Cells Expressing a Major Histocompatibility Complex Class I Molecule with a Single Covalently Bound Peptide Are Highly Immunogenic

By Estelle Mottez,* Pierre Langlade-Demoyen,* Hélène Gournier,† Frédéric Martinon,‡ Janet Maryanski,§ Philippe Kourilsky,* and Jean-Pierre Abastado*

From *Unité de Biologie Moléculaire du Gène, INSERM U277, and †Immunité Cellulaire Antivirale, Institut Pasteur, 75724 Paris; §Institut Cochin de Génétique Moléculaire, Hôpital Cochin, 75013 Paris, France; and the ‡Ludwig Institute for Cancer Research, Lausanne Branch, CH-1066 Epalinges, Switzerland

Summary

The major histocompatibility complex (MHC) class I molecules expressed at the cell surface are associated with a large number of different peptides so that the density of a given MHC–peptide complex is relatively low. Here we describe the properties of MHC class I molecules genetically attached to a single antigenic peptide. Cells expressing these fusion proteins are recognized by T cells specific for the particular MHC–peptide complex. Coculture of naive splenocytes with cells expressing these MHC–peptide fusion proteins and the B7.1 antigen allows the induction of primary cytotoxic T lymphocytes (CTL) in vitro. Injection of these cells into naive mice enhances the frequency of specific CTL precursors and protects against a subsequent challenge with a tumor or a virus bearing the antigenic peptide. Soluble MHC–peptide fusions were also produced in which all three components, that is, the heavy chain, β2-microglobulin and the peptide, have fused into a single-chain protein. The availability of MHC class I molecules bound to a single peptide provides valuable tools for the manipulation of CTL responses and the analysis of the selection processes in the thymus.

The MHC class I molecules present peptides which are mainly derived from endogenously synthesized proteins (1, 2). These MHC-peptide complexes can be recognized by T cells bearing specific TCRs (for review see reference 3). A given cell expresses 10^8–10^9 MHC class I molecules (4) and probably presents up to 10^4 different peptides or perhaps even more (5). A specific MHC–peptide complex is therefore present in small amounts per cell. 100–200 complexes are sufficient to sensitize a target cell for lysis by an already activated CTL clone (6). This number, however, is too low to activate CTL precursors from a naive mouse. CTL have often proven to be both efficient and specific against viruses. Eliciting CTL against tumors has also been a major goal of immunologists. Since tumors are variants of normal cells, most peptides presented by a tumor cell are self-peptides. Self-specific CTL are normally deleted in the thymus through a process called negative selection. The only immunogenic peptides in a tumor are actually often poorly presented, either because they are derived from poorly expressed proteins or because they bind poorly to the MHC molecules. Increasing the density of a specific MHC–peptide may help induce specific CTL, especially against a tumor, and different strategies have been taken. Mutant cell lines deficient in the translocation of endogenous peptides into the lumen of the endoplasmic reticulum (ER) can be efficiently loaded with exogenous peptides and have been used to induce specific CTL. Their use in vivo, however, is limited by allogenic or xenogenic reactivity.

MHC class I molecules are heterodimers composed of a polymorphic transmembrane heavy chain noncovalently associated with β2-microglobulin (β2m). We took advantage of their known tridimensional structure (7–9) to produce soluble K^d, K^b, and HLA-A2 molecules by connecting through an appropriate peptidic link the COOH terminus of the third domain of the corresponding heavy chains to the NH2 terminus of β2m (10, 11). These single chain MHC class I molecules are soluble in the absence of detergent (12). The single chain K^d molecule has been more extensively studied and found to bind the same repertoire of peptides as its native cell surface–expressed counterpart (13), to be functional, and has provided useful tools to study T cell activation (14). The tridimensional structure of several MHC class
I–peptide complexes has been determined by crystallography (15–20). The peptide lies in a groove made of a platform of β sheets and two α helices encoded by the second and third exons of the gene encoding the heavy chain. The overall topology of these different complexes is similar, most of the interaction resulting from conserved contacts between the residues of the MHC groove and conserved structures of the peptide.

In this study, we used this information to genetically connect an antigenic peptide to the heavy chain of the mouse Kd molecule. The purpose was to produce cell lines in which the density of the chosen MHC–peptide complex would be considerably increased. The expectation was that such cells would be potent immunogens capable of inducing specific CTL from naïve mice. We show here that this is the case for three Kd–peptide fusions and that the corresponding transfected cells are recognized by all specific CTL tested and are highly immunogenic in vitro and in vivo. Mice vaccinated with these cells are specifically protected against a subsequent virus or tumor challenge. In addition, a soluble peptide–Kd fusion was also constructed and shown to activate in vitro a specific T cell hybridoma. Thus, the extracellular part of MHC molecules can be manipulated at both ends. Such single-chain MHC–peptide complexes could be useful in vivo, especially when the native complex has a rapid dissociation rate.

Materials and Methods

Cell Lines. L (CCL1.3, American Type Culture Collection [ATCC], Rockville, MD), LKd, and P815-Cw3 have been previously described (21) and were grown in DME supplemented with 10% FCS. CHO cells or CHO cell transfectants were grown in αMEM supplemented with 10% FCS or only 2% for soluble MHC production.

Plasmid Construction and Transfection. Unique XhoI and SmaI sites were introduced by site directed mutagenesis into the Kd sequence at the ApaI site located at the 3' end of the leader sequence. Double strand oligonucleotides encoding the various antigenic peptides were cloned between these two sites (see Fig. 2). The resulting constructs were transfected into mouse L cells (ATCC CCL 1.3). Stable transfectants expressing high levels of the fusion proteins were selected by flow cytometry sorting by use of the SF1-1.1.1 mAb (ATCC HB159) in a FACStar® station (Becton Dickinson & Co., Mountain View, CA).

Soluble single-chain Kd-Cw3-N10 was obtained by replacing the 5' moiety (a NotI-EcoRV fragment) of the soluble single-chain Kd (10) with that from the membrane-bound Kd-Cw3-N10. The resulting construct was inserted into pFRSV (22) and transfected into CHO cells. Stable transfectants were selected and cultivated in increased concentrations of methotrexate as described (22). Culture supernatants were tested for Kd or peptide–Kd secretion by immunoassay.

Immunoassay for Soluble Kd. 96-well plates (CML-Microtest Luxlon, Nemours, France) were coated for 2 h with SF1-1.1.1 (anti-α2) at 5 μg/ml in 100 mM carbonate buffer, pH 9.5. After washing and blocking with PBS containing 0.5% gelatin, serial dilutions of culture supernatants were added to the wells and incubated for 1 h. After washing with PBS, 50 μl of 1 μg/ml biotinylated 20-8-4S (anti-α1-α2) (23) was added. Immune complexes were revealed with horseradish peroxidase-conjugated streptavidin by use of o-phenylenediamine dihydrochloride (P8806; Sigma Chemical Co., St. Louis, MO) as substrate.

Immunoassay for Peptide Elution. Cells (10⁶) were metabolically labeled with [35S]methionine (10 μCi) for 1 h, washed, lysed, and immunoprecipitated as previously described (13). Immunoprecipitates were analyzed by SDS-PAGE and revealed by fluorography.

T Cell Hybridoma. The T cell hybridoma 9.4 (14), product of the fusion of the Cw3/1.1 CTL clone with the 58 TCR-αα–ββ-hybridoma (29), has been previously described. T cell response was determined by measuring IL-2 secretion monitored by its capacity to sustain the proliferation of the IL-2–dependent cell line CTLL-2.

Primary CTL Induction. Marine nucleoprotein (NP) and Cw3 primary specific CTL were generated in 5-d MLC by incubating 3 × 10⁷ splenocytes with 5 × 10⁶ irradiated (10,000 rad) transfected L cells. Lymphocytes were harvested on day 5 and assayed for cytolytic activity on 51Cr-labeled target cells.

Cytotoxicity Assays. Cell-mediated cytolytic activities were detected in a 6-h 51Cr assay. Percent specific lysis of 10⁶ 51Cr-labeled target cells in 200 μl was determined for various lymphocyte-to-

### Table 1. Peptide-Kd Fusions Used in this Study

| Construct | Peptide | Spacer | Cells |
|-----------|---------|--------|-------|
| Kd        |         |        |       |
| Kd-A2[170-185] | RYLENGKETLQRTDAP | GG | LKd  |
| Kd-Cw3[170-185] | RYLKNGKETLQRAEHP | GG | L-Kd-A2.1, -A2.2, -A2.3 |
| Kd-Cw3-N10 | RYLKNGKETL | GGPGGGSGGGG | L-Kd-Cw3[170-185] |
| Kd-Cw3-N20 | RYLKNGKETL | GGPGGGSGGGG | L-Kd-Cw3, LKd-Cw3-B7 |
| Kd-Cw3-N30 | RYLKNGKETL | GGPGGGSGGG | L-Kd-Cw3-N20 |
| Kd-Cw3-β3m | RYLKNGKETL | GGPGGGSGGGG | L-Kd-Cw3-N30 |
| Kd-NP | TYQRTRALV | GGPGGGSGGGG | Secreted by CHO |
|         |         |        | L-Kd-NP-B7 |

MHC Class I with Covalently Bound Peptide
target cell cell ratios. Spontaneous $^{31}$Cr release values varied between 6 and 15% of total incorporated label. The number of cytototoxic cells generated by coculture was quantitated in terms of lytic units (30).

Tumor Challenge. P815-Cw3 tumors were induced in syngeneic F1 mice (C3H-H • × C3H) by s.c. injection of 7 × 10^6 cells above the right shoulder blade. Tumor growth was determined by measuring tumor dimension at regular time intervals.

Limiting Dilution Analysis. Microcultures were inititated under limiting dilution analysis conditions in 24-well replicates, as previously described (31). Each microculture received 10^5 irradiated splenocytes as feeder cells in 200 µl RPMI 1640 medium supplemented with 10% FCS, 50 U of IL-2, and 5 µg/ml of con A. On day 10, each well was split and assayed for cytotoxicity on $^{51}$Cr-labeled P815-Cw3 and P815 target cells in parallel in a 6-h $^{51}$Cr release assay. Individual microcultures were considered positive when $^{51}$Cr release exceeded three standard deviations of the spontaneous release. CTL precursor frequencies were estimated by Poisson distribution analysis.

Vaccination Mice were inoculated three times intraperitoneally with 10^6 transfected L cells at 7-d intervals. 10 d after the last injection, mice were challenged with three LD50 doses of influenza virus A/PR/8 in a nasal inoculum.

Results

Construction and Cell Surface Expression of H-2K<sup>d</sup> Molecules with Covalently Bound Peptides. Oligonucleotides coding for different antigenic peptides were inserted into the K<sup>d</sup> cDNA sequence (32) between the signal sequence and the α1 domain (Figs. 1 and 2). At the 3' end, a linker encoding a peptidic link were made of small side chain residues was added. As listed in Table 1, four different K<sup>d</sup>-restricted antigenic peptides were used: A2[170-185] derived from HLA-A2 (25), Cw3[170-185] and the optimal 10-mer Cw3[170-179], both derived from HLA-Cw3 (33), and NP[147-155] derived from the nucleoprotein of the influenza virus (34). The spacer between the antigenic peptide and the NH2-terminal end of the α1 domain of K<sup>d</sup> was composed of two Gly residues for the 16-mers, A2[170-185] and Cw3[170-185]. For the Cw3 10-mer, three spacers were compared: a 10-, 20-, and a 30-residue spacer based on the Gly-Gly-Gly-Ser motif that we and others (10, 35, 36) previously used to produce single chain MHC molecules or immunoglobulins. In the construct with NP, the 10-residue spacer was used.

The constructs were transfected into mouse L cells, and stable transfectants were selected. Using flow cytometry and the SF1-1.1 anti-K<sup>d</sup> mAb, we determined if the different fusion proteins were expressed at the cell surface. As shown in Fig. 3 A for the K<sup>d</sup>-A2[170-185] fusion, transfected cells expressed high levels of the fusion at their surface. Similar results were found for the other fusions. The same conclusion was drawn by use of a different anti-K<sup>d</sup> mAb, 34-1-2. This suggests that the overall structure of the K<sup>d</sup> portion was not altered, as two antibodies specific for two different epitopes could recognize the fusion proteins.

Some fusion proteins have been reported to be cleaved when expressed in vivo. We wanted therefore to determine if the peptidic part of the fusion was expressed. We did this experiment for the K<sup>d</sup>-A2[170-185] fusion. Since we had no antibody specific for this peptide, we metabolically labeled LK<sup>d</sup>A2.3 cells and immunoprecipitated the cell extract by use of the SF1-1.1 antibody. After electrophoresis, a single band was observed at an apparent molecular mass of 40 kD (Fig. 4). Comparison with an immunoprecipitate from L cells transfected with the wild-type K<sup>d</sup> (LK<sup>d</sup>) showed a molecular mass increase of ~2 kD, consistent with the addition of 18 amino acids. This indicates that the antigenic peptide A2[170-185] was not cleaved from the fusion protein. Taken together, these data indicate that the fusions between the different antigenic peptides and the K<sup>d</sup> molecule were expressed at the cell surface of the transfected cells.

Production and Purification of Soluble K<sup>e</sup> Molecules with Covalently Bound Antigenic Peptide and β<sub>2m</sub>. We have previously described the construction of a single-chain K<sup>e</sup> molecule (SC-K<sup>e</sup>) with covalently attached β<sub>2m</sub>. It was produced by
connecting the COOH terminus of the α3 domain of K\(^d\) to the NH\(_2\) terminus of β\(_{2\text{m}}\) (10). This fusion was shown to specifically bind peptides (12), select the same repertoire of peptides as the native K\(^d\) molecule (13), and specifically interact with its cognate TCR (14).

We therefore produced a soluble form of one of the peptide–K\(^d\) fusion proteins, K\(^d\)–Cw3-N10. For this purpose, the COOH-terminal end of the α3 domain of K\(^d\)–Cw3-N10 was connected to the NH\(_2\)-terminal end of β\(_{2\text{m}}\) (Fig. 1, right) through a 15-residue spacer. This construct was transfected into CHO cells with a modified DHFR used as marker. Transfected clones were selected and cultivated with increasing concentrations of methotrexate. One clone resistant to 1 mM methotrexate was selected and subcloned. The culture medium of the subclone was tested for the presence of the fusion protein by immunoassay, as described in Materials and Methods. Between 1 and 3 mg/liter was detected in a 2- to 3-d culture supernatant. The peptide–K\(^d\) protein was purified from culture supernatant by immunoaffinity as previously described for SC-K\(^d\). Thus, it is possible to produce single-chain MHC–peptide complex in which all components of the natural heterotrimer, that is, the heavy chain, β\(_{2\text{m}}\), and the peptide, have been fused through appropriate peptidic spacers.

**Recognition of the Cell Surface-expressed Peptide–K\(^d\) Fusion Proteins by Peptide-specific K\(^d\)-restricted CTL Clones.** To determine if the peptide fusions displayed a structure similar to that of the corresponding K\(^d\)-peptide complexes, we tested the recognition of L cells expressing the K\(^d\)-A2-[170-185] or K\(^d\)-Cw3-[170-185] fusions by CTL clones specific for the A2 or Cw3 peptides and restricted by K\(^d\). Three independent transfectants expressing different level of the K\(^d\)-A2-[170-185] fusion were tested for specific recognition by an A2-specific K\(^d\)-restricted CTL clone (332/2A). As shown in Fig. 3 B, all three transfectants were lysed in the absence of added peptide, while L cells expressing the native K\(^d\) were not lysed. Furthermore, the level of lysis paralleled the level of cell surface expression of the fusion protein (compare Fig. 3, A and B).

A panel of seven additional independent CTL clones was then tested with one of the transfected L cell clones (Fig. 5). A specific lysis was observed in all cases. For most CTL clones, the percentage of lysis was >80% at an E/T ratio of 10:1. Similarly, two independent CTL clones specific for Cw3 and restricted by K\(^d\) lysed L-Ka-Cw3-[170-185] cells (Fig 5). In summary, all nine CTL clones tested recognized the fusion proteins in a way similar to the K\(^d\) molecule loaded with the corresponding peptide. This recognition was dose dependent.

**Optimization of the Spacer Length.** Four different fusions had been constructed with the Cw3 peptide (Fig. 1 and Materials and Methods). Three of them used the optimal Cw3-[170-179] and different spacers of 10, 20, and 30 residues. We wanted to compare the CTL recognition of these different fusions with that of LK\(^d\) cells loaded with exogenous Cw3 peptide.

We chose transfectant clones that expressed similar levels of the different fusions as judged by flow cytometry and recognition by the SF1-1.1.1 mAb. This mAb was chosen because it recognizes an epitope in the α3 domain (11) and was therefore expected to be less sensitive to the fused peptide or spacer. Fig. 6 shows the lysis of these different cells by the CTL clone CAS20. All cells were efficiently recognized and only small differences could be noted. While LK\(^d\) loaded with the Cw3 peptide and LK\(^d\)-Cw3-[170-185] cells were lysed with similar efficiency, the best recognized construct

![Figure 3](image.png)  
*Figure 3.* Cell surface expression of K\(^d\)-Cw3-[170-185] parallels recognition by a specific CTL clone. L cells were transfected with the K\(^d\)-A2-[170-185] construct. Three independent transfectants were analyzed by flow cytometry using the anti-K\(^d\) SF1-1.1.1 mAb (A) or for lysis by the 332/2A CTL clone specific for the A2 peptide presented by K\(^d\) (B).

![Figure 4](image.png)  
*Figure 4.* Immunoprecipitation of the K\(^d\)-A2-[170-185] fusion protein. L cells expressing the native K\(^d\) or the K\(^d\)-A2-[170-185] fusion protein were metabolically radiolabeled. After lysis, the extract was immunoprecipitated with the anti-K\(^d\) SF1-1.1.1 mAb and analyzed by gel electrophoresis on a 12% SDS-polyacrylamide gel. Immunoprecipitation products were revealed by fluorography.
was K<sup>d</sup>-Cw3-N10. Recognition of K<sup>d</sup>-Cw3-N20 was slightly better than that of K<sup>d</sup>-Cw3[170-185], while recognition of K<sup>d</sup>-Cw3-N30 was slightly less efficient. This suggested that the optimal spacer length was 10 residues; all subsequent experiments were performed with the K<sup>d</sup>-Cw3-N10 fusion, hereafter referred to as K<sup>d</sup>-Cw3.

**T Cell Hybridoma Stimulation by Soluble MHC Molecules with Covalently Bound Antigenic Peptide and β<sub>2</sub>m.** We previously showed that a single-chain K<sup>d</sup> in which the heavy chain had been connected to β<sub>2</sub>m could be loaded with exogenous peptides, and that the resulting complexes could specifically interact with their cognate TCR (14). Since L cells expressing the different peptide-K<sup>d</sup> fusions could be recognized by specific CTL, we anticipated that the soluble K<sup>d</sup>-Cw3-β<sub>2</sub>m could specifically trigger a K<sup>d</sup>-restricted Cw3-specific T cell hybridoma.

T cells were incubated for 24 h with purified K<sup>d</sup>-Cw3-β<sub>2</sub>m and SF1-1.1.1 mAb in half stoichiometric amount. Culture supernatant was tested for IL-2 content by its capacity to sustain IL-2-dependent CTLL proliferation. As shown in Table 2, K<sup>d</sup>-Cw3-β<sub>2</sub>m but not SC-K<sup>d</sup> induced IL-2 secretion by the 9.4 T cell hybridoma. Antibody alone had no effect. We concluded that a heterotrimer made of the MHC heavy chain, β<sub>2</sub>m, and the antigenic peptide can therefore be mimicked by a single-chain triple fusion (all in one).

**No Cw3 Peptide Can Be Eluted from Cells Expressing the K<sup>d</sup>-Cw3 Fusion Protein.** A small number of MHC-peptide complexes presented at the cell surface is sufficient to trigger activated T cells (6). Although we could not detect cleaved fusion protein after metabolic labeling and immunoprecipitation (Fig. 4), one could argue that CTL clones lysing the L-K<sup>d</sup>-Cw3 cells were not recognizing the uncleaved fusion protein but rather a peptide derived from the fusion protein and presented by the cleaved fusion protein (i.e., K<sup>e</sup>). Minute quantities of cleaved fusion protein could be sufficient for CTL triggering but still undetectable biochemically.

To rule out this hypothesis, we extracted the peptides from L-K<sup>d</sup>-Cw3 cells and showed that this peptide mixture did not contain the antigenic Cw3 peptide. One billion L-K<sup>d</sup>-Cw3 cells were lysed and their extract immunoprecipitated by use of the anti-K<sup>d</sup> SF1-1.1.1 antibody. After acid extraction of the immunoprecipitate, peptides were purified and...
concentrated by ultrafiltration and loaded on P815 cells. In parallel, peptides were extracted from the same number of P815 cells transfected by the HLA-Cw3 gene (hereafter referred as P815-Cw3) known to present the Cw3 peptide in the context of Kd. As shown in Table 3, peptide extracted from L-Kd-Cw3 cells did not sensitize P815 to lysis by the CAS20 CTL clone, while peptides extracted from P815-Cw3 did. This demonstrates that the antigenic peptide is not presented by L-Kd-Cw3 cells and, therefore, that the CTL clone CAS20 recognizes the uncleaved fusion protein.

**Coculture of Naive Splenocytes with L-Kd-Cw3 Cells Allows the Generation of Primary Specific CTL.** Cells expressing a high number of a unique MHC–peptide complex on their surface have been shown to activate specific CTL in vitro (37). As L-Kd-Cw3 cells express a high level of fusion protein at their surface, and this fusion mimics the authentic Kd-Cw3 complex, we tested the capacity of these cells to induce in vitro-specific CTL. However, when L-Kd-Cw3 cells were cocultivated with naive splenocytes from C3H-OH x C3H origin, few lytic units were recovered (Fig. 7). We next transfected L-Kd-Cw3 cells with a cDNA encoding the B7.1 costimulator molecule. As shown in Fig. 7, these cells (L-Kd-Cw3-B7) could now elicit primary CTL in vitro. In contrast, L-Kd cells expressing B7.1 and loaded with exogenous Cw3 peptide were not able to activate specific CTL in primary cultures.

**Table 3. Absence of Antigenic Peptide in Extract from L-Kd-Cw3 Cells**

| Cell extract | Specific lysis |
|--------------|---------------|
| None         | 5.4 ± 2.1     |
| P815-Cw3     | 27.6 ± 2.2    |
| P815         | 1.9 ± 1.7     |
| L-Kd-Cw3     | 2.5 ± 3.3     |

P815, P815-Cw3, and L-Kd-Cw3 cells (10⁶) were lysed and cell extracts were immunoprecipitated by use of the anti-Kd SF1-1.1.1 mAb. Peptides were acid extracted from the immunoprecipitates, and presence of the Cw3 peptide was assayed by measuring P815 cell lysis by the CAS20 CTL clone in the presence of peptide extract at an E/T cell ratio of 3. Results shown are means of triplicates ± SD.

We next extended this observation to L cells expressing Kd fused to a viral peptide (L-Kd-NP-B7). As found for the Kd-Cw3 fusion, L cells expressing both Kd-NP and B7.1 were able to initiate the activation of naive CTL, while L-Kd cells expressing B7.1 and loaded with exogenous NP peptide had no effect. These results indicate that cells expressing different Kd–peptide fusions and the B7.1 molecule acquire the ability to specifically activate T cells in vitro; both the B7.1 molecule and the fusion protein play crucial roles in this process. Neither alone is sufficient to activate primary CTL in vitro.

**In Vivo Priming of Naive Mice with Cells Expressing the Kd Protein Fusion.** To assess if cells expressing an MHC class I molecule with a single covalently bound peptide were able to prime and protect mice against a subsequent tumoral challenge or an acute viral infection, we injected F1 mice (C3H-OH × C3H) intraperitoneally with L cells expressing B7.1 and the Kd-Cw3 or the Kd-NP fusions. We compared their ability to elicit specific cytolytic activity in vivo.

As shown in Fig. 8, mice injected with L-Kd-Cw3-B7 were able to reject a lethal dose of P815-Cw3 in <13 d, while mice preimmunized with either the irradiated tumor P815-Cw3 or L-Kd-Cw3 rejected the tumor in 20 and 25 d,
respectively. Mice injected with L cells or LKd cells expressing B7.1 and loaded with exogenous Cw3 peptide were not able to reject the P815-Cw3 tumor cells and died within 25 d after the tumor challenge. Therefore, exogenous peptide loading is not sufficient to induce protection, even in the presence of B7.1.

To test whether LKd-Cw3-B7 injection elicits the induction of specific CTL in vivo, limiting dilution analysis was performed 10 d after the injection. A higher frequency (1/600) of Cw3-specific CTL precursors was found in mice immunized with LKd-Cw3-B7 compared with control mice injected with LKd-NP-B7 (<10^-4) (data not shown). Mice immunized with LKd-Cw3 cells that did not express B7.1 had an intermediate frequency of CTLp (1/2,000), in agreement with the observed delayed rejection of the tumor. We therefore concluded that immunization of syngeneic mice with L cells expressing B7.1 and Kd covalently attached to Cw3 induced specific CTL and protected against the tumor.

We next extended this observation to the immune response against influenza virus. Mice (C3H-OH × C3H) received three i.p. injections of LKd-NP-B7 and were then challenged with 3 LD50 of influenza virus A/PR/8 by nasal injection. As shown in Fig. 9, all immunized mice survived without any symptom of disease for >14 d, while all control mice injected with LKd-Cw3-B7 died within 7 d. This observation suggests that immunization with cells expressing MHC class I molecules with covalently bound peptide is of general interest.

**Discussion**

In this study we have constructed several fusion proteins in which we connected a Kd-restricted peptide (Cw3, A2, or NP) to the mature Kd protein. After transfection of L cells, these fusion proteins were expressed at the cell surface and recognized by Kd-specific antibodies. Transfected L cells were lysed by all tested Kd-restricted CTL specific for the fused antigenic peptide. This indicates that the fusion proteins display a structure very similar to that of the natural Kd-peptide complexes.

The crystal structures of several MHC class I molecules loaded with peptides have been determined (16, 17, 19, 38). The topology of the antigenic peptide within the binding site is similar in all cases: all peptides display the same NH2–COOH orientation in the cleft, and the peptide is
bound to the class I molecule through mostly conserved interactions. The NH₂ terminus is deeply buried into the groove, while the COOH-terminus is more accessible. As a result, addition of a single residue at the NH₂ terminus of an optimal antigenic peptide is very deleterious to the peptide–MHC interaction, while addition of few residues at the COOH terminus of the peptide is less disturbing for the interaction (39). Moreover, relatively long peptides (up to 33 residues) have been isolated from MHC class I molecules. Pool sequencing of these peptides and comparison with the known allelic motifs suggest that some of them may extend out of the site on their COOH-terminal end (40, 41). Our finding that the COOH-terminal end of the peptide could be extended with a peptidic link without altering the overall structure of the Kd–peptide complex is compatible with these observations.

The NH₂-terminal end of the MHC class I molecule lies under the platform made of β sheets from the α₁ and α₂ domains and is therefore unlikely to be seen by the TCR. Mage et al. (42) produced a single-chain Dd molecule in which β₂m has been connected to the NH₂ terminus of the mature heavy chain. Our finding that peptides could be linked to the NH₂ terminus of the mature Kd extends this observation. Preliminary modeling of our fusion proteins suggests that the linked antigenic peptide lies in the groove of the MHC molecule, bulges out at its COOH terminus, and then passes under the β sheet platform. According to this model, the fusion protein would look similar to the authentic Kd–peptide complex.

In the different structures of MHC class I molecules determined by crystallography, the COOH terminus of the peptide and the NH₂ terminus of the α₁ domain are spaced by about 37 Å. This distance could theoretically be spanned by an 11-residue connecting peptide. In agreement with this prediction, we found that the best spacer length was 10 amino acids. It should be noted, however, that other spacers (20 and 30 residues) were also efficiently recognized, suggesting that the fusion protein can accommodate different spacer lengths. This is reminiscent of what we previously found when we used the same type of spacers to connect the ectodomain of Kd to β₂m. As long as the spacer was ≥5 amino acids long, the fusions were correctly folded (10).

While this work was in progress, Kozono et al. (43) fused antigenic peptides to the β chain of soluble MHC class II–molecules. They showed that such MHC class II-peptide fusions are recognized by specific T cells. While the peptide cleft of MHC class I is more narrow at each end, our results show the feasibility of this approach for class I. Therefore, for both class I and class II, it is possible to produce molecules presenting a single peptide.

We also produced a soluble form of the Kd–Cw3 fusion by connecting the COOH-terminal end of the α₁ domain of the Kd–Cw3 fusion to the NH₂ terminus of β₂m. This triple fusion was capable of triggering a specific T cell hybridoma. The extracellular part of MHC class I molecules can therefore be manipulated at both ends simultaneously. There is a potential advantage in using peptide–MHC fusions over peptide–MHC complexes. Peptide–MHC complexes are quite stable at 4°C or at room temperature. However, some dissociate rapidly at 37°C. Their half life in injected animals is therefore expected to be short. Having the peptide covalently bound to the MHC molecules should stabilize these labile complexes and increase their biological effects.

Two lines of evidence suggest that CTL recognized the uncleaved fusion protein. First, when L cells transfected with the Kd–A2[170-185] fusion were radiolabeled and the fusion protein precipitated with an anti–Kd antibody, a single band at the expected molecular weight was observed after electrophoresis and autoradiography. No degradation product was detected. Second, when we extracted the peptides from L-Kd–Cw3 cells, we could not detect any Cw3 peptide using a CTL clone as a readout. This is somehow surprising since the fusion protein is an endogenous protein and should be processed following the MHC class I pathway. Thus, the Cw3 peptide either is not produced by the processing machinery or is not translocated by the Tap gene products into the ER; alternatively, it is produced and degraded, presumably because it does not bind any class I molecule within the ER (44). However, when it is attached to Kd, it is protected and therefore presented at the cell surface.

Cells expressing peptide–Kd fusions were found to be highly immunogenic in two experimental systems involving the P815-Cw3 tumor and the influenza virus. The immunogenicity was tested in vitro by measuring the generation of primary CTL and in vivo by testing the protection against a lethal challenge and measuring the frequency of CTL precursors. In these experiments, cells expressing the peptide–Kd fusions were compared with cells expressing Kd and loaded with exogenous peptides. Exogenous peptide loading was found incapable of inducing CTL in vitro or in vivo. In vitro, but not in vivo, the B7.1 molecule was required for CTL induction. However, in vivo, a more rapid rejection of the tumor and a higher frequency of CTL precursors was found when the cells expressed both the B7.1 and fusion molecules.

Several hypotheses could account for the increased immunogenicity of cells expressing the peptide–MHC fusions over cells loaded with exogenous peptides: (a) cells expressing the peptide–MHC fusions express a higher number of specific MHC–peptide complexes per cell. As already mentioned, the number of MHC–peptide complexes per cell required for the recognition by an already activated CTL and for the induction of naive T cells. High numbers of peptide–MHC complexes per cell, which can be achieved by use of different strategies, including acid treatment (45) or use of tap gene mutants (46, 47), results in primary CTL induction. (b) The peptide–MHC fusions are more stable than natural peptide–MHC complexes. This explanation applies particularly for in vivo experiments where natural MHC–peptide complexes are susceptible to dissociation. In these situations, peptide–MHC fusions open new possibilities. (c) The peptide–MHC fusions are continually renewed. This is not the case when animals are injected with cells loaded with exogenous peptides. Experiments using minigenes to drive the ex-
pression of antigenic peptides suggest that continuous production of new complexes is an important parameter of in vivo immunogenicity. (d) In cells transfected with the peptide-K^d fusions, K^d is exclusively or preferentially associated with the fused peptide. No cleavage product of the fusion could be detected, but it remains possible that a number of peptides still bind in the groove of the fusion protein. Furthermore, only a fraction of the fused peptide may display the immunologically competent conformation. It should also be noted that transfected cells express K^k and D^k in addition to the peptide-K^d fusion protein so that, in any case, the fused peptide is not the only one presented by the transfected cells. If TCR aggregation is important for T cell triggering, specific MHC–peptide complex density rather than number per cell may be an important parameter. However, as previously noted by De Bruijn et al. (47), a relatively small increase in the density of MHC–peptide complexes is probably sufficient for the induction of a T cell response. More work is needed to discriminate between these different explanations.

In conclusion, single-chain MHC–peptide fusions can be produced for both class I and class II. These fusions may prove useful for manipulating the immune response, in particular when the antigenic peptide has a low affinity for the MHC molecule, as may often be the case for tumor-derived antigenic peptides. Thymic injection of cells expressing an MHC class I molecule bound to a single antigenic peptide or production of mice transgenic for such fusions would be invaluable for studying positive selection of T cells.

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Address correspondence to Jean-Pierre Abastado, Unité de Biologie Moléculaire du Gène, INSERM U277, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris, Cédex 15, France.

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