As with studies on the Na^+/K^+ pump[24], early results on Na^+/Ca^{2+} exchange appeared inconsistent with consecutive ion exchange models (see ref. 5 for review). Our work provides strong support for a consecutive Na^+/Ca^{2+} exchange mechanism and should facilitate structure-function studies of the cloned Na^+/Ca^{2+} exchanger. The results of both ion jump experiments and steady-state I_{NaCa} measurements are inconsistent with Ca^{2+} translocation involving net negative charge movement[8]. We conclude from our site-density estimates and I_{NaCa} measurements that maximum exchanger turnover rates are about 5,000 s^{-1}. As already proposed for the Na^+/K^+ pump[12-17] and rod outer segment Na^+/Ca^{2+}, K^+ exchanger[2,22], voltage-dependence must reside in the binding and release of extracellular Na^+ or a closely associated occlusion/deocclusion reaction. □

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Negative and positive selection of antigen-specific cytotoxic T lymphocytes affected by the α3 domain of MHC I molecules

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THE α1 and α2 domains of major histocompatibility complex (MHC) class I molecules function in the binding and presentation of foreign peptides to the T-cell antigen receptor and control both negative and positive selection of the T-cell repertoire[1-3]. Although the α3 domain of class I is not involved in peptide binding, it does interact with the T-cell accessory molecule, CD8 (refs 4, 5). CD8 is important in the selection of T cells as anti-CD8 antibody injected into perinatal mice interfers with this process[6]. We previously used a hybrid class I molecule with the α1/α2 domains from L^d and the α3 domain from $Q7^b$ and showed that this molecule binds an L^d-restricted peptide but does not interact with CD8-dependent cytotoxic T lymphocytes[7]. Expression of this molecule in transgenic mice fails to negatively select a subpopulation of anti-L^d cytotoxic T lymphocytes. In addition, positive selection of virus-specific L^d-restricted cytotoxic T lymphocytes does not occur. We conclude that besides the α1/α2 domains of class I, the α3 domain plays an important part in both positive and negative selection of antigen-specific cells.

To examine the role of the α3 domain of class I molecules in the responses of alloreactive and antigen-specific cytotoxic T lymphocytes (CTL), we generated two transgenic mouse strains, C3H.L^d and C3H.L^{Q3}, derived from C3H/HeJ (H-2^k) mice. C3H.L^d express intact L^d, whereas C3H.L^{Q3} express an L^d molecule whose α3 domain is switched with $Q7^b$. The $Q7^b$ α3 domain differs from L^d at five residues and in addition has a unique three-amino-acid insert at positions 275-277 (ref. 8). We showed that this molecule (L^{Q3}) binds the same viral peptide as L^d but is not recognized by peptide-specific CD8-dependent CTL (ref. 7). Similar observations have been reported with α3 mutant class I molecules not recognized by CD8-dependent CTL, the defect being due to an alteration in the binding site for CD8 (refs 4, 5). Both L^d and L^{Q3} genes contain the same

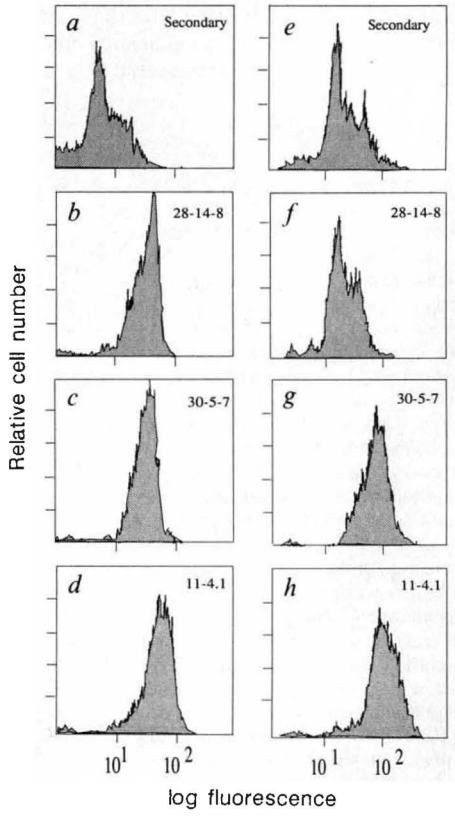

FIG. 1 Cell-surface expression of endogenous or transgene class I expression on spleen cells of C3H.L^d (*a-d*) and C3H.L^{Q3} (*e-h*) transgenic mice was confirmed by fluorescent antibody staining and analysis on a FACScan flow cytometer (Becton Dickinson). Antibodies used in these analyses include: *a* and *e*, fluorescein isothiocyanate-goat anti-mouse IgG; *b* and *f*, 28-14-8 (anti-L^d α3 domain); *c* and *g*, 30-5-7 (anti-L^d α2 domain); *d* and *h*, 11-4-1 (anti-K^k) (American Type Culture Collection). C3H.L^d and C3H.L^{Q3} express comparable levels of L^d or L^{Q3}, respectively. A similar distribution of antigen is also observed in thymus (data not shown).
METHODS. C3H.L^d and C3H.L^{Q3} mice were generated by injection of C3H/HeJ oocytes with either a 13.2-kilobase (kb) *PvuI/SalI* fragment containing the 27.5.27 L^d gene[8], or a 9.9-kb *SphI* fragment of the L^{Q3} hybrid class I gene construct[7]. Each transgene includes the 5' upstream regulators and promoters for wild-type L^d, and expression is detected on lymphoid tissues as expected. Mice carrying the transgene were identified by dot-blot analysis using a pBR322 probe.

L^d promoter, and antigen expression by these transgenic strains is similar (Fig. 1).

We tested the ability of C3H spleen cells to respond to L^d or L^{Q3}. C3H anti-C3H.L^d primary lymphocyte culture (MLC) effector cells recognize L^d but not L^{Q3}, and this lysis is readily inhibited by anti-CD8, in agreement with our previous findings[7] (Fig. 2*a*). When C3H.L^{Q3} spleen cells are used to stimulate a primary *in vitro* MLC, recognition of L^d does occur, and this response is also inhibited by anti-CD8 (Fig. 2*b*). Although no lysis is detected on L^{Q3} targets, in some experiments a low response is seen to L^{Q3} (data not shown). Thus, spleen cells from L^{Q3} mice do stimulate a primary *in vitro* anti-L^d response. The ability of L^{Q3} transgenic cells to sensitize anti-L^d CTL is probably due to the relatively high levels of this molecule on antigen-presenting dendritic cells[9]. This would be consistent with the data of Alexander *et al.*[10], who show that antigen density affects the extent of CD8 dependency of T-cell recognition. Accordingly, the CTL generated should be biased towards more efficient recognition of L^d versus L^{Q3} on L-cell targets that express relatively low levels of antigen, presumably because the molecule has a wild-type L^d $\alpha 3$ domain.

Secondary C3H anti-L^d recognize L^d, and unlike primary cultures, these cells are relatively resistant to blocking by anti-CD8 (ref. 7) (Fig. 2*c*). This is a characteristic of many secondary alloreactive CTL[11,12]. L^{Q3} targets are also recognized by these effectors[7], but are readily blocked by anti-CD8 (Fig. 2*c*). This finding is similar to that reported by Salter *et al.*[5], who noted that T-cell recognition of $\alpha 3$ mutant class I molecules is particularly sensitive to anti-CD8 blocking. Secondary C3H anti-L^{Q3} CTL recognize both L^d and L^{Q3} and these responses are resistant to blocking by anti-CD8 (Fig. 2*d*). Thus, stimulation with these molecules illuminates a hierarchy of CD8 dependence, presumably reflecting differences in T cell-receptor affinities for antigen. Only relatively high-affinity anti-L^d CTL recognize L^{Q3}.

As our data show that only relatively high-affinity CTL recognize L^{Q3}, we investigated whether expression of the L^{Q3} molecule during T-cell development in C3H.L^{Q3} animals eliminates all L^d-reactive T cells from the repertoire. We therefore cultured L^{Q3} responder cells with L^d stimulators and found that they do respond to L^d, albeit weakly (Fig. 3*a*). Addition of supernatant containing interleukin-2 (IL-2) to this culture enhanced this response (Fig. 3*e*). We restimulated these cultures for two further weeks and found strong L^{Q3} anti-L^d CTL activity (Fig. 3*c*). These CTL are very sensitive to anti-CD8. A dilution of anti-CD8 monoclonal antibody, which fails to inhibit a CD8-dependent primary anti-L^d response (Fig. 3*f*), completely blocks L^{Q3} anti-L^d CTL, suggesting that receptors on these cells have a very low affinity for L^d (Fig. 3*c*). Culture of L^d responder cells with L^{Q3} for six days or for more than three weeks with additional stimulators plus IL-2 does not generate CTL activity, as we

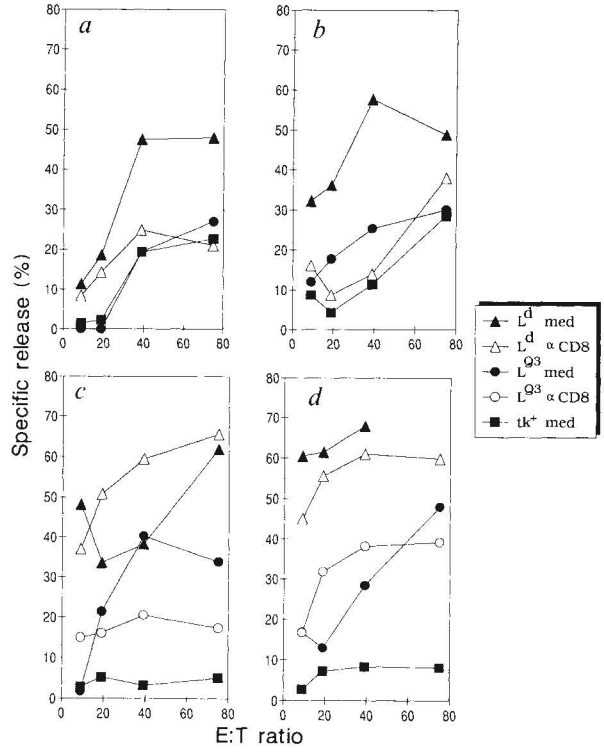

FIG. 2 C3H.L^d or C3H.L^{Q3} spleen cells stimulate anti-L^d responses. *a*, C3H anti-C3H.L^d or *b*, C3H anti-C3H.L^{Q3} primary *in vitro* MLC, d6 assay. *c*, Secondary C3H anti-C3H.L^d, or *d*, C3H anti-C3H.L^{Q3} CTL generated from C3H mice that rejected L^d or L^{Q3} skin grafts (about day 13), were immunized intraperitoneally with 50×10^6 C3H.L^d or C3H.L^{Q3} spleen cells, respectively, after >3 weeks and restimulated *in vitro* on day 7 or 14 and assayed 6 days later. Targets of a standard 6-h ^{51}Cr release assay are L-cell transfectants that express L^d (▲), L^{Q3} (●), or control (■) untransfected cells (thymidine kinase, tk^+), assayed in medium alone or L^d (△) or L^{Q3} (○) in the presence of a 1:20 dilution of culture supernatant of anti-CD8 monoclonal antibody YTS169.4 (YT) (ref. 21). L-cell targets were matched for roughly equivalent expression of transfected class I gene products[7].

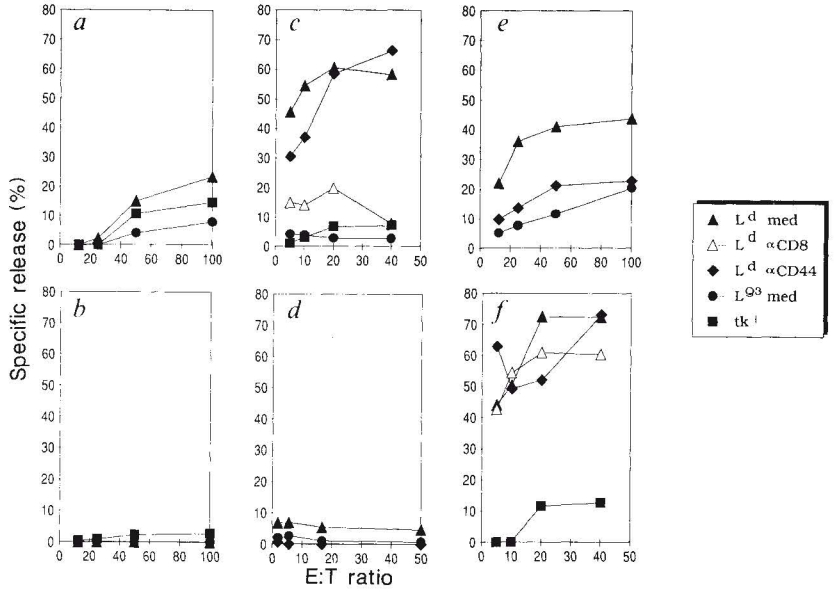

FIG. 3 C3H.L^{Q3} responder cells recognize C3H.L^d in a CD8-dependent manner. *a*, C3H.L^{Q3} anti-C3H.L^d or *b*, C3H.L^d anti-C3H.L^{Q3} primary *in vitro* MLC generated in the absence of exogenous IL-2, day-6 assay. *c*, C3H.L^{Q3} anti-C3H.L^d or *d*, C3H.L^d anti-CH3.L^{Q3} CTL lines generated from primary *in vitro* MLC and fed on days 7, 14, and 21 with C3H.L^d or C3H.L^{Q3} stimulator cells, respectively, and fresh medium plus IL-2 (ref. 22) and assayed on day 24. *e*, C3H.L^{Q3} anti-C3H.L^d primary *in vitro* MLC generated in medium plus IL-2, day-6 assay. *f*, C3H anti-C3H.L^d CTL line from primary *in vitro* MLC fed on days 7, 14 and 21 and assayed on day 24. Targets of a standard 6-h ^{51}Cr release assay are L-cell transfectants that express L^d (▲), L^{Q3} (●), or untransfected control (tk^+) (■), assayed in medium alone; of L^d in the presence of a 1:40 dilution of anti-CD8 culture supernatant (△), or L^d in the presence of a 1:500 dilution of control monoclonal anti-CD44 (◆), which reacts with a molecule present on CTL, which is not involved in killing[23]. Similar results were seen using concanavalin A blast target cells (data not shown).

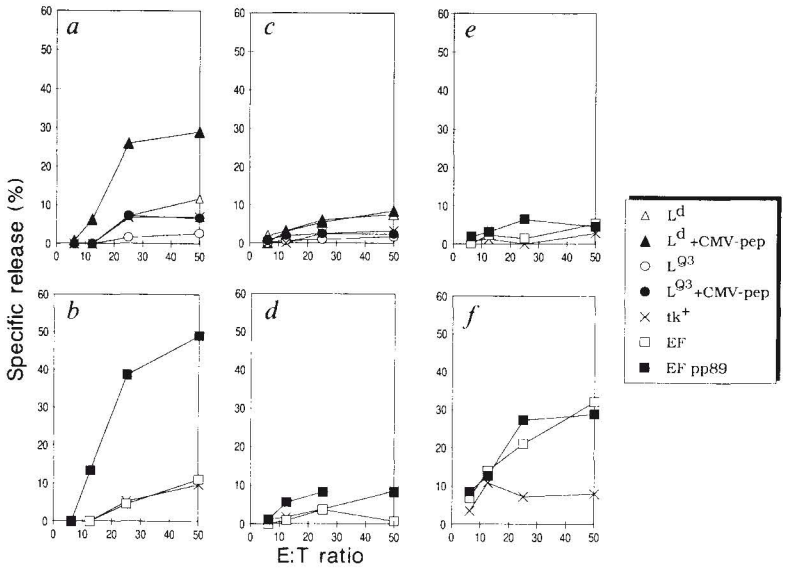

FIG. 4 C3H.L^{Q3} spleen cells do not respond to MCMV nonamer peptide (CMV-pep) presented by C3H.L^{Q3} or C3H.L^d stimulator cells. *a* and *b*, C3H.L^d anti-C3H.L^d + CMV-pep or *c* and *d*, C3H.L^{Q3} anti-C3H.L^{Q3} + CMV-pep or *e* and *f*, C3H.L^{Q3} anti-C3H.L^d + CMV-pep CTL were generated by primary *in vitro* MLC followed by two weekly passages with stimulator cells plus CMV-pep in IL-2-containing medium[7]. In *e* and *f*, data represent two independent experiments. Target cells of a standard 6-h ^{51}Cr release assay were control L cells (×), or L cells that express L^d (△) or L^{Q3} (○) cultured in the presence (filled) or absence (open) of MCMV peptide (YPHFMPTNL; one-letter amino-acid code) at 500 μg ml^{-1} for 15 h at 37 °C[14]; or SV40-transformed BALB/c embryonic fibroblast cells (EF) untransfected (□) or transfected with the *ie1* gene encoding the MCMV pp89 protein (■)[24].

expected (Fig. 3*b*, *d*). Thus, although expression of the L^{Q3} molecule eliminates high-affinity anti-self T cells, a population of low-afinity L^d-reactive T cells, revealed by their reactivity on L^d but not L^{Q3}, evades this negative selection.

As class I molecules function in positive selection of the antigen-specific repertoire[2], we next examined the effect of the Q7^b α3 domain on the generation of virus-specific L^d-restricted T-cell responses. We used a synthetic peptide (designated CMV-pep) derived from the immediate early protein pp89 of murine cytomegalovirus (MCMV) which binds to both L^d and L^{Q3} (see legend to Fig. 4)[7,13,14]. Primary L^d anti-L^d-CMV-pep CTL recognize CMV-pep-pulsed L^d target cells (Fig. 4*a*). This response is blocked by anti-CD8 and these CTL fail to recognize peptide pulsed L^{Q3} targets (Fig. 4*a* and ref. 7). But no anti-CMV-pep activity is detected from L^{Q3}mice (Fig. 4*c*). Further, L^d-CMV-pep, which expresses a native L^d α3 domain and therefore might be recognized by low-affinity L^{Q3}-restricted anti-CMV-pep CTL, is not recognized by L^{Q3} cells (Fig. 4*c*).

We also tested CTL reactivity with target cells that express equivalent levels of L^d, but which differ as to whether they express the MCMV pp89 antigen. L^d anti-L^d-CMV-pep CTL recognize L^d + pp89 cells, but not untransfected cells (Fig. 4*b*), indicating that recognition of L^d-CMV-pep is not due to increased expression of L^d in CMV-pep-treated cells. L^{Q3} anti-L^{Q3}-CMV-pep cultures do not generate anti-L^{d}pp89 activity illustrating further the inability of these cells to stimulate L^{Q3}-restricted antigen-specific responses effectively (Fig. 4*d*).

As the presence of the L^d α3 domain might allow low-affinity CTL to respond to CMV-pep, we co-cultured L^{Q3} responders with L^d-CMV-pep antigen-presenting cells. These cultures do not generate L^d-restricted CMV-pep-specific CTL (Fig. 4*e*, *f*). In some experiments we noted L^{Q3} anti-L^d alloreactivity (Fig. 4*f*), as already described (Fig. 3*a*, *c*, *e*). But this alloreactivity is reduced compared with cultures in which peptide is omitted (compare Figs 4*f* and 3*c*; also data not shown), presumably because CMV-pep pre-empts self peptides from gaining access to L^d, which contributes to the bulk of alloreactive specificities. This is in agreement with the finding that binding of specific peptides to the L^d molecule influences L^d antigenic structure[15]. Regardless of whether an anti-L^d response was detected in these cultures, L^{Q3} anti-L^d-CMV-pep did not elicit detectable antigen-specific responses (Fig. 4*e*, *f*).

Thus, stimulation of CTL precursors with L^d and L^{Q3} allows us to illustrate a hierarchy of CTL affinities and CD8 interactions *in vitro*, and use of transgenic mice demonstrates that a similar *in vivo* hierarchy of affinities, one of which determines the T-cell repertoire, is also influenced by the α3 domain of class I. Previous data indicate that the specificity of the T cell receptor plays a major part in determining the T-cell repertoire[3]. The function of CD8 has been addressed in peripheral T cells where its accessory and co-receptor functions have been demonstrated[16,17]. CD8 participates in the generation of the repertoire because anti-CD8 antibodies injected into perinatal mice prevent selection of CD8$^+$ T cells[6]. Downregulation of CD8 expression has also been noted in transgenic mice bearing anti-self receptors[18]. Further, using a mutant mouse strain lacking cell-surface CD8 expression, Fung-Leung *et al.*[19] have shown that CD8 is necessary for the development of functional alloreactive or antigen-specific class I, but not class II, MHC-restricted T cells.

Our data indicate that the α3 domain of class I controls positive selection of the TCR repertoire. This suggests that although CD8-independent CTL with high-affinity receptors for antigen can function in the periphery, their generation is dependent on this co-receptor during ontogeny. This requirement could reflect the action of CD8 as an accessory molecule with low-density class I on thymic stromal cells where education occurs, but we favour CD8 as a co-receptor required for signalling[20]. The finding that anti-L^d alloreactive T cells are not completely eliminated in L^{Q3} animals could reflect either the retention of low-affinity cells requiring CD8 as an accessory molecule, or a CD8-dependent co-receptor signalling event required for efficient negative selection. □

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Co-engagement of CD8 with the T cell receptor is required for negative selection

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ALTHOUGH it is established that the CD8 and CD4 co-receptors are involved in T-lymphocyte recognition and activation in the periphery, it is less clear whether these molecules participate in thymic selection events. Analysis of thymic selection in mice transgenic for T cell-receptor genes[1-4] or for major histocompatibility complex (MHC) genes[5], or mice injected with antibodies against CD8, CD4 or MHC molecules[6-8], is consistent with the participation of CD8 and CD4 in thymic selection. But antibody-mediated crosslinking of surface receptors in thymic organ cultures[9] has indicated that CD8 is not involved in thymic deletion. We show here that mice transgenic for a mutant MHC class I molecule that cannot interact with CD8 do not delete CD8-dependent T cells reactive with the wild-type molecule. This finding unequivocally establishes that for negative selection in the thymus, CD8 must interact with the same MHC class I molecule as the T cell receptor.

The role initially proposed for CD8 and CD4 was to bind to MHC class I and class II molecules, respectively, and so increase the avidity of the interaction between effector T cells and antigen-presenting cells[10,11]. The association of the protein tyrosine kinase $p56^{lck}$ with CD8 and CD4 (refs 12-15) indicates that these coreceptors could also have a function in signal transduction. We have focused on the identification of the binding site for CD8 on MHC class I molecules, and have shown that substitutions at amino-acid residue 227 (refs 16-18) and at other residues between 222 and 229 (ref. 19) of class I molecules prevents this molecule from interacting with CD8. A similar result was obtained using a conjugate binding assay[20]. We concluded that for effector cell function of CD8-dependent cytotoxic T lymphocytes (CTL), the T cell receptor (TCR) $\alpha\beta$ molecule and CD8 must interact with the same MHC class I molecule. This finding is one of the criteria for distinguishing between the terms co-receptor and accessory molecule[21].

To establish unequivocally a role for CD8 in thymic selection, we derived mice transgenic for a gene encoding the H-$2K^b$ α1 and α2 domains and the α3 domain from either the wild-type *H-2D*^{d} gene (this line is designated 28.1) or a mutant *H-2D*^{d} gene (this line is designated 29.7) which has a single amino-acid substitution at residue 227 (Glu → Lys) that destroys the interaction with CD8 (ref. 18). The genes used to derive these mice were identical in the 5′ and 3′ flanking regions to the *H-2D*^{d} gene used to demonstrate that transgene-encoded class I molecules function identically to endogenously encoded class I molecules[22,23]. Our transgenic mice were originally derived on a (C57B1/6 × BALB/c) F2 background and were backcrossed to B10.D2 for two generations to obtain *H-2*^{d} homozygosity (confirmed by analysis using the polymerase chain reaction (PCR) and oligonucleotides specific for *H-2*^{b} or *H-2*^{d} class II genes). These mice expressed the product of the transgene on spleen cells at a level slightly above the expression of H-$2K^b$ on spleen cells of *H-2*^{b}/*H-2*^{d} F1 mice (Fig. 1). Analysis by two-colour fluorescence activated cell sorting (FACS) of class I expression on thymocytes established that cells expressing high levels of *H-2*^{d}-encoded class I molecules also expressed a high level of the transgene-encoded class I molecule, and that the ratios of T : B cells and of $CD4^+$: $CD8^+$ T cells did not alter in the peripheral lymphoid organs of these mice (data not shown). To examine reactivity for H-$2K^b$ in the absence of any other known MHC gene disparity, the transgenic mice were mated with B10.AKM ($K^kI^kD^q$) and the response to B10.MBR ($K^bI^kD^q$) was analysed. *In vitro* stimulation of spleen cells from (B10.AKM × 29.7)F1 mice (transgenic for the mutant *H-2K*^{b} gene) with irradiated B10.MBR spleen cells elicited anti-H-$2K^b$ CTL that were cytotoxic for EL4 (H-2^b) cells (Fig. 2*a*). As these CTL also killed M12.C3 cells transfected with the *H-2K*^{b}*α1α2/H-2D*^{d} wild-type *α3* gene (M12.28K^b; Fig. 2*b*), the determinants recognized by these CTL were present on both wild-type H-$2K^b$ molecules and H-$2K^b$/H-$2D^d$ exon-shuffled molecules. This CTL population was not cytotoxic for M12.C3 transfectants expressing the H-$2K^b$ molecule in association with the mutated H-$2D^d$α3 domain (M12.29K^b; Fig. 2*c*). To confirm that mice transgenic for the gene with the mutant α3 domain (29.7) were not tolerant to H-$2K^b$α1/α2 determinants expressed in association with a wild-type α3 domain from either H-$2D^d$ or H-$2K^b$, spleen cells from the 29.7 mice were stimulated *in vitro* with spleen cells from the 28.1 mice (transgenic for the *H-2K*^{b}*α1/α2 H-2D*^{d} α3 wild-type gene). In this strain combination, the only difference in MHC genes is the substitution at residue 227 in the transgene of the responder. CTL generated under these conditions killed both EL4 and the M12.28K^b cells (Fig. 3*a* and *b*), establishing that the mutation in the α3 domain of the 29.7 mice resulted in the failure of these mice to delete T cells alloreactive to H-$2K^b$ α1/α2 determinants expressed in association with a wild-type α3 domain from either H-$2D^d$ or H-$2K^b$. These CTL were completely inhibited by antibody to CD8 and did not kill the M12.29K^b cells (Fig. 3*c*), suggesting that there were no autoreactive CD8-independent T cells present in the 29.7 mice. Co-culture of spleen cells from the (B10.AKM × 28.1)F1 mouse with B10.MBR spleen cells did not generate any

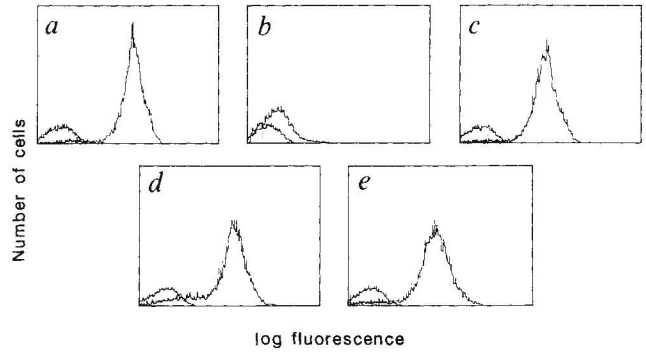

FIG. 1 Flow microfluorometric analysis of H-$2K^b$ expression on spleen cells from *a*, C57BL/6; *b*, B10.D2; *c*, (C57BL/6 × DBA/2)F1; *d*, *e*, two of the H-$2K^b$ transgenic lines. The background staining for each population is represented in the same panel.
METHODS. Spleen cells (10^6) were incubated with biotinylated monoclonal antibody EH144 (anti-H-$2K^b$) for 30 min on ice, washed twice and then incubated for a further 30 min with fluoroscein isothiocyanate-conjugated avidin. After staining, cells were washed and the fluorescence of 10,000 viable cells was measured on an EPICS Profile analyser on a logarithmic scale.