Brief Definitive Report

Efficient Loading of Identical Viral Peptide Onto Class II Molecules by Antigenized Immunoglobulin and Influenza Virus

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Summary

Several prior reports have identified peptides that are naturally associated with major histocompatibility complex (MHC) class II molecules on presenting cells. We have examined the delivery of a peptide from exogenous sources to MHC class II molecules. The peptide derives from the influenza virus hemagglutinin (HA) and activates a $CD4^+$ T cell hybridoma. In functional assays of antigen presentation, this epitope is delivered effectively to T cells either in the context of influenza virus or chimeric immunoglobulin (Ig) molecules (Ig-HA) in which the peptide has replaced the CDR3 loop of the heavy chain. We find that the identical 11-mer peptide can be isolated from mouse MHC class II antigens whether the exogenous source of peptide is free HA peptide, the Ig-HA chimera, or ultraviolet-inactivated PR8 influenza virus. The Ig-HA chimera proves to be the most efficient vehicle for charging class II molecules via the exogenous route. Given the fact that self Igs represent natural long-lived carriers, we suggest that antigenized Igs have considerable potential for peptide delivery to MHC molecules in situ.

MHC-restricted responses by T lymphocytes require recognition of short peptides bound to externally displayed grooves of the MHC gene products. These peptides can be isolated from APCs by immunoselecting the MHC molecules, eluting the bound peptides, and chemically characterizing the eluted materials. This was accomplished first for MHC class I molecules (1–4) and more recently for MHC class II molecules (5–8). But for a single report with a high dose of exogenous antigen (9) most of the studies with MHC class II have concentrated on peptides that are naturally associated with APCs.

Here we assess delivery of an exogenous influenza hemagglutinin (HA) peptide, corresponding to residues 110–120, onto the I-E subset of mouse MHC class II molecules. Our goal was to analyze the presentation of the HA110–120 peptide directly at the level of signal one, i.e., the formation of the MHC peptide complex, and to evaluate the efficacy of different carriers in delivering this peptide to class II molecules. Functional assays with an HA-specific, I-E restricted, $CD4^+$ T cell hybridoma had shown that the HA peptide could be delivered to T cells not only in the context of influenza virus but also in that of self Ig molecules (10). These Ig-HA chimeras were obtained by replacing the CDR3 loop of a mouse H chain Ig gene with sequences encoding the HA peptide. The efficacy of presentation in functional assays depends not simply on the formation of the appropriate MHC–peptide complex "signal one" but also on other so-called "second signals" that can be influenced by the vehicle used to deliver the peptide. In the case of Ig-HA for example, it is known that presentation requires an interaction with the Fc receptor on APCs (10).

By isolating peptides from class II MHC molecules, it should now be possible to assess directly the efficacy with which different forms of a foreign antigen are converted to MHC–peptide complexes. This is of some interest in the design of peptide-based immunization and tolerization regimens.

Here we compare the delivery to class II molecules of a specific HA peptide within different contexts: free HA peptide, intact influenza virus, or Ig-HA chimeric molecules. The identical 11-mer peptide can be isolated from the I-E molecules of APCs in each instance, but Ig-HA proves to be the most efficient vehicle for the formation of signal one, the MHC–peptide complex.

Materials and Methods

Antigens. A synthetic peptide corresponding to amino acid residues 110-120 (SFERFEIFPKE) of PR8 virus HA was obtained from IAF Biochem (Laval, Quebec, Canada). Reverse phase (RP)-HPLC of this peptide showed a single peak. The purity and molecular weight of the peptide was confirmed by mass spectrometry performed by Dr. B. Chait (The Rockefeller University).

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Chimeric Ig (Ig-HA) expressing the HA110-120 epitope in the CDR3 loop of an antiarsonate mAb, 91A3, was produced by SP2/0 cells doubly transfected with 91A3 chimeric H-HA and wild-type L chain genes (10). Chimeric Ig-HA molecules that were produced by the transfectomas were purified on a rat anti-murine κ chain mAb-Sepharose column.

PR8 influenza virus was grown in the allantoic cavities of embryonated eggs and purified on a sucrose gradient. The PR8 virus preparation was UV inactivated before use in experiments.

Antigen Pulse. APCs (10^9) of the 2PK3 B lymphoma cell line (H-2^d haplotype) were suspended in 500 ml DMEM supplemented with 5% FCS and pulsed with 30 mg of HA110-120 synthetic peptide for 3 h, 30 mg of chimeric Ig-HA for 18 h, or 30 mg of UV-inactivated PR8 virus for 18 h. APCs were washed three times with PBS at 4°C and used for extraction of class II–peptide complexes.

Extraction of I-E^d-peptide Complexes and Peptide Elution. Pulsed APCs were hypotonically lysed on ice for 10 min under gentle agitation in 10 ml of 10 mM Tris-HCl, pH 7.5, and a cocktail of protease inhibitors: 16 mM EDTA, 2.6 mM 1, 10-phenanthroline, 15 mM Pepstatin A, and 2 mM PMSF. The lysate was cleared of nuclei and debris by centrifugation at 2,000 *g* for 20 min, and the membranes were collected from the supernatant by centrifugation at 100,000 *g* for 90 min at 4°C. The membrane pellets were washed twice in cold PBS at 100,000 *g*, resuspended in 100 ml cold PBS, and solubilized for 60 min on ice with gentle agitation using an equal volume of PBS containing 2% NP-40 and the cocktail of inhibitors.

I-A^d–peptide complexes were depleted by passage through a column of anti-I-A^d Ab (25-9-17S; American Type Culture Collection [ATCC], Rockville, MD) coupled to Sepharose. The effluent of this column was then repassed through an anti-I-E^d Ab (14-4-4, ATCC) column. The anti-I-E^d Ab-Sepharose column was first washed with 15 vol of PBS, pH 7.4, containing 0.5% NP-40 and 0.1% SDS, and then PBS, pH 7.4, containing 1% *n*-octyl-β-D-glucopyranoside (OG) until the A_{280} nm reached a plateau. Bound I-E^d–peptide complexes were eluted with a solution containing 0.15M NaCl and 0.1% OG adjusted to pH 11 with diethylamine. The eluate was collected in an equal volume of 1 M Tris-HCl, pH 7, and dialyzed overnight at 4°C against PBS in 6,000–8,000 molecular weight cut off (MWCO) bags (Spectrum Co., Houston, TX). Peptides were then dissociated for 30 min at 37°C in 2.5 M acetic acid. The peptides were separated by ultrafiltration on membranes of 5,000 MWCO (Amicon Corp., Danvers, MA), and the filtrates were dialyzed for 4 d in 500 MWCO bags (Spectrum Co.) against large volumes of distilled water. Dialyzed peptides were lyophilized, redissolved in 1 ml of 0.1% TFA (Sigma Chemical Co., St. Louis, MO), 2% 1-propanol (vol/vol) in deionized water, and centrifuged for 10 min at 15,000 *g*. The supernatant was collected and peptides were separated by RP-HPLC.

Isolation of Peptides by RP-HPLC. Peptides were chromatographed on a RP-HPLC C2/C18 column (SuperPac Pep-S, 250 × 4 mm, 5-μm particles; Pharmacia LKB, Piscataway, NJ) equilibrated in solvent A (0.1% TFA, 2% 1-propanol [vol/vol] in water). The peptides were eluted using an 80-min linear gradient from 2 to 98% solvent B (0.1% TFA, 2% 1-propanol, 30% water in acetonitrile [vol/vol]) at a flow rate of 0.5 ml/min. Fractions were collected at 1-min intervals and analyzed for the presence of HA peptide using a competitive RIA (see below).

In the case of the pulse with PR8 virus, three successive fractions (see Fig. 1) were found to have HA activity. These fractions were pooled, dried by rotary vacuum, resuspended in solvent A, and rechromatographed on a C4 column (250 × 4 mm, 5 μm particle, Millipore Co. Waters Chromatography Div., Milford, MA). The gradient conditions for the C4 column were identical to those described for the C2/C18 column. Fractions eluted from the C4 column were reassayed for HA activity by competitive RIA.

Identification of HA Peptides by Competitive RIA. All RP-HPLC fractions were tested for HA activity as described (11). Briefly, the fractions were dried in a rotary vacuum, resuspended in PBS, and one tenth of each fraction was incubated at 37°C for 2 h with affinity-purified rabbit anti–HA110-120 Abs (2 ng) in 100 μl PBS containing 1% BSA and 0.05% Tween 20. The mixture was then transferred into microtiter plates coated with HA110-120-BSA, and residual rabbit Abs that bind to the plates were revealed with ^{125}I-labeled goat anti–rabbit Ig.

Sequences of I-E^d–associated Peptides. RP-HPLC fractions that showed HA activity, as well as fractions that did not contain HA activity, were analyzed using a gas phase microsequencer (model 2090E; Porton Instruments, Tarzana, CA). The amounts of sequenced peptides were estimated according to repetitive yield analysis.

T Cell Activation Assay. Irradiated (2,200 rad) 2PK3 APCs (10^4) were incubated with the different antigens and 2×10^4 HA110-120–specific LD 1-24 T cell hybridoma (12) in round-bottomed 96-well plates for 48 h. Supernatants were then collected and tested for IL-3 production as a measure of T cell activation, using the IL-3–dependent DA-1 cells (15×10^3) and the colorimetric MTT assay as described (10, 12).

Results and Discussion

The peptide corresponding to amino acid residues 110-120 of influenza virus HA is a helper epitope recognized by $CD4^+$ T cells in association with I-E^d MHC class II molecules (12). A T cell hybridoma specific for this epitope can be activated by a synthetic HA110-120 peptide, by purified HA, by influenza virus, and by peptides displayed within the CDR3 loop of a chimeric self Ig carrier, Ig-HA (Table 1).

We have previously identified (10) several features of the Ig-HA delivery system: Ig-HA is at least 1,000-fold more potent as an antigen than free peptide; Ig-HA is delivered to APCs via the Fcγ receptor; a chloroquine-sensitive processing step by live APCs is required; and presentation is blocked by mAb to I-E^d but not to I-A^d class II molecules.

In this study we eluted and analyzed HA peptides delivered to I-E^d molecules by Ig-HA and PR8 virus so that we could dissect the efficacy of T cell activation at the level of MHC–peptide complexes. 10^9 2PK3 lymphoma B cells were pulsed with HA (110-120) peptide, chimeric Ig-HA, or PR8 virus, each at a constant dose by weight, i.e., 30 mg in a 500-ml vol of culture media. I-E^d class II molecules were extracted. The peptides bound to I-E^d molecules were acid eluted and fractionated by RP-HPLC.

To identify HA peptides in the HPLC fractions, we used a sensitive method that detects picomoles of peptide (11). In this technique, fractions were assayed by competitive RIA for inhibition of the binding of affinity-purified rabbit anti–HA(110-120) Abs to wells coated with HA(110-120)-BSA conjugate. As evident in Fig. 1, *a–c*, respectively, one major fraction was identified from cells pulsed with HA(110-120) peptide, two fractions from cells pulsed with Ig-HA, and several fractions from those pulsed with PR8 virus. These

Table 1. *Activation of LD 1-24 T cells by Ig-HA*

Antigen	Concentration	Activation (A_{570} nm)
	μg/ml	
Nil		0.215 ± 0.03*
PR8 virus	10	0.962 ± 0.05
HA(110-120)	143	0.954 ± 0.07
BHA	10	0.805 ± 0.09
Ig-HA	10	0.842 ± 0.17
Ig-NP	10	0.207 ± 0.02
NP(147-161)	208	0.228 ± 0.03

Stimulation of the LD 1–24 Th hybridoma, specific for amino acid residues 110-120 of site 1 of influenza A virus HA (12), was evaluated with various antigens and 2PK3 B lymphoma cells as APCs (see Materials and Methods). The activation of T cells was assessed by measuring IL-3 release using the colorimetric MTT assay and the IL-3–dependent DA-1 cells as described (10, 12). The antigens were: PR8, UV-inactivated influenza A virus; HA(110-120), synthetic peptide corresponding to residues 110-120 of influenza A virus HA; Ig-HA, a chimeric Ig carrying HA(110-120) within the CDR3 loop of the H chain (10); BHA, bromelain-released PR8 virus HA; NP(147-161), synthetic peptide corresponding to amino acid residues 147–161 of influenza virus nucleoprotein; and Ig-NP, a chimeric Ig carrying NP(147-161) peptide also within the CDR3-loop of the H chain (13).

* Mean ± SD of quadruplicates. The absorbance was measured at 570 nm (reference wavelength of 630 nm).

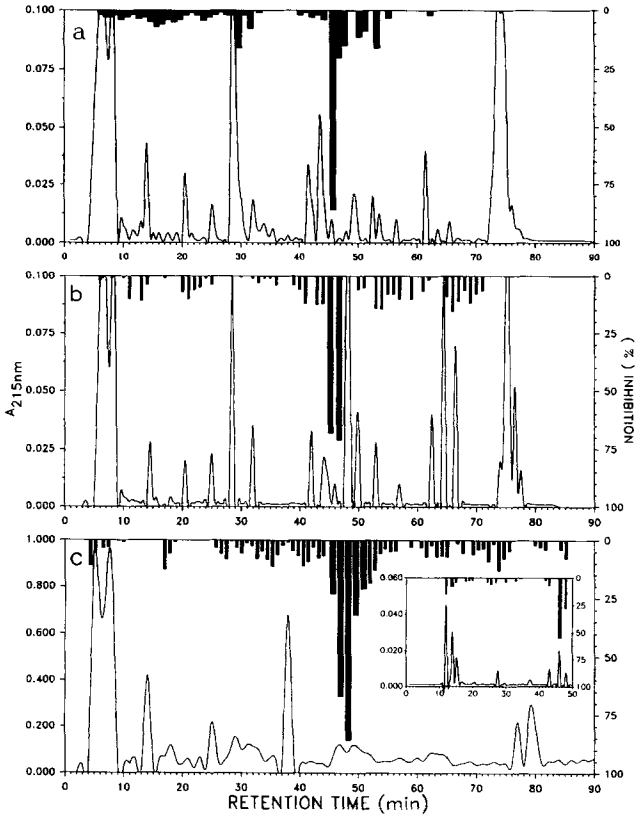

Figure 1. Identification of HA(110-120) and related peptides among those eluted from I-E^d molecules. Peptides eluted from I-E^d molecules of 2PK3 cells that were previously pulsed with HA(110-120) synthetic peptide (*a*); Ig-HA (*b*); or UV-inactivated PR8 virus (*c*) were chromatographed on a C2/C18 RP-HPLC column, and fractions were assayed for inhibition of the binding of rabbit anti-HA(110-120) Abs to HA(110-120) peptide-BSA conjugate by solid phase RIA as described in Materials and Methods. RIA inhibition experiments were repeated three times and indicated values are of one representative experiment. The percent inhibition of each fraction (*solid bars*) is superimposed over its corresponding retention time on the chromatograms. The peptides in fractions 47–49 (*c*) were pooled, rechromatographed on a C4 column (5-μm particles), and reassayed for inhibition (*inset, c*). Significant inhibition was obtained with only a single subfraction.

fractions (solid bars in Fig. 1, *a-c*) inhibited significantly the binding of rabbit anti–HA(110-120) Abs to HA(110-120)-BSA conjugates. In the case of PR8 virus, three major inhibitory fractions were pooled and refractionated on a C4 column. Only one repurified fraction showed significant inhibitory activity (Fig. 1 *c*, inset).

Sequence analyses revealed that one inhibitory fraction from each of the forms used to deliver antigen to APCs (HA peptide, Ig-HA, PR8 virus) had an identical sequence to the canonical synthetic peptide that stimulated the T cell hybridoma (Table 2). The additional inhibitory fraction from cells pulsed with Ig-HA contained an octamer corresponding to the eight NH_2-terminal residues of HA(110-120). Truncated class II–restricted peptides have been isolated from processed foreign (9) and self (6) antigens. A synthetic peptide corresponding to the octamer isolated from I-E^d of APCs pulsed with Ig-HA does not activate the specific Th hybridoma (data not shown), suggesting that it lacks critical amino acid residues required for interaction with the TCR.

Analysis of peptides from those fractions that did not inhibit the RIA revealed sequences that did not match any protein sequence in any database (Table 2, fractions 42 and 38) and one fraction that corresponded to amino acids 405–422 of BSA (a component of the culture medium).

The recovery of peptide from APCs pulsed with known amounts of peptide, Ig-HA, and PR8 virus was estimated by repetitive yields from the peptide sequencer (Table 3). Ig-HA was the most effective delivery system. The Ig-HA delivered four times more peptide to MHC class II molecules than a pulse of free HA(110-120) peptide, even though the latter was administered at a 50-fold-higher dose (compare lines 1 and 2 of Table 3). Whereas 470 pmoles of HA(110-120) was recovered following a pulse of 400,000 pmoles of peptide within the context of self Ig, only 10 pmoles of peptide was recovered from an estimated pulse of 100,000 pmoles of peptide within PR8 virus (compare lines 2 and 3 of Table 3).

Table 2. *Sequences of Peptides from RP-HPLC Fractions*

Antigen	Retention time	Inhibition	Structure	Homology with
	min	%		
HA(110-120)	46	85	SFERFEIFPKE	HA
	45	67	SFERFEIF	HA
Ig-HA	47	70	SFERFEIFPKE	HA
	42	13	XKPNAAEDPGLAXQAAK	Unknown
	50	9	VDEPQNLIKQNXDQFEKLG	BSA
PR8 virus	47–49	87	SFERFEIFPKE	HA
	38	5	XRPNAGEDPGLARQAPK	Unknown

Peptide fractions that were inhibitory in the competitive RIA, as well as some noninhibitory fractions, were analyzed using a gas phase microsequencer (model 2090E; Porton Instruments). Shown are the percent inhibition of binding of rabbit anti-HA(110-120) Abs to HA(110-120)-BSA and the sequence (single-letter code) of peptide found in the indicated fractions. Peptides of fractions 42 and 38 did not match any protein sequence in the Genpept, Protein Information Resources, and Swiss-Prot databases (14), whereas the peptide of fraction 50 matched with residues 405-423 of BSA.

Table 3. *Efficient Generation of HA (110-120) Peptide from Ig-HA*

Pulse with 30 mg of	HA(110-120) offered to cells	HA(110-120) recovered from class II molecules
	pmole	
HA(110-120)	20.0×10^6*	102‡
Ig-HA	0.4×10^6	470
PR8 virus	0.1×10^6	10

The molecular weights used to calculate the molarities of Ig-HA and HA were 150 and 215 kD, respectively.

* The peptide content of Ig-HA was estimated on the basis that 1 pmole of Ig-HA contains 2 pmole of HA (110-120). For PR8 virus, the peptide content was estimated on the basis that HA represents 25% of total viral protein (15) and that 1 pmole of HA contains 3 pmole of HA (110-120).

‡ Peptide recovery was estimated using repetitive yield data from the microsequencer.

These data reveal that peptides generated from different forms of an external antigen (free peptide, peptide carried in the context of self Ig, and free virus) have identical structures when associated with MHC class II molecules. This may reflect a fine-tuning by the processing and presentation machinery to select optimally fitting peptides from the multitude of peptides that might be generated by proteolysis of natural proteins. As in the case of class I–restricted peptides (13, 16, 17) flanking regions need not play an important role in processing. However, different carriers can play a major role in the efficacy with which antigenic peptides are internalized and routed by APCs.

The generation of identical peptides from Ig-HA and PR8 virus, and the high efficacy of Ig-HA in activating T cells, imply that antigenized self Ig molecules represent an effective carrier for delivery of peptides to MHC class II molecules, as in vaccination or tolerization protocols, and a carrier that has a potential to be long-lived and devoid of side effects.

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References

1. Van Bleek, G.M., and S.G. Nathenson. 1990. Isolation of an endogenously processed immunodominant viral peptide from the class I H-2K^b molecule. *Nature (Lond.).* 348:214.
2. Falk, K., O. Rotzschke, and H.-G. Rammensee. 1990. Cellular peptide composition governed by major histocompatibility complex class I molecules. *Nature (Lond.).* 348:248.
3. Falk, K., O. Rotzschke, K. Deres, J. Metzger, G. Jung, and H.-G. Rammensee. 1991. Identification of naturally processed viral nonapeptides allows their quantification in infected cells and suggests an allele-specific T cell epitope forecast. *J. Exp. Med.* 174:425.
4. Jardetzky, T.S., W.S. Lane, R.A. Robinson, D.R. Madden, and D.C. Wiley. 1991. Identification of self peptides bound to purified HLA-B27. *Nature (Lond.).* 353:326.
5. Rudensky, A.Y., P. Preston-Hurlburt, S.-C. Hong, A. Barlow, and C.A. Janeway, Jr. 1991. Sequence analysis of peptides bound to MHC class II molecules. *Nature (Lond.).* 353:622.
6. Rudensky, A.Y., P. Preston-Hurlburt, B.K. Al-Ramadi, J. Rothbard, and C.A. Janeway, Jr. 1992. Truncation variants of peptides isolated from MHC class II molecules suggest sequence motifs. *Nature (Lond.).* 359:429.
7. Hunt, D.F., H. Michel, T.A. Dickinson, J. Shabanowitz, A.L. Cox, K. Sakaguchi, E. Appella, H.M. Grey, and A. Sette. 1992. Peptides presented to the immune system by the murine class II major histocompatibility complex molecule I-A^d. *Science (Wash. DC).* 256:1817.
8. Chicz, R.M., R.G. Urban, J.C. Gorga, D.A.A. Vignali, W.S. Lane, and J.L. Strominger. 1993. Specificity and promiscuity among naturally processed peptides bound to HLA-DR alleles. *J. Exp. Med.* 178:28.
9. Nelson, C.A., R.W. Roof, D.W. McCourt, and E.R. Unanue. 1992. Identification of the naturally processed form of hen egg white lysozyme bound to the murine major histocompatibility complex class II molecule I-A^k. *Proc. Natl. Acad. Sci. USA.* 89:7380.
10. Zaghouani, H., R. Steinman, R. Nonacs, H. Shah, W. Gerhard, and C. Bona. 1993. Presentation of a viral T cell epitope expressed in the CDR3 loop region of a self immunoglobulin molecule. *Science (Wash. DC).* 259:224.
11. Brumeanu, T.D., R. Kohanski, C.A. Bona, and H. Zaghouani. 1993. A sensitive method to detect defined peptide among those eluted from murine MHC class II molecules. *J. Immunol. Methods.* 160:65.
12. Haberman, A.M., C. Moller, D. McCreedy, and W.V. Gerhard. 1990. A large degree of functional diversity exists among helper T cells specific for the same antigenic site of influenza hemagglutinin. *J. Immunol.* 145:3087.
13. Zaghouani, H., M. Krystal, H. Kuzu, T. Moran, H. Shah, Y. Kuzu, J. Schulman, and C. Bona. 1992. Cells expressing an H chain Ig gene carrying a viral T cell epitope are lysed by specific cytolytic T cells. *J. Immunol.* 148:3604.
14. Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403.
15. Compans, R.W., and P.W. Chopin. 1975. Reproduction of myxoviruses. *In* Comprehensive Virology. Vol. 4. H. Fraenkel-Contrat, and R.R. Wagner, editors. Plenum Press, New York. 179.
16. Chimini, H., P. Pala, J. Sire, B.R. Jordan, and J.L. Maryanski. 1989. Recognition of oligonucleotide-encoded T cell epitopes introduced into a gene unrelated to the original antigen. *J. Exp. Med.* 169:297.
17. Hahn, Y.S., V.L. Braciale, and T.J. Braciale. 1991. Presentation of viral antigen to class I major histocompatibility complex–restricted cytotoxic T lymphocyte. Recognition of an immunodominant influenza hemagglutinin site by cytotoxic T lymphocyte is independent of the position of the site in the hemagglutinin translation product. *J. Exp. Med.* 174:733.