H2-O Inhibits Presentation of Bacterial Superantigens, but Not Endogenous Self Antigens

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Ling Qi and Suzanne Ostrand-Rosenberg

H2-O/HLA-DO are MHC class II accessory molecules that modulate exogenous Ag presentation. Most class II accessory molecules are expressed in all professional APC; however, H2-O is only expressed in B cells and medullary thymic epithelial cells. Because B cells present exogenous Ags and superantigens (SAgs), and medullary thymic epithelial cells are specialized APC for self Ags during negative selection in the thymus, we have hypothesized that H2-O might play a role in MHC class II-restricted SAg and self Ag presentation. In this study, we demonstrate that H2-O expression inhibits presentation of the bacterial SAg staphylococcal enterotoxins A and B to four SAg-reactive T hybridoma cells. In contrast, H2-O has no effect on presentation of endogenous self Ags, as measured by tumorigenicity in vivo and Ag presentation to three self Ag-specific T hybridoma cells. Additional experiments suggest that H2-O inhibits presentation of exogenous Ags by both newly synthesized and recycling MHC class II molecules. These data suggest H2-O may have a physiological role in tolerance induction and SAg-mediated toxic shock. The Journal of Immunology, 2001, 167: 1371–1378.

We have tested this hypothesis by generating H2-O-transfected cells and using the transfectants as APC for SAg and self Ags. Because earlier reports showed that H2-O also interacts with Ii (2, 8), we have assessed the effects of H2-O in the presence or absence of Ii. Our results demonstrate that H2-O inhibits SAg presentation, but does not affect endogenous self Ag presentation. We have also examined H2-O effects on exogenous Ag presentation and find that H2-O inhibits presentation of exogenous peptides associated with both newly synthesized and recycling MHC class II molecules. In no case does Ii expression alter the functions of H2-O, suggesting that H2-O is a potent inhibitor of SAg and exogenous Ag presentation via an Ii-independent mechanism.

Materials and Methods

Cell lines and transfections

Sal is a MHC class II-negative A/J-derived spindle cell sarcoma (11). Sal/Aα, Sal/Aα/DM, Sal/Aα/Ii, Sal/CITTA (MHC class II transcriptional activator), and their hen egg lysozyme (HEL)-transfected Sal cells (Sal/Aα/DM/HEL and Sal/Aα/Ii/HEL) were previously described. In these transfected, HEL is tagged with a (KDEL) signal which causes HEL to be retained in the ER (12, 13). H2-O transfectants were generated as described (13) using the pCMU/Oa and pCMU/Ob plasmids encoding the H2-Oa- and H2-Oβ-chains, respectively. Transfectant nomenclature uses “DM” and “DO” for H2-DM and H2-O, respectively. Cells expressing H2-O were selected in 1.5 μg/ml puromycin (BD Biosciences, San Jose, CA) for Sal/Aα/DM/DO and Sal/Aα/DM/HEL/DO cells, or 400 μg/ml hygromycin (Calbiochem, La Jolla, CA) for Sal/CITTA/DO cells, tested by flow cytometry, and then cloned by limiting dilution.

Reagents

Brefeldin A (BFA), chloroquine, staphylococcal enterotoxins A and B (SEA and SEB), HEL, and RNase were purchased from Sigma (St. Louis, MO). HEL-46-68 peptide (NTDGSTDYGILQINSR) was purified by HPLC. Mouse mAbs 10-2.16 (anti I-Ak) (14), hyHEL7 and hyHEL10 (anti-HEL) (15), rat mAb In-1 (anti-Ii) (16), and rabbit antiserum K553 (anti-H2-DM) were previously described (17). Mouse mAb 9E10 (anti-c-myc) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA, and Johns Hopkins University, Baltimore, MD). Rabbit antiserum K553 against the C-terminal tail of the H2-β-chain was previously described (5).
Flow cytometry

Tumor cells were stained by indirect immunofluorescence either internally or externally, and analyzed on an Epics XL flow cytometer, as previously described (13).

RT-PCR

Total RNA was extracted using a RNasy MiniKit (Qiagen, Valencia, CA), according to the manufacturer’s directions. A total of 100 ng RNA was amplified using a one-step RT-PCR kit (Qiagen) and the following conditions: 50°C × 30 min and 95°C × 15 min, followed by 30 cycles of 95°C × 30 s, 60°C × 30 s, and 72°C × 30 s. H2-Oa 5’ primer, CTCCTTACCA ATCTTCAGGC, and 3’ primer, GTGTGCCTGATCATGACGAC; H2-Ob 5’ primer, TGGTCTAAACGTATGGCTTTCTG, and 3’ primer, GTTCCACCAAAGTGGTCCAGTAC.

Cell surface biotinylation

Log-phase cells (> 95% viability) were washed twice with cold PBS containing 0.1 mM CaCl2 and 1 mM MgCl2, and resuspended at 10⁶/ml in 0.5 mg/ml sulfo-N-hydroxy succinimide–long chain–biotin (Pierce, Rockford, IL) for 30 min at 4°C with gentle shaking. The reaction was stopped by addition of glycine to a final concentration of 10 mM. Biotinylated cells were washed twice with excess PBS and subjected to immunoprecipitation.

Immunoprecipitation

All procedures were conducted at 4°C. A total of 3 × 10⁶ tumor cells/ml or 3 × 10⁶ splenocytes/ml was incubated in lysis buffer (20 mM Tris, pH 8, 150 mM NaCl, 5 mM EDTA, and 1% Nonidet P-40) containing protease-inhibitor cocktails (Roche, Indianapolis, IN) for 40 min, and lysates were microcentrifuged for 30 min to remove nuclei and cellular debris. The resulting supernatants were divided into 100-μl aliquots and were pre-cleared with 0.2 μl rabbit serum and twice with 50 μl 10% protein G-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ). Precleared supernatants were incubated with 0.2 μl K535 or 3 μg 9E10 for 1 h, followed by addition of 200 μl 10% protein G-Sepharose for another 1 h. The resulting immunoprecipitates were pelleted by microcentrifugation and washed four times with wash buffer (50 mM Tris, pH 8, 150 mM NaCl, or 0.1% Nonidet P-40).

Endoglycosidase H (endo H) assay

Immunoprecipitated beads were resuspended in 40 μl endo H buffer (20 mM Na2PO4, pH 6.5, 0.1% SDS, and 0.02% NaN3) and boiled for 5 min. After microcentrifugation at room temperature, the supernatants were divided into two aliquots. Recombinant endo H (2 μu/ml) was added to one aliquot, and both aliquots were incubated overnight at 37°C and then boiled for 5 min to inactivate endo H before analysis by Western blot.

Western blot

Western blot analyses were performed as described (18) using 12% SDS-PAGE. For c-src or H2-ØJ analysis, incubation with 9E10 (1:500) or K535 (1:3000) was followed by sheep anti-mouse or donkey anti-rabbit HRP (1:10,000; Amersham), respectively. In surface biotinylation assays, biotinylated proteins were visualized with streptavidin–HRP (1:20,000; Pierce). Bands were quantified by densitometry using National Institutes of Health Scion Image software (http://www.scioncorp.com).

SAg-binding analysis

APC at 5 × 10⁶/ml in PBS/2% FCS were incubated with 10 μg/ml biotinylated SEB (Toxin Technology, Sarasota, FL) for 45 min at 37°C and washed four times with PBS before incubation with 30 μl 2.5 μg/ml streptavidin–PE (BD Biosciences) for 20 min at 4°C. Following labeling, the cells were washed at 4°C four times with PBS, and SAg binding was quantitated by flow cytometry. Fluorescence intensity = (anti-log of mean channel fluorescence with biotin-SEB + streptavidin–PE) – (anti-log of mean channel fluorescence with streptavidin–PE).

Ag presentation assays

SAg-reactive and Ag-specific CD4⁺ T hybridoma cells used in this study are shown in Table I. Exogenous and endogenous Ag presentation assays were performed as described (13). For SAg presentation assays, 5 × 10⁵ APC were incubated with 5 × 10⁴ T hybridoma cells plus SAg for 18–20 h at 37°C. For exogenous Ag presentation assays with chloroquine or BFA, 1.5 × 10⁶ Sal/A⁺/DM or Sal/CIITA cells were incubated at 37°C with chloroquine for 30 min or BFA for 15 min, followed by addition of native HEL, RNase, or HEL46–61 peptide for an additional 4–5 h. Chloroquine or BFA was present during the incubation with Ag. To ensure adequate T cell responses, different concentrations of Ag were used for different APC and hybridoma cells. Sal/A⁺/DM and Sal/CIITA cells were pulsed with HEL at 1 mg/ml for 3A9 and 3B11.1, RNAse at 10 μg/ml for TS12, or HEL46–61 peptides at 100 μg/ml for 3A9. Sal/A⁺/DM cells were pulsed with HEL at 8 mg/ml for 2B6.3, 2D4.1, and 1B9. Sal/CIITA cells were pulsed with HEL at 20 mg/ml for 2B6.3, 3A9, and 3B11.1, or with RNAse at 10 ng/ml for TS12. Sal/A⁺/DM/DO.7 cells were pulsed with HEL at 10 mg/ml for 2B6.3, 3B11.1, and 1B9, with HEL at 2 mg/ml for 3B11.1 and 3A9, or with RNAse at 10 mg/ml for TS12. Following incubation with Ag, the APC were fixed with 0.5% glutaraldehyde for 30 s on ice, quenched in 200 mM glycine, and washed twice with PBS and once with culture medium. A total of 5 × 10⁶ Ag-loaded, fixed APC was then incubated with 7 × 10⁵ T hybridoma cells for 18–20 h at 37°C. Ag-specific and SAg-mediated T cell activation was measured by quantifying the amount of IL-2 in the assay supernatants using an IL-2 ELISA (Pierce), as previously described (12). Values are the average of triplicate cultures ± SD. In experiments using chloroquine or BFA, percentage of response = 100% × ((IL-2 with drug)/(IL-2 without drug)).

Mice and tumor challenges

C57BL/6 H2-O⁻ (H2-Oα⁻ β⁻) and H2-Oα⁻ (H2-Oα⁻ β⁻) mice were previously described (5). AJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed and bred in the Biology Department animal facility. Six- to eight-week-old male and female mice were used. Tumor challenges were performed as previously described (12). All animal procedures have been approved by the University of Maryland Baltimore County Institutional Animal Care and Use Committee.

Results

Characterization of H2-O-expressing Sal cells

To study the functions of H2-O in the absence or presence of Ii, Sal/A⁺/DM and Sal/CIITA cells were transfected with plasmids encoding H2-Oα⁻ and H2-ØJ-chains, respectively (Sal/A⁺/DM/DO and Sal/CIITA/DO cells). Fig. 1, A and B, shows the flow cytometry profiles of three Sal/A⁺/DM/DO clones (clones 4, 5, and 7) and two Sal/CIITA/DO clones (clones A10 and G1), respectively, stained for surface MHC class II (I-Å⁻) and internal H2-DM, H2-O, and/or Ii. In CIITA-transduced cells, MHC class II, Ii, and H2-DM, but not H2-O, are coordinately up-regulated (26).

Table I. T hybridoma cells used in this study

<table>
<thead>
<tr>
<th>T Hybridoma Cells</th>
<th>Reactivity</th>
<th>TCR Vβ Specificity or MHC Restriction/Ag Specificity</th>
<th>Reference</th>
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<tbody>
<tr>
<td>3B11.1</td>
<td>SEB-reactive</td>
<td>Vβ8</td>
<td>19</td>
</tr>
<tr>
<td>3A9</td>
<td>SEB-reactive</td>
<td>Vβ8</td>
<td>20</td>
</tr>
<tr>
<td>SKK45.10</td>
<td>SEB-reactive</td>
<td>Vβ8</td>
<td>21</td>
</tr>
<tr>
<td>A2.A2</td>
<td>SEA-reactive</td>
<td>Vβ1</td>
<td>22</td>
</tr>
<tr>
<td>2B6.3</td>
<td>Ag-specific</td>
<td>I-Å⁻/HEL&lt;sub&gt;25–43&lt;/sub&gt;</td>
<td>19</td>
</tr>
<tr>
<td>3B11.1</td>
<td>Ag-specific</td>
<td>I-Å⁻/HEL&lt;sub&gt;34–45&lt;/sub&gt;</td>
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<tr>
<td>3A9</td>
<td>Ag-specific</td>
<td>I-Å⁻/HEL&lt;sub&gt;46–61&lt;/sub&gt;</td>
<td>20</td>
</tr>
<tr>
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<td>I-Å⁻/HEL&lt;sub&gt;12–124&lt;/sub&gt;</td>
<td>24</td>
</tr>
<tr>
<td>1B9</td>
<td>Ag-specific</td>
<td>I-Å⁻/HEL&lt;sub&gt;116–129&lt;/sub&gt;</td>
<td>19</td>
</tr>
<tr>
<td>TS12</td>
<td>Ag-specific</td>
<td>I-Å⁻/RNase&lt;sub&gt;43–56&lt;/sub&gt;</td>
<td>25</td>
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</tbody>
</table>

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H2-O IN SAg AND SELF Ag PRESENTATION
H2-O expression in all clones was confirmed by Western blot analysis (Fig. 1C) and RT-PCR (data not shown). An H2-Oαβ2 clone (SaI/CIITA/DO.3) was also obtained, and its phenotype was confirmed by RT-PCR and immunoprecipitation (data not shown). To ascertain that H2-Oαβ complexes leave the ER and medial Golgi, endo H assays were performed on H2-O immunoprecipitates. Treatment with endo H reduces the molecular mass of H2-O by removing N-linked high mannose, but not complex-type oligosaccharides. Protein glycosylation with high mannose or complex-type oligosaccharides occurs in the ER/medial Golgi or the trans Golgi/trans Golgi network, respectively. Therefore, when proteins leave the ER and medial Golgi, they are endo H resistant. As shown in Fig. 1D, ∼90 and 60% of H2-Οβ-chains of SaI/β2/DO/DO.5 and SaI/CIITA/DO.A10 cells (H2-Oαβ2), respectively, are resistant to endo H digestion. In contrast, virtually all H2-Οβ-chains in SaI/CIITA/DO.3 cells (H2-Oαβ2) are endo H sensitive. Similar results were obtained for splenocytes of H2-Oαβ and H2-Oαβ mice, respectively. These results demonstrate that H2-Οβ-chains that are associated with H2-Oα-chains in H2-Oαβ transfectants mature through the ER and Golgi.

Correct MHC class II conformation is critical for effective Ag presentation. One measure of class II conformation is MHC class II/peptide complex stability in 1–2% SDS (27). The role of H2-O in modulating MHC class II SDS stability is controversial (3–7). To determine the effect of H2-O on I-Ak molecules in the SaI transfectants, boiled and nonboiled cell extracts were Western blotted and probed for I-Aκ. As shown in Fig. 1E, the percentage of SDS-stable MHC class II dimers in SaI/Ak and SaI/CIITA transfectants, respectively. Therefore, H2-O does not significantly alter SDS stability of I-Ak molecules.

H2-O inhibits presentation of bacterial SAg to T cells

SEB activates murine T cells by binding to the Vβ7, Vβ8, or Vβ17 region of the TCR and α-chain of the MHC class II of the APC (28). To determine whether H2-O affects SEB presentation, Sal

FIGURE 1. Characterization of H2-O-transfected SaI/Aκ/DM and SaI/CIITA cells. SaI/Aκ/DM/DO (A) and SaI/CIITA/DO (B) cells were stained for surface I-Aκ (10-2.16) and internal expression of H2-DM (K553), H2-O (K535), or Ii (In-1). Solid lines represent Ab-specific staining; dotted lines represent staining with fluorescent conjugate without specific Ab. C, Whole cell lysates were boiled before 12% SDS-PAGE, and membranes were probed for H2-Oβ (K535, top) and I-Aκβ (10-2.16, bottom). Lysates from A/J splenocytes were included as a positive control. D, Endo H sensitivity of H2-O. H2-O immunoprecipitates from H2-Oαβ2-transfectants (lanes 1–4), H2-Oαβ1-transfectants (lanes 5 and 6), or H2-Oαβ2-splenocytes (lanes 7 and 8) or H2-Oαβ1-splenocytes (lanes 9 and 10) were mock treated (−) or treated with endo H (+) before SDS-PAGE and immunoblotting for H2-Oβ (K535). L, Ig light chain; s, endo H sensitive; r, endo H resistant. E, Expression of H2-O does not reduce the percentage of SDS-stable MHC class II dimers in SaI/Aκ/DM/DO and SaI/CIITA/DO cells. Western blots of boiled (B) or nonboiled (NB) cell extracts were probed for I-Aκβ (10-2.16). C, Compact I-Aκ dimers; β, free I-Aκβ chain; Aggr., aggregates; C% = 100% × ((density of C)/(density of (C + β + Aggr.))). These data are representative of at least two independent experiments.
cells with or without H2-O were incubated with two Vβ8 T hybridoma cells (Table I) that release IL-2 in response to SEB presentation. As shown in Fig. 2A, presentation of SEB by the three SaI/Ak/DM/DO clones to T hybridoma cells 3B11.1, 3A9, or SKK45.10 is reduced compared with presentation by parental SaI/Ak/DM cells. Similarly, as shown in Fig. 2B, SEB activation of T hybridoma cells 3B11.1, 3A9, and SKK45.10 by the two SaI/CIITA/DO clones is reduced relative to presentation by parental SaI/CIITA cells. To ascertain that the reduced presentation is not due to plasmid vector sequences and requires both the H2-Oα- and H2-Oβ-chains, SaI/CIITA/DO.3 (H2-Oα2β1) cells were used as APC for SEB. Hybridoma responses to SEB plus SaI/CIITA/DO.3 or SaI/CIITA cells were equivalent (data not shown).

To determine whether H2-O has the same effect on other SAggs, the same transfectants were tested as APC for activation of the SEA-reactive Vβ1 T hybridoma cell A2.A2. As shown in Fig. 3, presentation of SEA to A2.A2 T cells is uniformly inhibited by H2-O. Therefore, presentation of SAggs is markedly reduced if the APC expresses H2-O, regardless of expression of Ii.

H2-O prevents binding of SAggs to MHC class II molecules

H2-O may inhibit SAg presentation by reducing SAg binding to surface class II molecules. To test this hypothesis, SEB binding to H2-O2 and H2-O3 transfectants was quantified by flow cytometry using biotinylated SEB. As shown in Fig. 4, SEB binding to MHC class II molecules of SaI/Ak/DM/DO and SaI/CIITA/DO cells is 3- to 10-fold decreased compared with SEB binding to SaI/Ak/DM and SaI/CIITA cells. Therefore, expression of H2-O partially blocks SAg binding to MHC class II molecules, suggesting that H2-O-mediated inhibition of SAg presentation may be the result of decreased SAg binding to surface MHC class II molecules.

H2-O does not alter tumorigenicity nor affect presentation of endogenous self Ags

MHC class II molecules also present endogenous self Ags (29). We have exploited this phenomenon and used SaI/Ak sarcoma cells with or without H2-O were incubated with two Vβ8 T hybridoma cells (Table I) that release IL-2 in response to SEB presentation. As shown in Fig. 2A, presentation of SEB by the three SaI/Ak/DM/DO clones to T hybridoma cells 3B11.1 and 3A9 is reduced compared with presentation by parental SaI/Ak/DM cells. Similarly, as shown in Fig. 2B, SEB activation of T hybridoma cells 3B11.1, 3A9, and SKK45.10 by the two SaI/CIITA/DO clones is reduced relative to presentation by parental SaI/CIITA cells. To ascertain that the reduced presentation is not due to plasmid vector sequences and requires both the H2-Oα- and H2-Oβ-chains, SaI/CIITA/DO.3 (H2-Oα2β1) cells were used as APC for SEB. Hybridoma responses to SEB plus SaI/CIITA/DO.3 or SaI/CIITA cells were equivalent (data not shown).

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H2-O does not alter tumorigenicity nor affect presentation of endogenous self Ags

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Table II.  **H2-O does not alter tumorigenicity**

<table>
<thead>
<tr>
<th>Tumor Cells</th>
<th>Tumor Incidence&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>SaI</td>
<td>5/5</td>
</tr>
<tr>
<td>SaI/Ak</td>
<td>0/10</td>
</tr>
<tr>
<td>SaI/Ak/DM</td>
<td>0/13</td>
</tr>
<tr>
<td>SaI/Ak/DM/DO.4</td>
<td>0/8</td>
</tr>
<tr>
<td>SaI/Ak/DM/DO.5</td>
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</tr>
<tr>
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<td>7/7</td>
</tr>
<tr>
<td>SaI/CIITA/DO.G1</td>
<td>7/7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Syngeneic A/J mice were inoculated i.p. with 10<sup>6</sup> tumor cells and followed for 2 months for tumor development. Tumor incidence is the number of mice with palpable tumor/total number of mice injected.

**FIGURE 2.** H2-O inhibits SEB presentation. SaI/Ak/DM/DO (A) or SaI/CIITA/DO (B) transfectants were incubated with SEB-reactive T hybridoma cells (3B11.1, 3A9, or SKK45.10) in the presence of SEB. These data are representative of three independent experiments.

**FIGURE 3.** H2-O inhibits SEA presentation. SaI/Ak/DM/DO (left) or SaI/CIITA/DO (right) transfectants were incubated with SEA-reactive T hybridoma cells A2.A2 in the presence of SEA. These data are representative of three independent experiments.

**FIGURE 4.** H2-O decreases SEB binding to cell surface MHC class II. SaI transfectants were incubated with biotinylated SEB, followed by streptavidin-PE. The data are representative of two independent experiments.
cells as cancer vaccines (30). The vaccines were originally designed to directly present endogenous tumor Ags to host CD4+ T cells. In vitro Ag presentation (12, 13) and in vivo immunization (18, 31) experiments support the hypothesis that the class II-transfected tumor cells with or without coexpression of H2-DM are APC for tumor-encoded self Ags. If H2-O inhibits presentation of self Ags, then H2-O-transfected SaI/Ak/DM cells might regain their malignant phenotype and cause tumors in syngeneic, immunocompetent mice. To test this hypothesis, syngeneic A/J mice were inoculated i.p. with SaI/Ak/DM/DO transfectants. H2-O2 SaI/Ak and SaI/Ak/DM, and MHC class II SaI cells served as controls. As shown in Table II, the three SaI/Ak/DM/DO clones are rejected, as are SaI/Ak and SaI/Ak/DM tumor cells, while SaI cells are malignant in 100% of mice. Therefore, H2-O does not inhibit presentation of endogenous tumor Ags, as measured by tumor rejection.

H2-O+ APC have been reported to have enhanced loading and presentation of exogenous Ags relative to H2-O− APC (5, 6). If expression of H2-O also enhances presentation of self Ags, then nonimmunogenic tumor cells may be converted to an immunogenic phenotype by expression of H2-O. To test this hypothesis, groups of A/J mice were challenged i.p. with SaI/CIITA or two clones of SaI/CIITA/DO cells. As shown in Table II, SaI/CIITA cells are lethal in A/J mice, regardless of whether they express H2-O. Therefore, H2-O does not enhance presentation of endogenous tumor Ags, as tested in this in vivo system.

Although the tumor rejection experiments suggest that H2-O does not alter presentation of self Ags, H2-O may exert subtle effects that are not detected by this assay. Therefore, we have generated SaI/Ak/DM/DO and SaI/Ak/DM cells expressing KDEL-tagged HEL as a self Ag (SaI/Ak/DM/HEL/DO and SaI/Ak/DM/HEL cells) and tested them as APC to a panel of HEL-specific,

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** H2-O does not affect self Ag presentation. A, SaI/Ak/DM/HEL/DO clones 3, 17, and 22 and SaI/Ak/DM/HEL cells were stained for cell surface expression of I-Ak and internal expression of H2-DM, H2-O, or HEL (hyHEL 7 and 10), as described in Fig. 1. B, HEL-transfected clones were incubated with HEL-specific I-Ak-restricted T hybridoma cells (2B6.3, 3B11.1, or 3A9). These data are representative of three independent experiments.

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** H2-O inhibits presentation of exogenous Ags. H2-O+ or H2-O− SaI/Ak/DM (A) or SaI/CIITA (B) cells were incubated with HEL-specific or RNase-specific, I-Ak-restricted T hybridoma cells (3B6.3, 3B11.1, 3A9, 2D4.1, 1B9, or TS12) in the presence of exogenous native HEL or RNase, respectively. These data are representative of at least three independent experiments with each hybridoma.
which the three Sal/A/DH/HEL/DO clones, along with Sal/A/DM/HEL cells, were tested as APC for HEL epitopes 25–43, 34–45, and 46–61. Sal/A/DH/HEL cells are included as a negative control because Ii blocks presentation of self Ags (12, 18). In all cases, the Sal/A/DM/HEL/DO clones are comparable with Sal/A/DM/HEL cells in presenting three HEL epitopes, confirming that H2-O expression does not alter presentation of endogenous self Ags.

H2-O affects presentation of exogenous Ags by newly synthesized and recycling MHC class II molecules

The effect of H2-O on presentation of exogenous Ags has been previously tested, and the results are controversial. In some studies, H2-O expression inhibits exogenous Ag presentation (5, 7), while in other studies H2-O enhances presentation (5, 6). Using five HEL-specific and one RNase-specific, I-A<sup>k</sup>-restricted T hybridomas (Table I), we have tested H2-O transfectants as APC for exogenous HEL and RNase, respectively. As shown in Fig. 6, three Sal/A/DM/DO clones (Fig. 6A) and two Sal/CIITA/DO clones (Fig. 6B) are inhibited for presentation of all tested HEL and RNase epitopes relative to parental Sal/A/DM and Sal/CIITA cells. The inhibition is not due to dysfunctional MHC class II molecules because H2-O<sup>−</sup> clones efficiently present exogenously pulsed peptides (data not shown). Therefore, H2-O consistently inhibits presentation of exogenous Ags by both I<sup>+</sup> and I<sup>−</sup> APC.

Previous studies have established that both newly synthesized and recycling MHC class II molecules present exogenous Ags (32–34). To determine whether H2-O differentially affects presentation of these two pools of MHC class II molecules, we have treated APC with drugs that selectively inhibit Ag processing and presentation. Chloroquine, a lysosomotropic amine, interferes with the acidification of MIICs, which in turn blocks presentation of exogenous Ags in these compartments (35). As shown in Fig. 7B, presentation of all epitopes by Sal/CIITA and Sal/A/DM, except HEL<sub>25–43</sub>, is inhibited by chloroquine, suggesting endosomal trafficking is required for most epitopes. BFA prevents transport of newly synthesized proteins from the ER, and hence blocks trafficking of newly synthesized MHC class II molecules, while having minimal effect on recycling class II (33, 36). As shown in Fig. 7A, Sal/A/DM and Sal/A/DM/DO cells treated with BFA are not inhibited for presentation of RNase<sub>43–56</sub> or HEL<sub>34–45</sub>, suggesting that presentation of these epitopes may use recycling MHC class II molecules. In contrast, presentation of HEL<sub>25–43</sub>, HEL<sub>46–61</sub>, HEL<sub>112–124</sub>, and HEL<sub>116–129</sub> by Sal/A/DM and Sal/A/DM/DO cells and epitopes HEL<sub>25–43</sub>, HEL<sub>34–45</sub>, HEL<sub>46–61</sub>, and RNase<sub>34–56</sub> by Sal/CIITA and Sal/CIITA/DO cells is inhibited by BFA, suggesting that newly synthesized MHC class II molecules are required for presentation of these epitopes. Because presentation of all of these epitopes is inhibited by H2-O (Fig. 6), H2-O appears to inhibit presentation of exogenous Ags by both newly synthesized and recycling class II molecules.

H2-O is also present at the cell surface

Previous studies have established that the majority of H2-O is localized to the MIICs by tightly associating with H2-DM (2). However, H2-O may affect Ag presentation by its expression at the cell surface. To determine whether H2-O is expressed on the outer face of the plasma membrane, surface proteins of Sal/A/DM and Sal/A/DM/DO cells were biotinylated, and Western blots of streptavidin (SA)-HRP or 9E10 (anti-c-myc) were probed with streptavidin-HRP. As shown in Fig. 7C, H2-O is expressed at the cell surface. Immunoprecipitation of HEL proteins in these transfectants with specific T hybridoma cells (Table I). Previous studies demonstrated that the HEL proteins in these transfectants are retained in the ER and are not secreted or present at the cell surface (18). However, HEL expression is not inhibited by BFA, suggesting that HEL presentation may use recycling MHC class II molecules. It is possible that HEL presentation is inhibited by BFA because H2-O affects presentation of exogenous Ags by newly synthesized and recycling MHC class II molecules (Fig. 6), while H2-O expression is inhibited by chloroquine, suggesting endosomal trafficking is required for most epitopes. BFA prevents transport of newly synthesized proteins from the ER, and hence blocks trafficking of newly synthesized MHC class II molecules, while having minimal effect on recycling class II (33, 36). As shown in Fig. 7A, Sal/A/DM and Sal/A/DM/DO cells treated with BFA are not inhibited for presentation of RNase<sub>43–56</sub> or HEL<sub>34–45</sub>, suggesting that presentation of these epitopes may use recycling MHC class II molecules. In contrast, presentation of HEL<sub>25–43</sub>, HEL<sub>46–61</sub>, HEL<sub>112–124</sub>, and HEL<sub>116–129</sub> by Sal/A/DM and Sal/A/DM/DO cells is inhibited by BFA, suggesting that newly synthesized MHC class II molecules are required for presentation of these epitopes. Because presentation of all of these epitopes is inhibited by H2-O (Fig. 6), H2-O appears to inhibit presentation of exogenous Ags by both newly synthesized and recycling class II molecules.
Ag was immunoprecipitated from biotinylated cells. As shown in Fig. 7C, c-myc is only detected by blotting with c-myc Ab (right panel) and not with streptavidin-HRP (middle panel). Therefore, H2-O is present in the MIICs and at the cell surface.

Discussion
Expression of H2-O in MHC class II+ tumor cells dramatically inhibits the presentation of the SAgs SEB and SEA. Recent experiments with splenic B lymphocytes from wild-type and H2-O knockout mice confirm the inhibitory effect of H2-O expression on SEB presentation (V. Clements, L. Qi, and S. Ostrand-Rosenberg, unpublished results). Although the inhibition could be the result of several factors, the observation that H2-O+ cells bind less SEB than H2-O− cells suggests that the inhibition may be due to reduced cell surface SAg/MHC class II complexes. There are several possible mechanisms for the decrease in SAg binding to MHC class II: 1) Peptide binding alters MHC class II conformation (27). Because H2-O alters the repertoire of exogenous peptides by interacting with H2-DM in the MIICs, it may modify the conformation of MHC class II molecules via an H2-DM-dependent mechanism and thereby reduce SAg binding. 2) SAgs binding may be sterically blocked by long peptides bound in the peptide groove of class II molecules (37). If H2-O preferentially mediates binding of longer peptides, then steric interference from longer peptides could alter SAg binding to MHC class II molecules. The data of van Ham et al. (7) do not favor this hypothesis. 3) H2-O may bind to MHC class II molecules at the cell surface. We and others have shown there is H2-O at the plasma membrane (Fig. 7C) (8), although it remains unclear whether surface H2-O interacts with H2-DM or MHC class II molecules. Recently, Arndt et al. (38) reported that 10–15% of total HLA-DM is functional at the cell surface, binding to MHC class II molecules. Therefore, surface class II/H2-DM/ H2-O complexes may exist, which alter the conformation of MHC class II or directly compete for SAg binding sites, and thereby lead to a reduction in SAg binding. 4) Recent studies have shown that in the MIICs, HLA-DO may directly interact with MHC class II and other tetraspan proteins, such as CD82 and CD63, independent of HLA-DM binding (39). Therefore, H2-O may affect SAg presentation by its interaction with proteins other than H2-DM and via as yet uncharacterized mechanisms.

Regardless of the mechanism by which H2-O reduces SAgs-induced T cell activation, the dramatic reduction suggests that H2-O may play a role in vivo in presentation of SAgs. Bacterial SAgs are immunostimulatory proteins that cause significant diseases in humans, ranging from toxic shock syndrome to autoimmune pathologies (28). These diseases occur when MHC class II+ host APC bind and present SAgs to host T cells. Although B cells present SAgs (40), they are less efficient presenters of SAgs than H2-O− APC, such as dendritic cells (DC) and macrophages (41). The discrepancy in SAg presentation between B cells, macrophages, and DC was originally attributed to differences in levels of expression of costimulatory and/or MHC class II molecules (41). However, given the inhibitory effects of H2-O on SAg presentation, it is also possible that DC and macrophages are better SAg presenters because they lack H2-O.

H2-O appears to selectively affect Ag presentation based on the origin of the Ag. That is, presentation of exogenous Ags such as soluble HEL and RNase, is inhibited, whereas presentation of endogenous self Ags, such as self HEL and tumor Ags, is not affected. This dichotomy may reflect differences in where exogenous vs endogenous peptides are loaded onto MHC class II molecules. Recent studies suggest that the binding of endogenous self peptides to MHC class II occurs in the endocytic pathway, as does binding of exogenous peptides (13, 42). However, binding of endogenous vs exogenous peptides may occur in different compartments of MIICs (13, 18), and different pHs in these compartments may account for differential effects of H2-O (5, 7).

H2-O expression in mTEC may also play a role in T cell selection in the thymus. mTEC together with bone marrow-derived APC (DC) are major APC for negative selection of developing T lymphocytes in the thymus (10). mTEC are H2-O+, while DC are H2-O− (8, 9). If H2-O functions in mTEC as it does in the SaI transfectants, then mTEC may be inhibited for presentation of exogenous Ags, but not endogenous self Ags. Therefore, mTEC may preferentially delete autoreactive T cells specific for endogenous self Ags, but not T cells reactive to exogenous self Ags. In contrast, because DC are H2-O−, they may preferentially delete T cells reactive to exogenous self Ags presented by cross-presentation. The combination of H2-O+ (mTEC) and H2-O− (DC) APC may complement each other and delete autoreactive T cells specific for both endogenous and exogenous Ags, and thereby minimize overall autoreactivity. The expression of H2-O in mTEC and its absence in DC may also explain the observation that unlike DC, mTEC do not induce full tolerance (43).

Note added in proof. A recent study (44) demonstrated that HLA-DR3 conformation could be altered by HLA-DM in a peptide-independent manner. Therefore, H2-O may reduce SAg presentation in a peptide-independent fashion by either binding directly to class II or by interacting with H2-DM.

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