Presentation of Antigen by Mixed Isotype Class II Molecules in Normal H-2^d Mice

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Summary

A panel of DBA/2 T cell hybridomas specific for the sperm whale myoglobin epitope 110-121 was found to recognize antigen presented by the mixed isotype class II molecule Eα^dAβ^d. The response was blocked by monoclonal antibodies specific for Eα and Aβ^d chains; in addition, the hybridomas responded to antigen presented by L cells expressing EαAβ^d molecules, and made no response with L cells expressing I-A^d or I-E^d molecules. Two more groups of hybridomas isolated from DBA/2 and B10.D2 mice immunized with myoglobin also recognized peptide 110-121 presented by Eα^dAβ^d. Thus, although it is expressed at biochemically undetectable levels on spleen cells, the Eα^dAβ^d molecule is an important presenting element in normal H-2^d mice making a conventional immune response to a protein antigen. These results suggest that high levels of class II expression are not a prerequisite for T cell activation.

Class II MHC-encoded molecules are heterodimeric membrane proteins that bind peptides and display them on the cell surface for recognition by T lymphocytes. The α and β chains of class II MHC molecules show very strong preferences for matched isotypic pairing (1, 2). High level expression of mixed isotype molecules can be achieved after transfection of certain combinations of α and β gene pairs into L cells (3–5). There is also biochemical, serological, and functional evidence for low-level expression on some B cell tumors (4, 6) and human EBV-transformed cells (7). EαAβ molecules can be detected on the spleen cells of mouse strains with defective Eβ expression (8), and also in mice expressing high levels of an Eα transgene (8–10,) where they can be recognized both as alloantigens (9) and as antigen-presenting molecules (10). Despite these latter results, it was thought that mixed isotype molecules were expressed at levels too low to play any physiological role in individuals with balanced class II chain expression. Here we show that the biochemically undetectable mixed isotype molecule Eα^dAβ^d constitutes a major presenting element in H-2^d mice for a determinant on sperm whale myoglobin (SpW Mb).[1] The quantitative aspects of this mixed isotype molecule suggest that the very low number of MHC molecules expressed is not limiting in T cell activation, and may have important implications for understanding certain autoimmune states.

Materials and Methods

Animals. DBA/2 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) or from the National Cancer Institute (Bethesda, MD). Males, 8–12 wk of age, were used for all experiments.

Antigens. SpW Mb was purchased from Serva (Heidelberg, Germany). SpW Mb peptides and 110-121 SpW Mb long chain biotinylated (LCB) peptide at the NH_2 terminus were synthesized using standard solid phase methods as previously described (11).

Antigen-presenting Cells. The L cell transfectants RT 7.7H7 14.3 (EαAβ^d) (3), RT 10.3 C1 (EαEβ^d) (3), RT 2.3.3H D6 (Aα^dAβ^d) (12), and CA36.2.1.Ii (Eα^dEβ^d and invariant chain) (13), and the A20 B cell lymphoma line (14) have been previously described. APC were exposed to 3,000 rad from a cesium source before coculture with T cells.

T Cell Hybridomas. The T cell hybridomas in Table 1, *A* and *D* were obtained by fusion of SpW Mb–specific T cell clones (15, 16) with the BW5147 TCR-α/β^- cell line (17) as previously described (18). Briefly, 3 d before fusion, the T cell clones were restimulated as previously described (16) in the presence of 5 U/ml of human rIL-2 (Cetus Corp., Emeryville, CA). After removal of dead cells on a Ficoll-Paque gradient, 2–10 $\times$ 10^6 cells were fused with 40 $\times$ 10^6 BW5147 cells as described (18). The antigen fine specificity of the T cell hybridomas was indistinguishable from that

[1] *Abbreviations used in this paper:* LCB, long chain biotinylated; SpW Mb, sperm whale myoglobin.

of the original T cell clones. The T cell hybridomas in Table 1, *B* and *C* were produced by fusion of SpW Mb–immune lymph node cells stimulated once in vitro and will be reported elsewhere (K. Sellins et al., manuscript in preparation).

IL-2 Assays. 0.1–1 × 10^5 hybridoma cells and 0.5–1 × 10^5 irradiated APC were cultured with serial dilutions of antigen in flat-bottomed microtiter wells. After a 24-h incubation at 37°C, 100 μl supernatant was harvested from each well, frozen, thawed, and added to 0.5–1.0 × 10^4 HT-2 cells in 100 μl medium to measure IL-2 production as previously described (18). Plates were pulsed with [^{3}H]TdR (1 μCi/well) at 20 h, and harvested at 24 h. Results are given as the mean of triplicate wells ± SD.

Antibody Blocking. A20 APC (10^5/well) were incubated at room temperature for 30 min with serial twofold dilutions of mAb. 10^4 hybridoma cells/well and antigen were then added, and the plates incubated for 24 h at 37°C. 100 μl supernatant was harvested from each well and assayed for IL-2 as described above.

Peptide Binding Assays. Binding assays with SpW Mb 110-121 LCB peptide were performed as previously described (11, 19). Briefly, class II MHC-negative cells; DAP3; EαEβ^d, Aα^dAβ^d, and EαAβ^d L cell transfectants (3 × 10^5) were incubated in Dulbecco's PBS containing biotinylated peptide at different concentrations at 37°C for 4 h in 96-well round-bottomed plates. Subsequently, the cells were stained with successive layers of fluoresceinated avidin D (10 μg/ml in PBS/0.1% BSA; Vector Laboratories, Burlingame, CA), biotinylated anti-avidin D (10 μg/ml in PBS/0.1% BSA; Vector Laboratories), and again fluoresceinated avidin D, each for 30 min at 4°C. The mean fluorescence of 5,000 stained cells was determined by flow cytometry on a FACScan® analyzer (Becton Dickinson Immunochemistry Systems, Mountain View, CA). The background, measured in the absence of peptide, as well as nonspecific binding signal to a class II MHC-negative fibroblast cell line, DAP3, was subtracted from each signal.

Results and Discussion

Analysis of a panel of DBA/2 (H-2^d) SpW Mb–specific T cell clones (20) originally suggested that six independent clones recognized the determinant 110-121 when presented by the Eα^dEβ^d class II molecule. First, the response could be blocked by the Eα-specific mAb 14.4.4S, and was apparently unaffected by the Aβ^d-specific antibody MKD6. Second, there was no response to antigen presented by D2.GD spleen cells; the D2.GD strain has functional $A\beta^d$, $A\alpha^d$, and $E\beta^d$ genes, but carries a defective $E\alpha$ allele (21), and is therefore Aα^dAβ^d (I-A)$^+$, and Eα^dEβ^d (I-E)$^-$.

Many of these clones were subsequently fused with the TCR-α/β^- BW5147 variant fusion partner (17) to generate T cell hybridomas, which were assayed on class II–expressing B cells and L cell transfectants. The Eα^dEβ^{d+} L cell line CA 36.2.1Ii, transfected with the $E\alpha^dE\beta^d$ and invariant chain genes (13), presented antigen very efficiently to the hybridoma 13.26.8-H6, specific for an epitope within residues 132–147, confirming the Eα^dEβ^d presentation of antigen to T cells specific for this epitope (22). However, CA 36.2.1.Ii cells were unable to present myoglobin or peptide to the 110-121-specific hybridoma 12.2.9-H7. Both hybridomas made excellent control responses to antigen presented by the BALB/c (H-2^d) B lymphoma cell line A20 (data not shown). Similar results were obtained using several other 110-121-specific hybridomas and Eα^dEβ^d-transfected L cell lines (G. Ruberti, unpublished results).

These experiments suggested that the 110-121-specific hybridomas did not recognize 110-121 presented by Eα^dEβ^d, even though the Eα^d chain clearly formed part of the presenting molecule. Eα^d chains expressed in H-2^d cells associated preferentially with Eβ^d chains, but co-transfection experiments have shown that Eα^d can pair with Aβ^d to be expressed as an isotype-mismatched class II molecule (2–4). Since all our data were consistent with Eα^dAβ^d-associated recognition of the 110-121 epitope, the antibody inhibition experiments were repeated using higher concentrations of MKD6 and 14.4.4S. The specificity of the antibody preparations is shown in Fig. 1, *A* and *B*. MKD6 blocked the response of an Aα^dAβ^d/106-118-specific hybridoma (11.12.8b-H5.1), but had no effect on the response of the Eα^dEβ^d/132-147-specific hybridoma 13.26.8-H6.1. Conversely, 14.4.4S

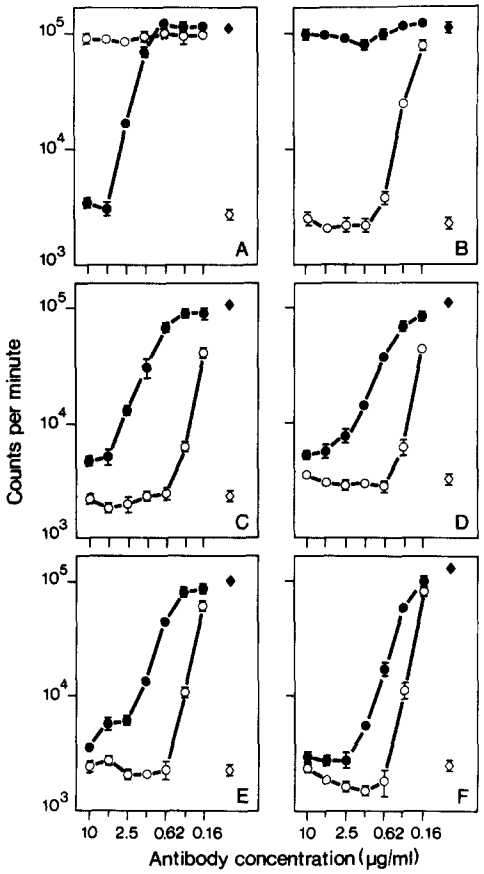

Figure 1. mAbs specific for Aβ^d and Eα block the T cell response of T cell hybridomas specific for peptide 110-121. T cell hybridomas were stimulated with SpW Mb presented by A20 cells in the presence of serial twofold dilutions of the Aβ^d-specific antibody MKD6 (●) or the Eα-specific antibody 14.4.4S (○); control wells contained medium alone (◇) or antigen without antibody (◆). Hybridomas were: (*A*) 11.12.8b-H5.1; (*B*) 13.26.8-H6.1; (*C*) 8.2.1d-Ha1.5; (*D*) 9.4.23.H1b.2; (*E*) 11.3.7e-H2.2.3; and (*F*) 12.2.9-H7.2. Those hybridomas are subclones of those in Fig. 2 and Table 1. Antigen concentrations, chosen to give slightly suboptimal proliferation, were 0.16 μM (*B* and *C*), 0.31 μM (*A* and *E*), 0.62 μM (*F*), or 1.25 μM (*D*). Results are given as the mean of triplicate wells ± SE.

Table 1. *Response of DBA/2 and B10.D2 T Cell Hybridomas to Peptide 110-121 Presented by EαAβ^d, EαEβ^d, and Aα^{d}Aβ^d L Cell Transfectants*

	Hybridomas	EαAβ^d	EαEβ^d	Aα^{d}Aβ^d
A	11.3.7e-H2	152,354	274	529
	8.2.1d-H1a	129,437	440	927
	12.2.9-H7	97,202	923	228
	9.4.23-H1b	141,817	2,456	493
	14.12.21-H10	119,136	2,410	2,425
	14.16.12-H7b	131,807	1,791	2,683
B	DB10 10.H7	115,140	2,924	1,590
	DB7 2.E2	133,117	1,591	1,339
	DB10 9.C6	84,063	2,751	1,227
C	B17 9.B4	129,299	2,410	2,324
	B17 9.C9	140,681	1,378	1,234
	B17 9.A10	195,889	722	1,226
	B13 5.G5	133,383	1,910	2,312
	B13 5.D10	82,717	780	210
	B13 5.C11	123,662	1,386	2,624
	B13 5.B8	141,378	440	927
	B19 4.E8	139,560	960	1,110
D	13.26.8-H6	2,090	101,170	2,241
	11.12.8b-H5	1,378	1,110	108,768

(*A*) 110-121-specific DBA/2 hybridomas derived by fusion of T cell clones isolated from long-term SpW Mb-specific T cell lines, as previously described (15, 16); (*B*) 110-121-specific DBA/2 hybridomas produced by fusion of SpW Mb-immune lymph node cells stimulated once in vitro; (*C*) 110-121-specific B10.D2 hybridomas produced by fusion of SpW Mb-immune lymph node cells stimulated once in vitro; (*D*) control responses of the DBA/2 hybridomas 13.26.8-H6 and 11.12.8b-H5 (15). All the hybridomas in *B* and *C* appear to be independent, as determined by TCR Vβ sequence or antigen fine specificity. (*A*, *B*, and *C*) hybridomas were stimulated with peptide 110-121 (50 μM); (*D*) 13.26.8-H6 and 11.12.8b-H5 were stimulated with peptides 132-147 (50 μM) and 102-120 (50 μM), respectively. Peptide was presented by the L cell transfectants RT 7.7H7 14.3 (EαAβ^d) (3), RT 10.3 C1 (EαEβ^d) (3), and RT 2.3.3H D6 (Aα^{d}Aβ^d) (12). Representative results of four experiments are shown. Results are expressed as the mean of triplicate wells in counts per minute. The SDs were within 10% of the mean values.

completely blocked the response of 13.26.8-H6.1, but did not inhibit the response of 11.12.8b-H5.1. Both antibodies blocked the response of four independent 110-121-specific hybridomas, 8.2.1d-H1a.5, 9.4.23-H1b.2, 11.3.7e-H2.2.3, and 12.2.9-H7.2 (Fig. 1, *C–F*). It was essential to assay antibody blocking at limiting antigen concentrations; at higher antigen concentrations, 14.4.4S still blocked the response almost completely, whereas MKD6 gave little if any inhibition. It has been shown that MKD6 binds poorly to EαAβ^d, compared with Aα^{d}Aβ^d (3, 4); this could explain why we previously saw no inhibition of the 110-121 response with MKD6, under conditions where conventional Aα^{d}Aβ^d-controlled responses were blocked quite efficiently.

To test directly whether Eα^{d}Aβ^d could present the 110-121 determinant, the hybridomas were assayed on L cells transfected with Aα^{d}Aβ^d (RT 2.3.3H D6) (12), EαEβ^d (RT 10.3 C1) (3), or EαAβ^d (RT 7.7H7 14.3) (3). Table 1 *A* shows that six independent 110-121-specific hybridomas responded to peptide 110-121 presented by the EαAβ^d-expressing L cells, but were not stimulated by this peptide on Aα^{d}Aβ^d or EαEβ^d transfectant cells. The control Aα^{d}Aβ^d (11.12.8b-H5)- and Eα^{d}Eβ^d (13.26.8-H6)-restricted hybridomas were stimulated by peptides 102-120 and 132-147 on Aα^{d}Aβ^{d+} and EαEβ^{d+} L cells, respectively, but were unable to recognize peptide presented by EαAβ^d transfectants (Table 1 *D*).

These six DBA/2 110-121-specific hybridomas were made from clones isolated from T cell lines stimulated several times in vitro before cloning. A second set of hybridomas was made from myoglobin-immune DBA/2 lymph node cells stimulated just once in vitro before fusion (K. S. Sellins et al., manuscript in preparation). Those hybridomas that made no response to antigen presented by D2.GD spleen cells were also

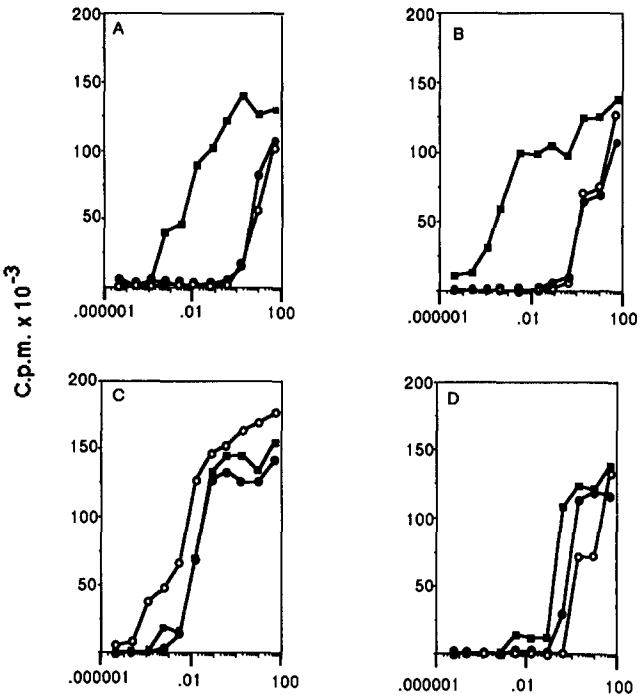

Figure 2. Comparison of antigen presentation by spleen cells, A20 cells, and class II MHC-transfected L cells. T cell hybridomas were cultured with serial fivefold dilutions of peptides 110-121 (*A* and *B*), 132-147 (*C*), or 102-120 (*D*), presented by DBA/2 spleen cells (○) A20 (●) or L cells (■) expressing EαAβ^d (*A* and *B*), EαEβ^d (*C*), or Aα^{d}Aβ^d (*D*); details of these L cell lines are given in the legend to Table 1. Hybridomas were: (*A*) 9.4.23-H1b; (*B*) 11.3.7e-H2; (*C*) 13.26.8-H6; and (*D*) 11.12.8b-H5. Representative results are shown from three experiments. Results are represented as counts per minute. Standard deviations were within 10% of the mean values. Similar results were obtained with the 110-121-specific hybridomas 8.2.1d-H1a and 14.16.12-H7b (data not shown).

found to recognize antigen presented by $E\alpha A\beta^d$, with no crossreactive recognition of peptide presented by $A\alpha^d A\beta^d$ or $E\alpha E\beta^d$ (Table 1 *B*). Finally, 110-121-specific hybridomas from the H-2^d strain, B10.D2, also made after a single in vitro restimulation and unresponsive to antigen presented by D2.GD spleen cells (K. S. Sellins, et al., manuscript in preparation), were again found to recognize antigen presented by $E\alpha A\beta^d$ (Table 1 *C*). Thus, presentation of peptide 110-121 by the mixed isotype molecule $E\alpha^d A\beta^d$ was a reproducible phenomenon that could be observed in two different H-2^d mouse strains.

In normal H-2^d cells, with balanced expression of the four class II chains, it has been estimated that $E\alpha^d A\beta^d$ molecules account for <5% of the class II molecules (2). To test this, L cells ($A\alpha^d A\beta^d$, $E\alpha E\beta^d$, or $E\alpha A\beta^d$) expressing MHC molecules at levels similar to that on A20 and H-2^d spleen cells were compared for their ability to present myoglobin peptides to the various hybridomas. For $A\alpha^d A\beta^d$- (Fig. 2 *D*) and $E\alpha^d E\beta^d$- (Fig. 2 *C*) restricted responses, the L cells gave dose response curves very similar to those seen with A20 and H-2^d spleen cells. In contrast, the $E\alpha A\beta^d$-transfected L cells allowed half-maximal responses at peptide concentrations three to five orders of magnitude lower than with A20 or H-2^d spleen cells (Fig. 2, *A* and *B*). These results were consistent with very low expression of $E\alpha^d A\beta^d$ relative to $A\alpha^d A\beta^d$ and $E\alpha^d E\beta^d$ on H-2^d cells, and demonstrated the efficiency of T cell recognition of peptide 110-121 presented by $E\alpha^d A\beta^d$ molecules.

Given the very low level of $E\alpha^d A\beta^d$ expression on H-2^d cells, the repeated isolation of 110-121-specific T cells that recognized the antigen presented by this molecule was rather surprising. One possibility was that peptide 110-121 bound very efficiently to $E\alpha^d A\beta^d$, so that the density of 110-121/$E\alpha^d A\beta^d$ complexes on the antigen-presenting cell surface was high enough to trigger T cell activation. Thus, although $E\alpha^d A\beta^d$ expression was low relative to $A\alpha^d A\beta^d$ or $E\alpha^d E\beta^d$, the number of 110-121/$E\alpha^d A\beta^d$ complexes could have approached that of peptides 102-120 and 132-147 bound to $A\alpha^d A\beta^d$ and $E\alpha^d A\beta^d$, respectively. This possibility was tested by measuring the mean fluorescent signals generated by the binding of LCB 110-121 peptide to $E\alpha E\beta^d$, $A\alpha^d A\beta^d$, and $E\alpha A\beta^d$ L cell transfectants. As demonstrated by data presented in Fig. 3, this peptide did not bind with detectably higher affinity to the hybrid MHC molecule than to the $A\alpha^d A\beta^d$ or $E\alpha^d E\beta^d$ molecules. Another possibility was that those T cells specific for 110-121/$E\alpha^d A\beta^d$ expressed TCRs with high affinity for this ligand. Among the 17 cell lines described here, all but one expressed Vβ8.2; of these, 14/15 Vβ8.2$^+$ chains sequenced were Vβ8.2/Dβ2.1/Jβ2.6, while the remaining clone expressed Vβ8.2/Dβ2.1/Jβ2.5 (15, 16, and K. S. Sellins et al., manuscript in preparation). The Vβ8.2/Dβ2.1/Jβ2.6 chain can pair with at least four different TCR-α chains to form TCR-α/β heterodimers specific for 110-121/$E\alpha^d A\beta^d$ (16), and it is tempting to speculate that this TCR β chain confers particularly high TCR affinity for peptide 110-121 presented by $E\alpha^d A\beta^d$.

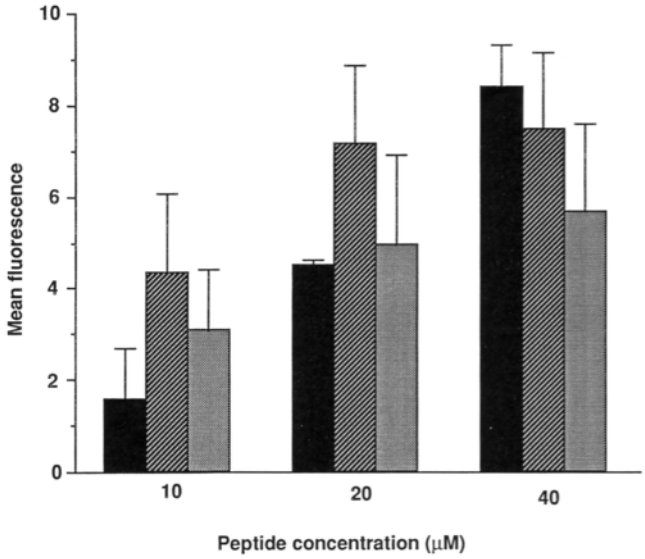

Figure 3. Mean fluorescent signals generated by the binding of LCB SpWMb 110-121 peptide to cells expressing $E\alpha E\beta^d$ (RT 10.3 C1) (■), $A\alpha^d A\beta^d$ (RT 2.3.3H D6) (■), and $E\alpha A\beta^d$ (RT 7.7H7 14.3) (▨) class II MHC molecules. The biotinylated peptide was assayed as described in Materials and Methods.

There has been considerable debate about any function in vivo for mixed isotype class II molecules (2, 23). The crucial issue concerns the level of cell surface expression, rather than any special properties of mismatched α/β pairs. Activation of class II–specific T cells in the periphery depends critically on class II density on the APC (24–26). Mixed isotype class II molecules, when expressed at sufficiently high levels, appear to have properties indistinguishable from those of isotype-matched molecules. They can be recognized as alloantigens (9), present conventional protein antigens (4, 6, 10), and can also cause partial deletion of T cell subsets in Mls-positive mice (8). However, the association of class II chains is influenced by strong isotype and haplotype preferences in α/β pairing (2–4, 27–29), so that cells with balanced Aα, Aβ, Eα, and Eβ chain synthesis express low or undetectable amounts of mixed isotype molecules. In previous reports, mixed isotype molecules have been recognized by T cells only when their expression is favored by co-transfection of particular class II α or β chains (4), by unbalanced synthesis of the isotypically matched α or β chain (6–8), or by the introduction of Eα transgenes expressed at high levels (8–10).

The present study shows that normal mice with balanced class II α and β chain synthesis can use isotypically mismatched molecules in a conventional immune response, even though these molecules are expressed at such low levels, relative to the isotypically matched class II molecules, that they can be biochemically undetectable on spleen cells (2, and R. Germain, unpublished results). These results suggest that high levels of class II expression are not a prerequisite for T cell activation. It is not possible to say from these experiments whether the low level of class II expression required for acti-

vation in the periphery reflects equally low thresholds for positive or negative selection in the thymus; it has been shown by others that levels of class II expression too low to stimulate antigen-specific or allogeneic responses nevertheless induce tolerance to much higher levels of the same class II molecule (9, 10, 30). These data raise questions about the role that mismatched MHC molecules might play in a physiological immune or autoimmune response without the necessity of invoking any unbalanced expression of isotype-matched chains (2, 23). Antigen presentation by mixed isotype molecules may well depend both on the particular antigen and on the available TCR repertoire. Presentation by mixed isotype molecules in vivo would greatly increase the repertoire of class II–restricted T cell responses, especially in humans, where individuals heterozygous for DR, DQ, and DP alleles potentially express a very large number of different class II molecules. Certain autoimmune diseases correlate with extended HLA-D haplotypes, rather than with particular DR, DQ, or DP alleles (31–33). Our results support the possibility that these diseases are induced or maintained by responses involving mixed isotype molecules (23).

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