Analysis of H2-O Influence on Antigen Presentation by B Cells

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J Immunol 2003; 171:2331-2337; doi: 10.4049/jimmunol.171.5.2331
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Analysis of H2-O Influence on Antigen Presentation by B Cells

Christopher Alfonso,1 G. Stuart Williams, Jung-Ok Han, Johan A. Westberg, Ola Winqvist,2 and Lars Karlsson3

HLA-DM (DM; in mouse H2-DM) promotes the exchange of MHC class II-associated peptides, resulting in the accumulation of stable MHC class II-peptide complexes. In naive (but not germinal center) B cells, a large part of DM is tightly associated with HLA-DO (DO; in mouse H2-O), but the functional consequence of this association for Ag presentation is debated. Here, we have extended previous studies by examining the presentation of multiple epitopes after Ag internalization by fluid phase endocytosis or receptor-mediated uptake by membrane Ig (mIg) receptors. We find that the effects of H2-O are more complex than previously appreciated; thus, while only minor influences on Ag presentation could be detected after fluid phase uptake, many epitopes were substantially affected after mIg-mediated uptake. Unexpectedly, the presentation of different epitopes was found to be enhanced, diminished, or unaffected in the absence of H2-O, depending on the specificity of the mIg used for Ag internalization. Interestingly, epitopes from the same Ag did not necessarily show the same H2-O dependency. This finding suggests that H2-O may control the repertoire of peptides presented by B cells depending on the mIg-Ag interaction. The absence of DO/H2-O from germinal center B cells suggests that this control may be released during B cell maturation. The Journal of Immunology, 2003, 171: 2331–2337.

Peptide binding to MHC class II molecules occurs in the endosomal/lysosomal system and requires degradation and removal of the invariant chain, an intracellular chaperone. The final step in this process, the removal of the class II-associated invariant chain peptide (CLIP)4 fragment as well as the loading of antigenic peptides, is mediated by DM, an MHC-encoded resident of the endosomal/lysosomal system (1). APCs from mice (or cell lines) lacking functional DM have a phenotype where the peptide class II repertoire presented at the cell surface is changed as a reflection of both poor CLIP release and poor ability to edit the repertoire of class II-associated peptides in general (2–6).

In dendritic cells and macrophages the majority of DM appears to be present as a free heterodimer, but in naive B cells this molecule is instead tightly associated with HLA-DO (DO; H2-O in the mouse), another MHC class II-encoded, nonpolymorphic heterodimeric protein (7). This association is initiated immediately after synthesis within the endoplasmic reticulum and is maintained during the transport of DO/H2-O into the endosomal system. Indeed, the intracellular transport of DO/H2-O requires DM such that the absence of DM results in degradation of DO/H2-O without any signs of transport out of the endoplasmic reticulum. The functional consequences of DM-DO association are debated. Data have been published suggesting that DO can enhance DM function (8, 9), but the majority of publications have supported the interpretation that DO inhibits DM function (9–13). The function of DO has been shown to be pH dependent, such that the peptide exchange activity of DM-DO complexes is limited to a more acidic pH window than the peptide exchange activity of DM alone (8, 9, 12, 13). These data suggest that DO may function to limit DM activity to more acidic environments corresponding to lysosomes.

The physiological role of DO/H2-O is still poorly understood. Recent publications have demonstrated decreased DO expression in germinal center (GC) B cells (14, 15), and since DO largely appears to inhibit Ag presentation, it has been suggested that the absence of DO may improve Ag presentation and promote GC B cell differentiation, but no in vivo evidence for this hypothesis has yet been demonstrated. Here we have more extensively analyzed the role of H2-O in Ag presentation using H2-O-deficient mice. We found that the influence of H2-O on Ag presentation was difficult to detect after fluid phase uptake of protein Ags. In contrast, after receptor-mediated uptake (by membrane Ig (mIg)), H2-O modulated the presentation of Ags such that the presentation of different epitopes derived from individual Ags could be promoted or suppressed, or remained unaffected. Furthermore, Ag presentation (and the H2-O dependency of the presentation) was strongly influenced by the nature of the Ig-Ag interaction, suggesting that this interaction in B cells may be a dominant factor that dictates whether individual epitopes are presented and thus, ultimately, the outcome of Ag processing.

Materials and Methods

Mice

H2-O−/−, H2-DM−/− deficient mice (H2-O+/− and H2-DM−/−, respectively) and wild-type littermates have been described previously (4, 9). Mice transgenic for mlg M receptors recognizing phosphorylcholine (PC) (16) or trinitrophenol (TNP) (17) were obtained from Dr. J. Kenny. All transgenic mice were used as heterozygotes at 6–10 wk of age.

T cell hybridomas and Ag presentation assays

OVA- and hen egg lysozyme (HEL)-specific, H2-A−/−-restricted T cell hybridomas (6) are shown in Table I. The peptide specificity of several OVA-specific hybridomas has not been defined, but responses to HPLC-separated tryptic digests suggest the hybridomas have distinct specificities. BO4 was obtained from A. Rudensky. All cell culture was performed in RPMI with 10% FBS, 2-ME, penicillin, and streptomycin (complete medium).

Received for publication December 30, 2002. Accepted for publication June 6, 2003.

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4 Abbreviations used in this paper: CLIP, class II-associated invariant chain peptide; GC, germinal center; HEL, hen egg lysozyme, mlg, membrane Ig; PC, phosphorylcholine; TNP, trinitrophenol.

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0002-2331/03/$02.00
For Ag presentation assays, primary B cells (purification described previously, (9)) were cultured (4 x 10^7 cells/well) with T cell hybridomas (2 x 10^5 cells/well). Overnight supernatants were assayed for IL-2 production by ELISA. For mIg-mediated presentation, B cells were pulsed with Ag on ice for 30 min. Non-Ig-bound Ag was removed by centrifugation through an FBS density gradient. Ag-pulsed B cells were split equally between the T cell hybridomas used (giving 4 x 10^5 B cells/well) and cultured overnight. Data (IL-2, nanograms per milliliter) are mean values of triplicate cultures ± SD. TNP-conjugated OVA (TNP-OVA) was obtained from Biosearch Technologies (Novato, CA). PC-conjugated OVA (PC-OVA) and PC-HEL were prepared as previously described (18). For kinetic studies, Ag-pulsed B cells were washed with PBS and fixed for 1 min with 0.4% paraformaldehyde in PBS at room temperature. Fixation was stopped with excess 0.2 M glycine in complete medium. Fixed cells were centrifuged through an FBS gradient and washed twice with PBS before overnight culture with T cell hybridomas. Only hybridomas Ob4, Ob15, 426.6, and H30.44 consistently recognized Ag on fixed APCs (data not shown).

**Table 1. H2-A^b-restricted T cell hybridomas used in this study**

<table>
<thead>
<tr>
<th>T Cell Hybridoma</th>
<th>Specificity</th>
</tr>
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<tbody>
<tr>
<td>Ob2</td>
<td>OVA</td>
</tr>
<tr>
<td>Ob4</td>
<td>OVA266–277</td>
</tr>
<tr>
<td>Ob15</td>
<td>OVA</td>
</tr>
<tr>
<td>Ob27</td>
<td>OVA</td>
</tr>
<tr>
<td>426.6</td>
<td>OVA323–339</td>
</tr>
<tr>
<td>Hb1.9</td>
<td>HEL20–35</td>
</tr>
<tr>
<td>H30.44</td>
<td>HEL30–35</td>
</tr>
<tr>
<td>H46.13</td>
<td>HEL46–61</td>
</tr>
<tr>
<td>BO4</td>
<td>HEL74–90</td>
</tr>
</tbody>
</table>

**Determination of pH sensitivity**

TNP- or PC-conjugated OVA were coated onto 96-well ELISA plates (1 μg/ml in PBS). After blocking with 5% BSA in PBS, serum from TNP- or PC-conjugated OVA was coated onto the relevant APC plate. After a 1-h incubation at room temperature, the wells were washed three times over a 1-h period with 50 mM citrate/phosphate buffer of defined pH ranging from 4.0–7.4. Alkaline phosphatase-conjugated, goat anti-mouse IgG was used as the secondary Ab, and plates were developed with Sigma 104 in 50 mM ethanolamine buffer, pH 9.0. Plates were read at 405 nm, and binding is expressed as a percentage of the maximal binding.

**Results**

**Ag presentation by H2-O^-/- B cells after fluid phase uptake**

Earlier studies have investigated the presentation of epitopes from a limited number of Ags by H2-O-deficient B cells and an HLA-DO-transfected cell line (9, 11, 19). To examine Ag presentation more extensively, we used a number of H2-A^b-restricted T cell hybridomas recognizing multiple epitopes within OVA and HEL (Table I). Splenic B cells were purified and cultured with intact protein Ags or peptides. Fig. 1 shows IL-2 responses after an overnight culture with T cell hybridomas. The vast majority of T cell hybridomas did not recognize Ag presented by H2-DM^-/- B cells, indicating that presentation by H2-A^b molecules is highly DM dependent, as described previously (6). A single hybridoma (Hb1.9) recognized HEL presented by H2-DM^-/- B cells, but we have recently shown this hybridoma to be an exception and HEL 20–35-specific T cells generally do not recognize this epitope after culture of H2-DM^-/- cells with intact HEL (6).

Presentation of protein Ag by H2-O^-/- or wild-type B cells gave similar dose-dependent responses for most T cell hybridomas (Fig. 1, a and c). This was observed for both Ags (OVA and HEL), suggesting either that the processing of Ags within B cells is largely independent of H2-O function or, alternatively, that the assay set-up used in these experiments was not sufficiently sensitive to detect differences in presentation. We have previously reported a small difference (~3-fold) in the presentation of HEL to hybridoma BO4 after fluid phase uptake (9), but additional experiments suggest that this difference may not be significant, and only for a single T cell hybridoma (Ob27) was a 10-fold difference in Ag presentation consistently observed such that presentation of OVA was enhanced by H2-O^-/- B cells. Also, presentation of synthetic peptides was unaffected by the absence of H2-O, showing that peptide exchange at or close to the cell surface was largely independent of this molecule (Fig. 1, b and d). This phenotype is
not shared by H2-DM−/− mice, in which presentation by H2-DM−/− B cells does occur but is notably reduced for all peptides examined (except HEL 20–35; see above).

The increased presentation by H2-O−/− B cells to Ob27 is in agreement with previous studies showing that HLA-DO can inhibit Ag presentation (10, 11), but these data also show that under the conditions used here, the absence of H2-O does not radically alter the ability of B cells to present Ags.

**Ag presentation by H2-O−/− B cells after mlg-mediated uptake**

Since fluid phase uptake by B cells is both inefficient and most likely of limited physiological relevance, we investigated whether H2-O influences presentation of epitopes derived from Ags internalized using surface mlg receptors. To study this process, Ig transgenic mice encoding mlg specific for the hapten TNP were bred with H2-O−/− and H2-DM−/− mice. Ig transgenic B cells were pulsed with TNP-OVA on ice for 30 min, separated from excess Ag, then split and cultured overnight together with different OVA-specific H2-A∗-restricted T cell hybridomas. IL-2 responses of T cell hybridomas to Ag-pulsed B cells were shown in Fig. 2a. Individual hybridomas varied greatly in the responses to the Ag-pulsed B cells, with the responses of Ob4 and Ob15 being ~10-fold greater than those observed after fluid phase uptake (note the 100-fold Ag dilutions for Ob4 and Ob15). The response of hybridoma 426.6 (specific for OVA323–339) was enhanced roughly 103-fold, whereas Ob2 generally gave similar responses whether Ags were internalized via fluid phase- or receptor-mediated uptake. Hence, epitope-specific differences appear to exist in the processing of Ags after internalization via mlg-mediated uptake. In contrast to the strong responses detected against most epitopes after mlg-mediated uptake of TNP-OVA, we observed poor presentation of different HEL epitopes after mlg-mediated uptake of TNP-conjugated HEL (TNP-HEL) by transgenic B cells (Fig. 2b). This is not a reflection of a direct destruction of epitopes by haptenation, since all four HEL epitopes examined were presented equally well after fluid phase uptake of TNP-HEL and nonhaptenated HEL (Fig. 2c).

In contrast to the situation after fluid phase uptake, several H2-O-dependent differences in Ag presentation were observed for mlg-mediated uptake. Thus, at low Ag doses, better responses were elicited from hybridomas Ob4 and in particular from Ob15 when the presenting B cells lacked H2-O expression, implying that the presence of H2-O did not favor Ag presentation of these epitopes after anti-TNP-mediated uptake (Fig. 2a; note the 100-fold Ag dilutions for Ob4 and Ob15). In contrast, presentation of the OVA323–339 epitope to hybridoma 426.6 by H2-O−/− B cells was weaker than that by wild-type cells, showing that H2-O could also promote Ag presentation. Presentation to hybridoma Ob2 appeared to be H2-O independent. Thus, H2-O skewed the presentation of OVA epitopes internalized by anti-TNP mlg receptors, resulting in either enhancement or suppression depending on the epitope. None of the OVA-reactive hybridomas examined recognized Ag after Ig-mediated uptake by H2-DM−/− B cells. Therefore, while the presentation of these epitopes is clearly dependent on H2-DM, within a given Ag individual epitopes appear to vary in their dependency on H2-O.

**Kinetics of Ag presentation by anti-TNP transgenic B cells**

To analyze the kinetics of presentation after mlg-mediated uptake, primary B cells from anti-TNP Ig transgenic mice were pulsed with 100 μM TNP-OVA on ice, separated from excess Ag, and cultured at 37°C. At the indicated times, B cells were gently fixed, then split and cultured overnight with different T cell hybridomas. Fig. 3 shows the response of OVA-specific T cell hybridomas to Ag-pulsed fixed B cells. Although some presentation was clearly detected within 1 h, maximal presentation by the TNP-transgenic cells occurred after 4–6 h of processing, presumably reflecting increased density of relevant class II-peptide complexes at the B cell surface. Responses were diminishing by 8 h, possibly as a result of internalization of class II complexes from the cell surface or dissociation of the antigenic peptide from the class II molecules. For hybridomas Ob4 and Ob15, the kinetics of presentation by H2-O−/− B cells was similar to those of wild-type B cells, although the amount of IL-2 secretion was higher, which is consistent with the dose-response curves (Fig. 2a). The presentation kinetics of the OVA323–339 epitope to hybridoma 426.6 was similar to that seen for the other hybridomas, but in this case the H2-O dependency was striking, with no presentation being detectable for H2-O−/− B cells, again demonstrating that H2-O was required for normal presentation of this epitope.
Ag-Ig receptor interaction influences H2-O-dependent Ag presentation

To determine whether the H2-O-dependent differences in Ag presentation we observed after receptor-mediated uptake could be generalized or were restricted by the specificity of the transgenic Ab used, we bred mice encoding a second independent transgenic Ab with a distinct specificity, recognizing the hapten PC (16), to the H2-O/– and H2-DM/– mice. Our original model of DO/H2-O function was in part based on observations using PC-specific B cells, and we sought to extend these observations. Thus, transgenic PC-specific B cells were pulsed with PC-OVA on ice and, after removal of excess Ag, cultured overnight with the panel of OVA-specific T cell hybridomas (Fig. 4a). We found enhanced responses from some hybridomas (Ob4, Ob15, and 426.6), but not others (Ob2 and Ob27) compared with fluid phase uptake, again suggesting that internalization by PC-specific mlg altered the relative presentation of different OVA-derived epitopes compared with the presentation after fluid phase uptake. As expected, no response was elicited after culture with H2-DM/– B cells, showing that also in this case presentation by H2-Aa was highly dependent on H2-DM after mlg-mediated uptake. In this case the responses from Ob4 and Ob15 were markedly decreased when H2-O/– B cells rather than wild-type cells were used as APCs, while Ob27 and 426.6 showed more modestly decreased responses, and Ob2 appeared unaffected by the presence or the absence of H2-O. Intriguingly, for hybridomas Ob4 and Ob15, the influence of H2-O in the presentation of PC-OVA was reversed compared with that on TNP-OVA, demonstrating that differences in the properties of the different Abs analyzed, the Ag-Ab interaction(s), or the antigenic structure (due to differences caused by haptenation or hapten structure) changed the H2-O dependency in presentation. In contrast, 426.6 and Ob2 showed the same H2-O requirement independently of which Ag-Ab pair was analyzed; thus, 426.6 was presented less well by H2-O/–/– B cells in both cases, whereas Ob2 was presented equally well, independently of whether the cells expressed H2-O.

We also studied the uptake and presentation of PC-conjugated HEL (PC-HEL; Fig. 4b) to the same panel of HEL-specific T cell hybridomas as that described in Fig. 1. The hybridomas responded after uptake of PC-HEL (in contrast to the situation with TNP-HEL), but the responses of individual hybridomas after uptake of PC-HEL were generally not strikingly enhanced compared with fluid phase uptake. Indeed, the response of one hybridoma (Bo4) was diminished after uptake of Ag using PC-reactive mlg receptors. For hybridomas H30.44 and H46.13, which recognize epitopes 30-53 and 46-61, presentation by H2-O/–/– B cells was weaker (roughly 10-fold) after pulsing with Ag, showing that H2-O was required for normal presentation of these two epitopes. Presentation of HEL/20–35 to hybridoma Hb1.9 by wild-type or H2-O/–/– B cells was very similar, but as mentioned above, Hb1.9 is an unusual hybridoma that recognizes HEL/20–35 after processing of intact HEL by H2-DM/–/– cells (6).

Kinetics of Ag presentation by anti-PC transgenic B cells

The kinetics of PC-OVA presentation were also analyzed, similarly to that of TNP-OVA (Fig. 5a). Again, presentation to hybridomas could be observed after 1 h, but in this case maximal presentation appeared by to occur later than after TNP-mediated uptake. Thus, presentation of PC-OVA did not diminish significantly by 8 h, suggesting either that class II-peptide complexes were removed or destroyed less rapidly, or that altered processing resulted in the continued production of new complexes that replenished the cell surface repertoire. The H2-O dependency of the response by Ob4 and Ob15 was quite dramatic, confirming the dose-response curves (see Fig. 4a). Presentation by H2-O/–/– B cells could not be readily detected at early time points, and although some presentation was detected at 4–8 h, this was drastically lower than that by wild-type B cells. Unfortunately, the responses of other OVA-reactive hybridomas (including 426.6) were not strong enough to permit this type of analysis after PC-mediated uptake.

H30.44 was the only HEL-specific hybridoma to consistently recognize Ag after fixation of primary B cells; therefore, kinetic data of HEL presentation are shown for this single hybridoma. Fig. 5b shows that the presentation of epitope HEL/30–53 by H2-O/–/– B cells was extremely poor, whereas wild-type B cells presented efficiently. Thus, also in this case, the kinetic differences in epitope appearance seem to mirror the differences between H2-O/–/– and wild-type cells seen after Ag pulsing.
Dissociate from the Ag at pH effectively bound to the hapten at neutral pH, the anti-PC Ab began to washes using alkaline phosphatase-conjugated secondary Ab. The pH sensitivity of the hapten-Ab interaction. Diluted tides from/to class II molecules (20–22), and the protective effects on class II molecules during this peptide exchange, maintaining the empty groove in a peptide-receptive state (23, 24). DM activity is regulated by pH, and although peptide exchange can occur at neutral pH, optimal activity is achieved at pH 6.5 to 4.5 (20, 21, 25). This is consistent with the fact that DM is predominantly localized intracellularly within the acidic endosomal system (26, 27), although DM has also been shown to be present at the cell surface in dendritic cells (28, 29). The relevance of this finding is unclear, however, since the physical interaction between DM and DR molecules has been reported to be much more readily detectable at acidic pH than at neutral pH (30).

Recent studies have shown DM activity to be regulated by DO/H2-O, findings initially prompted by the physical association of the two molecules within the endosomal system of B cells (7). DO/H2-O function remains controversial, with both positive and negative effects on DM function being described, in terms of detected CLIP levels and in vitro peptide binding (8–11). Mass spectrometric analysis of class II-associated peptides has shown that DO/H2-O influences the class II-associated peptide repertoire (12). Although ~90% of the cell surface repertoire was shown to be unaffected in DO-transfected cell lines compared with DO-negative cells, the latter population contained qualitatively different peptides and peptides of unusually large size (>18 kDa), leading to the suggestion that DO/H2-O inhibits the presentation of large peptides. Modulation of the class II-peptide repertoire would predictably lead to an altered Ag presentation, and preliminary data assigning a role for DO/H2-O in this aspect have been described (9, 11, 13, 19). In this study we found that, with a single exception, the presentation of a number of different epitopes derived from two protein Ags (OVA and HEL) was largely unaffected by the presence or the absence of H2-O after fluid phase uptake. This extended study partly contrasts with our own published data (9) and with findings reported using transfected cell lines (11, 13). Since the lack of influence by H2-O applied to different Ags and class II molecules, it is unlikely to be an exceptional finding, although this suggestion needs to be confirmed using other Ags. In contrast to what has been reported previously (19), we also did not find that H2-O influenced Ag processing of the Ags used in this study differently in backcrossed mice (five generations to C57BL/6) than in F2 (129/Ola × C57BL/6) mice (not shown). A number of factors may explain the differences between the observations described here and previously published data. First, transfection of DO is likely to result in nonphysiologically high protein levels, which may give an artificially strong effect on Ag presentation. Second, different class II molecules may be differentially influenced by DO/H2-O. Third, there may be differences between species. The last explanation is supported by the finding that CLIP levels appear to correlate with the expression of DO in human B cells, while this is not always the case in mouse B cells (9, 14, 15, 31).

Ag presentation after fluid phase uptake is widely used in many experimental systems (involving both B cells and other cell types as APCs), but may not be physiologically relevant to B cells, since this cell type in vivo mainly presents cognate Ags to receive T cell help. Receptor-mediated uptake using surface mlg is usually very efficient and is likely to be the dominant mechanism for Ag capture by B cells in vivo (32). Using two different Ig transgenic systems, we found that H2-O affected the presentation of many epitopes after Ag internalization using mlg, as our preliminary data had suggested. Surprisingly, in the extended analysis presented here, we found that the effect of H2-O varied depending on the epitope as well as the receptor mediating the Ag uptake. H2-O did promote, suppress, or was neutral with respect to Ag presentation in

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C. Alfonso, G. S. Williams, and L. Karlsson. H2-O influence on antigen presentation in H2-E-expressing mice. Submitted for publication.
an epitope-dependent manner. The molecular mechanisms underlying these results are not clear, but are likely to reflect altered DM-mediated loading of peptides onto class II molecules within the endosomal system. In vitro, DO/H2-O lowers the optimal pH for DM-mediated peptide exchange (to pH <5.5), and treatment of APCs with bafilomycin A1 (which inhibits endosomal acidification) reduces Ag presentation in DO-expressing, but not DO-deficient, cells, suggesting that the presence of DO/H2-O may favor the presentation of peptides generated within late endosomes or lysosomes (9, 12). The loading of antigenic peptides is influenced by a number of factors, including the structure of the Ag and its resistance to proteolysis, the dwell-time of the Ag in a given proteolytic compartment, and the availability of accessible class II binding sites. While different Ag-mIg combinations (such as those analyzed here) are likely to be different with regard to antigenic structure and the dwell-time in a given compartment, H2-O is unlikely to affect these parameters, but could be expected to alter the conditions for class II-peptide interactions. In the absence of H2-O, DM would be active throughout the endosomal system, resulting in class II molecules being occupied with high affinity peptides that are generated in earlier endosomal compartments. These peptides would not normally be able to efficiently associate with class II molecules due to low DM activity, but in a situation where these peptide-class II complexes have been formed, they would effectively inhibit the formation of other peptide-class II complexes that are normally generated in later compartments, such as lysosomes. Thus, the absence of H2-O may change the relative contributions of different processing compartments to the collection of antigenic peptides loaded onto class II molecules, resulting in the pool of intracellular class II molecules being occupied with a quantitatively different repertoire of peptides. DM-dependent epitopes that are presented equally well by normal or H2-O−/− cells may reflect those epitopes that are loaded onto class II molecules in multiple compartments.

It has been well established that Ig molecules impart stability on bound Ags, affecting subsequent degradation (33, 34). Stable Ig-Ag complexes have different degradation patterns compared with free Ag (35), and Ig molecules have been shown to promote or suppress the presentation of different peptides derived from Ig-bound Ags (36). These data have been interpreted to reflect protection from proteolysis due to Ab-mediated stabilization of certain parts of the Ag. In the systems we used here we had not expected to see large epitope-specific differences after receptor-mediated uptake (compared with fluid phase uptake), since haptenation is likely to occur randomly at surface-exposed amino acid residues (mainly tyrosine residues in the case of PC coupling, lysine residues in the case of TNP coupling). Thus, Ab binding to the haptenated Ag could be predicted to result in more or less random protection from proteolysis, which should minimize Ab-mediated changes in the presented repertoire, resulting in similar relative presentation of different epitopes as after fluid phase uptake, even though the overall presentation may be more effective. Contrary to expectation, we found that the relative presentations of different epitopes from the same Ag were dramatically different after either TNP- or PC-mediated Ag uptake compared with fluid phase uptake. It is difficult to directly compare the presentation by PC- and TNP-conjugated Ags, since the exact Ag structure is neither homogenous nor well defined, but in both cases large epitope-specific differences in dose-response curves were observed after Ig-mediated uptake of haptenated OVA compared with the uptake by fluid phase, showing that hapten-specific Ig molecules also influence the processing of Ags by selectively favoring the presentation of certain epitopes over others. Interestingly, all four HEL epitopes tested were extremely poorly presented after Ig-mediated uptake of TNP-HEL, while three of four epitopes were presented to some extent after uptake of PC-HEL. This is not a consequence of epitope destruction by haptenation, since both TNP-HEL and PC-HEL are present well after fluid phase uptake (Fig. 2c and not shown), but reflects Ig-mediated inhibition of Ag presentation. The poor or absent presentation of HEL epitopes after Ig-mediated uptake may reflect a property of HEL itself, since receptor-mediated Ag uptake of non-haptenated HEL by anti-HEL specific MD4 transgenic B cells does not significantly increase presentation above the fluid phase level for any of the hybridomas used in this study (37) (C. Alfonso and L. Karlsson, unpublished observations).

In vitro assays showed that both the anti-hapten Abs used here were stably bound to Ag at neutral pH, yet at acidic pH the PC-Ab complexes were found to dissociate more readily than the TNP complexes. It is possible that the degradation of Ig-bound TNP- and PC-OVA differed in part as a consequence of differential dissociation of Ig from the bound Ag at different stages of the endosomal system, although structural differences between the two Ags may also be important in this respect, as discussed above. Batista et al. (38) have suggested that the kinetics of Ag delivery to relevant processing compartments greatly influences the efficiency of Ag processing and that avidity between Ag and Ab largely determines the kinetics of Ag delivery. The pH stability of the TNP-Ig complex suggests that TNP-conjugated Ags may have higher effective avidity in acidic endosomal compartments than the PC-Ig complexes, and this could contribute to the observed kinetic differences in the presentation to Ob4 and Ob15 between the two receptor-Ag combinations. Alternatively, differences in receptor signaling or intracellular targeting may also contribute to the observed differences in Ag presentation, particularly since B cells from PC transgenic animals (in contrast to B cells from TNP transgenic mice) do not have a strictly naive phenotype (39) (although they express H2-O, not shown).

We and others have previously suggested that DO/H2-O would promote the presentation of Ags that are internalized via mIg (1, 9, 12). The current data suggest that this hypothesis may have some validity, but is likely to be an oversimplification and does not appear to be broadly applicable to all epitopes or combinations of Ig-Ag complexes. H2-O was found to have a minor impact on presentation of Ags internalized by fluid phase endocytosis, yet had a dramatic effect on the presentation of epitopes derived from internalized via mIg, resulting in the promotion or suppression of different epitopes. These differences may partly reflect kinetic differences between the experiments using fluid phase or receptor-mediated Ag uptake. The bolus of Ag moving through the endosomal system after receptor-mediated uptake is likely to reveal differences better than the continuous presence of Ag after a long fluid phase uptake. Fluid phase Ag-pulsing experiments (up to 3 h) did not reveal any differences between the wild-type and H2-O-deficient B cells for Ob4 and Ob15, the only two hybridomas in which any response could be detected (data not shown). However, even this relatively short incubation with Ag could potentially be long enough to obscure kinetic differences in presentation. The effect of H2-O was greatly influenced by the Ig-Ag interaction, suggesting that the presence of DO/H2-O may serve to promote T cell help to B cells that express receptors with certain characteristics. DO has recently been shown to be down-modulated in GC B cells (14, 15), suggesting that DO (or rather the absence of it) may be involved in the affinity maturation process. Ag processing within GC B cells is poorly characterized, but is likely to differ from the process in primary follicular B cells, not least due to the differences in receptor affinity and receptor isotypes. The anti-TNP receptor appears to have higher avidity than the anti-PC Ig receptor (at least at acidic pH), and it is interesting to note that in contrast
to other epitopes, the best-presented epitopes after receptor-mediated uptake of TNP-OVA (i.e., the epitopes recognized by hybridomas Ob4 and O15) are actually better presented in the absence than in the presence of H2-O. While the expression of DO/H2-O may promote the presentation of many or most epitopes after uptake by B cells with low or medium affinity receptors, the expression of high affinity receptors may decrease the dependence on DO/H2-O and thus provide a competitive advantage for high affinity B cells in the GC reaction. The validity of this hypothesis is being investigated.

It is likely that the down-regulation of DO/H2-O in GC B cells is related to the affinity maturation process, but the data presented here also suggest that it is not simply a question of removing an inhibitory influence of DO/H2-O on Ag processing, as has been suggested previously. The effect of DO/H2-O on Ag presentation will require further study, including the analysis of affinity maturation in vivo.

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