Low temperature and peptides favor the formation of class I heterodimers on RMA-S cells at the cell surface

(major histocompatibility complex/H-2/β2-microglobulin/cytotoxic T lymphocyte)

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ABSTRACT RMA-S murine cells have a mutation that interferes with the assembly of class I major histocompatibility complex (MHC) heterodimers and are deficient in the expression of class I molecules on the cell surface. The mutant phenotype has been reported to be normalized upon incubation of RMA-S cells at 25°C. We find that much of the increased expression of class I heterodimers is dependent on cultivating RMA-S cells in bovine serum or with purified bovine β2-microglobulin. Furthermore, epitopes that are associated with class I MHC molecules that have bound xenogeneic β2-microglobulin are preferentially formed on RMA-S cells cultured at 25°C. These heterologous class I molecules are ther- molabile. Increased expression of class I molecules has also been observed on RMA-S cells incubated at 37°C in the presence of I-restricted peptides. We find that the increased expression of Dp molecules induced by influenza virus nucleoprotein residues 365–380 is similarly dependent on culturing RMA-S cells in bovine serum or with purified bovine β2-microglobulin.

The RMA-S cell line is an immunoselected variant of the RBL-5 murine lymphoma that is deficient in the expression of class I major histocompatibility complex (MHC) molecules on the cell surface (1). This cell line synthesizes class I heavy and light chains but fails to assemble class I heterodimers that are stable (2). RMA-S cells can be induced to express higher levels of class I molecules on the cell surface under two experimental conditions. First, the expression of a particular class I molecule is increased on RMA-S cells that are incubated with peptides that bind to that class I molecule (3). Second, incubation of RMA-S cells at 22–28°C leads to nearly normal expression of class I molecules (4). The low-temperature-induced class I heterodimers are believed to lack associated peptides and are unstable at 37°C (4, 5). These and other observations have led to the view that peptides are essential for the initial assembly and subsequent transport of class I heterodimers to the cell surface. In the absence of these peptides the class I heterodimers either do not form or are highly unstable under physiological conditions (3, 4, 6). These phenomena should be reexamined in the light of recent observations on the role of exogenous β2-microglobulin in function of class I molecules on the cell surface (7–10).

The association of peptides with class I MHC molecules on the cell surface is dependent on exogenous β2-microglobulin (7–10). We have also detected a large pool of free class I heavy chain on the surface of primary and cultured cells (29). These latter molecules arise from the dissociation of β2-microglobulin from class I heterodimers at the cell surface.

In the course of these studies we were surprised to find that RMA-S cells expressed a sizable pool of free heavy chains that were capable of binding exogenous β2-microglobulin.

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Abbreviations: FITC, fluorescein isothiocyanate; β2M, β2-microglobulin; mAb, monocalon antibody; MHC, major histocompatibility complex.

MATERIALS AND METHODS

Antigens and Reagents. NP-(365–380), a synthetic peptide corresponding to residues 365–380 of the nucleoprotein of influenza virus (1968 A strain), was synthesized in the molecular biology core facility of the Dana–Farber Cancer Institute. Purified bovine β2-microglobulin was kindly provided by Merve Groves (U.S. Department of Agriculture). Human β2-microglobulin was purchased from Sigma.

Cell Lines. EL4 cells were originally derived from a chemically induced lymphoma of C57BL/6 mouse origin. RMA-S cells (1) were kindly made available by K. Kärre (Karolinska Institute, Stockholm) and A. Townsend (John Radcliffe Hospital, Oxford). Cells were passaged in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g of glucose per liter, supplemented with fetal calf serum (10%) or iron-supplemented calf serum (10%) and antibiotics (Irvine Scientific).

Monoclonal Antibodies (mAbs). The hybridomas MKD6 (anti-IA<sup>5</sup>) (12), S19.8.503 (anti-murine β2-microglobulin) (13), BBM.1 (anti-human β2-microglobulin) (14), M1/42 (15) (pan-reactive anti-murine class I molecules, specific for class I heterodimers with murine β2-microglobulin) (16), Y3 (anti-K<sup>β</sup>) (17), AF6-88.5 (anti-K<sup>α</sup>) (18), 28.11.5S (anti-D<sup>β</sup>) (19), and 28.14.8S (anti-D<sup>α</sup>) (19) were kindly made available by the laboratories that developed them and in some cases were obtained from the American Type Culture Collection.

Immunofluorescence. Indirect immunofluorescence was performed as described (20), using saturating concentrations of mAb followed by fluorescein isothiocyanate (FITC)-conjugated rabbit antiserum to mouse immunoglobulin. Samples were analyzed on a FACScan flow cytometer (Becton Dickinson).

Cell Culture. RMA-S cells were cultured at 37°C or room temperature (24–26°C) for 24 hr in the presence or absence of bovine (fetal or calf) serum (10%). In some experiments RMA-S cells were cultured with NP-(365–380) peptide (100 μM) for 24 hr at 37°C. Opti-MEM medium (GIBCO) supplemented with Nutridoma (1%, Boehringer Mannheim), 1-glutamine, and antibiotics was used in all experiments in which cells were cultured under serum-free conditions. Complete DMEM was used in all other experiments. In some experi-
ments purified bovine β₂-microglobulin (2.5 µg/ml) was added to the culture medium.

**Results**

**Binding of Xenogeneic β₂-Microglobulin Affects Selected Epitopes on Class I Molecules.** Xenogeneic β₂-microglobulin from the extracellular fluids can associate with murine class I molecules on cells by exchanging with endogenous β₂-microglobulin (16, 24) or binding to free heavy chains on cells (29). In examining class I molecules, we identified mAbs that could distinguish between Kb molecules with murine versus xenogeneic β₂-microglobulin. The Y3 and AF6-88.5 mAbs were used to immunoprecipitate Kb from EL4 cells that either had been labeled with ¹²⁵I by a lactoperoxidase reaction or had been incubated with ¹²⁵I-labeled xenogeneic β₂-microglobulin. The Y3 mAb can immunoprecipitate Kb molecules that are associated with ¹²⁵I-labeled β₂-microglobulin of bovine, human, or murine origin (Fig. 1, lanes H, L, and D, respectively). The immunoprecipitation of the xenogeneic light chain is specific, as it is not observed with several other mAbs (Fig. 1, lanes E, F, and J). In contrast, xenogeneic β₂-microglobulin is not immunoprecipitated by the AF6-88.5 mAb (Fig. 1, lanes G and K). The AF6 mAb is active in this experiment, as it immunoprecipitates Kb molecules from ¹²⁵I surface-labeled EL4 cells (Fig. 1, lane C). The M1/42 mAb, which recognizes a nonpolymorphic determinant on all class I molecules, is similarly specific for class I molecules with a murine light chain (ref. 16 and unpublished data).

Anti-murine β₂-microglobulin (S19.8) immunoprecipitates class I heavy and light chain from EL4 cells (Fig. 1, lane B). However, this mAb does not immunoprecipitate labeled light chain from EL4 cells incubated with ¹²⁵I-labeled bovine (Fig. 1, lane F) or human (Fig. 1, lane J) β₂-microglobulin. This demonstrates that the binding of bovine and endogenous β₂-microglobulin to class I heavy chains is mutually exclusive. These results confirm previous findings with murine-human class I heterodimers (29).

These results, and other similar findings (25, 26), are of interest because they demonstrate a serological alteration in class I molecules that have bound xenogeneic β₂-microglobulin. The present results imply that the polymorphic residues in β₂-microglobulin affect the conformation of the distal domains of the class I heavy chain. This conformational alteration is more subtle than the gross serological changes that are observed with free class I heavy chains (3). As a consequence of these effects, these mAb reagents permit us to analyze the composition of class I molecules in situ on cells.

**Incubation of RMA-S Cells at Low Temperature Differentially Affects Specific Epitopes on Class I Molecules.** The expression of class I epitopes on RMA-S cells that were cultured for 18 hr at 37°C or 25°C was analyzed by immunofluorescence and flow fluorocytometry. There was increased expression of all class I epitopes on RMA-S cells cultured in bovine serum at 25°C as compared with 37°C (Fig. 2), as was reported (4). However, there were considerable differences between particular epitopes in their magnitude of increased expression. There was a marked increase in the binding of the anti-Kb mAb, Y3, and the anti-D8 mAbs, 28.11.5S and 28.14.8S, to RMA-S cells cultured at low temperature (Fig. 2). In contrast, there was only a modest increase in the binding of the anti-Kb mAb, AF6-88.5; the anti-β₂-microglobulin mAb, S19.8 (Fig. 2), and the anti-class I (nonpolymorphic) mAb, M1/42 (Fig. 2). Previous studies did not describe this quantitative variation in the level of expression of different class I epitopes (4). We have observed some greater expression of murine β₂-microglobulin in occasional experiments; however, it has always been less than the induction of Kb and D8 epitopes associated with class I-xenogeneic β₂-microglobulin heterodimers (unpublished data).

**The Increase in Certain Class I Epitopes on RMA-S Cells Cultured at Low Temperature Is Largely Dependent on Xenogeneic β₂-Microglobulin in Serum.** The observation that the large increase in certain class I epitopes occurred without a corresponding increase in murine β₂-microglobulin on RMA-S cells cultured at low temperature suggested that the majority of these class I molecules did not contain a murine light chain. Similarly, the two other mAbs that displayed only a modest increase in binding to the induced-RMA-S cells

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**Fig. 1.** Different mAbs can distinguish class I heterodimers that are associated with murine versus xenogeneic β₂-microglobulin. EL4 cells were surface-labeled with ¹²⁵I by a lactoperoxidase reaction (lanes A–D) or were incubated with ¹²⁵I-labeled bovine β₂-microglobulin (10 µg/ml) (lanes E–H) or ¹²⁵I-labeled human β₂-microglobulin (10 µg/ml) (lanes I–L) for 1 hr at 37°C and washed. These cells were solubilized in isotonic buffer containing Nonidet P-40 (1%) and immunoprecipitated with anti-human β₂-microglobulin (BBM1), anti-murine β₂-microglobulin (S19.8, S503), anti-Kb (Y3), or anti-Kb (AF6-88.5) antibodies. Immunoprecipitated proteins were resolved by electrophoresis on a 14% polyacrylamide gel under reducing conditions and labeled proteins were visualized by autoradiography. Molecular masses are indicated in kDa. Lanes A–H are derived from the same experiment. Lanes I–L are from a separate experiment.
(M1/42 and AF6-88.5) recognize only class I molecules with an endogenous light chain (Fig. 1 and ref. 16). These results raised the possibility that a significant component of the increased binding of Y3 and 28.11.5S was dependent on the formation of class I heterodimers with xenogenic \( \beta_2 \)-microglobulin from the culture medium. To examine this possibility, we analyzed the expression of class I epitopes on RMA-S cells cultured at 25°C or 37°C in the presence or absence of serum. The large increase in the binding of Y3 and 28.11.5S was dependent on culturing the RMA-S cells in serum-containing media (Fig. 3). Under serum-free conditions the level of expression of all epitopes was similar (Fig. 3). The modest increase in the epitopes detected by the S19.8, AF6-88.5 (Fig. 3), and M1/42 (data not shown) mAbs on induced-RMA-S was similar in the presence or absence of serum.

To verify that \( \beta_2 \)-microglobulin was the component in serum that was necessary for the effects observed above, RMA-S cells were cultured at 25°C with purified bovine \( \beta_2 \)-microglobulin in the absence of serum. As shown in Fig. 3B, bovine \( \beta_2 \)-microglobulin was sufficient to cause the same pattern of increased class I epitopes as observed in serum.

**Thermolability of Low-Temperature-Induced Class I Molecule with Endogenous Versus Exogenous \( \beta_2 \)-Microglobulin.** The determinants detected by the Y3 and 28.11.5S mAbs on RMA-S cells incubated in bovine serum at 25°C are rapidly lost when these cells are incubated at 37°C (Fig. 4; ref. 4). In contrast, when the RMA-S cells are first incubated at 25°C under serum-free conditions, there is only a minor reduction in the Y3 and 28.11.5S epitopes upon incubation at 37°C (Fig. 4). There is a similar minor reduction in the expression of S19.8, AF6-88.5, and M1/42 epitopes on these RMA-S cells whether or not bovine serum is present (Fig. 4). Together these data demonstrate that the majority of thermolabile class I molecules on the RMA-S cells cultured at low temperature are heterologous class I heterodimers with a bovine light chain. The smaller number of murine–murine class I molecules that are induced at low temperature also appear to dissociate at physiological temperature (Fig. 4). These findings are consistent with the model that empty class I molecules are unstable at 37°C (4). We have observed that human \( \beta_2 \)-microglobulin associates more stably with murine heavy chains under comparable conditions (unpublished data). This may explain why transfected murine class I molecules are
Fig. 5. Increased expression of D\textsuperscript{b} molecules on RMA-S cells incubated with NP-(365-380) is dependent on exogenous bovine \(\beta_2\)-microglobulin. RMA-S cells were incubated for 24 hr at 37°C in the presence or absence of NP-(365-380) (100 \(\mu\)M) in complete Opti-MEM (serum free), complete Opti-MEM supplemented with fetal calf serum (FCS) (10%), or complete Opti-MEM (serum free) supplemented with bovine \(\beta_2\)-microglobulin (2.5 \(\mu\)g/ml). The cells were subsequently stained with 28.11.5S (anti-D\textsuperscript{b}), Y3 (anti-K\textsuperscript{b}), and S19.8 (anti-murine \(\beta_2\)-microglobulin) and were analyzed as described for Fig. 2. Data are expressed as fluorescence histograms of cell number (ordinate) versus fluorescence intensity (abscissa; note logarithmic scale). The specific epitope that is stained and the culture conditions (presence or absence of fetal calf serum or bovine \(\beta_2\)-microglobulin) are indicated at the top of each histogram. The thin and thick lines indicate cells cultured with or without NP-(365-380), respectively. The shaded histograms represent the background fluorescence of cells stained with an irrelevant mAb (MKD\textsubscript{6}, anti-1\textsuperscript{A\textsubscript{b}}).

Expressed on human T2 cells (27), which are thought to have a defect similar to that in RMA-S cells (28).

The Increase in D\textsuperscript{b} Molecules on RMA-S Cells Incubated with NP-(365-380) is Dependent on Exogenous \(\beta_2\)-Microglobulin in Serum. The results obtained above led us to examine the role of exogenous \(\beta_2\)-microglobulin in the expression of class I molecules on RMA-S cells exposed to high concentrations of peptides (3). The expression of D\textsuperscript{b} molecules is selectively increased on RMA-S cells that are incubated with the NP-(365-380) peptide for 18 hr at 37°C (Fig. 5A and B), as expected. However, there is no corresponding increase in the expression of murine \(\beta_2\)-microglobulin on these cells (Fig. 5C). Furthermore, the induction of D\textsuperscript{b} requires the presence of serum during the exposure to peptide (Fig. 5D). The addition of purified bovine \(\beta_2\)-microglobulin to serum-free medium is sufficient to allow the induction of D\textsuperscript{b} molecules by peptide (Fig. 5E).

**DISCUSSION**

It was previously reported and we confirm that incubation of RMA-S cells at low temperature results in an increase in the expression of class I molecules (4). However, our data illustrate that a major component in the overall increased expression of class I heterodimers reflected the stabilization of the binding of bovine \(\beta_2\)-microglobulin from the extracellular fluids to murine class I heavy chains. There was increased expression of murine \(\beta_2\)-microglobulin but its magnitude, in our hands, was modest. These data imply that low-temperature incubation permitted only a minor increase in the transport of intact class I heterodimers to the cell surface and/or that the murine–murine heterodimers that were induced were still relatively unstable at 26°C. Consistent with this latter possibility, we detect an increased pool of free class I heavy chains on RMA-S cells cultured at low temperature when human \(\beta_2\)-microglobulin is used as a probe (unpublished data), and pulse–chase experiments had previously demonstrated increased sialylation of class I molecules under these conditions (4).

Increased levels of class I molecules are present on RMA-S cells that are incubated with exogenous immunogenic peptides (3). It was originally suggested that exogenous peptides entered RMA-S cells and induced the assembly of class I molecules in the endoplasmic reticulum. It was subsequently suggested that peptides might be stabilizing "empty" class I molecules that were transported to the cell surface (4). The findings that exogenous \(\beta_2\)-microglobulin is required for this effect and that the "induced" class I molecules have a xenogeneic light chain strongly argue against the possibility that the peptide is exerting its major effects in the endoplasmic reticulum. Similarly, it is unlikely that peptide is stabilizing empty class I molecules as they emerge on the cell surface. The data favor the interpretation that this phenomenon, in large part, reflects another facet of peptide binding to class I molecules at the cell surface upon \(\beta_2\)-microglobulin reassociation (7–10).

The previous characterization of the RMA-S cell line has been important in establishing the current concepts of biosynthetic assembly of class I molecules and their instability in the absence of bound peptide. However, at least some of these phenomena are clearly associated with mature molecules and, accordingly, their relevance to biosynthesis depends on the extent to which the assembly of heterodimers at the cell surface parallels biosynthetic assembly.

The findings with RMA-S cells are potentially important for understanding the behavior of class I molecules on the cell surface. Intact class I heterodimers on nonmutant cells appear to be unreactive to binding peptides (7–10). These findings have implied that when peptides dissociate there is an alteration in the class I binding site such that it becomes unreactive to binding new peptides. The instability of class I molecules that lack bound peptides on RMA-S cells suggests that this alteration may arise from the loss of \(\beta_2\)-microglobulin.

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