Brief Definitive Report

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

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Summary

Class I major histocompatibility complex (MHC) restricted T lymphocytes preferentially recognize fragments of polypeptides processed through a nonendosomal presentation pathway. At present the intracellular compartment(s) in which polypeptide fragmentation occurs and factors which influence the formation of an antigenic epitope are not well understood. To assess the role of residues flanking an antigenic site in the generation of the antigenic moiety recognized by class I MHC restricted T lymphocytes we have moved the coding sequence for an immunodominant H-2K^d restricted site on the influenza A/JAPAN/57 hemagglutinin (residues 202-221) by site-directed mutagenesis to six different positions along the coding sequence of the hemagglutinin gene. We have found that all six classes of mutants are recognized by MHC class I restricted T cells as efficiently as the wild type hemagglutinin gene product. Thus neither N-terminal to C-terminal position within the translation product nor sequences flanking the antigenic site influence processing.

Major histocompatibility complex class I restricted T lymphocytes appear to play a central role in the recovery from viral infection (1). Unlike the antigen-specific receptor on B lymphocytes which recognizes free antigens, the antigen receptor on these T lymphocytes recognize non-native fragments of large polypeptides complexed to MHC molecules (2). Fragments of both nonglycosylated cytosolic proteins (3) and membrane glycoproteins (4) can be recognized by MHC class I restricted T cells in virally infected cells. These processed viral antigens appear to preferentially associate with nascent class I MHC molecules in a pre-Golgi compartment before export of the peptide MHC complex to the cell surface (5).

At present very little information is available on the nature of the fragmentation process yielding the antigenic epitopes, the specific protease(s) involved or the mechanism of transport of antigen fragments between compartments within a cell. In particular, it is not known if factors such as location of a site within a protein or flanking residues affect the processing and presentation of a site and therefore the immunogenicity of a site. In this report we have examined the influence of flanking sequences bordering an immunodominant antigenic site in the A/JAPAN/57 influenza hemagglutinin (HA) on the processing of the antigenic epitope recognized by H-2K^d restricted CTL after de novo HA expression in target cells. Our strategy, to re-insert in frame the coding sequence for the immunodominant HA 202-221 site at different locations within the HA gene yielded mutant HAs which are recognized by HA 202-221 specific CTL as efficiently as the wild type HA translation product. The implications of these observations are that highly specific processing information is inherent in the HA 202-221 site and not in neighboring amino acid residues, or that some aspects of processing are fairly nonspecific.

Materials and Methods

Generation of HA Mutant Constructs. To construct plasmids containing the repositioned HA 202-221 epitope, the epitope site was first deleted from the A/JAPAN/57 HA cDNA by site-directed, loopout mutagenesis in M13 bacteriophage using a double-primer for the annealing step and extension of the primer with the klenow fragment of *Escherichia coli* polymerase (6). The sequence of the HA 202-221 deletion mutant and the corresponding oligonucleotide are shown in Fig. 1 *a*. The mutant HA gene was inserted into the shuttle vector pMT21, to facilitate subsequent insertion of the coding sequence for HA 202-221 at specific restriction endonuclear

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/91/09/0733/04 $2.00
Volume 174 September 1991 733-736

cleavage sites in the HA coding sequence. This plasmid was then digested at selected restriction sites within the HA coding sequence. Synthetic oligonucleotides encoding the HA 202-221 epitope and several flanking bases were inserted in frame into the existing HA open reading frame to produce the antigenic site. A Hind III-SspI fragment containing the HA gene with the prepositioned site was excised from the plasmid and inserted into the vaccinia expression vector under the control of a vaccinia virus promoter (7.5 kD) using the vaccinia psc11.3 insertion vector. The vaccinia virus recombinants encoding the altered HA mutants were generated by insertion at the vaccinia virus thymidine kinase locus using standard techniques.

T Cell Clones, Cell Lines. The cloned T lymphocytes were derived from BALB/c or BALB/cxC57BL/6(CB6) mice. Clones have been established and maintained as previously described (7). The clones were passaged weekly in the presence of A/JAPAN/57-infected irradiated syngeneic murine splenocytes and exogenous source T cell growth factors of rat splenocyte origin. The P815 (H-2^d) mastocytoma cell line was maintained in DME supplemented with 10% (vol/vol) fetal bovine serum and 1% (wt/vol) glutamine.

Assay for Cell-mediated Cytotoxicity. P815 was used as targets in standard ^{51}Cr-release assays as described (7). Target cells were infected with 20 MOI vaccinia recombinants and labeled with 100 μCi ^{51}Cr. Labeled target cells were washed twice with media and incubated with effector cells at 37°C for 6 h. Values for percent specific release represent the mean counts per min of quadruplicate cultures.

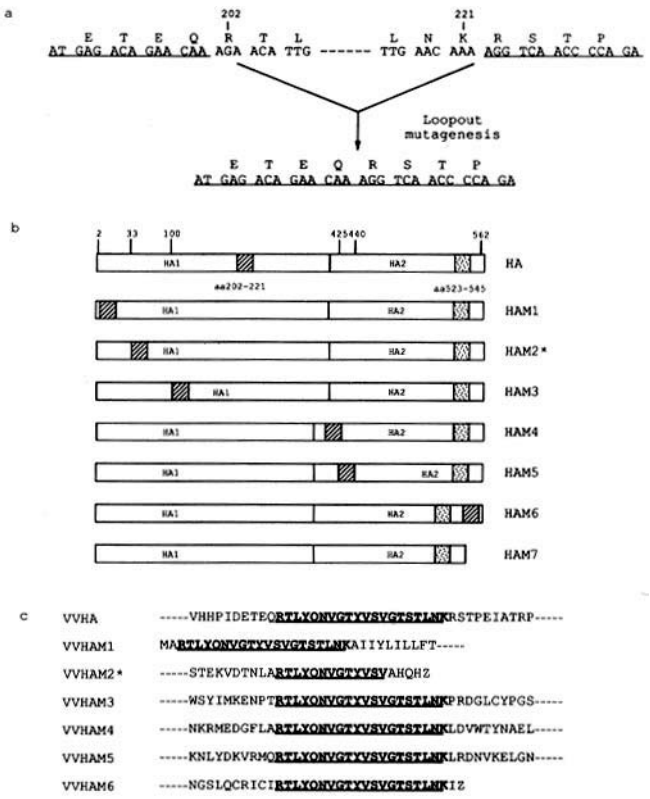

Figure 1. Localization and sequence of the repositioned HA 202-221 site. The coding sequence for the HA 202-221 site was deleted from influenza HA gene cDNA by loopout mutagenesis. The oligonucleotide used to generate the inframe mutant is underlined and included along with the derived amino acid sequence of the resulting deletion mutant (*a*). The HA 202-221 coding sequence was repositioned in the HA coding sequence at the indicated sites (*b*). The sequence of the HA translation product flanking the repositioned epitope is as shown (*c*). The HAM2 mutant which has a frame shift mutation within the coding sequence for the repositioned HA 202-221 site is shown as indicated by an asterisk (*).

Results and Discussion

The A/JAPAN/57 HA contains two immunodominant sites recognized by $CD8^+$ T lymphocytes in association with the H-$2K^d$ molecule. These sites span residues 202-221 in the HA1 portion of the HA and residues 523-545 within the transmembrane domain of the HA (8). The N terminal 202-221 site consists of two overlapping subsites, HA 202-212 and HA 211-221 which are recognized by K^d restricted CTL clones 11-1 and 40-2 respectively (9).

To examine the influence of amino acids neighboring an antigenic epitope on the processing and presentation of the site to MHC class I restricted T lymphocytes we constructed a panel of HA mutants in which the HA 202-221 site was repositioned within the coding sequence of the HA. Our strategy was first to delete the coding sequence for residues 202-221 by loopout mutagenesis and then to reinsert the coding sequence for the 202-221 site at different locations within the HA gene. Using this approach we have inserted HA 202-221 in frame at amino acid positions 2, (in the signal sequence), 33 and 101 in the HA1 and at HA2 positions 430, 466 and at the end of the cytoplasmic tail of the HA (i.e., residue 563, Fig. 1 *b*). These mutant HA genes designated HAM1 through HAM6 were sequenced in the region surrounding the repositioned 202-221 site and the derived amino acid sequence of these flanking sequences is as shown (Fig. 1 *c*). Mutant HAM2 has undergone a frame shift mutation within the repositioned 202-221 site resulting in a loss of residues 215 through 221 of the immunodominant site and the formation of a stop codon in the new reading frame four residues downstream of the insert (Fig. 1 *c*).

The mutant HA genes containing the repositioned 202-221 sites were expressed in H-2^d haplotype P815 target cells using recombinant vaccinia virus expression vectors and CTL recognition was determined using two K^d restricted clones 11-1 and 40-2 which recognize two overlapping sites. Functional recognition of these mutant HA expressing cells by HA 523-545 specific clones 14-7 and C-1 (10) provided an assessment of the processing and presentation of an independent site.

As Fig. 2 *a* shows, repositioning of the immunodominant 202-221 site had no effect on the presentation of the epitope to the clone 11-1. This clone recognized the HA mutants with similar efficiency. As expected, this CTL clone did not recognize the HAM7 deletion mutant which lacks the 202-221 site. Clone 40-2 which is directed to the C-terminal 211-221 subsite (9) recognizes five of the six repositioned mutants to the same extent as the wild type HA. It does not, however, recognize target cells expressing the HAM2 mutant which lacks the coding sequence for HA residues 215 through 221.

Repositioning of the 202-221 site along the coding sequence

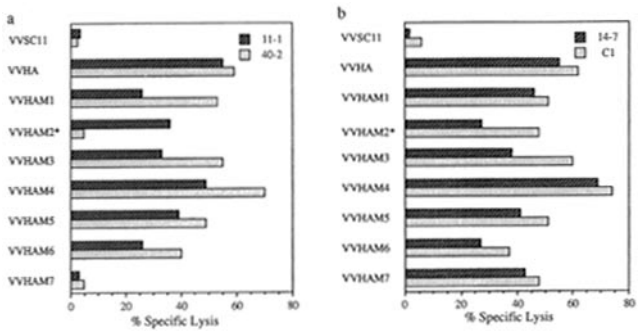

Figure 2. Class I MHC restricted recognition of the repositioned HA 202-221 site. The P815 (H-2^d) mastocytoma cell line was infected with vaccinia recombinants encoding the β-galactosidase gene (VVSC11), the A/JAPAN/57 HA (VVHA) or altered HA mutants (VVHAM1, 2, 3, 4, 5, 6, and 7) at a multiplicity of infection of 20 and used as targets in a standard 6-h ^{51}Cr release assay. Effector to target ratio was 5:1. Values are the means of quadruplicate assay. (*a*) CTL clones 11-1 and 40-2 which recognize the N- and C-terminal portions of the HA 202-221 site were used in the cytotoxicity assay. (*b*) Clones 14-7 and C1 which are directed to the HA 523-545 anchor site were used in the assay.

of the HA gene had no effect on the processing and presentation of the C-terminal 523-545 site for recognition by site-specific T cell clones (14-7 and C1) which lysed target cells expressing wild type HA and the HA mutants with comparable efficiency. It is noteworthy that the HAM2 mutant which is in a different reading frame after the insertion of HA 202-221 at position 33 is also recognized by the C-terminal 523-545 reactive clones when expressed in H-2^d haplotype P815 cells.

These studies demonstrate that neither the position of the epitope or the flanking sequence affected the presentation of the newly synthesized antigenic moiety to MHC class I restricted T lymphocytes. The cytoplasm has been implicated as a major site of processing of antigens for subsequent charging of class I MHC molecules and recognition by MHC class I restricted T lymphocytes for cytoplasmic proteins (3), protein antigens which are microinjected into the cell cytoplasm (11), and for cells expressing minigenes whose peptide products are expressed in the cell cytoplasm (12). Indeed, a putative transporter gene has been recently identified (13). In the case of glycoprotein antigens, early evidence suggested that glycoproteins were presented to class I restricted T cells through a nonendosomal presentation pathway (14). Both the cytoplasm and the endoplasmic reticulum could serve as potential sites of nonendosomal proteolysis of glycoproteins. Evidence in support of the cytoplasm as the site of glycoprotein fragmentation first came from the observation that class I MHC restricted T cells could recognize target cells expressing the signal minus form of the hemagglutinin (15). This is further supported by observations reported here that there is efficient recognition of the HAM6 mutants by CTL since it has a signal sequence and the repositioned HA 202-221 site is located in the cytoplasmic tail region of the HA molecule translocation of the mutant HA into the endoplasmic reticulum (Fig. 1 *b*).

We cannot formally exclude the possibility that HA fragments generated in the endoplasmic reticulum can provide a source of peptide fragments for class I MHC charging. Indeed there is evidence that nascent class I MHC molecules resident in an endoplasmic reticulum compartment may be capable of directly binding free peptide fragments (5). As noted above, it is not easy to explain the recognition of the HAM6 mutant or existence of sites which map to the transmembrane domain (4) or the signal sequence of glycoproteins by a mechanism involving fragmentation of the HA translation product in the endoplasmic reticulum. It may be that both the cytoplasm and the endoplasmic reticulum can provide peptide fragments for MHC class I charging.

In conclusion, our findings suggest that the processing and presentation machinery involved in fragmentation of antigens for presentation to MHC class I restricted T cells are not influenced by residues outside the antigenic site recognized by the MHC class I restricted T cells. This is in contrast to observations on the processing and presentation of antigen to MHC class II restricted T cells where residues outside of the antigenic site appear to alter processing and presentation (16). Although exceptions have been reported (17), it appears that the proteases carrying out processing in the class I presentation pathway act nonspecifically. This particular overlapping double site therefore permits further studies as to the presence of specific versus random cleavage sites within the HA 202-221 region.

We wish to thank Mrs. Cora Arthur for dedicated technical assistance and Mrs. Jerri Smith for expert secretarial support.

This study was supported by United States Public Health Service grants AI-15608, HL-33391, and AI-28317. Young S. Hahn is a fellow of the Cancer Research Institute, Inc.

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Received for publication 8 April 1991 and in revised form 13 June 1991.

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