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Immunology

Analysis of the association of peptides of optimal length to class I molecules on the surface of cells

(antigen presentation/major histocompatibility complex/H-2/β_2-microglobulin/cytotoxic T lymphocyte)

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ABSTRACT **The association of major histocompatibility complex (MHC) class I molecules on the surface of cells with synthetic antigenic peptides of eight or nine amino acid residues was examined. Peptides were synthesized that correspond to the antigenic sequences from ovalbumin and influenza nucleoprotein believed to be naturally processed and presented by cells with K^b and D^b MHC class I molecules, respectively. Consistent with the results of others, these peptides were 10^3–10^5 times more active in stimulating specific T cells as compared to peptides of longer sequences. When cells are incubated with these peptides at <0.01–0.1 μM, the association of the peptides with class I molecules is dependent on (*i*) the reassociation of free β_2-microglobulin from the extracellular fluids, (*ii*) a process that requires cells to be metabolically active, or (*iii*) stabilization of class I heterodimers by chemical crosslinking. In contrast, when cells are incubated with these peptides at >0.1–1.0 μM, the peptides associate with class I molecules in the absence of exogenous β_2-microglobulin, energy, or chemical crosslinking. Antigen competition experiments suggest that the class I molecules that bind peptides offered at high concentration become only transiently receptive to binding peptide. The concentration of peptides required for presentation to T cells under these conditions corresponds to those that stabilize K^b molecules on the surface of RMA-S mutant cells in the absence of exogenous β_2-microglobulin. These results support the concept that the receptivity of class I molecules on cells is determined by the dissociation of β_2-microglobulin from MHC class I that lacks bound peptides.**

The class I molecules of the major histocompatibility complex (MHC) are heterodimers composed of transmembrane heavy chains that are noncovalently associated with β_2-microglobulin. The N-terminal domains of the heavy chain form a cleft that binds peptides (1, 2). During biosynthesis of class I molecules, peptides promote the folding of heavy chains and stabilize the association between the two chains of the class I heterodimer (2, 3). Peptide-occupied class I molecules are transported to the plasma membrane where they are available for recognition by T lymphocytes (2).

It is also possible to form antigen (Ag)–class I complexes by incubating cells with peptides in the extracellular fluids (2, 4). The binding of peptides to class I molecules on cells can be promoted by the addition of free β_2-microglobulin to the extracellular fluids (5–8). Under these conditions, the binding of peptide occurs as a consequence of the reassociation of exogenous β_2-microglobulin with heavy chains. Peptides in the extracellular fluids can also bind to class I molecules through a process that requires metabolic activity (5, 6, 9, 10). In the absence of exogenous β_2-microglobulin and cellular metabolism, few Ag–class I complexes form when cells are incubated with peptides (5–11). We and others have suggested that this paucity of peptide-receptive class I molecules reflects that most class I heterodimers that are transported to the cell surface are initially occupied with endogenous peptides (11), and if these peptides are lost the heterodimer dissociates into an inactive free heavy chain (12–14).

Peptides bound to class I molecules have recently been eluted and characterized. These peptides are remarkably uniform in length, being composed of 8–10 amino acid residues, depending on the particular MHC molecule (2, 15, 16). Although longer peptides can bind to class I MHC molecules, those of the optimal length bind with the highest affinity (2, 17). Many of the previous analyses of peptide binding to class I molecules on cells, including our own, have utilized peptides that are longer than the optimal length. The studies described in this report were initiated to examine how exogenous peptides of optimal length associate with class I molecules on cells.

MATERIALS AND METHODS

Reagents. Chicken ovalbumin (OVA) and human β_2-microglobulin were purchased from Sigma. Bovine β_2-microglobulin was a kind gift from M. Groves (U.S. Department of Agriculture). OVA was treated with cyanogen bromide as described (18). The $OVA^{258-276}$ (residues 258–276 of OVA), $OVA^{257-264}$, influenza nucleoprotein $NP^{365-380}$, influenza $NP^{366-374}$, and Sendai virus $NP^{324-332}$ peptides were synthesized at the Dana–Farber Cancer Institute or purchased from Multiple Peptide Systems (San Diego, CA).

Cell Lines and Monoclonal Antibodies (mAbs). The following T-T hybridomas were used: RF33.70 [C57/BL6 (anti-OVA plus K^b) × BW.CD8.7] (19) and RF36.84 [C57/BL6 (anti-NP plus D^b) × BW.CD8.7] (6). The LB27.4 cell line is an Ia-positive, H-$2^{b\times d}$ B-B hybridoma (20). The EL4 cell line is a T-cell lymphoma of C57/BL6 (H-2^b) origin. EG7 is a clone of EL4 that is transfected with chicken OVA (21). RMA-S is an immunoselected variant of the RBL-5 lymphoma that is deficient in the expression of class I MHC molecules on the cell surface due to a mutation in the TAP-2 peptide transporter (22). mAb-containing culture supernatants were prepared from the hybridoma cell lines Y3 (anti-K^b MHC class I molecules) (23) and S19.8.503 (anti-murine β_2-microglobulin) (24).

Cell Culture. LB27.4 or EL4 Ag presenting cells (APCs) were passaged in Dulbecco's modified Eagle's medium or RPMI 1640 medium supplemented with bovine serum (10%) or in Opti-MEM medium (GIBCO) supplemented with 1% SP Nutridoma (Boehringer Mannheim) and 1% normal mouse serum (NMS), as indicated in the respective experimental protocols. It should be noted that if the APCs are previously exposed to xenogeneic β_2-microglobulin (e.g., passaged in

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Abbreviations: Ag, antigen; APC, antigen presenting cell; MHC, major histocompatibility complex; mAb, monoclonal antibody; NMS, normal mouse serum; NP, nucleoprotein; OVA, ovalbumin.

fetal calf serum) then receptive class I heterodimers can be found on the cell surface (ref. 7; K.L.R., unpublished data) apparently due to greater stability of empty heterodimers containing xenogeneic light chains (12). APCs were Ag exposed in Opti-MEM medium in the presence or absence of azide (15 mM) and/or purified β_2-microglobulin essentially as described (5) and as detailed in the respective experimental protocols. APCs were fixed with paraformaldehyde as described (13). Hybridoma cell cultures and interleukin 2 assays were done as described (5). RMA-S cells were incubated with Ag with or without bovine β_2-microglobulin in the absence of serum as described (25).

Immunofluorescence. Indirect immunofluorescence was performed as described (25). Samples were analyzed on a FACScan flow cytometer (Becton Dickinson).

RESULTS

Presentation of Octameric and Nonameric Antigenic Peptides with MHC Class I Molecules. Recently, the sequence of the naturally processed peptide from OVA that is presented with the K^b MHC class I molecule was predicted to encompass residues 257–264 (15). To examine whether this sequence would in fact stimulate an (OVA + K^b)-specific T cell, we examined the reactivity of the (anti-OVA + K^b)-specific hybridoma RF33.70 to a synthetic peptide corresponding to this sequence. We have previously shown that this hybridoma recognizes a synthetic peptide corresponding to residues 258–276 of OVA in association with K^b, a sequence originally defined by others (21). As shown in Fig. 1*A*, RF33.70 hybrid recognizes the octameric peptide $OVA^{257-264}$ in association with the appropriate APC, as predicted.

The sequence of a naturally processed peptide from the influenza nucleoprotein presented with D^b molecules was reported to span residues 366–374 (15). We have previously described the RF36.84 T-cell hybrid that recognizes a synthetic peptide corresponding to an immunodominant epitope originally defined by Townsend that spans residues 360–385 of influenza NP (4). We synthesized the nonameric peptide $NP^{366-374}$ and found that this peptide stimulated the RF36.84 T-cell hybrid in the presence of appropriate APCs (Fig. 2*A*).

The octameric or nonameric peptides that are generated and presented by cells have been described to be the most active sequences in binding to class I MHC molecules and stimulating T cells. We similarly find that these short peptides are several orders of magnitude more potent than longer peptides in stimulating the RF33.70 and RF36.84 T-cell hybrids (Figs. 1*A* and 2*A*).

We next examined the conditions that favored association of the short synthetic peptides with class I molecules on the surfaces of cells. Previously, we and others had described that free β_2-microglobulin promoted the association of exogenous peptides with class I molecules. EL4 APCs were incubated with azide to inhibit metabolic activity and were then incubated with various concentrations of $OVA^{257-264}$ peptide in the presence or absence of purified human β_2-microglobulin (5–8). The cells were subsequently washed and fixed with paraformaldehyde and assayed for the presence of peptide–MHC class I complexes with RF33.70 cells. As shown in Fig. 1*B*, when cells were incubated with 0.01 μM peptide, the association of peptide with class I molecules was dependent on the presence of β_2-microglobulin in the extracellular fluids. Similar results were obtained with the $NP^{366-374}$ peptide and the D^b class I molecule (Fig. 2*B*). These results indicate that the reassociation of β_2-microglobulin promotes binding of the octameric and nonameric peptides.

We (5, 6) and others (9, 10) had observed that some peptide–class I complexes could be formed on cells incubated with high concentrations of exogenous peptides in the absence of free β_2-microglobulin. However, this association of peptides required that the APCs be metabolically active. Similar results are observed with the short optimal peptides. As shown in Figs. 1*C* and 2*C*, when cells are incubated with 0.1 μM $OVA^{257-263}$ or 0.01–0.1 μM $NP^{366-374}$ peptide, class I

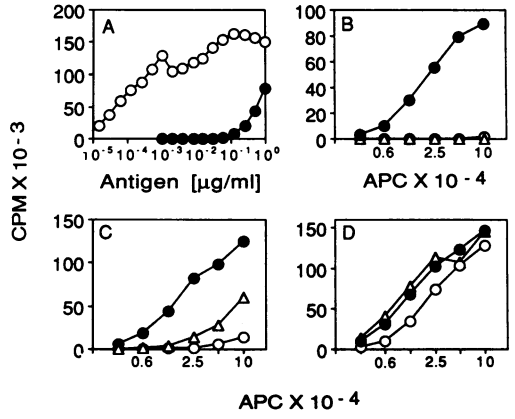

FIG. 1. Presentation of exogenous OVA peptides with K^b class I molecules on cells. (*A*) Microcultures were prepared with 10^5 RF33.70 cells (anti-OVA + K^b-specific T-T hybridoma), EL4 (5×10^4 cells) previously grown in bovine serum, and the indicated amounts of $OVA^{257-276}$ (●) or $OVA^{257-264}$ (○) as described. After 18 hr of incubation at 37°C, 100 μl of supernatant was removed, freeze-thawed, and assayed for interleukin 2 content with HT-2 cells. Data represent mean incorporation of [^{3}H]thymidine (cpm) into DNA of HT-2 cells stimulated with supernatants from duplicate cultures. (*B*) LB27.4 cells (3×10^6 cells) previously grown in 1% NMS were incubated with or without azide (15 mM) in serum-free Opti-MEM medium (1 ml). After 30 min of incubation at 37°C, $OVA^{257-264}$ (0.01 μM) was added to cells without azide (△), or to cells treated with azide either by itself (○) or with human β_2-microglobulin (10 μg/ml) (●). After an additional 2 hr of incubation at 37°C, the cells were washed and fixed with paraformaldehyde. The indicated number of treated LB27.4 cells were incubated with 10^5 RF33.70 cells and microcultures were handled and assayed as described in *A*. (*C*) Same as *B*, except $OVA^{257-264}$ was used at 0.1 μM. (*D*) Same as *B*, except $OVA^{257-264}$ was used at 1 μM. Data in *B–D* are from the same experiment; data in *A* are from an independent experiment.

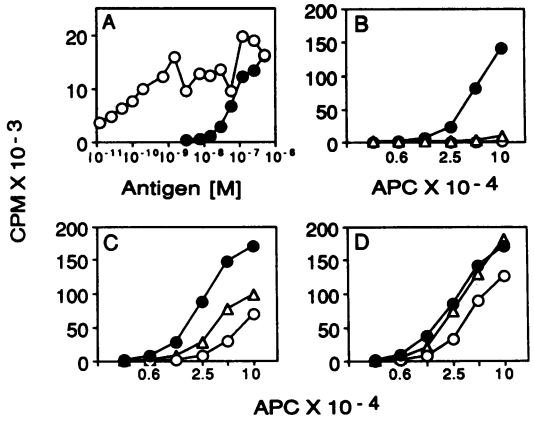

FIG. 2. Presentation of exogenous influenza NP peptides with D^b class I molecules on cells. (*A*) Microcultures were prepared with 10^5 RF36.84 cells (anti-NP + D^b-specific T-T hybridoma), paraformaldehyde-fixed EL4 (5×10^4 cells) previously grown in bovine serum, and the indicated amounts of $NP^{365-380}$ (●) or $NP^{366-374}$ (○) as described in Fig. 1*A*. Cultures were assayed as described in Fig. 1*A*. (*B*) Microcultures were prepared similar to Fig. 1*B*, except with EL4 previously grown in NMS instead of LB27.4 as APCs, RF36.84 instead of RF33.70 as the T-cell hybrid, and $NP^{366-374}$ (0.01 μM) instead of $OVA^{257-264}$ as the Ag. Assay conditions and symbols are the same as in Fig. 1*B*. (*C*) Same as *B*, except $NP^{366-374}$ was used at 0.1 μM. (*D*) Same as *B*, except $NP^{366-374}$ was used at 1 μM. Data in *B–D* are from the same experiment; data in *A* are from an independent experiment.

complexes can be formed in the absence of exogenous β_2-microglobulin, and this process is inhibited by azide.

When cells were incubated with the short optimal peptides at a concentration of 1 μM, we could detect the formation of peptide–class I complexes in the absence of exogenous β_2-microglobulin and cellular metabolism (Fig. 1*D*). This concentration of Ag was >1000-fold higher than was needed to sensitize the same cells in the presence of β_2-microglobulin (K.L.R., unpublished data). This was observed for both $OVA^{257-264}$ (Fig. 1*D*) and $NP^{366-374}$ (Fig. 2*D*), which bind to K^b and D^b molecules, respectively. This ability of class I molecules to bind peptides in the absence of both exogenous β_2-microglobulin and cellular respiration was not previously observed with long peptides. This may simply reflect that it was not possible to reach the same effective concentration of peptide because the long peptides are so much less potent (see Figs. 1*A* and 2*A*). Alternatively, it is possible that this reflects a difference in how class I molecules bind long versus short peptides. We do not favor this latter explanation, because K^b molecules on cells that are incubated with long peptides from Sendai virus (26) or OVA (K.L.R. and Ying Li, unpublished observations) preferentially bind short contaminants in the peptide preparations. Thus, previous studies with long peptides at high concentration may actually have been measuring the presentation of short peptides.

Effect on the Presentation of Short Peptides of Stabilizing Class I Heterodimers Through Chemical Crosslinking. We have previously found that stabilizing the association between β_2-microglobulin and heavy chain by chemical crosslinking results in the appearance of receptive class I molecules on cells that can bind peptides (13). We examined the effect of this stabilization on the presentation of short peptides of optimal length. In these studies, concentrations of peptides were used that are unable to sensitize APCs for recognition by T cells in the absence of β_2-microglobulin or energy. APCs were treated with buffer or aldehyde (to crosslink class I molecules *in situ*) and then exposed to peptide. After exposure to peptide, the cells were washed and the buffer-treated cells were fixed with aldehyde. As shown in Fig. 3*A*, chemical crosslinking of cells before exposure to peptide markedly increases the presentation of $OVA^{257-264}$ with K^b relative to cells that were crosslinked after exposure to this peptide. The fact that the peptide is binding to the K^b molecules was confirmed in biochemical studies (K.L.R. and Ying Li, unpublished observations). Identical results are observed for the presentation of $NP^{366-374}$ by D^b molecules on chemically crosslinked cells (Fig. 3*B*). It should be noted that

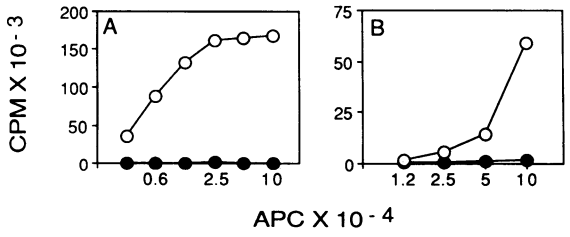

FIG. 3. Chemical crosslinking of class I molecules on cells generates class I molecules that are receptive to presenting optimal antigenic peptides. (*A*) EL4 (3×10^6 cells) grown in Opti-MEM with 1% NMS were washed and either treated with paraformaldehyde (○) as described or incubated with azide (15 mM) for 60 min at 37°C (●). These cells were subsequently exposed to $OVA^{257-264}$ (0.01 μM) for 1 hr at 37°C and then washed. The azide-treated group was then fixed with paraformaldehyde. The indicated number of treated APCs were cultured and assayed with RF33.70 cells as described in Fig. 1. (*B*) Similar to *A* except that $NP^{366-374}$ (1 μM) was used instead of $OVA^{257-264}$ and RF36.84 was used instead of RF33.70. The activity of RF36.84 was low in this experiment and required high concentrations of peptide for stimulation. Data in *A* and *B* are from independent experiments.

exogenous β_2-microglobulin was not present in these experiments and that the cells were not metabolically active. These results are identical to our previous observations with longer peptide preparations (13) that stabilizing class I heterodimers by chemical crosslinking creates receptive class I molecules on cells.

Short Peptides Compete for Binding to Class I Molecules but Do Not Displace Prebound Peptides. We next examined whether short peptides could displace peptides previously bound to class I molecules in Ag competition experiments (27). In these experiments, we used a Sendai $NP^{324-332}$, which binds to K^b molecules (26) but is not recognized by RF33.70 cells. As shown in Fig. 4*B*, the Sendai $NP^{324-332}$ peptide (20 μM) completely inhibits presentation of the $OVA^{257-264}$ peptide (1 nM) when APCs are incubated with the two peptides at the same time. This result indicates that the Sendai $NP^{324-332}$ peptide can efficiently compete with $OVA^{257-264}$ for binding to K^b molecules. However, when $OVA^{257-264}$–K^b complexes were first formed on cells that were then exposed to Sendai $NP^{324-332}$, no inhibition of presentation occurred (Fig. 4*A*). Identical results were obtained when the $OVA^{257-264}$–K^b complexes were formed through endogenous processing (Fig. 4*A*) or by incubation of APCs with high concentrations of exogenous peptide in the absence of β_2-microglobulin (K.L.R., unpublished data).

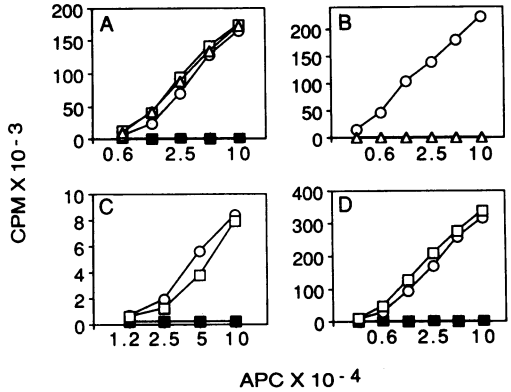

FIG. 4. Analysis of Ag competition with optimal-length antigenic peptides. (*A*) EG7 (6×10^6 cells) previously grown in bovine serum were washed and resuspended in azide (15 mM) in Opti-MEM (0.5 ml). After 30 min of incubation at 37°C, nothing (○), Sendai $NP^{324-332}$ (20 μM) (□), or Sendai $NP^{324-332}$ (20 μM) with human β_2-microglobulin (10 μg/ml) (△) was added. After an additional 2 hr of incubation at 37°C the cells were washed and fixed with paraformaldehyde. EL4 cells (■) were subjected to the same treatments without Ag. The indicated number of treated APCs were cultured with RF33.70 cells as described in Fig. 1. (*B*) Similar to *A*, except the APC was EL4 previously grown in bovine serum that was incubated with $OVA^{257-264}$ (1 nM) and human β_2-microglobulin (10 μg/ml) in the presence (△) or absence (○) of Sendai $NP^{324-332}$ (20 μM). (*C*) Paraformaldehyde-treated EL4 (5×10^6 cells) previously grown in bovine serum were resuspended in $OVA^{258-276}$ (100 μM) in Opti-MEM (1 ml). After 2 hr of incubation at 37°C the cells were washed and resuspended in Opti-MEM with (□) or without (○) Sendai $NP^{324-332}$ (20 μM). EL4 cells (■) were also subjected to the same treatments without Ag. After an additional 2 hr of incubation at 37°C the cells were washed and cultured with RF33.70 cells as described in Fig. 1. (*D*) EL4 (10^7 cells) grown in Opti-MEM with 1% NMS were washed and resuspended in Opti-MEM medium containing azide (15 mM) with (□) or without (○) Sendai $NP^{324-332}$ (20 μM). After 2 hr of incubation at 37°C, the cells were washed and resuspended in Opti-MEM/azide with $OVA^{257-264}$ (0.1 μM). After 1 hr of incubation at 37°C, the cells were washed and treated with paraformaldehyde. EL4 cells (■) were also subjected to the same treatments without Ag. The APCs were then cultured with RF33.70 cells as described in Fig. 1. Data in *A* and *B* are from the same experiment; data in *C* and *D* are from independent experiments.

Using the Ag competition assay, we find no evidence that a nonameric peptide is able to displace octameric peptide that was previously bound to class I molecules. In these experiments, we are attempting to displace a peptide that is the optimally sized ligand for K^b molecules and is likely to be bound with high affinity. It is conceivable that peptides that are bound to class I molecules with lower affinity might be susceptible to Ag competition. To examine this point, we incubated fixed APCs with the $OVA^{258-276}$ peptide. This peptide is longer than the optimal size for binding to K^b and lacks the N-terminal anchor residue at position 257 (15). It is 10^4- to 10^5-fold less active than $OVA^{257-264}$ in stimulating RF33.70 cells. As shown in Fig. 4C, Sendai $NP^{324-332}$ does not inhibit the presentation of Ag by the APCs previously incubated with $OVA^{258-276}$. Thus, even when a suboptimal peptide is bound to K^b, we are unable to detect displacement by a nonameric (optimal) peptide species.

In related experiments, we examined whether preincubation of APCs with Sendai $NP^{324-332}$ might block subsequent binding of $OVA^{257-264}$ to K^b. In these experiments, the APCs were treated with azide to prevent the appearance of new class I molecules at the cell surface. EL4 APCs were first incubated with or without Sendai $NP^{324-332}$ (20 μM), washed, and then incubated with $OVA^{257-264}$. As shown in Fig. 4D, previous exposure to Sendai $NP^{324-332}$ did not inhibit subsequent binding of OVA to K^b. This result suggests that the class I heterodimers that bind $OVA^{257-264}$ actually became receptive to binding peptide during the second incubation, presumably from the dissociation of bound peptides over this time interval.

Effect of Octameric Peptides on Class I Molecules on the RMA-S Mutant Cell Line. The cell line RMA-S (22) has a mutation in the TAP-2 gene (28) that results in an apparent deficiency of peptides for binding to newly assembled class I molecules. Consequently, the class I heterodimers that are assembled in this cell are largely unstable and there is a marked decrease in class I heterodimers on the cell surface (3, 14). Incubation of these cells with long peptides results in an increase of class I heterodimers on cells (3, 25). Therefore, we analyzed the ability of short peptides to increase MHC class I molecules on the cell surface of RMA-S. RMA-S cells were incubated with $OVA^{257-264}$ in the presence or absence of purified bovine β_2-microglobulin. As shown in Fig. 5 E and F, in the presence of bovine β_2-microglobulin, an increase in the expression of K^b is observed when RMA-S cells are incubated with 0.01–1.0 μM $OVA^{257-264}$. In the absence of β_2-microglobulin, ≈100-fold more $OVA^{257-264}$ is required to see an increase in the expression of K^b on the cell surface (Fig. 5 C and D). At these high concentrations of peptide, there is also a concomitant increase in the amount of murine β_2-microglobulin (Fig. 5 A and B). Similar experiments were performed with the short influenza $NP^{366-374}$ peptide. The increase in D^b expression on RMA-S cells is primarily observed on RMA-S cells that were incubated with this peptide in the presence of exogenous bovine β_2-microglobulin (K.L.R., unpublished data). This result is similar to our previous analysis of RMA-S cells incubated with $NP^{365-380}$ (25). We observed no increase or only a marginal increase in the expression of D^b and murine β_2-microglobulin on RMA-S cells incubated with high concentrations of the octameric/nonameric peptides in the absence of exogenous β_2-microglobulin (K.L.R., unpublished data).

DISCUSSION

This report analyzes how exogenous peptides of optimal size associate with class I MHC molecules on APCs. When APCs are exposed to concentrations of short peptides ranging from <0.01 to 0.1 μM, we do not detect binding of peptide to preexisting class I MHC heterodimers on the cell surface. Under these conditions, the presentation of peptide is dependent on exogenous β_2-microglobulin of the APC. These findings with the octameric/nonameric peptides are identical to findings with longer versions of these and other peptides (5–11). This result is not surprising, because when cells are incubated with long peptides, the species of peptide that actually binds to the class I molecule may be an octamer or a nonamer (26). These shorter peptides may be present as contaminants in the initial peptide preparation (26) or they may be generated through the action of proteases (29–32). The free β_2-microglobulin is likely to act by reassociating with free heavy chains on the cell surface (12). It is possible that peptide binding is facilitated through the exchange of exogenous β_2-microglobulin with bound light chains. If the latter were the case, it might be expected that exogenous β_2-microglobulin would facilitate the loss or exchange of previously bound peptides; however, we do not observe such effects.

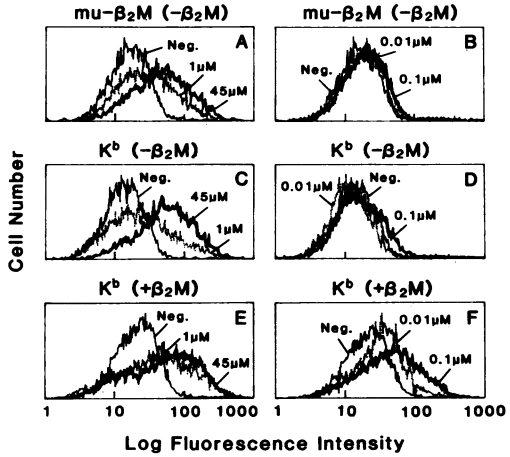

FIG. 5. Effects of optimal antigenic peptides on K^b class I molecules on RMA-S cells. (A and B) RMA-S cells (3×10^6 cells per ml) were resuspended without (Neg.) or with the indicated concentrations of $OVA^{257-264}$ in Opti-MEM Nutridoma (serum free). After 18 hr at 37°C, the cells were washed and stained with anti-murine β_2-microglobulin (β_2M) mAb (S19.8.503) followed by fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin. Fluorescence was quantitated on a FACScan flow cytometer and is displayed as histograms of log relative fluorescence (abscissa) vs. linear cell number (ordinate). (C and D) Same as A and B except the cells were stained with anti-K^b mAb (Y3). (E and F) Same as C and D except bovine β_2-microglobulin (2 μg/ml) was added during the 18-hr incubation. Data in all panels are from the same experiment.

When APCs are exposed to concentrations of short peptides >0.1–1 μM, we detect the formation of class I–peptide complexes in the apparent absence of exogenous β_2-microglobulin or metabolic energy. Several experiments were performed that give some insight into the basis for these results.

We found that competing peptides are ineffective in blocking class I binding sites if added before, but not during, the exposure of APCs to immunogenic peptide. These results imply that receptive class I molecules are not preexisting on the cell surface. These same competing peptides will inhibit if added simultaneously with the immunogenic peptide. That the binding of peptides to class I molecules can occur on cells in the absence of cellular respiration indicates that exocytosis of empty class I molecules is not necessary for this phenomenon. Taken together, these findings imply that previously unreceptive class I molecules become receptive during the incubation period with the immunogenic peptide and that this receptivity is transient.

An explanation for these findings is suggested from our observations with the RMA-S cell line. The concentrations of peptides that are required to sensitize APCs in the absence of exogenous β_2-microglobulin and energy are similar to those that are required to bind to and stabilize K^b molecules con-

taining a light chain of murine origin on RMA-S cells. Thus, when peptides dissociate from class I molecules on nonmutant cells, very high concentrations of peptide may be required for rebinding of peptide and stabilization of the heterodimer. In the absence of exogenous peptides, the empty class I molecules would dissociate as discussed below. The fact that peptides continuously dissociate from class I molecules is suggested by the finding of free heavy chains on the cell surface (12, 33). An alternative mechanism that is not mutually exclusive is that the high concentration of high-affinity peptide in the extracellular fluids is displacing bound peptides from class I heterodimers. We find no evidence for this effect. However, it is difficult to exclude the possibility that such displacement is occurring with peptides that we are unable to measure. If such displacement is not occurring, our results may imply that a conformation change in the class I binding site accompanies the dissociation of peptide. However, our results with RMA-S cells and K^b versus D^b molecules are somewhat discordant. This may indicate that there is a difference in the efficiency of peptide binding for K^b and D^b under our experimental conditions. If this is correct, then the fact that these two class I molecules behave similarly in Ag presentation assays may reflect that the T-cell assay is very sensitive and can detect a few hundred peptide–class I complexes.

Several lines of investigation have indicated that the paucity of peptide-receptive class I molecules on the cell surface is attributable to the instability of the class I heterodimer in the absence of a bound peptide. Thus, class I molecules that are formed on the mutant RMA-S cells in the absence of peptide dissociate at 37°C and can be stabilized by the addition of peptide (14, 34). On nonmutant cells, preventing the dissociation of the class I heterodimers on the cell surface through chemical crosslinking preserves peptide-receptive molecules (13). Without chemical crosslinking, a large pool of free heavy chains is found, which arises from the dissociation of previously assembled and transported heterodimers (12). In the absence of endogenous peptides, the ratio of dissociated to intact heterodimers is increased (12). Our findings with the short peptides are consistent with these observations and conclusions.

In the absence of exogenous β_2-microglobulin, both the short optimal peptides and long suboptimal peptides can associate with class I molecules via a process that requires metabolic activity (5, 6, 9, 10). Why metabolic activity is required for binding of peptides in the absence of β_2-microglobulin is not clear. We currently favor the possibility that the peptide is binding to newly exported class I molecules that are either empty or have bound a rapidly dissociating species of peptide. However, it is also possible that metabolic activity is required for the internalization of peptide into cells, where it binds to class I molecules.

The present results indicate that some receptive class I molecules can be found on cells but exist only transiently. Our results indicate that high concentrations of the optimal length peptides are needed for these molecules to bind and effectively present peptides. We and others have observed that such peptides are inactivated by serum proteases (29–32). They should also diffuse rapidly throughout the extracellular fluids. Thus, it is unlikely that significant binding of peptides to these receptive class I molecules would occur under physiological conditions. We have previously suggested that the dissociable light chain of class I molecules may have been selected during evolution to prevent peptide exchange on the cell surface and thereby preserve the immunological identity of cells (12). The present results are consistent with this hypothesis.

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