EXPRESSION OF H-2D\textsuperscript{b} ON THE CELL SURFACE IN THE ABSENCE OF DETECTABLE β2 MICROGLOBULIN

By TERRY A. POTTER, CLAUDE BOYER, ANNE-MARIE SCHMITT VERHULST, PIERRE GOLSTEIN, and T. V. RAJAN

From the *Departments of Pathology and Genetics, Albert Einstein College of Medicine, Bronx, New York 10461; and the Centre d'Immunologie INSERM CNRS de Marseille-Luminy, 13288 Marseille Cedex 9, France

Class I molecules of the major histocompatibility complex (MHC) are expressed on the cell surface as a complex of two noncovalently associated units, a 40-45,000 dalton subunit called the heavy chain and an 11,000 dalton subunit called β2 microglobulin (β2m) (1). In the mouse this appears to be true for molecules encoded in the Qa-Tla region as well as for molecules encoded by the K, D, or L loci (2, 3). In contrast to the 45,000 dalton class I chain, β2m is not integrated into the membrane. Similarly, it is believed that the heavy chains of class I molecules are not expressed in the absence of β2m. Evidence for this comes primarily from the study of two cell lines. The Burkitt lymphoma line, Daudi, expresses neither class I HLA antigens nor β2m on the cell surface (4). In this cell line the primary defect is the inability to synthesize β2m, as shown by biochemical studies and by somatic cell hybridization experiments in which the expression of Daudi HLA antigens could be rescued either by mouse or human β2m, provided by the normal partner in the hybrid (4). Essentially the same kind of data have been obtained by Hyman and his collaborators (5, 6) using a somatic cell variant of the C3H (H-2\text{K}) thymoma, R1. These studies led to the conclusion that class I proteins have to be associated with β2m for expression on the cell surface. We report here on a spontaneous variant of the B6 lymphoma EL4, which in contrast to the wild-type cell line, expresses neither H-2K\textsuperscript{b} nor β2m but does express H-2D\textsuperscript{b}, which can be detected by serological reactivity as well as by cytotoxic T lymphocytes (CTL).

Materials and Methods

Antibodies. The monoclonal antibodies to H-2 specificities and to β2m listed in Table I were obtained from various investigators. Rabbit anti-mouse β2m was a kind gift from Dr. E. Appella.

Tumor Cell Lines. The original EL4 cell line was isolated by Dr. Peter Gorer (7) in 1945. We have examined two sublines: EL4/NY, which has been maintained in our laboratory for several years, and EL4/Mar, which is a variant that arose in the laboratory of Dr. P. Golstein. RDM-4 is an AKR/J thymoma cell line maintained in vitro and

This work was supported by grant 1R01 CA-29194 to T. Rajan from the National Institutes of Health, Cancer Core Grant CA-13350 and Cell Biology of Lymphoid Cells Program Project Grant AI-20702 from the National Cancer Institute, and by CNRS and INSERM. T. Potter is the recipient of a fellowship from the Cancer Research Institute, Inc. T. Rajan is a Scholar of the Leukemia Society of America.
Reactivity of Monoclonal Anti-H-2 and Anti-β2m Antibodies on EL4/NY and EL4/Mar

<table>
<thead>
<tr>
<th></th>
<th>Y-3 (Kb)</th>
<th>EH-144 (Kb)</th>
<th>28-14-8 (Db)</th>
<th>B22/249 (Dp)</th>
<th>H141/51 (Dp)</th>
<th>Lym-11 β2m</th>
<th>Clone 23 β2m</th>
</tr>
</thead>
<tbody>
<tr>
<td>EL4/NY</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>EL4/Mar</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Reactivity was assessed by microcytotoxicity using monoclonal antibodies at a concentration of 1:100 and rabbit complement at a final dilution of 1:9, as previously described (9). +++, 99% of cells dead after 60 min incubation; –, 3% of cells dead after 60 min incubation. Monoclonal antibodies were obtained as follows: B22/249 and H141/51, Dr. G. Hammerling; 28-14-8, Dr. T. Hansen; Y-3, Dr. S. Nathenson; Lym-11, Dr. U. Hammerling; clone 23, Dr. F. W. Shen.

Cytotoxic T Cell Lines and Clones. The H-2Kb-specific polyclonal T cell line (KB5) of B10.BR origin and the cytotoxic T lymphocyte (CTL) clones derived from it (KB5-B1 and KB5-C20) were established and maintained as previously described (8). H-2Dq-specific CTL clones (K4R-E and K4R-G series) were derived in a similar manner using T cells from B10.BR mice immunized with B10.A (4R) spleen cells that were restimulated in vitro every 7 d with B10.A (4R) spleen cells and cloned by limiting dilution (8).

Immunoprecipitation and Electrophoresis of Radiolabeled Surface Molecules. Labeling of cell surface molecules with 125I was performed by the lactoperoxidase-catalyzed reaction in the presence of hydrogen peroxide as described previously (9). After preclearing the membrane lysate, immunoprecipitates were prepared by adding monoclonal or xenogeneic antibodies to mouse β2m, followed by a suspension of Staphylococcus aureus. The precipitates were subjected to electrophoresis on a 10-cm 15% acrylamide slab gel as described by Laemmli (10).

Results

Serological Analysis of H-2 and β2m. The examination of the cytotoxicity of several monoclonal antibodies for the two EL4 sublines showed that these two cell lines differed in the expression of cell surface molecules (Table 1). The anti-Kb (Y-3, EH-144), anti-Db (28-14-8, H-141/51), and the two anti-β2m (Lym-11, clone 23) monoclonal antibodies were all cytotoxic for EL4/NY, whereas only the anti-Db antibodies were cytotoxic for EL4/Mar. The inability of anti-Kb and anti-β2m antibodies to kill EL4/Mar was also observed at dilutions of the antibodies lower than those presented in Table 1.

The reactivity of anti-H-2 and anti-β2m antibodies was also examined by immunofluorescence on the fluorescence-activated cell sorter (FACS). The reactivity observed was essentially the same as that observed in microcytotoxicity testing. EL4/NY reacted with anti-Kb (EH-144) (Fig. 1) anti-Db (28-14-8, H-141/51) (Fig. 1) and anti-Lym-11 (Fig. 2) antibodies; on the other hand, only the anti-Db antibodies (28-14-8, H-141/51) (Fig. 1) but not anti-Kb (Fig. 1) or anti-Lym-11 (Fig. 2) antibodies showed any reactivity with EL4/Mar. In addition, it was apparent that the level of reactivity of the Db antibodies with EL4/Mar was essentially the same as that seen with EL4/NY. Thus, by both serological criteria, microcytotoxicity and immunofluorescence, EL4/NY was Kᵇ⁺ Dᵇ⁺ Lym-11 (β2m)⁺ whereas EL4/Mar was Kᵇ⁻ Dᵇ⁻ Lym-11⁻.

Immunoprecipitation of β2m from Cell Surfaces. The failure of EL4/Mar to react with the anti-Lym-11 antibody may have been due to a mutation resulting in the loss of allotypic specificity. To test this possibility, we immunoprecipitated β2m from lysates of cells labeled with 125I, using xenogeneic as well as allogeneic antibodies. Precipitation with either the allotypic antibodies, Lym-11 or clone 23 (Fig. 3), or the xenogeneic antibody (rabbit α-β2m) (Fig. 3) to mouse β2m,
brought down β2m from EL4/NY but not from EL4/Mar lysates (Fig. 3). Since the xenogeneic antibody recognizes determinants distinct from the allotypic specificity on β2m (e.g., this antibody can precipitate β2m from both C57BL/6 and BALB/c cell lines), the failure to precipitate β2m from EL4/Mar using the xenoantiserum is strong evidence that this cell line does not express β2m on the cell surface.

Cell-mediated Cytotoxicity. The reactivity of previously characterized (8) anti-K\(^b\)- and anti-D\(^b\)-specific alloreactive CTL was also examined on the EL4 sublines. The anti-K\(^b\)-specific polyclonal (line KB5) or monoclonal (clones KB5.B1 and C20) B10.BR CTL lysed EL4/NY but not EL4/Mar (Table II). By contrast,
FIGURE 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of molecules precipitated from EL4/NY and EL4/Mar. Membrane preparations labeled with 125I were precipitated with anti-β2m antibodies, rabbit α-β2m or clone 23 (monoclonal anti-β2m); 10-2-16 is a monoclonal anti-Ia reagent used as a negative control. β2m runs slightly faster than the 12,000 dalton marker and can be identified in the third and fifth lanes only. NRS, normal rabbit serum.

TABLE II

H-2D<sup>b</sup>-specific, But Not H-2K<sup>b</sup>-specific CTL Clones Lyse the EL4/Mar Tumor Target Cells

<table>
<thead>
<tr>
<th>Effector cells*</th>
<th>Percent specific ⁵¹Cr release on target cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B6&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>CTL line KB5</td>
<td>75</td>
</tr>
<tr>
<td>Clone KB5-B1</td>
<td>70</td>
</tr>
<tr>
<td>Clone KB5-C20</td>
<td>68</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>K&lt;sub&gt;4&lt;/sub&gt;R Clones</th>
<th>B10.BR&lt;sup&gt;+&lt;/sup&gt;</th>
<th>B10.A(4R)&lt;sup&gt;+&lt;/sup&gt;</th>
<th>EL4/NY</th>
<th>EL4/Mar</th>
<th>RDM4</th>
</tr>
</thead>
<tbody>
<tr>
<td>-E1</td>
<td>4.3</td>
<td>48</td>
<td>74</td>
<td>71</td>
<td>1.1</td>
</tr>
<tr>
<td>-E4</td>
<td>2.8</td>
<td>44</td>
<td>81</td>
<td>79</td>
<td>4.2</td>
</tr>
<tr>
<td>-E8</td>
<td>7.1</td>
<td>65</td>
<td>74</td>
<td>76</td>
<td>14</td>
</tr>
<tr>
<td>-G6</td>
<td>-1.4</td>
<td>57</td>
<td>70</td>
<td>76</td>
<td>3.2</td>
</tr>
</tbody>
</table>

* Effector cells: H-2K<sup>b</sup>-specific CTL line KB5 and clones KB5-B1 and C20 have been described (8). H2D<sup>b</sup>-specific CTL clones (K<sub>4</sub>R series) are from a B10.BR anti-B10.A (4R) immunization. Results shown are for an effector to target cell ratio of 2 to 1. Cell-mediated cytotoxicity was performed and the percent specific release calculated as previously described (8).

** Effector cell: Con A-induced splenic blast cells.

# Not tested.

| Lipopolysaccharide-induced splenic blast cells. |

All CTL clones derived from a B10.BR anti-B10.A (4R) immunization (anti-D<sup>b</sup>) lysed the EL4/Mar target cells as well as EL4/NY (Table II).

Discussion

Previous studies (1–3) have demonstrated that there is an association between MHC class I heavy chains and β2m. The observations that the Daudi cell line does not express any HLA class I antigens (4) and that the R1 variant is H-2<sup>−</sup>TL<sup>−</sup> (5, 6) due to defects in β2m synthesis suggest that β2m is required for cell surface expression of class I molecules. The data presented in this paper, using several criteria for detection of surface molecules, demonstrate that on a variant of the EL4 tumor line, EL4/Mar, H-2D<sup>b</sup> is expressed on the cell surface in the absence of detectable β2m. In the human MHC, results of sequential immuno-
precipitation of HLA molecules with monoclonal antibodies directed against the HLA heavy chain or β2m suggested that a subset of cell surface HLA heavy chains is not associated with β2m (11). Thus, the expression of H-2Kb on EL4/Mar in the absence of β2m, while novel for the murine system, may not be entirely unique for MHC class I antigens.

The failure to express both Kβ and β2m on the cell surface could be due to a primary defect either in β2m or Kβ synthesis; however, it is clear that if there is defect in β2m synthesis this does not affect Dβ expression. If the defect actually resides in Kβ heavy chain synthesis and β2m is produced, the failure to express β2m on the cell surface in association with H-2Dβ raises the possibility that there is no association between Dβ and β2m, and that H-2Dβ does not require β2m for membrane insertion. Alternatively, it may be that H-2Dβ is inserted in association with β2m but the complex dissociates soon after the appearance of the antigen on the cell membrane. In either case, it would seem that the association of H-2Dβ with β2m is more tenuous than that of other class I antigens. It is of interest to note that in contrast to Kβ, Kk, and Dd, which have two carbohydrate side chains, there are three associated with the Dβ molecule (12). This additional glycosylation site is on the C2 domain of the molecule, which has been suggested to be the β2m-binding region (13). In addition, precipitation with anti-Dβ brings down very little β2m compared with precipitation with anti-Kβ (14). It is therefore tempting to speculate that the third carbohydrate chain may replace β2m on Dβ.

It is noteworthy that the β2m-free Dβ molecule expressed by EL4/Mar could be detected by the monoclonal antibody 28-14-8. The epitope recognized by this antibody has been mapped to the C2 domain of H-2Ld (15). As noted above, this is also the domain that has been suggested to be the β2m-binding domain (13). Whether the additional carbohydrate group present in the C2 domains of the Dβ and Ld molecules influences β2m association is not known.

The results presented in this report may have some relevance to the attempts of groups studying the nature of the many class I genes in the murine MHC. One assay involves the transfection of an unknown class I gene into LTK− cells, which are then evaluated for increased expression of β2m (16). The failure of the transfected gene to cause an increase in β2m expression may be interpreted to mean that the gene is a “pseudogene” or otherwise unable to direct synthesis of a cell surface class I protein. The fact that H-2Dβ (this paper) and some human class I gene products (11) can be expressed on the cell surface without detectable β2m raises the possibility that other murine class I antigens may also exhibit this property. Therefore, a negative result in the β2m assay (16) must be interpreted with caution.

Summary

In this report we describe a variant of the C57BL/6 T lymphoma EL4 (EL4/Mar) which, in contrast to the parental cell line, expresses neither H-2Kb nor β2m-microglobulin (β2m) but which does express H-2Dβ detectable by serology and by alloreactive cytotoxic T lymphocytes (CTL). This observation raises the possibility that H-2Dβ and perhaps other major histocompatibility complex class I molecules are normally not associated with β2m on the cell surface. In addition, this report is the first to indicate that alloreactive CTL can interact with a β2m-free class I antigen.
We are indebted to Drs. U. Hammerling, G. Hammerling, F. W. Shen, T. Hansen, S. Nathenson, and E. Appella for generously providing the monoclonal antibodies and antisera used in this study. We also thank Ms. Kathy Meringolo, M. F. Luciani, and M. Buferne for excellent technical help. In addition we thank Ms. Rosina Passela for secretarial assistance in the preparation of this manuscript.

Received for publication 21 February 1984 and in revised form 19 March 1984.

References