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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 PK8 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 Ig's represent natural long-lived carriers, we suggest that antigenized Ig's have considerable potential for peptide delivery to MHC molecules in situ.

M HC-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).
Chimeric Ig (Ig-HA) expressing the HA110-120 epitope in the CDR3 loop of an anti–influenza 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^b 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^b–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 7.5, and the 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 100,000 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 4°C, 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.

1-A^b–peptide complexes were depleted by passage through a column of anti-1-A^b Ab (25-9-17S; American Type Culture Collection [ATCC], Rockville, MD) coupled to Sepharose. The eluent of this column was then repassed through an anti-bE d Ab (14-4-4, St. Louis, MO), 2% 1-propanol (vol/vol) in deionized water, and peptides were separated by RP-HPLC.

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 0.1% 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 125I-labeled goat anti–rabbit Ig.

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^b 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 Fcy receptor; a chloroquine-sensitive processing step by live APCs is required; and presentation is disected at the level of CD83-positive T cells when Ig-HA is delivered to CD8^+ T cells in association with I-E^b MHC class II molecules. In this study we eluted and analyzed HA peptides delivered to I-E^b 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^b class II molecules were extracted. The peptides bound to I-E^b 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–HA110-120 Abs to wells coated with HA110-120-BSA conjugate. As evident in Fig. 1, a-c, respectively, one major fraction was identified from cells pulsed with HA110-120 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 (µg/ml) | Activation (A₅₇₀ nm) |
|--------------|-----------------------|----------------------|
| Nil          | 0.215 ± 0.03*         |
| PR8 virus    | 0.962 ± 0.05          |
| HA(110-120)  | 0.954 ± 0.07          |
| BHA          | 0.805 ± 0.09          |
| Ig-HA        | 0.842 ± 0.17          |
| Ig-NP        | 0.207 ± 0.02          |
| NP(147-161)  | 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 assayed 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).

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 NH₂-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 E B cells 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.

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

| Antigen       | Retention time | Inhibition | Structure       | Homology with |
|---------------|----------------|------------|-----------------|---------------|
| HA(110-120)   |     min        |     %      | SFERFEIFPKE     | HA            |
|               | 46             | 85         | SFERFEIF        | HA            |
|               | 45             | 67         |                 |               |
| Ig-HA         | 47             | 70         | SFERFEIFPKE     | HA            |
|               | 42             | 13         | XKPNAEDPGLAXQAAK| Unknown       |
|               | 50             | 9          | VDEPQNLIKQNXDQF| BSA           |
| PR8 virus     | 47-49          | 87         | SFERFEIFPKE     | HA            |
|               | 38             | 5          | XRPNAGEDPGLARQ  | 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) recovered from class II molecules |
|--------------------|-----------------------------------------------|
| HA(110-120)        | 20.0 x 10^6 pmole                             |
| Ig-HA              | 0.4 x 10^6 pmole                              |
| PR8 virus          | 0.1 x 10^6 pmole                              |

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

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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