Levels of Peripheral T Cell Tolerance Induced by Different Doses of Tolerogen

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Antigen-specific immunosuppression requires an understanding of the parameters that control peripheral T cell tolerance. A liver-specific inducible promoter was used to drive the expression of the major histocompatibility complex antigen K^b in transgenic mice. Minute amounts of K^b, expressed exclusively on hepatocytes, induced tolerance by partial down-regulation of the T cell receptor (TCR) on the self-reactive $CD8^+$ cells. Contact of these tolerant T cells with high concentrations of K^b after induction led to complete down-regulation of TCR. Thus, tolerant T cells are susceptible to further tolerogenic signals and reach different levels of tolerance depending on antigen dose.

Self-tolerance is achieved mainly by deletion of autoreactive T cells during development in the thymus (*1*). Whether and how tolerance is established in mature peripheral T cells is of importance to antigen-specific immunosuppression for autoimmune disorders and transplantation (*2*). There have been several studies in transgenic mice in which nonthymic cells express a "foreign" protein under the control of tissue-specific promoter elements. In some of these studies tolerance was induced (*3–5*), whereas in others the tissue-specific antigen had no effect on the immune system; the outcome depended on the nature of the antigen and the site of expression (*6*). Thus, the consequences of extrathymic antigen expression are difficult to predict. An understanding of peripheral tolerance requires experimental systems in which all but one of the important parameters are kept constant. Here we describe a mouse system in which the major histocompatibility complex (MHC) class I antigen K^b is expressed exclusively on hepatocytes under the control of the inducible promoter of the human complement-reactive protein (CRP). The influence of antigen concentration on peripheral tolerance induction can be studied in this system since lipopolysaccharide (LPS) treatment enhances K^b expression. Thus, the issue of whether tolerant T cells that have already been exposed to small amounts of antigen are susceptible to further tolerizing signals by encounter with high levels of antigen can be addressed.

CRP is an acute phase reactant secreted exclusively by hepatocytes 15 hours after induction with LPS (*7*). Liver-specific K^b expression in CRP-K^b transgenic mice was extremely low before induction; it could be detected by mRNA analysis using the highly sensitive reverse transcription polymerase chain reaction (RT-PCR) (Fig. 1A), but not by immunohistology or cytofluorimetry of hepatocytes, regardless of the age of the mice (*8*). After induction, strong expression was found in the liver but not in the thymus or any other organ (Fig. 1A). The expression pattern was confirmed on the protein level by staining of isolated hepatocytes (Fig. 1B) and by immunohistology. This is in agreement with previous studies on CRP transgenic mice (*7*). The untreated CRP-K^b mice (H-2^{dxk}) were tolerant to K^b. They accepted skin grafts from transgenic CBA mice (H-2^k) expressing the genomic K^b gene (CBK mice) (*9*), whereas nontransgenic littermates rejected the grafts (see Table 1). This suggests that the extremely low amounts of antigen expressed on hepatocytes are sufficient to induce and maintain tolerance.

CRP-K^b mice crossed with mice transgenic for a TCR against K^b (Des-TCR), which contain large numbers of K^b-reactive cells, were also tolerant. It was important to determine that the tolerance was induced in the periphery by K^b on hepatocytes and not by minute amounts of K^b in the thymus that may have escaped detection. To this end, adult thymectomized CRP-K^b transgenic mice were grafted with the thymi of nontransgenic newborn mice of the same H-2 haplotype, irradiated, and reconstituted with bone marrow cells from Des-TCR mice (atxbm chimeras). These chimeric mice accepted K^b-positive skin grafts (Table 1). Although no K^b expression was detectable on bone marrow cells of untreated and LPS-treated CRP-K^b mice, we wanted to exclude the possibility that in the chimeras potentially K^b-positive bone marrow cells from the CRP-K^b mice could have repopulated the nontransgenic thymus, thereby leading to thymic tolerance induction. DBA/2 × CBA F_1 (H-2^{dxk}) mice were lethally irradiated, reconstituted with bone marrow of different origin, and skin grafted 6 to 8 weeks later (Table 1). Mice reconstituted with bone marrow of Des-TCR (H-2^{bxk}) origin accepted K^b-bearing skin grafts, because bone marrow–derived K^b-positive cells in the thymus deleted the K^b-reactive T cells. In contrast, reconstitution with bone marrow of either Des-TCR (H-2^{dxk}) or CRP-K^b × Des-TCR (H-2^{dxk}) mice led to skin graft rejection. These results demonstrate the absence of K^b-positive cells in the bone marrow of CRP-K^b ×

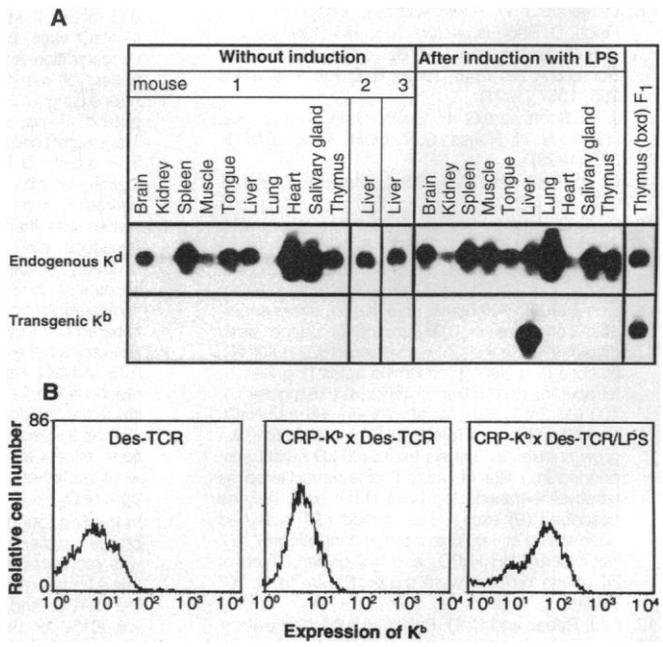

Fig. 1. Inducible liver-specific expression of K^b in CRP-K^b transgenic mice. (**A**) PCR analysis of reverse-transcribed mRNA isolated from various organs of CRP-K^b transgenic mice (H-2^{dxk}) in the absence (left) and presence (right) of induction by LPS (*19*). One out of three experiments is shown. In addition, mRNA from the liver of five other uninduced CRP-K^b (H-2^{dxk}) transgenic animals has been analyzed in the same way. Two of these experiments are also depicted (mouse 2 and 3, left). (**B**) Surface expression of K^b on hepatocytes of Des-TCR single-transgenic mice, CRP-K^b × Des-TCR double-transgenic mice without and with LPS treatment as described in (A). One out of five experiments is shown. The protocols for isolation and staining of hepatocytes are given in (*20*).

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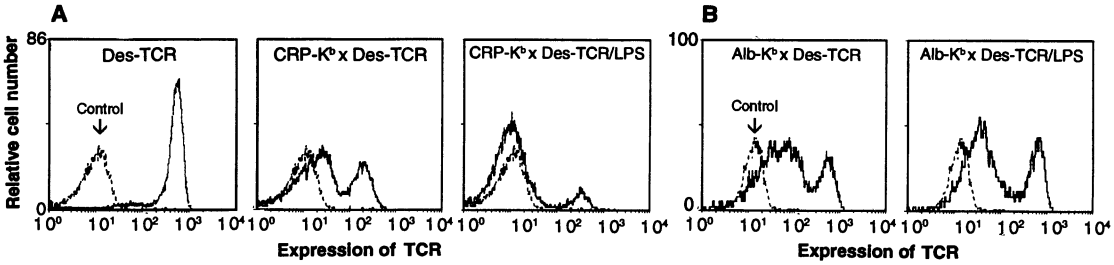

Fig. 2. The degree of TCR down-regulation is controlled by the amount of K^b antigen. Splenocytes from Des-TCR, and CRP-K^b × Des-TCR without and with induction by LPS (as indicated in Fig. 1) were depleted of B cells and labeled with monoclonal antibodies specific for CD2 (as a T cell marker), CD4, and TCRαβ. The TCR expression of $CD2^+CD4^-$ cells was then compared to that of $CD2^-CD4^-$ cells as an endogenous negative control (**A**). The decrease in the density of TCR molecules on the cell surface and the simultaneous increase in the number of T cells with strongly down-regulated TCR after treatment with LPS was observed only in CRP-K^b × Des-TCR but not in Alb-K^b × Des-TCR animals (**B**). The histograms shown are representative of seven similar experiments. For each graph, 5000 events were collected. The reagents used are described in (*21*).

Des-TCR mice. Therefore, the observed tolerance in the atxbm-CRP-K^b chimeras shows that K^b expressed exclusively on hepatocytes can lead to tolerance without any antigen-specific contribution by the thymus.

The CRP-K^b × Des-TCR mice were used to study the influence of antigen concentration on peripheral tolerance induction. In previous studies, use of the albumin promoter to direct K^b expression in the liver caused down-regulation of the TCR on peripheral K^b-reactive cells (*4*). We therefore investigated TCR expression on peripheral T cells in the CRP-K^b × Des-TCR mice before and after LPS induction. Since down-regulation of TCR is often accompanied by down-regulation of CD8 (*4*), CD2 was used as a marker for T cells and TCR expression measured on $CD2^+CD4^-$ cells, which contain the CD8 population. As a negative control, the $CD2^-CD4^-$ population was included (dotted line in Fig. 2). In Des-TCR single transgenic mice, 88% of the $CD2^+CD4^-$ T cells showed high expression of TCR molecules (Fig. 2A). In contrast, in uninduced CRP-K^b × Des-TCR mice only 30% of the $CD2^+CD4^-$ T cells expressed normal amounts of TCR molecules, and the remaining cells had reduced levels (Fig. 2A). These data demonstrate that minute amounts of K^b on hepatocytes are sufficient to cause down-regulation of the TCR. After induction of the K^b antigen with LPS, the fraction of T cells with normal TCR expression was further decreased, to 12% (Fig. 2A); the remainder had only background staining with the antibody to TCR, indicating complete down-regulation of the TCR. These data suggest that increased expression of K^b on the hepatocytes led to a more profound down-regulation of the TCR on the tolerant T cells. After LPS induction there was a decrease in the $CD2^+CD4^-$ population in comparison with $CD2^+CD4^+$ and $CD2^-CD4^-$ cells in CRP-K^b × DES-TCR animals (reduction of 30 to 50% of $CD2^+CD4^-$ cells in 70% of the animals tested). No such reduction of $CD2^+CD4^-$ cells occurred in LPS-treated control animals (Alb-K^b × DES-TCR; see below). These results are compatible with the proposal that additional contact with the K^b antigen results in deletion of some of the tolerant T cells. We confirmed these data in the CRP-K^b-atxbm chimeras after induction of K^b antigen with LPS.

Table 1. Acceptance of K^b-bearing skin grafts by CRP-K^b chimeric mice (*23*). Treatment 1: Transgenic mice were grafted with skin from CBA mice transgenic for the K^b-antigen (CBK) (*9*). Treatment 2: Bone marrow cells of CRP-K^b × Des-TCR mice did not enable DBA/2 × CBA F_1 mice to accept skin grafts.

Recipient H-2^{dxk}	Donor for bone marrow reconstitution	Number of mice/total	Survival time (days)
	Treatment 1		
CRP-K^b	–	11/11	>60
Des-TCR	–	0/10	8 ± 2
CRP-K^b × Des-TCR	–	7/7	>60
CRP-K^b-atxbm chimera	Des-TCR (H-2^{dxk})	6/6	>60
	Treatment 2		
(DBA/2 × CBA) F_1	Des-TCR (H-2^{dxk})	0/7	8 ± 2
(DBA/2 × CBA) F_1	Des-TCR (H-2^{bxk})	7/7	>60
(DBA/2 × CBA) F_1	CRP-K^b × Des-TCR (H-2^{dxk})	0/8	8 ± 2

To strengthen these conclusions, we performed several controls. First, it had to be excluded that the complete down-regulation of TCR molecules was caused by an indirect effect of LPS on tolerant T cells, possibly via a lymphokine cascade initiated by B cells. For this purpose, transgenic mice were used with constitutive K^b expression on hepatocytes under the control of the albumin promoter. Injection of LPS into Alb-K^b × Des-TCR mice had no effect on the constitutive expression of the K^b antigen, the fraction of T cells with down-regulated TCR, or the level of TCR expression (Fig. 2B).

It is important to know whether the increased K^b expression caused the already tolerant T cells to completely down-regulate their TCR or whether the down-regulated cells observed after induction were recent thymic emigrants that immediately encountered large amounts of K^b on hepatocytes. Thymectomized adult CRP-K^b × Des-TCR transgenic mice were assessed for TCR down-regulation before and after induction, and the same results were obtained as for the nonthymectomized animals, excluding a contribution by fresh thymic emigrants (Fig. 3). Both an antibody to TCR

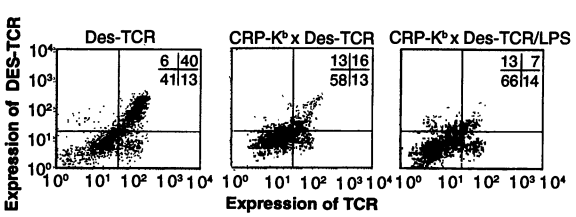

Fig. 3. Down-regulation of TCR molecules on Des-TCR^+ but not on Des-TCR^- T cells in thymectomized adult CRP-K^b × Des-TCR mice. Splenocytes from Des-TCR as well as CRP-K^b × Des-TCR without and with induction by LPS (as indicated in Fig. 1) were depleted of B cells and triple-stained with mAbs specific for only the transgenic Des-TCR (vertical axis), all TCRαβ (horizontal axis), and CD4. In this cytoflourimetric analysis, only $CD4^+$ cells were gated out. Therefore, the Des-TCR and TCR expression pattern of $CD2^+CD4^-$ cells as well as $CD2^-CD4^-$ cells is seen. The latter population was negative for the TCR (see Fig. 2) and accounts for the relatively high percentage of TCR^- cells depicted here. For each dot plot, 10,000 events were acquired. The results depicted are representative of four similar experiments. The reagents used are as described in (*22*).

that reacts with all TCRαβ molecules and an antibody to clonotype that reacts with the transgenic TCR were used. The antibody to TCR and the Désiré-1 antibody to clonotype can simultaneously bind to the same TCR molecule. By double staining, the expression of the Des-TCR on anti-K^b–specific T cells and TCR expression on T cells of other specificities was compared. As shown in Fig. 3, only Des-TCR T cells down-regulated their receptors in the thymectomized animals; T cells with receptors of other specificities were unaffected. These experiments indicate that the rapid increase in K^b expression on the hepatocytes led to complete down-regulation of TCR on already tolerant cells. Four weeks after LPS injection, K^b levels on hepatocytes were low again and the level of Des-TCR was up-regulated, demonstrating the reversibility of the down-regulation.

Whereas in other studies the expression of small amounts of foreign antigen, including K^b, on pancreatic islet β cells was ignored by the immune system (6), expression of even lower amounts of K^b on hepatocytes in the uninduced mice rendered the T cells tolerant and partially down-regulated the TCR. It is possible that the different anatomical sites are responsible for this discrepancy. The use of thymus chimeras ruled out any contribution by the thymus. The rapid increase of K^b on hepatocytes was accompanied by a further down-regulation of TCR, possibly by deletion. These findings demonstrate not only that the dose of antigen influences the degree of tolerance on a single-cell level but also that tolerant T cells are not necessarily refractory to further contact with the tolerogen—they can still be driven into a deeper state of tolerance. Thus, tolerance may be the result of multiple interactions with antigen.

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8. K^b expression was monitored during ontogeny from 3 days until 4 months after birth. In none of the 15 untreated CRP-K^b animals tested were high levels of antigen expression found.
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19. LPS (10 mg) was injected intraperitoneally on days 1 and 3 (mRNA was isolated on day 4). The oligonucleotides used for amplification annealed to conserved H-2K allele sequences. After Southern (DNA) blot, PCR products derived from endogenous K^d (upper row) and transgenic K^b (lower row) were identified by specific oligonucleotides. Details of the PCR protocol have been published elsewhere (*3*). The CRP gene was within a 13-kb fragment containing 9 kb of the 5′ untranslated region. A 5.5-kb fragment containing the coding region of the K^b gene was inserted into the Eco RI site 5′ to the ATG of the CRP gene. This construct was used to establish CRP-K^b transgenic mice. To exclude elevated LPS-levels as a result of infection, we housed the CRP-K^b animals under specific pathogen-free conditions.
20. Hepatocytes were isolated after liver perfusion according to the method of Seglen (*10*). After staining with the biotinylated antibody to K^b (B8-24.3) (*11*) followed by fluoroscein isothiocyanate (FITC)–labeled streptavidin (Boehringer Mannheim, Germany), 10,000 events were collected in a FACScan Cytometer (Becton Dickinson).
21. For staining, the following antibodies were used: FITC-labeled anti-CD2 monoclonal antibody (mAb) RM2.-5 (*12*), phycoerythrin (PE)-conjugated anti-CD4 mAb GK1.5 (*13*), and biotinylated mAb H57-597 (*14*) to all β chains of TCRs. Cy-chrome–conjugated streptavidin (Pharmingen, San Diego) was used as a secondary reagent.
22. The FITC-conjugated mAb Désiré-1 (*15*) was used to detect the transgenic Des-TCR. Biotinylated mAb H57-597 (*14*) followed by Cy-chrome–conjugated streptavidin served as a pan-TCRαβ reagent, and the PE-conjugated mAb GK1.5 (*13*) was used for gating out $CD4^+$ cells.
23. Donor tail skin, including the epidermis and dermis, was transferred onto the tail of a recipient from which an appropriate piece of skin had been removed (*16*). If 75% of a graft was necrotic it was considered as rejected. To establish atxbm chimeras, we removed the thymuses of adult transgenic mice when the mice were 6- to 8-weeks-old under Ketanest-Rompun anesthesia using an adaptation of the method of Lo and Sprent (*17*). After 2 weeks the mice were grafted with two to four neonatal thymus lobes ($H\text{-}2^{dxk}$) under the kidney capsule followed 2 to 4 weeks later by lethal irradiation (9.5 Gy) and reconstitution with 5×10^6 T cell–depleted bone marrow cells as indicated [anti Thy-1.2 treatment with the specific antibody 30H12 (*18*) and complement-mediated lysis]. Eight weeks later the mice were skin grafted.

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Natural Vertical Transmission of Western Equine Encephalomyelitis Virus in Mosquitoes

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The mechanism by which western equine encephalomyelitis (WEE) virus and other mosquito-borne alphaviruses (Togaviridae) survive during periods of vector inactivity is unknown. Recently, three strains of WEE virus were isolated from adult *Aedes dorsalis* collected as larvae from a salt marsh in a coastal region of California. This provides evidence of vertical transmission of WEE virus in mosquitoes in nature. Vertical transmission in *Ae. dorsalis* and closely related mosquito species may be an important mechanism for the maintenance of WEE virus in temperate regions in North America where horizontal transmission of the virus is seasonal.

Western equine encephalomyelitis virus (Togaviridae: *Alphavirus*) has been recognized as a cause of summertime epidemics and epizootics of encephalitis in humans and horses, respectively, in the western United States and Canada since the 1930s (*1*, *2*). The summertime transmission cycle of WEE virus in rural agricultural areas of western North America principally involves *Culex tarsalis* mosquitoes as vectors and passerine birds as amplifying hosts (*1*). In temperate climates, annual transmission of the virus ends when vector mosquitoes cease blood-feeding in autumn, which coincides with a decrease in ambient temperature and daylight. There is evidence that WEE virus persists in endemic areas between transmission seasons (*1*, *2*). Howev-

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