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H2-DM (DM, previously H2-M) facilitates the exchange of peptides bound to MHC class II molecules. In this study, we have used H2-DM-deficient (DM<sup>−/−</sup>) mice to analyze the influence of DM in the priming of B cell responses in vivo and for Ag presentation by B cells in vitro. After immunization, IgG Abs could be raised to a T-dependent Ag, 4-hydroxy-5-nitrophénylacetyl-OVA, in DM<sup>−/−</sup> mice, but closer analysis revealed the IgG response to be slower, diminished in titer, and composed of low-affinity Abs. The Ab response correlated with a vast reduction in the number of germinal centers in the spleen. The presentation of multiple epitopes by H2-A<sub>b</sub> from distinct Ags was found to be almost exclusively DM-dependent whether B cells internalized Ags via fluid phase uptake or using membrane Ig receptors. The poor B cell response in vivo could be largely, but not completely restored by expression of a H2-Ea<sub>d</sub> transgene, despite the fact that Ag presentation by H2-Ea<sub>d/b</sub> molecules was found to be highly DM dependent. Hence, while substantial Ab responses can be raised in the absence of DM, this molecule is a crucial factor both for Ag processing and for the normal maturation of T-dependent humoral immune responses in vivo. The Journal of Immunology, 2001, 167: 6348–6355.

Upon translocation into the endoplasmic reticulum MHC class II molecules associate with the invariant chain (Ii) (reviewed in Refs. 1 and 2). Occupancy of the peptide-binding groove by the class II-associated invariant chain-derived peptide (CLIP; aa 81–104) region of Ii results in the promotion of complex assembly and prevention of immediate and inappropriate peptide (and polypeptide) binding within the endoplasmic reticulum. Furthermore, Ii binding directs the intracellular transport of class II molecules into the endosomal/lysosomal pathway where proteolytic activity results in degradation of Ii while leaving the peptide-binding groove of newly synthesized class II molecules occupied with CLIP. CLIP is subsequently exchanged for other peptides generated within the endosomal system. This peptide exchange is catalyzed by H2-DM (mouse) or HLA-DM (human) (together referred to as DM) (3–6), a resident of the endosomal/lysosomal system within APCs (7, 8). DM also serves as a molecular chaperone that stabilizes empty class II molecules and preserves their ability to bind peptides (9, 10). Mice (11–15) or human cell lines (16–18) lacking functional DM have a phenotype where class II-CLIP complexes remain largely intact and dominate the cell surface class II-peptide repertoire. In mice expressing only H2-A<sub>b</sub> (which binds CLIP with high affinity), thymic selection does occur but is ineffective, and the number of CD4<sup>+</sup> cells in peripheral organs is only 30–50% of normal (11–13). Interestingly, expression of transgenic H2-A<sub>b</sub> (which has very low affinity for CLIP) did not restore T cell numbers (14), suggesting that although the release of CLIP from class II molecules is essential for Ag presentation, the ability of DM to maintain class II molecules in a peptide-receptive state may be more important than its ability to catalyze the release of CLIP. This conclusion is further supported by the fact that DM edits the peptide repertoire also in mice lacking Ii expression (19, 20).

In this study, we have analyzed the extent and quality of the humoral responses in DM<sup>−/−</sup> mice. We found that after immunization these animals were capable of mounting T cell-dependent Ig production in vivo, although the quality of the responses in terms of kinetics, titers, and affinity maturation were decreased. The responses were improved, but not normalized in DM<sup>−/−</sup>-deficient mice expressing an H2-Ea<sub>d</sub> transgene. To investigate the molecular basis for the T-B interaction resulting in Ab production, we analyzed the presentation of multiple epitopes derived from the two protein Ags used for the immunization studies, OVA and hen egg lysozyme (HEL), and show that presentation by H2-A<sub>b</sub> and H2-Ea<sub>d/b</sub> molecules in general is highly dependent on DM, whether B cells internalized Ags by fluid phase or after uptake via membrane IgM receptors. Together the data presented demonstrate an essential role for DM in humoral immune responses including affinity maturation, but confirms that different class II molecules show different sensitivities to the absence of this molecule.

Materials and Methods

Mice and immunization

Generation of DM-deficient (DM<sup>−/−</sup>) mice has been described (11). Mice transgenic for membrane IgM receptors recognizing phosphorylcholine (21) or trinitrophenyl (22) were obtained from Dr. J. Kenny (Gerontology Research Center, National Institute on Aging, National Institutes of Health, Baltimore, MD). Mice transgenic for H2-Ea<sub>d</sub> were obtained from C. Surh (Department of Immunology, The Scripps Research Institute, La Jolla, CA). Such mice predominantly express H2-A<sub>b</sub> and H2-Ea<sub>d/b</sub> MHC class II molecules. All transgenic mice were used as heterozygotes at 6–10 wk of age.

Mice were immunized with 100 μg of 4-hydroxy-5-nitrophénylacetyl-OVA (NP-OVA) or NP<sub>e</sub>-HEL (NP-HEL; Biosearch Technologies, Novato, CA) in alum (Sigma-Aldrich, St. Louis, MO) i.p. Mice were eye-bled under anesthetic, sera isolated, and stored at −20°C.

Immunoprecipitations

Splenocytes were labeled with [35S]methionine as indicated before lysis in 1% Triton X-100, PBS, and complete proteinase inhibitor (Roche Diagnostics, Indianapolis, IN). H2-Ea<sub>d/b</sub> was immunoprecipitated with mAb 14-4-4S (23). Immunoprecipitates were harvested with protein A, washed, and...
resuspended in SDS-PAGE sample buffer with 2% SDS and 5% 2-ME. Samples were boiled for 5 min, then separated on 7.5–12.5% polyacrylamide gels. Gels were fixed, dried, and autoradiographed. Autoradiographs were scanned using an Agfa Arcus II scanner (Ridgefield Park, NJ). Composites were printed on a Kodak XLS 8600 printer (Kodak, Rochester, NY).

**T cell hybridomas**

DM+/− mice were immunized in the hind footpad with 50 μg of OVA (Sigma-Aldrich) or HEL (Calbiochem, La Jolla, CA). After 7–10 days, draining lymph node (LN) cells were restimulated in vitro with OVA or HEL (50 μg/ml) and T cell blasts were expanded in RPMI 1640 with 10% FBS and 5% supernatant from Con A-stimulated mouse splenocytes. T cells were restimulated in vitro and fused with BW5147/TCR α β thymoma cells (24). Ag-specific T cell hybridomas were selected and expanded using standard protocols. The exact epitopes have not been mapped for all OVA-specific hybridomas, but HPLC-fractionated tryptic digests of OVA define distinct specificities between the individual hybridomas. HEL-specific T cell hybridomas H30.44 and H46.13 were made after immunization of DM+/− mice with peptides HEL 30–53 and 46–61, respectively. LN cells were stimulated in vitro with HEL and subsequently fused with BW5147/TCR α β cells. OVA-specific hybridomas 426.6 was generated after fusion of activated CD4+ T cells from OT-2-transgenic mice. T cell hybridoma BO4 was obtained from A. Rudensky (Department of Immunology and Howard Hughes Medical Institute, University of Washington School of Medicine, Seattle, WA). H2-Eα-restricted T cell hybridomas were generated after immunization of H2-Eα/H2-A−/− mice (generated by breeding H2-Eα-transgenic mice with H2-Ab−/−deficient mice) with HEL or OVA.

**Ag presentation assays**

Primary B cells (purification described previously (25)) were cultured (4 × 10^5 cells/well) in triplicate with T cell hybridomas (2 × 10^5 cells/well) and Ag. Supernatants from overnight cultures were assayed for IL-2 production by ELISA. For Ig receptor-mediated uptake and presentation to T cell hybridomas, B cells were incubated on ice for 30 min, followed by a 30-min pulse with Ag on ice. Non-Ig-bound Ag was removed by centrifugation through a FBS density gradient. Ag-pulsed B cells were split equally between the T cell hybridomas used (giving 4 × 10^5 B cells/well) and cultured overnight. Data (IL-2, ng/ml) are expressed as mean values of triplicate cultures ± SD. Titrinophenyl (TNP)-conjugated OVA (TNP-OVA) was obtained from Biosearch Technologies. Phosphorycholine (PC)-conjugated OVA (PC-OVA) and PC-HEL were prepared as described elsewhere (26).

For recall responses in vitro, mice were immunized in the hind footpad with peptide HEL 20–35 in IFA. After 8 days, draining LN cells were isolated and cultured (2 × 10^5/well) with Ag for 72 h. Cell proliferation was determined by 3[H]thymidine incorporation during the final 16 h of culture. Cells were counted using a Topcount (Packard Instrument, Meriden, CT) with liquid scintillation (Microscint; Packard Instrument).

Data are expressed as mean cpm ± SD.

**Ab assays**

NP-specific Abs were measured by ELISA using plates coated with NP35-BSA or NP2-BSA (1 μg/ml; Biosearch Technologies) in PBS to determine total or high-affinity Abs, respectively. Bound Ig was detected using alkaline phosphatase-conjugated goat anti-mouse IgM or IgG subclass Abs (Southern Biotechnology Associates, Birmingham, AL) and assays were developed using Sigma 104 (Sigma-Aldrich) in diethanolamine substrate buffer (Pierce, Rockford, IL). OD 405 nm was measured and values are expressed as arbitrary units after reference to standard hyperimmune anti-NP sera.

**Immunohistochemistry and immunofluorescence**

Mice were immunized with 100 μg of NP-OVA in alum (i.p.). Spleens were removed, embedded in OCT Tissue-Tec (Sakura Fine Tek, Torrance, CA) and frozen on dry ice 7 or 14 days after immunization. After acetone fixation, cryosections were incubated with fluorescein-conjugated peanut agglutinin (FITC-PNA) (Vector Laboratories, Burlingame, CA) or biotinylated NP35-BSA followed by Alexa 594-streptavidin (Molecular Probes, Eugene, OR). For the immunohistochemistry, biotin-PNA or biotin-conjugated primary Abs and an avidin-biotin complex kit with diaminobenzidine were used (Vector Laboratories) along with hematoxylin counterstaining.

**Results**

**T-dependent Ab production in DM-deficient mice**

Ab responses have not been extensively studied in DM-deficient mice, but immunized animals have been reported to be able to generate IgG responses to keyhole limpet hemocyanin (KLH), as well as to vesicular stomatitis virus (27). This is somewhat surprising considering that presentation of internalized Ags in the absence of DM is usually poor, and thus we decided to investigate T-dependent Ab responses more extensively.

DM+/− mice or wild-type littermates were immunized i.p. with a high dose (100 μg) of NP-OVA or NP-HEL in alum. Mice were bled at the time points indicated in Fig. 1. After immunization, DM+/− mice generated a substantial NP-specific IgG1 response in the NP-OVA immunized mice, although the primary IgG1 response was diminished and delayed compared with that of wild-type mice (Fig. 1, a and b). At later stages of the response, the magnitude of the IgG1 response showed a 10- to 20-fold reduction in DM+/− mice compared with wild-type mice. This reduction remained also after a booster immunization (data not shown). The poor serum IgG responses in DM+/− mice were, at least in part, caused by a reduced number of plasma cells, since ELISPOT analysis showed a marked reduction in the number of NP-specific Ab-secreting cells (data not shown).

In contrast to the immunization with NP-OVA, DM−/− mice immunized with NP-HEL were unable to mount a detectable IgG1 Ab response to NP (Fig. 1, c and d), yet the IgM response was normal, showing the immunization to be successful (data not shown).

The ability to produce anti-NP Abs in the NP-OVA-immunized DM-deficient mice suggests that for this Ag sufficient Ag processing occurs to allow productive, although inefficient T-B cell interaction. Severely impaired affinity maturation correlates with aberrant germinal center formation in DM−/− mice

The delayed and reduced Ab response to NP-OVA prompted us to investigate whether DM influenced the generation of high-affinity

**FIGURE 1.** T-dependent Ab production in DM−/− mice. DM+/− (a and c) or DM−/− (b and d) were immunized with 100 μg of NP-OVA (a and b) or NP-HEL (c and d) in alum (i.p.). Serum IgG1 Abs binding NP2-BSA were measured on the indicated days. NP2-specific IgG Abs are expressed as arbitrary units after reference to a standard hyperimmune anti-NP serum.
Abs. Levels of high-affinity NP-specific Abs were determined by binding sera from NP-OVA-immunized mice to plates coated with a sparsely haptenated substrate (NP$_2$-BSA) (Fig. 2). Strikingly, high-affinity NP-specific IgG1 Abs were virtually undetectable throughout the primary response of DM$^{+/+}$ mice, whereas the characteristic delayed production of high-affinity Abs during the primary response was observed in wild-type mice. The almost complete absence of NP$_2$-binding Abs shows that DM is essential for this process.

The absence of hapten-specific high-affinity Abs in DM$^{-/-}$ mice may reflect absent or abnormal germinal center reactions. To investigate this possibility, splenic architecture was examined in cryosections of DM$^{+/+}$ or wild-type mice. As shown in Fig. 3A, a and d, and B, a and c, analysis of spleen sections from nonimmunized mice showed a few small germinal centers. After NP-OVA immunization, large germinal centers were abundant (using PNA staining) in wild-type spleen both at days 7 and 14 (Fig. 3A, b and c, and Bb). In contrast, germinal centers were virtually undetectable in splenic sections from DM$^{-/-}$ mice (Fig. 3A, e and f, and Bd). Using high magnification, small numbers of atypically organized PNA-reactive cells were occasionally detected in the sections from DM$^{+/+}$ mice (Fig. 3Bb). Analysis of Ag-specific B cells at day 7 (using NP-staining) revealed few Ag-specific cells in sections from DM$^{-/-}$ mice (Fig. 3B, i and j), whereas such cells were readily detectable in germinal centers in the wild-type spleen sections (Fig. 3B, e–g). Similar results were obtained at day 14 (data not shown). These data show that both germinal center formation and the resulting maturation of Ab affinity are significantly reduced in DM$^{-/-}$ mice.

DM$^{-/-}$ mice expressing H2-Ea$^{a/b}$

Two reports have described H2-E-expressing DM-deficient mice, either by expression of a H2-Ea$^{a}$ transgene (14) or by deletion of DM in BALB/c stem cells (15). H2-E molecules from these mice had decreased SDS stability, as well as an increased content of CLIP peptides when compared with H2-E molecules from wild-type mice. Pulse-chase analysis of metabolically labeled splenocytes show that also H2-Ea$^{a/b}$ molecules expressed in DM$^{+/+}$ mice (generated by breeding with H2-Ea$^{a}$-transgenic mice) are to a larger extent occupied by CLIP (Fig. 4A). A large part of the H2-Ea$^{a/b}$ molecules were associated with CLIP after 2 h of chase and the CLIP content did not substantially decrease after a 24-h chase, suggesting that CLIP-H2-Ea$^{a/b}$ complexes are very stable under physiological condition. However, the complexes are not SDS stable even if boiling is omitted before gel analysis (data not shown).

To address the influence of H2-E expression on Ab responses, H2-Ea$^{a}$-transgenic mice on a DM$^{+/+}$ or DM$^{-/-}$ background were immunized with 100 µg of NP-OVA in alum i.p., and sera were collected on the days indicated. Fig. 4B, a and b, show that the presence of the H2-Ea$^{a}$ transgene resulted in DM$^{+/+}$ mice mounting IgG1 responses comparable to those of wild-type mice; both the kinetics and the magnitude of the primary response were restored to near normal levels. Moreover, high-affinity IgG1 Abs were readily detectable during the primary response (Fig. 4B, c–f), although the restoration of affinity maturation in H2-Ea$^{a}$-transgenic DM$^{-/-}$ mice was partial rather than complete.

Germinal center formation in H2-Ea$^{a}$-transgenic mice was investigated 7 or 14 days after immunization with NP-OVA in alum (i.p.). Fig. 5 shows that few if any PNA-positive cells were detectable in the sections from the DM$^{-/-}$ H2-Ea$^{a}$-transgenic mice, either at day 7 or day 14.
Thus, although expression of H2-Ea does improve the Ab response to NP-OVA in the absence of DM, both germinal center formation and affinity maturation remain significantly impaired.

**Ag presentation by DM+/− B cells**

Several reports have demonstrated that normal MHC class II-restricted Ag presentation is dependent on DM (reviewed in Ref. 6), and this is particularly true in the case of H2-A, which appears to be exquisitely DM sensitive. However, with a couple of exceptions (14, 28), most studies to date have described the presentation of single epitopes from different Ags, and it is not clear that different epitopes in a particular Ag are equally DM dependent.

The Ab responses to NP-OVA in the DM-deficient H2-Ab-expressing mice, as well as the H2-Ea-transgenic animals, led us to investigate whether some epitopes were presented by H2-A or H2-Eb on B cells in the absence of DM. To do this, we generated H2-A- or H2-Eb-transgenic T cell hybridomas against several distinct epitopes of OVA and HEL and several H2-Eb-restricted hybridomas against OVA. Fig. 6 shows the IL-2 response of OVA- and HEL-specific T cell hybridomas after overnight culture with intact protein together with primary B cells from wild-type or DM+/− mice (Fig. 6, a, c, and e). All of the T cell hybridomas recognized processed Ag presented by wild-type B cells in a dose-dependent MHC-restricted manner. In contrast, with one exception, all H2-A- or H2-Eb-transgenic T cell hybridomas examined completely failed to recognize Ag presented by DM+/− B cells even at the highest doses, indicating a strict DM dependency for presentation of different epitopes by H2-A or HEL. Surprisingly, we found that a single T cell hybridoma (Hb1.9) recognized HEL processed and presented by DM+/− B cells equally well as HEL presented by wild-type cells, suggesting that this epitope (HEL 20–35) was presented by H2-A independently of DM. Three of the four H2-Eb-restricted OVA-specific T cell hybridomas examined were essentially unable to recognize OVA presented by DM+/− B cells, yet responded well to OVA presented by DM-expressing B cells (Fig. 6e). A small response could be reproducibly elicited from T cell hybridoma OEB14 at a high Ag dose by DM+/− B cells, although this response was greatly reduced compared with the response to DM+/+ B cells. This result suggests that, similar to the presentation of OVA by H2-A, the presentation by H2-Eb molecules is predominantly dependent on DM.

Similar responses were observed from all OVA- or HEL-specific T cell hybridomas when splenocytes rather than B cells were used as APCs or when NP-OVA or NP-HEL were used as Ags instead of OVA and HEL (data not shown).

Presentation of exogenous peptides has also been reported to be altered for DM-defective cells (12–16). In this study, culturing synthetic peptides of OVA or HEL with DM+/− H2-A-expressing B cells generally elicited 10- to 100-fold weaker responses from T cell hybridomas than when the peptides were presented by wild-type cells (Fig. 6, b and d). In contrast to other peptides, the presentation of peptide HEL 20–35 to H2-Ab transgenic spleens prepared either before (a and d), 7 days (b and e), or 14 days (c and f) days after immunization with NP-OVA in alum (i.p.). Sections were stained as described in Fig. 3A legend.

**FIGURE 4.** Influence of H2-Ea transgene on humoral immune responses in DM+/− mice. A, Immunoprecipitation from 35S-labeled H2-Ea-transgenic spleen cells on DM+/− (left) or DM+/− (right) background. Splenocytes were labeled for 30 min, then either analyzed immediately (0 min) or after chase in nonradioactive medium for the indicated time (in hours), H2-Eb was immunoprecipitated and samples were analyzed after boiling and reduction. Size markers are in kilodaltons. B, H2-Ea-transgenic mice on DM+/− (a, c, and e) or DM+/− (b, d, and f) background were immunized with 100 μg of NP-OVA in alum (i.p.). Sera were measured for IgG1 Abs binding NP23-BSA (a and b) or NP2-BSA (c and d). Serum Abs are expressed as arbitrary units after reference to a standard hyperimmune anti-NP serum. The increased NP2/NP23 ratio over time (e and f) is a measure of the degree of affinity maturation in the immune response.

**FIGURE 5.** Absence of germinal centers in immunized H2-Ea-transgenic DM+/− mice. Sections of DM+/− (a–c) or DM+/− (d–f) H2-Ea-transgenic spleens prepared either before (a and d), 7 days (b and e), or 14 days (c and f) days after immunization with NP-OVA in alum (i.p.). Sections were stained as described in Fig. 3A legend.
Together, these data show that DM is essential for the processing and presentation of a number of different H2-A\(^{b}\)- and H2-E\(^{d/b}\)-restricted epitopes derived from OVA and HEL. We were unable to find any H2-A\(^{b}\)-restricted OVA epitopes that were presented by the DM-deficient cells and only one H2-E\(^{d/b}\)-restricted epitope that was recognized, although very poorly. In contrast, one H2-A\(^{b}\)-restricted HEL-reactive T cell hybridoma appeared to recognize the relevant epitope in a DM-independent manner.

**Poor recognition of processed HEL 20–35 in DM\(^{-/-}\) mice**

Hybridoma Hb1.9 appeared to recognize an epitope from HEL independently of DM, yet no IgG response against NP-HEL could be detected after immunization. If hybridoma 1.9 was representative of HEL 20–35-reactive T cells, then the lack of an Ab response to HEL would suggest that no HEL 20–35-reactive T cell precursors were present in the DM\(^{-/-}\) mice. To address whether this was the case, DM\(^{+/+}\) and DM\(^{-/-}\) mice were immunized with peptide HEL 20–35 in IFA. Draining LN cells were isolated 8 days later and assayed in vitro for recall responses to HEL. Fig. 7 shows that both primed DM\(^{-/-}\) and DM\(^{+/+}\) LN cells proliferated vigorously to the immunizing peptide, demonstrating that HEL 20–35-reactive T cell precursors were present in the DM\(^{-/-}\) mice. In contrast, only a minimal response by the DM\(^{-/-}\) cells was observed after culture with intact HEL, suggesting that the repertoire of HEL 20–35-specific T cells was essentially unable to recognize endogenously processed HEL presented by DM\(^{-/-}\) LN cells in vitro. In contrast, wild-type LN cells recognized intact HEL very well, implying this HEL epitope to be efficiently processed by DM\(^{+/+}\) LN cells. The lack of reactivity of the DM\(^{-/-}\) T cells in response to intact HEL is likely to reflect poor presentation by the DM\(^{-/-}\) APCs, suggesting that hybridoma 1.9 is not representative of HEL 20–35-reactive T cells in DM\(^{-/-}\) mice.

Ig-mediated internalization of Ag does not overcome DM-dependent presentation on H2-A\(^{b}\) or H2-E\(^{d/b}\) molecules

It has previously been reported that receptor-mediated uptake of Ag can overcome the HLA-DM dependency of Ag presented by HLA-DR4 molecules (29), and thus we investigated the DM dependence of presentation after Ig receptor-mediated uptake of Ag by B cells. Mice bearing transgenes encoding one of two independent membrane IgM receptors specific for the haptens TNP (22) or PC (21) were bred together with DM\(^{-/-}\), H2-E\(^{d}\)-transgenic DM\(^{-/-}\), or control mice. Transgenic B cells from the different mouse strains were analyzed for their ability to present multiple epitopes after Ig-mediated uptake of hapten-conjugated Ags (Fig. 8). Primary splenic B cells were pulsed with Ag for 30 min at 4°C, separated from excess non-Ig-bound Ag, and cultured overnight with T cell hybridomas at 37°C. The majority of H2-A\(^{b}\)-restricted
T cell hybridomas examined recognized Ag presented by Ig-transgenic wild-type B cells after Ig-mediated uptake, although variations were observed in the responses of individual hybridomas (Fig. 8, a–c). None of the H2-A\textsuperscript{b}-restricted hybridomas were able to recognize Ag after Ig-mediated uptake by DM\textsuperscript{−/−} B cells regardless of the specificity of the Ig-transgenic B cells, suggesting that internalization of Ag by membrane Ig was unable to overcome the DM dependency of presentation by H2-A\textsuperscript{b} on B cells. This indicates a strict DM dependency for presentation by H2-A\textsuperscript{b} molecules whether Ag is internalized via fluid phase or via membrane Ig receptors.

B cells doubly transgenic for anti-PC Ab and H2-E\textsuperscript{a} on either DM\textsuperscript{−/−} or DM\textsuperscript{+/+} backgrounds were examined for their ability to stimulate OVA-specific H2-E\textsuperscript{a/b}-restricted T cell hybridomas after Ig-mediated uptake of PC-OVA (Fig. 8d). All T cell hybridomas examined recognized OVA presented by H2-E\textsuperscript{a/b} molecules after Ig-mediated Ag uptake. Analogous to the H2-A\textsuperscript{b}-restricted hybridomas, variations in the dose-response curves of individual H2-E\textsuperscript{a/b}-restricted hybridomas could be detected which may reflect differential presentation of individual epitopes or variations in hybridoma sensitivity. However, internalization of Ag via the Ig receptor by DM\textsuperscript{−/−} B cells could not overcome the defect in presentation to the H2-E\textsuperscript{a/b}-restricted hybridomas. Thus, as was the case for H2-A\textsuperscript{b}, Ag presentation on H2-E\textsuperscript{a/b} molecules showed strict DM dependency whether Ag was internalized via fluid phase or by membrane Ig receptors.

Discussion

A number of studies have shown that DM is essential for normal presentation of MHC class II-restricted Ags to CD4\textsuperscript{+} T cells (6, 11–13, 30, 31). However, despite the very poor ability to process and present protein Ags by APCs from DM-deficient H2-A\textsuperscript{b}-expressing animals, IgG responses can clearly be raised against some protein Ags after immunization, illustrated by KLH (27) and NP-OVA (this report). However, the onset and amplitude of the responses were markedly delayed and decreased when compared with the responses in DM-expressing mice, illustrating the poor efficiency of B cell-T cell interaction. This is likely to be a direct reflection of the poor ability of APCs (including B cells) to present the immunized Ag, but it is also unclear to what extent the T cell repertoire in DM-deficient mice would be able to support a normal humoral response if the Ag presentation by APCs was restored to normal levels. The CD4\textsuperscript{+} T cells in DM-deficient mice expressing only H2-A\textsuperscript{b} are present at 30–50% of the normal levels and many of these cells are reactive with self-Ags (11–13). In bone marrow chimeras where positive selection is mediated by DM-deficient thymic epithelium, while negative selection is mediated by DM-expressing bone-marrow derived cells, only 5% of the normal
number of cells survived (27, 32, 33). The repertoire of these surviving cells is likely to be more limited than in normal animals, yet these cells can recognize some presented peptide epitopes, including epitopes from KLH and OVA (27, 33). Our failure to detect any IgG response against HEL, despite the fact that immunization with the peptide HEL 20–35 raised a healthy response in DM-deficient mice, also suggests that the poor presentation of Ag per se may be the main reason for the poor Ab response.

The almost complete absence of affinity maturation in the DM-deficient H2-A^b-expressing mice, as well as the absence of normal germinal centers, indicates that the interaction between B cells and T cells which results in the initial production of class-switched IgG1 Ab is less DM dependent than the later interaction which results in the formation of germinal centers. This may simply be a reflection of the amount of processed Ag that is available; immunization with a small dose of NP-OVA (1 µg) is not sufficient to raise an IgG Ab response in DM-deficient animals (data not shown), and the formation of germinal centers (in the absence of T cells) has been shown to require higher Ag doses than extracellular responses (34). However, affinity maturation of the Ab response is largely driven by the competition for increasingly scarce Ag and since there is clearly sufficient Ag present to initiate a response after high-dose immunization (100 µg), the lack of affinity maturation and germinal centers was somewhat unexpected. Our data suggest that germinal center formation involves quantitatively (or qualitatively) more stringent T cell-B cell interactions than the interactions resulting in Ig subclass switching. It is unlikely that the IgG production in these mice reflects bystander activation of B cells as a result of T cell priming by dendritic cells, since IgG production after OVA immunization requires Ag presentation by the B cells themselves (35).

The Ab response after immunization prompted us to analyze Ag presentation in more detail. Different epitopes from a single Ag may display different DM requirements (14, 28), and it was possible that one or several epitopes were still presented relatively efficiently by DM-deficient APCs. Nanda and Sant (28) have suggested that dominant epitopes presented by H2-A^d are DM dependent, while cryptic epitopes are not. This hypothesis could not be tested in the case of OVA presentation by H2-A^d, since cryptic and dominant epitopes have not been characterized and, indeed, only few epitopes have been defined (36, 37). However, despite the fact that DM-deficient mice do raise Ab responses to OVA (which does suggest that one or more epitopes are presented to CD4^+ T cells), we were unable to find any differences in the presentation of different OVA epitopes; none of the analyzed epitopes were presented at detectable levels by DM-deficient B cells, whether the Ag was internalized by fluid phase uptake or by receptor-mediated uptake (after haptenation). In contrast, although we could not detect any IgG response against HEL, one of the three HEL-reactive hybridomas, Hb1.9, apparently recognized processed HEL equally well whether this was presented by wild-type or DM-deficient cells. However, Hb1.9 may be exceptional rather than representative for T cells recognizing epitope HEL 20–35.

The inability to detect presentation of any OVA epitope presented by H2-A^d may suggest that the assays we have used are not sensitive enough to detect small, but sufficient amounts of epitopes presented in vivo. Alternatively, other OVA epitopes that we have not analyzed may be better presented than these. The T cell hybridomas in this study were all generated using DM-expressing APCs and thus they are likely to be biased toward recognition of epitopes that are favored by the presence of DM. It is possible that DM-deficient APCs present partly or totally different OVA-derived peptides than DM-expressing APCs.

H2-E^k/b molecules expressed in DM-deficient mice are CLIP-associated to a large extent (14) and we find that this is true also for H2-E^k/b (Fig. 4A). The Ag-presenting capacity of H2-E^k/b by BALB/c splenocytes has been reported to be relatively less DM dependent than the presentation by H2-A^d (15), and expression of functional H2-E^k/b in DM−/− mice led to improved positive selection, resulting in normalized numbers of thymocytes and peripheral CD4^+ T cells. In H2-E^a/d-transgenic DM-deficient mice, CD4^+ T cell numbers are also normalized, yet like CD4^+ T cells from DM-deficient mice expressing only H2-A^d, the CD4^+ T cells from H2-E^a/d-transgenic mice react vigorously against wild-type APCs (6). As shown here, this is likely to be a reflection of the poor capacity to present protein Ags by H2-E^k/b, resulting in suboptimal negative selection. The ability of DM-deficient H2-E^k/b-transgenic B cells (and spleen cells, data not shown) to present Ag to several different OVA-restricted hybridomas was found to be severely decreased when compared with wild-type cells, but a weak response could be detected (Fig. 6e), in contrast to the situation when the presentation by H2-A^d was analyzed (Fig. 6, a–d).

Considering the apparent abnormalities in the repertoire of selected T cells and the poor ability to present OVA-derived peptides, it was somewhat unexpected to find that the kinetics and amplitude of the primary Ab response to NP-OVA were almost normalized in the DM-deficient H2-E^k/b-transgenic mice. A degree of affinity maturation was also apparent, despite the fact that germinal centers were largely absent in these mice, as in the non-transgenic DM-deficient mice. The correlation between affinity maturation and the presence of germinal centers is not absolute since affinity maturation can occur also in mice lacking germinal centers (38, 39). In addition, affinity maturation can continue long after the germinal center reaction has ceased (40). On the other hand, NP-Ficoll can induce germinal center formation in the absence of T cells after immunization of mice expressing a transgenic B cell receptor reactive with NP (34), despite the fact that somatic affinity maturation does not occur after NP-Ficoll immunization (41). Thus, it is possible that very few germinal centers present in H2-E^a/d-transgenic DM−/− mice are adequate to support the degree of affinity maturation seen in the transgenic mice, but it is also possible that affinity maturation may occur outside morphologically distinguishable germinal centers.

In conclusion, we were unable to find any consistent signs of Ag presentation in H2-A^d mice lacking DM, yet these mice were able to mount an IgG1 response to at least some protein Ags. The lack of affinity maturation of the Ab response reveals different requirements for Ag presentation for the T cell-B cell interaction resulting in isotype switching, and the subsequent interactions resulting in affinity maturation of the Ab response. Expression of H2-E^k/b molecules improved the Ab response and allowed a degree of affinity maturation, despite the fact that Ag presentation by H2-E^k/b was almost as DM dependent as the presentation by H2-A^b. Thus, T cell-dependent humoral responses to protein Ags may occur even under conditions where Ag presentation is severely restricted to the point of being undetectable.

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References


