RESCUE OF DAUDI CELL HLA EXPRESSION BY TRANSFECTION OF THE MOUSE β_2-MICROGLOBULIN GENE

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β_2-Microglobulin (β2m)[1] is the light chain (12 kD) of class I MHC proteins. It is expressed on the surface of almost all nucleated cells in noncovalent association with a transmembrane heavy chain (~42 kD) glycoprotein encoded within the MHC (1, 2). The gene encoding a β2m is unlinked to the MHC and is located on chromosome 2 in mouse (3, 4) and chromosome 15 in man (5). While β2m shows very little variation within a species, the associated heavy chain molecules are extremely polymorphic (6).

Cell surface expression of the heavy chains of class I MHC molecules is generally thought to require concomitant expression of β2m (7–13). This conclusion has been based on studies of the human Daudi cell line (7–9) and of mutants of the mouse R1 cell line (10–13). In both systems mutations in the β2m gene are accompanied by lack of expression of β2m protein and lack of cell surface class I molecules. Cell surface expression of class I proteins was shown to be restored in each of these mutant cell lines after fusion to cells that express normal β2m protein (7, 8, 11, 13). Although these studies provide strong support for the requirement of β2m protein for cell surface class I expression, they are not conclusive, and recent data have suggested that the H-2D^b molecule can be detected on the cell surface in the absence of β2m (14–16). We therefore attempted to correct the presumed defect in the Daudi cell line in a more direct way by transfecting the mouse β2m gene into this cell line. Our results conclusively show that β2m protein is essential for the cell surface expression of the MHC class I antigens of Daudi cells.

Materials and Methods

Cell Lines. Daudi, a human Burkitt lymphoma cell line (17), was maintained in RPMI 1640 supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, and 10% heat-inactivated FCS or human serum. CTL lines were generated and characterized essentially as described (18). Briefly, PBLs from a normal donor (HLA A3,3; B7,7; DR6,6) were separated on Ficoll-hypaque and stimulated in primary culture with irradiated (10,000 rad) EBV-transformed B cell lines that express HLA-A2 and/or HLA-B17. Cells were

This work was supported by National Institutes of Health grant GM-34991 to J. R. Parnes. J. R. Parnes was the recipient of a John A. and George L. Hartford faculty fellowship award. Address correspondence to Jane R. Parnes, Department of Medicine, Division of Immunology, Stanford University Medical Center, Stanford, CA 94305.

[1] *Abbreviation used in this paper:* β2m, β_2-microglobulin.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/88/02/0288/12 $2.00
Volume 167 February 1988 288–299

stimulated in secondary culture with a different HLA-A2$^+$ and/or HLA-B17$^+$ B lymphoblastoid cell and cloned on a third cell line. Clones arising at 1 cell/well were tested for lysis of a panel of 11 target cells expressing HLA-B17, seven targets expressing HLA-A2, and six targets expressing other HLA types. Clones exhibiting desired specificities were subcloned at 0.3 cells/well.

Cotransfection of the Mouse β2m Gene with pSV2neo. A 14-kb Xho I fragment containing the β2m gene from a C57BL/6 mouse (*b* allele of β2m) was isolated from the phage clone Ch4A.B2-C57 (12) and was cotransfected into Daudi cells with pSV2neo (19), which contains the pBR322 origin of replication, the β-lactamase gene, and the neomycin resistance gene. Daudi cells were transfected by electroporation (20) essentially as described (21). 2×10^7 cells, 30 μg of the 14-kb Xho I fragment containing the β2m gene, and 10 μg of linearized (Bam HI) pSV2neo were mixed in 0.5 ml of 140 mM NaCl, 25 mM Hepes, and 0.75 mM Na_2HPO_4, pH 7.15. A bank of capacitors (effective capacity 14 μF) charged to 1,100 V was discharged via an electronic switch (model ZA 1,000; Prototype Design Service, Madison, WI) through the sample using a cell chamber (Prototype Design Service) with a length of 5 mm and a cross-sectional area of 1 cm^2 at 4°C. After the shock, the cells were left 10 min on ice, then 30 min at 37°C. The cell suspension was then dispersed into four culture flasks with 20 ml of medium per flask. 2 d after transfection, the antibiotic G418 was added to the flasks to a final concentration of 1 mg/ml. 3-wk after transfection, an aliquot of the G418-resistant cells was stained with mAbs for immunofluorescence analysis.

mAbs for Immunofluorescence Staining. mAbs W6/32 (22) and PA2.6 (23), which identify framework components of HLA-A, -B and -C heavy chains, MA2.1 (24), which reacts specifically with HLA-B17 and HLA-A2, and BBM.1 (25), which reacts with human β2m, were kindly provided by Dr. P. Parham, Stanford University, Stanford, CA. mAb specific for the *b* allele of mouse β2m was purchased from New England Nuclear, Boston, MA.

AH7.2 (IgG2) and G12.2 (IgG1) are mouse anti–rabbit IgG mAbs used as isotype-matched controls for W6/32 (specific mAb is IgG2, but an IgG1 mAb is also secreted from the fusion partner), BBM.1 (IgG2b), PA2.6 (IgG1), and MA2.1 (IgG1). Anti-Leu-2b (IgG2a) (Becton Dickinson & Co., Mountain View, CA) is a mouse mAb specific for human CD8 and was used as an isotype-matched control for the mouse anti-$\beta 2m^b$ mAb (IgG2a).

Immunofluorescence Staining. Cells were stained with the mAbs described above followed by fluorescein-conjugated goat anti–mouse IgG antibodies as a second-stage reagent (Jackson Immunoresearch Laboratories, West Grove, PA) and analyzed or sorted on the FACS.

DNA Probes. cDNA probes for the human β2m and HLA-A,B,C mRNA were as previously described (26). A 600-bp Sac I–Kpn I genomic fragment containing exon II of the β2m gene and flanking intron sequence was used as a probe for mouse β2m (12). All probes were isolated and labeled with ^{32}P by random hexamer priming (27).

RNA Gels and Hybridization. RNA was extracted from cells using the method of Chirgwin et al. (28). 10-μg RNA samples were subjected to electrophoresis through a 1.5% agarose gel containing 2.2 M formaldehyde and transferred to nitrocellulose (29). Blots were hybridized as described (30) to ^{32}P-labeled HLA-A, -B, -C, human β2m, or mouse β2m DNA probes. The filters were washed as described (30) and exposed to XAR-5 film (Kodak) at −70°C overnight. The positions of the 18S and 28S ribosomal RNA markers were determined by the ethidium bromide–staining pattern of the gel before blotting. For rehybridization, the blot was boiled in 0.1× SSC and 0.1% SDS for 5 min three times to remove hybridized probe and then was checked by exposure to XAR-5 film (Kodak) at −70°C overnight.

Cytotoxicity Assay. ^{51}Cr-release assays were performed in triplicate in V-bottomed wells as described (31).

Results

Surface Expression of HLA Molecules on Daudi Cells. Although Daudi cells do not express class I molecules (HLA-A, -B, or -C) on the cell surface, the heavy chain specificities of this cell line have been identified as A1, A26, B17, and B38

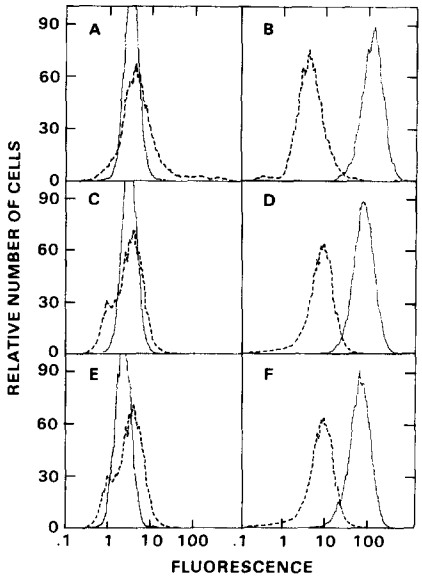

FIGURE 1. Daudi cells transfected with the mouse β2m gene express surface class I MHC molecules. Untransfected Daudi cells (*A*, *C*, and *E*) and mouse β2m-transfected Daudi cells (mβ2m-Daudi-1A) (*B*, *D*, and *F*) were stained with an HLA class I–specific mAb (*solid lines*) or an isotype-matched control mAb (*dotted lines*), followed by FITC-conjugated goat anti–mouse antibodies. Specific mAbs used were the monomorphic anticlass I mAbs W6/32 (*A* and *B*) and PA2.6 (*C* and *D*), or mAb MA2.1, which is specific for the HLA-B17 heavy chain (*E* and *F*).

by their expression on somatic cell hybrids between Daudi and human or mouse cell lines (8). If the lack of cell surface class I expression of Daudi cells is solely due to the absence of β2m protein, then transfection and expression of either the human or mouse β2m gene should rescue expression of these HLA specificities. We therefore cotransfected a DNA fragment containing the mouse β2m gene and linearized plasmid pSV2neo into Daudi cells by electroporation. Transfectants were selected by resistance to the antibiotic G418, stained with an mAb (W6/32) specific for a monomorphic determinant on class I HLA molecules, followed by a fluoresceinated goat anti–mouse Ig second-stage reagent, and then analyzed on the FACS. 1–5% of the total transfected Daudi cells stained brightly at the first FACS analysis. ~2,000 positive cells were sorted sterilely from each of four flasks and maintained independently in culture. One such sorted line, mβ2m-Daudi-1A, was used for further study. This line was stained with a series of mAbs that detect surface expression of class I HLA molecules. As shown in Fig. 1, the transfected Daudi cells (mβ2m-Daudi-1A) stained brightly with two mAbs specific for monomorphic determinants on class I HLA molecules, W6/32 (Fig. 1 *B*) and PA2.6 (Fig. 1 *D*), and with an mAb specific for HLA-B17 or HLA-A2, MA2.1 (Fig. 1 *F*) as compared with isotype-matched control mAbs. In contrast, there was no difference between the staining of untransfected Daudi cells with these mAbs as compared with the isotype-matched control mAbs (Fig. 1, *A*, *C*, and *E*). Surprisingly, we did find greater staining of the transfected mβ2m-Daudi-1A cells than the untransfected Daudi cells with the irrelevant IgG1 control mAb (G12.2) (Fig. 1, *C–F*), but not with the IgG2 control (AH7.2) (Fig. 1, *A* and *B*). We do not yet know the mechanism for this, but it is possible that expression of β2m protein or of surface class I molecules increases the expression of Fc receptors for IgG1. This possibility is currently being investigated.

mβ2M-Daudi-1A Express Mouse β2m Molecules on Cell Surface. If the expression

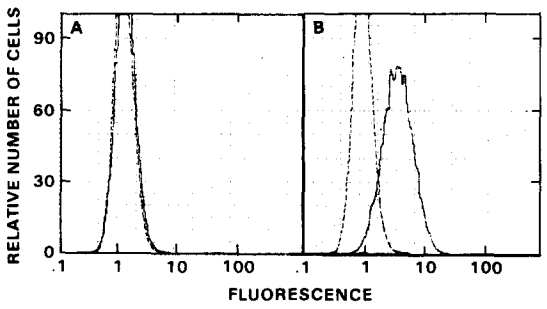

FIGURE 2. Daudi cells transfected with the β2m gene express cell surface mouse β2m. Daudi cells were cotransfected with the mouse (β2m) gene and pSV2neo. Transfectants were selected by resistance to the antibiotic G418. Untransfected Daudi cells (*A*) and transfected (*B*) mβ2m-Daudi-1A cells were stained with an mAb specific for the *b* allele of mouse β2m (*solid lines*) or an isotype-matched control (*dotted line*), followed by FITC-conjugated goat anti-mouse antibodies.

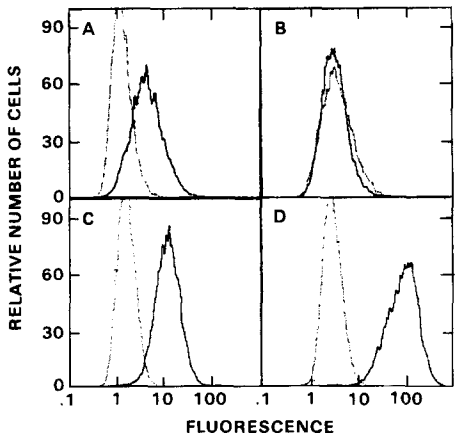

FIGURE 3. Mouse β2m can exchange with human β2m in human serum. Untransfected (*dotted lines*) and mβm-Daudi-1A cells (*solid lines*) were grown in either FCS (*A* and *B*) or human serum (*C* and *D*). Cells were then stained with a mAb specific for the *b* allele of mouse β2m (*A* and *C*) or mAb BBM.1, specific for human β2m (*B* and *D*), followed by FITC-conjugated goat anti-mouse antibodies.

of HLA class I molecules on the surface of mβ2m-Daudi-1A results from expression of the transfected mouse β2m gene, then the transfectants should also express cell surface mouse β2m. Fig. 2 shows that an mAb specific for the *b* allele of mouse β2m stained the transfectants brightly (Fig. 2*B*) as compared with untransfected Daudi cells (Fig. 2*A*). These results confirm that human HLA class I surface expression can be rescued in Daudi cells by the provision of a source of β2m within the cell.

Exchange of Mouse β2m with Human β_2m in Serum. Class I MHC molecules are anchored to the cell by the heavy chain, which fully traverses the plasma membrane. In contrast, β2m is located entirely outside the cell with no direct attachment to the lipid bilayer. Furthermore, the association between β2m and class I heavy chains is noncovalent. It is therefore not entirely surprising that β2m associated with class I heavy chains on the cell surface has been shown to exchange with free β2m present in serum used to grow cultured cells (32–34). We examined whether mouse β2m expressed on the surface of the mβ2m-Daudi-1A transfectants could exchange with human β2m by growing the cells in medium containing human serum instead of FCS. When mβ2m-Daudi-1A cells were grown in medium containing FCS, they stained positively only with an mAb specific for mouse β2m and not with mAb BBM.1, specific for human β2m (Fig. 3, *A* and *B*). In contrast, when these transfected cells were grown in medium containing human serum, they were stained by both of these mAbs (Fig. 3, *C* and *D*). Interestingly, mβ2m-Daudi-1A cells stained more brightly with the anti-mouse β2m mAb when grown in human serum as compared with FCS. This is

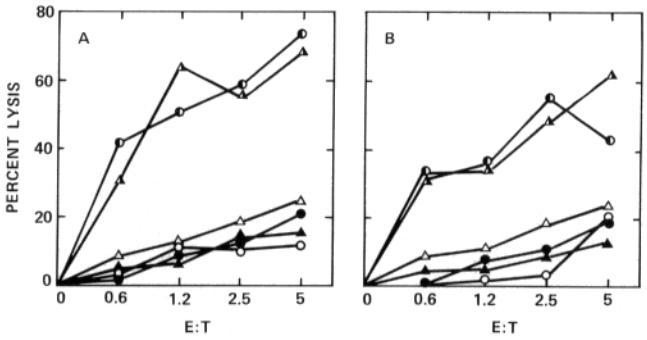

FIGURE 4. Transfected Daudi cells are recognized by CTLs for HLA-B17. Reactivities of two class I–specific CTL clones for β2m-Daudi-1A and untransfected Daudi cells were assessed in a chromium release assay. Clone AMSH.10 (*A*) is specific for HLA-B17, while clone AM-8B.2 (*B*) is specific for a determinant shared by HLA-B17 and HLA-A2. Target cells included untransfected Daudi cells (*open symbols*), Daudi cells transfected with pSV2neo alone (*solid symbols*), and Daudi cells cotransfected with mouse β2m and pSV2neo(mβ2m-Daudi-1A) (*half-filled symbols*). Cells were grown either in medium containing FCS (*circles*) or human serum (*triangles*).

likely to be a consequence of the greater concentration of free β2m in FCS as compared with human serum (33). As a result, once steady state is achieved, a greater percentage of cell surface β2m (mouse) will have exchanged with serum β2m (fetal bovine or human β2m, respectively) when the cells are grown in FCS as compared with human serum.

Cell-mediated Cytotoxicity. Since the human class I HLA/mouse β2m heterodimers on the surface of mβ2m-Daudi-1A cells retained serological reactivity with mAbs specific for human class I molecules, we next examined their functional role as targets for cytotoxicity. Two human CTL clones were used, AMSH.10, which is specific for HLA-B17, and AM8B.2, which is specific for a determinant shared by both HLA-A2 and HLA-B17. Both of these CTL clones lysed the mβ2m-Daudi-1A cells but not untransfected cells or Daudi cells transfected with pSV2neo alone (Fig. 4). Furthermore, there was no significant difference in cell-mediated cytotoxicity whether the target mβ2m-Daudi-1A cells were grown with FCS or with human serum (Fig. 4).

Expression of β2m and HLA Heavy Chain mRNA in Daudi Cells and β2m Transfectants. The expression of human and mouse β2m and HLA-A,B,C mRNA in mβ2m-Daudi-1A was examined by Northern blot analysis. As shown in Fig. 5, equal levels of class I heavy chain mRNA were present in untransfected Daudi cells and in mβ2m-Daudi-1A (Fig. 5, *A* and *B*). As expected, only mβ2m-Daudi-1A expressed mRNA that hybridized to a mouse β2m probe under high stringency conditions (Fig. 5, *C* and *D*). The two mouse β2m transcript sizes detected in mβ2m-Daudi-1A correlate with those seen in normal mouse cells which express β2m. The Northern blot hybridized to the mouse β2m probe was boiled to remove hybridized probe and rehybridized at high stringency to a human β2m cDNA probe (Fig. 5, *E* and *F*). A transcript of the appropriate size was detected both in untransfected Daudi cells and in mβ2m-Daudi-1A. Surprisingly, the level of human β2m mRNA in mβ2m-Daudi-1A was decreased severalfold compared with that in the untransfected cells. Since the mβ2m-Daudi-1A line represents a pool rather than a clone of transfectants, this is not likely to be a result of clonal variation. We do not yet know the mechanism for this decrease in endogenous β2m RNA. It is possible that synthesis of a functional β2m protein or a fully assembled heterodimeric class I molecule in some way results in

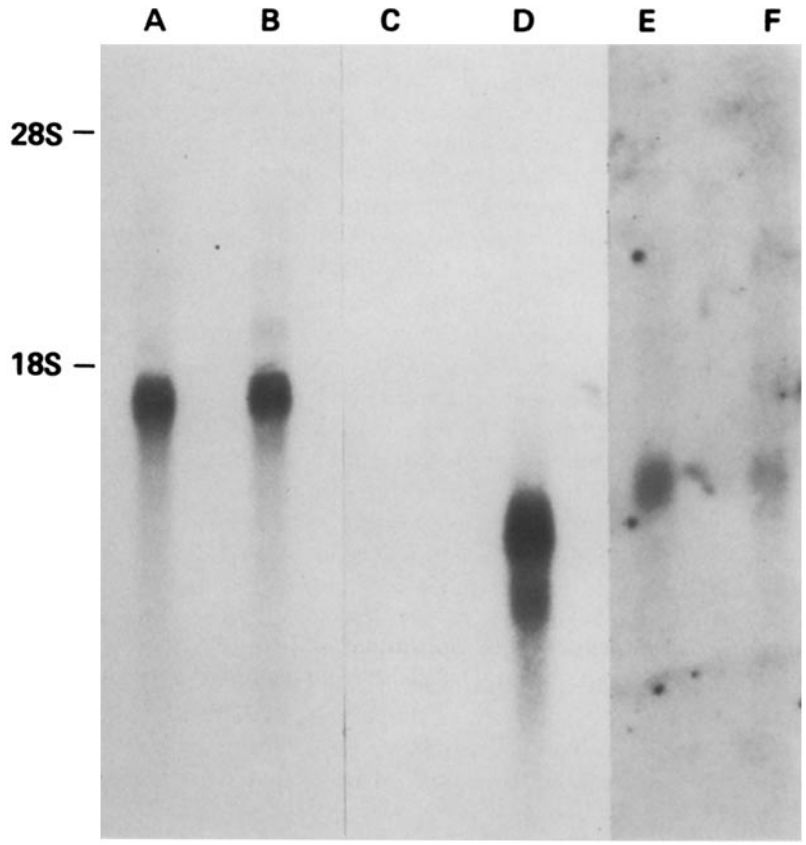

FIGURE 5. Expression of HLA heavy chain and β2m mRNA in Daudi cells and transfectants. Total RNA was isolated from untransfected (*A*, *C*, and *E*) and transfected mβ2m-Daudi-1A cells (*B*, *D*, and *F*) and a Northern blot was prepared. Lanes *A* and *B* were hybridized to a human HLA class I cDNA probe. Lanes *C* and *D* were hybridized to a mouse β2m genomic probe, then rehybridized, after boiling off the probe, to a human β2m cDNA probe (lanes *E* and *F*).

feedback inhibition of transcription of the endogenous β2m gene, or perhaps the transfected mouse β2m gene competes with the endogenous human gene for specific transcriptional factors. However, other transfected Daudi lines will need to be examined to determine whether this is a general phenomenon.

Discussion

The biosynthesis and assembly of class I MHC heavy chains and β2m have been studied both in mouse (35), and in greater detail, in human systems (9, 36–39). In similar fashion to other cell surface and secretory proteins, both the heavy chain and β2m are synthesized on membrane-bound polysomes, and in both cases the primary translation products contain NH_2-terminal signal sequences that direct the segregation of these proteins in the endoplasmic reticulum (9, 35). The signal peptides are cleaved off sometime after synthesis of the polypeptide chains. While the heavy chain becomes anchored in the membrane by means of its hydrophobic transmembrane sequence near the COOH-terminus, β2m lacks such a sequence and is expressed on the cell surface only by virtue of

its association with class I heavy chains. *N*-linked glycosylation of the heavy chain begins cotranslationally, but studies with tunicamycin indicate that it is not required for membrane insertion, association with β2m, or surface expression (9, 35, 37). Studies in human lymphoblastoid cell lines have shown that completed heavy chains bearing the high mannose (endoglycosidase H–sensitive) form of *N*-linked oligosaccharide are initially found unassociated with β2m immediately after synthesis (36). Association with β2m, which is not glycosylated, occurs soon thereafter (within 5–15 min), and this results in a change in conformation that alters the antigenic properties of the heavy chain (36, 39). The heavy chain can associate with a pool of presynthesized β2m (37). After association, the high-mannose form of oligosaccharide on the heavy chain is converted to the complex form (endoglycosidase H–resistant), a modification that takes place in the Golgi complex (36, 37, 39), and finally mature heterodimeric class I molecules are found on the cell surface from 30 to 60 min after initial synthesis, depending on the cell line (36, 37). Analysis of the biosynthesis and assembly of mouse and human heavy chain and β2m in cell-free translation systems supports the conclusions of the in vivo studies (9, 35, 38). Studies of the intracellular transport of human class I proteins after mRNA translation in *Xenopus laevis* oocytes have further shown that in this in vivo, albeit nonmammalian system, β2m is secreted into the medium when translated in the absence of heavy chain, while heavy chains are retained in the endoplasmic reticulum if translated in the absence of β2m (39). In contrast, when β2m is present, the heavy chains are transported at least as far as the *cis*-Golgi where the *N*-linked oligosaccharides are converted to an endoglycosidase H–resistant form (38). These findings suggest that β2m is required for the intracellular transport of class I heavy chains in the oocyte system.

Studies of the Daudi cell line have added greatly to the understanding of class I protein biosynthesis. Daudi is a Burkitt lymphoma–derived lymphoblastoid cell line (17) which lacks cell surface expression of class I HLA molecules (40). Daudi cells have been shown to contain mRNA for both class I heavy chains and for β2m (9, 41, 42), but the latter is not translatable because of a mutation in the initiation codon (43). As a result, no human β2m protein can be synthesized. In contrast, the heavy chain mRNA can be translated in vitro and in vivo, and cytoplasmic heavy chain can be immunoprecipitated by antiserum specific for dissociated HLA-A and -B heavy chains but not by alloantibodies or the mAb W6/32 (9). None of this heavy chain can be detected on the cell surface (9). The intracellular Daudi heavy chains appear to be processed normally and glycosylated (9), but the *N*-linked oligosaccharides remain in the endoglycosidase H–sensitive form (37). These results suggest that β2m is required for the heavy chain to be transported to the Golgi region, where the carbohydrate is converted to the complex form (37). Addition of purified β2m to whole cell lysates of Daudi does not result in association of the two chains (36), perhaps because of an alteration in conformation or glycosylation as compared to heavy chains in the presence of β2m (36). In contrast, the heavy chains synthesized by Daudi are capable of associating with human or mouse β2m in somatic cell hybrids, and such hybrids express Daudi-specific HLA-A and -B molecules on the cell surface (7, 8). These studies have led to the conclusion that β2m is required for heavy chains to be transported to the cell surface. A similar conclusion has been reached

in studies of mutants of the mouse R1 thymoma cell line (10–13). Four such mutants have been examined and each lacks expression of cell surface class I molecules (H-2K, -D, and TL) as determined by quantitative cytotoxic immunoabsorption. For one of these mutants an antiserum specific for isolated H-2 heavy chains has been used, and this reagent did not immunoprecipitate any heavy chain from the cell surface (16). Each of the four mutants has been shown to have defects in both chromosomal copies of the β2m gene, thereby preventing synthesis of β2m protein (12, 13). As in the case of Daudi, cell surface expression of class I molecules can be rescued by somatic cell fusion to a mouse cell line expressing β2m (11, 13).

Although the studies with Daudi and the R1 mutants support the hypothesis that β2m is required for cell surface expression of class I molecules, they do not constitute proof, because more than one defect may be complemented in the generation of somatic cell hybrids. These arguments have taken on greater significance because of recent studies indicating that the H-2D^b molecule can be expressed on the surface of mouse cells in the absence of β2m. Such expression was first suggested by Potter et al. (14, 15) as a result of their analysis of a mutant cell line (EL4/MAR) derived from the EL4 thymoma line. However, EL4/MAR clearly expresses intracellular β2m, and the conclusion that D^b is expressed on the surface of this cell without β2m has been challenged (16, 44). While providing an alternative explanation for the results of Potter et al. (14, 15) with EL4/MAR, Allen et al. (16) have provided strong evidence that D^b can indeed be expressed on the cell surface as an isolated heavy chain after transfection of the D^b gene into one of the β2m-deficient R1 mutant cell lines. However this molecule could not be recognized by mAbs specific for domains 1 or 2 of the D^b molecule, or by D^b-restricted CTLs. In contrast, a mAb specific for domain 3 (membrane-proximal domain) of D^b and the rabbit antiserum specific for isolated heavy chains could immunoprecipitate large amounts of the isolated D^b chain from the cell surface. It was concluded that the conformation of isolated cell surface D^b is very different from that present in heterodimers with β2m. These results correlate well with studies of changes in antigenicity of human heavy chains during biosynthesis and assembly (9, 36, 39). The fact that the putative isolated D^b heavy chain expressed by EL4/MAR was recognized both by allospecific CTLs and by a D^b domain 1–specific mAb that did not react with the D^b-transfected R1 mutant cell (14) led Allen et al. to question the interpretation of the EL4/MAR results (16). They suggested that endogenous β2m is probably used to transport D^b to the surface in EL4/MAR, and then is exchanged essentially completely with fetal bovine β2m in the growth medium. In any event, the results of Allen et al. (16) indicate that at least for D^b, a class I heavy chain that is not associated with β2m can be transported to the cell surface. We therefore felt it important to reexamine the defect in Daudi cells by establishing whether the lack of cell surface class I molecules was solely a result of the absence of β2m. Our results show that introduction of the isolated mouse β2m gene is indeed sufficient to rescue surface expression of Daudi class I molecules. The antihuman class I mAbs used all recognized the human class I molecule associated with mouse β2m (and/or fetal bovine β2m from serum). Similarly, two CTL clones specific for an HLA specificity encoded by Daudi were capable of killing the transfected Daudi cells, and this killing was indistinguishable whether the

cells were grown in FCS or human serum to allow exchange for human β2m. These results prove conclusively that at least in the case of the class I molecules of Daudi, β2m is required for cell surface expression. It may well be that D^b is a unique or at least unusual class I molecule with regard to its ability to be expressed on the cell surface without β2m.

Summary

The Daudi cell line is a B-lymphoblastoid line derived from a Burkitt lymphoma. Daudi cells lack cell surface expression of class I HLA molecules despite the presence of intracellular class I heavy chains. They have a defect in the gene encoding β_2-microglobulin (β2m), resulting in lack of translatable mRNA for this protein. It has been thought that this deficiency is responsible for the lack of cell surface class I expression. However, data have recently been presented demonstrating that at least one mouse class I heavy chain can be expressed on the cell surface in the absence of β2m. These results raised the questions of whether the lack of β2m is the only defect in Daudi and whether transfer of this single gene could restore surface class I expression. We found that transfection of the mouse β2m gene into Daudi indeed rescued cell surface expression of class I HLA molecules, and that these molecules could be recognized both by monomorphic and allospecific mAbs. CTL clones specific for HLA-B17 or a determinant shared by HLA-B17 and HLA-A2 killed the Daudi cells transfected with the β2m gene, but not untransfected Daudi or Daudi transfected with vector alone. Mouse β2m on the transfected Daudi cells could exchange with human β2m when the cells were incubated in human serum. This exchange did not alter the ability of the cells to be killed by the specific CTLs. These results demonstrate that the lack of β2m is the sole reason for lack of surface class I molecules in Daudi cells, and that β2m is required for cell surface expression of the specific class I heavy chains of Daudi.

We would like to thank Dr. Peter Parham for supplying mAbs and Diane Bet and Kim Brown for preparation of the manuscript.

Received for publication 4 August 1987.

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