Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 11396–11400, December 1993
Immunology

Amino acid substitutions in the floor of the putative antigen-binding site of H-2T22 affect recognition by a γδ T-cell receptor

(major histocompatibility complex)

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ABSTRACT **We have previously identified a self-reactive γδ T-cell clone (KN6) specific for the *H-2T* region gene product $T22^b$. Now we have investigated by an *in vitro* mutagenesis analysis of the *T22*^{b} gene the possibility that the interaction between the KN6 γδ T-cell receptor and $T22^b$ involves a peptide. The results demonstrate that mutations at the floor of the putative antigen-binding groove of $T22^b$ affect recognition by the γδ T-cell receptor. Furthermore, we have shown that KN6 cells react with cells that are deficient in the class I peptide transporter TAP1/TAP2. These results suggest that peptide is involved in the interaction of the KN6 T-cell receptor with T22 and that loading of T22 with the putative peptide is TAP1/TAP2-independent.**

T cells expressing αβ heterodimeric antigen receptors (αβ T cells) recognize antigen-derived peptides presented by major histocompatibility complex (MHC) class I or class II molecules. Less is known about the ligand specificity of the second type of T cells, bearing γδ receptors. Although γδ T cells recognizing classical class I or class II molecules have been identified (1–4), these T cells seem to recognize more commonly nonclassical class I (1, 5–7) or class I-like (8–10) molecules. Only in one case (6) has the involvement of peptide in the recognition been documented.

We have previously demonstrated that the product encoded by the *H-2T22* gene is recognized by the T-cell receptor (TCR) derived from the mouse KN6 γδ T-cell hybridoma (7). Here, we present a mutational analysis of the *T22* gene. The results demonstrate that mutations in the putative antigen-binding groove of T22 affect recognition by the KN6 γδ TCR. We also demonstrate that the KN6 cells can be activated by the TAP2-deficient cell line RMA-S or by splenocytes from TAP1 mutant mice. TAP1/TAP2 is a peptide transporter that lies in the major route for the assembly and cell surface expression of the MHC class I-peptide complex. Our results suggest that peptide is involved in the interaction of KN6 γδ TCR with T22 molecules and that this putative peptide is loaded to a T22 molecule by a TAP1/TAP2-independent mechanism.

MATERIALS AND METHODS

Cells and Mice. A20 is a mouse B-lymphoma cell line from BALB/c (*H-2*^{d}) origin and was obtained from the American Type Culture Collection. Monkey COS cells were from B. Seed (Massachusetts General Hospital, Boston). RMA and RMA-S cell lines (obtained from K. Kärre, Karolinska Institute, Stockholm, Sweden) were derived from the Rauscher virus-transformed mouse *H-2*^{b} lymphoma cell line RBL-5 (11, 12). RMA-S is a class I-negative variant from RMA with a mutant TAP2 gene (13). The KN6 transgenic mice have been described (5, 14). The generation of TAP1-deficient mice (*H-2*^{b}) has also been described (15).

Plasmid Construction and Transfection. The $T22^b$ cDNA has been described (7); $T22^k$ cDNA isolation will be described elsewhere. Mutations were introduced by the polymerase chain reaction (PCR) method (16) or with an oligonucleotide-directed *in vitro* mutagenesis system (Amersham). (See Fig. 1 for positions of residues that were mutated.) The wild-type or mutated T22 cDNAs were cloned into the expression vector pSVL (Pharmacia) containing the simian virus 40 late promoter. Transfections were performed by the DEAE-dextran method (18) with 10 μg of DNA. Transfection efficiencies were monitored by cotransfection with a human growth hormone (hGH) cDNA plasmid and measuring the activity of hGH in the medium with an Allegro hGH transient gene expression assay system (Nichols Institute, San Juan Capistrano, CA).

KN6 Recognition Assay. Recognition by KN6 γδ T cells was assayed with γδ T cells from transgenic mice that express productively rearranged KN6 γ and δ TCR genes (5, 14). $CD4^-8^-$ splenic T cells, isolated as described (19), were used as responders. Responders (5×10^4) were cocultured with various irradiated stimulator cells for 72 hr. Cell lines and transfectants used as stimulators were irradiated at 18,000 rads (1 rad = 0.01 Gy), and spleen stimulator cells were irradiated at 3000 rads. Various dilutions of stimulator cells were used in each experiment. The proliferating cells were labeled with [^{3}H]thymidine (1 μCi per well; 1 μCi = 37 kBq) for 6 hr and harvested for measurement of radioactivity in a scintillation counter. The result for only one dilution of stimulator cells is shown. The results shown are the mean cpm of triplicate cultures.

RESULTS

The Difference in the KN6-Stimulatory Activities of $T22^b$ and $T22^k$ Transfectants Resides in Residue 25. KN6 γδ T cells proliferate in the presence of spleen cells from many different mouse strains (7). We have isolated T22 cDNAs from various mouse strains and tested transient transfectants of these cDNAs for the recognition by KN6 γδ T cells in the proliferation assay. $T22^b$ transfectants of mouse A20 (*H-2*^{d}) cells and monkey COS cells strongly stimulated KN6 cells, whereas $T22^k$ transfectants were poorer stimulators (Fig. 2). In the α1 and α2 domains, $T22^b$ and $T22^k$ differ in two residues, the valine/phenylalanine difference at residue 25 and the arginine/glutamine difference at residue 107 (Fig. 1). If T22 adopts an essentially same three-dimensional structure as MHC class I molecules (17), residue 25 would be located at the bottom of the putative antigen-binding groove and

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Abbreviations: MHC, major histocompatibility complex; TCR, T-cell receptor.

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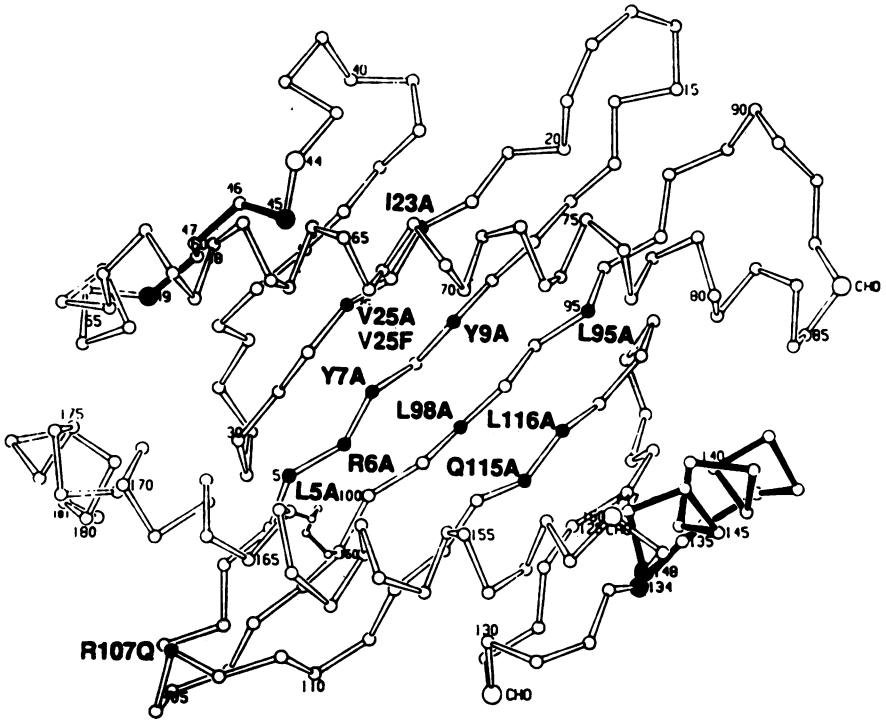

FIG. 1. Proposed model for the three-dimensional structure of $T22^b$ $\alpha1/\alpha2$ domains and location of amino acid substitutions. The model is based on the three-dimensional structure of HLA-A2 (17). Numbers designate α carbons of HLA-A2 amino acids. With the assumption that the polypeptide chain folding is similar, the possible locations of deletions present in $T22^b$ are indicated on the HLA-A2 structure (solid bonds). Large filled circles indicate the ends to be joined after deletion. Large open circles with a label CHO indicate the locations of potential N-linked glycosylation sites in $T22^b$. Filled circles with abbreviations indicate the locations of various mutations. Mutants are named according to residue positions and amino acid substitutions; e.g., mutant L98A has an alanine-for-leucine substitution in position 98.

therefore could affect peptide binding but would be unlikely to make direct contacts with TCR. Residue 107 would be positioned in one of the loops connecting β-strands and would be unlikely to affect peptide binding.

***In Vitro* Mutagenesis Analysis Suggests That a Peptide Is Involved in the KN6 $\gamma\delta$ TCR–T22 Interaction.** We generated two $T22^b$ variants in which the residue at position 25 or 107 was converted to the corresponding residue of $T22^k$, transfected A20 and COS cells with each of the two variants, and tested the transfectants in the KN6 proliferation assay. The V25F transfectants stimulated KN6 poorly, to a similar extent as $T22^k$ transfectants, whereas the R107Q transfectants were as good stimulators as $T22^b$ transfectants (Fig. 2). These results suggest that the valine/phenylalanine difference at residue 25 is critical in determining the difference in the KN6 stimulatory activities of $T22^b$- and $T22^k$-expressing cells. In contrast, the arginine/glutamine difference at residue 107 seems to be irrelevant. Since the residue 25 is at the bottom of the putative peptide-binding groove, the data are consistent with the hypothesis that peptide is involved in the KN6 TCR–T22 interaction.

To investigate this hypothesis further, we generated a set of 10 $T22^b$ variants in which one of the residues at the bottom of the putative $T22^b$ peptide-binding groove was altered to an alanine and tested them by the same assay. Four mutations (Y9A, V25A, I23A, and Q115A) did not affect the KN6-stimulatory activity, while another 4 mutations (Y7A, L95A, L98A, and L116A) reduced the KN6-stimulatory activity drastically (Fig. 3). The 9th mutation, L5A, greatly reduced the KN6-stimulatory activity when tested as A20 transfectants but exhibited only a mildly negative effect as COS transfectants. The 10th mutation, R6A, had a weak effect in A20 and no significant effect in COS. It is interesting that the V25A replacement did not alter the stimulatory activity of the transfectants whereas the V25F replacement did (see above). This is consistent with the view that the bulky side chain of phenylalanine but not the small side chain of alanine interferes with the binding of the putative peptide with the $T22^b$ molecules. Taken together these data support the hypothesis that a peptide is involved in the interaction between KN6 $\gamma\delta$ TCR and T22.

TAP1- or TAP2-Deficient Cells Can Stimulate KN6 $\gamma\delta$ T Cells. Most peptides bound to classical class I molecules are generated in the cytosol and transported into the lumen of the endoplasmic reticulum by an ATP-dependent heterodimeric peptide transporter encoded by the TAP1 and TAP2 genes (20–22). Cells with a mutation in the TAP1 or TAP2 gene fail to stimulate many MHC class I-restricted $\alpha\beta$ T-cell clones, because the stable cell surface expression of the MHC class I–peptide complexes recognized by these T cells requires peptide translocation mediated by the TAP1/TAP2 transporter. To examine whether loading of T22 by the putative peptide recognized by KN6 TCR is also dependent on the TAP1/TAP2 transporter, we attempted to stimulate KN6 cells with TAP1- or TAP2-deficient cells. As shown in Fig. 4, KN6 cells responded to the TAP2-deficient RMA-S cells as well as to RMA cells with the intact TAP2 gene. Likewise, the response of KN6 cells to the spleen cells from TAP1 mutant mice was similar to the response of those T cells to TAP1-positive spleen cells. These results indicate that the TAP1/TAP2 transporter function is not required for the stimulation of KN6 cell proliferation.

DISCUSSION

The specificity of $\gamma\delta$ T cells is poorly understood. We (7, 23, 24) and others (6, 25–27) have proposed that $\gamma\delta$ T cells might be specific for nonclassical class I molecules. For instance, the KN6 T-cell clone used in this study is specific for the *H-2T22*^{b} gene product (7). Other examples are as follows. An

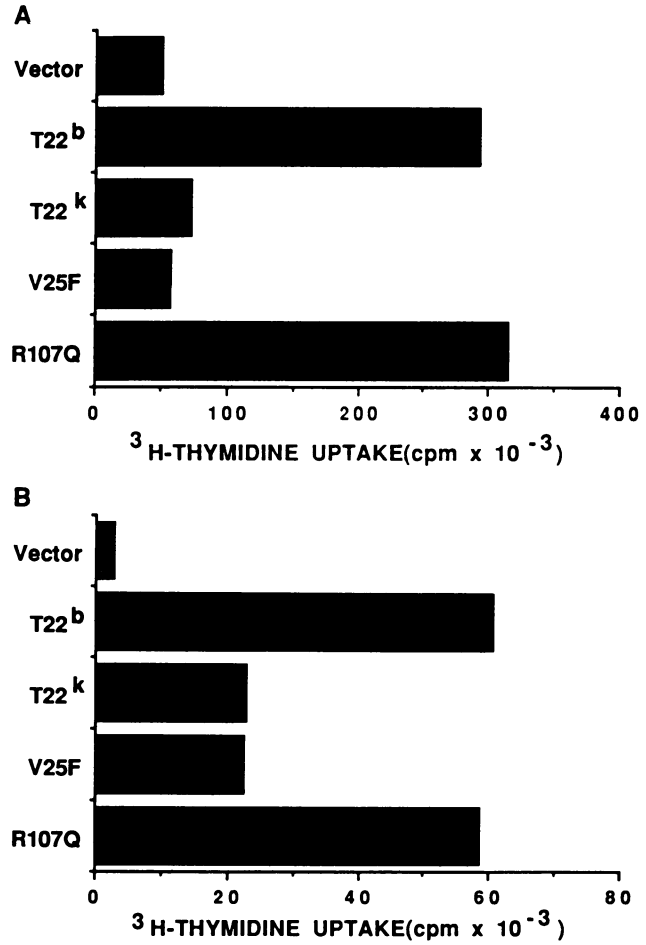

FIG. 2. Reactivity of γδ T cells against A20 (*A*) or COS (*B*) cells transfected with $T22^b$, $T22^k$, and $T22^b$ mutants. $CD4^-8^-$ splenic T cells (5×10^4) from KN6 transgenic mice were cultured for 3 days with the indicated irradiated transfectants; 3×10^4 A20 stimulator cells or 1.5×10^4 COS stimulator cells were used per well. One representative experiment of three separate transfections is shown.

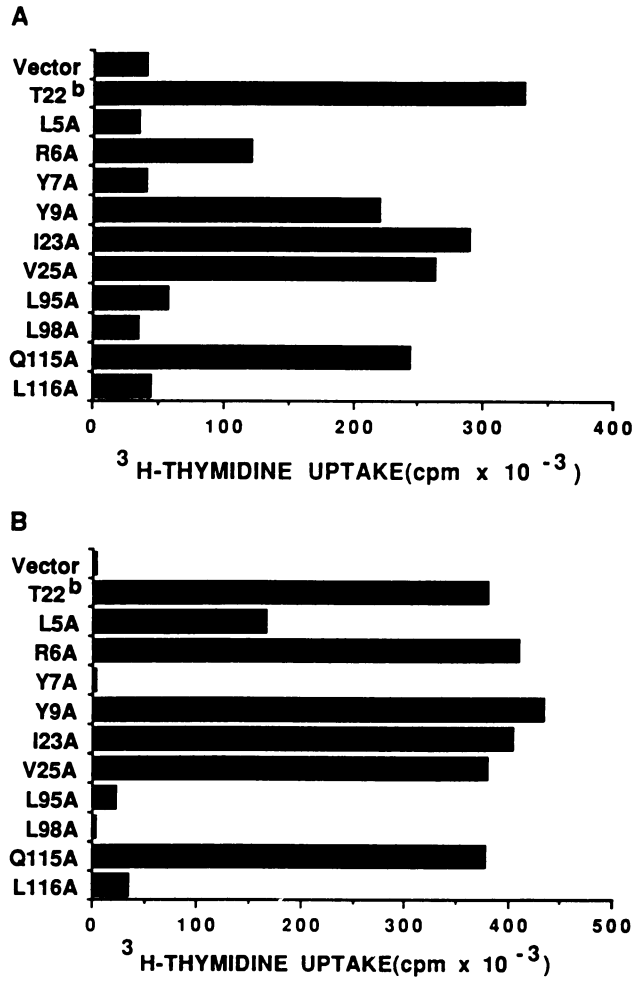

FIG. 3. Reactivity of KN6 γδ T cells against A20 (*A*) or COS (*B*) cells transfected with site-specific mutants at the putative groove of $T22^b$. The same method was used as in Fig. 2.

alloreactive γδ cytotoxic T-cell line was isolated that recognizes an *H-2T* region-controlled determinant, possibly identical to H-2T22 (1, 28). A γδ T helper hybridoma was shown to recognize the product of the *T23*^{b} gene ($Qa\text{-}1^b$) (6). Two human γδ T-cell clones were found to recognize CD1c (8), which is related to MHC class I molecules (29).

Only in one of these cases has the involvement of peptide in the interaction between γδ T cells and a nonclassical MHC been documented. The $Qa\text{-}1^b$-specific hybridoma mentioned above was obtained by fusion of poly(Glu,Tyr)-primed lymph node cells from DBA/2 mice with BW5147 thymoma cells, and was shown to recognize the Glu,Tyr copolymer in the context of $Qa\text{-}1^b$ (6). It is tempting to interpret the results of our mutant study as indicating that KN6 γδ TCR recognizes peptide. However, we cannot exclude the possibility that nonpeptide moieties such as carbohydrates are embedded in the T22 molecule and recognized by the TCR. T cells that modulate the antibody response against carbohydrates in a (classical) MHC-independent fashion have previously been identified (26, 30, 31).

Although the putative T22-binding moiety recognized by KN6 γδ TCR is unknown, the following observations are relevant in deducing its characteristics. First, KN6 recognizes syngeneic cells as well as some allogeneic cells (7). Second, cells from a variety of tissues tested are capable of stimulating KN6 (7, 24). Third, KN6 reacts with some monkey (this study) and human (L.V.K. and S.T., unpublished work) cells transfected with $T22^b$ cDNA. Finally, KN6 reacts with cells deficient in the TAP1/TAP2 transporter (this study). Thus, the putative T22-binding moiety is a self component commonly expressed by a variety of cells and conserved among various mammalian species and is, if it is peptide, brought to the T22 molecule via a pathway separate from the major class I pathway dependent on the TAP1/TAP2 transporter.

TAP-independent mechanisms for peptide presentation by class I molecules have been described before. For instance, a polypeptide chain cotranslationally translocated into the endoplasmic reticulum by a signal peptide can be a source of peptide that is presented by class I molecules in a TAP-independent manner (32–35). Another TAP-independent mechanism of peptide acquisition has been proposed for the class I-like molecule CD1b which is sensitive to chloroquine and therefore likely to involve an endocytic route of antigen provision as in the major peptide loading pathway for MHC class II molecules (36). This mechanism might also be responsible for the presentation of some viral antigens to class I molecules by TAP-deficient RMA-S or T2 cells (37–41).

While the reason why KN6 response is TAP independent is unknown, an attractive possibility would be that the putative peptide recognized by KN6 γδ TCR is derived from highly conserved stress proteins which are present in the lumen of the endoplasmic reticulum. Reactivity of some γδ TCRs with heat shock proteins has been reported (reviewed in ref. 42), and some of these proteins have been shown to be located in the lumen of the endoplasmic reticulum (reviewed

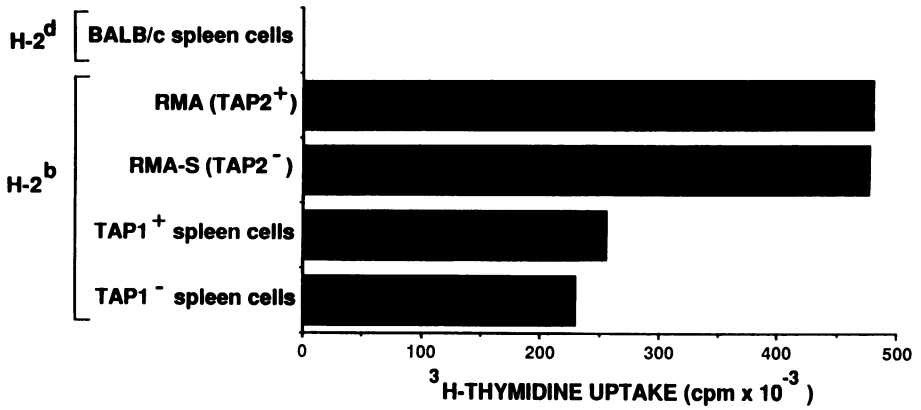

FIG. 4. KN6 cells recognize cells that are deficient in the presentation of peptides to MHC class I-specific $\alpha\beta$ T cells. KN6 cells from KN6 transgenic mice were cultured with the indicated irradiated stimulator cells (5×10^4 per well) for 3 days. BALB/c stimulator spleen cells are not recognized by KN6 (5, 7) and were used as negative control.

in ref. 43). Another possibility, which is not exclusive of the first one, is that nonclassical class I molecules may generally tend to be loaded by a TAP-independent pathway by antigen. In addition to the human CD1b case mentioned above, Qa-1^b-specific alloreactive cytotoxic T cells that recognize RMA-S have been identified (44, 45). Qa-1 is encoded by another nonclassical class I gene, *T23*, that is linked to *T22* (7, 24, 46).

We wish to thank Dr. B. Seed for COS cells, Dr. K. Kärre for RMA and RMA-S cells, members of our laboratory for helpful discussion, and Hala Bakhsh and Elly Basel for secretarial assistance. S.M. and Y.I. were supported by Kao Corporation, and B.S.K. was supported by the Howard Hughes Medical Institute. This work was funded by the Howard Hughes Medical Institute, the National Institutes of Health, and NEC Corporation.

1. Bluestone, J. A., Cron, R. Q., Cotterman, M., Houlden, B. A. & Matis, L. A. (1988) *J. Exp. Med.* **168,** 1899–1916.
2. Matis, L. A., Fry, A. M., Cron, R. Q., Cotterman, M. M., Dick, R. F. & Bluestone, J. A. (1989) *Science* **245,** 746–749.
3. Ciccone, E., Viale, O., Pende, D., Malnati, M., Ferrara, G. B., Barocci, S., Moretta, A. & Moretta, L. (1989) *Eur. J. Immunol.* **19,** 1267–1271.
4. Spits, H., Paliard, X., Engelhard, V. H. & de Vries, J. E. (1990) *J. Immunol.* **144,** 4156–4162.
5. Bonneville, M., Ito, K., Krecko, E. G., Itohara, S., Kappes, D., Ishida, I., Kanagawa, O., Janeway, C. A., Jr., Murphy, D. B. & Tonegawa, S. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 5928–5932.
6. Vidovic, D., Roglic, M., McKune, K., Guerder, S., MacKay, C. & Dembic, Z. (1989) *Nature (London)* **340,** 646–650.
7. Ito, K., Van Kaer, L., Bonneville, M., Hsu, S., Murphy, D. B. & Tonegawa, S. (1990) *Cell* **62,** 549–561.
8. Porcelli, S., Brenner, M. B., Greenstein, J. L., Balk, S. P., Terhorst, C. & Bleicher, P. A. (1989) *Nature (London)* **341,** 447–450.
9. Spits, H., Paliard, X. & de Vries, J. E. (1989) *J. Immunol.* **143,** 1506–1511.
10. Lam, V., DeMars, R., Chen, B. P., Hank, J. A., Kovats, S., Fisch, P. & Sondel, P. M. (1990) *J. Immunol.* **145,** 36–45.
11. Ljunggren, H.-G. & Kärre, K. (1985) *J. Exp. Med.* **162,** 1745–1759.
12. Kärre, K., Ljunggren, H. G., Piontek, G. & Kiessling, R. (1986) *Nature (London)* **319,** 675–678.
13. Yang, Y., Früh, K., Chambers, J., Waters, J. B., Wu, L., Spies, T. & Peterson, P. A. (1992) *J. Biol. Chem.* **267,** 11669–11672.
14. Ishida, I., Verbeek, S., Bonneville, M., Itohara, S., Berns, A. & Tonegawa, S. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 3067–3071.
15. Van Kaer, L., Ashton-Rickardt, P. G., Ploegh, H. L. & Tonegawa, S. (1992) *Cell* **71,** 1205–1214.
16. Hemsley, A., Arnheim, N., Toney, M. D., Cortopassi, G. & Galas, D. J. (1989) *Nucleic Acids Res.* **17,** 6545–6551.
17. Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennett, W. S., Strominger, J. L. & Wiley, D. C. (1987) *Nature (London)* **329,** 506–512.
18. Templeton, D. & Eckhart, W. (1984) *Mol. Cell. Biol.* **4,** 817–821.
19. Maeda, K., Nakanishi, N., Rogers, B. L., Haser, W. G., Shitara, K., Yoshida, H., Takagaki, Y., Augustin, A. A. & Tonegawa, S. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 6536–6540.
20. Monaco, J. J. (1992) *Immunol. Today* **13,** 173–179.
21. Shepherd, J. C., Schumacher, T. N. M., Ashton-Rickardt, P. G., Imaeda, S., Ploegh, H. L., Janeway, C. A., Jr., & Tonegawa, S. (1993) *Cell* **74,** 577–584.
22. Neefjes, J. J., Momburg, F. & Hammerling, G. J. (1993) *Science* **261,** 769–771.
23. Tonegawa, S., Berns, A., Bonneville, M., Farr, A., Ishida, I., Ito, K., Itohara, S., Janeway, C. A., Jr., Kanagawa, O., Katsuki, M., Kubo, R., Lafaille, J., Mombaerts, P., Murphy, D., Nakanishi, N., Takagaki, Y., Van Kaer, L. & Verbeek, S. (1989) *Cold Spring Harbor Symp. Quant. Biol.* **54,** 31–44.
24. Van Kaer, L., Wu, M., Ichikawa, Y., Ito, K., Bonneville, M., Ostrand-Rosenberg, S., Murphy, D. B. & Tonegawa, S. (1991) *Immunol. Rev.* **120,** 89–115.
25. Janeway, C. A., Jr., Jones, B. & Hayday, A. C. (1988) *Immunol. Today* **6,** 73–76.
26. Strominger, J. L. (1989) *Cell* **57,** 895–898.
27. Born, W., Hall, L., Dallas, A., Boymel, J., Shinnick, T., Young, D., Brennan, P. & O'Brien, R. (1990) *Science* **249,** 67–69.
28. Houlden, B. A., Matis, L. A., Cron, R. Q., Widacki, S. M., Brown, G. D., Pampeno, C., Meruelo, D. & Bluestone, J. A. (1989) *Cold Spring Harbor Symp. Quant. Biol.* **54,** 45–55.
29. Calabi, F. & Milstein, C. (1986) *Nature (London)* **323,** 540–543.
30. Letvin, N. L., Benacerraf, B. & Germain, R. N. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 5113–5117.
31. Shapiro, M. E., Kasper, D. L., Zaleznik, D. F., Spriggs, S., Onderdonk, A. B. & Finberg, R. W. (1986) *J. Immunol.* **137,** 341–346.
32. Henderson, R. A., Michel, H., Sakaguchi, K., Shabanowitz, J., Appella, E., Hunt, D. F. & Engelhard, V. H. (1992) *Science* **255,** 1264–1266.
33. Wei, M. L. & Cresswell, P. (1992) *Nature (London)* **356,** 443–446.
34. Buchmeier, M. J. & Zinkernagel, R. M. (1992) *Science* **257,** 1142.
35. Hammond, S. A., Bollinger, R. C., Tobery, T. W. & Siliciano, R. F. (1993) *Nature (London)* **364,** 158–161.
36. Porcelli, S., Morita, C. T. & Brenner, M. B. (1992) *Nature (London)* **360,** 593–597.
37. Zhou, X., Glas, R., Momburg, F., Hämmerling, G. J., Jondal, M. & Ljunggren, H. G. (1993) *Eur. J. Immunol.* **23,** 1796–1801.
38. Zhou, X., Glas, R., Liu, T., Ljunggren, H. G. & Jondal, M. (1993) *Eur. J. Immunol.* **23,** 1802–1808.

39. Hosken, N. A. & Bevan, M. J. (1992) *J. Exp. Med.* **175,** 719–729.
40. Esquivel, F., Yewdell, J. & Bennink, J. (1992) *J. Exp. Med.* **175,** 163–168.
41. Sijts, A. J. M., De Bruijn, M. L. H., Nieland, J. D., Kast, W. M. & Melief, C. J. M. (1992) *Eur. J. Immunol.* **22,** 1639–1642.
42. Haas, W., Pereira, P. & Tonegawa, S. (1993) *Annu. Rev. Immunol.* **11,** 625–673.
43. Gething, M.-J. & Sambrook, J. (1992) *Nature (London)* **355,** 33–45.
44. Hermel, E., Grigorenko, E. & Fischer Lindahl, K. (1991) *Intl. Immunol.* **3,** 407–412.
45. Aldrich, C. J., Waltrip, R., Hermel, E., Attaya, M., Fischer Lindahl, K., Monaco, J. J. & Forman, J. (1992) *J. Immunol.* **149,** 3773–3777.
46. Wolf, P. R. & Cook, R. G. (1990) *J. Exp. Med.* **172,** 1795–1804.