Impaired antigen presentation by murine I-A\textsuperscript{d} class II MHC molecules expressed in normal and HLA-DM-defective human B cell lines

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Abstract

The inability of certain antigen processing mutant cell lines to present intact proteins to T cells and to form SDS-stable MHC class II dimers has been shown to result from defective expression of HLA-encoded DMA and DMB genes. We have utilized some of these mutants to determine species compatibility of antigen presentation components. Mouse MHC class II I-A\textsuperscript{d} cDNA was transfected into the human B cell lymphoblastoid cell lines 8.1.6, 7.9.6 (a mutant cell line derived from 8.1.6) and an independent deletion mutant T2 (called 8.1.6d, 7.9.6d and T2.d respectively). These cells were then examined for various functions in antigen presentation. Interestingly, none of the cells transfected with I-A\textsuperscript{d} presented peptides derived from intact proteins to specific T cell hybridomas. However, presentation of synthetic peptides by these cells was normal. The ability to form SDS-stable dimers was dramatically reduced in the transfectants. In addition, I-A\textsuperscript{d} molecules at the cell surface appeared loaded predominantly with the invariant chain peptides, CLIP. These properties of the I-A\textsuperscript{d} transfectants are identical to those described for HLA class II molecules expressed in HLA-DM mutants. Perhaps the most interesting finding was the inability of I-A\textsuperscript{d} in 8.1.6 to present protein antigens. Since 8.1.6 cells present antigens to HLA-DR, DP, DO-restricted T cells and also have intact HLA-DM and invariant chain (Ii) functions, these results argue that some component of human antigen processing machinery is incompatible with I-A\textsuperscript{d} molecules.

Introduction

MHC class II molecules present peptides derived from an exogenous source to T cells of helper subtypes (1). However, before presentation of peptides to T cells, the internalized protein antigens require a great deal of intracellular processing within the antigen-presenting cells (APC). MHC class II molecules are synthesized within the endoplasmic reticulum where the binding groove of the MHC class II becomes blocked by a trimeric form of invariant chain (Ii) (2,3). This event ensures that the majority of MHC class II molecules do not bind endogenous peptides that are destined for MHC class I molecules. MHC class II and Ii together migrate via the Golgi apparatus to endocytic compartments (4–6) where Ii is proteolytically cleaved with class II molecules still associated with class II-associated Ii peptides (CLIP) (9–13). MHC molecules become receptive for binding peptides derived from endocytosed proteins once CLIP have been removed in a process that for some alleles appears to require the assistance of the gene products of the DM locus (14–16). Mutant B lymphoblastoid cell lines such as 7.9.6 (17,18) and T2 (13) lack the DM function required for normal presentation
I-A<sup>d</sup>-transfected human B cell lines are defective in antigen presentation

of exogenous protein antigens to class II-restricted T cells. Cell lines carrying defective DM genes fail to present intact antigens (17,18), lose expression of certain mAb (e.g. 16.23) epitopes (17–19), fail to form SDS-stable αβ dimers and express HLA class II molecules that are predominantly occupied with CLIP (11,13). Recent studies have provided convincing evidence that the transfection of functional copies of DM genes into these cell lines restores a normal antigen presentation phenotype (19,20).

Exactly how DM perform their function in MHC class II-mediated antigen presentation is still being debated. However, recent studies have indicated that the primary function of DM appears to be in catalysing CLIP removal from class II molecules (14–16). This process appears to require direct contact with MHC class II molecules (15). A possibility also exists whereby DM molecules may retain empty class II molecules in early MIC compartments until a suitable peptide has become bound to class II molecules. This class II–DM association may also prevent aggregation of empty class II molecules and rescue class II molecules from being degraded.

Our aim is to determine whether there is a species incompatibility between certain MHC class II molecules and antigen processing machinery for efficient antigen presentation by MHC class II molecules. To answer this, we have utilized certain well-characterized human cell lines, 8.1.6, 7.9.6 (17,18) and T2 (13,21), that are either normal or mutant in their ability to present antigens to HLA class II-restricted T cell clones respectively. These cells were then transfected with either I-A<sup>k</sup> (22) or I-A<sup>d</sup> molecules (this manuscript).

We have shown recently that murine MHC class II molecules I-A<sup>d</sup> transfected into these cell lines is able to present peptides derived from most intact proteins to I-A<sup>k</sup>-restricted T cell hybridomas (22). This suggests that either the presentation of I-A<sup>d</sup>-restricted epitopes is DM independent or that I-A<sup>d</sup> may have a different functional property such as a weaker association with CLIP (23). To further examine this issue we have now transfected murine MHC class II molecule I-A<sup>d</sup> into these cell lines. Like I-A<sup>k</sup>, I-A<sup>d</sup> has been used extensively to study antigen presentation. This allowed us to test a panel of antigens in antigen presentation assays to a number of well-characterized T cell hybridomas. In contrast to previously published results by Stebbins et al. (24), we find that none of the human cell lines transfected with I-A<sup>d</sup> present peptides derived from whole proteins to T cell hybridomas. However, these I-A<sup>d</sup>-transfected cells retain the ability to present synthetic peptides. This inability to present intact proteins correlates with the following properties of I-A<sup>d</sup>. Firstly, I-A<sup>d</sup> molecules in these cells are recognized poorly by the conformation-dependent antibody, MKD.6, indicating poor loading with antigenic peptides. Secondly, I-A<sup>d</sup> molecules in transfected human B cell lines fail to form SDS-stable dimers. Finally, the majority of I-A<sup>d</sup> molecules in human cell lines are occupied with I<sub>i</sub> peptides CLIP. These properties of I-A<sup>d</sup> resemble the features described for HLA class II molecules (e.g. HLA-DR3) in murine cell lines 9.5.3 and 7.9.6 (17,18,21). A surprising finding is that I-A<sup>d</sup> molecules also fail to function normally in a DR<sub>i</sub> normal cell line, 8.1.6. This implies that I-A<sup>d</sup> molecules are incompatible with some of the intracellular proteins of human origin that are normally required for antigen processing and presentation by these molecules.

**Methods**

**Cell lines and culture conditions**

All cell lines were maintained in RPMI 1640 medium supplemented with 10% FCS, 0.05 mM 2-mercaptoethanol, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The cell lines 8.1.6, 7.9.6 and T2 were transfected with I-A<sup>d</sup> α and β chains cDNA cloned into a SRα neo vector with an SV40 promoter (a gift from Dr Mark Davis, Stanford University, CA). A gene pulser and capacitance extender (Bio-Rad, Richmond, CA) set at 230 V and 960 µF was used to introduce DNA into the cells. Growth medium for the cell lines transfected with cDNA was also supplemented with G418 (400 µg/ml). Cells used in the assay were sorted for I-A<sup>d</sup> expression using a FACStar (Becton Dickinson, Mountain View, CA). A20, a B cell lymphoma cell line, was used as a control cell line. All cells were cultured at 37°C in a 5% CO₂ atmosphere. Cells were grown in G418-free medium for at least 24 h before using them in a T cell hybridoma assay.

T cell hybridomas used in this studies are as follows: ovalbumin (OVA) 323–339-specific and I-A<sup>d</sup>-restricted T cell hybridoma 3D0.548 was obtained from Dr P. Marrack (National Jewish Centre, Denver, CO), hen egg lysozyme (HEL) 8–29-specific, I-A<sup>d</sup>-restricted T cell hybridoma F1.2 and a micrococal nuclelease (Nase) 61–80-specific and I-A<sup>d</sup>-restricted T cell hybridoma 4H2.C9 were provided by Dr Luciano Adorini (Roche Milano Ricerche, Milan, Italy).

**T cell hybridoma assay**

Antigen-presentation assays were performed as described previously (25). Briefly, Peptide-specific T cell hybridomas (2.5×10<sup>4</sup>) were co-incubated with APC (2.5×10<sup>4</sup>), and various doses of OVA, HEL and Nase (all from Sigma) or synthetic peptides (Biomolecular Resource Facility, JCSMR, Australia) for 20–24 h in flat-bottom 96-well plates. After which, 50 µl of supernatant was harvested from each well and tested for IL-2 activity using the IL-2-dependent cell line, HT-2. Proliferation of HT-2 was measured from [³H]thymidine incorporation as previously described (25).

**Immunofluorescence and flow cytometry**

Surface expression of I-A<sup>d</sup> was assessed by MKD.6–FITC (anti-A<sub>i</sub><sup>d</sup>, 31) and unconjugated M5/114 (anti-A<sup>d</sup>, E<sup>d</sup>, E<sup>a</sup>, A<sup>d</sup>, A<sup>a</sup>, 32) followed by anti-rat IgG–FITC (Pierce, Rockford, IL). CLIP expression on the cell surface was determined by a biotinylated anti-CLIP antibody, CerCLIP (kindly provided by Dr Peter Cresswell, Yale University) (21), followed by labelling with streptavidin–FITC. Expression of DR3 molecules on the cell surfaces was measured using unconjugated mAb 16.23 (17, 18) and V1.15 (17,18) followed by anti-mouse IgG–FITC (Pierce). Briefly, cells were stained with an anti-class II antibody for 45–60 min in 1% BSA/PBS/0.01% NaN₃ solution at 4°C. Cells were washed twice in FACS medium and were subsequently incubated with secondary antibody labelled with fluorescein (where necessary) for a further 30–45 min. Cells were washed at least three times with the last wash
being in FACS medium containing propidium iodide (PI) to stain the dead cells. Ten thousand PI-negative cells were then analysed by flow cytometry in a FACScan (Becton Dickinson). The data is expressed as median fluorescence.

**Biosynthetic labelling and immunoprecipitation**

Cells were washed in methionine-free RPMI 1640 medium followed by two 45 min incubations at 37°C. Cells were pulsed-labelled in fresh methionine-free medium containing 0.2–0.5 mCi/ml [35S]methionine (Amersham, Amersham, UK) for 30 min. Cells were then washed three times in ice-cold PBS and chased with methionine-containing RPMI for various time points. At each time point, cells were lysed in 1% (v/v) NP-40 in 50 mM Tris–HCl, pH 7.5, containing 0.3 M NaCl, 5 mM EDTA, pepstatin, aprotinin and leupeptin (10 μg/ml each). After 15 min in ice (with occasional gentle shakes), nuclei were removed by centrifugation at 14,000 g for 15 min and the supernatant material was precleared by rotating at 4°C for 2h with 1 μl of normal rabbit serum and Protein A-Sepharose beads (50 μl). Supernatants were collected and I-A^d^ molecules were immunoprecipitated with a combination of MKD.6 and M5/114 mAb with 25 μl of Protein G beads (Sigma) for at least 4 h. Beads were washed four times in cold lysis buffer and immunoprecipitated material was collected by addition of SDS sample buffer and by incubating samples at 37°C for 30–45 min. Samples were analysed by 12.5% SDS–PAGE. Dried gels were exposed generally for 1–2 days with Hyperfilm (Amersham).

**Immunoblot analysis**

For immunoblots, cell lysates were prepared from 5–20 × 10^6^ cells in a lysis buffer containing 1% NP-40, 0.006 M CHAPS, 50 mM Tris (pH 8.0), 0.15 M NaCl, 5 mM EDTA and 1.5% aprotinin (Sigma). Nuclei and insoluble debris were removed by centrifugation at 16,000 g for 20 min. An equal volume of sample buffer containing 5% SDS, 62 mM Tris (pH 6.8) and 10% glycerol was added, and the samples were then incubated for 30 min at room temperature. Just prior to separation of proteins by SDS–PAGE, samples were either boiled for 2 min or were left unboiled. Electrophoresed samples were then transferred to nitrocellulose using a Novex apparatus. Nitrocellulose filters were blocked for 1 h in PBS containing 3% low fat milk, then incubated for 2 h with an appropriate unlabelled mAb (see figure legend). Nitrocellulose papers were washed four or five times in wash buffer (PBS, 3% low fat milk and 0.5% Tween 20) and probed with horseradish peroxidase-conjugated sheep anti-mouse Ig (Silenus, Melbourne, Australia). Antibody binding was detected by enhanced chemiluminescence (Amersham). Pre-stained rainbow markers were used to estimate molecular mass.

**Results**

**Cell surface expression of I-A^d^ on normal and mutant human B cell lines**

B lymphoblastoid cell line 8.1.6 is hemizygous and has been extensively described previously (17–20). Mutant cell line 7.9.6 was derived by chemical mutagenesis of 8.1.6, followed by selection with an anti-HLA-DR3-specific mAb 16.23 (17, 18). The other mutant cell line used in this study, T2, was derived after X-irradiation of lymphoblastoid cell line, LCL721.174 (13), that carries a large homozygous deletion across the MHC region and is therefore missing most of the MHC genes including the MHC class I and class II antigen processing genes (13, 21). Class II MHC molecules, I-A^d^, were stably transfected into cell lines 8.1.6, 7.9.6 and T2 to create 8.1.6.d, 7.9.6.d and T2.d respectively. mAb MKD.6 and M5/114 recognizing polymorphic and monomorphic determinants of I-A^d^ respectively were used to determine the cell surface expression of I-A^d^ class II molecules. As can be seen in Fig. 1, I-A^d^ expression on 7.9.6.d and T2.d was barely detected by MKD.6 antibody. Surprisingly, MKD.6 also failed to detect I-A^d^ in 8.1.6.d cells; however, the monomorphic antibody, M5/114, detected substantial levels of I-A^d^ in all the transfected cell lines (Fig. 1a). These results show that I-A^d^ molecules are expressed in these cells but are conformationally altered compare to wild-type.

mAb 16.23 and V1.15 recognize polymorphic and monomorphic determinants of DR3 respectively (17, 18). In order to compare the HLA-DR3 expression in I-A^d^-transfected 8.1.6 and 7.9.6, these cells were labelled with antibodies 16.23 and V1.15 (Fig 1b). As expected, expression of 16.23 epitope was reduced in 7.9.6.d, although DR expression detected by V1.15 was intact. These experiments show that transfection of I-A^d^ in 8.1.6 and 7.9.6 cells does not affect the usual expression of DR3 molecules in these cells.

**I-A^d^-transfected normal and mutant human cells fail to present processed determinants from intact antigens to specific T cell hybridomas**

The data presented in Fig. 1 indicated that I-A^d^ class II molecules in these transfecants display a mutant phenotype similar to the one described by Mellins and Pious for HLA-DR3 molecules in certain mutant cell lines (17, 18). In order to test how I-A^d^-transfected human cells present antigens and their peptides to T cells, a panel of I-A^d^-restricted T cell hybridomas specific for OVA, HEL and Nase was used. A mouse B cell line, A20, was used as a control APC. As can be seen in Fig. 2, while A20 presented all proteins and peptides to various T cell hybridomas, the I-A^d^-expressing 8.1.6.d, 7.9.6.d and T2.d failed to present whole OVA and Nase proteins. However, all transfecteds presented synthetic peptides at levels generally comparable to A20 cells (Fig. 2b, d and f). A very high concentration of HEL was required by 8.1.6.d and 7.9.6.d to stimulate HEL-specific T cell hybridoma (Fig. 2c). Interestingly, similar concentrations of HEL were required by fixed A20 to stimulate these T cells, suggesting that there may be low levels of contamination with HEL fragments in the whole protein resulting in low level stimulation (data not shown). It is also conceivable that the HEL 8–29 epitope can be generated from HEL without extensive intracellular processing and that some HEL antigen is denatured. Overall, these results show that transfected human cell lines are clearly defective in I-A^d^-mediated antigen presentation.

Since I-A^d^-transfected human cell lines can effectively present synthetic peptides to mouse T cell hybridomas, this also excludes the possibility that the lack of T cell stimulation
by these cells is due to species incompatibility between human APC and mouse T cell hybridomas. This argument is further supported by our previous experiments where we have shown that I-A<sup>d</sup>-transfected 8.1.6, 9.5.3 and T2 cells can effectively present whole proteins and peptides to I-A<sup>k</sup>-restricted mouse T cell hybridomas (22). It is also important to note that this defect in I-A<sup>d</sup>-mediated antigen presentation is not due to mutations in I-A<sup>d</sup> α or β genes as the same constructs behave normally when transfected into either the class II-deficient mouse cell line, M12.C3, or fibroblasts (data not shown). In addition, the sequences of A<sub>α</sub><sup>d</sup> and A<sub>β</sub><sup>d</sup> genes from 8.1.6d are wild-type (data not shown).

I-A<sup>d</sup> class II molecules fail to form SDS-stable dimers in human B cell lines

The ability of α and β chains to form SDS-stable heterodimers (~60 kDa) has been attributed to an antigen (or a subset of endogenous peptides) peptide-bound conformation of the MHC class II molecules (18,26). However, in antigen processing mutant cell lines, such as 9.5.3 and 7.9.6, HLA class II molecules fail to form SDS-stable dimers (18). We therefore asked whether I-A<sup>d</sup> molecules in 8.1.6d, 7.9.6d and T2.d would form SDS-stable dimers (Figs 3 and 4). We were particularly interested in 8.1.6d cells that normally form HLA-DR3 stable dimers in SDS (18).

Cells were pulsed labelled with [35S]methionine and chased for 2–6 h. I-A<sup>d</sup> molecules were immunoprecipitated from 8.1.6d, 7.9.6d and T2.d cell lysates with a combination of mAb MKD.6 and M5/114. Figure 3 shows that while there is a considerable amount of SDS-stable I-A<sup>d</sup> class II dimers at 6 h after chase in A20, there were no detectable SDS-stable I-A<sup>d</sup> dimers present in any of the human cell lines even after longer chase periods (Fig. 3).

The SDS stability of HLA-DR molecules in 8.1.6d and 7.9.6d cells was tested by mAb RM7.153 (33) and DA6147 (34) (Fig. 4). As expected, endogenous DR3 molecules expressed in 8.1.6d formed SDS-stable dimers, whereas DR3 molecules expressed in 7.9.6d failed to do so (Fig. 4). This pattern of HLA-DR SDS stability in 8.1.6d and 7.9.6d is identical to that seen in untransfected 8.1.6 and 7.9.6 cells (18 and data not shown).

I-A<sup>d</sup> molecules in 8.1.6d, 7.9.6d and T2.d are predominantly occupied with the invariant chain peptides, CLIP

The majority of class II molecules in antigen processing mutant cell lines have been shown to be associated with the β peptides, CLIP (11,13). It is this association of CLIP with MHC class II molecules that has been implicated in conferring SDS instability in MHC class II dimers (27–30). We therefore examined whether I-A<sup>d</sup>-transfected 8.1.6, 7.9.6 and T2 cell lines have CLIP associated with MHC class II molecules on their cell surfaces. A mAb, CerCLIP.1 (21), was used to stain the cells. Interestingly, when compared with wild-type 8.1.6 cells, 8.1.6d cells express much higher levels of CLIP associated with I-A<sup>d</sup> in transfected 7.9.6d cells was essentially identical. Finally, T2 cell lines transfected with I-A<sup>d</sup> also express very high levels of CLIP at the cell surface (Fig. 5). Once again this expression of CLIP correlated with the high expression of M5/114 in T2.d (see Fig. 1). The controls include cell line T2 transfected with either DR3 (T2.DR3) or murine I-A<sup>k</sup> (T2.k). Expression of HLA-DR on
I-Ad-transfected human B cell lines are defective in antigen presentation

Fig. 2. I-A\(^d\)-transfected human cell lines are defective in presenting intact antigens. 8.1.6d, 7.9.6d and T2.d were used to present three separate protein antigens to three I-A\(^d\)-restricted T cell hybridomas. Cell line A20 was used as a control. Hybridoma used were as follows: 3D0.548, OVA 323–339-specific (a,b); F1.2, HEL 8–29-specific (c,d); and 4H2.C9, Nase 61–80-specific (e,f). T cell hybridoma cells (2.5\( \times \)10\(^4\)) were incubated with various APC (2.5\( \times \)10\(^4\)) and antigens for 24 h. Culture supernatants (50 \(\mu l\)) were then analysed for IL-2 production using HT-2 cell line as described in Methods.

Discussion

T2.DR3 (Fig. 5) is very similar to that in the mutant cell line 7.9.6 and is predominantly recognized by monomorphic antibodies, such as V1.15 (data not shown). Like 7.9.6, T2.DR3 also fails to present intact proteins to T cells (21). T2.k, on the other hand, is able to present certain proteins to specific T cells and also forms SDS-stable class II dimers (22). As expected, high levels of CLIP were detected by the CLIP-specific antibody on T2.DR3 but not T2.k which has lower affinity for CLIP (22,23) (Fig. 5).

Studies by Mellins and Pious have shown that while 8.1.6 cells present both intact proteins and synthetic peptides to T cells, the 7.9.6 cell line only presents peptides (17,18). This defect in protein presentation by 7.9.6 appears to be due to a point mutation in the HLA-DMB gene (19,20) and results in several phenotypic changes in these cells. For example, HLA class II molecules in 7.9.6 are unstable under SDS conditions (18), cell surface class II expression is detected mainly by
I-Ad-transfected human B cell lines are defective in antigen presentation

Fig. 3. I-Ad molecules fail to form SDS-stable dimers in human cell lines. The cellular proteins of 8.1.6d, 7.9.6d and T2.d cells (3–5×10⁶) were pulse labelled with [³⁵S]methionine for 30 min and chased for indicated times before immunoprecipitation with a combination of I-A<sup>d</sup>-specific mAb MKD.6 and M5/114 antibodies described in Methods. Beads were left at 37°C for 30 min to dissociate the bound proteins in a non-reducing SDS-containing buffer and loaded directly on to 12.5% SDS–PAGE gels. Positions of αβ dimers, monomers and li are shown together with positions of mol. wt markers.

Fig. 4. Expression of SDS-stable HLA-DR dimers in 8.1.6d and 7.9.6d. Cell lysates from 8.1.6d and 7.9.6d (8×10⁶ each) were incubated in lysis buffer containing 2.5% for 20–30 min and then were either boiled (b) or unboiled (u) as described in Methods. Samples were fractionated on 10% SDS–PAGE and transferred onto nitrocellulose paper. Electroblotted samples were then analysed by immunoblotting with anti-DR mAb RM7.153 (1/250 diluted culture supernatant) and DA6147 (1/2 diluted culture supernatant). Antibody binding was detected by chemiluminescence and fluorography as described in Methods.

Fig. 5. Increased expression of CLIP in I-Ad-transfected human cells. 8.1.6, 7.9.6 and T2 cells transfected with either I-A<sup>d</sup>, I-A<sup>k</sup> or HLA-DR3 were stained with biotinylated CerCLIP.1 antibody (21) as described in the Methods. Binding of this antibody was detected by a streptavidin–FITC reagent (Pierce). Results are expressed as median fluorescence. Background median fluorescence with streptavidin–FITC alone ranged from 3 to 10 fluorescence units in all experiments.

monomorphic antibodies (17,18) and class II molecules in these mutant cell lines are predominantly associated with CLIP (this study). It has been demonstrated that these defects in 7.9.6 can be restored by transfecting the DMB gene (19).

With an eventual aim to understand the role of various components that regulate antigen processing for MHC class II-mediated antigen presentation and also to further develop a murine class II-based system to study antigen processing in mutant cell lines, we have now transfected I-A<sup>d</sup> class II molecules in 8.1.6 (normal), 7.9.6 (DBM mutant) and T2 (deletion mutant). These cell lines were then tested in various biological assays to examine I-A<sup>d</sup>-mediated antigen processing and presentation. We observed that I-A<sup>d</sup> transfected in either DMB or DMA/DMB mutant cell lines displayed a similar phenotype as seen for HLA-DR in these cell lines (17,18,21). However, to our surprise, 8.1.6 cell lines transfected with I-A<sup>d</sup> (8.1.6d) also displayed the mutant phenotype. In contrast, the levels of endogenous HLA-DR3 and their structural integrity are not altered in 8.1.6d (Figs 1b and 4).

The data show that despite functional DM and li molecules in 8.1.6d, these cells are unable to present proteins to I-A<sup>d</sup>-restricted T cells. While the defects in antigen presentation by 7.9.6d and T2.d might be explained by their defective expression of DM molecules, it is not clear why I-A<sup>d</sup>-mediated
antigen presentation is defective in 8.1.6d. We hypothesize that certain components in the antigen processing pathway have to be of mouse origin for I-Ad to function properly.

These results are also interesting in light of our previous experiments where we have shown that 8.1.6 (DM normal) and 9.5.3 (DM mutant) expressing I-Ak molecules are able to present most determinants derived from intact proteins to T cells (22). What this suggests is that while certain murine class II molecules are able to function essentially normally in these human cell lines, other molecules, such as I-Ak, cannot.

One possible explanation for this paradox may simply be the affinity of CLIP for I-A\textsuperscript{d} molecules, that we know is significantly higher than for I-A\textsuperscript{k} molecules (23). In this scenario, a self release mechanism for CLIP proposed by Kropshofer et al. (35) may operate in an I-A\textsuperscript{k} system independently of DM. However, in an I-A\textsuperscript{d} system, this self release mechanism for CLIP dissociation may not operate without some assistance from compatible DM or other molecules. Therefore, transfection of murine DM, li and other MHC-linked molecules in human cells expressing I-A\textsuperscript{d} molecules will form the next line of experiments to understand species compatibility in antigen presentation by MHC class II molecules.

Presentation of peptides to T cells is sometimes enhanced in DM-defective cell lines (18,22) although we did not observe this enhancement by I-A\textsuperscript{d} molecules in transfected cell lines. This is most likely due to the higher affinity of CLIP for I-A\textsuperscript{d} molecules, compared with I-A\textsuperscript{k} or HLA-DR3 class II molecules (23). Therefore, it is likely that in the face of strong CLIP binding, synthetic peptides may have reduced access to I-A\textsuperscript{d} so that the presentation of antigenic peptides is not particularly enhanced in the absence of functional DM.

The results presented here differ from the results of Stebbins et al. (24), who reported that irrespective of whether a human cell line has a mutation in the DM genes or not, I-A\textsuperscript{d} molecules function normally in these cell lines. However, these authors also showed that I-A\textsuperscript{d} molecules fail to form SDS-stable dimers in DM normal and DM mutant cell lines (24) consistent with the observations made here (Fig. 3). These observations imply the presence of defects in I-A\textsuperscript{d} -mediated function in I-A\textsuperscript{d}-transfected human cell lines. Moreover, Stebbins et al. have recently shown that I-A\textsuperscript{d} molecules do require mouse DM for the efficient formation of I-A\textsuperscript{d} SDS-stable dimers and the release of CLIP (36).

While we are unable to explain all the quantitative differences seen in our antigen presentation experiments and those of Stebbins et al. (24), it is possible that these differences could be due to a clonal variation in the cells used for transfection, differences in vectors used for introducing the transgenes or variations in the levels of I-A\textsuperscript{d} expression. Indeed, our transfectants showed greatly reduced levels of MKD.6 and increased levels of M5/114 reactivity at the cell surface when compared with the results previously published by Stebbins et al. (24). Perhaps the defect in I-A\textsuperscript{d}-mediated antigen presentation in Stebbins cells is masked by the high levels of I-A\textsuperscript{k} expression (24).

None of our transfectants were able to present peptides derived from intact OVA and Nase proteins to antigen-specific T cells hybridomas. In addition, the presentation of determinants derived from intact HEL was greatly reduced in 8.1.6d and that was even lower in 7.9.6d. HEL was not presented by T2.d at all. We believe that our results are internally consistent, noting (i) SDS instability of I-A\textsuperscript{d}, (ii) expression of CLIP–I-A\textsuperscript{d} complexes at the cell surface of transfected human cells and (iii) the inability of these cells to present protein antigens. Therefore, we conclude that despite intact antigen processing machinery of human origin in 8.1.6d, the mouse class II molecule I-A\textsuperscript{d} fails to function normally in these cells.

Antigen processing mutants have been shown to be occupied predominantly with CLIP from the exon three region of lI (11,13) DM molecules can assist dissociation of CLIP from certain HLA class II molecules (14–16). Our data have demonstrated that a large quantity of I-A\textsuperscript{d} molecules in 8.1.6d, 7.9.6d and T2.d were loaded with CLIP consistent with defective function for I-A\textsuperscript{d} molecules discussed above (Fig. 5).

It is conceivable that I-A\textsuperscript{d} molecules fail to traffic normally or do not interact properly with human antigen processing machinery in the appropriate endocytic compartments. This could account for the defective dissociation of CLIP from I-A\textsuperscript{d}, abnormal class II expression and impaired antigen presentation as observed in this study. It is also possible that the human li may not associate entirely normally with I-A\textsuperscript{d} molecules. However, this seems unlikely because we have shown in this study that human li can be immunoprecipitated with I-A\textsuperscript{d} molecules (Fig. 3) and that I-A\textsuperscript{d} are occupied with human CLIP in these cells (Fig. 5). Whether this li association with I-A\textsuperscript{d} molecules is in any way different from the manner in which human li interacts with human class II molecules is another interesting possibility. Similarly, CLIP dissociation from I-A\textsuperscript{d} molecules may not be efficiently catalysed by human DM molecules. There may also be other yet undefined component(s) in antigen processing and presentation that may also be crucial to maintain species compatibility.

Overall, this demonstration that I-A\textsuperscript{d}-expressing normal and mutant human B cell lines fail to present intact proteins to T cells, and that the I-A\textsuperscript{d} molecules in these cells display a mutant phenotype, presents us with a challenge to identify components that may be contributing in these defects in I-A\textsuperscript{d}-mediated antigen presentation. Elucidation of these mechanisms and the interaction of I-A\textsuperscript{d} molecules with key proteins in antigen processing pathways will provide crucial information to further unravel the complexities of class II-mediated antigen presentation.

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Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
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<tr>
<td>CLIP</td>
<td>class II-associated invariant chain peptides</td>
</tr>
<tr>
<td>li</td>
<td>invariant chain</td>
</tr>
<tr>
<td>HEL</td>
<td>hen egg lysozyme</td>
</tr>
<tr>
<td>Nase</td>
<td>micrococcal nuclease</td>
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<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
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I-Ad-transfected human B cell lines are defective in antigen presentation. Reference genes are both required for MHC class II/peptide complex formation in antigen-presenting cells. Nature 368:554. 1


