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Naturally processed heterodimeric disulfide-linked insulin peptides bind to major histocompatibility class II molecules on thymic epithelial cells

(thymic antigen-presenting cells)

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ABSTRACT **We determined whether disulfide-linked insulin peptides that are immunogenic *in vitro* for CD4$^+$ T cells bind to major histocompatibility complex class II *in vivo*. Radiolabeled recombinant human insulin (rHI) was injected into BALB/c mice, and processed rHI peptides bound to I-A^d molecules on different thymic antigen-presenting cells were characterized. The A6–A11/B7–B19 and A19–A21/B14–B21 disulfide-linked I-A^d-bound rHI peptides were isolated from thymic epithelial cells but not dendritic cells. While both thymic epithelial cells and dendritic cells present rHI to HI/I-A^d-specific T cells, these antigen-presenting cells do not present the reduced or nonreduced forms of the disulfide-linked rHI peptides. Thus, a naturally processed disulfide-linked peptide can bind to major histocompatibility complex class II *in vivo*. The potential role of these peptides in immunological tolerance is discussed.**

Naturally processed linear antigenic peptides of 13–25 aa bind to major histocompatibility complex (MHC) class II molecules on B-cell antigen-presenting cells (APCs) (1–3). We previously demonstrated that heterodimeric disulfide-linked insulin peptides are presented by B cells to CD4$^+$ T cells *in vitro* (4–6). Since this suggested that a disulfide-linked peptide binds to MHC class II, we analyzed whether human insulin (HI) is processed into a disulfide-linked peptide that binds to MHC class II *in vivo*. We investigated whether the same or different disulfide-linked HI peptides are bound to MHC class II on different thymic APCs. This was of interest, since it was proposed that thymic epithelial cells (TNCs) and dendritic cells (DCs) express a different array of MHC class II-bound antigenic peptides and mediate positive and negative T-cell selection, respectively (7). Our data show that naturally processed heterodimeric disulfide-linked HI peptides can be eluted from MHC class II on TNCs but not DCs. They also indicate that T-cell selection may be influenced by the processing of an antigen into different MHC class II-bound peptides by different thymic APCs.

MATERIALS AND METHODS

Recombinant HI (rHI) purified from *Escherichia coli* K-12 strain CA7233 transformed with plasmid plac 9/4 PI (Fig. 1*A*) was radiolabeled (≈3 mCi/mmol; 1 Ci = 37 GBq) at 19 distinct amino acids (Fig. 1*B*) (8). Labeled rHI was indistinguishable from commercially available HI (8) and was injected into 4- to 6-week-old female BALB/c (H-2^d) mice.

Cortical epithelial cells (thymic nurse cells; TNCs), corticomedullary DC rosettes (T-ROS), and a separate MHC class II$^+$ medullary DC/macrophage (Mϕ) population were isolated (9). The DC/Mϕ population was recovered as low-density cells (20–30% Percoll). Remaining tissue fragments were digested with collagenase type XI (0.5 mg/ml, from *Clostridium histolytum*; Sigma) to obtain T-ROS and with 0.02% trypsin (0.5 ml per thymus; Sigma) in Hanks' balanced salt solution (HBSS) containing DNase I (8 μg/ml; Sigma) to purify TNCs. Before assay, TNCs were allowed to reexpress surface MHC class II during culture (16 hr, 37°C) in RPMI 1640 (GIBCO) containing 10 mM Hepes (pH 7.4), 2 mM L-glutamine, 50 μM 2-mercaptoethanol, penicillin (100 units/ml), streptomycin (100 μg/ml), and 5% (vol/vol) fetal calf serum (CRPMI) and supplemented with interleukin 2 (IL-2; 15 units/ml).

APC yields (per mouse) were 4.2×10^4 for DCs/Mϕs, 1.4×10^5 for T-ROS, and 3.5×10^4 for TNCs. Enrichment of APCs was assessed by immunofluorescence using the anti-I-A^d MK-D6 (10), J11D and 33D1 (both detect thymic DCs) (11), anti-CD4 GK 1.5 (12), or anti-CD8 53-6.62 (13) monoclonal antibodies. The relative I-A^d surface densities were TNCs > T-ROS > DCs/Mϕs, with the density on TNCs being ≈2-fold greater than that of DCs/Mϕs. APCs represented ≈60% of each preparation, and their relative purity was 85–90%, as reported (14).

Noncovalently linked membrane-associated peptides were acid eluted (15 min, 4°C; HBSS at pH 3.6) from thymic APCs and resolved by reversed-phase C_{18} HPLC (15). Intracellular peptides were extracted from the acid-treated cell pellet, passed through Sephadex G-50 to obtain "insulin-sized peaks," and analyzed by C_{18} HPLC (15). Peptides (picomolar amounts) were analyzed for their amino acid composition using a Waters Pico·Tag HPLC system.

rHI peptides were purified from ≈8×10^5 TNCs, and DCs/Mϕs derived from 22 BALB/c mice injected with labeled rHI. To provide a source of unlabeled I-A^d and inhibit interactions of unoccupied monoclonal antibodies with labeled thymic APC intracellular I-A^d molecules, ≈8×10^6 TA3 (H-2^d × H-2^a) B-lymphoma cells (16) were added to the APCs in phosphate-buffered saline (PBS). Immunoprecipitation (30 min, 4°C) of membrane-associated peptides was carried out using 2 μg of either the MK-D6 (IgG2b, κ), VC6 [anti-(anti-Ia.2), isotype-matched to MK-D6; ref. 17], or 15-5-5S (anti-H-2K^d; ref. 18) monoclonal antibody; rabbit anti-mouse IgG (20 μg; 60 min); and protein A-Sepharose (0.1 ml, 60 min; Pharmacia). After cell lysis and washing of the Sepharose beads in PBS containing 0.2% digitonin, immune complexes were dissociated with 8 M urea (pH 4.0) and passed through Sephadex G-25 to fractionate "insulin-sized" peaks. Labeled

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Abbreviations: MHC, major histocompatibility complex; APC, antigen-presenting cell; HI, human insulin; TNC, thymic epithelial cell; DC, dendritic cell; T-ROS, corticomedullary DC rosettes; IL-2, interleukin 2; BI, beef insulin; Mb, myoglobin; OVA, ovalbumin; HIV, human immunodeficiency virus; rHI, recombinant HI; Mϕ, macrophage.

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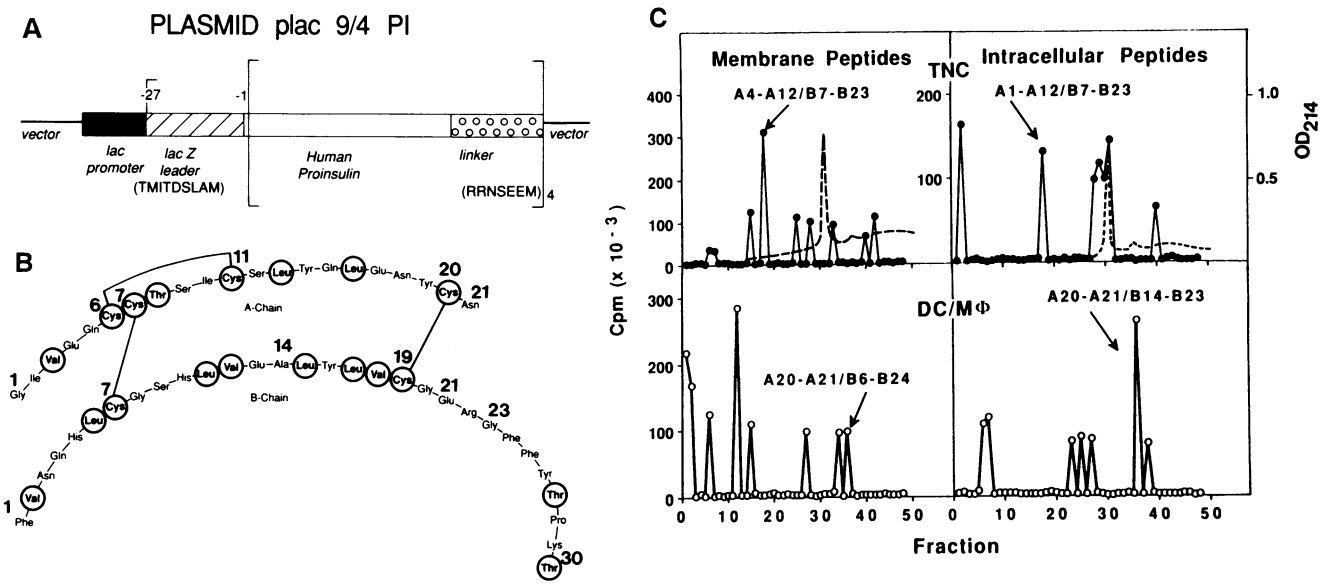

FIG. 1. Preparation and processing of labeled rHI. (*A*) Plasmid plac 9/4 PI, which carries a *lac* promoter but lacks a functional repressor gene, encodes a human proinsulin precursor fusion protein having the structure TMITDSLAM-(proinsulin-RRNSEEM)$_4$GS. This protein (42.3 kDa) makes up 55% (wt/wt) of rHI and is convertable to authentic rHI (8). (*B*) HI was biosynthetically labeled at 19 sites (circled) using [^{35}S]cysteine, [^{3}H]valine, [^{3}H]leucine, and [^{3}H]threonine (8). (*C*) Thymic TNCs and DCs/Mϕs were isolated from 12 BALB/c mice injected i.v. with labeled rHI. Membrane-associated peptides were acid eluted from 4–5 × 10^5 APCs, and intracellular peptides were extracted with 8 M urea/3 M HOAc/0.2% Triton X-100. Radiolabeled HI peptides processed by TNCs (●) or DCs/Mϕs (○) were resolved by C_{18} HPLC. Amino acid compositions of some peptides are indicated by arrows. The HPLC chromatogram (OD_{214}) of unlabeled HI (30 μg) used as an internal standard is shown.

rHI peptides were separated by C_{18} HPLC and analyzed for their amino acid composition (15).

BALB/c thymic APCs (10^5) were cultured with rHI and either the B8.P4.10 (B8; ref. 4) and 3D4.4 (19) HI/I-A^d-reactive T-cell hybrids (10^5) or nylon wool-purified lymph node T cells (5 × 10^5) isolated from HI-primed BALB/c mice. Test peptides used were HI A1–A14/B1–B16 (4) and the synthetic HI A6–A11/B7–B19 (A6) and A19–A21/B14–B21 (A19) peptides (20); control peptides were beef insulin (BI) A chain (Sigma) and myoglobin (Mb)-(108–121) (21). IL-2 activity in culture supernatants was assayed by stimulation of [^{3}H]thymidine incorporation into CTLL-2 cells (4).

Thymic APCs (5 × 10^5) prepulsed (4 hr, 37°C) with rHI (25 μM) were cultured with HI A6 or A19 (50 μM) and B8 T cells (10^5). Alternatively, HI-pulsed thymic APCs or TA3 cells were fixed with glutaraldehyde [0.2% (vol/vol)] and incubated (1 hr, 37°C) with HI A6 or A19 before adding B8 T cells. Control peptides that do [(ovalbumin (OVA)-(323–336) and Mb-(108–121)] or do not [Mb-(132–151), human immunodeficiency virus (HIV) Gp17-(65–80), BI A chain] bind to I-A^d were also added (50 μM) at the start of culture to displace I-A^d binding HI peptides. After 24 hr, supernatants were assayed for their IL-2 content. The percent inhibition of presentation by a competitor peptide was calculated by comparison to the maximal (100%) response obtained using APCs stimulated with rHI alone.

RESULTS

Processing of Labeled rHI *in Vivo* by Thymic APCs. BALB/c mice were injected i.v. with the unlabeled HI B23–B30 synthetic peptide (2 μg; Fig. 1*B*) and 5 min later with labeled rHI (2 μg, ≈2.2 × 10^6 cpm). About 30% of the rHI-associated radioactivity localized to the thymus by 2 min postinjection and decreased rapidly thereafter. If preinjection of HI B23–B30 was omitted, only 3% of the rHI-associated radioactivity localized to the thymus. Since HI B23–B30 contains residues that bind to the HI receptor (22), it may inhibit HI–HI receptor interactions on hepatocytes and adipocytes and enhance the routing of rHI to the thymus.

TNCs and DCs/Mϕs were isolated from 12 BALB/c mice injected with labeled rHI. Although it takes only 2 min for labeled rHI to reach the thymus after i.v. injection, another 30 min to 1 hr at 23°C is required to isolate these APCs during which more internalization and processing of HI can occur. Thus, thymic APC-derived HI peptides were characterized ≈1 hr after injection. About 25% of the rHI-associated radioactivity in the thymus was in TNCs, and about half of this activity was equally distributed between the plasma membrane and intracellular compartments. A similar amount (32%) and distribution of the rHI-associated radioactivity was found in DCs/Mϕs.

Membrane-associated and intracellular peptides in ≈5–6 × 10^6 thymic TNCs and DCs/Mϕs were resolved by C_{18} HPLC. Since peptides were not detectable by their OD_{214}, we monitored HI peptide-associated radioactivity. A partial overlap was observed for the intracellular and membrane peptides from each APC (Fig. 1*C*). Six distinct membrane-associated peptides were found on TNCs and DCs/Mϕs, and only one peptide (fraction 15) was common to each profile. Amino acid analyses showed that some intracellular (A1–A12/B7–B23) and membrane (A4–A12/B7–B23) TNC peptides contain A-loop residues disulfide-linked to B-chain residues (Fig. 1*C Upper*). These peptides were not detected on DCs/Mϕs (Fig. 1*C Lower*). Rather, some DC/Mϕ peptides consist of C-terminal A-chain residues A20 and A21 disulfide-linked to core B-chain residues, and such peptides are not immunogenic (4, 22). It is unlikely that the disulfide-linked peptides detected result from digestion by collagenase and trypsin used for APC isolation, since trypsin cleaves HI only between the B22—B23 and B29—B30 bonds and collagenase does not hydrolyse HI even in the presence of trypsin (F.F. and M.H., unpublished data). Thus, after ≈1 hr of processing, different patterns of membrane and intracellular HI peptides are evident in TNCs and DCs.

MHC Class II-Bound HI Peptides on Thymic APCs. TNCs and DCs/Mϕs were isolated from 22 BALB/c mice injected

with HI B23–B30 and labeled rHI. Their membrane-associated rHI peptides were immunoprecipitated and separated by gel filtration and C_{18} HPLC. A peak of radioactivity, representing ≈0.1% of total rHI-associated radioactivity in the thymus at 2 min postinjection, was found in fractions 19 and 20 of the Sephadex G-25 chromatogram of the specific MK-D6 (anti-I-A^d)-immunoprecipitated, but not control VC6- and 15-5-5S-immunoprecipitated, TNC samples (Fig. 2*A*). This peak was absent from precipitated DC/Mϕ samples (Fig. 2*B*). Thus, rHI is processed *in vivo* into peptides that bind to MHC class II on TNCs but not DCs/Mϕs.

Two I-A^d-bound rHI peptides on TNCs were resolved by HPLC at fractions 15 and 25 (Fig. 2*C*). Amino acid analyses established that they contain residues A6–A11 and B7–B19 (fraction 15) and A19–A21 and B14–B21 (fraction 25). The synthetic HI peptides A6–A11, B7–B19, A19–A21, and B14–B21 each elute in different fractions from C_{18} (data not shown). Moreover, the synthetic disulfide-linked peptides A6–A11/B7–B19 and A19–A21/B14–B21 coelute with the radiolabeled HI peptides in fractions 15 and 25, respectively. Although two peptides that coelute from C_{18} may differ in structure, these observations indicate that the two HI peptides eluted from I-A^d are the disulfide-linked A6–A11/B7–B19 (A6) and A19–A21/B41–B21 (A19) peptides.

Presentation of rHI by Thymic APCs. BALB/c TNCs, T-ROS, and DCs/Mϕs present HI to BALB/c-derived HI/I-A^d-reactive T-cell hybrids and peripheral lymph node T cells (Fig. 3). However, T-ROS present HI less efficiently than do DCs/Mϕs and TNCs. Addition of rHI to culture was necessary to detect efficient presentation even by thymic APCs from HI-primed mice. This suggests that HI peptide–I-A^d complexes, if preformed on TNCs and DCs/Mϕs, were either absent or present in amounts below that required for spontaneous presentation to T cells.

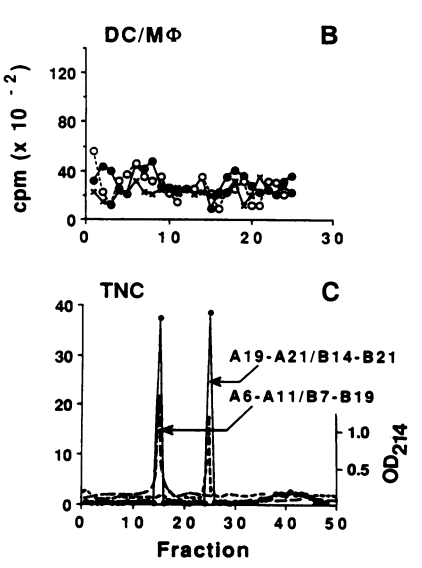

FIG. 2. MHC class II-associated rHI peptides processed by TNCs and DCs/Mϕs. TNCs and DCs/Mϕs were isolated from BALB/c mice injected i.v. with labeled rHI. Membrane-associated rHI peptides from TNCs (*A*) or DCs/Mϕs (*B*) were immunoprecipitated by MK-D6 (anti-I-A^d; ●), VC6 [anti-(anti-Ia.2); ○], or 15-5-5S (anti-H-$2K^d$, ×) and fractionated on Sephadex G-25 (*A* and *B*); the MK-D6-precipitated TNC peptides in *A* were resolved by C_{18} HPLC in *C*. C_{18} HPLC profiles (OD_{214}) of the unlabeled HI A6–A11/B7–B19 and A19–A21/B14–B21 synthetic peptides are presented in *C*. Results from one of three representative experiments are shown.

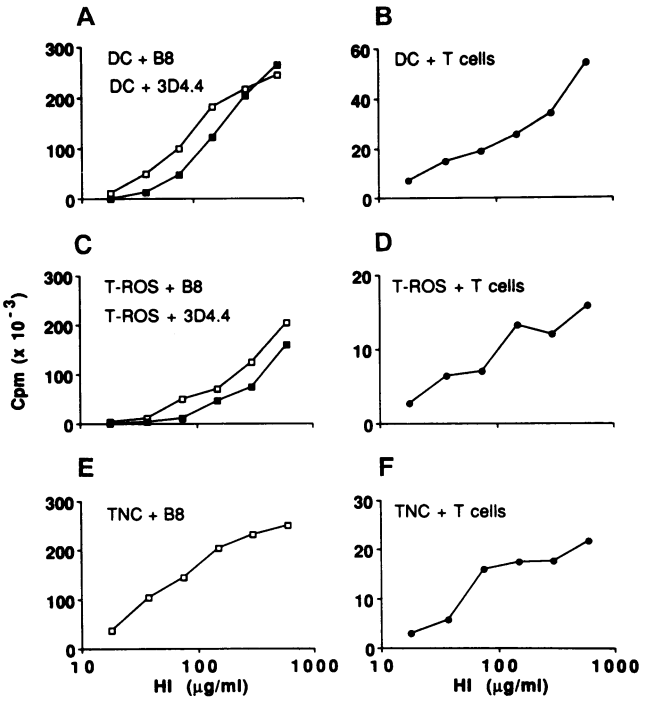

FIG. 3. Presentation of HI by thymic APCs to HI-specific T cells. DCs/Mϕs (*A* and *B*), T-ROS (*C* and *D*), or TNCs (*E* and *F*) (10^5) from naive BALB/c mice were incubated with different concentrations of rHI in the presence of the HI-reactive B8 or 3D4.4 T hybridoma cells (10^5) (*A*, *C*, and *E*) or lymph node T cells (5×10^5) isolated from HI-immune BALB/c mice (*B*, *D*, and *F*). After 24 hr, supernatants were assayed for their IL-2 content. Results are expressed as the mean cpm of [^{3}H]dT incorporated by triplicate samples. SD were ≤10%.

Presentation of HI A6 and A19 Peptides by Thymic APCs. The I-A^d-bound HI A6 and A19 peptides on TNCs were acid-eluted in insufficient quantity (≈0.1 nM) to determine if they can be presented to T cells. Therefore, synthetic A6 and A19 peptides, which possess the same amino acid composition and disulfide bonds as the naturally processed ones, were used in APC assays. These peptides were not presented by either TNCs (Fig. 4 *A* and *B*), T-ROS (Fig. 4 *C* and *D*), DCs/Mϕs (Fig. 4 *E* and *F*), or splenic DCs or B cells (data not shown) to B8 T cells (Fig. 4 *A*, *C*, and *E*) or lymph node T cells from HI-primed mice (Fig. 4 *B*, *D*, and *F*). Thus, HI A6 and A19 are not immunogenic. Prior reduction (2 mM dithiothreitol) of these peptides before or after binding to MHC class II did not enable them to be presented by either the thymic or splenic APCs (data not shown).

Inhibition of Presentation of HI by HI A6 and A19 Peptides. We verified that the inability of HI A6 and A19 to be presented was not due to their lack of binding to MHC class II. TNCs, T-ROS, and DCs/Mϕs were pulsed with HI (25 μM) to "saturate" MHC class II with HI peptides, washed, and incubated with either HI A6 or A19 (50 μM) or the control Mb-(108–121) and BI A-chain peptides (50 μM) in the presence of B8 T cells. The presentation of HI by TNCs (Fig. 5*A*), T-ROS (Fig. 5*B*), and DCs/Mϕs (Fig. 5*C*) was inhibited by 55%, 36%, and 24%, respectively, in the presence of HI A6. HI A19 and Mb-(108–121), but not BI A-chain, also competed effectively for presentation of HI. To further establish that HI A6 and A19 bind as disulfide-linked peptides in the groove of

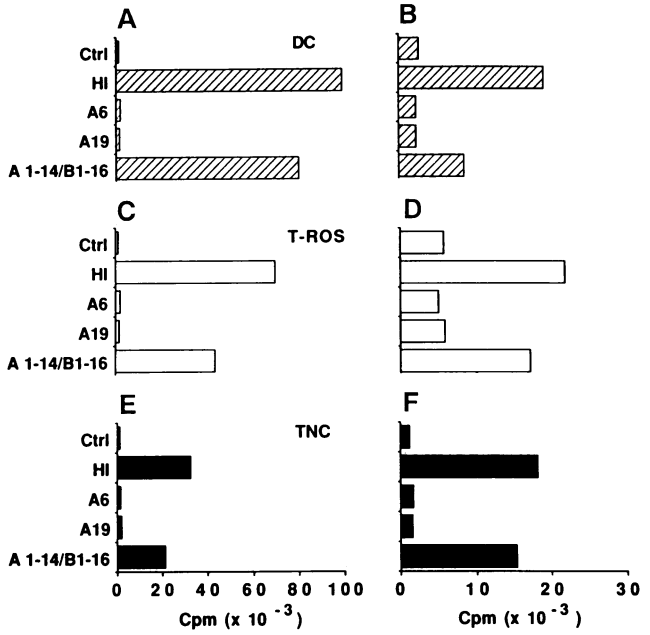

FIG. 4. HI A6 and A19 peptides are not presented by thymic APCs to HI-specific T cells. DCs/Mϕs (*A* and *B*), T-ROS (*C* and *D*), or TNCs (*E* and *F*) (10^5) from naive BALB/c mice were incubated with HI (25 μM) or the HI A6, HI A19, and Mb-(108–121) peptides (50 μM) in the presence of B8 T cells (*A*, *C*, and *E*) or BALB/c lymph node T cells (*B*, *D*, and *F*). IL-2 assays and expression of results were as in Fig. 3. Ctrl, Control.

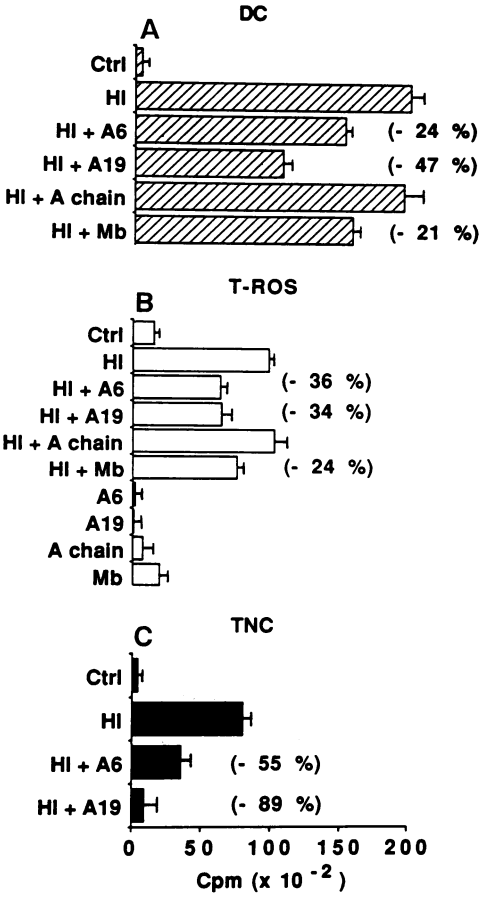

FIG. 5. HI A6 and A19 peptides compete with HI for binding to MHC class II. DCs/Mϕs (*A*), T-ROS (*B*), and TNCs (*C*) (10^6) were pulsed (4 hr, 37°C) with rHI (25 μM), and the APCs (5×10^5) were then incubated with the HI A6 or A19 synthetic peptide (50 μM) in the presence of B8 T cells (10^5). In control experiments, Mb-(108–121) (50 μM) was used as an I-A^d binding competitor and BI A-chain was used as an I-A^d nonbinding competitor. IL-2 assays and expression of results were as in Fig. 3. Percent inhibition was calculated as described in *Materials and Methods*. Ctrl, Control.

MHC class II, similar competition assays were performed using fixed APCs. HI A6 inhibited the presentation of HI by fixed T-ROS (Fig. 6*A*) and TA3 cells (Fig. 6*B*) by 75% and 71%, respectively. A similar inhibition was observed with the HI A19 and positive control OVA-(323–336) peptides but not the Mb-(132–151), HIV Gp17-(65–80), and BI A-chain negative control peptides, suggesting that the A6, A19, and OVA peptides bind in the groove of I-A^d.

DISCUSSION

Thymic T-cell selection may result from TCR recognition of different peptide–self-MHC complexes on various thymic APCs (7), but evidence for the presence of such complexes on these APCs has not yet been found. We show that insulin, an extrathymic antigen, circulates rapidly to the thymus and is processed into predominantly different peptides by TNCs (mediate positive selection) and DCs/Mϕs (mediate negative selection). Qualitative and quantitative differences were detected in TNC and DC/Mϕ membrane-associated HI peptides. Only two of greater than eight major HI membrane-associated TNC peptides bound to MHC class II. Limitations in the sensitivity of peptide detection preclude us from excluding the presence of other MHC class II-bound HI peptides on TNCs and DCs/Mϕs. Nonetheless, it is interesting that only five hen egg lysozyme (HEL) peptides bind to I-A^k and four of them contain the dominant HEL-(52–61) T-cell epitope (3). This agrees with the suggestion that peptides presented by MHC class II at a density sufficient to activate T cells may be of limited complexity for a given antigen (1, 2). While TNCs, T-ROS, and DCs/Mϕs present HI to HI-specific T cells, the I-A^d-bound HI A6 and A19 peptides were detected only on TNCs. Thus, HI may be processed differently and/or to different extents in TNCs and DCs/Mϕs, and these APCs may vary in the HI peptide–MHC class II complexes they present to T cells.

The detection of TNC-derived A6–I-A^d and A19–I-A^d complexes indicate that heterodimeric disulfide-linked HI peptides bind to MHC class II *in vivo*. The ability of peptides A6 and A19 to inhibit the presentation of HI by fixed APCs demonstrates that they bind in the groove of I-A^d without the need for further processing. We (22) and others (23) proposed that B-chain residues of this type of HI peptide bind MHC class II and that A-chain residues (A-loop residues) engage the T-cell receptor. If so, the length of the A6 B-chain component (13 aa) would correspond to the average length (15 aa) of class II-bound peptides (2–4). However, only A-chain peptides with free thiol groups (N-terminal 14 aa of BI A-chain) stably associate with MHC class II and activate T cells (24, 25). In the absence of B-chain residues, only 6 and 3 aa of HI A6 and A19, respectively, may bind to MHC class II, which is much shorter than the 13–25 aa present in naturally processed class II-bound peptides (1–3). Thus, the disulfide-linked A6 and A19 peptides may bind to MHC class II via their B-chain amino acids. In HI A6, the linked A7 and B7 cysteine amino acids represent the penultimate N-terminal cysteine and N-terminal cysteine residues, respectively. Similarly, in HI A19, the cysteine amino acids are at the N terminus of the A-chain component and close to the C terminus of the B-chain component. Since an MHC class II peptide binding groove is more open than that of an MHC class I groove and can bind longer peptides (26), these disulfide-linked HI peptides may bind to class II with a kink

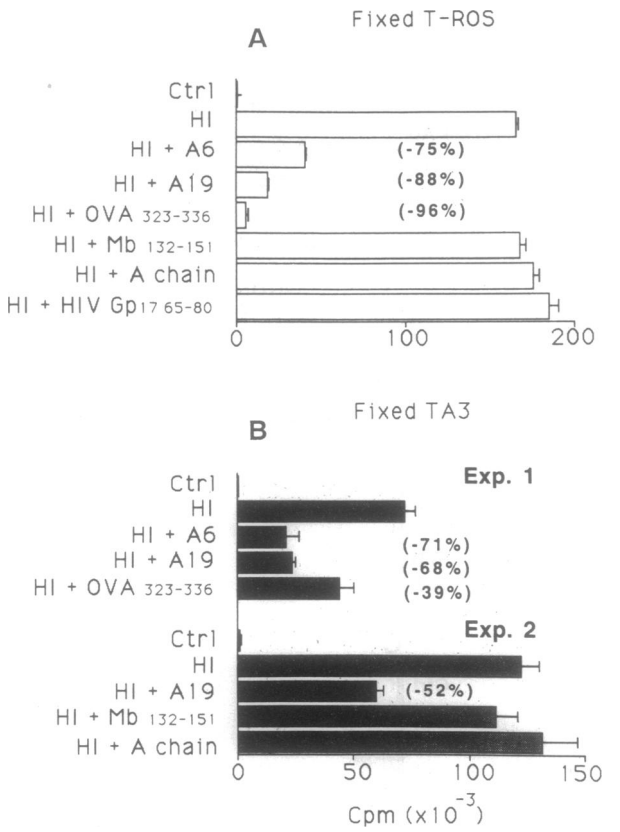

FIG. 6. HI A6 and A19 peptides compete with HI for binding to MHC class II on fixed APCs. T-ROS (*A*) and TA3 (*B*) cells (10^6) were pulsed (4 hr, 37°C) with rHI (25 μM), fixed with glutaraldehyde, and then incubated (5×10^5 APCs) with HI A6 or A19 (50 μM) for 1 hr before addition of B8 T cells (10^5). In control experiments, OVA-(323–336) (50 μM) was used as an I-A^d binding competitor (Exp. 1) and Mb-(132–151) and BI A-chain were used as I-A^d nonbinding competitors (Exp. 2). IL-2 assays, expression of results, and calculation of percent inhibition were as in Fig. 5. Ctrl, Control.

in their structure that might allow some A-chain residues to extend beyond the groove.

If disulfide bond reduction mediates antigen processing of insulin *in vivo*, the presence of disulfide-linked HI peptides bound to MHC class II on TNCs suggests that this reduction occurs at the cell surface after the formation of peptide–MHC class II complexes. Removal of the proposed kink in the disulfide-linked peptide structure upon reduction might promote a better induced fit of the peptide in the MHC class II groove and enhanced T-cell receptor recognition. However, reduction of disulfide bonds in peptides A6 and A19 after they bound to MHC class II did not enable them to be presented to T cells. This lack of T-cell immunogenicity of A19 was expected since it is identical in sequence to its self-mouse insulin homolog. The inability of A6 to be presented may be due either to its lack of or weak immunogenicity. Although it consists of an immunodominant A-chain loop containing T-cell epitope, it lacks some loop-flanking residues required for its immunogenicity (24, 25). This peptide differs from its self-mouse insulin homolog only at residue B9 (20), which may result in the absence or low frequency of A6-specific peripheral T cells.

T-cell unresponsiveness to HI A6 and A19 may arise from the striking structural similarity between the B7–B19 and B14–B21 B-chain components of these peptides and the B8–B18 and B10–B22 peptides demonstrated by Muir *et al.* (28) and H. Weiner (29), respectively, to suppress the incidence of type I diabetes in nonobese diabetic mice after oral administration of HI. Oral administration of either HI A-chain or various A-chain peptides did not prevent diabetes onset. Thus, processed insulin B-chain linear peptides or heterodimeric disulfide-linked peptides that contain these linear B-chain fragments may elicit the T-cell unresponsiveness to HI observed here and the induction of oral tolerance to insulin and suppression of type I diabetes previously reported (27).

Our observations illustrate the importance of examining the T-cell immunogenicity of MHC-bound peptides physiologically processed *in vivo*, since only a fraction of these peptides may be immunogenic and involved in positive T-cell selection. They may also explain why a nonimmunogenic peptide(s) of an antigen is bound to and cotransported with MHC class II molecules to the surface of a given APC in the thymus and/or periphery (i.e., to induce tolerance). Finally, they may identify naturally processed peptides of a given autoantigen (e.g., HI) that could prove efficacious in the prevention of an autoimmune disease.

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