Biochemical characterization of activation-associated bovine class I major histocompatibility complex antigens

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Summary. Utilizing a 'sandwich' ELISA assay we have been able to demonstrate that mAb W6/32, B1G6 and IL-A19 are reactive with three different monomorphic determinants on bovine class I major histocompatibility complex (MHC) molecules. Sequential immunoprecipitations performed with the mAb revealed that class I molecules on PBM comprise a single population with respect to reactivity with the mAb in that the β2m-associated proteins bear all three epitopes. By contrast, TCGF-driven lymphoblasts and cells transformed by Theileria parva (Tp) additionally express molecules of Mr 45000 bound to β2m which are recognized by mAb B1G6 and IL-A19 but not by W6/32. These two subclasses of molecules were further distinguished on the basis that, when tunicamycin was added to cultures in the preparation of cells for analysis, mAb W6/32 precipitated class I heavy chains of Mr 39000 while the extra molecules detected only by mAb B1G6 and IL-A19 were of Mr 37000 and 39000. On thymocytes, the mAb W6/32-non-reactive class I molecules are present in low amounts and are expressed by cells in the medulla area, unlike BoT1 (analogous to human CD1) molecules which are expressed by the cortical cells. Our studies also revealed that the supposed β2m-specific mAb B1G6 does not recognize the β2m-associated molecules (BoT1) precipitated by mAb TH97A and thus the specificity of mAb B1G6 in cattle is for an epitope on bovine β2m which is strongly influenced by the nature of the heavy chain with which the β2m is associated.

Keywords: monoclonal antibodies, sandwich ELISA, sequential immunoprecipitations, class I MHC antigens

Introduction

Monoclonal antibodies (mAb) W6/32 and B1G6, directed against HLA-A, B and C products and human β2-microglobulin (β2m) respectively, have been invaluable tools in the definition of class I major histocompatibility complex (MHC) antigens in cattle (Brodsky et al. 1981; Chardon et al. 1983). As previously described, mAb W6/32 and B1G6 detect the same set of at least five BoLA class I molecules expressed at the surface of bovine peripheral blood mononuclear cells (PBM) derived from a
heterozygous animal (Bensaid et al. 1988). Here we describe studies utilizing mAb IL-A19, detecting a monomorphic epitope of BoLA class I proteins, and mAb TH97A, defining BoT1 molecules analogous to human CD1 (MacHugh et al. 1988). We have compared, with histo- and immunochemical techniques, the properties of the molecules carrying the determinants recognized by these mAb with those of the molecules carrying determinants for the cross-reacting mAb W6/32 and B1G6 in order to gain further insights into the diversity, structure and expression of class I MHC molecules in cattle.

Materials and methods

Animals
A 3-month-old Boran (Bos indicus) calf was used as a donor of thymus cells. Peripheral blood mononuclear cells were collected from a 4-year-old Boran steer.

Monoclonal antibodies
MAb W6/32 (IgG2a) recognizes an epitope on all HLA-A, B and C heavy chains associated with β2m (Barnstable et al. 1978; Parham et al. 1979). The mAb B1G6 (IgG2b) is directed against human β2m and recognizes this molecule both free and complexed with HLA heavy chains (Liabeuf et al. 1981). B1G6 has also been found to be reactive with bovine β2m (Chardon et al. 1983).

The mAb IL-A19 (Ig2a) was derived in our laboratory from a hybridoma produced by fusion of the spleen cells of a Balb/c mouse with myeloma X63.Ag. Prior to fusion, the mouse had received multiple inoculations with PBM from B. indicus and B. taurus animals. Hybridoma selection and cloning was by standard methods. This mAb reacts with an epitope present on the surface of PBM of all cattle studied (more than 2000). The reactivity of mAb TH97A (IgG2a) has been described previously (MacHugh et al. 1988). It recognizes a molecule in cattle analogous to human CD1. Throughout this study, the IgG2a myeloma mAb UPC10 (Bionetics, Karsington, Maryland) was used as a negative control antibody and mAb J5 (IgG2a) (Naessens et al. 1985), which is not reactive with bovine cells following solubilization in detergent, was used as a preclearing antibody.

Purification of mAb
Ascites containing mAb were centrifuged at 10000 g for 15 min. To 1 ml of swollen packed protein A-sepharose (Pharmacia, Uppsala, Sweden) (PrA-S), 1 ml of ascites was added. After 2 h incubation at 4°C, the PrA-S with bound mAb was washed. When used for immunoprecipitations washing was three times in NET1 [50 mM Tris-HCl pH 7.4, 5 mM EDTA, 0.5 M NaCl, 1 mg/ml bovine serum albumin (BSA), 0.5% NP40] followed by three times in NET3 [50 mM Tris-HCl pH 7.4, 5 mM EDTA, 0.15 M NaCl, 0.5% NP40]. The washed material was then resuspended at 50% v/v in NET3. When used for biotinylation, mAb-PrA-S was washed three times in NET1 without detergent and BSA and three times in NET3 without detergent.
Elutions of mAb from the PrA-S were performed with 1 ml of 0·1 M glycine/HCl pH 2·5. Eluates were neutralized to pH 7·5 with a 5% NaHCO₃ solution prior to dialysis against phosphate buffered saline (PBS) pH 7·4.

_Biotinylation of mAb_  
To a solution of purified mAb at 1 mg/ml in PBS pH 7·4, 150 µg of NHS-biotin (Pierce, Rockford, Illinois, USA) were added and incubated with constant shaking for 1 h at room temperature. Following biotinylation, mAb were diluted to 0·5 mg/ml, dialysed for 24 h against PBS and then stored at 4°C with sodium azide (added to a final concentration of 0·1% w/v).

_Preparation of PBM_  
PBM were prepared from jugular venous blood collected into an equal volume of Alsever's solution by centrifugation on Ficoll-Paque (Pharmacia) at 800 g for 30 min at room temperature. Cells harvested from the interface were washed three times in PBS.

_Preparation of TCGF-driven lymphoblasts_  
TCGF-driven lymphoblasts were prepared as described by Brown & Grab (1985). Cultures were maintained for more than 2 months prior to biochemical analysis.

_Preparation of T. parva-transformed cells_  
PBM were infected and transformed _in vitro_ by the method of Stagg _et al._ (1981), with sporozoites of _Theileria parva_ obtained from the salivary glands of infected _Rhipicephalus appendiculatus_ ticks. The resulting parasitized cell lines were maintained in RPMI 1640 (Gibco, Paisley, UK) containing 10% foetal bovine serum (FBS). Such cell lines may be maintained indefinitely in culture and, in most cases, without changes in their BoLA-A phenotype (Teale _et al._ 1985).

_Preparation of thymocytes_  
Cell suspensions of bovine thymus were prepared by gently flushing small pieces of thymus with RPMI 1640 medium containing 10 µg/ml deoxyribonuclease (Sigma, St Louis, Missouri, USA) and 10 iu/ml heparin. Cells were then washed in PBS before radio-iodination.

_Radio-iodination of cells_  
Cells washed in PBS were surface-labelled with Na¹²⁵I (Amersham International, UK) by the lactoperoxidase (Sigma)-catalysed reaction in the presence of hydrogen peroxide, as described previously (Cone & Marchalonis 1974). The reaction was terminated by washing the cells twice in PBS containing 0·1% w/v sodium azide.
Metabolic labelling of activated cells

TCGF-driven lymphoblasts and Tp-transformed cells were washed once in leucine-free RPMI 1640 growth medium containing 10% dialysed FBS. Cells were suspended at 10^6/ml in leucine-free medium. Concanavalin A (Type V, Sigma) was added to TCGF-driven lymphoblasts at a concentration of 2.5 μg/ml. Cultures were preincubated for 1h at 37°C in 5% CO_2 in air, then ^14^C-leucine (Amersham) was added to a concentration of 50 μCi/ml. Cultures were maintained for a further period of 16h at 37°C in 5% CO_2 in air, following which cells were washed twice in PBS containing 0.1% sodium azide. In some experiments, tunicamycin (Sigma) was added to 1 μg/ml. Tunicamycin fraction A was repurified by HPLC according to the method of Mahoney & Duksin (1980).

Detergent solubilization of cells

Cells were lysed for 45 min at 10^7/ml in lysis buffer [10 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.15 M NaCl, 5 mM iodoacetamide, 40 μg/ml leupeptin (CRB, Cambridgeshire, UK), 2.5 μg/ml antipain (CRB), 1 mM TLCK, 1 mM PMSF, 5 μg/ml aprotinin (Boehringer, Mannheim, FDR) and either 0.5% NP40 or 0.5% TX114 (Sigma)]. After solubilization, lysates were centrifuged for 15 min at 10000g. When used for sandwich ELISA studies, T. parva-infected cells were solubilized at 10^8/ml in lysis buffer with 0.5% NP40 for 1h. Samples lysed with TX114 were treated as described by Bordier (1981). Briefly, in an Eppendorf 1.5-ml tube, 500 μl of the detergent lysate was overlaid on 250 μl of lysis buffer containing 10% (w/v) sucrose but without detergent. Tubes were warmed for 5 min at 37°C and centrifuged for 5 min at 250g at room temperature. The pellet (50 μl) containing integral or strongly associated membrane proteins was brought to 500 μl with lysis buffer containing 0.5% NP40.

Sandwich ELISA

The test was performed in 96-well flat-bottomed ELISA plates (Gibco). Purified mAb (20 μg in 100 μl of PBS) were dispensed into each well and plates incubated overnight at 4°C. Wells were then blocked with 200 μl of 5% BSA in PBS for 4h at room temperature or overnight at 4°C and then washed three times with PBS. Fifty μl of detergent-solubilized cells were added to each well and incubated for 1h. After three washes with PBS containing 0.1% Tween 20 (Sigma), 2.5 μg of the appropriate biotinylated mAb in 50–100 μl of PBS/0.1% Tween 20 was added to each well. Following 1h incubation at 4°C and three washes with PBS/0.1% Tween 20, 50 μl of streptavidin-lactoperoxidase complex (Amersham) in PBS/0.1% Tween 20/1% BSA were added to each well and the plates were then incubated for 1h at 4°C. Wells were washed three times in PBS/0.1% Tween 20 prior to dispensing 100 μl/well of ABTS substrate (Amersham). Optical densities at 412 nm were determined with an ELISA reader after 15–30 min.
**Immunoprecipitations**

Immunoprecipitations were performed with mAb preabsorbed on PrA-S. Each detergent lysate (500 μl) was incubated for 45 min at 4°C with 50–100 μl of packed PrA-S with preabsorbed mAb J5 (J5-PrA-S). After a 5-min centrifugation at 10000 g, supernatants were cleared again with the same amount of PrA-S. This cycle of preclearing was repeated once before the addition to the lysates of the relevant or control mAb-PrA-S. Following incubation for 1 h, antigen-mAb complexes were centrifuged and washed three times with NET1, once with NET2 [50 mM Tris-HCl pH 7.4, 5 mM EDTA, 0.25 M NaCl, 0.1% NaN₃] and three times with NET3.

**Sequential immunodepletions**

Several rounds of precipitation, as described above, were performed on the same lysate aliquot with a given mAb prior to incubation with a second mAb. When 125I-proteins were immunoprecipitated, immune complexes bound to PrA-S were counted in a gamma counter during the last wash. Successive additions (three to six times) of the same antibody were made until the radioactivity in the pellet equalled that obtained in negative controls. The second antibody was then added, beginning a new cycle. For 14C-labelled lysates, the same procedure was followed but a small aliquot of sepharose from the final wash was dissolved in Aquasol (New England Nuclear, Boston, Massachusetts, USA) and counted in a β scintillation counter.

**Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)**

PrA-S pellets were heated to 90°C for 5 min with 35–50 μl of sample buffer [180 mM Tris-HCl pH 6.8, 6% (w/v) SDS, 22.5% glycerol, 15% (v/v) 2-mercaptoethanol and 0.6% (w/v) bromophenol blue]. For some precipitates, sample buffer without 2-mercaptoethanol was used. Each sample was loaded on the top of a straight (12.5%) or a gradient (7.5–17.5%) polyacrylamide gel containing 0.1% SDS (Maizel 1971). A mixture of 14C-methylated proteins (Amersham) provided molecular mass markers. When precipitates labelled with 14C were analysed, gels were treated for fluorography (Chamberlain 1979). Dried gels were applied to Fuji RX films for fluorography or autoradiography.

**Histochemistry**

Cryostat sections (6–8 μm) of bovine thymus were prepared as described elsewhere (MacHugh *et al.* 1988). After fixation for 5 min in acetone, sections were stained by the indirect immunoperoxidase technique with an anti-mouse Ig conjugated with peroxidase (Serotec, Blackthorn, UK). The dianinobenzidine reaction was enhanced by exposing the sections to 1% osmium tetroxide for 30 s and the contrast was further improved for photography by using a 490-nm interference filter.
Table I. Molecular mapping of BoLA class I monomorphic epitopes. Numbers indicate OD readings obtained in a sandwich ELISA of class I molecules of NP40-solubilized Tp-transformed cells

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<th>Second biotinylated mAb layer</th>
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<td>B1G6</td>
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<td>IL-A19</td>
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Results

Molecular mapping of BoLA class I monomorphic epitopes
The results of sandwich ELISA studies are shown in Table 1. When mAb W6/32 was first coated onto plastic wells and incubated with a lysate of Tp-transformed cells, no binding of second biotinylated mAb IL-A19 or B1G6 could be detected. Negative results were also obtained in all cases when first and second mAb were identical. However, plastic wells in which mAb B1G6 or IL-A19 were fixed showed a positive reaction when further incubated with biotinylated mAb W6/32 or IL-A19 and W6/32 or B1G6, respectively. Highest optical density readings were recorded when mAb IL-A19 was used as a first layer antibody. These data indicate that the three mAb recognize three distinct epitopes localized on at least one identical set of BoLA class I molecules. Furthermore, using lysates of surface-radio-iodinated cells and SDS-PAGE analysis of captured material, we were able to determine that negativity in the wells in which mAb W6/32 formed a first layer was not due to failure to catch the specific antigen (result not shown).

Identification of BoLA 'class I-related' molecules appearing upon cell activation
Sequential immunoprecipitations were performed as previously described (Krangel et al. 1978; Bensaid et al. 1988). All cells were surface radio-iodinated. TCGF-driven lymphoblasts and Tp-transformed cells were also internally labelled with 14C-leucine.

Following depletion of mAb W6/32-reactive molecules present on 125I-PBM lysates (Fig. 1, lanes B and C), a further incubation of mAb B1G6 (Fig. 1, lane E) did not reveal a further population of molecules, while mAb IL-A19 precipitated only very small amounts of polypeptides of Mr 45000 associated with β2m (Fig. 1, lane D). Molecules not removed by mAb W6/32, but reactive with mAb IL-A19, represented less than 10% of the total proteins binding to mAb W6/32. Thus, on PBM, a single set of molecules carries the epitopes defined by mAb W6/32, B1G6 and IL-A19. The same experiments were performed with TCGF-driven lymphoblasts (Fig. 2) and Tp-transformed cells (Fig. 3). After molecules binding to mAb W6/32 were removed from lysates of 125I-labelled material (Fig. 2a, lanes B and C; Fig. 3a, lanes B and C
BoLA activation molecules

Figure 1. Cell surface radio-iodinated PBM were solubilized in NP40 detergent. After preclearing with irrelevant mAb J5, the cell lysate was divided into two aliquots in which were respectively incubated a control mAb UPC10 (lane A) and mAb W6/32 (lane B), both previously absorbed on PrA-S. Clearing of mAb W6/32-reactive molecules required three consecutive rounds of precipitation with W6/32-PrA-S (lane C). This lysate was then divided into two aliquots in which were respectively incubated IL-A19-PrA-S (lane D) and B1G6-PrA-S (lane E). Samples were run in a 12.5% polyacrylamide gel. Marker protein relative masses are indicated to the left of lane A: ovalbumin, 45000; carbonic anhydrase, 31000; lysozyme, 14000.

and Fig. 3b, lanes D and E), consecutive immunoprecipitations performed with mAb B1G6 (Fig. 2a, lane D and Fig. 3a, lane D) or with mAb IL-A19 (Fig. 3b, lane F) revealed a substantial population of dimeric molecules analogous to class I MHC antigens. When lysates were depleted of mAb IL-A19-reactive molecules (Fig. 3b, lanes G and H), nothing could be detected following precipitation with mAb W6/32 and B1G6 (Fig. 3b, lanes I and K).

The existence of BoLA ‘class I-like’ molecules not detected by mAb W6/32 was confirmed by using TCGF-driven lymphoblasts and Tp-transformed cells metabolically labelled with \(^{14}\)C-leucine. Invariably, on both cell lysates, following depletion of mAb W6/32 binding molecules (Fig. 2c and 3c, lanes B and C), incubation and precipitation with mAb B1G6 revealed large amounts of \(\beta_2m\)-associated molecules of Mr 45000 (Fig. 2c and 3c, lane D).

In order to further characterize the diversity of these ‘class I-like’ molecules, \(^{125}\)I-labelled lysates from TCGF-driven cells and Tp-transformed lymphoblasts were immunodepleted with mAb B1G6 (Fig. 2b, lanes B and C, Fig. 3b, lanes A and B) prior to precipitation with mAb IL-A19 (Fig. 2b, lane D, Fig. 3b, lane C). In each case, mAb B1G6 left Mr 45000/12000 dimers detected by mAb IL-A19. On Tp-transformed cells, the remaining molecules binding to IL-A19 were in sufficient amounts to suspect that these ‘class I-like’ antigens are in fact composed of two subpopulations only one of which reacts with IL-A19. The same experiment (Fig. 3d) performed on internally labelled Tp-transformed cells did not confirm these results, however.
Figure 2. In two independent cell surface radio-iodinations of TCGF-driven lymphoblasts, NP40 lysates were precleared and each divided into two aliquots.

(a) In the first, mAb UPC10 was used as a control (lane A) and precipitation was with W6/32-PrA-S (lane B). After four immunoprecipitations with this mAb (lane C), B1G6-PrA-S was incubated in the same lysate (lane D). Samples were run under reducing conditions in a 12.5% polyacrylamide gel.

(b) In the second aliquot, UPC10 was used as a control (lane A). In lane B is shown the results of immunoprecipitation with B1G6-PrA-S. Further clearing of molecules reacting with mAb B1G6 was achieved by five consecutive rounds of precipitation (lane C). To the same lysate was then added IL-A19-PrA-S (lane D). Samples prepared in the presence of 2-mercaptoethanol were run in a 12.5% polyacrylamide gel. Lanes A and B represent the autoradiograph obtained following exposure for 16h while for lanes C and D the films were exposed for 3 days. Note in lane C the absence of β2m.

(c) Bovine TCGF-driven lymphoblasts were metabolically labelled with 14C-leucine. After solubilization in TX114 and membrane protein extraction, the lysate was divided into two samples. In the first sample mAb UPC10 was used as a control (lane A). The second sample was incubated with W6/32-PrA-S (lane B). Following four immunoprecipitations with W6/32-PrA-S (lane C), B1G6-PrA-S was added (lane D). Immunoprecipitates were prepared in reducing conditions and run in a 7.5%-17.5% gradient gel.

Biochemical differences between ‘classical’ BoLA class I molecules and class I activation antigens

To further investigate the biochemical properties of these ‘class I-like’ antigens, TCGF-driven lymphoblasts and Tp-transformed cells were grown in the presence of tunicamycin, which inhibits the attachment of N-linked glycosyls to proteins. Lysates were depleted of mAb W6/32-reactive molecules (Fig. 4c, lanes C, D, H and G) and then precipitations were performed with mAb B1G6 (Fig. 4b, lanes E and F). While heavy chains bound to mAb W6/32 were resolved at Mr 39000, molecules further precipitated with mAb B1G6 were distinct at Mr 37000 and 39000.

Class I activation antigens reactive with mAb B1G6 are not BoTI-related molecules

MAb TH97A, which detects a bovine CD1-like antigen, has been found to be reactive with cortical thymocytes and dendritic cells but not with Tp-transformed cells (MacHugh et al. 1988). Monoclonal antibody TH97A was compared to the other antibodies, and in particular to mAb B1G6, which has been described previously as specific for bovine β2m (Chardon et al. 1983). As shown in Fig. 5a, 125I-thymocyte lysates were depleted of mAb W6/32-reactive molecules (lanes B and C) and then incubated with mAb B1G6 (lane D) which precipitated Mr 45000
Figure 3. Sequential immunoprecipitations performed on Tp-transformed cells.

(a) Transformed lymphocytes were cell surface radio-iodinated, lysed with NP40 and divided into two aliquots. In lane A, mAb UPC10 was used as a control. In lane B is the result of an immunoprecipitation with W6/32-PrA-S in the second aliquot. Lanes C and D represent respectively the last cycle of precipitation with W6/32-PrA-S and the incubation of mAb B1G6-PrA-S on the same lysate.

(b) NP40 lysates of cells surface radio-iodinated Tp-transformed cells were divided into three samples (lanes A–C, D–F and G–K). Lanes A, D and G are the results of direct immunoprecipitations performed with B1G6-PrA-S, W6/32-PrA-S and IL-A19-PrA-S, respectively. Lysates were cleared with 5 to 6 rounds of precipitations with B1G6-PrA-S (lane B), W6/32-PrA-S (lane E) and IL-A19-PrA-S (lane H). Then, to the cleared samples were added IL-A19-PrA-S (lanes C and F) and W6/32-PrA-S (lane I). The third aliquot in which mAb IL-A19-reactive molecules were removed was further treated with another cycle of precipitation with W6/32-PrA-S (lane J) prior to addition of B1G6-PrA-S (lane K). All immunoprecipitates were prepared under reducing conditions and run in a 12.5% polyacrylamide gel.

(c) Sequential immunodepletions were performed on TX114 lysates of 14C-leucine labelled Tp-transformed cells. MAb UPC10 was used as a negative control (lane A). MAb W6/32 reactive molecules (lane B) were removed (lane C), following which, B1G6-PrA-S was added to the lysate (lane D).

(d) Two aliquots of 14C-leucine-labelled membrane proteins from Tp-transformed cells were respectively subjected to immunoprecipitation with the UPC10-PrA-S (lane A) and B1G6-PrA-S (lane B). After four cycles of immunoprecipitations with B1G6-PrA-S, the last cycle being analysed in lane C, IL-A19-PrA-S was added (lane D). For gels represented in c and d, samples were treated with 2-mercaptoethanol and run in a 12.5% polyacrylamide gel.
Figure 4. TCGF-driven lymphoblasts (lanes B, C, D and E) and Tp-transformed cells (lanes F, G and H) were labelled with \textsuperscript{14}C-leucine in the presence of tunicamycin and lysed in TX114. Sequential immunoprecipitations were performed with W6/32-PrA-S and B1G6-PrA-S. In lanes C and H, W6/32-PrA-S was first reagent. After the last incubation with W6/32-PrA-S (lanes D and G), mAb B1G6 was applied (lanes E and F). In lane B, TCGF-driven lymphoblasts labelled without tunicamycin were reacted with W6/32-PrA-S. Lane A shows the results of the reaction of control mAb UPC10 with a mixture of labelled TCGF-stimulated lymphoblasts and Tp-transformed cells. Only heavy chains are shown. The arrow indicates a Mr 37000 product recognized by mAb B1G6.

Figure 5. Cell surface radio-iodinated thymocytes were lysed in NP40 and subjected to sequential immunoprecipitations. Lanes A are in both cases results of control precipitations with UPC10-PrA-S.

(a) The lysate was divided into two aliquots. Lanes B and C are the results of the first and last cycle of precipitations performed with W6/32-PrA-S on the first aliquot. B1G6-PrA-S was then added (lane D). Molecules precipitated by B1G6-PrA-S in a second lysate are analysed in lane E. After immunodepletion with B1G6-PrA-S (lane F), W6/32-PrA-S was added (lane G). Immunoprecipitates were treated under reducing conditions and run in a 12.5% polyacrylamide gel.

(b) The thymocyte lysate was divided into two aliquots. Lanes B and C are the results of the first and last cycles of precipitation performed with B1G6-PrA-S on the first aliquot. Subsequently, TH97A-PrA-S (lane D) as incubated in the same lysate. In the second aliquot, molecules reactive with mAb TH97A were precipitated (lane E). Samples were boiled without 2-mercaptoethanol and run in a 12.5% polyacrylamide gel.
proteins associated with $\beta_{2m}$. In another lysate, class I molecules were removed with mAb B1G6 (lanes E and F). In this case in addition to a major Mr 45000 protein, small amounts of Mr 42000 products were apparent. Subsequently, lysates were incubated with mAb W6/32 (lane G). As observed on PBM, there are no class I or class I-like molecules bearing only the mAb W6/32-defined epitope. The same immunodepletion experiment was performed (Fig. 5a) but using as a first step mAb B1G6 (lanes B and C) and as second step, mAb TH97A (lane D). Samples were run under non-reducing conditions. While proteins reacting with mAb B1G6 were resolved as a broad band spanning Mr 42000 to 45000 (lanes B and C), mAb TH97A precipitated heavy chains of Mr 47000 (lane D). Surprisingly, the BoT1 heavy chain was co-precipitated with a light chain of Mr 12000 migrating in an identical manner to $\beta_{2m}$.

To address the question of which type of cells are recognized by the different mAb, thin tissue sections of thymocytes were probed with all four mAb presented in this study. Medullary thymocytes were stained by mAb W6/32 (Fig. 6a), B1G6 (Fig. 6b) and IL-A19 (Fig. 6c) while mAb TH97A reacted only with cortical thymocytes (Fig. 6d). We conclude that mAb B1G6 does not react with all molecules associated with $\beta_{2m}$ and furthermore, does not apparently recognize a BoT1 subset.

Discussion

The ‘sandwich’ ELISA is a sensitive technique which provides information on the epitope composition of molecules. However, negative results are not always informative. They may be due to an inability of the first mAb layer to catch antigens. However, we were able to show that all three mAb, W6/32, B1G6 and IL-A19, when bound to plastic wells, were efficient in binding BoLA class I molecules in the assay.
A. Bensaid et al.

Another possibility is the destruction of the antigen binding site of a second layer antibody during the biotinylation procedure. For this reason, all biotinylated mAb were assessed in cell surface binding assays in which reactivity was detected with anti-mouse Ig reagents and a fluorescence-activated cell sorter. As a general rule, when compared to the non-treated mAb, biotinylated mAb had a decreased reactivity but they were never negative or ‘weak’. With this knowledge we could justifiably conclude that the failure of mAb B1G6 and IL-A19 to recognize the molecules captured by mAb W6/32 is due to conformational changes induced by binding to mAb W6/32. This is consistent with the ability of mAb W6/32 to inhibit a wide range of alloreactive and MHC-restricted cytotoxic T cells (Goddeeris et al. 1986; Teale et al. 1986). Since mAb W6/32 reacts with proteins bound either to mAb B1G6 or IL-A19 and combinations of the latter two mAb give positive sandwich ELISAs, we conclude that the three mAb define different monomorphic epitopes clustered on a single set of BoLA class I molecules.

Sequential immunodepletion is a useful method for distinguishing different molecules co-expressed on the same cell. For cell surface proteins it is an alternative to the co-capping technique which is subject to many criticisms. It is important with this technique, however, that the affinities of mAb for the antigens studied do not differ dramatically. If, on PBM, differences in affinity may explain the remaining small quantities of mAb IL-A19-reactive molecules following immunodepletion with mAb W6/32 and B1G6, such an interpretation is not possible when considering results obtained with proliferating cells. Indeed, β2m-associated molecules remaining in lysates following W6/32 depletion, and recognized by mAb IL-A19 and B1G6, are in at least equal amounts to those cleared by mAb W6/32. This phenomenon is enhanced by a factor of 3 to 4 when cells are internally labelled. This suggests that both expression and turn-over of proteins precipitated by mAb IL-A19 and B1G6, but unreactive with mAb W6/32, are favoured in activated cell populations.

When mAb B1G6 and IL-A19 were compared in sequential immunodepletions, paradoxical results were obtained. Thus, both in TCGF-driven lymphoblasts, and in particular, in parasitized lymphocytes, surface labelled with 125I, mAb B1G6 left a significant population of molecules to be precipitated by mAb IL-A19, whereas mAb B1G6 did not precipitate proteins following immunodepletion with IL-A19. However, when parasitized cells were internally labelled, only a very small population of mAb IL-A19-reactive molecules was detected following immunodepletion with mAb B1G6. Indeed, these 14C-leucine labelled molecules were only detected in the autoradiograph after 2 weeks of exposure while the same radio-iodinated molecules were detected after just a few days. In the light of such an apparent contradiction, caution has to be exercised before concluding that there is a population of β2m-associated molecules lacking the epitope defined by mAb B1G6. This apparent paradox could be the result of technical factors, such as differences in affinity of antibodies and/or sensitivity of the X-ray film to gamma and beta radiation.

Definitive proof of the existence of class I-related activation molecules expressed on TCGF-activated and Tp-transformed cells was provided by inhibiting glyco-
BoLA activation molecules

lation with tunicamycin. Molecules binding to mAb W6/32 were of Mr 39000 while additional molecules precipitated by mAb B1G6 were of Mr 39000 and 37000. This result suggests that there are two populations of 'class I-related' antigens, assuming that all glycosyls are N-linked.

To investigate further whether these molecules are equivalent to the human CD1 or mouse 'TL antigens, thymocytes were tested. Results were clear and revealed that 'class I-related' antigens are expressed only on medullary thymocytes and have equal levels of expression to BoLA class I molecules whereas BoT1 molecules were detected on cortical thymocytes. Furthermore, BoT1 heavy chains were of Mr 47000 while heavy chains precipitated by mAb W6/32 and B1G6 were of Mr 44000 to 45000. These results also show that B1G6 does not recognize β2m associated with the BoT1 heavy chain. Therefore, it is likely that the BoT1 heavy chain influences the conformation of β2m to which it is associated in such a manner that mAb B1G6 becomes unreactive with these heterodimers. MAb B1G6 reacts with a human cortical thymocyte molecule (D47) belonging to CD1 (Khan-Perles et al. 1985), therefore bovine 'class I-related' antigens are unlikely to be strictly analogous to D47 molecules. In the mouse, rat and guinea pig, molecules of Mr 43000 to 41000 associated with β2m but different from H-2 and TL antigens have been described and named Qa (reviewed by Harris et al. 1984; Widacki et al. 1985). One of their main features is expression on peripheral T cells. Furthermore, Qa molecules have a protein backbone smaller than H-2 or TL antigens. In this respect, the bovine glycoproteins that we describe here resemble Qa antigens since they had a smaller protein backbone size than classical BoLA class I products, although they have a different tissue distribution and very low, if any, expression on PBM. Studies of human material have failed so far to demonstrate definitively molecules analogous to Qa. However, Gazit et al. (1984) described an alloantiseraum which did not react with human PBM but was reactive with mitogen-stimulated PBM. They assumed that the antigens detected by this reagent were analogous to Qa molecules. The additional β2m-associated molecules described here, which are expressed at high levels on activated cells, may be analogous to those described by Gazit et al. (1984).

In summary, β2m-associated glycoproteins of Mr 45000, distinct from classical BoLA class I or BoT1 products, are detected on medullary thymocytes, mitogen-activated and Tp-transformed lymphoblasts of cattle.

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