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Immunology

Expression in transgenic mice of class I histocompatibility antigens controlled by the metallothionein promoter

(major histocompatibility complex/tolerance/hepatitis)

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ABSTRACT **To study the effects of increased expression of major histocompatibility complex class I molecules on the development of self-tolerance, transgenic mice were produced that expressed the *H-2K*^{b} gene under the control of the metallothionein promoter. Administration of zinc enhanced transgene expression in liver, kidney and exocrine pancreas. No evidence suggestive of an autoimmune response was found in transgene-expressing tissues in mice otherwise allogeneic to *H-2K*^{b}. Despite this lack of responsiveness *in vivo*, T cells could be stimulated *in vitro* to lyse *H-2K*^{b}-bearing target cells. No infiltration was detected in transgenic mice after irradiation and reconstitution with bone marrow cells. When spleen cells were used for reconstitution, however, dense lymphocytic infiltration was seen, particularly in the portal tracts of the liver, and this was accompanied by piecemeal necrosis and apoptosis of periportal hepatocytes. This aggressive response progressively diminished with time, and by 12 weeks after reconstitution many of the portal tracts were free of infiltration while the others showed no accompanying necrosis. The picture at this stage was similar to that seen in chronic persistent hepatitis. These results suggest that, in addition to negative selection in the thymus, peripheral mechanisms not involving clonal deletion or permanent clonal anergy can prevent immune responses to self molecules.**

Class I and class II major histocompatibility complex (MHC) antigens are cell membrane glycoproteins that function by presenting protein fragments to antigen-specific T cells (1, 2). They may also be recognized as foreign by alloreactive T cells, which occur at high frequencies (3, 4) and cause rapid rejection of grafts between individuals differing at the MHC.

Various mechanisms have been proposed to account for the imposition of tolerance to MHC molecules. Compelling evidence is mounting in favor of the hypothesis (reviewed in ref. 5) that thymocytes reactive to self-MHC are deleted (6–9). If negative selection operates on those thymocytes that encounter antigen in the thymus, how is tolerance imposed to molecules unique to extrathymic tissues?

To address this question, it would be advantageous to have a system in which strong antigens, such as MHC molecules, could be expressed extrathymically and without the complications associated with grafting tissues. This may be achieved by using transgenic mice (10), which offer many advantages in investigating immunological self-tolerance (11), as when the rat insulin promoter was used to direct the expression of MHC molecules to pancreatic beta cells (12–14). Overexpression of these molecules led to beta-cell death despite the absence of immune involvement (13, 14). Although the transgene product was undetectable in the thymus, the mice were tolerant.

To study further the effects of MHC molecules on the cells that express them and on the development of self-tolerance, we used the zinc-inducible metallothionein (MT) promoter to direct expression of the class I H-2K^b molecule to various cell types including hepatocytes that express H-2 antigens at levels undetectable by immunohistological methods (15). The sheep (rather than mouse) MT promoter (sMTp) (16) was used, given its lower basal expression and unresponsiveness to glucocorticoids (17).

MATERIALS AND METHODS

Mice. Mice were obtained from the specific pathogen-free colonies of The Walter and Eliza Hall Institute or from the Animal Resources Centre (Perth, Western Australia). Irradiation (750 R; 1 R = 0.258 mC/kg) was administered from a ^{60}Co source.

Production of Transgenic Mice. The sMTp was isolated as an *Eco*RI/*Bam*HI fragment from pMT010/A$^+$, provided by Choo *et al.* (16); this fragment was blunted, coupled to *Sal* I linkers (Promega Biotec), and subcloned into pK^{b}6 (14) upstream of the genomic *H-2K*^{b} gene (18). The resulting sMTp-K^b construct was purified after *Eco*RI/*Hin*dIII digestion and injected into (C57BL/6 × SJL)F_2 eggs (10). Progeny were screened at weaning by hybridizing tail blot DNA to a ^{32}P-labeled sMTp probe. Molecular biology methods were as described (19).

Histology. Organs were fixed in buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin. Staining for *H-2K*^{b} was performed with the monoclonal antibody B8-24-3 (20) as described (14).

Cytolytic T-Lymphocyte (CTL) Assays. Spleen cells from transgenic *H-2*^{s} mice or their nontransgenic littermates were cocultured with irradiated (2000 R) B10.A(5R) (*K*^{b}*D*^{d}) spleen cells in RPMI 1640 medium supplemented with 10% fetal calf serum and 2-mercaptoethanol. No lymphokines were added. After 4 days, the cultures were harvested, counted, and added to two round-bottom microtiter plates in 1:2 dilutions (each in triplicate) from an effector/target cell ratio of 50:1. *H-2K*^{b} (EL4) or *H-2D*^{d} (P815) target cells were labeled with ^{51}Cr (New England Nuclear) and added to each plate. After 4.5 hr, 100 μl of supernatant was collected from each well and counted. The following standard formula was used: % specific lysis = (cpm of test wells − cpm of background wells)/(cpm of detergent-treated wells − cpm of background wells) × 100.

Skin Grafts. Transgenic mice of the 43-5 line were mated with B10.H-2^{bm1} (bm1), which differs from the congenic C57BL/10 strain only at the *H-2K* locus. Progeny were screened for the transgene and grafted with *H-2K*^{b}-bearing C57BL/10 skin (21).

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Abbreviations: MHC, major histocompatibility complex; MT, metallothionein; sMTp, sheep metallothionein promoter; GVHD, graft-versus-host disease; CTL, cytotoxic T lymphocyte; CAH, chronic active hepatitis; CPH, chronic persistent hepatitis.

RESULTS

Specificity of Transgene Expression. Five transgenic founders, identified from a total of 37 mice produced as described above were backcrossed to SJL and their homozygous *H-2*^{s} progeny were characterized. Various organs were collected from transgenic mice that were either uninduced or had received an injection of $ZnSO_4$ 24 hr previously. RNA was extracted and subjected to Northern blot hybridization with a transgene-specific oligonucleotide (14). Of the five lines, two did not express the transgene, one had a high constitutive level of expression, and the other two had Zn-enhanced transcription from the sMTp-K^b gene (Fig. 1*A*). Similar tissue specificity of transgene expression was observed in these two lines, as shown (Fig. 1*B*) for the 43-5 line, which was chosen for further experiments. The highest levels of transgene-derived RNA were found in the liver of the Zn-treated mice and expression was also induced in the pancreas and kidney. Liver and kidney showed some constitutive expression. Very low level transgene expression was observed in the thymus while other organs, such as heart, lung, and spleen, had low to undetectable levels.

Production of *H-2K*^{b} Molecules in Transgenic Mice. Tissues from Zn-treated and from uninduced transgenic mice were analyzed immunohistochemically with B8-24-3 (20). Staining was observed in those tissues that had sMTp-K^b transcripts (Fig. 2), indicating that there was no posttranscriptional impediment to synthesis of the transgene product. The liver was the only site of detectable *H-2K*^{b} membrane expression in the uninduced transgenic mice. Zn treatment induced *H-2K*^{b} in the proximal tubules of the kidney and exocrine pancreas (but not in the islets) and increased it in the liver. All the hepatocytes stained, with the reaction product localized to the sinusoidal and lateral domains of the plasma membranes. No staining of these cells was observed on tissues from Zn-treated nontransgenic *H-2*^{s} littermates or with control monoclonal antibody on transgenic tissues.

Further evidence for membrane expression was obtained from experiments in which transgenic mice, control nontransgenic littermates, and C57BL/6 mice were irradiated and reconstituted with *H-2K*^{b}-primed spleen cells—i.e., with cells from SJL mice injected previously with C57BL/6 splenocytes. Signs of graft-versus-host disease (GVHD) were not seen in nontransgenic mice but were apparent in both the transgenic and control C57BL/6 mice. The latter showed classic GVHD (22): dense lymphocytic infiltration of portal tracts, piecemeal necrosis and apoptosis of periportal hepatocytes, lymphocytes infiltrating the biliary epithelium, and endothelialitis of both the hepatic and portal venules; there was minor lobular apoptosis. In contrast, the livers of transgenic mice showed only some of the above changes (Fig. 3*B*): marked hepatocellular death, intense lobular infiltration, focal necrosis, and more extensive periportal necrosis. Damage to the bile ducts and vasculature was diminished or absent. Therefore, the transgene product was both accessible to T cells and presented in a recognizable form.

Effect of Transgene Expression on Responsiveness to *H-2K*^{b}. Tissues from transgenic mice given Zn were examined as an initial approach to investigating the effects on the immune system of otherwise allogeneic class I molecules. Even after >5 months of dietary Zn, the transgene-expressing cells appeared normal. This contrasted with the deleterious effects of class I overexpression in pancreatic β cells (14). No lymphocytic infiltrate was detected in the tissues of the induced transgenic mice, which, at this operational level, were tolerant of *H-2K*^{b}. This tolerance was not abrogated by a variety of maneuvers, including treatment with cyclophosphamide, immunization with C57BL/6 splenocytes, and administration of exogenous interleukins (results not shown). Definitive evidence of tolerance to *H-2K*^{b} came from skin graft experiments. Transgenic 43-5 mice were crossed to bm1 and their offspring were grafted with C57BL/10 skin. Typical of a class I difference, all the nontransgenic littermates rejected their grafts, while the transgenic mice accepted theirs (Table 1).

Lymphocytes from transgenic mice were tested for their responsiveness to *H-2K*^{b}-bearing cells *in vitro*, by stimulation with B10.A(5R) (*K*^{b}*D*^{d}) splenocytes. CTLs were assayed for ability to lyse *H-2*^{b} and *H-2*^{d} target cells, the response to *H-2D*^{d} serving as an internal control. CTLs generated from transgenic spleen were equally as effective as nontransgenic CTLs in lysing *H-2K*^{b} targets (Fig. 4). Hence, tolerance to the transgene product was apparent *in vivo* but not *in vitro*.

Reconstitution of Irradiated Transgenic Mice with Bone Marrow or Spleen Cells. To examine the basis for this *in vivo* specific unresponsiveness, spleen cells or T-cell-depleted bone marrow from SJL mice were used to reconstitute irradiated recipients. Whether Zn-induced or not, no evidence of GVHD was seen in mice receiving the bone marrow

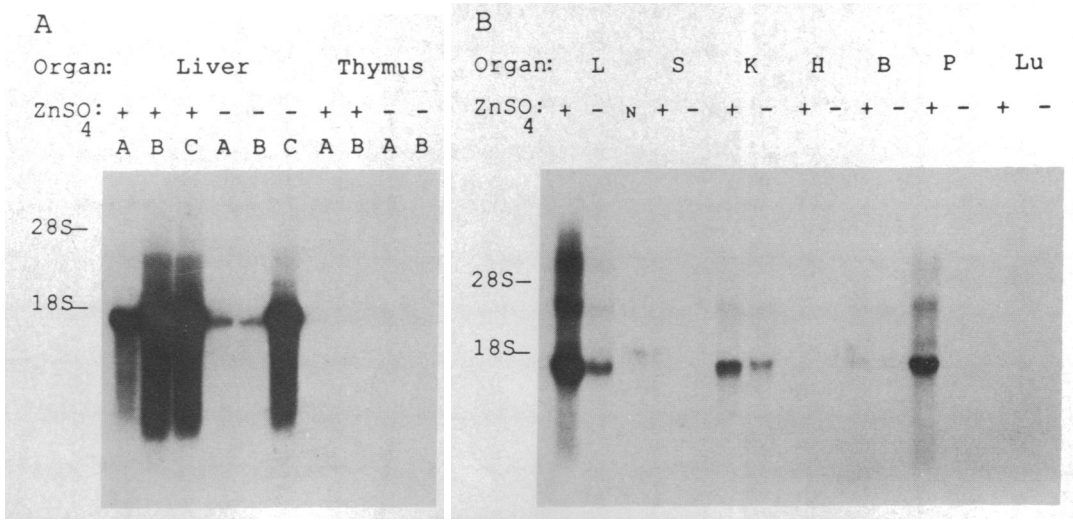

FIG. 1. Transcription of the sMTp-K^b transgene. Transgenic mice were either untreated (−) or were administered $ZnSO_4$ (+) at 5 mg/kg 16 hr before collection of the organs indicated. RNA was isolated, fractionated by denaturing gel electrophoresis, and transferred to nylon membranes, which were probed with a ^{32}P-labeled transgene-specific oligonucleotide (14). (*A*) Comparison of transgene expression in the liver and thymus of different transgenic lines. (*B*) Tissue distribution of transgene expression in the 43-5 line. L, liver; S, spleen; K, kidney; H, heart; B, brain; P, pancreas; Lu, lung; N, nontransgenic littermate mouse treated with $ZnSO_4$.

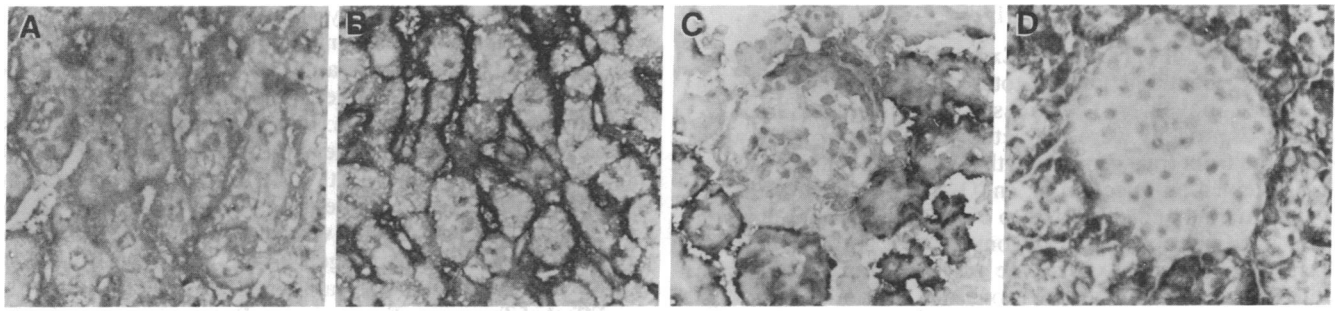

FIG. 2. Immunocytochemical detection of the *H-2K*^{b} transgene product in $ZnSO_4$-treated 43-5 transgenic mice. Frozen sections were prepared from various tissues and stained with control (*A*) or *H-2K*^{b}-specific monoclonal antibodies (*B–D*). (*A* and *B*) Liver. (*C*) Kidney. (*D*) Pancreas. (×280.)

inoculum. Thus, differentiation of nontransgenic T cells within the transgenic recipients resulted in an inability to respond to *H-2K*^{b}. In contrast, transgenic recipients of mature T cells (see below) developed GVHD lesions in those organs shown to express the transgene product (liver, kidney, and pancreas), while other organs including the thymus and

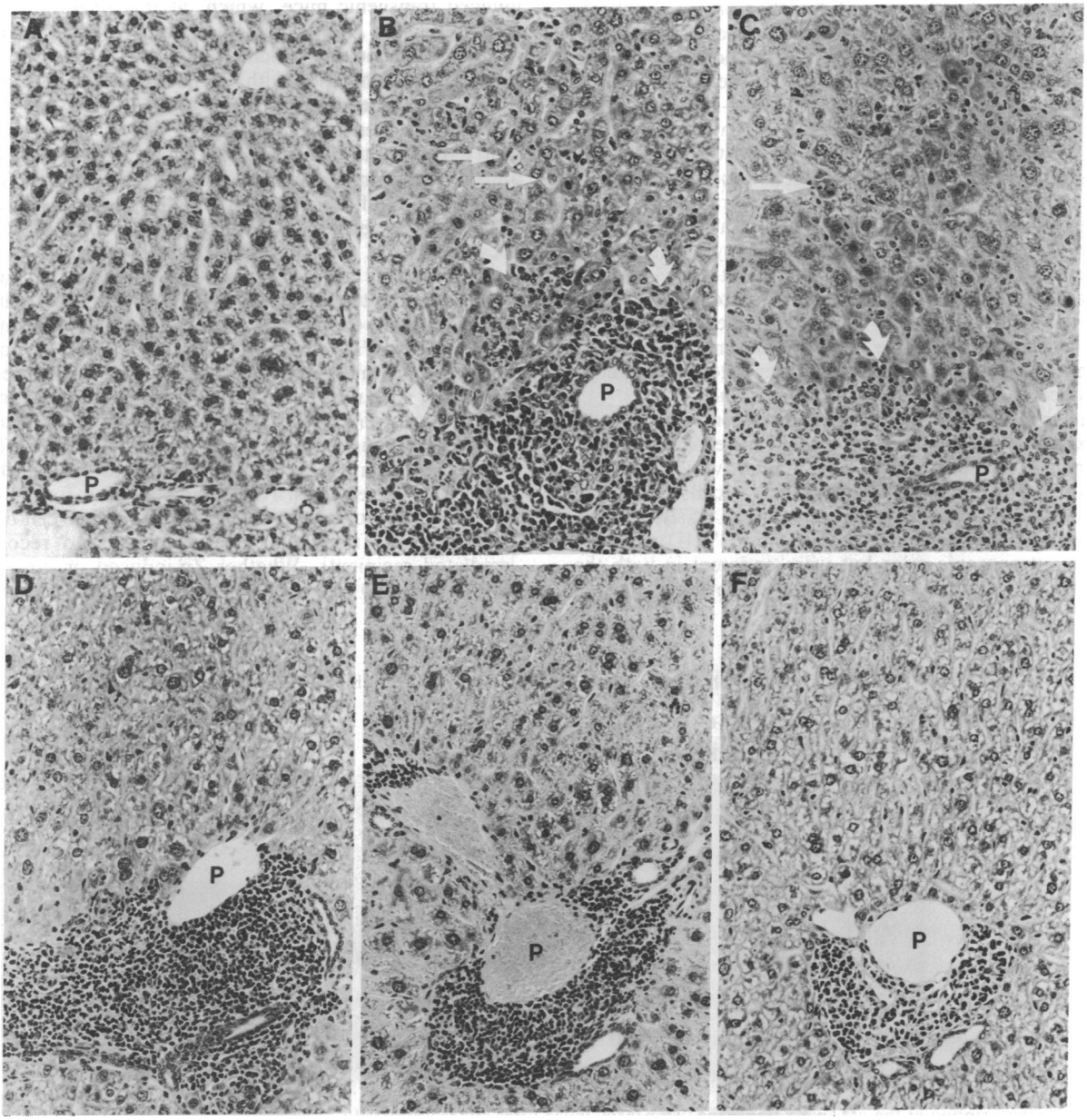

FIG. 3. Liver histology in spleen cell reconstituted transgenic mice. Irradiated *H-2*^{s} nontransgenic (*A*) and transgenic (*B–F*) mice were reconstituted with 3–5 × 10^7 spleen cells from SJL mice that had been previously immunized with C57BL/6 splenocytes (*B*) or from untreated SJL mice (*C–F*). Liver histology was examined at various times after reconstitution: 2 wk (*B*); 3 wk (*A* and *C*); 6 wk (*D*); 12 wk (*E*); 24 wk (*F*). P, portal tract; straight arrows, apoptotic bodies; curved arrows, piecemeal necrosis. (Hematoxylin and eosin; ×150.)

Table 1. Survival of C57BL/10 skin grafts on transgenic and nontransgenic mice

Mice	*n*	Graft survival, days <13	<16	>50
Transgenic	9	0	0	9
Nontransgenic	4	3	1	0

Transgenic 43-5 mice were crossed to bm1 and the progeny were grafted with C57BL/10 skin. Hair growth was noted on surviving grafts.

spleen were histologically normal after hemopoietic reconstitution.

Resolving Nature of the GVHD in Spleen Cell-Reconstituted Transgenic Mice. Irradiated transgenic and nontransgenic littermates were reconstituted with normal SJL spleen cells. Tissues were collected and examined at various times afterwards (Fig. 3). After 3 wk, the transgenic recipients showed evidence of GVHD regardless of induction status (Fig. 3*C*), unlike their littermate controls (Fig. 3*A*). While this GVHD was much less aggressive than in the primed-cell reconstituted mice, lobular hepatitis was apparent as evidenced by apoptosis, focal necrosis, and infiltrating lymphocytes. Mitotic figures indicated some degree of liver cell regeneration. Activated lymphocytes expanded the portal tracts, mediating piecemeal necrosis of the periportal hepatocytes, reminiscent of human chronic active hepatitis (CAH) (23). Although the infiltrates around the portal tracts had increased after a further 3 wk, the lobular infiltrates had decreased and there was less hepatocellular death (Fig. 3*D*). The histology resembled chronic persistent hepatitis (CPH) (23). At this time, most of the periportal infiltrating cells were Thy 1^+; both $CD4^+$ and $CD8^+$ T cells were observed (data not shown). Many of the portal tracts were free of lymphoid infiltrates 12 and 24 wk after reconstitution (Fig. 3 *E* and *F*), although large numbers of small lymphocytes surrounded some portal tracts. Both piecemeal necrosis and lobular lymphocytic infiltration were greatly reduced or absent. These observations suggest that, despite a persisting infiltrate, tolerogenic mechanisms limited the immune response to the transgene-expressing cells.

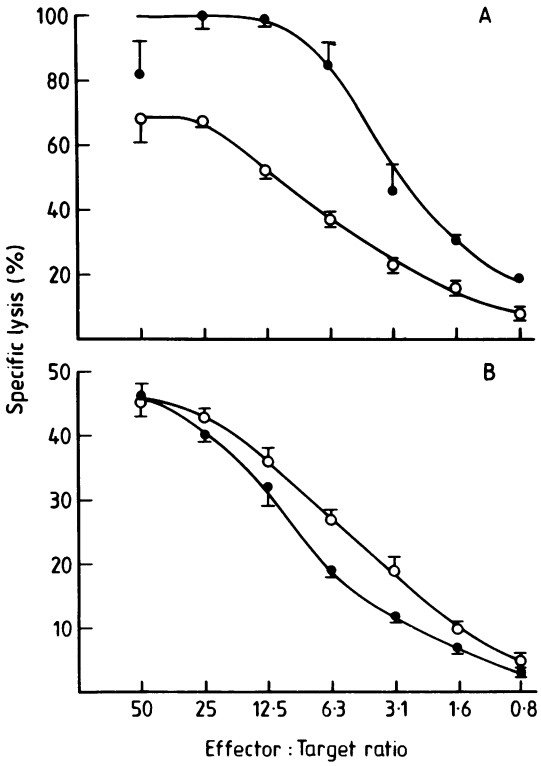

FIG. 4. Responsiveness of CTLs from transgenic mice. Spleen cells from transgenic (●) or nontransgenic littermate (○) mice were stimulated *in vitro* with irradiated B10.A(5R) spleen cells. After 4 days, the responder cells were collected and tested for their ability to lyse ^{51}Cr-labeled target cells. (*A*) P815, *H-2*^{d} target. (*B*) EL4, *H-2*^{b} target.

DISCUSSION

The sMTp directed expression of the transgene faithfully, as specific RNA was found in those tissues that synthesize MT (24) and *H-2K*^{b} was found in the same cell types shown to produce MT (25): hepatocytes and renal tubular cells. In addition, exocrine pancreas expressed the transgene (Fig. 2).

There are marked differences between the sMTp-K^b mice and those expressing MHC molecules in pancreatic β cells, which showed dysfunction and loss of these cells at an early age (12–14). By contrast, despite the elevated production of *H-2K*^{b} in the liver of the Zn-treated sMTp-K^b mice, no adverse effect was noted even after many months, strengthening the conclusion (12–14) that the pancreatic β cells are unusually sensitive to the effects of overexpression of MHC molecules. A further difference between these transgenic mice was in their immunological response to the transgene product. Unresponsiveness was evident in the latter group both *in vivo* and *in vitro* (refs. 12–14; G.M., unpublished data). Although no lymphocytic infiltration was seen in sMTp-K^b mice and they did not reject *H-2K*^{b} grafts, they mounted unimpaired CTL responses to *H-2K*^{b} targets *in vitro*. Thus, there may be more than one mechanism whereby tolerance can be imposed upon T cells reactive to self molecules expressed on nonlymphoid cells in the periphery. Neither deletion nor permanent silencing of reactive clones can, however, account for the tolerant state of the sMTp-K^b mice. Unresponsiveness *in vivo* but not *in vitro* has been observed previously in other systems, although these have not involved the responses of intact animals to authentic self antigens but have used radiation chimeras (5), surgically created chimeras in frogs (26) and chickens (27), and kidney transplants in "tolerized" rats (28).

Of the many possible mechanisms that could be invoked to establish tolerance to peripheral antigens, some can be eliminated as explanations for the current model. For example, the "veto effect" (29) is unlikely to occur because the transgene is not expressed in lymphoid organs. Furthermore, irradiated transgenic mice reconstituted with nontransgenic bone marrow cells displayed no signs of GVHD. Another possible mechanism involves the postulated "second signal" required to activate T cells; its absence leads to tolerance, so that removal of resident leukocytes from grafted tissues enables transplantation across MHC barriers, but subsequent immunization with donor leukocytes leads to graft rejection (reviewed in ref. 30). Lack of a second signal cannot explain the tolerance in the sMTp-K^b mice because immunization with C57BL/6 spleen cells did not induce lymphoid infiltrates into transgene-expressing tissues.

When irradiated transgenic *H-2*^{s} mice were injected with SJL splenocytes, lymphoid infiltration was observed in the liver and to a lesser extent in the kidney and pancreas. Amongst the infiltrating lymphocytes were $CD8^+$ cells, as expected of a response to class I antigens (31). The additional presence of $CD4^+$ cells may be secondary, due to presentation of antigens released by cells lysed by the original $CD8^+$ CTLs. Another possibility is that the $CD4^+$ cells may themselves have an ameliorating effect on the immune response. In this context, it has been shown (31) that injection of purified $CD8^+$ cells with large numbers of $CD4^+$ cells into class I disparate recipients prevented lethal GVHD.

The liver infiltrate was accompanied by focal hepatocyte necrosis as in classic GVHD. The reaction became less severe with time so that by 12 wk after reconstitution, there were only small lymphocytes in the portal tracts without lobular infiltration. It is remarkable that even such an aggressive response as that to class I antigens, which often leads to lethal GVHD (31), can be curtailed. This suggests that if a graft could survive the early ravages of the immune response, tolerogenic mechanisms may be invoked for its survival. Survival of the transgene-expressing tissues would be enhanced by the lack of expression of *H-2K*^{b} by the vascular endothelium and by the regenerative capacity of liver cells.

The reconstituted transgenic mice had liver histology similar to that of human chronic hepatitis (23), a disease of unknown pathogenesis showing a varied range of expression. Shortly after reconstitution, the pathology resembled that of CAH and, with time, CPH. This raises the possibility that individuals with CPH are in the course of resolving an autoimmune response by mechanisms similar to those operating in our transgenic mice. Thus, an insult to the liver may result in the expression of molecules that may be recognized by T cells of patients in whom tolerance is defective. In some of these individuals, as in the transgenic model, tolerogenesis may result in CPH. Otherwise, the lesion will continue to develop as CAH. From studies on human CAH, there is evidence for an immunoregulatory defect (32) but there has been no valid animal model for this disease. The reconstituted sMTp-K^b mice may provide a system for investigating the potential immunoregulatory defect leading to the human disease.

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