Life Technologies). After incubation at 37°C for 7 days, the supernatant was harvested by centrifugation, passed through a 0.2-μm filter, and stored at −20°C. Human PHA-LCM was purchased from the Terry Fox Media Preparation Service. Recombinant human GM-CSF was a gift from Sandoz Pharmaceuticals Corp. (Hanover, NJ). Both the human IL-3/GM-CSF fusion molecule PIXY321 and the recombinant murine KL were gifts from Immunex Corp. (Seattle, WA). Colony assay results were compared using a two-tailed Student's *t* test assuming unequal variance.

BMC transduction and transplantation. Total BMC were prepared from partially inbred miniature swine as described (*17, 18*). The resulting cavernous bone was cut into small pieces, suspended in RPMI 1640 medium (Life Technologies) containing 5 μg/ml deoxyribonuclease (Sigma), and mechanically agitated for 1–2 hr. Bone fragments were then removed by passing through a nylon mesh, and the resulting single-cell suspension was depleted of contaminating erythrocytes with ACK lysing buffer (B&B Research Labs, Fiskeville, RI) and finally washed. The BMC were incubated at 33°C in 5% CO_2 at approximately 5×10^5 cells per ml in 15-cm tissue culture dishes (Falcon Labware, Cockeysville, MD) each containing 120 ml of freshly thawed virus supernatant supplemented with 2 mM L-glutamine, 6 μg/ml polybrene (hexademethrine bromide; Sigma), 1 μg/ml gentamicin (Life Technologies), and 2–3% conditioned medium from swine long-term BM culture (*16*). After 18–20 hr, the medium, minus the nonadherent cells, was replaced with freshly thawed virus supernatant supplemented as above. After an additional 20-hr period, cells were collected, washed in Hanks' balanced salt solution (Life Technologies), and resuspended in 10% autologous serum diluted in Hanks' balanced salt solution.

Miniature swine BMT procedures have been reported previously (*17, 19, 20*). Briefly, autologous BMC were harvested 2 days before BMT (day −2) from pigs that subsequently received 10 Gy of whole body irradiation in two fractions of 5 Gy each on day −1 and day 0. After transduction, BMC were infused intravenously (day 0) at a dose of 0.4 to 1.3×10^8 viable nucleated cells/kg. Blood product transfusions were administered only if recipients experienced severe thrombocytopenia (platelets less than 20,000/mm^3) or severe anemia (hematocrit below 20). Intravenous hyperalimentation formulated from dextrose, amino acid (FreeAmine III(R); generously provided by McGaw Laboratories, Irvine, CA), lipid (NutriLipid(R); McGaw), and multivitamin solutions was given to those animals that remained anorectic for more than 4 days during the aplastic period.

Detection of retrovirus-related sequences and products. At approximately 5 months after BMT, recipients were screened for the presence of replication-competent helper virus in serum samples by the marker-rescue assay (*14, 15*). Serial progenitor analysis was performed on BMC collected by flushing and centrifugation from fragments (2–3 cm) harvested from open rib biopsies. Aliquots were then assayed for progenitor colony formation in the absence and presence of G418 drug selection as described. All cultures were plated in parallel with unmanipulated BMC to control for the efficiency of selection.

RNA was prepared from peripheral blood samples collected on heparin and total whole blood cells (WBC) prepared by hypotonic shock of the buffy coat. The cells were then disrupted in 4 M guanidine thiocyanate, and RNA and DNA were isolated after ultracentrifugation on a cesium chloride gradient essentially as described (*21*), with the modification that genomic DNA was physically isolated from the CsCl interface, precipitated with EtOH, resuspended in 1 mg/ml proteinase K, incubated overnight at 37°C, and extracted with phenol and precipitated with EtOH again. Northern blot analysis was performed according to standard methods (*22*). Class II transcripts were detected with a full-length ^{32}P-labeled DRB cDNA probe (*11*). Vector-specific transcripts were identified with a probe for the 3′ untranslated (UT) portion of the viral long-terminal repeat (LTR) (Fig. 1), which was generated by polymerase chain reaction amplification of vector plasmid in the presence of [^{32}P]dCTP, [^{32}P]dATP, and primers 5′-CGTGCTAGCTTAAGTAACGCC-3′ and 5′ TACCACAGATA TCCTGTTTGGCC-3′. Filters were finally washed in 0.1× saline-sodium phosphate-EDTA (SSPE) buffer at 60°C for 30 min.

Kidney transplantation (KTx). Allogeneic KTtx was performed according to the protocol described previously (*23*) at 152–162 days after BMT (Table 1). In one case (animal 10660), bilateral nephrectomy was performed at the time of KTx. In all other cases, the left ureter was ligated, leaving the nonfunctioning native kidney in place to allow the possibility of recipient survival by removal of the suture in the event of early allograft rejection. Left nephrectomy was then performed at 30–45 days after transplant. Immunosuppression after KTx was with intravenous CsA (Sandimmune IV; kindly provided by Sandoz Pharmaceuticals Corporation, Hanover, NJ) at an initial dose of 10 mg/kg/day, adjusted twice a week as necessary to maintain whole blood trough levels of 600–800 ng/ml. Daily doses were given for 12 days, with the first dose given approximately 2 hr before the graft revascularization. KTx recipients were monitored daily for CBC, serum creatinine levels, and blood urea nitrogen (if creatinine was elevated).

Pathology. An open KTx biopsy was performed between postoperative day (POD) 20 and POD 30 in all animals. Biopsies were classified according to the "Banff Schema" of the International Society of Nephrology as applied to clinical KTx (*24*). All animals that died underwent complete postmortem examinations.

Mixed lymphocyte reaction (MLR). One-way MLR were carried out using mononuclear cells obtained by gradient centrifugation with Lymphocyte Separation Medium (Organon Teknika, Durham, NC) as described (*10*). Blocking MLR assays were performed with the use of monoclonal antibodies (mAbs) against different SLA class II gene products. mAbs used were: 4OD (mouse anti-pig SLA-DR monomorphic) (*25*), TH16 (mouse anti-pig SLA-DQ monomorphic) (*26*), and 36-7-5 (mouse anti-mouse H2 K^k) (*27*), which was used as a negative control. These antibodies were used at an optimal concentration of 5% (v/v) of ascites titrated in buffered medium. In some assays,

normal mouse ascites replaced 36–7-5 mAb at the same concentration as the positive control. Relative responses were calculated for each responder in reference to the allogeneic response in the presence of 36–7-5 or normal mouse ascites.

RESULTS

Effect of cytokines on transduction of porcine BMC. Because of the demonstrated usefulness of recombinant cytokines for increasing the transduction rate in many other systems (*28, 29*), and because of the lack of recombinant swine cytokines at the time, a panel of murine and human recombinant cytokines was screened for activity on swine BMC (*16*). We focused on the use of recombinant murine KL, which has been demonstrated previously to cross-react with swine BMC (*16*) and the human IL-3/GM-CSF fusion molecule PIXY321 (*30*). This latter molecule was investigated as an alternative to IL-3, which has been found to greatly enhance the rate of BMC transduction in several models (*28, 29*). The activity of PIXY321 on swine BMC was compared with its standard activity on human BMC using titrated amounts of PIXY321 and human GM-CSF in a progenitor colony assay. As seen in Figure 2A, human GM-CSF (500 U/ml) induced a similar frequency of colonies from swine BMC as from human BMC ($P=0.95$), although maximal induction levels were only 70% of those observed with the LCM control ($P<0.05$ for being the same as LCM). Similarly, saturating doses of PIXY321 induced comparable levels of both human and swine colony-forming cells (CFC) ($P=0.85$). However, in contrast to GM-CSF, PIXY321 was as effective as LCM at inducing progenitor colony formation ($P=0.85$ for human BMC and $P=0.41$ for swine BMC), despite the fact that human IL-3 alone exhibits no activity in swine progenitor colony assays (*16*). This observation suggests that PIXY321 has more activity on swine BMC than can be attributed to the GM-CSF moiety alone.

The effects of the KL + PIXY321 cytokine mixture on the rate of gene transfer into swine BMC was examined by plating transduced BMC in progenitor colony assays in the presence or absence of selecting amounts of G418 (Fig. 2B). Transduction rates were calculated using the ratio of the number of colonies that grew in the presence of G418 to the number of colonies that grew in the absence of selection. Results from this set of experiments indicated that the percentage of G418^r CFC observed in three independent experiments was 2.8±1.1 times higher when the transduction cultures were carried out in the presence of KL and PIXY321.

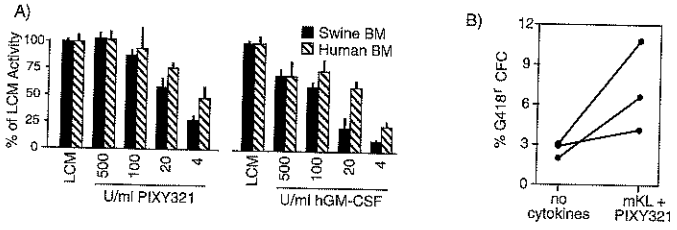

FIGURE 2. Increased transduction rate with PIXY321 and KL. (A) Effects of PIXY321 and/or human GM-CSF on swine and human progenitor colony formation. The efficiency of colony induction was calculated as a percentage of the colony formation achieved with 7.5% of species-specific LCM. Each data point represents the average of two independent experiments with samples plated in duplicate, and error bars represent 1 SD. (B) Effects of PIXY321 or human GM-CSF on swine BMT rate. BMC were transduced at a multiplicity of infection of approximately 2 in the absence or presence of PIXY321 (100 U/ml) and murine KL (20 U/ml), and then collected and plated in progenitor colony assays either with or without selecting amounts of G418. The percentage of G418^r CFC was then calculated by dividing the number of colonies that grew under selection by the number that grew without selection, and multiplying by 100.

BMT and engraftment. Freshly harvested BMC were transduced as described above with an estimated multiplicity of infection between 1.5 and 2, and then infused back into the donor preconditioned with whole body irradiation. As seen in Table 1, the procedure was performed on a total of five young animals (15±2 weeks): three with vectors for allogeneic DRB genes in the absence of cytokines, one with a vector for a syngeneic DRB gene in the absence of cytokines, and one with a vector for an allogeneic DRB gene in the presence of KL and PIXY321. They received $9\pm3\times10^7$ cells from the transduction culture per kilogram of body weight, and engrafted within 12±4 days based on our criteria of a white blood cell count of 1000/mm^3, with only minimal blood product transfusion support. This engraftment rate is consistent with previous results using uncultured BMC (*17, 31*), indicating that the reconstituting ability of the BMC were not significantly compromised by the time in culture or exposure to the virus supernatant.

Presence and expression of the DRB somatic transgene in BM and blood cells. Progenitor colony formation was estimated, under drug selection, on aliquots of BM taken before infusion and later from serial rib biopsies (Table 2). These data indicate that the ratio of myeloid progenitors in the initial BMC inoculum (week 0) that were transduced and that expressed the Neo gene ranged between 6.5% and 25.9%, with the highest gene transfer efficiency observed for animal 10736, the recipient of BMC transduced in the presence of growth factors. The initial transduction rate observed for this animal was 2.8 times higher than the average of 9.1±2.2 observed for the other four animals, a result that was consistent with the pilot transduction studies with the growth factors presented in Figure 2B. The initial levels of G418^r CFC were maintained for at least 12–22 weeks after BMT. A substantial decline was observed after this period, with the frequencies of G418^r CFC decreasing to about half of the original values by 28 weeks in the three animals surviving at this latter time point. The fact that a similar trend was observed in animal 10807, which received BMC transduced with a syngeneic DRB gene (Table 2), suggested that the decrease of G418^r CFC was not due to reactivity against the transduced DR antigens.

The presence and transcription of the allogeneic DR cDNA were also assessed by Southern and Northern blotting analysis, respectively. Samples were collected every 1–4 weeks for the first 3 months after BMT, at 22 weeks just before transplantation of the renal allograft, and every few weeks thereafter for up to 28 weeks (except for animal 10660, which was killed shortly after renal transplantation). Presented in Figure 3 are Northern blots, corresponding to each experimental animal, which were hybridized with the virus-specific 3′ UT LTR probe described in Figure 1. Specificity of probing was demonstrated by the absence of a specific signal in the lane of WBC from naive swine (Fig. 3, (−)WBC, top panel), as well as by the distinct hybridization pattern for RNA made by cells that produced DRB-recombinant viruses and that included transcripts arising from the DRB transcription cas-

TABLE 2. Frequency of G418^r CFC from serial rib biopsies

Animal (genetics)[a]	Week after BMT	CFC count ± G418[b]		Net percent G418^r CFC[c]
		Experimental	Control	
10660	0	51/218	5/203	10.5
(d → cc)	5	25/115	0/110	10.9
	8	10/85	0/126	5.9
	12	58/214	3/119	12.3
	22	na[d]	na	
10680	0	27/171	0/194	7.9
(c → dd)	5	42/159	3/119	11.9
	8	27/122	0/167	11.1
	12	23/124	1/67	8.5
	23	77/278	9/181	11.4
10697	0	15/115	0/108	6.5
(c → dd)	5	12/73	0/81	8.2
	8	na[d]	na	
	12	na[d]	na	
	22	20/243	2/185	3.6
	28	8/192	1/186	1.8
10807	0	33/146	0/167	11.3
(c → cc)	5	27/106	0/71	12.7
	8	74/238	0/178	15.5
	12	82/256	1/220	15.8
	22	51/217	0/201	11.8
	28	26/235	0/167	5.5
10736	0	148/272	3/119	25.9
(d → cc)	5	68/155	1/111	21.5
	8	48/120	0/71	20.0
(cytokines)[e]	12	63/130	0/178	24.2
	22	87/306	2/177	13.7
	28	38/141	2/145	12.8

[a] Genetics: vector DRB haplotype → recipient haplotype.

[b] Number of colonies counted in cultures with G418/the number of colonies counted in cultures without G418. Twice as many cells were plated in cultures with G418 as in cultures without G418 to increase the assay sensitivity. Control cultures were set up in parallel with untransduced marrow to monitor the efficiency of drug selection.

[c] Net percent G418^r CFC=ratio of colonies grown with versus without G418 from the experimental sample, minus the same ratio from the control sample, multiplied by 0.5 (to account for the fact that twice as many cells were plated in the cultures with G418 compared with the cultures plated without G418), and multiplied by 100.

[d] na, not available due to technical problems.

[e] Cytokines include KL and PIXY321.

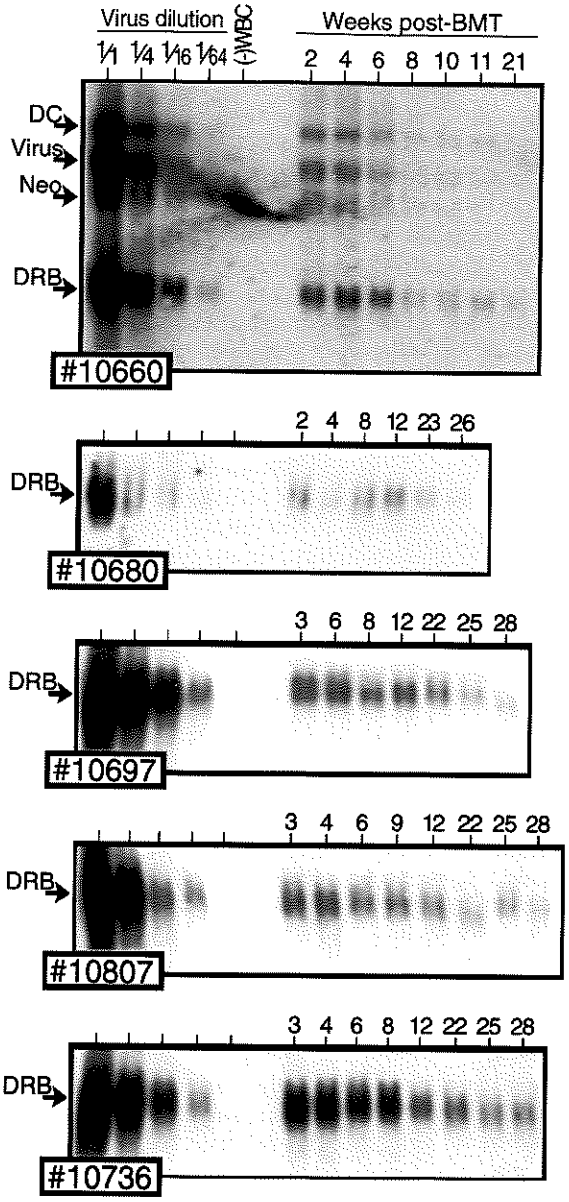

FIGURE 3. Northern blot analysis of serial whole blood samples. Eight micrograms of RNA from transduced WBC and an untransduced control animal (−)WBC were loaded per lane. Controls included titrating amounts of RNA from vector producer cells diluted with RNA from (−)WBC to a final amount of 8 μg per lane. Gels were stained with ethidium bromide to confirm equal loading (not shown), blotted onto nylon membranes, and hybridized with the LTR probe (detailed in Fig. 1). Positions of transcripts are indicated in the top panel. DC: large read-through transcript presumably originating from the double-copy DRB transcription cassette located in the proviral 5′ LTR (*9*); Virus: genomic viral transcript; Neo: spliced Neo transcript; DRB: vector-derived DRB transcript. The other panels represent the region around the virus-derived DRB-specific RNA band.

sette (DRB); the full-length virus and spliced Neo transcripts originating from the promoter in the 5′ viral LTR (virus, neo); and a large "read-through" transcript presumably originating from the double-copy DRB transcription cassette located in the proviral 5′ LTR (*9*). Detectable DRB-viral signals were observed in WBC from all the animals studied. Considering that the amount of RNA loaded in each lane was constant, as estimated by the levels of hybridization obtained with an ubiquitous DRB probe (not shown), it seems that the intensity of virus-DRB signal decreased with time but remained visible in samples from animals 10807 and 10736 over a 28-week period after BMT (Fig. 3). In addition, densitometric analysis, performed to evaluate the intensity of the transferred DRB signals, indicated that the levels of vector-derived DRB transcripts in the animals for which cytokines were not included in the transduction cultures were initially about 2–4% of endogenous DRB levels for the first few weeks, and then decreased over time to about 1% at the last time points tested. Similar studies done on animal 10736 showed that cytokine-treated BMC had initial levels of virus-DRB

transcription at approximately 5–6% of the levels of endogenous DRB for the first 8 weeks before declining to approximately 2% after week 12.

To determine whether the gradual decrease in vector RNA levels resulted from transcriptional quiescence, DNA samples from animal 10736 were analyzed by Southern blotting with a neo probe and showed discernible proviral signals until 28 weeks after BMT (data not shown). Densitometric analysis suggested that the intensity of the signal diminished over time with kinetics similar to those observed for the corresponding RNA samples. The correlation between RNA and DNA signal intensities suggests that the decline of transcript levels in WBC over time was probably not due to transcriptional quiescence, but rather to gradual loss of transduced cells.

Effects of allogeneic DR transfer on T-cell reactivity in vitro. Conventional one-way MLR assays were performed to assess the possible influence of virus-DRB expression on the class II-dependent immune response of pig recipients after reconstitution. Unfortunately, an analysis that was focused on only DR-dependent MLR was not achievable due to the fact that there are no animals with recombinant haplotypes separating DR and DQ loci. Therefore, lymphocytes from four of the experimental animals were tested in conventional MLR, 12 weeks after BMT, for a response to donor and third-party class II stimulators where the donor haplotype differed from the recipient by both the relevant DR allele and an irrelevant DQ allele. Although significant responses over the background were observed, no specific decrease in MLR to the haplotype matched to the transduced DR was observed (not shown). Lymphocytes from three of the engineered animals (10660, 10697, and 10736) were also used as stimulators of naive responder cells from the same nontransduced SLA haplotype. None of the PBL stimulators from these animals, tested 12 weeks after BMT, induced significant MLR of the naive responder cells, whereas all were capable of stimulating third-party responders (not shown).

Because the above results failed to demonstrate expression of the transduced DR antigen by conventional MLRs, blocking experiments with specific anti-class II DR and -DQ mAbs were carried out. Figure 4 shows results from a representative experiment involving responses from PBL of the experimental animal 10736 (top panel) and of control animal 10807 (bottom panel) to SLA^{dd} stimulators. The MLR of both animals, detected in the presence of a saturating amount of anti-DR blocking antibodies (first set of bars) was similar to the reactivity observed with naive SLA^{cc} controls, suggesting similar anti-DQ responses. In contrast, the presence of saturating levels of anti-DQ blocking antibodies during the reaction (second set of bars) revealed decreased (presumably DR dependent) reactivity in responder cells from the DR-engineered animal 10736 (Fig. 4A) but not in responder cells from the control animal 10807 (Fig. 4B), both with respect to the responses of a naive SLA^{cc} animal. Using both the anti-DQ and anti-DR antibodies in this assay confirmed the specificity of the blocking by showing almost complete inhibition of class II-mediated stimulation of all responder cells (third set of bars).

Survival of MHC fully mismatched renal allografts in genetically manipulated animals. To test whether allogeneic class II gene transfer into autologous BM might mimic the effects of class II matching for induction of specific unresponsiveness to renal allografts, four of the DRB-engineered recipients were subjected to renal allotransplantation using the CsA regimen previously shown to induce tolerance for class II-matched donor-recipient pairs (*4*). Five months later, the animals were challenged with renal allografts disparate for both class I and class II of the recipient, but DR matched to the transduced DRB (Table 1). The fifth animal (10807; SLA^{c}) was used as a control and received first autologous BMC transduced with DRB^{c} recombinant retroviruses followed by a fully mismatched KTx (SLA^{d}). The function of renal allografts was monitored by serum creatinine levels, as shown in Figure 5. Creatinine profile and rejection time for all rejector animals were comparable to those previously observed for rejection across full MHC disparities with this

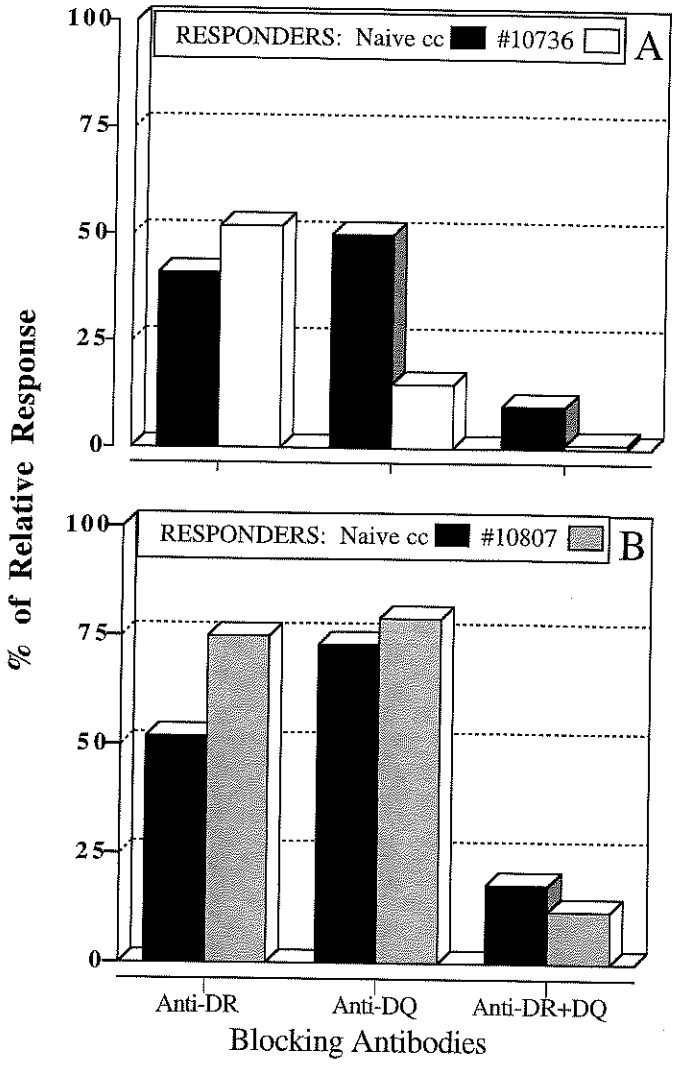

FIGURE 4. Blocking MLR analysis on lymphocytes from animals 10736 and 10807. (A) PBL from animal 10736 (SLA^{cc} with DRB^{d} transgene) and from a naive SLA^{cc} animal were stimulated with SLA^{dd} PBL in the presence of the mAbs indicated (see *Materials and Methods*). Responses were measured as a percent of the response observed in the presence of an irrelevant ascites fluid (which in some cases was nonspecifically stimulatory, leading to percent maximum responses less than 100%). Values are derived from results in triplicates. (B) The same analysis was performed using PBL from animal 10807 (SLA^{cc} with the DRB^{c} transgene).

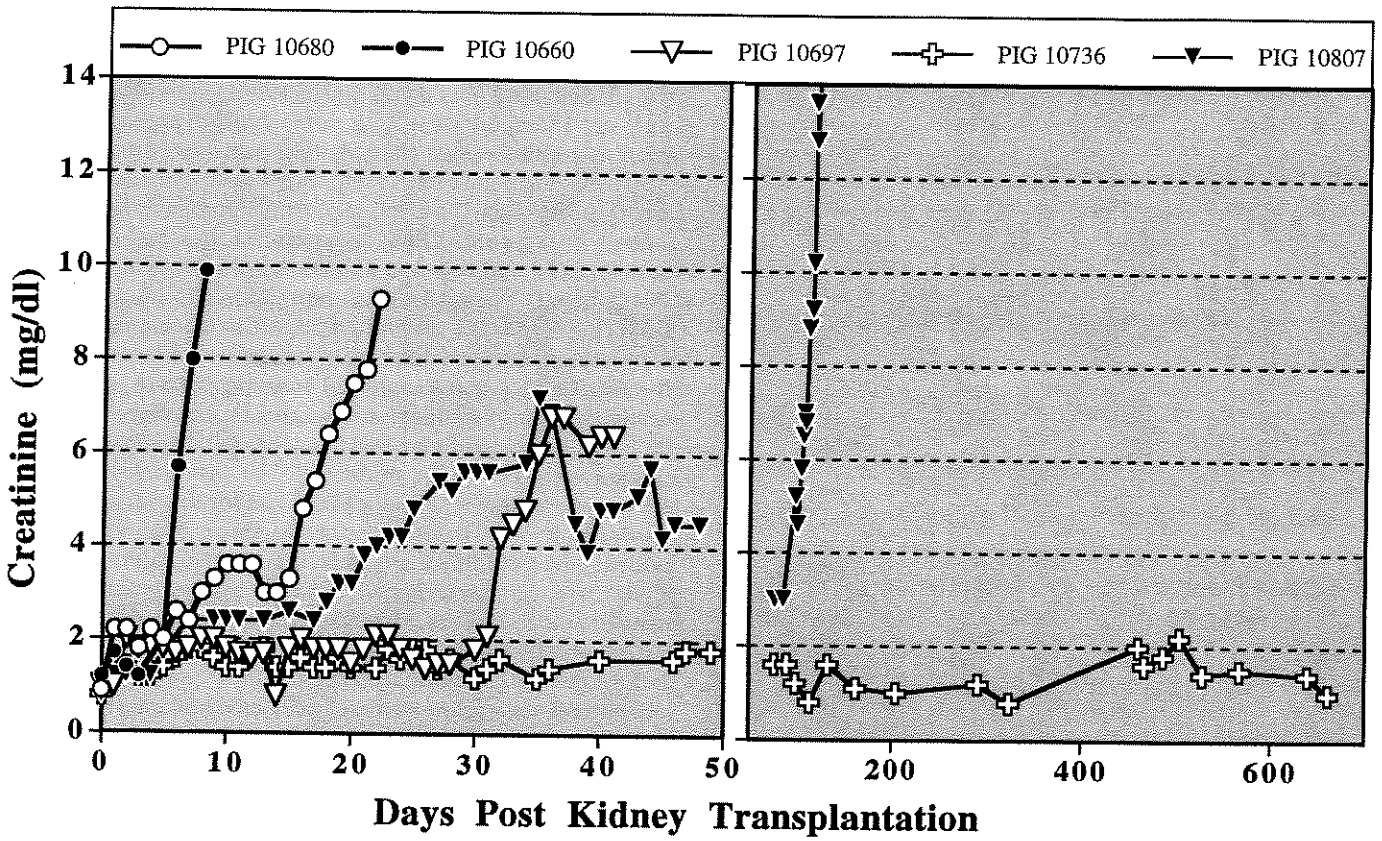

FIGURE 5. Serum creatinine levels in recipients of transduced marrow after fully mismatched KTx.

CsA treatment regimen (*23*), except for animal 10660, which rejected its transplanted kidney on day 8 while still receiving CsA. Histologic examination of its graft revealed a dense mononuclear cell infiltrate throughout the renal parenchyma, focal necrosis and hemorrhage, enlarged glomeruli, and substantial infiltrates in the intimal layer of renal vessels consistent with acute rejection. Two other animals (10680 and 10697) survived 22 and 40 days, respectively (Fig. 5). Allograft biopsy and autopsy findings indicated rejection with typical cellular infiltrates, tubular atrophy, and vascular changes. Control animal 10807 developed slow but progressive renal failure beginning shortly after transplantation and succumbed on day 120. Examination of the renal tissue at POD 120 revealed cellular rejection with massive infiltration (Fig. 6B). In contrast, pig 10736, the only animal whose marrow was transduced in the presence of growth factors, accepted its transplanted kidney long term and showed normal kidney functions and no signs of rejection, as illustrated in Figure 6B, until death at POD 955 after BMT.

DISCUSSION

As part of our program in transplantation immunology, we are testing the use of retrovirus vectors for the transfer of allogeneic MHC genes into BMC of partially inbred miniature swine (*1, 5, 32*) with the intent of inducing transplantation tolerance to subsequent primarily vascularized allografts (*6, 18*).

In an attempt to improve transduction rates, we initially evaluated the use of recombinant cytokines to induce cell cycling of the hematopoietic stem cell (HSC) targets (*33*). There are reports in both murine and human systems in which several cytokine combinations, most notably those including IL-3 and KL, have proven useful for this purpose (*28, 29*). Because recombinant forms of murine and human IL-3 showed virtually no activity in swine progenitor colony assays (*16*), and because the IL-3/GM-CSF fusion molecule PIXY321 showed enhanced hematopoietic properties on human progenitors (*30*), we evaluated this molecule as a possible alternative to IL-3. As seen in Figure 2, several aspects of the activity of this fusion molecule on swine BMC were demonstrated by comparing its ability to stimulate swine and human progenitor colony formation to the colony-stimulating ability of human GM-CSF alone. First, it was found that, at saturating doses, PIXY321 induced colony formation from both human and swine BM to a greater extent than human GM-CSF alone, indicating that this fusion molecule exhibited more activity on swine BM than could be attributed to the GM-CSF moiety alone. It is not clear whether this additional activity is due to enhanced signaling through the GM-CSF moiety of the fusion molecule, increased specificity for the swine IL-3 receptor, or some novel mechanism involving shared receptor subunits (*34, 35*). Second, although both PIXY321 and GM-CSF induced colony formation from both human and swine BM to an equal extent when used at saturating levels, it took approximately five times as much of either molecule to reach saturation on swine BMC compared with human BMC (Fig. 2). This would indicate either that these molecules are not fully cross-reactive in swine or that higher cytokine levels are required for activation of swine CFC. Nevertheless, the use of PIXY321 in combination with

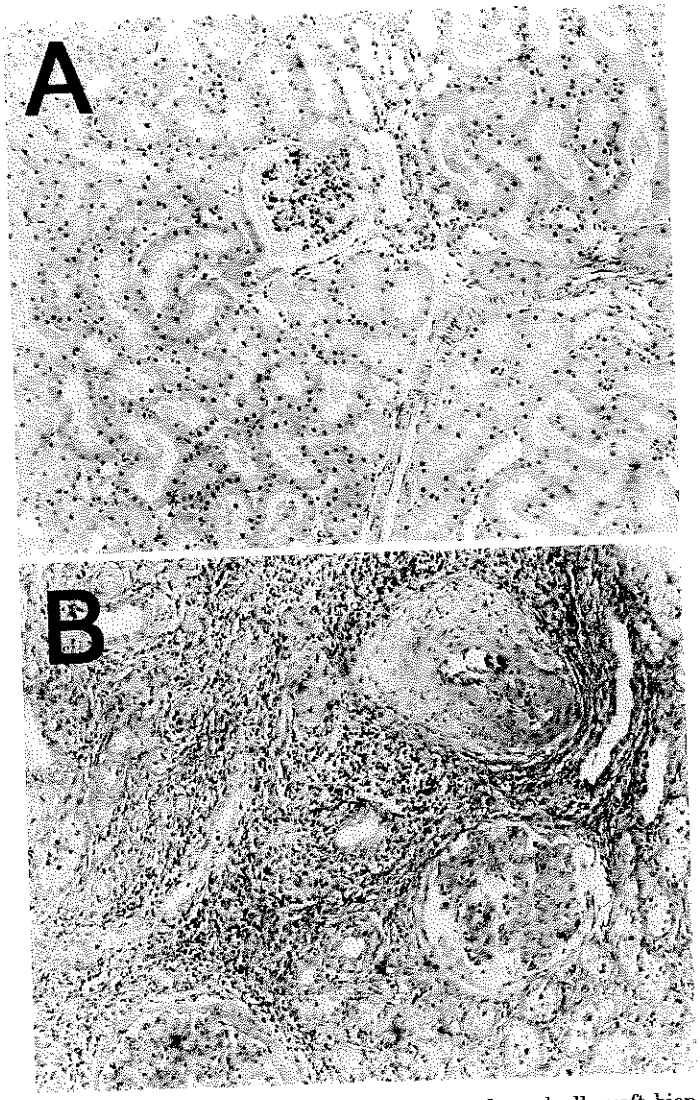

FIGURE 6. Hematoxylin and eosin staining of renal allograft biopsies. (A) Tolerant animal 10736: kidney at day 540 after KTx showing normal vessel, glomerulus, and tubules (×125). (B) Rejector animal 10807: kidney at day 120 after KTx showing severe rejection with vascular, glomerular, and tubular injury (×125).

murine KL seemed to nearly triple the rate of transduction into swine myeloid progenitors, consistent with results reported for human in vitro models with human IL-3 and KL (*29*).

There are several possible explanations for the high rate of transduced cells observed in this study compared with the results reported for other in vivo large animal models (*36*, *37*) and clinical trials (*38*, *39*). First, all of the animals used in this study were relatively young, ranging from 13 to 17 weeks of age. Previous studies have demonstrated that BMC from miniature swine in this age range are enriched for myeloid progenitors (*16*), and thus may contain better or more enriched targets for transduction. Second, the transduction cultures used in this study were carried out at a lower temperature, 33°C, than the 37°C commonly used by others (*37*). These conditions were chosen both to optimize the survival of HSC and to increase the half life of the virus particles from approximately 4 hr to approximately 24 hr (*40* and S. Shulman, unpublished results). Finally, the marrow cells were maintained ex vivo for less than 48 hr to assure the survival of a significant number of repopulating HSC (as confirmed by the efficient rate of engraftment; Table 1), and the recipients were conditioned with a fully myeloablative preparative regimen to minimize the chances that residual, untransduced HSC would compete with transduced cells for reconstitution and thus decrease the fraction of transduced cells by dilution.

Once integrated, DRB recombinant proviral sequences seemed to be expressed in vivo for the duration of the study. The efficient transcriptional expression of the transduced DRB cDNA could be attributed in part to the use of a double-copy vector configuration, which generates a second copy of the DRB cDNA transcriptional cassette outside of the LTR transcriptional boundaries thought to be responsible for transcriptional quiescence in mice (*41*, *42*), and the use of the ubiquitously active Tk promoter. The fact that viral LTR-driven expression was also maintained for the duration of the study, as indicated both by the Northern analysis of WBC and the drug selection analysis of BM progenitors, suggests that the mechanism for LTR-based transcriptional quiescence observed in mouse models, methylation of CpG units within the LTR (*41*, *42*), may not present a significant problem in this large animal model.

Because our earlier studies indicated that tolerance could be induced to renal allografts by a 12-day course of CsA uniformly only when class II antigens were matched (*4*, *18*), we anticipated that both DQ and DR antigens might have to be transduced into BM stem cells for this genetic engineering approach to fully succeed. However, because no recombinants between DR and DQ are available, we had never tested the effect of matching only for one or the other of these class II antigens, and it therefore remained possible that matching for only one of the two loci might suffice. We decided to test the effect of DR gene transfer on tolerance induction in the renal transplantation model. The first three animals tested by renal allografts all rejected. In animal 10660, rejection occurred by day 8 after KTx, suggesting that expression of the transferred allogeneic gene products may have led to sensitization, rather than to tolerance. However, no antidonor type antibodies were detected in the serum of this animal by days 6 and 8. Alternatively, early rejection in this animal may have been due to unusually low serum levels of CsA during the 12-day treatment period of 347±37 ng/ml, compared with the established range of 400–800 ng/ml, previously demonstrated to be required for tolerance induction in this model (*4*). Two other DRB-engineered pigs (animals 10680 and 10697) rejected their renal allografts in a time frame consistent with that expected for full MHC plus minor mismatched transplants treated with a high-dose of CsA for 12 days (reviewed in *3*), suggesting that the gene transfers performed 5 months earlier had achieved no lasting effects on the immune system.

Animal 10736, on the other hand, responded to the fully mismatched renal transplant as though it were matched for class II, becoming tolerant after the 12-day course of CsA. In fact, this animal has not shown any signs of rejection during more than 2.5 years of follow-up. In assessing differences between animal 10736 and the other two genetically engineered animals (10680 and 10697) that might account for this qualitative difference in results, we consider the fact that the transduction of BMC of animal 10736 was carried

out in the presence of growth factors (PIXY-321 and the murine stem cell factor) as the most likely explanation. The percentage of G418-resistant colony-forming units observed after transduction was clearly higher in this animal than in the others that rejected their grafts (Table 2). We hypothesize that these higher transduction rates led to increased levels of DR gene expression, passing the threshold required for tolerance induction.

Another implication of these results is that matching for a single class II gene may be sufficient for induction of tolerance in our CsA treatment protocol. Thus, the mechanism by which tolerance is induced may require, in the donor organ, the presence of a class II antigen shared with the recipient rather than the absence of any nonshared antigens. If the mechanism of spontaneous tolerance induction to vascularized allografts is similar in this respect, such a hypothesis may also explain the discrepancy between the data of Calne et al. (*43*) and our own on the requirement for class II matching. In our studies, spontaneous tolerance to renal allografts developed only when transplants were performed between class II matched animals (*44*), whereas Calne et al. (*43*) observed tolerance to liver allografts between some outbred swine that were mutually stimulatory in MLR, suggesting class II mismatch. If only some class II need to be matched for tolerance induction, then the data of Calne et al. (*43*) could be explained by the sharing of one class II gene allele but mismatching for another, which could lead to a positive MLR.

In summary, we consider these results very encouraging for the eventual use of genetic engineering as an approach to transplantation tolerance. By confirming the in vivo efficacy of these vectors and transduction schemes, these studies also serve as a basis for future testing of alternative vectors and transduction schemes in this genetically defined, preclinical large animal model. In this first study, we chose to use retrovirus-mediated transfer of allogeneic MHC class II DR cDNA both because: 1) clinical studies have indicated that DR matching in humans correlates with long-term survival of transplanted vascularized organs (*45*, *46*); and 2) expression of an allogeneic DR antigen in class II committed cells should require transfer of only the β chain gene, because the α chain sequence of the heterodimer is invariant in swine (*47*) as it is in man (reviewed in *48*). Additional studies on the transfer of DQ allelic genes have required the construction of a multigenic virus, because both the DQA and DQB sequences are polymorphic (*49*, *50*), and these studies are in progress. In addition, to render the approach more clinically acceptable, we are currently exploring the use of milder conditioning regimens.

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