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Ligand Exchange of Major Histocompatibility Complex Class II Proteins Is Triggered by H-bond Donor Groups of Small Molecules*

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Kirsten Falk‡§¶, Julie M. Lau‡¶, Laura Santambrogio‖, Viviana Marin Esteban§, Fabiola Puentes§, Olaf Rötzschke‡§, and Jack L. Strominger‡‖‡‡**

From the ‡Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts 02138, the §Max Delbrück Center for Molecular Medicine, Robert-Rössle-Strasse 10, 13125 Berlin, Germany, and the ‖Department of Cancer Immunology/AIDS, Dana Farber Cancer Institute, Boston, Massachusetts 02115

Hydrogen bonds (H-bonds) are crucial for the stability of the peptide-major histocompatibility complex (MHC) complex. In particular, the H-bonds formed between the peptide ligand and the MHC class II binding site appear to have a great influence on the half-life of the complex. Here we show that functional groups with the capacity to disrupt hydrogen bonds (*e.g.* -OH) can efficiently catalyze ligand exchange reactions on HLA-DR molecules. In conjunction with simple carrier molecules (such as propyl or benzyl residues), they trigger the release of low affinity ligands, which permits the rapid binding of peptides with higher affinity. Similar to HLA-DM, these compounds are able to influence the MHC class II ligand repertoire. In contrast to HLA-DM, however, these simple small molecules are still active at neutral pH. Under physiological conditions, they increase the number of "peptide-receptive" MHC class II molecules and facilitate exogenous peptide loading of dendritic cells. The drastic acceleration of the ligand exchange on these antigen presenting cells suggests that, in general, availability of H-bond donors in the extracellular milieu controls the rate of MHC class II ligand exchange reactions on the cell surface. These molecules may therefore be extremely useful for the loading of antigens onto dendritic cells for therapeutic purposes.

Peptide ligands bind to the peptide-binding groove of MHC[1] class II molecules by an array of intermolecular hydrogen bonds (H-bonds). These hydrogen bonds are mostly formed between the backbone of the peptide and conserved residues of the MHC class II molecule. Some of these H-bonds are particularly crucial for the stability of the ligand complex (1). It has been shown for a murine MHC class II molecule that the elimination of H-bonds between the ligand and residues His-81 or Asp-82 of the I-A^d β-chain results in a rapid loss of the bound peptide (2). Detailed kinetic studies with these mutated MHC molecules revealed peptide dissociation rates that were increased up to 200-fold (3). This increase was in the same range observed after addition of HLA-DM to the peptide complex of the nonmutated I-A^d molecule. It was therefore proposed that HLA-DM-mediated ligand release (4, 5) is also accomplished by the disruption of H-bonds (6), a hypothesis also introduced when the crystal structure of HLA-DM was published (7).

Because H-bonds appear to be fundamental in maintaining the stability of the MHC class II peptide complexes, we started to investigate small molecules capable of disrupting H-bonds with the goal of achieving an HLA-DM-like catalytic effect on the kinetics of peptide binding. H-bonds require a hydrogen donor and an acceptor group, which provides a free electron pair. Some of the functional groups that can fulfill this function are hydroxyl or amino groups. They are present in a variety of natural and synthetic molecules, such as lipids, metabolites, amino acids, and pharmaceutical drugs. One example is ethanol, where the well known physiological effects appear to result from subtle conformational changes of neurotransmitter receptors caused by the disruption of H-bonds (8, 9). To show that hydroxyl or other H-bond-forming functional groups (in conjunction with appropriate small carrier molecules) can in fact mimic the catalytic activity HLA-DM would not only provide additional support for the postulated function of H-bonds as a molecular basis of the mechanism (6, 7, 10), it would also suggest that in general the availability of molecules with H-bond forming capacity in the extracellular milieu controls the rate and extent of ligand exchange on the cell surface.

Small molecular compounds with peptide complex destabilizing capacity would also represent powerful tools to enhance the efficiency of HLA-DM-independent ligand exchange reactions, which take place, for example, on the cell surface of antigen presenting cells. A series of studies has already been published that describes successful immunotherapies based on the reinjection of antigen-loaded dendritic cells (DC) (11–15). The major goal of these attempts is to deliver high amounts of immunogenic peptide antigens derived from tumor-specific or tumor-associated proteins. In addition to the benefit of increased loading rates, these small molecular catalysts would also allow the bypassing of proteolytic endosomal processing compartments, which facilitates the presentation of epitopes sensitive to proteolytic degradation when following the classical MHC class II presentation pathway.

Previous studies have already shown that, under certain conditions, the stability of peptide-MHC complexes can be affected by detergents or detergent-like compounds (16, 17). The mechanism, however, remained obscure, and the chemical nature of these compounds did not allow any experiments with

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¶ The first two authors contributed equally to this work.

** To whom correspondence may be addressed: Max Delbrück Center for Molecular Medicine, Robert-Rössle-Str. 10, 13125 Berlin, Germany. Tel.: 49-30-9406-3664; Fax: 49-30-9406-2394; E-mail: roetzsch@mdc-berlin.de.

‡‡ To whom correspondence may be addressed. Tel.: 617-495-2733; Fax: 617-496-8351; E-mail: jlstrom@fas.harvard.edu.

[1] The abbreviations used are: MHC, major histocompatibility complex; PE, phycoerythrin; pCP, *p*-chlorophenol; tg, transgenic; H-bond, hydrogen bond; DC, dendritic cell; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; MBP, myelin basic protein; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting.

living cells. In this study we tested the effect of simple H-bond donor/acceptor molecules, such as ethanol, *n*-propanol, phenol, and aniline on the ligand exchange of MHC class II molecules. By utilizing both soluble HLA-DR molecules and cellular *in vitro* systems, we evaluated the effect on the molecular level as well as under physiological conditions in cell assays (including T cell stimulation experiments). Because of potential implications for the induction of autoimmune reactions, autoantigens, such as the encephalitogenic MBP86–100 epitope (derived from the myelin basic protein), together with allelic forms of MHC class II molecules associated with the development of autoimmune diseases (*e.g.* HLA-DR2) were included in this study.

EXPERIMENTAL PROCEDURES

Reagents and Peptide Ligands—Most reagents were obtained from Sigma (*n*-propanol, *n*-butanol, phenol, *n*-propylamine, aniline); *para*-chlorophenol was obtained from Fluka. The peptides IC106–120 (CLIP) (KMRMATPLLMQALPM) (18), MBP86–100 (NPVVHFFKNIVTPRT) (19), HA306–318 (PKYVKQNTLKLAT) (20), and PLP139–151(C140S) (HSLGKWLGHPDKF) (21, 22) were synthesized by using standard solid phase Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry. For biotinylated peptides the biotin was attached to the N terminus of peptides, which were extended by a short amino acid spacer sequence (SGSG). All peptides were obtained from the Biopolymers Laboratory at Harvard Medical School.

Production of Soluble MHC Class II Molecules—Soluble MHC class II molecules HLA-DR1 (DRA1*0101, DRB1*0101) (4) and HLA-DR2 (DRA1*0101, DRB1*1501) (23) and soluble HLA-DM molecules (4) were produced in S2 insect cells stably transfected with vectors encoding truncated MHC α and β chains, which lack cytoplasmic and transmembrane domains as described (4). S2 cells expressing HLA-DR1, HLA-DR2, and HLA-DM were provided by D. Wiley, K. Wucherpfennig, and D. Zaller, respectively.

SDS-PAGE Separation of Peptide-MHC Complexes—Complexes were formed in a volume of 6 μl by incubating 0.5 μg/ml HLA-DR with 0.16 μg/ml peptide ligand in the presence of 5% ethanol or 0.07 μg/ml soluble HLA-DM for 4 h at 37 °C at pH 5.0 (25 mM sodium acetate/sodium phosphate, 37.5 mM NaCl). After incubation pH was raised to 7.3, and after an additional 20 min at 37 °C the samples were separated by SDS-PAGE. SDS-PAGE separation was done on a 4–15% Tris-glycine gradient gel (Bio-Rad) at 4 °C. Samples were loaded without prior boiling using a nonreducing sample buffer. A protein standard of 25–150 kDa (Novagen) was used as a marker. Protein bands were visualized by silver stain.

ELISA Experiments—Preformed CLIP peptide-HLA-DR complexes were generated by incubating 10 μl of HLA-DR (1 mg/ml) with 0.5 μl of biotinylated CLIP peptide (1 mg/ml) for 18–24 h at 37 °C. For peptide release experiments, the reaction was diluted 1:5 with phosphate-buffered saline. 4 μl of the dilution were mixed with 4 μl of buffer (100 mM Na_2HPO_4, 100 mM sodium acetate, 150 mM NaCl; the pH was adjusted to 5.0 or 7.3). Depending on the experiment, indicated amounts of excess peptide and/or catalyst (HLA-DM or small molecule) were added to a total volume of 10 μl. The reaction mixture was incubated for 3 h at 37 °C. In some release experiments, high amounts of high affinity peptides were added to prevent binding of already released CLIP peptide. The release reaction was added to ELISA plates, previously coated with an α-HLA-DR monoclonal antibody (L243, American Type Culture Collection) and incubated for 1 h at 37 °C. Plates were washed and incubated for 30 min at room temperature with avidin coupled with peroxidase (Sigma). The amount of stable biotin-CLIP·HLA-DR complex was detected by using 3,3′,5,5′-tetramethylbenzidine substrate (Kirkegaard and Perry Laboratories) and measured at 450 nm with a microplate reader (Dynex Technologies). In some experiments Eu^{3+}-labeled streptavidin (DELFIA, Wallac) was used. In this case the detection was carried out by using a fluorescence enhancing solution (15 μM β-naphthoyltrifluoroacetone, 50 μM tri-*n*-octylphosphine oxide, 6.8 mM potassium hydrogen phthalate, 100 mM acetic acid, 0.1% Triton X-100), and the signal was measured with a Victor fluorescence reader (Wallac) using the time-resolved mode at an excitation wavelength of 340 nm and emission wavelength of 614 nm.

FACS Analysis of Empty MHC Class II Molecules on Dendritic Cells—Dendritic cells were isolated from the bone marrow of SJL/J mice (Jackson) and established by *ex vivo* differentiation of precursor cells as described (24). Briefly, cultures were maintained in DMEM plus 5% FCS supplemented every 2 days with 10 ng/ml granulocyte/macrophage colony-stimulating factor. B cells, T cells, and granulocytes were removed by using rat monoclonal antibodies (PharMingen) B220/CD45R, CD90.2/Thy 1.2, and Ly6GGR-1/RB6–8C5, in conjunction with magnetic beads coated with sheep anti-rat IgG (Dynal, Great Neck, NY), first on initial isolation from bone marrow and again immediately before staining. Cell surface expression of empty MHC class II molecules was determined by flow cytometry as described (25, 26). For peptide binding DC (5×10^5) were incubated at 37 °C in the absence or presence of peptide ligands in DMEM supplemented with or without 2% of *n*-propanol, 10 μM iodoacetamide, 1 μM EDTA, 0.02% NaN_3, 1 μM deoxyglucose for 4 h. For staining DC were incubated on ice with saturating amounts of primary antibody for 30 min in phosphate-buffered saline (150 mM NaCl, 10 mM sodium phosphate, 12 mM NaN_3, pH 7.2) containing 1 mg/ml bovine serum albumin, 10 μM iodoacetamide, 1 μM EDTA, 0.02% NaN_3, 1 μM deoxyglucose and then washed, incubated with phycoerythrin-conjugated $(Fab')_2$ secondary antibody (Jackson Immunoresearch) that had been preabsorbed with normal serum, washed again, and analyzed immediately by using a FACScalibur flow cytometer (Becton Dickinson). The primary antibody used in cytometry, KL-304 (anti-$IA^{s,k,u,f}$ β 57–68, specific for empty MHC class II molecules; Refs. 25–27) was produced in hybridomas (American Type Culture Collection) and purified by ammonium sulfate precipitation and protein A or protein G chromatography. Fcγ receptor binding was blocked by preincubation with 1 μg of rat monoclonal antibody CD16/CD32 (PharMingen).

Cell Surface Peptide Loading Assay—MHC class II negative mouse L cells L929 (American Type Culture Collection) and fibroblast cells transfected with HLA-DR1 (L57.23; HLA-DRA1*0101, HLA-DRB1*0101; provided by E. Rosloniec) or HLA-DR2 (L466; HLA-DRA1*0101, HLA-DRB1*1501; provided by K. Wucherpfennig) were incubated with titrated amounts of biotinylated peptide ligands in DMEM plus 5% FCS supplemented with or without 2% *n*-propanol for 4 h at 37 °C. Cells were then washed and stained with streptavidin-phycoerythrin and analyzed by flow cytometry using a FACScalibur instrument (Becton Dickinson). In some experiments, splenocytes from HLA-DR1 tg B10.M mice (provided by D. Zaller) were also used.

T Cell Assay—HLA-DR-expressing fibroblast cells L57.23 (HLA-DR1) and L466 (HLA-DR2) were incubated for 4–6 h with titrated amounts of peptide antigens in DMEM plus 5% FCS supplemented with or without 2% *n*-propanol (5×10^4 cells/well). The cells were then washed twice and incubated for 24 h with 5×10^4 cells/well of the MBP86–100-specific T cell hybridoma 08073 (provided by L. Fugger) or the HA306–318-specific CH7C17 T cells, a T cell receptor-transfected Jurkat cell line (provided by L. Stern with kind permission of L. R. Wedderburn) in DMEM plus 5% FCS. T cell response was determined by analyzing the interleukin-2 release in a secondary assay with CTLL cells (28).

RESULTS

Ethanol is one of the simplest small molecules with H-bond forming capability (8). To test the influence on peptide loading, soluble HLA-DR1 molecules were incubated with a high affinity peptide ligand (HA306–318) in the presence of ethanol or soluble HLA-DM. After 4 h of incubation, complex formation was analyzed by SDS-PAGE (Fig. 1). As expected the presence of HLA-DM significantly increased the amount of peptide complex (Fig. 1, *lane 4*). The band representing the SDS-stable HA306–318·HLA-DR1 complex was clearly evident in the HLA-DM-containing sample, whereas the band was much fainter when no catalyst was present (*lane 2*). However, the same effect was also evident when ethanol instead of HLA-DM was used (*lane 3*). The intensity of the staining of the band was even higher than in the HLA-DM sample, indicating that more peptide-MHC complex was generated by ethanol.

Because ethanol displayed some HLA-DM-like catalytic activity in the loading experiment, the effect of the small molecule and of HLA-DM was further compared in ELISA experiments. In the experiment shown in Fig. 2, the capacity of the two "catalysts" to exchange ligands was determined by the release of CLIP peptide from HLA-DR2 in the presence of titrated amounts of free peptides with different affinities to the HLA-DR2 molecule (MBP86–100, high affinity; IC106–120, medium affinity; HA306–318, low affinity). Because HLA-DM requires endosomal pH to exhibit optimal activity, the first part

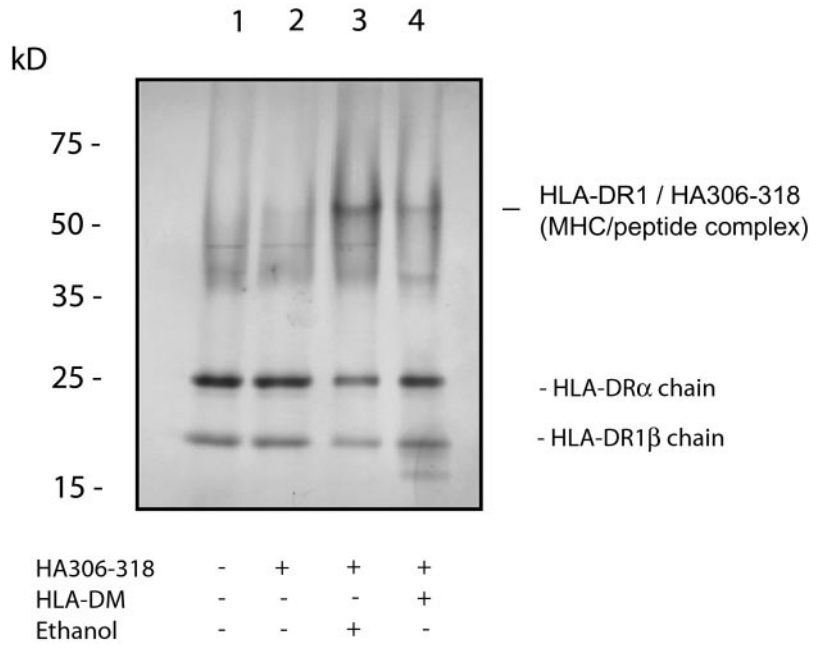

FIG. 1. **Loading of soluble HLA-DR1 with HA306–318 peptide in the presence of HLA-DM or ethanol.** Soluble HLA-DR1 molecules were incubated with HA306–318 peptide alone (*lane 2*), in the presence of 5% ethanol (*lane 3*) or soluble HLA-DM (*lane 4*). HLA-DR1 incubated without peptide was used as a control (*lane 1*). After incubation for 4 h at 37 °C at pH 5.0, the pH was adjusted to 7.3 and the reaction mixtures were separated by SDS-PAGE. Loaded MHC class II molecules are evident as 60-kDa bands (SDS-stable HLA-DR1-peptide complex); unloaded empty HLA-DR1 molecules dissociate and are detected as single chains with mass of approximately 25 kDa (α-chain) and 20 kDa (β-chain). The bands were visualized by silver staining.

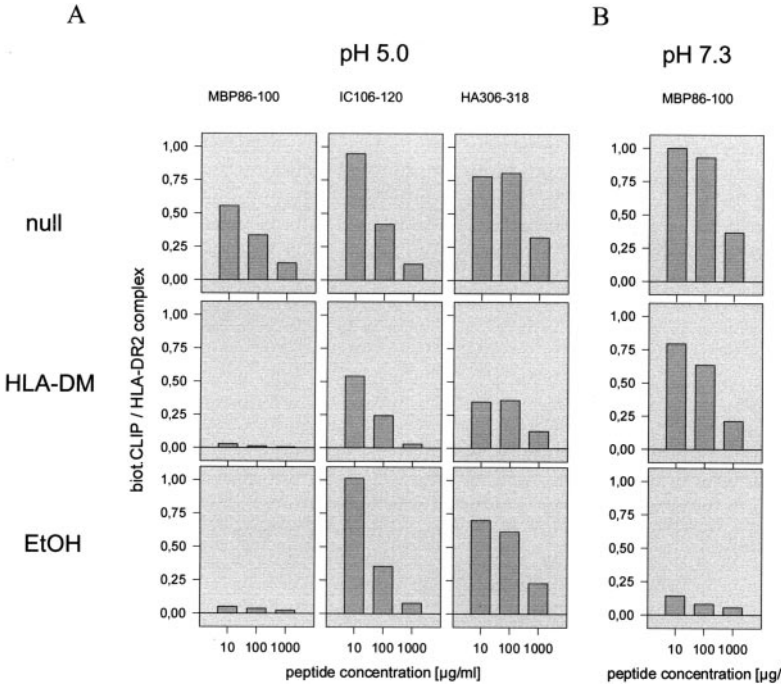

FIG. 2. **Comparison of the ligand exchange of soluble HLA-DR2 mediated by HLA-DM and by ethanol.** The release of biotinylated CLIP in the presence of peptides with different affinities to the MHC class II molecule was tested without any catalyst (*upper panels*), with 0.1 mg/ml soluble HLA-DM (*middle panels*), or with 5% ethanol (*lower panels*). Preformed complexes of biotin CLIP and soluble HLA-DR2 molecules were used. Values expressed on the *ordinate* represent the relative amount of biotin-CLIP·HLA-DR2 after the release reaction and were determined in a sandwich ELISA (immobilized anti-HLA-DR capture antibody and soluble avidin-peroxidase to detect the CLIP peptide). *A*, ligand exchange at pH 5.0. The release of CLIP was carried out for 3 h at 37 °C in a buffer adjusted to pH 5.0. The following peptides were present during the incubation: high affinity MBP86–100 (*left panel*), medium affinity IC106–120 (*middle panel*), or low affinity HA306–318 (*right panel*). *B*, exchange at pH 7.3. The same experiment was carried out as described under *A* except that the effect of HLA-DM and ethanol was compared at pH 7.3 in the presence of the high affinity MBP86–100 peptide only.

of the experiment was carried out at pH 5.0 (Fig. 2*A*). As expected the influence of HLA-DM (*middle panel*) was most evident in the presence of the high affinity peptide MBP86–100. An almost complete release of the biotinylated CLIP peptide was detected at a concentration of only 10 μg/ml MBP86–100, whereas in the presence of the IC106–120 or HA306–318 peptide little difference to the noncatalyzed control reaction (*upper panel*) was observed. However, the same result was also obtained with 5% ethanol (*lower panel*). Ethanol catalyzed the exchange of CLIP by MBP86–100 but did not enhance the exchange by IC106–120 or HA306–318. Thus, ethanol can mediate the replacement of peptides according to their affinity. In contrast to HLA-DR2, a release of CLIP from HLA-DR1 by ethanol was not seen, which was due presumably to the higher affinity of the CLIP peptide to HLA-DR1 (data not shown).

In the second part of this experiment, the effectiveness of HLA-DM and ethanol on the ligand release was tested under physiological conditions (Fig. 2*B*). It is known that HLA-DM molecules show little or no catalytic activity at pH 7.0 (4, 29–31). In accordance with these reports, almost no release of biotinylated CLIP peptide from HLA-DR2 molecules was observed at pH 7.3. Approximately 80% of the CLIP·HLA-DR2 complexes were still detectable after the incubation with HLA-DM and 10 μg/ml MBP86–100. Compared with HLA-DM the ligand exchange capacity of ethanol appeared to be only slightly reduced at pH 7.0. After incubation with ethanol and 10 μg/ml high affinity peptide, less than 12% of the HLA-DR2 molecules were still loaded with CLIP. The different exchange capacity of ethanol and HLA-DM at neutral pH is presumably because of the fact that the dipole moment of ethanol's hydroxyl group remains unchanged upon pH shifts from 5.0 to 7.0, whereas conformational transitions abrogate the catalytic activity of the HLA-DM molecule.

The previous experiments indicated that ethanol in principle

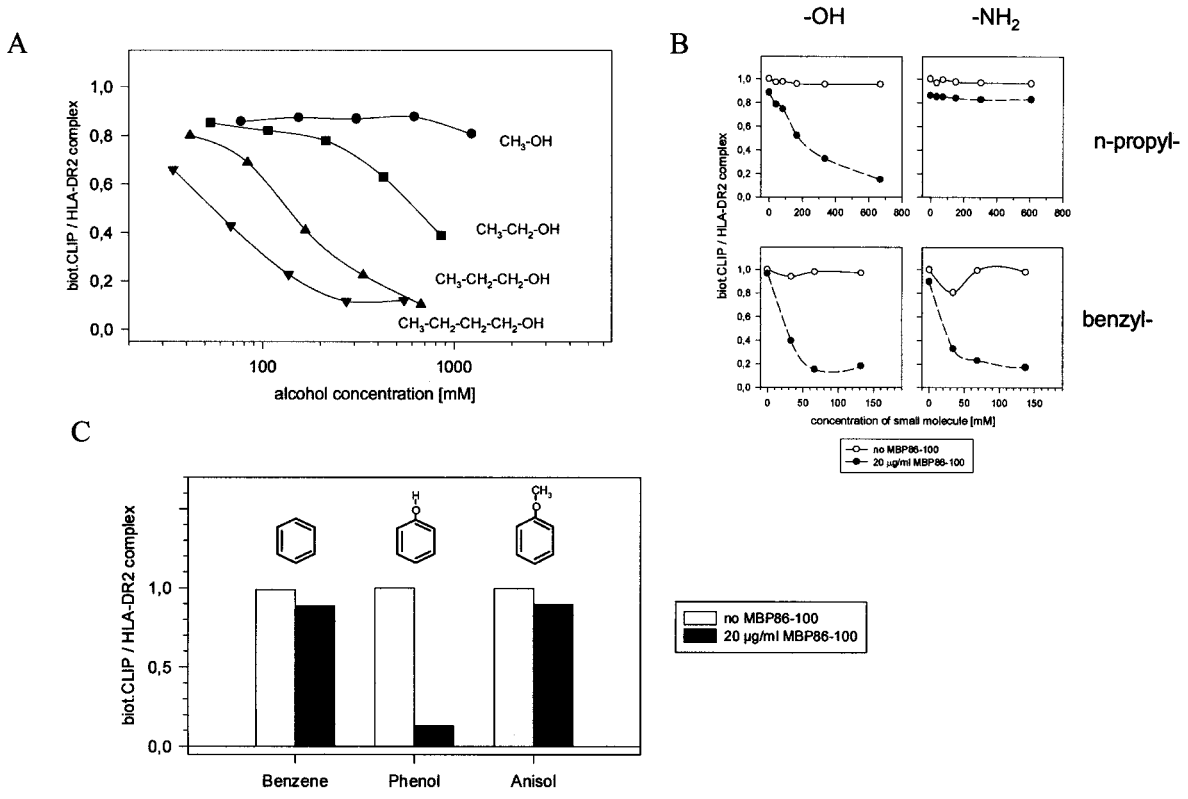

FIG. 3. **Influence of the structure of small molecular compounds on the ligand exchange.** The release of biotin-CLIP from soluble biotin-CLIP·HLA-DR2 was tested with several aliphatic and aromatic compounds. In all experiments the release reaction of biotin-CLIP·HLA-DR2 complexes was carried out for 3 h at pH 7.3. The amount of CLIP·HLA-DR2 remaining after the incubation was determined by ELISA as described in Fig. 2. *A*, influence of the chain length of aliphatic alcohols. Biotin-CLIP·HLA-DR2 was incubated with titrated amounts of methanol (CH_3-OH), ethanol (CH_3-CH_2-OH), *n*-propanol (CH_3-CH_2-CH_2-OH), and *n*-butanol (CH_3-CH_2-CH_2-CH_2-OH). The data shown in the figure represent the amount of stable complex after the release reaction and were generated in the presence of 20 μg/ml MBP86–100 (only little or no release was detected in the absence of the MBP peptide (data not shown)). *B*, effect of hydroxyl or amino groups in conjunction with propyl or benzyl residues. The release capacity of hydroxyl (*left panel*) and amino groups (*right panel*) was compared when attached to aliphatic *n*-propyl (*upper panel*) or to aromatic benzyl residues (*lower panel*). The experiments were carried out in the absence (*open symbols*) or presence of 20 μg/ml MBP86–100 (*closed symbols*). *C*, H-bond donor function. The effect of benzene, phenol, and anisol on CLIP·HLA-DR2 was compared. The release was carried out at a concentration of 60 mM small molecule in the absence (*open bars*) or presence (*filled bars*) of 20 μg/ml MBP86–100.

showed the HLA-DM-like catalytic activity anticipated from an H-bond disrupting small molecule. The amount needed, however, was still relatively high. To identify more effective compounds, organic small molecules with various functional groups were tested. Most effective were hydroxyl groups in conjunction with hydrophobic carrier residues. For aliphatic residues it was found that the catalytic activity increased with the length of the chain (Fig. 3*A*). Methanol (C_1) had virtually no effect, ethanol (C_2) triggered a 50% release of the CLIP peptide at a concentration of 600 mM, *n*-propanol at 140 mM, and *n*-butanol (C_4) at a concentration of 40 mM. The substitution of the hydroxyl group by (positively charged) amino groups resulted in a complete loss of catalytic activity. No release was therefore observed with *n*-propylamine (Fig. 3*B*, *upper panel*) or with *n*-octylamine (data not shown), one of the detergent-like compounds previously reported to affect full-length HLA-DR3 (16). Additionally, no release was seen with ethanolamine, amino acids, and other charged molecules, indicating that the presence of charged substituents abrogates the effect even when located distant to the H-bond donor group. Another group of molecules that failed to induce exchange reactions were the so-called "chemical chaperones" (32), which included compounds like Me_2SO, glycerol, and some other simple organic small molecules (data not shown).

Aromatic compounds tended to be more effective ligand exchangers than aliphatic molecules. The activity of two of these compounds is shown in the *lower panel* of Fig. 3*B*. Phenol triggered a 50% release at a concentration of about 30 mM, and a similar effect was also seen with aniline (which carries an uncharged amino group because of mesomeric resonance). The requirement of a functional group with H-bond forming capacity is illustrated in Fig. 3*C*. Removal of the hydroxyl group or substitution of the hydrogen completely abrogated the effect, indicating that the small molecule has to function as an H-bond donor to affect the exchange rate of MHC class II molecules.

Because small molecular H-bond donors retain effectiveness at neutral pH, they were tested in cell assays under physiological conditions. In contrast to the more potent phenol, *n*-propanol did not affect the viability of cells. A series of experiments was therefore conducted in which the influence of *n*-propanol on cell surface ligand exchange was determined with MHC class II-expressing antigen presenting cells such as dendritic cells or transfected fibroblast cell lines. First, whether or not the exposure of *n*-propanol results in the release of endogenous ligands was examined. Because such a release should lead to an increase in the amount of "empty" MHC molecules on the cell surface, the analysis was carried out by FACS using the conformation-specific KL304 antibody (27), which has been recently found to bind exclusively to empty MHC class II molecules (25, 26). FACS analysis of murine bone marrow-derived dendritic cells revealed that, after 4 h of incubation with 2% *n*-propanol, the amount of empty MHC class II molecules was indeed significantly increased (Fig. 4*A*). The median fluorescence of the KL304 staining was 81, whereas, in the absence of *n*-propanol, a value of only 38 was measured.

To ensure that the incubation with *n*-propanol generated

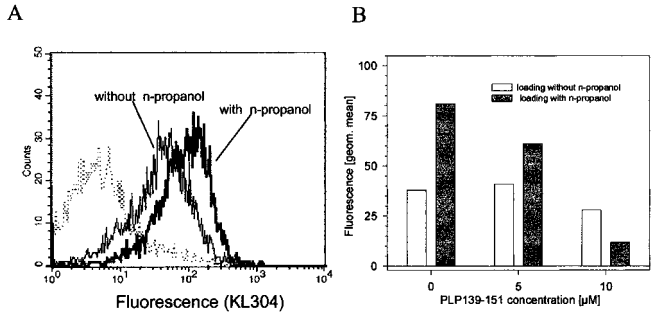

FIG. 4. **Detection of empty MHC class II molecules after exposure of dendritic cells to *n*-propanol.** Bone marrow-derived dendritic cells were incubated with medium containing 2% *n*-propanol in the absence or presence of high affinity binding peptides. Ligand exchange was detected by FACS analysis. *A*, generation of empty surface MHC class II molecules. After 4 h of incubation at 37 °C (in the absence of any peptides added), the amount of empty MHC molecules was determined by staining the dendritic cells with the conformation specific KL304 antibody (indicative for the empty conformation). The figure shows a FACS histogram generated in the absence (*thin solid line*) or presence of 2% *n*-propanol (*bold solid line*). An isotype control is represented as *dashed line*. *B*, incubation of surface MHC class II molecules with *n*-propanol and high affinity peptides. The incubation of dendritic cells (derived from SJL/J mice) with 2% *n*-propanol (*filled bars*) or without *n*-propanol (*open bars*) was repeated in the presence of indicated amounts of the I-A^s-restricted PLP139–151 peptide. Incubation and analysis were carried out as described for *A*. The *bars* indicate the median of the fluorescence of the KL304 staining.

functional "peptide-receptive" MHC class II molecules that can be reloaded with high affinity peptides, the exposure of I-A^s-expressing dendritic cells was repeated in the presence of the PLP 139–151 peptide (Fig. 4*B*). KL304 staining revealed that, in the absence of *n*-propanol, the peptide caused only a slight reduction in the amount of empty MHC molecules. Only at the highest peptide concentration used (10 μg/ml) was a 26% decrease in the KL304 staining from a fluorescence median of 38 to 28 detected. With 2% *n*-propanol, however, the amount of detectable empty MHC class II decreased by almost 85% when the peptide was present during the incubation. The geometrical mean of the fluorescence of the KL304 staining was reduced from 81 (detected in the absence of the peptide) to 12 (detected with 10 μg/ml PLP139–151). Thus, *n*-propanol is able to generate functional empty MHC class II molecules on the surface of antigen presenting cells, which can efficiently be loaded with high affinity peptide ligands.

The peptide loading was further analyzed with biotinylated peptides (Fig. 5*A*). The staining with phycoerythrin-labeled streptavidin allowed direct determination of the amount of peptides bound on the cell surface by FACS analysis. For these experiments fibroblast cells expressing HLA-DR1 or HLA-DR2 were incubated with titrated amounts of biotin-HA306–318 and of biotin-MBP86–100 peptide, respectively. In the presence of *n*-propanol, the staining of HA306–318 on HLA-DR1-expressing fibroblast cells increased ~10-fold (*upper panel*), whereas for MBP86–100 and HLA-DR2 the increase was even 20-fold (*lower panel*). Here, a median fluorescence of ~12 was determined at the highest peptide concentration used (1 μg/ml), whereas in the absence of *n*-propanol only a value of 3 was measured. This value, however, was already reached at a peptide concentration of about 50 ng/ml in the presence of 2% *n*-propanol. Notably, no surface staining was detected on fibroblast cells not expressing MHC class II molecules, which confirmed that MHC class II molecules were indeed loaded with the peptides.

As shown in Fig. 5*B*, T cell assays were used to test whether the increase in loading efficiency also translated into improved T cell responses. HLA-DR-expressing fibroblast cells were loaded with peptide antigens in the absence or presence of *n*-propanol and used to challenge CH7CH17 T cells, a Jurkat cell line transfected with T cell receptor specific for HLA-DR1/HA306–318 (33) (*upper panel*), or the mouse T cell hybridoma 08073 specific for HLA-DR2/MBP86–100 (*lower panel*). In accordance with the previous peptide loading experiment, the HLA-DR1-restricted T cell response was triggered at ~10-fold lower concentrations when 2% *n*-propanol was present during the incubation of the antigen presenting cell with the HA306–318 peptide. HLA-DR2-restricted T cells needed less than 1/20 of the peptide concentration to be stimulated when the loading of MBP86–100 was carried out in the presence of *n*-propanol.

The above cell experiments illustrate that the presence of *n*-propanol significantly amplifies the T cell response by lowering the threshold concentration of the free antigen. To identify even more effective compounds, several modified small molecules were tested. Fig. 6*A* shows a comparison between *n*-propanol and *p*-chlorophenol (pCP), an H-bond donor molecule where the ligand exchange activity could be improved by an additional substitution of the hydrophobic benzyl carrier residue. In this experiment the influence of the two compounds was tested on the loading of HLA-DR2-expressing fibroblast cells with biotinylated MBP86–100 peptide. To reach a fluorescence median of 10 requires only 1 mM pCP. This is 200-fold less than with *n*-propanol, where a concentration of 200 mM was needed to generate an equivalent signal. Specificity and efficiency of pCP is particularly evident in the loading of splenocytes from HLA-DR1 transgenic mice. The dot-plot chart in Fig. 6*B* demonstrates that efficient binding of biotinylated HA306–318 peptide to the HLA-DR1 molecule of these cells is possible only when 2 mM pCP is present during the incubation (*lower right panel*). Without the H-bond donor, almost no peptides were transferred onto the cell surface HLA-DR molecules (*lower left panel*). Notably, only the HLA-DR^+ cell population was affected by the treatment with pCP, which shows again the specificity of treatment with H-bond donor molecules.

DISCUSSION

In this study we showed that small molecular compounds with H-bond forming capacity can mimic the action of HLA-DM. They accelerate the release of low affinity peptide ligands, generate peptide receptive forms, and mediate the exchange with other peptides. As in the case of HLA-DM, the ligand exchange takes place according to the affinity of the peptide to the MHC class II molecule. "Peptide editing" (34) is therefore an inherent feature of the MHC class II molecule, and catalysts, such as HLA-DM and small molecular H-bond donors, convert the MHC molecule into a state that enables it to perform ligand selection.

Small molecular ligand exchange catalysts require an H-bond donor group to be functional. Blockage or replacement of this group results in the abrogation of the ligand release capacity. Crystal structure analysis of HLA-DM (7) and of a mutated I-E^k molecule (35) indicated that rapid ligand exchange is associated with alterations in the H-bond network between peptide and MHC molecule. A prior study with mutated I-A^d molecules (10) had already suggested that the "selective" state is characterized by the loss of one or more of the H-bonds connecting the ligand to the MHC binding site. It is therefore not unlikely that this H-bond network is the target for both HLA-DM and the small molecular compounds. Normal H-bonds can provide between 1 and 2 kcal/mol, and, depending on the chemical environment, the contribution can even increase to more than 5 kcal/mol (36). Thermodynamically a reduction of binding energy by 2 kcal/mol translates into a decrease in the affinity by ~100-fold (3), a decrease in affinity that is sufficient to trigger the release of weakly bound ligands.

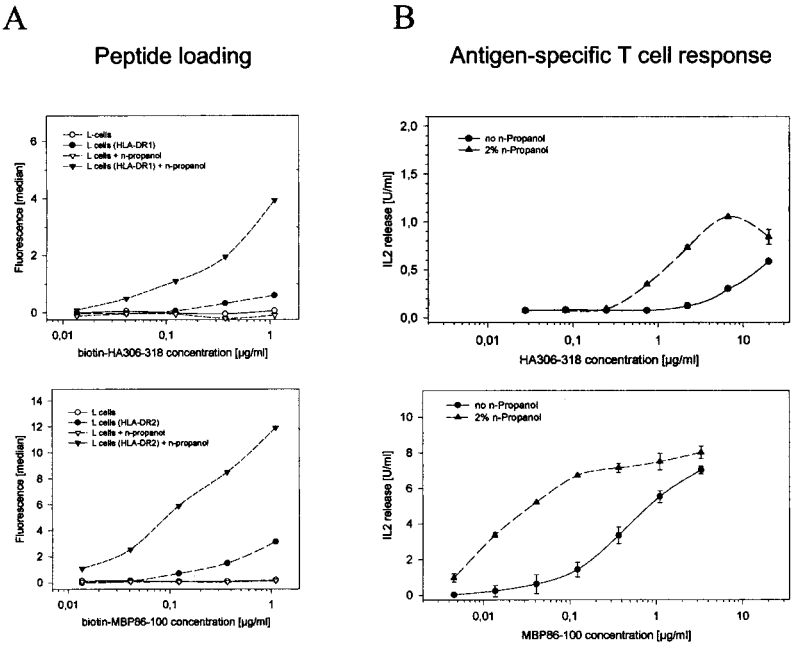

FIG. 5. **Enhanced antigen loading efficiency and increased T cell sensitivity after *n*-propanol treatment.** The loading of peptide antigens onto surface MHC class II molecules of antigen presenting cells and the T cell response triggered by these cells was determined. The antigen loading was carried out in the absence (*circles*) or presence (*triangles*) of 2% *n*-propanol. *A*, antigen loading. Fibroblast cells transfected with HLA-DR1 or HLA-DR2 (*solid symbols*) were incubated with titrated amounts of biotinylated HA306–318 peptide (for HLA-DR1; *upper left panel*) or biotinylated MBP86–100 (for HLA-DR2; *lower left panel*) for 4 h. The amount of peptide loading was determined by FACS analysis after staining the cells with phycoerythrin-labeled streptavidin. L cells, which do not express HLA-DR (*open symbols*), were used as controls for specific peptide binding. *B*, T cell response. HLA-DR1 or -DR2-expressing fibroblast cells were incubated for 4 h with titrated amounts of HA306–318 (*upper panel*) or MBP86–100 (*lower panel*) in the absence (*solid circles*) or presence (*solid triangles*) of 2% *n*-propanol. After incubation the cells were washed and used to challenge T cells. The specific T cell response was determined by using CH7C17, T cell receptor-transfected Jurkat cells (HLA-DR1/HA306–318), and the T cell hybridoma 08073 (HLA-DR2/MBP86–100). The response was measured by determining the interleukin-2 release.

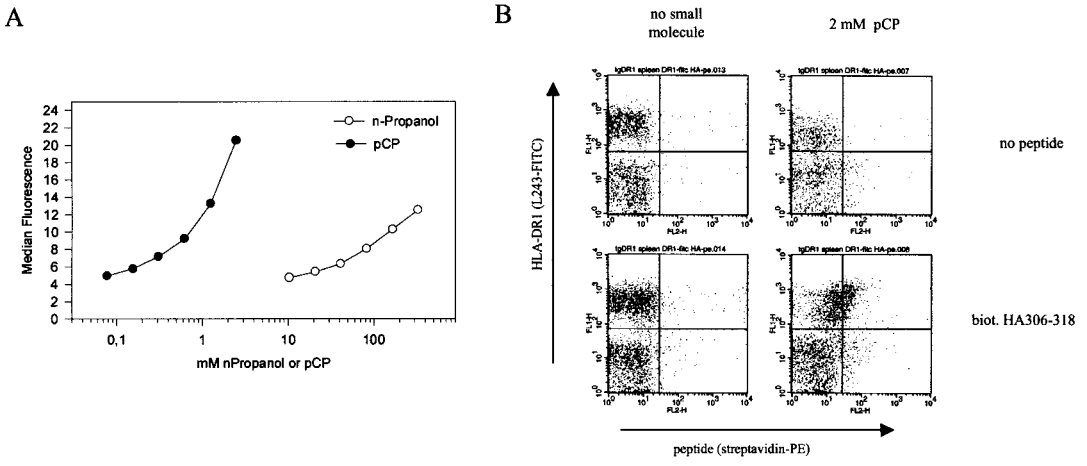

FIG. 6. **Peptide loading of antigen presenting cells with *n*-propanol or with pCP.** MHC class II-expressing fibroblast cells (*A*) and splenocytes from HLA-DR1 tg mice (*B*) were incubated for 3 h with biotinylated peptides in the presence of small molecular H-bond donors. The loading efficiency was determined by FACS analysis after staining with streptavidin-PE. *A*, comparison of *n*-propanol and *p*-chlorophenol for the peptide-loading of fibroblast cells. HLA-DR2-expressing fibroblast cells were incubated with 2.5 µg/ml biotinylated MBP86–100 peptide in the presence of titrated amounts of *n*-propanol (*open circles*) or *p*-chlorophenol (*closed circles*). Values expressed on the *ordinate* represent the median of the streptavidin-PE staining. *B*, loading of splenocytes by *p*-chlorophenol. Splenocytes derived from HLA-DR1 tg mice were incubated in the absence of *p*-chlorophenol (*left panel*) or in the presence of 2 µM *p*-chlorophenol (*right panel*) with 10 µg/ml biotinylated HA306–318 peptide (*lower panel*) or without any peptide (*upper panel*). After incubation the cells were stained with fluorescein isothiocyanate-labeled anti-HLA-DR antibody (L243) and with streptavidin-PE.

Furthermore, other H-bonds formed within or between the domains of the α and β-chains of the MHC class II molecule might be crucial for the stability of the complex. It remains to be seen whether the changes in the H-bond network induced by HLA-DM and small molecular compounds are identical or whether H-bonds at different sites are affected.

The exposure of cells to small molecular H-bond donors triggers conformational changes of cell surface MHC that can be detected directly by antibodies. We showed previously that the structural transitions occurring in the region of residues 57–71 of the β chain α-helix of the class II MHC molecule that could be sensed by a staining with KL304 are indicative for the formation of empty MHC molecules (25, 26). We now show that disruption of H-bonds increases KL304 reactivity on immature DC, which is most likely related to an increase in the number of "peptide-receptive" MHC class II molecules. Whether this is only a consequence of the release of previously bound ligands (37, 38) or, as shown for HLA-DM (30, 39), also a result of a

stabilization of empty but receptive MHC class II molecule is currently under investigation.

Although the ligand exchange by HLA-DM and by small molecular H-bond donors shares similarities, some differences are apparent. One striking difference is the pH dependence. HLA-DM was detected on the membrane of some antigen presenting cells (40, 41). Although it was reported to mediate some cell surface ligand exchange (41), its catalytic activity should be drastically reduced as the result of the neutral pH. In contrast to HLA-DM, the small molecules presented in this study are still very active under physiological conditions. They effectively catalyzed exchange reactions on soluble MHC molecules and also demonstrated that they can "edit" the composition of the ligand repertoire of antigen presenting cells. The classical MHC class II antigen processing and presentation pathway is designed to channel only peptide ligands on the surface, which primarily derive from exogenous proteins and are incorporated by defined endocytic or receptor-mediated uptake mechanisms. An alternative pathway seems to be the direct loading of surface MHC class II (42). This applies not only for fully processed peptide antigens but also for proteins or protein fragments. For several proteins, such as the encephalitogenic myelin basic protein (MBP) or hen egg lysozyme, it has been shown that they can bind directly to surface MHC class II molecules (43) and even extracellular processing mechanisms seem to exist (25). Hydroxyl groups or other functional groups capable of acting as H-bond donors are present in numerous natural and synthetic small molecules and macromolecules. The availability of these groups might therefore control rate and contribution of the HLA-independent presentation pathway.

Our data revealed some allele-specific differences in the sensitivity of peptide-MHC complexes to small molecular H-bond donors. CLIP was removed only very inefficiently from HLA-DR1, whereas the CLIP complex of HLA-DR2 and of HLA-DR4 was found to be particularly easy to exchange (data not shown). Both molecules, HLA-DR2 and -DR4, are frequently connected with autoimmune diseases such as insulin-dependent diabetes mellitus, multiple sclerosis, Goodpasture's syndrome, rheumatoid arthritis, and pemphigus vulgaris (44). Autoantigens transferred onto MHC molecules of dendritic cells can "accidentally" provoke autoimmune reactions, which potentially develop into severe autoimmune diseases (45). Although the concentrations needed are still relatively high, more effective H-bond donor molecules that act as mediators of this process might exist representing environmental risk factors that have not been considered yet. The susceptibility of MHC class II molecules toward the destabilization by these compounds could represent an alternative link, which could explain the genetic correlation beyond the presentation of mimicry antigens.

In conclusion, we provided evidence that small molecules capable of disrupting H-bonds are able to trigger ligand exchange of MHC class II molecules. All of the three alleles were sensitive to the exposure, suggesting that in general they have a profound effect on the peptide repertoire displayed at the cell surface. At this point the small molecular H-bond donors tested so far are still structurally relatively simple molecules. Nevertheless, they demonstrated surprisingly high, and specific, catalytic activity. Subtle modifications have already produced compounds that trigger ligand exchange at significantly reduced concentrations, and it is very likely that more optimally adapted compounds will be identified. Their use for external loading of peptide antigens could be particularly beneficial in immunotherapies, where specific epitopes must be loaded on antigen presenting cells to induce a T cell-specific immune response.

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