

## The Requirement for DM in Class II-restricted Antigen Presentation and SDS-stable Dimer Formation Is Allele and Species Dependent

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### Summary

Recently several cell lines have been identified with mutations in a major histocompatibility complex (MHC)-linked protein that lead to defects in class II-restricted antigen presentation and a defect in the formation of class II SDS-stable dimers. The defect in these cells has recently been shown to result from the inability to express the MHC-encoded nonclassical class II molecule called DM. To further examine the role of DM in class II-restricted antigen presentation, we asked if this defect would equally affect different allelic and species variants of class II molecules. To investigate this, we transfected the parent cell lines T1 and 8.1.6 and their respective antigen presentation mutants T2 and 9.5.3 with the genes encoding I-A<sup>d</sup> and examined the derived transfectants for their ability to present antigen, the conformation of I-A<sup>d</sup> at the cell surface, association of I-A<sup>d</sup> with invariant chain (Ii), and the ability to form I-A<sup>d</sup> SDS-stable dimers. The lack of functional DM expression did not affect any of the anti-I-A<sup>d</sup> monoclonal antibody (mAb) epitopes tested or the ability of I-A<sup>d</sup> to associate and dissociate with Ii. Surprisingly, these studies also revealed that the antigen presentation defect observed for DR in the 9.5.3 cells did not compromise I-A<sup>d</sup>-restricted antigen presentation. In addition, we found that the level of SDS-stable dimer formation did not correlate with antigen presentation capacity for I-A<sup>d</sup> and that the amount of SDS-stable I-A<sup>d</sup> dimer depends on the cellular context in which the class II molecule is expressed. Our results suggest that the ability to form SDS-stable dimer is not strictly correlated with class II-restricted antigen presentation. Finally, when two allelic forms of murine class II molecules were compared in the defective T2 cell line, it was found that I-A<sup>k</sup> but not I-A<sup>d</sup> forms SDS-stable dimers equivalent to that seen in the parental cell lines. Overall, our results suggest that DM may modulate rather than play a requisite role in I-A<sup>d</sup>-restricted antigen presentation and SDS-stable dimer formation and that dependency on DM may be allele or species specific.

MHC class II molecules have the ability to present a wide array of antigens derived from both exogenous and endogenous sources. Studies involving the use of lysosomotropic reagents (1), those that have examined the sites of class II localization within the APC (2–6), the biochemistry of invariant chain (Ii)<sup>1</sup> (reviewed in 7), and class II conformation (8–12) have led to a general model of class II-restricted antigen presentation. In this model, class II molecules bind Ii in the endoplasmic reticulum (ER), an event which blocks the loading of most endogenous peptides (13, 14). The class

II/Ii complex then traffics out of the ER through the Golgi and *trans*-Golgi network where it is sorted into the endocytic pathway. Within endocytic compartments, Ii is degraded and removed from the class II binding groove. Antigenic peptides produced from the proteolytic breakdown of internalized or endogenous antigens then bind class II molecules, an event that confers the SDS-stable phenotype. Finally, the class II/peptide complex is exported to the cell surface for recognition by T cells.

Despite knowledge of many of the elements that regulate the function of class II molecules, there remains considerable uncertainty on the exact sequence of events that lead to expression of functional peptide/class II complexes at the cell surface. Dissection of the intracellular mechanisms of antigen presentation has been greatly facilitated by the derivation of mutants. Several of these mutants have profound defects in

<sup>1</sup> Abbreviations used in this paper: ADH, alcohol dehydrogenase; c, chicken; CLIP, class II-associated invariant chain peptide; ER, endoplasmic reticulum; GAM, goat anti-mouse; HEL, hen egg lysosome; Ii, invariant chain; MYO, myoglobin; PAS, protein A-Sepharose; PINS, pork insulin; t, turkey.

class II-restricted antigen presentation (15–20). Studies on two of these mutant cell lines have now mapped the defect to the MHC-encoded nonclassical class II-like proteins termed DMA and DMB (21–23). Failure to express functional DM leads to a loss of two DR epitopes recognized by the antibodies 16.23 and 7.3.19 (17, 24), several epitopes expressed on DQ and DP (23), an inability to form SDS-stable class II dimers, the loss of intact antigen presentation to several T cell clones and the failure to stimulate several allospecific T cell clones. These results suggest that DM expression is required for antigen presentation by DR and that DM functions late in the class II pathway.

To further examine the requirement for DM in antigen presentation and SDS-stable dimer formation, we transfected both the 8.1.6 and T1 cell lines, and their respective mutants 9.5.3 and T2 that lack functional DM expression, with the genes encoding the murine MHC class II molecule I-A<sup>d</sup>. This allowed the study of DM's impact on the presentation of a wider panel of antigens than has been possible with human class II molecules and also allowed us to examine the class II I-A<sup>d</sup>-restricted presentation of an endogenous antigen (H-2L<sup>d</sup>) which is presented by a pathway distinct from the classical class I or class II pathways (25). Finally, this system permitted us to test the hypothesis that species- or allele-dependent differences in class II structure might modify the requirement for DM in the various activities of class II molecules.

Surprisingly, we have found that the lack of functional DM expression has no detectable effect on class II-restricted antigen presentation to our panel of I-A<sup>d</sup>-restricted T cells. In addition, through the analysis of several different cell types in which the murine class II molecule is expressed, we have found little correlation between the generation of SDS-stable dimer and the efficiency of antigen presentation. Together our results suggest that allelic polymorphism and/or species variation in class II may modulate the requirement for DM in class II-restricted antigen presentation, and that the levels of SDS-stable dimer achieved within a given cell do not necessarily predict antigen presentation capacity.

## Materials and Methods

**Cell Lines and Reagents.** Cell lines were maintained at 37°C and 5% CO<sub>2</sub> in either DMEM containing 5% FCS for the T cell hybridomas, RPMI 1640 containing 5% BCS for the 8.1.6 and 9.5.3 cell lines, and Iscove's containing 5% BCS for the 174XCEM.T1 (T1) and 174XCEM.T2 (T2) cells, all supplemented with 5 mM HEPES, 2 mM glutamine, 1 mM nonessential amino acids, and 5 × 10<sup>-5</sup> M 2-ME. All medium and supplements were purchased from GIBCO BRL (Gaithersburg, MD) unless otherwise noted. 8.1.6 and 9.5.3 were provided by Dr. E. Mellins (University of Pennsylvania, Philadelphia, PA) and T1 and T2 and the T1 and T2 I-A<sup>k</sup> transfectants were provided by Dr. P. Cresswell (Yale University, New Haven, CT). T1/T2 and 8.1.6/9.5.3 were transfected with genes encoding I-A<sup>d</sup> and H-2L<sup>d</sup> using electroporation. Briefly, 5 × 10<sup>6</sup> cells were washed and resuspended in 0.8 ml PBS without Ca<sup>2+</sup> or Mg<sup>2+</sup> containing 5 μg of each full-length cDNA encoding the I-A<sup>α</sup> and I-A<sup>β</sup> chains driven by the β-actin promoter (26), 10 μg of the plasmid encoding H-2L<sup>d</sup> (L<sup>d</sup>-4), kindly provided

by Dr. T. Hansen (Washington University, St. Louis, MO), and 0.5 μg of pSV2 neo as the selection marker (27). DNA was linearized before electroporation. Cells were electroporated at room temperature using a Gene Pulser and Capacitance Extender (Bio-Rad Laboratories, Richmond, CA) set at 210 V and 960 μF. Cells surviving drug selection (0.6 mg/ml G418) were sorted by selection for both I-A<sup>d</sup> and H-2L<sup>d</sup> cell surface expression using either Dynabeads (DynaL Inc., Great Neck, NY) or by FACStar<sup>Plus</sup>® (Becton Dickinson & Co., Mountain View, CA) and periodically resorted to maintain I-A<sup>d</sup> expression.

**T Cell Hybridomas.** The I-A<sup>d</sup>-restricted chicken OVA-specific T cell hybridomas 3DO 54.8, and DO 11.10 were obtained from Dr. P. Marrack (National Jewish Center, Denver, CO) (28); the pork insulin-specific hybridoma Pd 2.30 was provided by Dr. P. Jensen (Emory University, Atlanta, GA); the hen egg lysozyme-specific hybridomas GD3.25.5 and GD5.16 were provided by H. Dang and Dr. E. Sercarz (University of California at Los Angeles, Los Angeles, CA); the other T cells used in this study, ACh 27.7 and ACh 8.2, both recognizing horse myoglobin; ACh 48.2 and ACh A32, both recognizing alcohol dehydrogenase; and ACh T40 which recognizes turkey OVA, were produced in our laboratory as follows. BALB/c mice were primed in the rear foot pads with 50–100 μl of antigen emulsified in CFA, and 10 d later popliteal lymph nodes were harvested and purified T cells were plated at 5 × 10<sup>6</sup> in 24-well plates on feeder layers of irradiated syngeneic spleen cells plus antigen at 20–50 μg/ml. 4 d later, cells were replated at 5 × 10<sup>6</sup> in 24-well plates on feeder layers of irradiated syngeneic spleen cells without antigen. 7–10 d later, T cells were restimulated and T cell blasts were fused with BW5147 as previously described and cloned in HAT-containing media (25). The fine specificities of these T cell hybridomas have not been determined.

**mAbs.** The hybridomas producing mAbs reactive with I-A<sup>d</sup> (MKD6, M5/114, K24-199, 25.9.17 SII, 34.5.3S, and Bp107.2.2), and with I-A<sup>k</sup> (10.2.16), were acquired from American Type Culture Collection (ATCC; Rockville, MD). Their reactivity requirements have been previously described (29). The anti-I-A<sup>d</sup> rat mAb B21-2 (30), the anti-HLA-DR mAb L243 (31), anti-CD8 mAb 2.43 (32), and the anti-K<sup>k</sup> mAb 16.1.11 (33) were also obtained from ATCC. Other mAbs reactive against HLA-DR used in these experiments include HB10.A (34) (obtained from Dr. E. Mellins) and 16.23 (24) (obtained from Dr. R. DeMars, University of Wisconsin, Madison, WI).

**Antigen Presentation Assays.** APC co-cultures were performed as previously described (25). Briefly, 5 × 10<sup>4</sup> APCs were co-cultured with 5 × 10<sup>4</sup> T cells in 0.2 ml in a 96-well flat-bottomed plate with increasing concentrations of antigen. After 16–20 h, 50 μl of supernatant was removed, frozen, thawed, and then tested for lymphokine content by culture with lymphokine-dependent cell line CTLL-2 as previously described (35). For assays involving fixation of APCs, cells were harvested and washed 2× in PBS then fixed in 0.1% paraformaldehyde for 30 min at room temperature. Cells were then washed extensively and added to cultures at the doses indicated in the figure legends with 5 × 10<sup>5</sup> T cells/well, and supernatants were tested for lymphokine content. The T cells' dependence on Ii was assessed by antigen presentation assays using L cells transfected with I-A<sup>d</sup> with or without genomic Ii, kindly provided by Dr. J. Miller (University of Chicago, Chicago, IL) (36).

**Inhibition of Protein Synthesis and Antigen Presentation.** APCs were pretreated in the presence or absence of 55 μg/ml emetine (Sigma Chemical Co., St. Louis, MO) for 4 h before incubation with antigen, then pulsed with antigen for 6 h in the presence or absence of emetine. APCs pulsed with antigen in the absence of emetine were treated with 55 μg/ml emetine for 1 h at the end of the an-

tigen pulse to control for any nonspecific toxic effects of the emetine. All APCs were then washed and fixed as described above with an additional 2-h culture in complete medium to remove any residual emetine. Another set of APCs, used to test the presentation of peptide, were treated as above but in the absence of antigen.

**Flow Cytometry Analysis.** MHC cell surface expression was measured by staining with mAb followed by a secondary staining reagent, FITC-labeled goat anti-mouse Ig (FITC-GAM) (Cappel Laboratories, Cochranville, PA), as described previously (35), and analyzed on a FACScan<sup>®</sup> cytofluorimeter (Becton Dickinson & Co.). Background fluorescence was evaluated using an irrelevant isotype-matched mAb and FITC-GAM or medium alone and FITC-GAM, which gave equivalent background staining.

**Western Blot Analysis.** Cells were lysed at  $4 \times 10^7$  cells/ml on ice in lysis buffer containing 0.5% CHAPS, 0.5% NP-40, and 50  $\mu$ M iodoacetamide (Sigma Chemical Co.) and the protease inhibitors PMSF, leupeptin, and TPCK (Boehringer Mannheim Corp., Indianapolis, IN). Nuclei and insoluble debris were removed by centrifugation at 16,000 *g* for 30 min. Postnuclear supernatants were adjusted to 0.4% SDS, 10% glycerol, and 0.06 M Tris, pH 6.8. In some experiments, the class II molecules were isolated with mAbs/Protein A-Sepharose (PAS) before SDS-gel electrophoresis. Immunoprecipitated samples were eluted in 0.4% SDS, 10% glycerol, and 0.06 M Tris, pH 6.8, at room temperature for 30 min; one-half was boiled for 5 min at 100°C while the other half remained at room temperature. Proteins were then fractionated by SDS-10%-PAGE and were transferred onto nitrocellulose membranes at 70 V for 6 h. Membranes were blocked overnight in 5% dry milk with 0.04% Sigma Antifoam Concentrate and rinsed with Tris-buffered saline containing 0.05% Tween 20 (TBST). Class II molecules were detected with a 1:10 dilution of the anti-I-A<sup>d</sup> mAb M5/114 or anti-I-A<sup>k</sup> mAb 10.2.16 supernatant or a 1:3,000 dilution of the purified anti-DR mAb HB10.A, in a solution containing 1% dry milk for 4 h then washed extensively with TBST and then incubated with a 1:2,500 dilution of secondary antibody, either goat anti-rat horseradish peroxidase or GAM horseradish peroxidase (GIBCO BRL), in 2.5% milk for 1 h. Membranes were washed again as before and developed by chemiluminescence using ECL (Amersham Corp., Arlington Heights, IL).

**Laser Densitometry Measurements.** Density measurements were taken on a LKB Laser densitometer. The resulting density curves were weighed in order to determine the area beneath each curve. Measurements were averaged based on several different exposures of each Western blot. Results are presented as a range of values obtained from separate experiments. Ratios of SDS-stable class II dimer from the unboiled fraction vs. total class II from the boiled fraction were used to control for class II levels between cells. These ratios were used to obtain cell-to-cell comparisons and confirmed using the Ambis Optical Imaging System (Ambis, San Diego, CA).

**Metabolic Labeling, Immunoprecipitation, and SDS-PAGE.** Metabolic labeling and immunoprecipitation of proteins from transfected cells was performed as previously described (37). Briefly, cells were incubated for 4 h in leucine-free RPMI 1640 supplemented with 5% dialyzed FCS, then labeled in the same medium containing 0.25 mCi/ml of [<sup>3</sup>H]leucine (Amersham Corp.), at 37°C, 5% CO<sub>2</sub> for 45 min. Half of the sample was reserved and the remaining cells were washed and incubated in medium containing a twofold excess of unlabeled leucine for 4 h. Radiolabeled cells were pelleted and lysed at 10<sup>6</sup> cells/ml in a buffer containing 1% NP-40 (Sigma Chemical Co.) and protease inhibitors. Postnuclear supernatants were precleared with formalin-fixed staphylococcus A (Immunoprecipitin; GIBCO BRL), adjusted to 5% dry milk and then

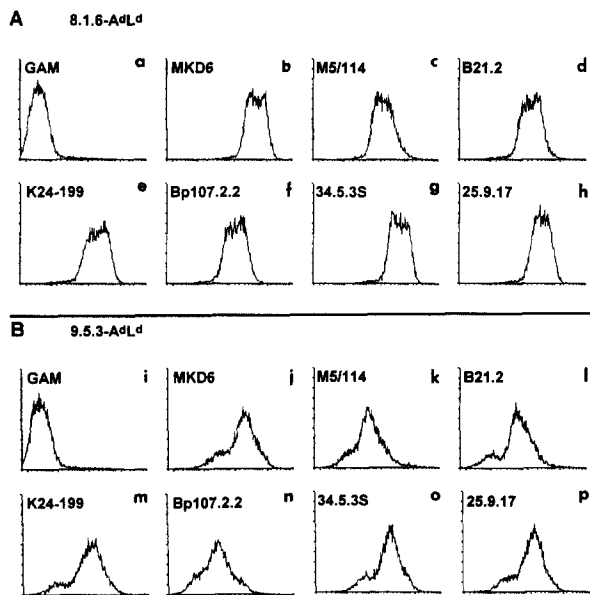
precleared further by incubation with 4:1 mixture of PAS/PGS prebound with irrelevant mAb. Aliquots of the lysate were then incubated with PAS/PGS prebound with either M5/114, L243, or BU45 (The Binding Site, San Diego, CA) and immunoprecipitated material was analyzed by 10% SDS-PAGE. Gels were treated with En<sup>3</sup>Hance (NEN, Boston, MA) and subjected to autoradiography at -80°C.

## Results

**Generation of I-A<sup>d</sup> Transfectants of the 8.1.6 and 9.5.3 and T1 and T2 APC.** There have been several cell lines identified with defects in an MHC-encoded gene(s) that controls class II-restricted antigen presentation (15-20). The 721.174 and closely related 174XCEM.T2 (T2) cell line, which was derived from the 174XCEM.T1 (T1) fusion, both have large homozygous deletions in their MHC regions. Another set of parent/mutant human cells (8.1.6 and 9.5.3, respectively) have been generated by chemical mutagenesis (17). The mutations in both the .174 and 9.5.3 cell lines have recently been mapped to DM, an MHC-encoded, nonclassical class II molecule (21-23). Both the T1 and T2 cell lines and the 8.1.6 and 9.5.3 cell lines were studied in this report, but due to the possible contribution of the other class II structural genes or other unknown factors missing in the larger T2 deletion, we focused the majority of our studies on the 8.1.6 and 9.5.3 cells. These cells were transfected with genes encoding the I-A<sup>d</sup> class II and H2-L<sup>d</sup> class I molecules.

**Surface Staining of 8.1.6-A<sup>d</sup>L<sup>d</sup> and 9.5.3-A<sup>d</sup>L<sup>d</sup> Cells with Either DR or I-A<sup>d</sup>-specific mAbs.** Although cell surface levels of human class II molecules in cells lacking DM expression are unchanged from their respective parent cell lines, the finding that the mutant cell lines show a marked reduction in staining with the DR-specific mAbs 16.23 and 7.3.19 as well as the loss of epitopes on DP and DQ suggest that the conformation of human class II molecules is altered (17, 23). It was therefore of interest to determine if the murine I-A<sup>d</sup> molecule would be similarly affected. Fig. 1 is representative of several immunofluorescence experiments and shows that there were no selective differences in cell surface staining for the I-A<sup>d</sup> molecules expressed on the parent 8.1.6-A<sup>d</sup>L<sup>d</sup> and mutant 9.5.3-A<sup>d</sup>L<sup>d</sup> transfectants. These results indicate that while the lack of DM affects the cell surface conformation of several human class II molecules as detected by mAbs, it was not possible to detect a conformational change for I-A<sup>d</sup> with the panel of mAbs tested.

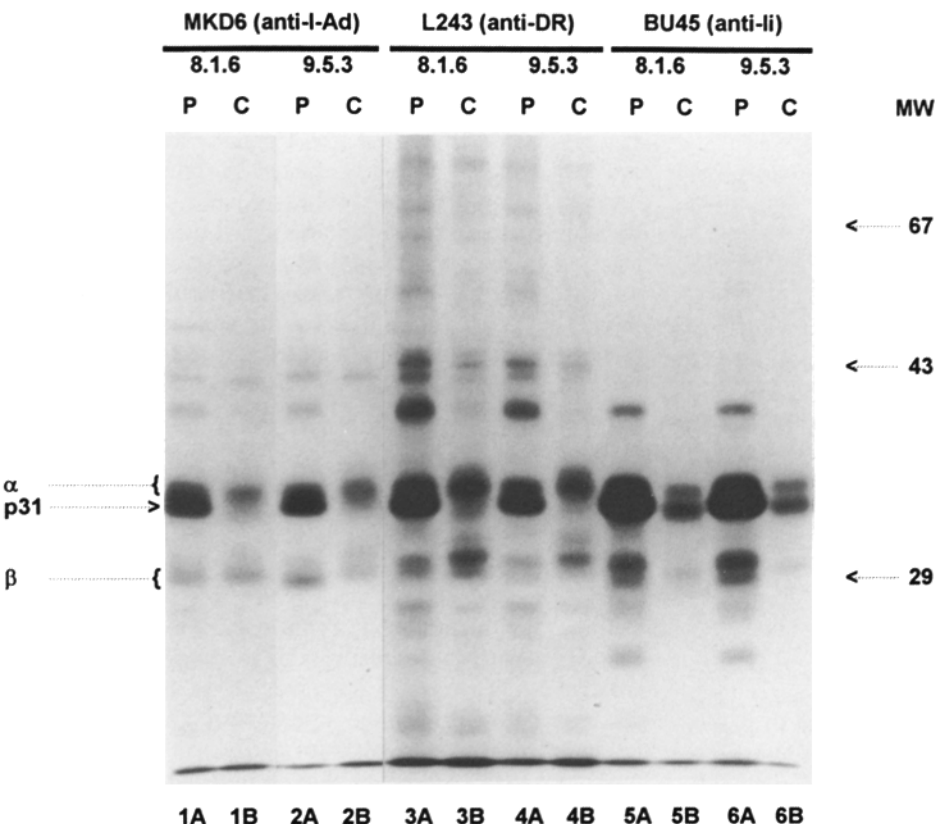
**Murine Class II Matures Normally and Associates with Ii in Both 8.1.6 and 9.5.3.** The immunofluorescence results in Fig. 1 show that I-A<sup>d</sup> expressed in the parent and mutant displayed similar reactivity with all mAbs, including the Ii-dependent mAb K24-199. The latter finding suggests that a productive interaction takes place between murine class II and human Ii molecules. To confirm that class II/Ii interaction was normal in these cells and that the species differences would not disrupt class II/Ii interaction, we performed pulse-chase immunoprecipitation experiments. Fig. 2 shows the results from a kinetic analysis of newly synthesized class II molecules with a pulse period of 45 min and a chase period of 4 h. The left panel confirms DR/Ii maturation and associ-



**Figure 1.** FACS<sup>®</sup> analysis of the surface murine class II expression on 8.1.6 and 9.5.3 transfected cell lines. Cells were stained with either MKD6 (b and j), M5/114 (c and k), B21.2 (d and l), K24-199 (e and m), Bp107.2.2 (f and n), 34.5.3S (g and o), or 25.9.17 SII (h and p). Staining obtained in the absence of primary antibody (a, i) is comparable to those obtained with irrelevant antibody. Panels a-h represent 8.1.6-A<sup>d</sup>L<sup>d</sup> while panels i-p represent 9.5.3-A<sup>d</sup>L<sup>d</sup>. Mean channel measurements indicate 9.5.3-A<sup>d</sup>L<sup>d</sup> has approximately two- to threefold less I-A<sup>d</sup> class II than 8.1.6-A<sup>d</sup>L<sup>d</sup> when measured with these various antibodies.

ation is normal in the 9.5.3 cell line, in agreement with previous findings (16). More importantly, this analysis revealed normal biosynthesis and transport of I-A<sup>d</sup>  $\alpha$  and  $\beta$  chains expressed in human cells. The murine  $\alpha/\beta$  dimers assemble with human Ii chain, mature coordinately with Ii, and eventually dissociate from Ii in a fashion similar to that observed for I-A<sup>d</sup>  $\alpha/\beta$  dimers and murine Ii and for DR and human Ii. In addition, these studies indicate that there is no detectable difference in the maturation patterns of I-A<sup>d</sup> in the parent 8.1.6 and mutant 9.5.3 human cell lines.

*Antigen and Peptide Presentation by the 8.1.6-A<sup>d</sup>L<sup>d</sup> and 9.5.3-A<sup>d</sup>L<sup>d</sup> Cell Lines to T Cell Hybridomas.* Results from the DR-restricted antigen presentation studies involving 8.1.6 and 9.5.3 have shown that DM-deficient cells are defective in the presentation of intact antigen to T cell clones for several antigens tested, although some uncloned antigen-specific lines displayed some reactivity with the mutant APC (17). Further studies also showed that 9.5.3 cells are defective in the stimulation of several allo-class II-specific T cell clones (18). To evaluate the relative ability of the mutant and parent APC to present antigens using the murine-A<sup>d</sup> molecule, we used a diverse panel of I-A<sup>d</sup>-restricted T cell hybridomas. Fig. 3 shows representative results of antigen presentation experiments involving the antigens chicken ovalbumin (cOVA), alcohol dehydrogenase (ADH), horse myoglobin (MYO), hen egg lysozyme (HEL), and pork insulin (PINS). Fig. 3 k shows the presentation of the endogenous antigen H-2L<sup>d</sup> to the T cell hybridoma BC7. Surprisingly, our experiments indicate



**Figure 2.** Pulse-chase immunoprecipitation of class II and Ii from 8.1.6 and 9.5.3 transfected cell lines. Cells were labeled with [<sup>3</sup>H]leucine for a 45 min pulse (a) and then chased in the presence of excess unlabeled leucine for 4 h (b). 8.1.6-A<sup>d</sup>L<sup>d</sup> (lanes 1, 3, 5) or 9.5.3-A<sup>d</sup>L<sup>d</sup> (lanes 2, 4, 6) were lysed and immunoprecipitated with the anti-DR mAb L243 (lanes 1a-2b), anti-I-A<sup>d</sup> $\beta$  mAb M5/114 (lanes 3a-4b), or anti-Ii mAb BU45 (lanes 5a-6b).

that under the conditions tested, there is no apparent difference in the ability of the 9.5.3 cell line to present exogenous or endogenous antigen relative to the 8.1.6 cell line.

These data suggest that I-A<sup>d</sup>-restricted antigen presentation may not be dependent on functional DM expression for the panel of T cells tested. To verify that the presentation seen was due to processing of internalized antigen and not due to contaminating peptides in our antigen preparation, we tested our cells' ability to present intact antigen when fixed. Fig. 4 shows representative results of the antigen presentation experiments done for all T cells with fixed APC. These results show that presentation of intact antigen is blocked by fixation, whereas presentation of exogenous peptide is not. This suggests that the stimulation of antigen-specific T cells by both cell lines is not due to contamination of our antigens by peptides but to the processing and presentation of intact antigen through a fixation-sensitive pathway.

To explain the absence of any DM requirement in I-A<sup>d</sup>-restricted antigen presentation, we considered that DM might not interact productively with the I-A<sup>d</sup> molecule. This would render both cells effectively mutants, since I-A<sup>d</sup> would essentially function without DM in both cells. This hypothesis predicts that the actual efficiency of antigen presentation in both cells would be quite low. To test this hypothesis, we compared the antigen presentation ability of the 8.1.6 transfectants with the murine B cell lymphoma A20. Fig. 5 shows that even across the species barrier, 8.1.6-A<sup>d</sup>L<sup>d</sup> is quite competent at antigen presentation. Presentation of peptide by the 8.1.6 transfectant was equal to or better than A20 for all T cells tested across a peptide dose-response curve (data not shown). These results argue against the possibility that the equivalent I-A<sup>d</sup>-restricted antigen presentation seen for 9.5.3 and 8.1.6 reflects the presence of a defect in both cells. Rather, our results support the notion that the functional competence of I-A<sup>d</sup> may be independent of DM.

*Equivalent Antigen Presentation Between Parent and Mutant Is Observed for Both Ii-dependent and Ii-independent Epitopes and Is Sensitive to Protein Synthesis Inhibitors.* One possible way in which class II antigen presentation may proceed in the absence of DM is through the use of recycled class II molecules. In fact, a recent study has shown that an epitope from a viral protein can be presented by recycled class II molecules in DM-deficient cell lines (38). To address this issue, we initially examined our panel of T cell hybridomas for dependence on Ii. Since Ii enhancement presumably occurs with newly synthesized class II molecules, we reasoned that Ii-dependent T cells would not be able to effectively use recycled class II molecules. We therefore tested our T cell panel for dependence on or enhancement by Ii, using L cells transfected with class II I-A<sup>d</sup> with or without expression of genomic Ii. The results of these studies, shown in Table 1, indicated that our T cell panel contained several T cells that show enhanced antigen presentation in the presence of Ii. Thus, in our system, Ii dependence is not an accurate predictor of dependence on DM.

We then considered that despite the ability of Ii to enhance I-A<sup>d</sup>-restricted antigen presentation to some T cells, it was possible that 8.1.6 and 9.5.3 are particularly capable

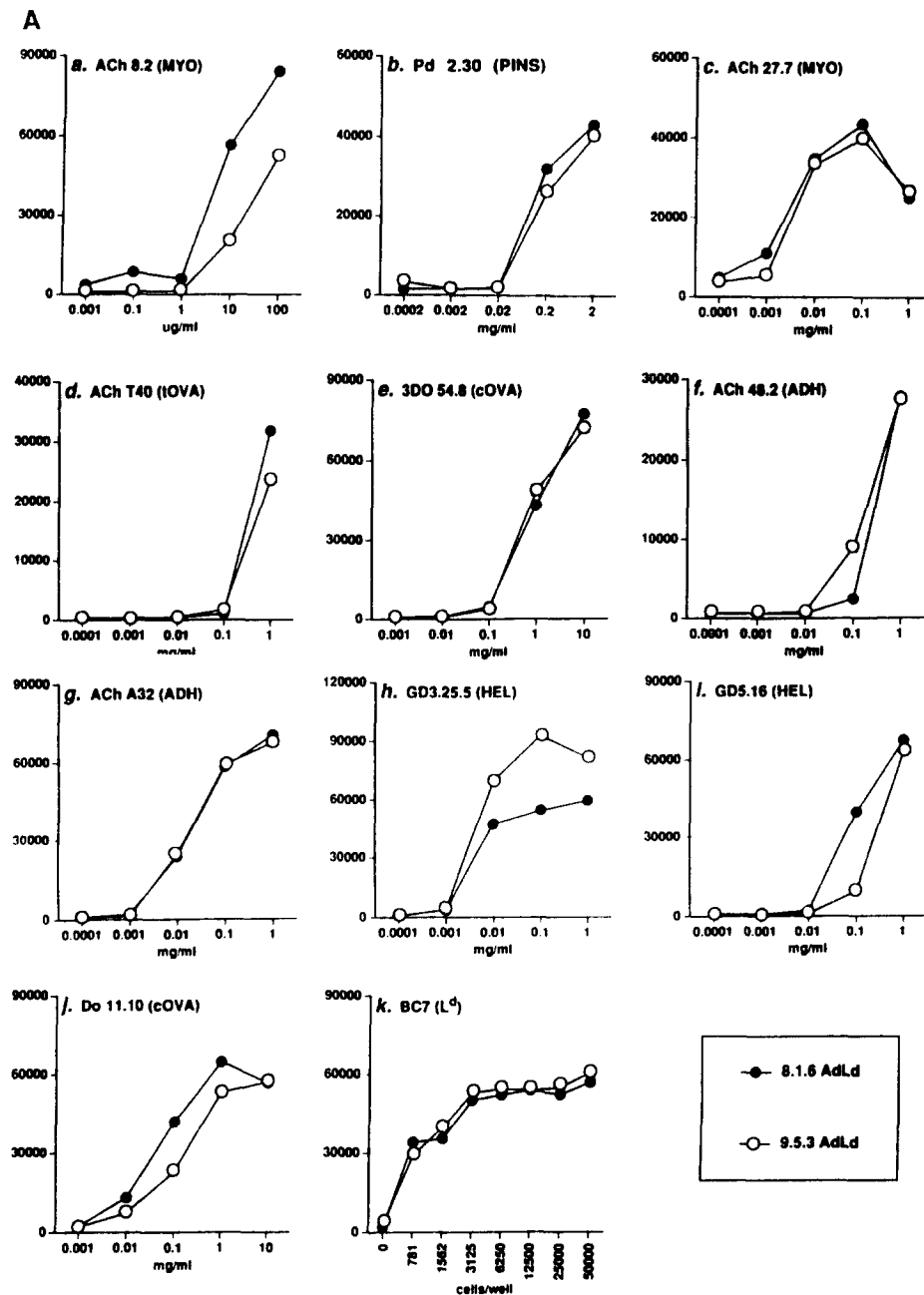
**Table 1.** *Ii Dependence for Antigen-specific T Cell Hybridomas*

| T cell   | Antigen | Effect of Ii |
|----------|---------|--------------|
| ACh A32  | ADH     | None         |
| ACh 48.2 | ADH     | + + +        |
| 3DO 54.8 | cOVA    | None         |
| DO 11.10 | cOVA    | None         |
| GD3.25.5 | HEL     | +            |
| GD5.16   | HEL     | +            |
| ACh 8.2  | hMYO    | None         |
| ACh 27.7 | hMYO    | +            |
| Pd 2.30  | PINS    | +            |
| ACh T40  | tOVA    | None         |

This table represents data showing either no effect of Ii (None), <5-fold enhancement (+), or a >20-fold enhancement (+ + +). Antigens are: cOVA, tOVA, ADH, HEL, hMYO, and PINS.

of using class II molecules from recycled sources and that this might account for our failure to observe an antigen presentation defect in 9.5.3. To test this possibility, we examined several of the T cells' requirements for newly synthesized class II molecules in antigen presentation using the transfected human lymphoblastoid cells. One of these was Ii independent (3DO 54.8) and two T cells that showed a slight Ii enhancement (GD3.25.5 and GD5.16). In the study by Pinet et al. (38), DM-independent presentation via recycled class II was not blocked by treatment with a protein synthesis inhibitor. We therefore tested the ability of both the 8.1.6 and 9.5.3 to present antigen to T cells after treatment with emetine. Fig. 6 shows that emetine treatment effectively inhibited antigen presentation to the T cells tested, while it did not inhibit peptide presentation. Collectively, these results suggest that antigen presentation in our system requires newly synthesized class II molecules or some other protein with a relatively short half-life. Together, our experiments suggest that the DM-independent antigen presentation seen in the 9.5.3 cell line is not due to a known alternative mechanism of antigen presentation.

*DR and I-A<sup>d</sup> SDS-stable Dimer Formation in the 8.1.6-A<sup>d</sup>L<sup>d</sup> and 9.5.3-A<sup>d</sup>L<sup>d</sup> Cell Lines.* To further examine the function of I-A<sup>d</sup> in the parent and mutant cells, we employed an additional assay that is thought to reflect peptide binding: an assay that shows the ability of class II molecules to form SDS-stable dimers (8-12). The ability of I-A<sup>d</sup> to form SDS-stable dimers in the 8.1.6 and 9.5.3 cell lines as well as in the T1, T2, and the murine B cell A20 was analyzed. In these experiments we also sought to confirm the observation that the mutant 9.5.3 and our 9.5.3-A<sup>d</sup>L<sup>d</sup> transfectants were deficient in the production of DR SDS-stable dimers. As shown in Fig. 7 A, 9.5.3 and 9.5.3-A<sup>d</sup>L<sup>d</sup> both show a marked decrease in the production of DR SDS-stable dimer relative to the parent cell line 8.1.6. Laser densitometry measurements, performed in several different experiments of this



**Figure 3.** Antigen presentation by 8.1.6 and 9.5.3 transfected cell lines to I-A<sup>d</sup>-restricted T cell hybridomas.  $5 \times 10^5$  APCs, either 8.1.6-A<sup>d</sup>L<sup>d</sup> (solid circles) or 9.5.3-A<sup>d</sup>L<sup>d</sup> (open circles) were cultured with  $5 \times 10^5$  T cell hybridomas. (A) Panels (a-j) represent presentation of exogenous native antigen present at the time of APC/T cell co-culture. Panel (k) represents the presentation of the endogenously synthesized L<sup>d</sup> epitope to BC7. (B) Peptide presentation by unfixed APC to a representative sample of T cells from (A). Peptides were prepared by trypsin digestion of each antigen. Abbreviations: cOVA, chicken ovalbumin; tOVA, turkey ovalbumin; MYO, horse myoglobin; PINS, pork insulin; HEL, hen egg lysozyme; ADH, alcohol dehydrogenase. During the course of the antigen presentation studies, parent and mutant cells either had the two- to threefold differences in cell surface density of I-A<sup>d</sup> (mutant two- to threefold lower than parent) shown in Fig. 1 or were indistinguishable in I-A<sup>d</sup> cell surface density, with the same results.

type quantified this as 15–30-fold reduction. Examination of the I-A<sup>d</sup> SDS-stable dimer in these cells led to two significant observations. First, in contrast to the results obtained with DR, I-A<sup>d</sup> SDS-stable dimer formation is decreased in the mutant cell line only by a factor of 2 to 4 as measured by densitometry, a result we have reproduced in numerous experiments (Fig. 7, B and C). These experiments suggest that while DM does affect I-A<sup>d</sup> SDS-stable dimer formation, it does so less severely than it does DR. Our second observation was that I-A<sup>d</sup> produces much less SDS-stable dimer in 8.1.6 than does DR, relative to total or unstable class II molecules.

**Comparison of SDS-stable Dimer Formation in Different Cellular Contexts.** To examine whether the overall deficiency

in the generation of I-A<sup>d</sup> SDS-stable dimer was due to an inherent property of I-A<sup>d</sup> or to a result of the species mixing of proteins from the human APCs and the murine class II molecules, we extended our studies to several additional cell types, using an immuno-Western procedure in which class II molecules are first isolated by immunoprecipitation and then prepared for a normal Western blot. Fig. 8 shows that although T1-A<sup>d</sup> produces SDS-stable dimer at levels comparable to the murine B cell line A20, both the 8.1.6 and 9.5.3 cell lines produce far less, a 20-fold reduction in 8.1.6 and an 80-fold reduction in 9.5.3 as compared with A20. In these experiments we also assessed whether allelic polymorphism might affect the formation of SDS-stable dimers. Fig. 8 shows that the mutant T2 cell line does not produce

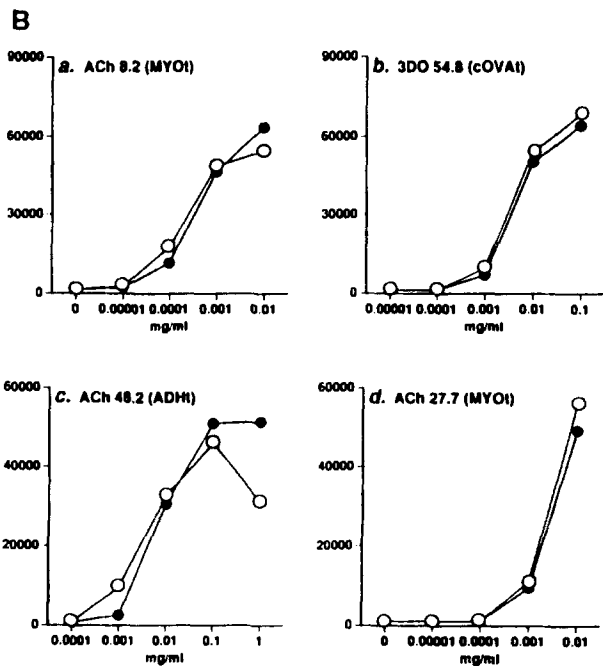


Figure 3.

significant levels of I-A<sup>d</sup> SDS-stable dimer. In contrast, I-A<sup>k</sup> molecules maintain high levels of SDS-stable dimer in the T2 cell line (Fig. 9). Although the total amount of I-A<sup>k</sup> is somewhat lower in the T2 transfectant when compared with the T1 transfectant, the amount of SDS-stable dimer relative to the total number of class II molecules is equal. The densitometry measurements revealed ratios of SDS-stable dimer to total class II in T1/T2 that ranged between 1:0.96 and 1:1.51 for I-A<sup>k</sup>, in contrast to the phenotype observed for I-A<sup>d</sup>, where SDS-stable dimer in the mutant was <2% of

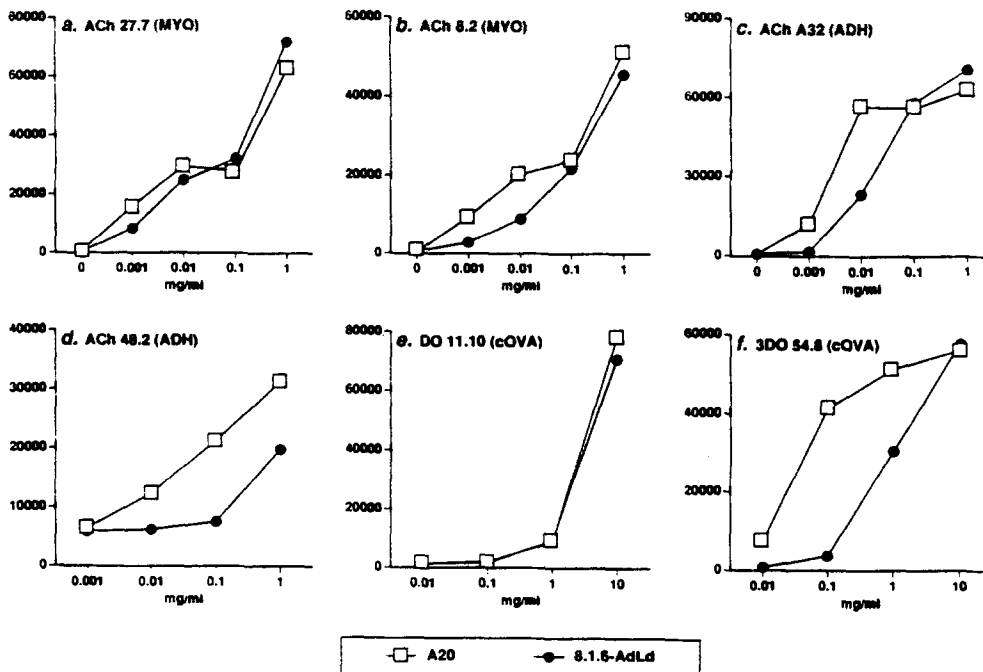


Figure 5. Comparison of antigen presentation ability of A20 and 8.1.6.  $5 \times 10^5$  APCs, either A20 (open squares) or 8.1.6-AdL<sup>d</sup> (solid circles), were cultured with  $5 \times 10^5$  T cell hybridomas. Indicated in each panel is the T cell used and its antigen specificity. Culture supernatants were tested for lymphokine content using the CTLL cell line as described in Materials and Methods. Data shown represent average cpm from triplicate cultures.

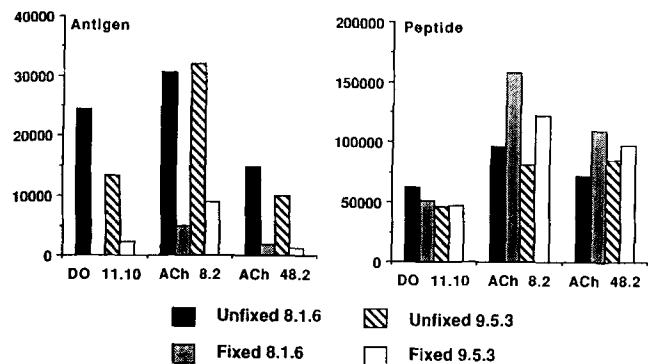
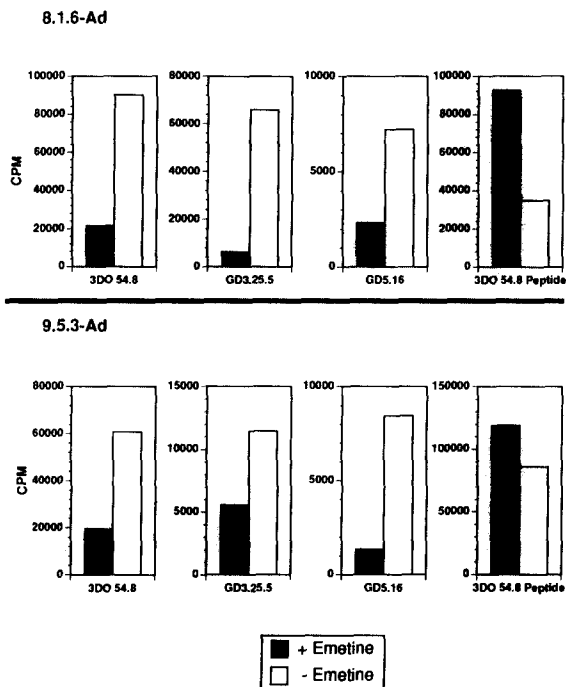


Figure 4. Effects of fixation on the ability of 8.1.6 and 9.5.3 transfected cell lines to present antigen and peptide.  $5 \times 10^5$  APCs, either fixed with 0.1% paraformaldehyde or unfixed, were cultured with  $5 \times 10^5$  T cell hybridomas and antigen or peptide at a concentration of 1 mg/ml except for intact OVA which was at 10 mg/ml. 8.1.6-AdL<sup>d</sup> (solid, unfixed; stippled, fixed) and 9.5.3-AdL<sup>d</sup> (striped, unfixed; open, fixed) presentation of antigen or peptide to three representative T cells from normal antigen presentation experiments.

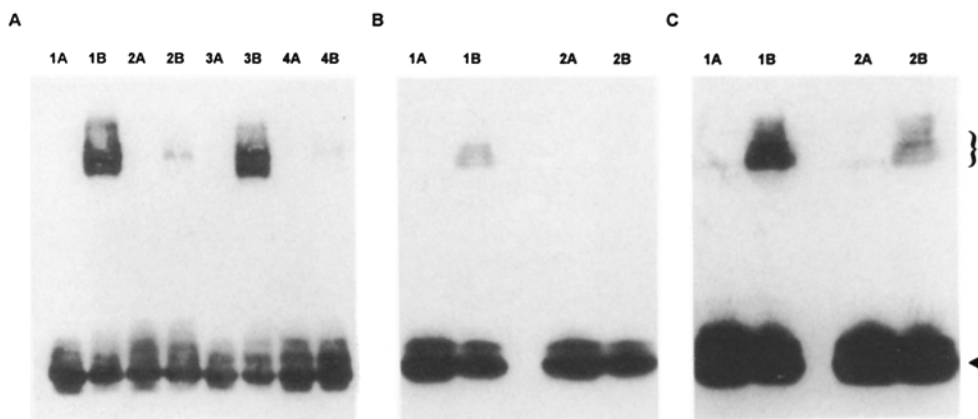
that observed in the parent. These findings suggest that different allelic forms of class II molecules are distinctive in their requirements for SDS-stable dimer formation and reinforce the hypothesis that DM gene products may not play a requisite role in many of the biological activities of the class II molecule.

### Discussion

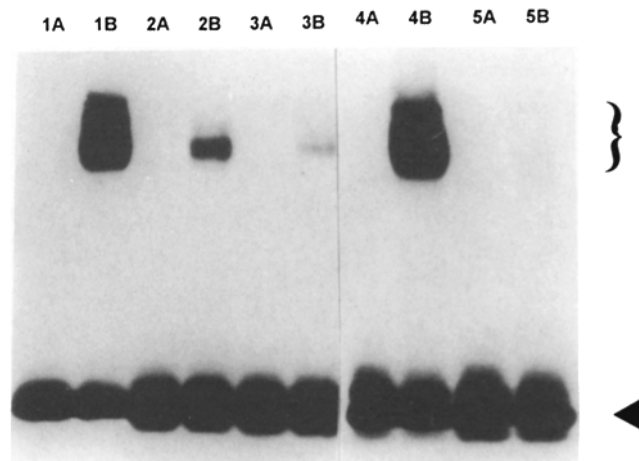
Previous studies have shown that the mutant cell line 9.5.3 has lost some conformational epitopes normally expressed on cell surface DR, lacks antigen presentation by DR, and the ability of DR and DP dimers to remain stable in SDS (16,



**Figure 6.** Effects of emetine on the ability of 8.1.6 and 9.5.3 transfected cell lines to present antigen and peptide. APCs were treated with 55  $\mu\text{g/ml}$  emetine for 4 h or cultured in medium alone. APCs were then pulsed with antigen for an additional 4 h in the presence or absence of emetine. APCs pulsed with antigen in the absence of emetine were treated with 55  $\mu\text{g/ml}$  emetine for 1 h at the end of the antigen pulse. Cells used for peptide control were treated in parallel without antigen.  $5 \times 10^5$  APCs either treated (solid bar) or untreated (open bar) were cultured with  $5 \times 10^5$  T cells. For GD3.25.5 and GD5.16, APCs were pulsed with 10 mg/ml HEL; for 3DO 54.8, APC were pulsed with 10 mg/ml cOVA. The peptide used for 3DO 54.8 was a tryptic digest of cOVA at 1 mg/ml. No enhancement of peptide presentation was seen for emetine-treated APC with several other T cells tested.



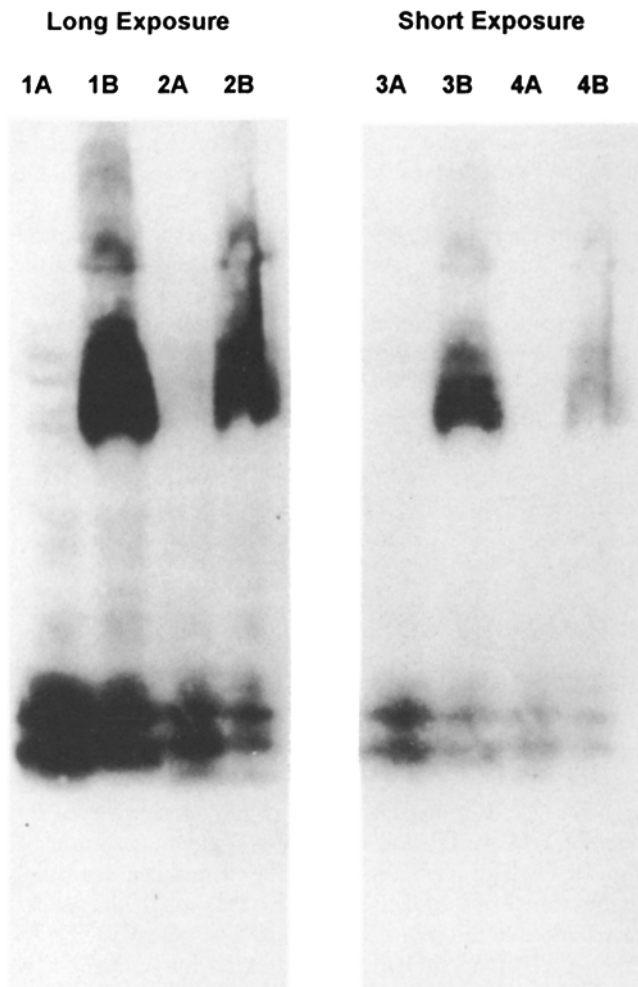
**Figure 7.** Analysis of class II dimer stability in the parent cell line 8.1.6 and mutant 9.5.3. Cell lines 8.1.6 and 9.5.3 were lysed in CHAPS/NP-40 lysis buffer. Post-nuclear supernatants were then adjusted to 1% SDS and incubated for 30 min at room temperature. Samples were split in half and either boiled for 5 min (lanes A) and separated by SDS/PAGE or loaded directly without boiling (lanes B). Proteins were transferred onto nitrocellulose membranes, probed with the anti-DR mAb HB10.A (panel A) or anti-I-A<sup>d</sup> $\beta$  mAb M5/114 (panels B and C), and developed by ECL. SDS-stable dimer is marked by the bracket and unstable class II by an arrow. (A) Western probed with HB10.A to determine DR stability of 8.1.6 (lanes 1A-1B), 9.5.3 (lanes 2A-2B), 8.1.6-A<sup>d</sup>L<sup>d</sup> (lanes 3A-3B), and 9.5.3-A<sup>d</sup>L<sup>d</sup> (lanes 4A-4B). Laser densitometry of autoradiograms indicated that 8.1.6 produces 15-30-fold greater levels of SDS-stable DR dimer than 9.5.3. (B) Western blot probed with anti-I-A<sup>d</sup> $\beta$  mAb M5/114 to determine I-A<sup>d</sup> stability on 8.1.6-A<sup>d</sup>L<sup>d</sup> (lanes 1A-1B), and 9.5.3-A<sup>d</sup>L<sup>d</sup> (lanes 2A-2B). Densitometry measurements indicate that the level of SDS-stable I-A<sup>d</sup> in 8.1.6 is only two- to fourfold greater than that of 9.5.3. (C) Long exposure of panel B.



**Figure 8.** Comparison of SDS-stable dimer levels between 8.1.6-A<sup>d</sup>L<sup>d</sup>, 9.5.3-A<sup>d</sup>L<sup>d</sup>, T1-A<sup>d</sup>L<sup>d</sup>, T2-A<sup>d</sup>L<sup>d</sup>, and A20. Cell were lysed in 0.5% CHAPS as for an immunoprecipitation experiment, as described in Materials and Methods. Class II molecules were isolated from the lysates using PAS-bound MKD6. Class II molecules were eluted from the PAS by incubation in sample buffer containing 1.0% SDS. Eluted samples were then split and half were subjected to boiling (A panels) or loaded directly (B panels). Lanes are A20 (1A-1B), 8.1.6-A<sup>d</sup>L<sup>d</sup> (2A-2B), 9.5.3-A<sup>d</sup>L<sup>d</sup> (3A-3B), T1-A<sup>d</sup>L<sup>d</sup> (4A-4B), and T2-A<sup>d</sup>L<sup>d</sup> (5A-5B). Laser densitometry measurements indicate that, when normalized to total class II molecules in the boiled sample, T1-A<sup>d</sup>L<sup>d</sup> produces 50% the stable dimer of A20, 5% 8.1.6-A<sup>d</sup>L<sup>d</sup>, and 1.25% 9.5.3-A<sup>d</sup>L<sup>d</sup>. T2-A<sup>d</sup>L<sup>d</sup> produces no detectable SDS-stable class II dimers.

17). Recent studies have shown that 9.5.3 has a point mutation in the DMB gene (22). To examine whether the phenotypes observed in these cell lines reflect a generalizable phenomenon, we undertook a series of studies to determine the biological impact of this mutation on I-A<sup>d</sup>. To accomplish this, we transfected the parent and mutant cell lines with





**Figure 9.** Western blot analysis of SDS-stable dimer formation in T1-A<sup>k</sup> and T2-A<sup>k</sup>. Cells were lysed for a normal Western blot, transferred to nitrocellulose, and probed with the anti-I-A<sup>k</sup> mAb 10.2.16. Lanes 1A–1B and 3A–3B represent T1-A<sup>k</sup> and lanes 2A–2B and 4A–4B are T2-A<sup>k</sup>. These cells are not matched for class II I-A<sup>k</sup> surface expression. Mean channel measurements from FACS<sup>®</sup> analysis indicate that T1-A<sup>k</sup> has two-fold more I-A<sup>k</sup> at the cell surface than T2-A<sup>k</sup>.

the genes encoding I-A<sup>d</sup> and examined a number of phenotypic characteristics that correlate with antigen presentation.

To determine if a change in class II conformation could be detected for I-A<sup>d</sup>, we used an extensive panel of I-A<sup>d</sup>-specific mAbs. These analyses indicated that the mutation in DM has no discernible effect on I-A<sup>d</sup> mAb epitopes, including one mAb epitope that is enhanced by Ii association with class II, K24-199. Although the use of a broad panel of mAbs that react with distinct class II epitopes suggests that I-A<sup>d</sup> conformation is not affected by the lack of functional DM, it is possible that there are segments of the I-A<sup>d</sup> molecule that are altered by the mutation in 9.5.3 but are not detected by the mAbs tested. The experiments described here also evaluated I-A<sup>d</sup>-restricted antigen presentation in the mutant cell lines. Our results, which were compiled using 11 different T cells and 7 different antigens, led to the conclusion that I-A<sup>d</sup>-restricted antigen presentation is not de-

tectably dependent on DM. Parent and mutant cell lines transfected with the murine class II molecule I-A<sup>d</sup> were able to process and present exogenously added antigen with similar efficiency. In addition, parent and mutant transfectants were equally effective at presenting an endogenous antigen, H2-L<sup>d</sup>. We have also shown that the ability to present antigen in the absence of DM holds for both Ii-dependent and Ii-independent T cells. Additionally, our studies revealed only a modest impact of the 9.5.3 defect on the ability of I-A<sup>d</sup> to form SDS-resistant dimers, significantly less than those detected for DR. It is interesting that the absolute levels of I-A<sup>d</sup> SDS-stable dimer appear to be quite low in the 8.1.6 cell. This could not be attributed to a generalizable property of I-A<sup>d</sup> because I-A<sup>d</sup> forms relatively high levels of SDS-stable dimers in the B cell line A20, nor to some consequence of a species mismatch of the murine class II structural proteins within a heterologous cellular context because I-A<sup>d</sup> forms SDS-stable dimers in T1 and loses this property in T2. Maturation of both human DR and murine I-A<sup>d</sup> appears normal in both parent and mutant cell lines, as do association and dissociation with Ii. Finally, we evaluated whether different allelic forms of class II molecules have similar requirements for SDS-stable dimer formation. Based on our observation that I-A<sup>k</sup> is capable of maintaining stable dimers in T2 while I-A<sup>d</sup> is not, it appears that DM is differentially required for expression of SDS-stable dimers by different class II molecules. Collectively, our results suggest that the requirement for DM may differ for structural variants of the class II molecule.

*I-A<sup>d</sup>-restricted Antigen Presentation by 8.1.6 and 9.5.3 Transfectants.* Our first finding, that there was no apparent difference in antigen presentation between 8.1.6 and 9.5.3, is of importance because it suggests that the function controlled by DM may not be ubiquitously required for presentation of all antigens and/or for all MHC class II molecules. We have considered a number of possible mechanisms to explain why DM is apparently not required for I-A<sup>d</sup>-restricted antigen presentation. Two observations suggest that it is unlikely that our results reflect a failure of murine class II molecules to interact productively with human DM-encoded proteins (thereby effectively making both 8.1.6 and 9.5.3 mutants for I-A<sup>d</sup>). First, 8.1.6 and the murine B cell line A20 present antigen equivalently for most T cells tested, and second, I-A<sup>d</sup> forms SDS-stable dimers in T1 at levels comparable to A20 and forms none in T2.

If in this system, I-A<sup>d</sup> does not require DM, how might it achieve this independence? One possibility is that the I-A<sup>d</sup>-restricted antigen presentation pathway has options for antigen presentation that do not rely on DM. This could be an alternative pathway of antigen presentation that I-A<sup>d</sup> can or does use that does not depend on DM. For example, DM may regulate access of class II molecules to an intracellular compartment that is requisite for DR-restricted presentation but not used extensively by I-A<sup>d</sup>. Alternatively, I-A<sup>d</sup> might have a structure that either by itself or through association with human Ii, mimics some conformation that is normally imparted by interaction with DM. Either scenario suggests a role for DM that is not critical for all class II-restricted antigen presentation. This type of activity would

thus be in contrast to the generalized need for TAP proteins in class I-restricted presentation.

A second possible explanation for the dichotomy between I-A<sup>d</sup>- and DR-restricted antigen presentation is that the antigens used for the I-A<sup>d</sup> and DR studies might be distinctive in some generalizable way. One difference in the two systems is the nature of the antigens and/or mode of immunization used to elicit the T cells. Typically in human studies, antigens are limited to those naturally encountered by an individual. Three of the antigens tested for presentation in the context of DR, gp 350, HBsAg, and purified protein derivative (PPD), are derived from pathogens that naturally access endogenous presentation routes, while the fourth, tetanus toxoid, is a bacterial product (17). In contrast, the T cells used in our I-A<sup>d</sup>-restricted model system were generated in response to soluble antigens in adjuvant introduced subcutaneously. Due to the differences in the primary stimulation involved in these responses, epitope generation might rely on different internal proteins. If this is true, we would then expect that I-A<sup>d</sup>-restricted presentation of pathogenic antigens to T cells generated in mice by normal parasitic infection might be more dependent on DM, a possibility we are currently investigating. Thus, it is possible that although we have used a broad panel of T cells and antigens, our panel nonetheless excludes epitopes that are DM dependent. The possibility that there is microheterogeneity in DM dependence even for a given class II restriction element has recently been suggested by Pinet et al. (38). Finally, it is possible that DM does participate in I-A<sup>d</sup>-restricted antigen presentation but its effect can only be detected under limiting conditions other than cell number and antigen concentrations, those tested in our system.

*Efficiency of Antigen Presentation Does Not Correlate with SDS-stable Dimer Formation.* Our second key observation is that across several different cell lines, there appears to be little correlation between antigen presentation ability and the extent of SDS-stable dimer formation. Our data have shown that SDS-stable dimer formation is reduced 4-fold between 8.1.6 and 9.5.3 and 20-fold between A20 and 8.1.6. Neither of these reductions had any observable effect on antigen presentation by the I-A<sup>d</sup> class II molecule expressed within these cells. We can hypothesize two separate explanations to account for this observed dichotomy. First, SDS-stable dimer formation for I-A<sup>d</sup> may be conferred primarily by a minor set of peptides that vary from cell to cell. This possibility is supported by the finding that the majority of peptides isolated from purified class II molecules belong to a small number of proteins that are primarily derived from within the APC and that vary depending on the particular allele of class II studied (39–41). Second, dissociation of SDS-stable dimer formation and antigen presentation could result if SDS-stable dimers are generated from the general pool of peptides within the cell but require precisely trimmed peptides. For example, longer peptides, although still capable of activating T cells, might not readily confer the SDS-stable phenotype. That only a subset of MHC binding peptides confer SDS-stable dimer is supported by recent *in vitro* binding studies as well as by studies with live APC (11, 42, 43). Certainly, the CLIP pep-

ptide(s) belong to the category of peptides that can bind to class II molecules yet fail to confer the SDS-stable phenotype (20, 44).

*The Function of DM in MHC Class II-restricted Antigen Presentation.* Upon the initial description of these mutants, a number of protein functions could be imagined that would lead to impaired SDS-stable dimer formation and antigen presentation. Among these might be proteins that mediate crucial class II trafficking events or loading of peptides onto the class II molecule. Any model that describes the function of DM in the mutants must now accommodate the following results. First, the ability of at least one MHC class II restriction element (I-A<sup>d</sup>) to present a variety of antigens is independent of DM. Second, in the T1/T2 cell lines using SDS-stable dimer as an assay for peptide binding to class II molecules, different alleles of murine class II are differentially dependent on DM. Third, T1 and 8.1.6 differ in their ability to form I-A<sup>d</sup> SDS-stable dimer. Finally, recent studies by other groups have shown that loss of DM results in an enrichment of an Ii fragment (CLIP) on the class II molecule (20, 44).

Most of these observations can be reconciled with a model in which DM keeps the MHC class II binding pocket free of CLIP. DM may function by extracting CLIP from the groove, removing CLIP from the peptide pool, or by mediating some transport event that leads to the separation of CLIP from the class II molecule. By any of these activities the absence of DM would lead to CLIP persistence on the class II molecule. This phenotype might be allele dependent based on the affinity of the CLIP peptide for the class II allele under examination. In this regard it is interesting to note that whereas I-A<sup>d</sup> and DR have similarly high affinities for human CLIP, I-A<sup>k</sup> is estimated to have as much as 1,000-fold lower affinity (Sette, A., personal communication). Consequently, I-A<sup>k</sup> may not need DM to have its binding pocket available for peptides that can confer SDS-stable dimers, which is consistent with its ability to form SDS-stable dimers in T2. Additionally, a variable impact of DM on antigen presentation can be explained if the persistence of CLIP on the class II molecule only affects presentation of those epitopes where the availability of "empty" class II *per se* is the limiting factor in antigen presentation. Thus, this model predicts that for I-A<sup>d</sup>-restricted antigen presentation, empty I-A<sup>d</sup> may not be the limiting element for epitope generation.

One prediction of this model is that the lack of DM would negatively affect SDS-stable dimer formation and antigen presentation only in the presence of Ii. In several models, class II and Ii molecules are introduced into cells that lack constitutive expression of class II, Ii, and possibly other coregulated molecules such as DM. Antigen presentation is frequently imparted by the expression of the class II molecules alone and can be significantly augmented by transfection with Ii (36, 45–48). If these cells lack expression of DM, one would anticipate that Ii expression would actually antagonize antigen presentation by effectively removing a significant amount of available class II molecules. This might certainly be expected in the case of a class II molecule that has a high affinity for CLIP, such as I-A<sup>d</sup> or DR. However antagonism of an-

tigen presentation by Ii has not been observed thus far (36, 45). One possible way to reconcile these results is the ability of Ii to enhance the export of class II molecules from the ER to endocytic compartments might balance the loss of available class II molecules later in the endosomal compartments due to CLIP binding (49, 50).

Regardless of the specific role or function of DM, these results show three clear observations. First, the antigen presentation defect seen for DR in the 9.5.3 cells does not detectably impair antigen presentation by I-A<sup>d</sup>. Second, changes in the level of SDS-stable dimer formation are not directly

related to changes in antigen presentation. Finally, different class II alleles have unique requirements for SDS-stable dimer formation and different cell types may vary in their ability to meet these requirements. These results question our current understanding of SDS-stable dimer formation and emphasize the need for greater understanding of the elements that are limiting and required for class II-restricted antigen presentation as a whole. Elucidation of these issues will be necessary to further unravel the mechanisms of class II-restricted antigen presentation.

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