Functionally conformed free class I heavy chains exist on the surface of beta 2 microglobulin negative cells.
Functionally Conformed Free Class I Heavy Chains Exist On The Surface Of $\beta_2$ Microglobulin Negative Cells

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Summary

Cytotoxic T lymphocytes (CTL) can recognize antigenic peptides bound in a groove formed by the $\alpha_1$ and $\alpha_2$ domains of the heterodimeric major histocompatibility complex class I molecule. Proper assembly, transport, and stability of functional class I molecules is thought to require $\beta_2$ microglobulin ($\beta_2m$), the light chain of the class I heterodimer. We show here that the requirement for $\beta_2m$ is not absolute. $\beta_2m^{-}$ cells can be stained by the $\alpha_1$ domain-specific B22-249.1 monoclonal antibody, which detects a conformation-dependent epitope. Furthermore, $\beta_2m^{-}$ Con A blast target cells can be lysed by alloreactive CTL, even in serum-free conditions. Contrary to previous reports, the expression of low levels of conformed $\beta^b$ heavy ($H$) chains is a property of both normal and transformed $\beta_2m^{-}$ cells. Finally, we present evidence that a subset of properly conformed $H$ chains, free of $\beta_2m$, may have almost equal representation on $\beta_2m^{+}$ and $\beta_2m^{-}$ cells.

Materials and Methods

Mice. All $\beta_2m^{-}$ strains (4) were bred at the Life Science Addition Animal Facility (University of California, Berkeley) or the
Center for Cancer Research at the Massachusetts Institute of Technology (Cambridge, MA). All other strains were purchased from The Jackson Laboratory (Bar Harbor, ME).

**Tumor Cells.** R1E/D" and R1E/D/F2m (6) were a generous gift from M. Zuffiga (University of California, Santa Cruz).

**Cell Culture Media.** Media containing bovine β2m: RPMI 1640, 5% FCS, 5 μg/ml gentamicin, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 mM Hepes, and 50 μM 2-ME (RP-5). β2m free media: AIM-V (Gibco Laboratories, Grand Island, NY), and 50 μM 2-ME.

**Antibodies.** Allophycocyanin-conjugated streptavidin was obtained from Molecular Probes (Eugene, OR). Fluorescein-conjugated goat anti-mouse Ig (cat. no. 1010-02) was obtained from Southern Biotechnology Associates (Birmingham, AL). Sheep anti-fluorescein-conjugated magnetic beads (cat. no. 4310D) were obtained from Advanced Magnetics (Cambridge, MA). Anti-CD8 (AD1[15]), anti-CD4 (RL174), anti-NK1.1 (PK136), and anti-Kk (16-1-2α) can be obtained from the American Type Culture Collection (Rockville, MD). Anti-Dα (B22-249.1) was a gift from P. Walden (Max Planck Institute, Tübingen, Germany) and was biotinylated using standard methods. Anti-Thy-1.2 (JIF) was a gift from J. Sprent (The Scripps Research Institute, La Jolla, CA).

**Radiation Bone Marrow Chimeras.** Bone marrow was prepared from female β2m- H-2Kd homozygous mice (5 mo-old) resulting from the intercross of the 4th backcross generation of B10.BR × (B6 × 129)F1, and was depleted of T cells by treatment with anti-Thy-1.2 (JIF) plus complement. Ten million T-depleted marrow cells were injected into female B10.BR mice (3-5 mo-old) irradiated (980 rad from a 137Cs source) ~2 h earlier. To prevent rejection of the grafts mice were injected into female fl2m- H-2 k homozygous mice (5 mo-old) resulting from the intercross of the 4th backcross generation of B10.BR × B10.BK (6) were a generous gift from P. Walden (Max Planck Institute, Tübingen, Germany) and were biotinylated using standard methods. Anti-Thy-1.2 (JIF) was a gift from J. Sprent (The Scripps Research Institute, La Jolla, CA).

**Immunofluorescent Staining Analysis.** Lymph node suspensions from individual mice or tumor cells were stained at 106 cells/25 μl for 20 min at 4°C in either PBS, 5% FCS, 0.2% NaN3 (medium containing β2m), or PBS, 14 μg/ml β2m-galactosidase (Sigma Chemical Co., St. Louis, MO), and 0.2% NaN3 (β2m-free staining media). Between stains, cells were washed two times. After the final wash, cells were fixed in 1% paraformaldehyde and stored, for analysis 12 wk later.

**Cytolysis Analysis.** Primary MLC were established essentially as described (8). Briefly, 25 × 106 responder splenocytes and 25 × 106 stimulator splenocytes (receiving 3,000 rad from a 137Cs source) were cultured together in 20 ml of RP-5 in upright T-25 flasks (Corning Glass Works, Corning, NY) for 5 d and then harvested for cytolytic assay. Cells harvested on day 9 or 10 from 1st MLC were reestablished in 2nd MLC (2-3 × 106 responder cells/culture) with fresh stimulator cells (25 × 106 cells/culture) and cultured for 4 d before harvesting for assay. The cytolytic assay was performed essentially as described (8) either in RP-5 or AIM-V, as indicated. For target cell preparation, splenocytes were cultured for 2 or 3 d in 24-well plates (Falcon Plastics, Lincoln Park, NJ) at 2 × 106 cells/ml with 2 μg/ml Con A (when culturing in RP-5) and 0.33 μg/ml Con A (when culturing in AIM-V).

**Immunomagnetic Depletion of B Cells.** β2m- spleen cells were stained with affinity-purified, fluorescein-conjugated, goat anti-mouse Ig antibody in β2m-free staining media for 20 min at 4°C, washed two times, then treated with sheep anti-fluorescein-conjugated magnetic beads (Advanced Magnetics) for 30 min at 4°C with gentle rocking. Ig+ (and hence, magnetic) cells were removed by exposing the cell suspension for 15 min at room temperature to a strong magnetic field using a Biomag device (Advanced Magnetics). Nonimmobilized cells were recovered and passed a second time through the Biomag device to remove any remaining Ig+ cells. Recovered cells were washed into AIM-V medium before the cytolytic assay.

**Antibody Plus Complement Depletion.** CTL populations were resuspended at 20 × 106 cells/ml in RP-5, and the optimal dilution of mAb and a mixture of rabbit and guinea pig complement. After incubation at 37°C for 40 min, viable cells were recovered by passage over a ficoll gradient and washed into the appropriate media.

**Results**

Polyclonal CTL populations specific for H-2Kd were generated in MLC, in which BALB/c (H-2Kd) or B10.BR (H-2Kd) spleen cells were stimulated with B6 (H-2Kd) stimulator spleen cells. In most cases, the CTL were restimulated with B6 spleen cells before assaying their activity. The CTL were tested for their capacity to lyse Con A blast targets from H-2Kd-type β2m− or β2m+ mice (Fig. 1). Corroborating our earlier study, β2m− target cells were lysed by the CTL, although substantially less efficiently than were β2m+ target cells. To achieve equivalent lysis of β2m− target cells, 50 times more CTL from primary MLC were usually required than for lysis of β2m+ target cells (Fig. 1A). CTL from secondary MLC appeared capable of more efficient relative lysis of β2m− target cells, since only 10-20 times more CTL from secondary MLC were typically required for lysis of β2m− target cells than for lysis of β2m+ target cells (Fig. 1B). There was no significant lysis of syngeneic (BALB/c or B10.BR) β2m− target cells by any of these CTL, demonstrating their specificity.

Previous studies have shown that bovine β2m, present in FCS, can associate with class I H chains on cells cultured in serum-containing medium (5). It was therefore possible that lysis of β2m− cells by CTL depended on the binding of serum β2m to free class I chains on the surface of β2m− cells. To test this possibility, we compared experiments performed in FCS-containing medium with those performed in AIM-V serum-free medium. The two media were used for generating target cell blasts and for the cytolytic assay. Because AIM-V medium failed to support the generation of high levels of CTL activity in MLC, the CTL were generated in FCS-containing medium, and were washed extensively in AIM-V medium before the cytolytic assay. As shown in Fig. 1, A and B, a similar extent of lysis of β2m− target cells was observed in FCS-containing medium as in AIM-V serum-free medium, indicating that serum β2m is not necessary for lysis of β2m− cells by CTL.

The lysis of β2m− target cells is not mediated by MHC class II (MHC-II)-specific CTL, because T-blast target cells, which do not express MHC-II in the mouse, were employed for all of these experiments. To rule out the possibility that contaminating B cell blasts in the Con A blast preparation are responsible for the observed lysis, we employed as target cells β2m− Con A blasts that had been depleted of class II+ B cells before culture, by immunomagnetic separation with
before the cytolysis assay. Treatment of the population with effector cell population with specific mAb and complement NKI.I+CDS- NK cells can also lyse by CTL of the conventional CDS+CD4-NKI.1- phenotype, lysis of B2m- cells by these cytolytic cells is mediated or anti-NK1.1 plus complement had no effect. There- fore, lysis of B2m- targets were from (B6 x 129)F2-4 animals, the H-2d targets were from B10.BR mice, and the H-2k targets were from B10.A(4R) responder mice.

The effector cells that lyse H-2b, B2m- target cells were typed for expression of cell surface markers by treating the effector cell population with specific mAb and complement before the cytolytic assay. Treatment of the population with anti-CD8 mAb plus complement strongly reduced lysis of B2m- cells, whereas treatment with anti-CD4 plus complement or anti-NK1.1 plus complement had no effect. Therefore, lysis of B2m- cells by these cytolytic cells is mediated by CTL of the conventional CD8+CD4- NK1.1- phenotype (Fig. 2). Although our previous results indicate that NK1.1+CD8+ NK cells can also lyse B2m- target cells (9), the conditions of the MLC apparently do not support the growth or activation of NK cells.

To identify the MHC molecule(s) responsible for lysis of B2m- target cells, we used spleen cells from MHC-recombinant congenic mice as responder cells to generate polyclonal CTL specific for Kk or Dd. By stimulating B10.A(18R) (KkA~EbD d) spleen cells with B6 cells, CTL specific for Dd were obtained. B10.A(4R) (KkA~EbD d) responder spleen cells stimulated with B6 cells resulted in a population containing Kk-specific CTL. Fig. 3 shows that both populations lysed B2m- cells, with less efficiency, B2m-, H-2b target cells. In this and other experiments, B10.A(18R) anti-B6 CTL achieved better and more reproducible lysis of B2m- target cells than did the B10.A(4R) anti-B6 CTL. These results suggest that anti-Dd CTL are more efficient than anti-Kk CTL at lysing B2m- target cells.

To directly test whether the B10.A(18R) anti-B6 CTL recognize Dd on B2m- target cells, antibody blocking experiments were performed. These experiments employed the mAb B22-249.1, which recognizes the α1 domain of Dd. Binding of B22-249.1 mAb has been used as evidence that the α1 domain of Dd is in a properly folded conformation (1, 3, 10). To minimize any sources of soluble B2m in the experiment, the target cells were generated by culture in AIM-V medium, the assay was performed in AIM-V medium, and purified B22-249.1 IgG was employed. Nonsaturating effector/target ratios were used, based on a preliminary titration experiment (Fig. 4 A). As shown in Fig. 4 B, B22-249.1 IgG, in a dose-dependent manner, inhibited lysis of both B2m+ and B2m- target cells by the B10.A(18R) anti-B6 CTL. The antibody had no effect on lysis of H-2k targets by B10.A(18R) anti-B10.BR CTL, demonstrating the specificity of the effect. These results demonstrate directly that allospecific CTL can recognize Dd molecule on B2m- cells. The results further suggest that the Dd molecules recognized are in a folded conformation detected by the B22-249.1 mAb.

Although the previous experiments were performed under
conditions minimizing sources of soluble $\beta_2\text{m}$, it remained possible that $\beta_2\text{m}$ from the CTL themselves was transferred to the target cells during the cytolysis assay. To address this possibility, we sought to employ a population of CTL that were $\beta_2\text{m}^-$, which are deficient in CD8$^+$ CTL. However, differentiation of $\beta_2\text{m}^-$ hematopoietic stem cells in irradiated $\beta_2\text{m}^+$ mice leads to the efficient differentiation of CD8$^+$ CTL precursors under the influence of the host thymus (Bix and Raulet, manuscript submitted for publication). Therefore, to generate $\beta_2\text{m}^-$, we produced radiation chimera by inoculating irradiated B10. BR mice with bone marrow cells from H-2$^k$, $\beta_2\text{m}^-$ donors. 13 wk later, spleen cells from the reconstituted chimeras were depleted of class I$^+$ cells (2), or not (1), and washed twice consecutively in MLC with B6 spleen cells to generate anti-H-2$^k$ CTL. To deplete the minor fraction of radioresistant hematopoietic cells of host (class I$^+$) origin often found in radiation chimeras, the chimeric CTL population was depleted of residual radioresistant $\beta_2\text{m}^+$, H-2$^k$ cells by treatment with anti-H-2$^k$ mAb (16-1-2n) plus complement on the day of the cytolysis assay. As before, the cytolysis assay and target cell cultures were performed in AIM-V medium. Thus, essentially no $\beta_2\text{m}$ was present in this experiment.

As shown in Fig. 5 B, the $\beta_2\text{m}^-$, anti-H-2$^k$ CTL lyed $\beta_2\text{m}^-$, H-2$^k$ target cells. Comparing data on 4 dose-response curves, the lysis of $\beta_2\text{m}^-$ versus that of $\beta_2\text{m}^+$ target cells by the $\beta_2\text{m}^-$ CTL was similar to that mediated by the $\beta_2\text{m}^+$ (B10.BR) CTL, although the latter population was somewhat more potent (compare Fig. 5, A and B). The depletion of residual H-2$^k$ cells from the chimeric CTL population made no difference in the dose-response curves. Depletion of H-2$^k$ cells from the control B10.BR CTL population eliminated CTL activity, demonstrating the effectiveness of the antibody plus complement treatment. These results suggest that lysis of $\beta_2\text{m}^-$ H-2$^k$ target cells by allo-specific CTL does not depend on $\beta_2\text{m}$.

The data in the previous experiments do not distinguish whether most anti-H-2$^k$ CTL in the populations lyse $\beta_2\text{m}^-$, H-2$^k$ targets poorly, or alternatively, that only a subset of anti-H-2$^k$ CTL lyse $\beta_2\text{m}^-$ targets. As one means of addressing this question, we generated CTL by stimulating B10.BR responder spleen cells twice consecutively with either $\beta_2\text{m}^-$ or $\beta_2\text{m}^+$ H-2$^k$ stimulator spleen cells in MLC. Although significant CTL activity was not induced after only one round of stimulation with $\beta_2\text{m}^-$ cells (4), significant activity was usually evident after two or more rounds of stimulation (Fig. 6). These effector cells lysed $\beta_2\text{m}^+$ and $\beta_2\text{m}^-$ H-2$^k$ targets with nearly equal efficiency, but did not lyse B10.BR target cells (Fig. 6 B). As in previous experiments, the CTL raised against $\beta_2\text{m}^+$, H-2$^k$ stimulator cells lysed $\beta_2\text{m}^+$ target cells much better than they lysed $\beta_2\text{m}^-$ target cells. These data suggest that a subset of anti-H-2$^k$ CTL lye $\beta_2\text{m}^+$ and $\beta_2\text{m}^-$ target cells with nearly equal efficiency.

To examine the expression of serologically detected class I epitopes on the $\beta_2\text{m}^-$ cells, we stained lymph node cells with a conformation-dependent anti-class I antibody (B22-249.1), and examined them by flow cytometry. To ensure that no $\beta_2\text{m}$ was present in the experiment, we employed purified antibody preparations, harvested the cells in serum-free (AIM-V) medium, and stained the cells in medium that contained H-2$^k$.
Figure 7. β2m− lymph node cells and β2m− tumor cells show cell surface expression of properly conformed class I Db molecules. Lymph node cells (a, b, and c) from β2m− H-2b (B6 × 129)F1 (a), or β2m− H-2b (B6 × 129)F1 (b), or control β2m− H-2b (B10.BR) (c) and β2m− tumor cells (R1E-Db/β2m) (d), (β2m− tumor cells (R1E-Db) (d), were stained with biotinylated anti-Db mAb (B22-249.1) in the presence of 5% FCS (dashed line) or in β2m−free medium (solid line). (Dotted line) Control staining with second reagent (APC-streptavidin) only. Data are presented as cell number vs log fluorescence intensity.

β-galactosidase as a source of protein, instead of serum. We compared the staining of β2m− lymph node cells to the staining of the β2m− R1E/Db tumor cells. As shown in Fig. 7, β2m− lymph node cells stained with B22-249.1 mAb, although the intensity of staining was about 1/20th that of β2m+ cells. This level of staining is similar to that observed in the presence of serum, which provides a source of β2m. The specificity of the staining is shown by the failure of the antibody to significantly stain B10.BR (H-2b cells).

To our surprise, β2m− R1E/Db cells also stained specifically with B22-249.1 mAb. The absolute intensity of staining was similar to that of β2m− lymph node cells. By comparison, the β2m+ variant of these cells, R1E/Db/β2m, stained more intensely than β2m− lymph node cells. Considering that the tumor cells are larger than lymph node cells, it appears that the β2m− tumor cells may express a lower density of Db on their surface than the β2m− lymph node cells.

Discussion

Our results suggest that the Db molecule can assume a functional conformation in the absence of β2m. This conclusion is based on the findings that β2m− cells were stained by the conformation-dependent B22-249.1 mAb in serum-free medium, and were lysed by allospecific β2m− CTL in serum-free medium. Furthermore, the Db-specific lysis of β2m− cells could be blocked with the conformation-dependent B22-249.1 mAb.

Our results with the R1E/Db cell line contrast with previous results suggesting that this cell line fails to present properly conformed H chain at the cell surface (1). The different results are not due to the use of different reagents, because the earlier studies, like ours, employed the B22-249.1 mAb. It is likely that previous studies with R1E/Db were not sufficiently sensitive to detect correctly conformed Db on the cells, since we find that successful detection requires a highly sensitive staining protocol. Although R1E/Db stains relatively weakly compared with the staining of R1E/Db/β2m, the results indicate that the expression of low levels of conformed Db H chains is a property of both normal and transformed β2m− cells.

We further find that a subset of allospecific CTL generated by multiple stimulations with β2m− cells, lyse β2m− and β2m+ cells equally well. This observation, taken together with other findings, is most simply explained by assuming that these effectors recognize a set of properly conformed H chains, free of β2m, that are roughly equally represented on β2m− and β2m+ cells. Whether these free H chains are associated with peptides is not known. Evidence for CTL recognition of peptide-free, β2m-containing class I molecules has been reported (11). However, it seems unlikely that H chains free of both peptides and β2m would maintain a functional conformation at the cell surface.

We favor an alternative possibility, that is suggested by recent findings that in the absence of β2m, free Db H chains incubated with physiologically relevant, nonamer peptides assume a conformation reactive with B22-249.1 mAb (3). High (micromolar) concentrations of peptide were required to observe this effect. Nonetheless, it is possible that some cellular peptides bind to free Db H chains in β2m− cells tightly enough to stabilize the properly folded conformation for transportation to the cell surface. Such complexes might be expected to have a short half-life in the absence of β2m. Alternatively, a subset of peptides may bind free H chains very tightly, resulting in stable complexes at the cell surface.

A great deal of recent effort has been devoted to attempts to understand the assembly of class I H chains with antigenic peptides, and β2m in vivo (3, 11–14). An important question is whether peptides associate with H chain before or after β2m, and the relative contribution of peptide binding and β2m binding to the stability of the trimolecular complex. Our results raise the possibility that, at least in some instances, peptides may bind H chains in the absence of β2m with sufficient affinity to establish a functionally conformed molecule on the cell surface.
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