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Immunology

A nondeletional mechanism of peripheral tolerance in T-cell receptor transgenic mice

(anergy/H-2 antigens/T lymphocytes)

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ABSTRACT **To investigate tolerance to extrathymic self molecules, we produced two groups of transgenic mice: one expressed the major histocompatibility complex molecule H-2K^b in pancreatic β cells, and the other expressed rearranged T-cell receptor genes encoding an anti-H-2K^b receptor. The transgenic T-cell receptor genes were shown to confer the correct specificity and to be expressed appropriately. T cells bearing this receptor were activated by H-2K^b *in vitro* and *in vivo*, and they underwent negative selection in mice expressing H-2K^b in the thymus. To determine the fate and function of these anti-H-2K^b T cells in mice expressing H-2K^b exclusively in the periphery, the two groups of transgenic mice were mated to produce double transgenic offspring. In these, transgene-expressing T cells were present in both thymus and periphery. Persisting T cells had not down-regulated either their antigen-specific receptors or their CD8 molecules. Despite the persistence of large numbers of potentially reactive T cells, the mice were tolerant of H-2K^b in that they could not reject H-2K^b-bearing skin grafts, although they did reject third-party grafts. The results show that peripheral T-cell tolerance, unlike that imposed in the thymus, does not involve deletion of T cells. The existence of T cells bearing receptors specific for self components raises the possibility that aberrant activation of such cells may lead to the development of autoimmune disease.**

The main mechanism by which tolerance is imposed upon self-reactive T cells operates intrathymically. It involves deletion of those cells that bear T-cell receptors (TCRs) with a sufficiently high avidity for antigens encountered within the thymus and presented by class I or class II molecules encoded by the major histocompatibility complex (1–4). Since not all self molecules can be expressed intrathymically, it is necessary to determine how T lymphocytes become tolerant to antigens expressed exclusively in the periphery. We have addressed this question by producing transgenic mice in which the class I antigen H-2K^b was expressed extrathymically under the control of the rat insulin promoter (RIP-K^b mice) (5, 6) or of the metallothionein promoter (7). In these, and in other transgenic models of extrathymic class II expression (8–11), tolerance of the transgene product was shown to be imposed in the periphery.

Two models were proposed to account for peripheral induction of tolerance (12). In one, tolerance would be imposed on the effector cells by functional silencing, not by elimination. Indeed, the cells may become reactivated in the presence of interleukin 2 (IL-2) (6). In the other, negative selection would affect only high-affinity effector cells, while the remaining low-affinity cells would require IL-2 for activation. It is difficult to distinguish between these alternatives, since the frequency of T cells reactive to a given antigen is generally too low to be detected physically, and there is no dominant clonotype in the response to these antigens. To overcome this problem, we have produced transgenic mice that have rearranged TCR genes encoding an anti-H-2K^b TCR. Monoclonal antibodies were used to follow the fate of anti-H-2K^b T cells in double transgenic mice expressing H-2K^b in the periphery. We show here that these mice did exhibit tolerance to H-2K^b. Their T cells were present in undiminished proportions and had not downregulated the TCR or the CD8 accessory molecule. Therefore, induction of peripheral tolerance need require neither clonal deletion nor TCR downregulation of potentially reactive T cells.

MATERIALS AND METHODS

Mice. Inbred mouse strains were obtained from the breeding facilities of the Walter and Eliza Hall Institute for Medical Research. Transgenic mice were bred and maintained under standard conditions.

Production of TCR Transgenic Mice. Rearranged TCR V_α and V_β variable region genes from the bm1 anti-C57BL/6 cytolytic T lymphocyte (CTL) clone F3 were generously provided by Denis Loh and William Sha (13). The rearranged genomic β gene was obtained as a 20-kilobase (kb) fragment after *Kpn* I digestion of the pV_β11(F3) plasmid. The rearranged genomic V_α8.1 gene was subcloned in the *Eco*RI–*Bam*HI site of pIC20H (14) and the genomic C_α constant region gene, including the enhancer element (15), was introduced as a *Bgl* II fragment into the *Bam*HI site of the resulting plasmid. Purified α and β constructs were microinjected together into fertilized (CBA × B10.BR)F_2 eggs, as previously described (5). Transgenic mice were identified by Southern hybridization using a V_α8 probe (13) and by flow cytometry on peripheral blood lymphocytes, using a monoclonal anti-V_β11 antibody, KT11 (16). The transgenic lines, termed F3 after the CTL clone from which the TCR genes were derived, were maintained by repeated crossing to the bm1 strain. In some experiments, progeny of F3 mice mated to C57BL/6 (B6) or RIP-K^b transgenic mice (5, 6) were used.

CTL and Limiting Dilution Assays. Graded numbers of lymph node cells were set up in 32 wells with 5×10^3 irradiated (1500 R) B6 or BALB/c stimulator cells and mouse recombinant IL-2 (DNAX) at 20 international units/ml. After 7 days, microcultures were split and cytotoxicity was determined on ^{51}Cr-labeled concanavalin A-stimulated blasts derived from B6 or BALB/c spleen cells and from bm1 cells as specificity control. Cultures with >3 SD of lytic activity over the spontaneous release were scored positive and the CTL frequency was determined by using the Poisson distribution. Bulk cultures of spleen cells were stimulated with irradiated B6 or B10.A(5R) (H-2K^{b}D^d) splenocytes and assayed on H-2K^b (EL4 or MC57G) or H-2^d (P815) target cells as described (6, 7).

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Abbreviations: CTL, cytolytic T lymphocytes; IL-2, interleukin 2; RIP, rat insulin promoter; TCR, T-cell receptor; V, C, and J, variable, constant, and joining regions.

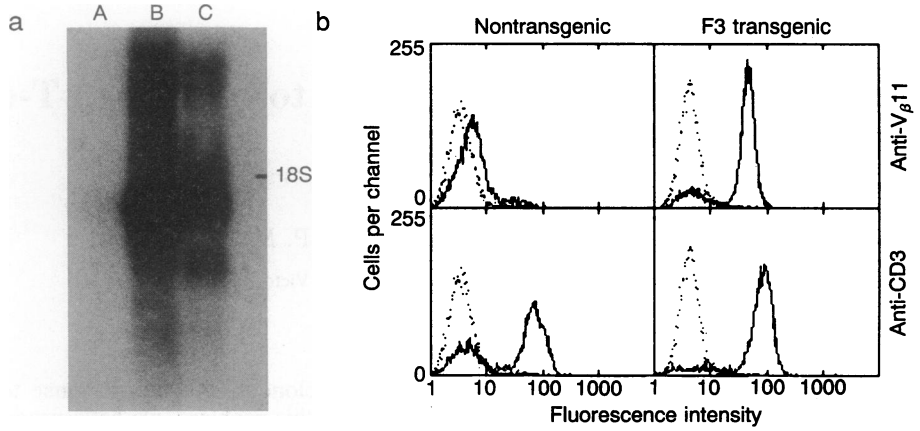

FIG. 1. Expression of transgenic TCR α- and β-chain genes. (*a*) Northern blot analysis of V_α transgene expression. RNA was extracted from spleens of nontransgenic (lane A) and F3 mice of the 127-2 (lane B) and 131-7 (lane C) lines. The position of 18S rRNA is indicated. (*b*) Flow cytometric analysis of V_β transgene expression. Dotted line, no primary antibody.

Skin Grafts. Recipient nontransgenic mice and single and double (F3 × RIP-K^b) transgenic mice, all deriving their H-2 from bm1, received skin grafts (17) from B6 mice, which differ from bm1 only at the H-2K locus, and from third-party BALB/c donors.

Flow Cytometry. Peripheral blood lymphocytes were collected from nontransgenic or F3 transgenic mice of the 131-7 line crossed to bm1 mice. The cells were allowed to react with monoclonal antibodies to $V_\beta11$ (16) or to CD3 (18), washed, and incubated with fluorescein-conjugated, mouse IgG-absorbed sheep antibodies to rat IgG (Silenus, Melbourne, Australia). After further washing, the cells were analyzed by using a FACScan flow cytometer (Becton Dickinson). Cell suspensions were prepared from thymus, spleen, or pooled lymph nodes of nontransgenic and single and double transgenic mice. The transgene status of these mice had been determined previously by assessment of urine glucose for the RIP-K^b gene and by $V_\beta11$ dominance amongst peripheral blood lymphocytes for the F3 TCR genes. Cells were incubated with biotinylated antibodies to $V_\beta11$ (16) or to CD3 (18), washed, and allowed to react with the fluorochrome-conjugated monoclonal antibodies anti-CD8 and anti-CD4 (Becton Dickinson) and with Texas red-conjugated streptavidin (Caltag, South San Francisco, CA). After further washing, the cells were analyzed on a FACStar$^+$ (Becton Dickinson). Dead cells were excluded by staining with propidium iodide. In some experiments, streptavidin-duochrome was used and analyses were performed on the FACScan.

Analysis of Transgene Expression. RNA extraction, electrophoresis, transfer to nylon membranes (Amersham), probe labeling, and hybridization were performed by using standard techniques (19). Expression of the V_α transgene was assessed by reverse-transcription PCR (20). RNA was extracted from CD8$^+$ $V_\beta11^+$ FACS-purified T cells from single or double transgenic mice, from CD8$^+$ sorted cells from a nontransgenic littermate mouse, or from whole bm1 spleen. RNA was reverse-transcribed and aliquots of cDNA from the equivalent of 10^4 cells were subjected to semiquantitative PCR analysis with V_α (CGGGATCCAGACAGAAGGCC) and J_α joining region (GGAATTCTGACCGTTAACCT) transgene-specific primers. After 15 rounds of amplification, aliquots were electrophoresed on an agarose gel, transferred to nylon membranes, and probed with a $V_\alpha8$ probe.

RESULTS

Production and Characterization of TCR Transgenic Mice. The rearranged α- and β-chain genes encoding the TCR from an anti-K^b CTL clone (13), F3, were used to generate transgenic mice. Three independent lines were obtained, each of which expressed the transgenic α-chain gene, as shown for two of these lines by Northern blot hybridization (Fig. 1*a*). Unless stated otherwise, mice of these lines were H-2K^{bm1} homozygotes. Expression of the $V_\beta11$ transgene was detected by flow cytometry analyses (Fig. 1*b*). The majority of CD3$^+$ lymphocytes reacted with the $V_\beta11$-specific antibody, KT11. In normal mice, <4% of lymph node cells were detected by this antibody (16).

Having established that the transgene constructs were expressed appropriately, it was necessary to demonstrate that the transgenic receptor conferred an H-2K^b specificity. This was achieved in three ways. First, offspring of the 127-2 F3 TCR transgenic mice crossed to B6 had no CD8$^+$ $V_\beta11^+$ cells in either thymus or periphery, showing that transgene-expressing T cells were subjected to negative selection in the thymus (Fig. 2). Second, as shown in Fig. 3, stimulation of lymph node cells under limiting dilution conditions showed that the CTL precursor frequency against H-2K^b targets was very much greater in F3 mice (1/7) than in nontransgenic mice (1/10^4), while reactivity to third-party haplotype was significantly reduced in F3 mice (<1/10^5) compared to nontransgenic mice (1/2381). Finally, F3 mice rejected B6 skin grafts more quickly than did nontransgenic mice, but they showed delayed rejection of BALB/c grafts (see Table 2).

Phenotype of Lymphocytes from F3 × RIP-K^b Double Transgenic Mice. The 131-7 mice were crossed to RIP-K^b

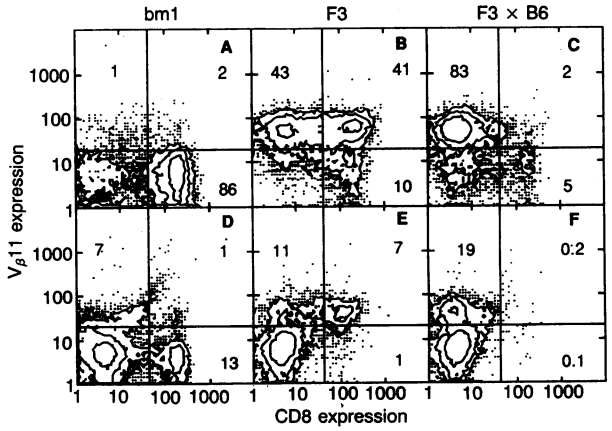

FIG. 2. Negative selection of $V_\beta11^+$ cells in F3 × B6 mice. Thymus (*A–C*) and spleen (*D–F*) cells from control or 127-2 F3 mice expressing either H-2K^{bm1} or H-2K^b were characterized for expression of CD8 and the $V_\beta11$ transgenic TCR. CD8$^+$ $V_\beta11^+$ cells are seen in F3 × bm1 mice but not in F3 × B6 mice. Numbers indicate percentage of cells in that quadrant.

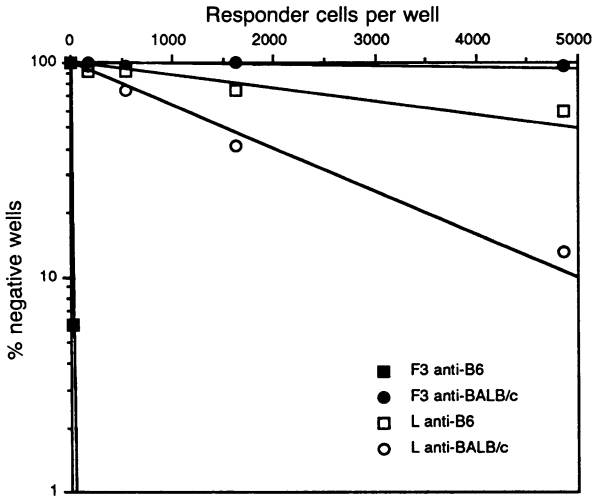

FIG. 3. CTL precursor frequency in lymph node cells, from F3 mice or nontransgenic littermates (L), cultured with B6 or BALB/c stimulators and IL-2 at 20 international units/ml. After 7 days, cytolytic activity was tested on ^{51}Cr-labeled B6 (■, □) or BALB/c (●, ○) concanavalin A-stimulated blasts. The frequencies of CTL precursors reacting to the respective targets are as follows: F3 anti-B6, 1/7; F3 anti-BALB/c, $<1/10^5$; littermate anti-B6, $1/10^4$; and littermate anti-BALB/c, 1/2381.

mice, which express H-2K^b in β cells (5), and their double transgenic (i.e., F3$^+$ RIP$^+$) progeny were analyzed. Unlike the results shown in Fig. 2, in which CD4$^-$ CD8$^+$ V$_\beta$11$^+$ cells were deleted, this population was found in the thymus of F3$^+$ RIP$^+$ mice and in proportion similar to those of F3 mice (Fig. 4). The decrease in CD4$^+$ CD8$^+$ thymocytes in the F3 mice may be due to the development of V$_\beta$11$^+$ CD8$^+$ cells directly from a CD4$^-$ CD8$^-$ precursor. The numbers of cells in the lymphoid organs of F3 and F3$^+$ RIP$^+$ mice were similar and, as shown in Fig. 5, V$_\beta$11-expressing T cells were found in unreduced numbers in the periphery of F3$^+$ RIP$^+$ mice. The levels of CD8 and TCR were not significantly down-regulated in these cells. As expected of mice transgenic for class I-restricted TCR (21), few CD4$^+$ cells were found in either F3 or F3$^+$ RIP$^+$ mice. There were CD4$^-$ CD8$^-$ V$_\beta$11$^+$ cells in both groups; the significance of this is unclear, although similar cells have been identified in other TCR transgenic mice (22).

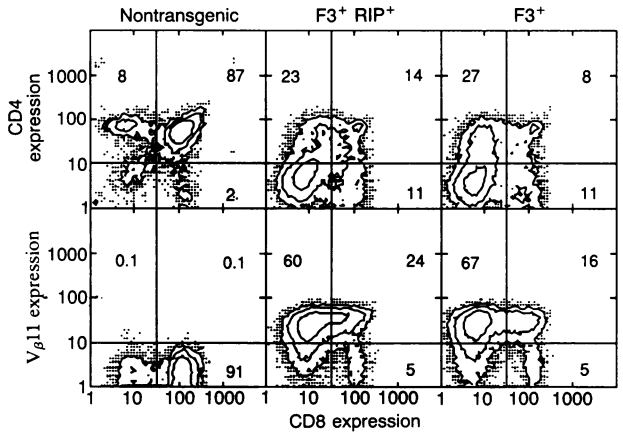

FIG. 4. Expression of TCR transgene in the thymus of single and double transgenic mice. Thymocytes were prepared from mice of the same litter from a cross between 131-7 F3 mice and RIP-K^b mice (5). Three-color analyses were performed, and the results are shown as plots of CD8 versus CD4 expression (*Upper*) and of CD8 versus V$_\beta$11 expression (*Lower*). Numbers indicate percentage of cells in that quadrant.

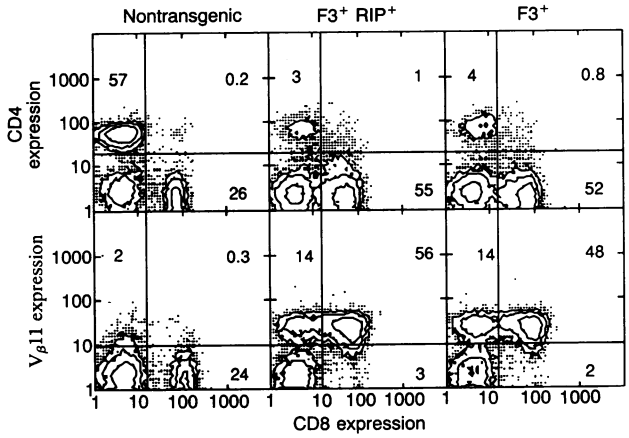

FIG. 5. Expression of TCR transgene in lymph nodes of double transgenic mice. Lymph node cell suspensions were prepared and the results are shown for each sample as in Fig. 4.

Responsiveness of F3 × RIP-K^b Double Transgenic Mice. It was necessary to determine the immune status of the F3$^+$ RIP$^+$ mice, given that they had large numbers of T cells specific for the class I antigen expressed in their β cells. CTL assays were performed on spleen cells taken from mice that displayed lymph node profiles similar to those shown in Fig. 5. F3 CTL killed the H-2K^b-bearing target cells very effectively, while the F3$^+$ RIP$^+$ mice had only marginal activity in this assay (Table 1). Furthermore, whereas the F3 mice rejected H-2K^b skin grafts rapidly, the F3$^+$ RIP$^+$ mice showed extended graft survival, most in excess of 100 days (Table 2). Therefore, unresponsiveness to H-2K^b was evident both *in vitro* and *in vivo* in the F3$^+$ RIP$^+$ mice. The eventual rejection of one B6 graft and the presence of some CTL activity in some F3$^+$ RIP$^+$ mice may be due to the loss of tolerogen caused by the nonimmune death of β cells, previously shown to result in loss of tolerance (6).

Expression of the Transgenic α Chain in Double Transgenic Mice. One objection to the interpretation of our results is that the persisting V$_\beta$11$^+$ cells in the F3$^+$ RIP$^+$ mice expressed the transgenic β chain but not the α chain, thus possessing a different specificity and escaping negative selection. If so, one would predict that endogenous V$_\alpha$ genes would contribute to the formation of class II-restricted TCRs (and therefore to the presence of CD4$^+$ cells in the periphery), and of TCR reactive to other alloantigens. To test this, we generated transgenic mice with only the rearranged F3 TCR β-chain gene (F3β mice). Comparison with the 131-7 line shows an increased frequency in the F3β mice of CD4$^+$ cells, the majority of which express V$_\beta$11 (Fig. 6). The ratio of CD4$^+$ to CD8$^+$ cells was 2:1 in nontransgenic mice, 1:1 in F3β mice, and 1:6 in 131-7 mice. Furthermore, F3β mice rejected H-2K^b grafts more slowly than did F3 mice, and third-party grafts more rapidly than either F3 single or double transgenic mice

Table 1. Immune status of double transgenic mice: Cytotoxicity *in vitro*

Genotype of CTL	% of splenocytes		% maximum lysis	LU per 10^7 spleen cells
	V$_\beta$11$^+$	V$_\beta$11$^+$ CD8$^+$		
F3	29	9	73	142
F3 × RIP-K^b	19	5	18	12
F3 × RIP-K^b	25	19	16	5
F3 × RIP-K^b	14	8	46	47

CTL assays were performed on B6-stimulated splenocytes from mice typed for transgene status. Percentages of total splenocytes are shown for the V$_\beta$11$^+$ and the V$_\beta$11$^+$ CD8$^+$ cells. Maximum lysis is shown for an effector-to-target ratio of 50:1. One lytic unit (LU) = the number of cells required for 10% specific lysis.

Table 2. Immune status of double transgenic mice: Skin graft survival

Recipient	Donor	No. of grafts	No. of grafts surviving			
			10–12 days	13–20 days	20–28 days	>100 days
F3	B6	10	10	—	—	—
F3	BALB/c	9	—	4	5	—
F3 × RIP-K^b	B6	7	—	—	1	6
F3 × RIP-K^b	BALB/c	4	—	4	—	—
F3β	B6	3	—	2	1	—
F3β	BALB/c	3	3	—	—	—
Nontransgenic littermates	B6	8	2	5	1	—
	BALB/c	8	7	1	—	—

(Table 2). These results strengthen our contention that endogenous V_α chain genes are not expressed significantly in the periphery of the F3 or $F3^+$ RIP^+ mice.

Nevertheless, to demonstrate that the double transgenic mice did indeed express the transgenic α-chain gene, RNA was extracted from small numbers of FACS-purified $CD8^+$ $V_\beta11^+$ cells from F3 mice that had rejected B6 skin grafts and from $F3^+$ RIP^+ mice that had accepted these grafts for >100 days. From this RNA, cDNA copies were made and limited cycles of PCR were performed with transgene-specific primers. PCR products were detected by hybridization to a $V_\alpha8$ probe. From such semiquantitative analyses (Fig. 7), specific bands were found in both the F3 and $F3^+$ RIP^+ lanes. At this level of detection, no signal was found in the $CD8^+$ cells isolated from bm1 mice. A faint band was detectable in the sample of whole bm1 spleen RNA, presumably originating from transcripts of T cells that had rearranged members of the $V_\alpha8$ gene family to J_α genes that could cross-hybridize to the primers used for PCR. These results indicate that the double transgenic mice had persisting $V_\beta11^+$ T cells that expressed the $V_\alpha8$ transgene.

DISCUSSION

The introduced TCR genes conferred an H-$2K^b$ specificity upon the majority of T cells present in the F3 transgenic mice. This was shown by *in vivo* and *in vitro* tests of T-cell specificity. F3 mice rejected H-$2K^b$-bearing skin grafts more rapidly than nontransgenic mice but rejected third-party grafts only slowly. Strong CTL responses were mounted to H-$2K^b$-bearing target cells but not to H-$2D^d$-bearing targets. Limit dilution analyses showed that as many as 1 in 7 lymph node cells was specific for H-$2K^b$. T cells expressing the transgene product were thus able to respond appropriately to H-$2K^b$. They could also be negatively selected, since $V_\beta11^+$ cells were deleted if H-$2K^b$ was expressed in the thymus.

Despite this large pool of potentially reactive cells, double transgenic mice were tolerant of H-$2K^b$. This was confirmed by the most stringent test of tolerance: the ability to accept H-$2K^b$-disparate skin indefinitely. Tolerance was also evident *in vitro*: K^b-reactive CTL were generated far less efficiently from cultures of spleen cells of double transgenic mice. However, a variable response to K^b was found and may have been due to newly emerging T cells, which had not yet been tolerized. Furthermore, as the pancreatic β cells progressively died from nonimmune mechanisms (5), the source of tolerogen decreased and tolerance was imposed less effectively.

The tolerant state was not due to deletion of potentially reactive T cells, either in the thymus or in the periphery. Transgene-expressing cells were detected in spleen, thymus, and lymph node populations of $F3^+$ RIP^+ mice by using anti-$V_\beta11$ antibodies. No significant difference was seen between single TCR and double transgenic mice with respect to either CD8 or $V_\beta11$ expression. Thus, neither deletion nor substantial down-regulation of these molecules was responsible for the induction of tolerance. It may be argued, however, that cells expressing the F3 TCR were deleted, while others used endogenous α-chain genes in association with the transgenic $V_\beta11$ gene to form receptors of other specificities. This is not likely to be the case. Endogenous α chains associating with transgenic β chains would form some TCRs that would react with alloantigen or be restricted to

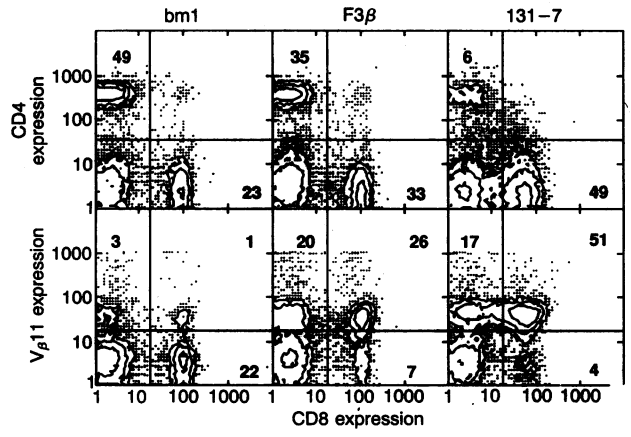

FIG. 6. Phenotypic comparison of lymph node cells from transgenic mice having the F3 β-chain gene or both F3 α- and β-chain genes. Lymph node cell suspensions were prepared from nontransgenic bm1 mice, from F3β mice (which have the rearranged $V_\beta11$ gene only), and from 131-7 mice. Analyses were conducted and the results are shown as described in the legend to Fig. 4.

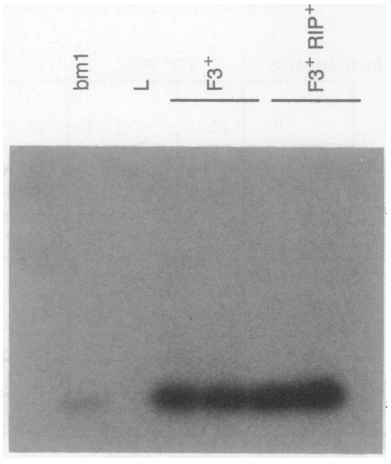

FIG. 7. Expression of the transgenic α chain. $CD8^+$ $V_\beta11^+$ cells were isolated from lymph nodes of F3 mice that had previously rejected K^b-disparate skin grafts and from $F3^+$ RIP^+ mice that retained intact grafts for >100 days. $CD8^+$ cells were also isolated from a nontransgenic littermate (L). The presence of $V_\alpha8$ transcripts in these cells and in whole bm1 spleen was detected as described in the text. Note the presence of transgene-specific bands in both F3 and $F3^+$ RIP^+ mice (two individuals each).

class II molecules (23, 24) and so would be found on $CD4^+$ cells. These predictions are valid, as they were borne out in studies of $V_\beta 11$-chain transgenic mice (Fig. 6 and Table 2). In contrast, $F3^+$ RIP^+ mice showed a preponderance of $CD8^+$ cells and rejected BALB/c grafts slowly.

The expression of the $V_\alpha 8$ transgene was demonstrated by both Northern blot hybridization and semiquantitative PCR, using purified $CD8^+$ $V_\beta 11^+$ cells from the double transgenic mice. Levels of expression were apparently similar to those of their single transgenic littermates. This was in stark contrast to the tolerance status of these mice: the F3 single transgenic mice had rejected H-$2K^b$-bearing grafts rapidly, while the $F3^+$ RIP^+ mice had surviving grafts in spite of the presence of circulating T cells expressing the transgenic α and β TCR chains encoding anti-H-$2K^b$ specificity. These results therefore support a model for tolerance induction by the inactivation, rather than elimination, of the relevant effector cells.

The results presented here differ from recent work with other double transgenic mice (25, 26). In one of these studies, tolerance was not apparent: T cells persisted, but they ignored the transgenic antigen unless it was presented in an immunogenic form (25). In the other report, tolerance occurred without T-cell deletion, but both CD8 and TCR were down-regulated (26). It is likely that mechanisms operating to induce peripheral tolerance differ according to the nature, site of synthesis, and amount of tolerogen and the frequency and avidity of reactive T cells.

It is impressive how tolerance in the $F3^+$ RIP^+ mice was imposed upon such a large pool of potentially reactive T cells by such a small source of tolerogen. How has anergy (27) been induced in these cells? Must all T cells proceed through the islets to become tolerized, or is some other mechanism involved? The homogeneous population of anergic T cells from double transgenic mice should be invaluable in examining the phenotype and molecular basis of T-cell unresponsiveness.

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