N-Linked Oligosaccharides of Murine Major Histocompatibility Complex Class II Molecule

ROLE IN ANTIGENIC PEPTIDE BINDING, T CELL RECOGNITION, AND CLONAL NONRESPONSIVENESS

To evaluate the functional role of the N-linked oligosaccharides of major histocompatibility complex (MHC) II molecules, affinity-purified murine I-A<sup>+</sup> class II molecules were deglycosylated in the presence of asparagine amidase enzyme. The deglycosylated I-A<sup>+</sup>-molecules were characterized by 12% SDS-polyacrylamide gel analysis under reduced and native conditions and the complete enzymatic removal of all three N-linked sugar components from the α/β heterodimer was confirmed by lectin-link Western blot analysis. Like the native I-A<sup>+</sup>-molecules, the deglycosylated I-A<sup>+</sup>-molecules were fully capable of binding an antigenic peptide from myelin basic protein MBP(89–101). The kinetics of dissociation of preformed complexes of I-A<sup>+</sup>-MBP(89–101) and deglycosylated I-A<sup>+</sup>-MBP(89–101) were compared at 4 and at 37 °C. Both complexes were equally stable at 4 °C; however, at 37 °C the deglycosylated I-A<sup>+</sup>-MBP(89–101) complexes showed an increased rate of dissociation as compared with the native I-A<sup>+</sup>-MBP(89–101) complexes. When tested for their ability to recognize the T cell receptor on T cells, both complexes bound to cloned HS-1 T cells that recognize and respond to I-A<sup>+</sup>-MBP(89–101). Finally, the complexes of deglycosylated I-A<sup>+</sup>-MBP(89–101) were tested for the induction of in vitro nonresponsiveness and compared with native I-A<sup>+</sup>-MBP(89–101) complexes. Both complexes were capable of inducing 95–100% nonresponsiveness in a proliferation assay. These results suggest that the N-linked oligosaccharide of MHC class II molecules may not be essential for either antigenic peptide binding or T cell recognition. In addition results obtained here provide evidence that the carbohydrate moieties of MHC class II molecules may not be involved in induction of T cell clonal anergy.

MHC II class II molecules (or Ia antigens) are cell surface glycoproteins which in conjunction with specific antigenic fragments are recognized by the TCR<sup>+</sup> on CD4 positive T cells. Most MHC class II molecules are heterodimeric structures consisting of a 34-kDa α polypeptide and a 29-kDa β polypeptide chains. The α polypeptide has two N-linked oligosaccharides located at position 82–84 in the α-1 domain and the other at position 122–124 in the α-2 domain (1). The β polypeptide chain possesses a single N-linked oligosaccharide at position 19–21 in the β-1 domain (2–4). It has been reported that the α/β chains expressed by various cell types or different cell lines show heterogeneity in the oligosaccharide patterns (5–6). The heterogeneity of carbohydrate components has been shown earlier to have some association with the differences in the capacity of antigen-presenting cells (APC) to activate T lymphocytes in an antigen-specific manner (7–9). In contrast, Krieger et al. (10) reported that neuraminidase-treated I-A<sup>+</sup> molecules did not show any alteration in their ability to present ovalbumin peptide to T hybridoma cells using a planar membrane system of antigen presentation. Several reports have been published indicating that the carbohydrate moieties of MHC class I antigens are not essential for their recognition by T lymphocytes (11–15). Although a number of studies have evaluated the role of N-linked oligosaccharides in the expression and function of MHC class I, relatively little is known about the role of N-linked sugar entities in MHC class II function. A study in this regard involved site-directed mutagenesis to selectively modify the N-linked acceptor sites of α and β chains, showing that the N-linked oligosaccharides may affect the secondary structure of class II molecules, and deletion of these oligosaccharides may have either a negative or positive effect on antigen presentation to T hybridomas (16). There is no evidence available that correlates the role of N-linked carbohydrate components of MHC class II and antigen-specific T cell tolerance or nonresponsiveness, a phenomenon referred to as clonal anergy (17). In this study, affinity-purified murine I-A<sup>+</sup>-molecules were deglycosylated with N-glycanase, and the deglycosylated I-A<sup>+</sup> was used to examine the role of N-linked oligosaccharide components in (i) antigenic peptide binding, (ii) stability of I-A<sup>+</sup>-MBP peptide complexes, (iii) binding of deglycosylated I-A<sup>+</sup>-MBP complexes to TCR on cloned T cells, and (iv) in vitro induction of T cell nonresponsiveness.

MATERIALS AND METHODS

Purification of Murine I-A<sup>+</sup>—I-A<sup>+</sup> was purified from an Nonidet P-40 extract of membranes prepared from SJL/J mouse spleen cells using an affinity support prepared by coupling monoclonal anti-body, 10-2.16 (specific for both I-A<sup>+</sup> and I-A<sup>+</sup>), with Sepharose 4B beads by the standard cyanogen bromide coupling method as described earlier (18, 19). Briefly, a high-speed (100,000 × g) membrane fraction was detergent-extracted in a buffer containing 10 mm Tris-HCl, pH 8.3, 0.5% Nonidet P-40, 0.1 m NaCl, 5 mM EDTA, 0.02% sodium azide, and 1 mM PMSF, and the lysate was recycled over the pre-equilibrated...
antibody column at 4 °C for 16 h. The column was washed with 10 bed volumes of deoxycholate buffer containing 10 mM Tris-HCl, pH 8.3, 0.5% deoxycholate, 0.1 M NaCl, 5 mM EDTA, 0.02% sodium azide, and 1 mM PMSF followed by 5 bed volumes of PBS containing 1% n-octyl-β-D-glucopyranoside (OG) detergent. Finally, IA* was eluted by 20 mM phosphate buffer, pH 7.5, containing 0.1 M NaCl, 1% OG, 0.02% sodium azide, and 1 mM PMSF. Each fraction was immediately neutralized with acetic acid to a final concentration of 12 mM, and the pooled IA* preparation was concentrated using an Amicon Centricon-10 concentrator. Affinity-purified IA* was characterized by 12% SDS-polyacrylamide gel electrophoresis under native conditions.

Removal of N-Linked Oligosaccharides by N-Glycanase—N-Glycanase is Genzyme's (Cambridge, MA) registered trademark name for peptide-N(α-D-glucosaminy1)asparagine amidase F; peptide-N-glycosidase F; PNGase F; peptide N-glycosylhydrolase F (EC 3.5.1.52). One mg of purified IA* at a concentration of 650 μg/ml (buffer: 20 mM phosphate, pH 8.0, 100 mM NaCl and 1% OG) was incubated at 95 °C for 5 min in the presence of 0.25% SDS and 5 mM 2-ME, cooled to room temperature, and then incubated with 150 units of N-glycanase for 16 h at 37 °C. Deglycosylated IA* was recovered by adding 2 volumes of ethanol at -20 °C. Protein solution was left at −20 °C for 2 h, recovered by centrifugation at 10,000 × g, and residual ethanol was removed by vacuum centrifugation. The deglycosylated IA* was then resuspended in 10 mM Tris-HCl, pH 8.3, 0.1 M NaCl, 0.02% sodium azide, 1 mM PMSF containing 0.5% Nonidet P-40 detergent and filtered through Millipore ultra-free filtration tubes.

Lectin-link Western Blot Analysis—Lectin-link Western blot kit was purchased from Genzyme Inc. Cambridge, MA, and the manufacturer's recommended protocol was followed with some modifications. Proteins (1 μg) were electrophoretically transferred onto a nitrocellulose membrane at 250 mA constant current for 3 h at 25 °C using Bio-Rad's miniblot apparatus in standard Tris/glycine/methanol buffer, pH 8.8 (1 liter of transfer buffer contains 2.4 g of Tris base, 11.52 g of glycine, and 200 ml of methanol). The nitrocellulose membrane containing protein bands was blocked with carbohydrate-free blocking reagent and was incubated with the biotinylated lectins at 25 °C for 1 h. The blot was washed with washing buffer and incubated with avidin-alkaline phosphatase for another 1 h at 25 °C. Following incubation, the blot was washed extensively with washing buffer and developed with the staining substrate. Among five different lectins tested for blot analysis, Datura stramonium agglutinin (DSA) worked best for our application.

Synthesis of Peptide Analogs—The bovine MBP peptide analogs with the sequence MBP(89–101)Y10 with the sequence Ac-YFKNIVTPRTPPP-NH2 was purchased from Pierce Chemical Co. IODO-ASQARPSQRHGSKY-NH2 and ovalbumin OVA(323–340)Y340 were synthesized by standard solid-phase methodology using side chain-protected Fmoc amino acids on an automated peptide synthesizer (Applied Biosystems 431 model). A tyrosine residue was added in both MBP(89–101) and OVA(323–340) peptides either to the N- or C-terminus of the respective peptides. Deglycosylated MBP(89–101) and OVA(323–340) peptides were purified by reverse-phase high performance liquid chromatography, and the homogeneity and the identity of the purified peptides were confirmed by mass spectrometric analysis.

Radiolabeling of Peptide and IA* Molecules—Radiolabeling of peptides was achieved by the standard chloramine-T labeling procedure (20). Typically, 2.5 mg of peptide in 500-μl volume was incubated with 2 mCi of Na125I in 0.1 M sodium phosphate buffer, pH 7.7. The labeled peptides were separated from free 125I by G-10 Sephadex gel filtration chromatography, and the specific activities calculated ranged from 0.5 to 6.8 × 106 cpm/μg. Labeling of IA* and deglycosylated IA* with 125I was achieved using Pierce Chemical Co. IODO-BEADS and the manufacturers recommended procedure. 100 μCi of Na125I in 0.1 M sodium phosphate buffer, pH 7.5, was added to 200 μg of IA molecules. Pre-loaded IODO-BEADS were prepared by washing with 50 mM phosphate buffer, pH 7.5 (1 ml of buffer for two beads) and dried on whatman paper. Two beads were added to the radioactive mixture and left at room temperature for 15 min. Labeled IA* or deglycosylated IA* was separated from the beads, and free 125I was removed by extensive dialysis against PBS containing 0.1% OG at 4 °C. Specific activities of IA* and deglycosylated IA* ranged from 6.5 to 6.8 × 106 cpm/μg.

Peptide Binding Assay—The binding of radiolabeled peptides was measured by the silica gel TLC plate assay method as described earlier (18, 19). Briefly, IA molecules at a concentration of 400–500 μg/ml were incubated with 30-fold molar excess of radiolabeled peptides at 37 °C for 48 h. In competition experiments, 300-fold molar excess of unlabeled peptide over IA was also added in the same reaction mixture prior to the incubation. The unbound peptide was removed by dialysis against PBS containing 0.1% OG, and 1-μl samples were applied to triplicate at the origin of silica gel TLC plate. Ascending TLC was performed using plastic-supported silica gel plates (DC-Plastifikolen Kieselgel 60 F254) obtained from EM Sciences Inc. The solvent system consists of 5% ammonium acetate in 50% aqueous methanol. The solvent front was allowed to run 5 cm from the origin in a standard TLC chamber. The plates were subjected to autoradiography, and the distribution of radioactivity was estimated by excising and γ counting strips at Rf (relative frequency) = 0–0.2 and Rf = 0.2–1.0. In parallel experiments, an equivalent amount of radiolabeled peptides incubated and dialyzed in the absence of IA were also spotted at the origin of the TLC plate in triplicates, and the radioactivity values were subtracted in calculating the percent peptide occupancy of IA molecules. The percent of IA* or deglycosylated IA*bound labeled peptides was calculated from the specific activities of the respective peptides.

Preparation of Class II-Peptide Complexes—Two types of complexes were prepared; unlabeled complexes of either IA* or deglycosylated IA* with MBP(89–101) or MBP(1–14) were prepared and purified as described earlier (21) with some modifications and were used for all in vitro T cell functional assays. Briefly, affinity-purified IA* or deglycosylated IA* molecules at 400–500 μg/ml were incubated with 48 h with 30-fold molar excess of unlabeled peptides in 10 mM Tris-HCl, pH 8.3, 0.02% sodium azide, 1 mM PMSF, and 1% OG. The unbound free peptide was removed by extensive dialysis of the complex against serum-free RPMI medium, and the final complex preparation was tested for endotoxin using limulus amebocyte lysate method. For T cell binding experiments, radiolabeled IA* or deglycosylated IA* was incubated with unlabeled peptides under identical conditions and purified. Specific activities of purified radiolabeled complexes were 6.7 × 106 and 6.83 × 106 cpm/μg for IA*-MBP(89–101) and deglycosylated IA*-MBP(89–101), respectively.

Induction of in Vitro T Cell Nonresponsiveness—Residual antigen-presenting cells were removed by subjecting cloned HS-1 T cells (10 days following antigen pulsing) to two rounds of 19% metrizamide density-gradient centrifugation, followed by two washes in RPMI 1640 medium. HS-1 cells (1 × 106) were cultured with either 5 or 20 μg of preformed IA*-MBP(89–101) or deglycosylated IA*-MBP (89–101) complexes for 26 h at 37 °C. A 10-fold molar excess of MBP(89–101) peptide, IA* alone, deglycosylated IA* alone, and IA*-MBP(1–14) or deglycosylated IA*-MBP (1–14) complexes were used as controls. The cells were washed four times and 8 × 105 T cells in triplicate were cultured with 5 × 104 freshly irradiated SJL/J spleen cells in the presence of 0.1 μCi of [3H] thymidine, and 1 μg (1 Ci = 37 GBq) of [3H] thymidine was added, and the degree of proliferation was measured by incorporated radioactivity.

RESULTS

In order to evaluate the potential role of N-linked oligosaccharides of MHC class II molecules in various in vitro studies, affinity-purified IA* from SJL/J mice was subjected to N-glycanase treatment. IA* eluted from a 10-2.16 monoclonal antibody-coated affinity column was concentrated to 400–500 μg/ml and denatured in the presence of SDS and mercaptoethanol. An attempt to remove N-linked sugar residues of IA* under fully native conditions (i.e. in the absence of SDS and ME) was totally unsuccessful even with a 20 times increased concentration of IA*. The incubation was carried out for 16 h at 37 °C. The deglycosylated IA* did not react with 10-2.16 monoclonal antibody when tested in a dot blot assay using peroxidase-conjugated anti-mouse IgG (data not shown). Therefore the recovery of deglycosylated IA* from the reaction mixture was achieved by ethanol precipitation at −20 °C followed by centrifugal vacuum concentration. Concentrated deglycosylated IA* was reconstituted by detergent buffer (20 mM phosphate, pH 8.0, 100 mM NaCl, and 1% OG) and was characterized on 12% SDS gel under reduced and nonreduced conditions (Fig. 1). Under reduced
conditions, both α and β chains showed increased sharpness of bands and migrated at lower molecular weight region as compared with glycosylated α and β chains (Fig. 1A). Similarly, faster migrating partially dissociated α and β chains were observed under nonreduced conditions (Fig. 1B). A significant amount (40%) of the deglycosylated IA* remained of bands and migrated at lower molecular weight region as compared with glycosylated IA*. Lanes 2 and 3 of the native gel were scanned on Microtek (model MRS-600 ZS) to calculate percent dimer in the preparation and are shown in C.

To ensure complete removal of N-linked sugar residues from IA* had occurred, lectin-link Western blot analysis was performed (Fig. 2). IA* and deglycosylated IA* were run on a 12% polyacrylamide SDS gel under reduced conditions. One set (A) was stained with silver nitrate to visualize the α and β chains. The other set was subjected to electro-transfer onto a nitrocellulose membrane. Transferred proteins on nitrocellulose were fixed and allowed to react with biotinylated lectin. The membrane was then allowed to react with alkaline phosphatase-coupled avidin to develop color with substrate. As shown in Fig. 2B, the deglycosylated IA* lane did not show any reactivity. IA* incubated under identical conditions but in the absence of N-glycanase, as well as standard glycoprotein mix, showed strong reactivity in the DSA-biotinylated lectin and avidin system.

Peptide binding to IA* or deglycosylated IA* was examined under nonreduced conditions (Fig. 1B). A significant amount (40%) of the deglycosylated IA* remained of bands and migrated at lower molecular weight region as compared with glycosylated IA*. Lanes 2 and 3 of the native gel were scanned on Microtek (model MRS-600 ZS) to calculate percent dimer in the preparation and are shown in C.

Fig. 1. Characterization of deglycosylated IA* on SDS-polyacrylamide gel. Affinity-purified IA* was deglycosylated using N-glycanase and recovered in detergent solution as described under “Materials and Methods.” One μg of IA* or deglycosylated IA* was applied on a 12% polyacrylamide gel under reduced (A) or nonreduced (B) conditions. Electrophoresis was carried out in Bio-Rad’s minigel apparatus at 100 constant voltage, and gels were silver-stained. Lane 1, molecular weight markers; lane 2, glycosylated IA*; and lane 3, deglycosylated IA*. Lanes 2 and 3 of the native gel were scanned on Microtek (model MRS-600 ZS) to calculate percent dimer in the preparation and are shown in C.

Fig. 2. Lectin-link Western blot analysis of deglycosylated IA*. One μg of either IA* and deglycosylated IA* along with 1 μg of glycoprotein standard (from Genzyme Inc.) and molecular weight markers were subjected to 12% polyacrylamide SDS-polyacrylamide gel electrophoresis analysis. At the end of electrophoresis, the gel was cut into two sections: one section (A) was silver-stained, and the other section (B) was transferred onto a nitrocellulose membrane at 250 mA for 3 h. The transferred proteins were probed for the presence of N-linked carbohydrate using the lectin-link (biotinylated lectin/ avidin-alkaline phosphatase system) assay as described under “Materials and Methods.”

Fig. 3. Binding of MBP(89-101) peptide to deglycosylated IA*. Radiolabeled MBP(89-101) or OVA(323-340) peptides at 30-fold molar excess over Ia molecules were incubated with 100 μg of IA* or deglycosylated IA* in 200-μl total volume at 37°C for 48 h. In another experimental set, 300-fold excess unlabeled MBP(89-101) was added along with 10-fold excess [125I]-MBP(89-101) peptide. The unbound peptide was removed by dialysis, and complexes were analyzed in triplicate on a silica gel TLC plate as described under “Materials and Methods.” Column 1 represents peptide binding to native IA*, and column 2 represents peptide binding to deglycosylated IA*.

by a silica gel TLC plate assay method as described earlier (18). Radiolabeled synthetic peptides [MBP(89-101)Yα and OVA(323-340)Yα] were used in peptide binding studies. IA* or deglycosylated IA* was incubated with 30-fold molar excess of labeled peptides at pH 8.0, an optimized pH for IA*-MBP(89-101) complex formation determined in our laboratory (19). In all peptide binding experiments, an equivalent amount of radiolabeled peptides incubated and dialyzed under identical conditions, but in the absence of class II molecules, were used as control, and counts appearing at the origin (usually <2%) were subtracted for calculating the percent occupancy of class II with labeled peptide. As shown in Fig. 3 (solid bar), both IA* (column 1) and deglycosylated IA* (column 2) were capable of binding MBP(89–101) peptide. The percent of these Ia molecules occupied with peptide ranged...
from 40 to 50 in various experiments. Specificity of the binding was confirmed by either using an irrelevant peptide like OVA(323–340)Y140, which is known to bind to IAd (22), or by competing out the radiolabeled MBP(89–101) peptide with excess unlabeled MBP(89–101) peptide. In all control experiments, the deglycosylated IA* behaved similar to native IA*

Next we compared the rate of dissociation of IA*–MBP(89–101) and deglycosylated IA*–MBP(89–101) complex to see whether sugar residues are critical in stabilizing the preformed complexes. Complexes of IA*–MBP(89–101) and deglycosylated IA*–MBP(89–101) with 125I-labeled peptides were prepared, purified, and at various times a 1-μl aliquot from the reaction mixture was loaded onto a silica gel TLC plate in triplicate and the percent of IA* or deglycosylated IA* with bound peptide was calculated. Data presented in Fig. 4A shows that the complexes of IA*–MBP(89–101) were very stable at 4 and at 37 °C. However, in the case of deglycosylated IA*–MBP(89–101) complex, the dissociation rate was relatively high at 37 °C as compared with 4 °C (Fig. 4B). Assuming a first order of kinetics, the slope from the inset figures was used to calculate the dissociation rate constant (Kd). The Kd values for IA*–MBP(89–101) complex at 4 and 37 °C were comparable (1.4 × 10−6 s−1 and 0.5 × 10−6 s−1, respectively). The deglycosylated IA*–MBP(89–101) complex, however, had a Kd value of 1.7 × 10−6 s−1 at 4 °C and 8.1 × 10−6 s−1 at 37 °C.

Having demonstrated that the deglycosylated IA* binds antigenic peptide, we sought to determine whether complexes of deglycosylated IA* and peptide can recognize TCR on cloned T cells. HS-1 T cells, which recognize MBP(89–101) of IA*–MBP(89–101) complexes as described in "Materials and Methods." The deglycosylated IA*–MBP(89–101) complex, however, had a Kd value of 1.7 × 10−6 s−1 at 4 °C and 8.1 × 10−6 s−1 at 37 °C.

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of 1 × 10^6 HS-1 T cells with 20 μg of either IA*-MBP(89-101) or deglycosylated IA*-MBP(89-101) resulted in 95-100% nonresponsiveness. An equivalent amount of IA* alone, deglycosylated IA* alone, or complexes of either IA*-MBP(1-14) or deglycosylated IA*-MBP(1-14) did not show any significant inhibition in the proliferative response. Similarly, cells exposed to a 10-fold molar excess MBP(89-101) peptide alone did not induce any significant nonresponsiveness in any experiment. Identical results were obtained in another experiment with a lower dose (5 μg) of IA*-MBP(89-101) or deglycosylated IA*-MBP(89-101) complexes (data not shown).

**DISCUSSION**

In this study, N-linked oligosaccharide moieties of murine IA* α/β heterodimeric molecules were enzymatically removed by asparagine amidase (N-glycanase) to examine the effect of the two N-linked oligosaccharides of the α chain and one N-linked oligosaccharide of the β chain of MHC class II molecules in antigenic peptide binding, T cell recognition, and T cell function. This enzyme catalyzes the hydrolysis of Asn-linked oligosaccharides at the β-asparagylglycosylamine bond between the innermost GlcNAc and the asparagine residue of glycoproteins. In general, the cleavage of N-linked sugar residues by N-glycanase is highly efficient when glycoproteins are fully denatured. Cleavage of N-linked oligosaccharides of native glycoproteins often results in incomplete digestion and requires 10-20 times more enzyme units. Our attempt to cleave N-linked sugar residues of murine IA* under fully native conditions was totally unsuccessful even at 20-fold excess enzyme units. Addition of mild reducing agent (up to 20 mM cysteine) along with 20-fold excess enzyme and increased incubation period did not improve the cleavage of oligosaccharides of these molecules under native conditions. Because of these reasons, IA* was denatured in the presence of SDS and ME prior to the digestion. Exposure of MHC class II molecules to SDS had no effect on peptide binding ability of these molecules. This was shown earlier (24) and was confirmed recently in our laboratory (25), because the α/β heterodimeric MHC class II molecules and their individual polypeptide chains electrosed from native SDS-polyacrylamide gels can equally bind antigenic peptides. Similarly, chains isolated from reduced SDS gels, where ME is added in the sample buffer, can equally bind antigenic peptide.2

As expected, both the α and β chains of deglycosylated IA* when analyzed on reduced silver-stained SDS gel showed a significant shift in their molecular weight and increased band sharpness with no detectable glycosylated chains. However, no significant difference in the mobility of the α/β heterodimer was observed when IA* and deglycosylated IA* were analyzed under nonreduced 12% SDS-polyacrylamide gel electrophoresis. To further demonstrate the complete removal of all three N-linked sugar residues from IA*, a highly sensitive Lectin-link Western blot analysis was performed that involves a biotin-avidin system. In this system lectins bind specifically to the sugar chains on the glycoproteins that are transferred onto nitrocellulose and easily visualized by enzyme-substrate color development. Detection of glycosylated IA* was optimized using five different biotinylated lectins: concanavalin A, Ricinus communis agglutinin (RCA), DSA, Phascolus vulgaris erythrobactin (PHA-E), and wheat germ agglutinin. Among all these, DSA was most sensitive and worked best for detecting IA* glycosylation in our experiments. The deglycosylated IA* recovered following N-glycanase treatment under the experimental condition, did not show any detectable reactivity with the DSA-biotin/alkaline phosphate-avidin system. Milligram quantities of IA* were subjected to N-glycanase treatment and the recovered deglycosylated IA* was used for various studies.

Binding of the MBP(89-101) peptide to deglycosylated IA* was examined to see the effect of oligosaccharide removal on peptide binding ability. Calculating the percent of MHC class II molecules with bound labeled peptide by the TLC method is highly sensitive and has been shown to correlate well with the standard dialysis method (19). In all peptide binding experiments, the deglycosylated IA* was able to bind antigenic peptide in quantities similar to the native IA* molecules. The binding data presented here suggest that the denatured MHC class II molecules in the presence of SDS and 2-ME are capable of binding antigenic peptide like the nonreduced and nondenatured preparations. This result is not surprising, since isolated α and β chains or dimers of MHC class II molecules eluted from SDS gels have been shown to bind antigenic peptides like the native dimer molecule (24, 25). Since there was no significant difference between the deglycosylated IA* and native IA* in their ability to bind antigenic peptide, we decided to compare the stability of these two complexes by measuring the dissociation kinetics.

Dissociation kinetics of several different MHC class II-peptide complexes indicate that the complexes of MHC class II and selected peptides are usually very stable (26-29). In fact, in a long term complex stability study using human MHC class II molecules and antigenic peptides, we have recently observed that preformed complexes are 95-100% stable for over a period of 6 months at 4 °C.2 In this study, the dissociation kinetics of deglycosylated IA*.MBP(89-101) complexes were measured at 4 and at 37 °C. No significant difference in the rate of IA*.MBP(89-101) and deglycosylated IA*.MBP(89-101) complex dissociation was observed at 4 °C. However at 37 °C, the deglycosylated IA*.MBP(89-101) complex showed a 4-fold increased rate of dissociation (Kd of 8.1 × 10^-6 s^-1 versus 1.4 × 10^-6 s^-1).

To compare the T cell recognition of IA*.MBP(89-101) and deglycosylated IA*.MBP(89-101) complexes in vitro, binding of preformed complexes to cloned HS-1 T cells was measured. In T cell binding experiments, radiolabeled MHC class II was complexed with unlabeled peptides and the binding was measured at 37 °C. The rate of complex association was initially slow in the case of deglycosylated IA*.MBP(89-101) complexes compared with the native IA*.MBP(89-101) complex. However at the end of a 6-h incubation, the number of bound complexes per T cell was almost identical. Both the dissociation kinetics and the T cell recognition data suggest that the N-linked sugar residues are perhaps important in stabilizing the MHC-peptide complexes and are involved in cell-cell interaction.

We have shown recently that the cloned HS-1 T cells when exposed to the complexes of IA* plus MBP(90-101) resulted in T cell nonresponsiveness to a subsequent challenge of MBP(90-101) along with fresh APC (30). A similar set of experiment was performed to determine the role of N-linked oligosaccharide components of MHC class II on T cell inactivation. APC-depleted HS-1 cells when incubated with either IA*.MBP(89-101) or deglycosylated IA*.MBP(89-101) complexes, and challenged with an increasing concentration of MBP(89-101) peptide, became almost completely nonresponsive. Lack of a proliferative response was not the result of either cell death or loss of viability, because in various control experiments cells exposed with IA* alone, deglycosylated IA* alone, or complexes of IA* and deglycosylated IA* with

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2 B. Nag, D. Passmore, and D. Kopa, unpublished results.
MBP(1–14) responded well in the proliferation assay. To ensure that the lack of proliferative response in the case of deglycosylated IA*-MBP(89–101) complexes is not due to release of bound peptide, a 10-fold molar excess of peptide MBP(89–101) was included in our control experiments that showed no effect on proliferative response.

In conclusion, these results demonstrate that the N-linked sugar residues of murine IA* molecules are not essential for peptide binding, T cell recognition, or in vitro induction of T cell nonresponsiveness. Under physiological conditions, the N-linked oligosaccharides moities may play an important role in stabilizing the MHC class II-peptide complexes on the surface of APC and perhaps are involved in cell-cell adhesion. Further studies with several different T cell clones are necessary to generalize the role of N-linked sugar residues of MHC class II molecules.

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