TABLE 2 Frequency of LCMV-specific cytotoxic T-cell percursors among $CD8^+$ T cells of TCR$\alpha\beta$ transgenic mice

| Mice | Reciprocal frequencies |
|---|---|
| C57BL/6 Uninfected | $>10^4$ (ref. 15) |
| C57BL/6 acutely infected with LCMV, day 8 | 3.0–4.1 |
| $\alpha\beta$-transgenic H-$2^b$, $Mls^b$ Uninfected | 1.8–4.1 |
| $\alpha\beta$-transgenic H-$2^{bd}$, $Mls^a$ Uninfected | 2.4–3.9 |
| $\alpha\beta$-transgenic H-$2^b$, $Mls^b$ Neonatally infected LCMV-carrier | $>10^4$ |

Limiting numbers of responder spleen cells were cultured in 96-well round-bottom plates with irradiated (3,000 rad) LCMV-infected peritoneal macrophages ($10^3$ cells per well) and with irradiated spleen filler cells from LCMV-infected mice ($10^5$ cells per well) in the presence of 25% (v/v) supernatant from concanavalin A-stimulated rat spleen cells. The precise number of $CD8^+$ T cells was determined by staining of an aliquot of the used responder spleen population with CD8-specific monoclonal antibody and cytofluorometric analysis. After 7 days, the cytolytic activities of the microcultures were tested on LCMV-infected and noninfected MC57G target cells in a $^{51}Cr$-release assay[12]. Cultures were scored as positives when LCMV-specific lysis was >15%. Frequencies were calculated according to Taswell[24].

Thus tolerance induction involving the same TCR can vary with the antigen involved. It could be that the time-dependent appearance or the localization of the two antigens (LCMV, $Mls^a$), or both of these factors, are different in the thymus. In LCMV-carrier mice, LCMV antigens have been detected very early throughout the thymus[16], so thymoctyes could meet LCMV antigen and MHC class I molecules in the thymic cortex and medulla. The distribution of the $Mls^a$ antigen is not known. But because $Mls^a$ antigen is recognized by T cells in the context of MHC class II molecules which are not detected in the outer cortex[18], deletion of most double $CD4^+CD8^+$ cells would not occur in $\alpha\beta$-transgenic $Mls^a$ mice. Alternatively, the affinity of the transgenic receptor for LCMV-H-$2D^b$ could differ from that for $Mls^a$. Double positive thymocytes having a high-affinity receptor for the appropriate antigen (LCMV-H-$2D^b$ in our model) are therefore deleted at an early state of development. Thymocytes expressing a low-affinity receptor to $Mls^a$ would be deleted at a later stage of development when TCR densities on these cells are increasing[19]. In conclusion, tolerance induction to the two antigens (LCMV, $Mls^a$) examined with the same transgenic receptor differed drastically. These findings indicate that T-cell tolerance by clonal deletion does not occur at one single discrete stage of T-cell development. □

*Note added in proof*: Since the submission of this manuscript, Berg *et al.*[20] have reported that $\alpha\beta$TCR ($V\alpha_{11}$, $V_{\beta 3}$) transgenic Mls-$2^a$ and -$3^a$ mice exhibit deletion of $CD4^+CD8^+$ thymocytes, whereas $\beta$TCR $V\beta_3$ transgenic Mls-$2^a$ and-$3^a$ mice do not delete $CD4^+CD8^+$ thymocytes. Because the TCR density on thymocytes of $\alpha\beta$-transgenic mice is higher than that in $\beta$-transgenic mice, it was argued that deletion of $CD4^+CD8^+$ thymocytes correlates wth TCR density and maturation state. Because we found that the same $CD4^+CD8^+$ thymocytes are deleted in the LCMV-carrier but not in $Mls^a$ mice, TCR density and maturation state alone cannot explain our findings, suggesting that affinity could also play a part. □

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# *In vivo* priming of virus-specific cytotoxic T lymphocytes with synthetic lipopeptide vaccine

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**CYTOTOXIC T lymphocytes (CTL) constitute an essential part of the immune response against viral infections[1]. Such CTL recognize peptides derived from viral proteins together with major histocompatibility complex (MHC) class I molecules on the surface of infected cells[2–4], and usually require *in vivo* priming with infectious virus[5]. Here we report that synthetic viral peptides covalently linked to tripalmitoyl-*S*-glycerylcysteinyl-seryl-serine ($P_3CSS$) can efficiently prime influenza-virus-specific CTL *in vivo*. These lipopeptides are able to induce the same high-affinity CTL as does the infectious virus. Our data are not only relevant to vaccine development, but also have a bearing on basic immune processes leading to the transition of virgin T cells to activated effector cells *in vivo*, and to antigen presentation by MHC class I molecules.**

The epitopes recognized by MHC class I-restricted, virus-specific CTL can be defined by short synthetic peptides (ref. 3; see also Fig. 1). These peptides are thought to bind to a groove on top of the MHC molecule, as indicated by its crystallographic structure[4]. CTL can recognize target cells incubated in picomolar concentrations of a given peptide (ref. 6; see also Fig. 2). Paradoxically, mice cannot be primed with the same peptide at any concentration tested (H. S. *et al.*, manuscript in preparation). An example for this failure is shown in Fig. 2*a*, *d*, *g*: BALB/c (H-$2^d$) mice were immunized with influenza nucleoprotein (NP) NP147–158 (R-) epitope (Fig. 1), which is the most efficient peptide to be recognized by H-$2^d$-restricted CTL specific for influenza nucleoprotein[6]. On stimulation of recipient spleen cells *in vitro*, no killing of peptide-incubated or virus-infected target cells was observed. By contrast, mice immunized with infectious influenza virus (Fig. 2*b*, *e*, *h*) responded well.

The failure of peptide to prime *in vivo* could be related to the non-physiological form of antigen presentation, because

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CTL usually recognize fragments derived from protein endogenously produced in the target cell[7,8], and/or due to a missing second signal, consisting of cytokines released by antigen-presenting cells[9]. We therefore attempted to prime virus-specific CTL *in vivo* with NP147–158 (R-) peptide conjugated to $P_3CSS$ (see Fig. 1). The latter was synthesized according to the immunologically active N-terminal sequence of the principal lipoprotein of *Escherichia coli*, also known as Braun's lipoprotein[10]. $P_3CSS$ mediates attachment to the cell membrane, internalization into the cytoplasm, and activates macrophages to secrete cytokines[11,12]. Priming with the NP147–158(R-)/$P_3CSS$-conjugate was indeed efficient, producing a virus-specific CTL response as strong as that induced by priming with infectious virus (compare Fig. 2*c, f, i* with 2*b, e, h*). CTL priming was also efficient with two other $P_3CSS$–peptides (Fig. 3). One contained the influenza NP147–158 peptide, the other influenza NP365–380, which are recognized by H-$2^d$ or H-$2^b$-restricted CTL, respectively[6,13]. Figure 4*a–c* shows that only $P_3CSS$-[NP147–158 (R-)], but not Ser-Ser-[NP147–158 (R-)] or NP147–158(R-), is efficient for priming. Lipopeptide priming is MHC class I-restricted, as shown for two lipopeptides in Fig. 4*d–i*.

CTL primed *in vivo* with lipopeptide and restimulated *in vitro* with native peptide have a remarkable ability to recognize virus-infected cells. This is in marked contrast to those peptide-specific CTL obtained by the *in vitro* stimulation of spleen cells from unprimed mice at high cell density with viral peptide. Such

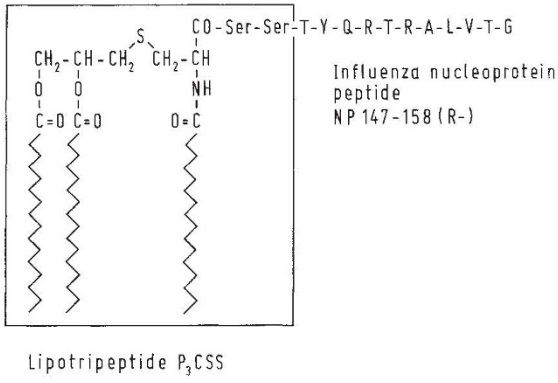



FIG. 1 Chemical formula of lipopeptide vaccine $P_3CSS$-[NP147–158 (R-)] of relative molecular mass ($M_r$) 2,332 (single-letter amino-acid code). Influenza NP peptide was synthesized by solid phase techniques on an Applied Biosystems 430A synthesizer using our own Fmoc program, Fmoc-L-amino acids and *p*-benzyloxybenzylalcohol-resin. The peptide was elongated with two additional serine residues followed by tripalmitoyl-*S*-glycerylcysteine (coupling procedures: (benzotriazolyloxy)tris-(dimethylamino)phosphonium hexafluorophosphate (BOP)/1-hydroxybenzotriazole (HOBt)), except for Gln coupled as symmetrical anhydride with diisopropylcarbodiimide (DIC), and $P_3C$ coupled with DCC/HOBt). Peptide and lipopeptide were removed from the resin with trifluoroacetic acid containing 2.5% thioanisole and *p*-thiocresol (1 h at 25 °C, and for complete deprotection of Arg(2,2,5,7,8-pentamethylchroman-6-sulphonyl [Arg(Pmc)] additional treatment for 30 min at 50 °C). Amino-acid analysis, enantiomer analysis by gas–liquid chromatography on Chirasil-Val and (+)-FAB mass spectrometry proved the identity of peptide and the peptide portion of the lipopeptide. Purity of lipopeptide was more than 90% as analysed by HPLC. The amino-acid sequence outside the box represents the peptide reported to be most efficiently recognized by H-$K^d$-restricted influenza-specific CTL (ref. 6). This sequence is analogous to amino acids 147–158 of NP of influenza strain A PR/8/34 (PR8), except that the Arg at position 156 has been deleted. Additional lipopeptide vaccines were synthesized by coupling the lipotripeptide $P_3CSS$ as shown inside the box to influenza NP peptide of residues 147–158 (TYQRTRALVRTG), which is also recognized by H-$K^d$-restricted CTL, albeit with 1,000-fold less efficiency as compared with NP147–158 (R-) (ref. 6), or to influenza NP peptide of residues 365–380 (IASNENMET-MESSTLE) which is recognized by H-$2^b$-restricted CTL (ref. 13). $M_r$ of $P_3CSS$-(NP147–158) is 2488, that of $P_3CSS$-(NP365–380), 2,853. Known biological activities of $P_3CSS$ are described in refs. 10–12, 18, 20–22.

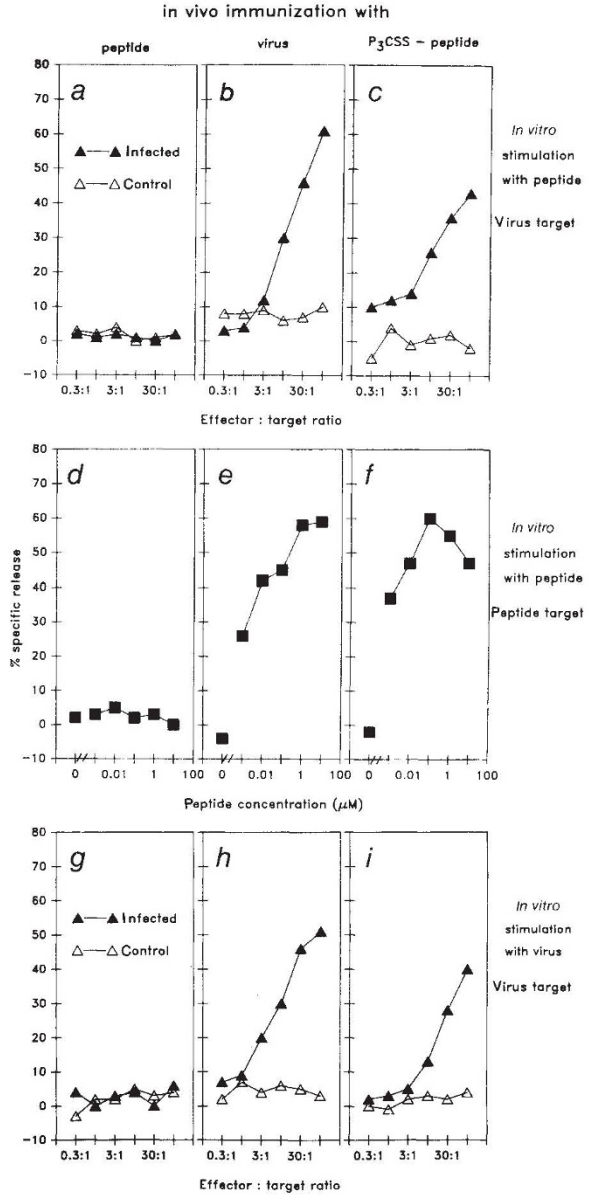
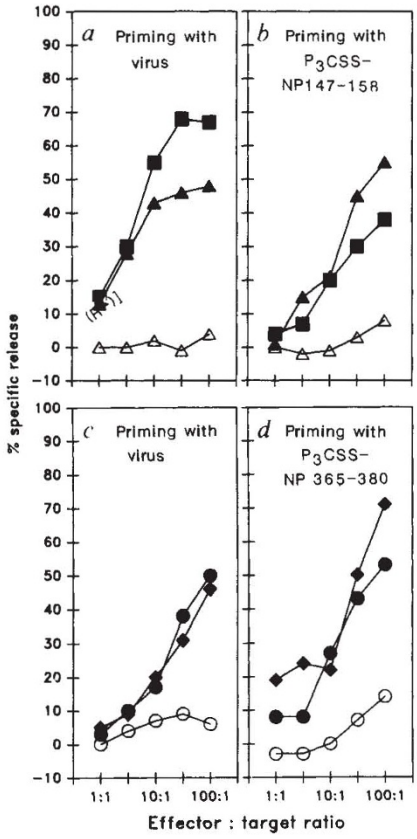



FIG. 2 CTL activity of spleen cells from BALB/c mice after immunization with peptide, virus, or $P_3CSS$–peptide. Adult BALB/c (H-$2^d$) mice were immunized with NP147–158 ($R^-$) peptide (*a, d, g*), or with influenza virus (*b, e, h*) or with $P_3CSS$–peptide (*c, f, i*). After 6 days, recipient spleen cells were stimulated for 5 days against peptide (*a–f*) or strain PR8-infected syngeneic stimulator cells (*g–i*). *a–c* and *g–i*, CTL activity on untreated (△) or strain PR8-infected (▲) P815 (H-$2^d$) target cells. *d–f*, CTL activity on P815 cells, preincubated with titrated concentrations of peptide. Effector:target ratio in *d–f*, 30:1. *a–g* represent one experiment; *h* and *i* are from an independent experiment. Spontaneous $^{51}Cr$-release of target cells was between 10–20%. METHODS. Adult mice received intravenous injections of $8\times10^7$ syngeneic spleen cells preincubated either with 1.6 μM NP147–158($R^-$) peptide (*a, d, g*) or with 160 μM of $P_3CSS$-[NP147–158($R^-$)] lipopeptide (*c, f*) or received 50 haemagglutinating units of influenza virus A strain PR/8/34 (PR8) (*b, e, h*) or 100 μg $P_3CSS$-[NP147–158 ($R^-$)] lipopeptide (*i*). Volume of inocula was adjusted to 0.3 ml phosphate-buffered saline in all cases. Recipient spleen cells ($2.5\times10^7$) were stimulated *in vitro* in volumes of 10 ml α-minimal essential medium (MEM; Gibco) supplemented with 10% fetal calf serum, 2-mercaptoethanol, glutamine and antibiotics against 80 nM NP147–158($R^-$) peptide (*a–f*) or against $5\times10^6$ PR8-infected, irradiated (2,000 rad) syngeneic spleen cells. CTL activity was determined in a standard $^{51}Cr$-release assay[23]. Infection of stimulator and target cells was as previously described[24]. Preincubation of target cells with peptide was for 30 min at 37 °C.



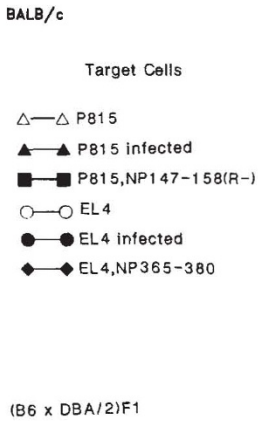




FIG. 3 CTL activity after priming of mice with $P_3$CSS-(NP147-158) or $P_3$CSS-(NP365-380) vaccines. BALB/c (*a*, *b*) or (B6 × DBA/2) $F_1$ ($H\text{-}2^b \times H\text{-}2^d$) (*c*, *d*) mice were immunized with influenza virus (*a*, *c*) or with 100 μg $P_3$CSS-(NP147-158) (*b*) or with 100 μg of $P_3$CSS-(NP 365-380) (*d*). After 6 days, recipient spleen cells were stimulated against 0.8 μM of NP147-158 peptide (*a*, *b*) or 0.8 μM NP365-380 peptide (*c*, *d*). CTL activity was determined on untreated P815 target cells, (△), PR8-infected P815 cells (▲), P815 cells preincubated with NP147-158 ($R^-$) (■), untreated EL4 ($H\text{-}2^b$) targets (○), PR8-infected EL4 (●) or EL4 cells preincubated with NP365-380 peptide (◆). Spontaneous $^{51}Cr$ release ranged between 11% and 25%.

METHODS. Preincubation of target cells with peptides was for 90 min at 37 °C using 0.8 μM of the respective peptide. Methods otherwise as in Fig. 2, legend.

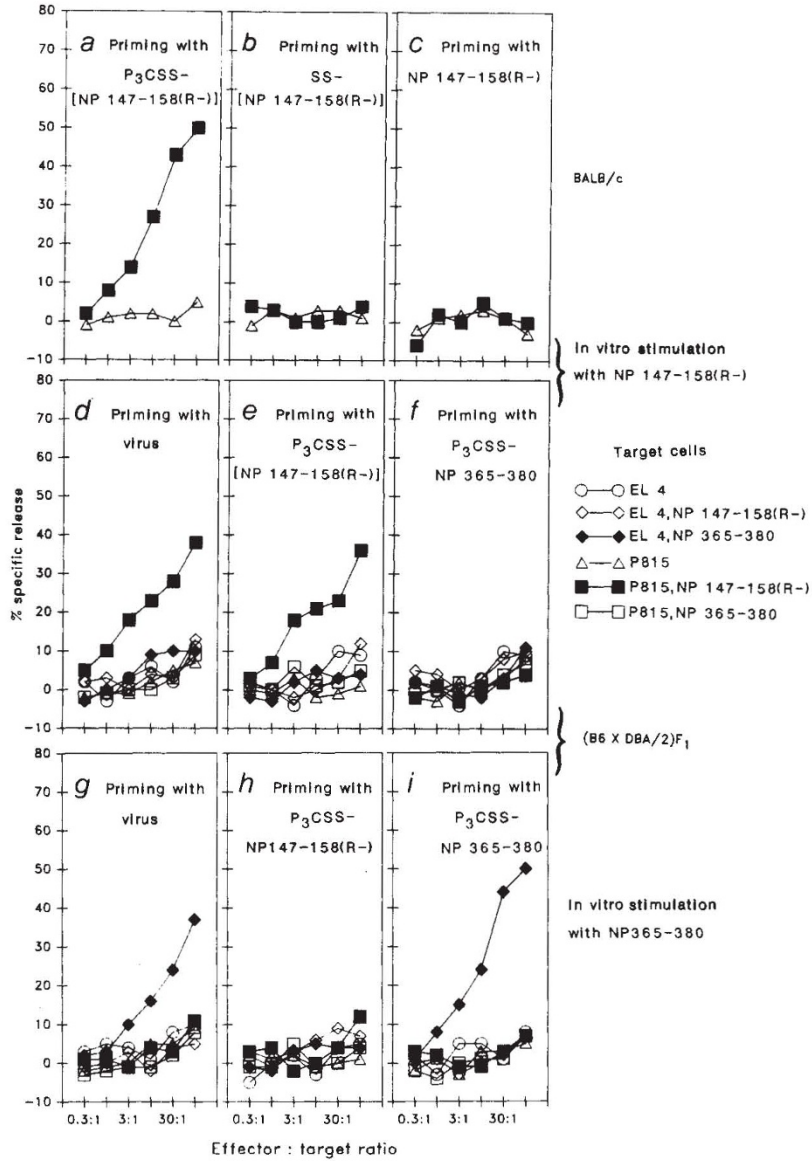



FIG. 4 Specificity and MHC class I-restriction of lipopeptide-priming. BALB/c (*a*–*c*) or (B6 × DBA/2) $F_1$ (*d*–*i*) mice received injections of 100 μg $P_3$CSS-[NP147-158 ($R^-$)] (*a*, *e*, *h*), 50 μg SS-[NP147-158 ($R^-$)] (NP147-158 ($R^-$) peptide elongated with two serine resides at the N terminus; $M_r$, 1,422) (*b*), 50 μg NP147-158 ($R^-$) ($M_r$, 1,247) (*c*), PR8 virus (*d*, *g*), or 100 μg $P_3$CSS-NP365-380 (*f*, *i*). Six days after injections, recipient spleen cells were stimulated against NP147-158 ($R^-$) peptide (*a*–*f*) or NP365-380 (*g*–*i*). CTL activity was determined on untreated P815 target cells (△), P815 cells preincubated with NP147-158($R^-$) (■), P815 cells preincubated with NP365-380 (□), untreated EL4 target cells (○), EL4 cells preincubated with NP147-158 ($R^-$) (◇) or EL4 preincubated with NP365-380 (◆). Note that neither P815 nor EL4 express MHC class II molecules. Spontaneous $^{51}Cr$ release of target cells was between 14% and 26%. Experiments performed as for Figs 2 and 3.

primary CTL do not, in general, recognize virus-infected cells, but do recognize peptide-incubated target cells. To explain this discrepancy it has been suggested that the affinity of these peptide-specific CTL primed *in vivo* is low compared with the affinity of CTL primed *in vivo* with infectious virus[14]. This assumes that virus-infected cells express the peptide-MHC complexes relevant for CTL recognition at very low density, as opposed to the density of such complexes presented by cells incubated with an exogenous source of peptide. Obviously, the lipopeptides that we used are able to induce the same high-affinity CTL as virus-infected cells do.

We do not know the exact mechanism of *in vivo* priming by $P_3$CSS-peptides. A trivial explanation would be based on the time of availability of the respective peptide preparation to be accessible for CTL; other possibilities could be based on the introduction and internalization of the conjugate into the normal MHC class I presentation pathway by means of membrane attachment[14,15], which has been suggested to be responsible for the occasional ability of complete proteins, or large fractions of them, to prime CTL *in vivo*[16,17]. A third possible mechanism might depend on the activating effect that the $P_3$CSS anchor has on certain cells including macrophages, inducing the release of cytokines serving as differentiation factors for CTL[9]. The insertion of variations into the lipoamino acid $P_3$C should establish which of these mechanisms are required for the transition of a virgin CTL *in vivo* to an active effector/memory cell, as well as for the introduction of a peptide into the MHC class I presentation pathway.

Our data show for the first time that virus-specific CTL, an important component of the immune response against viral infection, can be primed *in vivo* with a totally synthetic molecule, $P_3$CSS-peptide. Toxic effects of $P_3$CSS have not been observed *in vivo*[18]. A general problem limiting the use of peptide vaccines in outbred populations, however, is that T cells from MHC-different individuals recognize different peptides from viral proteins, always in conjunction with the respective self MHC (ref. 13). Thus, a universal synthetic vaccine to prime a population for CTL response against a given virus should contain all of the relevant epitopes recognized by T cells. Fulfilment of this requirement by using a mixture of lipopeptides seems not to be too far-fetched, given the relatively good accessibility of synthetic $P_3$CSS-peptide conjugates[19,20]. $P_3$CSS conjugated to peptides seems to be especially suited for vaccine design, because the induction of neutralizing antibodies, the other principal contribution to viral immunity, can also be achieved with $P_3$CSS conjugated to the relevant epitopes[18,20]. □

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# T-cell tolerance by clonal anergy in transgenic mice with nonlymphoid expression of MHC class II I-E

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**T-CELL reactivity to the class II major histocompatibility complex I-E antigen is associated with T-cell antigen receptors containing the *Vβ* gene segments *Vβ17a* (ref. 1) and *Vβ5* (ref. 2). Mice expressing I-E with the normal tissue distribution (on B cells, macrophages, dendritic cells and thymic epithelium) induce tolerance to self I-E by clonal deletion in the thymus[3]. By contrast, we find that transgenic INS-I-E mice that express I-E on pancreatic β-cells, but not in the thymus or peripheral lymphoid organs, are tolerant to I-E (ref. 4) but have not deleted Vβ5- and Vβ17a-bearing T cells. Moreover, whereas T-cell populations from nontransgenic mice proliferate in response to receptor crosslinking with Vβ5- and Vβ17a-specific antibodies, T cells from INS-I-E mice do not. Thus, our experiments provide direct evidence that T-cell tolerance by clonal paralysis[5–9] does occur during normal T-cell development *in vivo*.**

To determine whether the tolerance to I-E in our INS-I-E mice resulted from deletion of I-E-reactive T cells, we used the Vβ17a-specific monoclonal antibody KJ23 (ref. 1) and the MR9-4 monoclonal antibody that recognizes $V\beta5.1^+$ and $V\beta5.2^+$ T-cell receptors (TCR) (J. Bill, E. Palmer and O.K., manuscript submitted). INS-I-E transgenic mice analysed for the presence of $V\beta17a^+$ T cells were back-crossed several times to the SJL/J strain so that they were homozygous for the *Vβ17a* gene. INS-I-E mice analysed for the presence of $V\beta5^+$ T cells were back-crossed several times to the C57BL/6 strain[2], such that they were positive for the *Vβ5* gene and negative for the *Vβ17a* gene. Table 1 summarizes the results of immunofluorescent staining on peripheral lymph node cells of the INS-I-E mice. We found

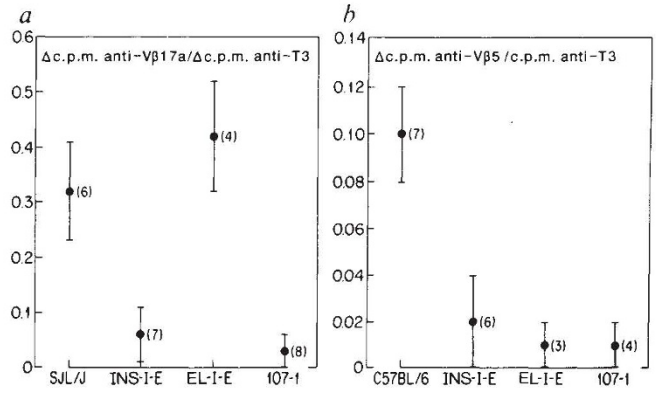


FIG. 1 Summary of T-cell proliferation induced by TCR crosslinking. Results are expressed as as *a*, Δc.p.m. anti-Vβ17a/Δc.p.m. anti-T3 and *b*, Δc.p.m. anti-Vβ5/Δc.p.m. anti-T3. Values shown indicate $\bar{X} \pm$ s.e.m. obtained for individuals tested with both anti-Vβ and anti-T3 mAbs (*n* value given in parentheses).