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Differential Effects of Flanking Residues on Presentation of Epitopes from Chimeric Peptides

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Chimeric peptides in which the optimal *H-2*^{d} mouse hepatitis virus nucleocapsid (pN) and human immunodeficiency virus type 1 (p18) epitopes, separated by 38, 7, or 2 amino acids, were expressed from a single open reading frame by using recombinant vaccinia viruses to analyze antigen processing of proximal class I-restricted epitopes. Recognition of the carboxy-terminal D^d-restricted p18 epitope was independent of the amino-terminal flanking residues. By contrast, proximity of the carboxy-terminal epitope decreased recognition of the amino-terminal L^d-restricted pN epitope. Immunization resulted in the induction of both p18- and pN-specific antiviral cytotoxic T lymphocytes, irrespective of the number of amino acids separating the epitopes.

Cytotoxic T lymphocytes (CTL) recognize processed antigen in the form of 8- to 10-amino-acid (aa) peptides in association with major histocompatibility complex (MHC) class I molecules (10, 18, 21). These peptides are generally derived from antigens which are proteolytically degraded in the cytosol and transported into the endoplasmic reticulum, where they assemble with class I heavy chains and β2 microglobulin to form a stable tripartite complex which is transported to the plasma membrane (11, 16, 21). Naturally processed class I binding peptides are characterized by a sequence motif specific for the allelic variations within the cleft of individual class I heavy chains (18, 21). However, despite the presence of numerous peptides with potential binding motifs in an antigen, CTL responses to only a very limited number of determinants are induced (2, 17, 28). Although the cytosol and the endoplasmic reticulum are sources for peptides (11), it is unclear whether class I molecules associate with mature peptides that have undergone cleavage to the optimal epitope or assemble with larger precursor peptides, which are trimmed during or after assembly (21). The highly selective cell surface presentation of individual peptides is regulated primarily by peptide-class I binding affinity (10, 11, 17, 28). However, accessibility to proteolysis and specificity of peptide transporter proteins provide additional limiting factors for peptide presentation (6, 11, 19, 22).

The primary role of residues within the CTL epitope for class I presentation has clearly been established (10, 13, 17, 28) and is supported by efficient presentation of endogenous epitopes lacking flanking sequences (2, 3, 8) or placed in the context of heterologous or mutated flanking residues (5–7, 12, 13, 27). The influence of flanking sequences on class I processing, however, remains controversial. Alterations in the 5 to 10 residues localized directly adjacent to an optimal epitope may cause defective presentation, as demonstrated by the reduced or inhibited presentation of natural L^d- and K^d-restricted determinants (7, 8, 13).

Efficient processing has implications for both the outcome of a CTL response following viral infection and the design of recombinant polyvalent vaccines containing multiple T-cell epitopes to provide protective immunity (6–8, 27). Several problems concerning class I antigen presentation may arise when multiple epitopes are linked. First, residues from one epitope may form flanking sequences deleterious to the presentation of adjacent epitopes. Second, the differential trafficking rate of individual class I molecules (1) may result in preferential induction of CTL specific for the epitope that is presented by a faster-trafficking class I molecule. Third, processing of the chimeric peptides may result in the formation of novel epitopes which may induce CTL that do not recognize native antigen (20).

To establish a model for analysis of antigen presentation and CTL induction within the *H-2*^{d} haplotype, we tested responses to two immunodominant viral epitopes expressed as chimeric peptides: one epitope comprises the D^d-restricted p18 sequence from the gp160 protein of human immunodeficiency virus type 1 (HIV-1) strain IIIB (26); the second epitope, designated pN, is L^d restricted and derived from the nucleocapsid protein (JN) of the JHM strain of mouse hepatitis virus (MHV; 2). The optimal peptides are a 10-mer (aa 318 to 327; p18-10) within the p18 epitope (3) and a 9-mer (aa 318 to 326; pN318-326) within the pN epitope (2). Both epitopes are efficiently recognized when expressed endogenously in either the presence (peptides comprising 51 to 67 aa) or the absence of native flanking sequences (9 or 10 aa) by CTL specific for native antigen (2, 3).

The influence of epitope proximity on presentation was investigated by using three minigenes expressing tandem peptides in which the HIV p18 and MHV pN epitopes were separated by 38 (tan38)-, 7 (tan7)-, and 2 (tan2)-aa spacers (Fig. 1). Immediate flanking sequences of both epitopes in tan38 and tan7 are composed of native sequences (Fig. 1); however, the p18-10 epitope in tan2 is directly linked to native JN residues. Genes encoding tan38, tan7, and tan2 with an initiation start codon and stop codons were cloned into vaccinia virus (Vac) vectors pTM1 (9) and pSC11ss-derived pK (3). Homologous recombination with the wild-type Vac WR strain as previously described (25) resulted in Vac recombinants vtan38, vtan7, and vtan2. The gene encoding tan38 was

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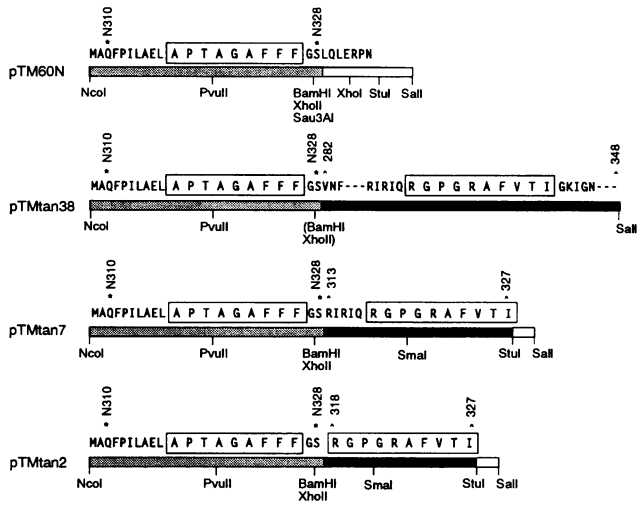

FIG. 1. Schematic of the minigenes and amino acid sequences of chimeric peptides. Minigenes are represented by bars. JN-specific coding sequences are shaded, gp160 is in black, and the vector-derived polylinker is in white. Restriction site positions are shown relative to the peptide sequence. Amino acids are in the one-letter code; the boxed amino acids represent the optimal peptide required for CTL recognition. The beginning and end of JN- and gp160-derived residues are marked by the symbols * and ^, respectively. The numbering of amino acids identifies their position in the native proteins. Designations of the chimeric constructs indicate the lengths of intervening flanking residues between the epitopes.

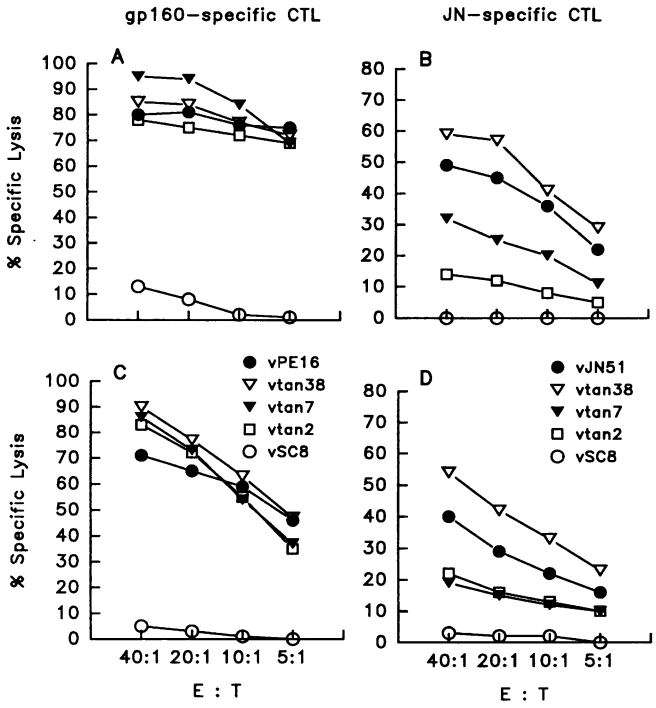

FIG. 2. Differential recognition of epitopes derived from endogenous chimeric peptides. J774.1 targets ($H\text{-}2^d$) (A and B) or L929 target cells ($H\text{-}2^k$) expressing either D^d (C) or L^d (D) molecules were infected with either vtan38, vtan7, or vtan2. Targets infected with Vac recombinants expressing either gp160 (vPE16) or aa 301 to 355 of the JN protein (vJN51) served as positive controls; vSC8 (expressing the *Escherichia coli lacZ* gene)-infected targets were negative controls. Presentation of p18 was tested with gp160-specific CTL (A and C), and that of pN was tested with JN-specific CTL (B and D). Symbol legends are identical for panels A and C and panels B and D. Cytolytic activity was measured in a 4-h ^{51}Cr release assay as previously described (2, 3). Effector cells were added at the effector/target cell ratios (E:T) indicated. The cytolytic activity shown is representative of three independent assays.

assembled by sequential cloning of a 64-bp fragment encoding JN aa 310 to 328 and a 210-bp fragment encoding gp160 aa 282 to 348 into pTM1 (9). The JN minigene was amplified by PCR as previously described (2), with primer oligonucleotides ON1011 (5'-ATA GGA TCC ATG GCA CAG TTC CCC ATT CTT GCA; nucleotides 1011 to 1026) and ON1427 (5'-TTA CCC GGG CAC ATT AGA GTC ATC TTC TAA C; nucleotides 1427 to 1447), which contained *Nco*I and *Sma*I sites to introduce an ATG start codon and allow unidirectional cloning. Following digestion with *Nco*I and *Sau*IIIA, the PCR product was cloned into the *Nco*I-*Bam*HI sites of pTM1 to yield plasmid pTM60N. Plasmid pTMtan38 was obtained by insertion of an *Xho*II-*Sal*I fragment encoding the gp160 V3 loop (3) into the *Bam*HI-*Sal*I sites of pTM60N. Minigene tan38 was excised from pTMtan38 with *Nco*I and *Hin*cII and ligated into the *Nco*I-*Stu*I sites of plasmid pK (3), resulting in plasmid pKtan38. Construct tan2 was generated by direct cloning of complementary oligonucleotides CB7 (5'-GATCCA GAG GAC CCG GGA GAG CAT TTG TTA CAA TAT AGG and CB10 (5'-CCT ATA TTG TAA CAA ATG CTC TCC CGG GTC CTC TG) into the *Bam*HI-*Stu*I sites of plasmid pTM60N downstream of the pN epitope minigene. The resulting plasmid, pTMtan2, contained a unique *Sma*I site within the coding region of the p18-10 epitope and a stop codon introduced by the oligonucleotides. Construct pTMtan7 was generated by inserting annealed oligonucleotides CB8 (5' GAT CCA GAA TCC GTA TCC AGA GAG GAC CC) and CB9 (5' GGG TCC CCT CTG GAT ACG GAT TCT G) encoding the 7-aa spacer region into the *Bam*HI-*Sma*I sites of pTMtan2. Plasmids pKtan2 and pKtan7 were generated by subcloning the respective *Nco*I and *Stu*I tan7 and tan2 fragments into the *Nco*I-*Sma*I sites of plasmid pK. Sequences were confirmed by using pSC11ss-specific primers as previously described (25).

The ability of the chimeric peptides to be processed for simultaneous presentation by both the D^d and L^d class I molecules was assessed by using J774.1 ($H\text{-}2^d$) cells. Targets were infected with Vac recombinants vtan38, vtan7, and vtan2 at a multiplicity of 5, incubated for 12 h at 37°C, and tested for recognition of the individual epitopes by both gp160 (Fig. 2A)- and JN (Fig. 2B)-specific CTL in a ^{51}Cr release assay as previously described (2, 3). Antigen-specific CTL populations were in vitro-restimulated cultures derived from BALB/c mice immunized with either JHM or a Vac recombinant expressing gp160 (vPE16) as previously described (2, 3). In numerous experiments, the p18 epitope was recognized equally well by gp160-specific CTL independent of the amino (N)-terminal sequences (Fig. 2A). However, parallel analysis of identical targets consistently showed a loss of pN-specific recognition with increased proximity to the carboxy (C)-terminal p18 epitope (Fig. 2B). To rule out a processing defect or preferential binding of the chimeric precursor peptide to D^d as a cause for decreased L^d-restricted presentation in $H\text{-}2^d$ cells, L929 cells ($H\text{-}2^k$) expressing either L^d (K2a7) or D^d (K8-30) class I molecules were tested. Figure 2C and D confirms the consistently higher levels of p18 recognition on D^d and the impaired presentation of the pN epitope on L^d targets when

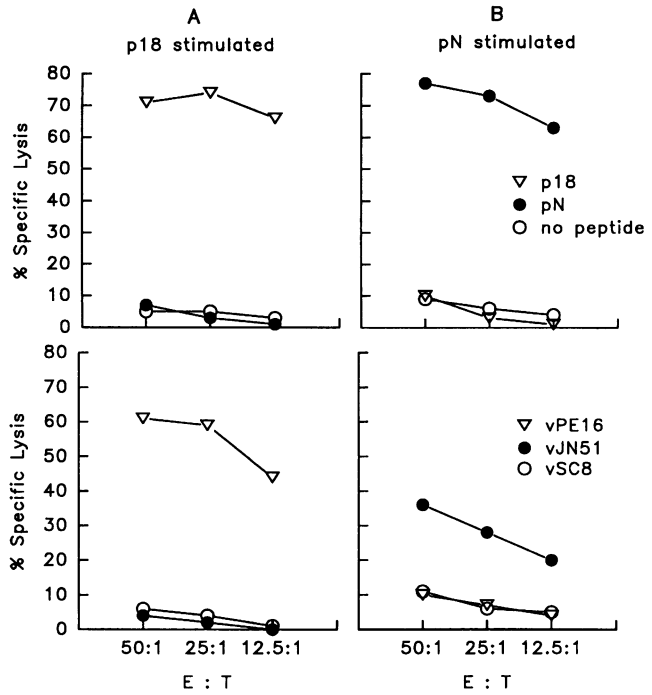

FIG. 3. Cytolytic activity of CTL from mice immunized with the Vac recombinant expressing chimeric epitope tan38. Polyclonal CTL derived from mice immunized with vtan38 were restimulated in vitro on either peptide p18 (A) or pN318-335 (B) and tested for recognition of exogenous (upper panels) and endogenous (lower panels) antigens. J774.1 target cells were coated with 1 μM peptide p18 or pN318-335 for 15 min at 37°C prior to the addition of effectors or infected with Vac recombinants expressing gp160 (vPE16), a truncated JN protein (vJN51), or a heterologous protein (vSC8). E:T, effector/target cell ratio.

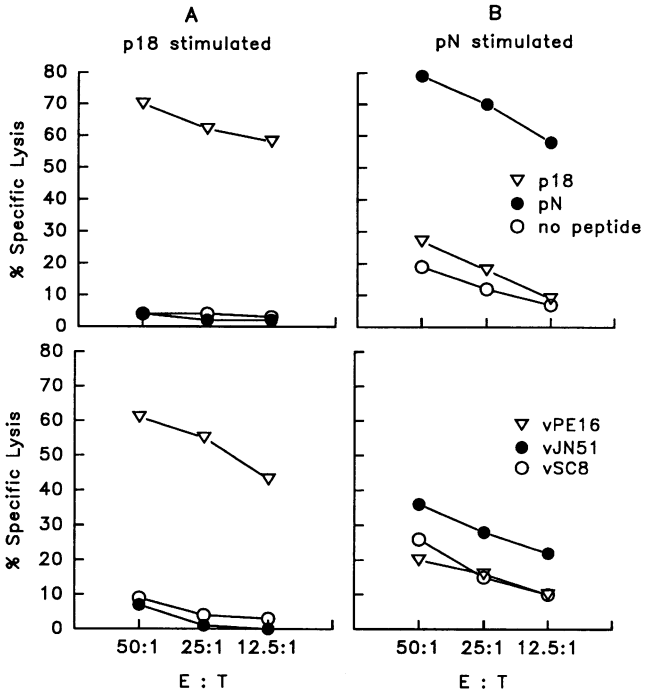

FIG. 4. Cytolytic activity of CTL from mice immunized with Vac recombinants expressing chimeric epitope tan2. Polyclonal CTL derived from mice immunized with vtan2 were restimulated in vitro on either peptide p18 (A) or pN318-335 (B) and tested for recognition of exogenous (upper panels) and endogenous (lower panels) antigens on J774.1 target cells as described in the legend to Fig. 3. E:T, effector/target cell ratio.

derived from vtan7 or vtan2. As the N-terminal sequences of the pN epitope were identical in all constructs (Fig. 1), reduced recognition can be attributed to either hindering C-terminal flanking residues introduced by the proximity of pN to p18 in the tan2 and tan7 chimeras or shorter overall peptide length. Reduced stability of the shorter peptides, comprising 31 (vtan2) and 36 (vtan7) aa, is unlikely to account for the inefficient presentation because the p18 epitope is presented efficiently in the identical targets (Fig. 2A and B). In addition, no differences in recognition were observed for a variety of JN protein truncations containing the pN epitope (2).

Efficient recognition is an insufficient criterion to ensure in vivo induction, as demonstrated by the failure of a minigene-encoded CTL epitope to induce antigen-specific CTL, despite its ability to sensitize targets (15). vtan38, which was efficient at sensitizing targets for lysis by both gp160- and JN-specific CTL (Fig. 2), was tested for the ability to prime secondary in vitro JN- and gp160-specific CTL. Spleen cells from vtan38-immunized mice were divided into three cultures and stimulated with either p18, pN318-335, or both peptides. Effectors from each group were tested for recognition of J774.1 targets infected with Vac recombinants expressing either gp160 (vPE16) or a truncated JN protein (vJN51; 2) and targets coated with either peptide. vtan38-induced CTL, restimulated with the individual peptides, were specific for the respective native antigens and corresponding peptides (Fig. 3A and B). Lysis of pN peptide-coated targets was consistently higher than that of targets sensitized with the endogenous JN epitope, suggesting that the endogenous concentration of the pN epitope is limiting (Fig. 3B). vtan38-induced effector cells stimulated with both peptides recognized targets expressing both native antigens but not targets infected with a heterologous Vac recombinant (data not shown). No differences were detected between the activity of CTL derived from vtan38-immunized mice and that of CTL from mice immunized simultaneously with Vac recombinants expressing gp160 aa 281 to 348 (v18-76; 3) and JN aa 301 to 351 (vJN51; 2) individually (data not shown). Furthermore, prior studies demonstrated that antigen-specific CTL were not primed in vitro by peptide stimulation (data not shown). Therefore, expression of both epitopes from a single gene was as effective as expression from the separate minigenes for CTL induction and the presence of two immunodominant epitopes did not inhibit CTL priming to either epitope.

JN-specific CTL recognized both $H\text{-}2^d$ and L^d targets infected with vtan2 weakly compared with vtan38-infected cells (Fig. 2). Therefore, vtan2 was tested for priming of both gp160- and JN-specific CTL. Effector cells were divided and restimulated in vitro with either peptide and tested for lysis of targets expressing the individual antigens or coated with peptide (Fig. 4). As expected, the cytolytic activity of p18-restimulated CTL (Fig. 4A) was comparable to that of vPE16-induced CTL (Fig. 2). No recognition of pN-coated targets was detected. The activity of pN-restimulated effectors (Fig. 4B) was similar to that of CTL from vtan38 (Fig. 3B) and coimmunized mice. As noted above, the L^d-restricted response for the cytosolic JN epitope was consistently lower than the D^d-restricted response

despite efficient recognition of peptide-coated cells. Although vtan2 was less efficient than vtan38 at sensitizing targets for lysis by JN-specific CTL (Fig. 2), no significant differences in the CTL activities of in vitro-restimulated spleen cells from vtan2-, vtan7 (data not shown)-, or vtan38 (Fig. 3)-immunized mice were observed. These results suggest that despite differential recognition of individual epitopes in vitro, the linkage of distinct epitopes restricted to different class I molecules can induce broadly reactive CTL within a single haplotype. Weak recognition in vitro does not necessarily correlate with a lack of CTL induction in vivo, consistent with results obtained by analysis of positional effects on class I presentation of a murine cytomegalovirus epitope (7). However, CTL activity from secondary cultures is the most sensitive CTL detection system (4, 23) and often does not reflect biologically relevant levels of CTL memory required for a protective response (4, 7, 23). Comparison of the threshhold required for recognition in vitro versus CTL induction in vivo is not feasible, as the local MHC-peptide complex concentration on the surface of virus-infected tissues in vivo is difficult to estimate. In addition, CTL induction in vivo requires accessory adhesion molecules, which may significantly lower the threshhold of MHC-peptide complexes required for induction (14, 24). In contrast to our findings, failure to induce CTL despite highly efficient recognition in vitro has also been described (15). These discrepancies between antigenicity and immunogenicity may reflect additional requirements for $CD4^+$ T-helper cells and/or the engagement of different subsets of antigen-presenting cells by the respective immunizing agent.

Flanking sequences may be critical in class I antigen presentation by hindering proteolysis and/or transport of peptides into the endoplasmic reticulum and thereby limiting the number of potential antigenic class I epitopes (6, 8, 19, 22). The proximal location of the heterologous N-terminal MHV epitope did not interfere with presentation of the C-terminal HIV epitope, confirming that altered N-terminal flanking sequences and the absence of C-terminal flanking residues have no effect on D^d-restricted presentation in vitro and CTL induction in vivo. Conversely, C-terminal sequences flanking the N-terminal MHV epitope exerted a negative effect on the efficiency of presentation. Additional analysis of a chimeric peptide containing the epitopes in reverse order with a single-residue spacer, namely, the HIV epitope in the N-terminal position and the MHV epitope in the C-terminal position (MRGPGRAFVTI_E_APTAGAFFF), demonstrated highly efficient recognition of the MHV epitope and very poor recognition of the HIV epitope, confirming preferential presentation of the C-terminal epitope. Similar to other constructs, CTL induction to both epitopes was detected by using secondary stimulated cultures (data not shown). Consistent with previous reports, these results imply that processing of the C-terminal flanking residues is more selective than events at the N terminus (8, 13). This study supports the relevance of flanking sequences for optimal presentation of proximal epitopes. The induction of CTL specific for both native MHV N protein and HIV gp160 by chimeric peptides containing as little as one or two intervening residues supports the feasibility of a multi-T-cell epitope minigene approach to providing broad protective immunity. Direct correlation between in vitro efficiency of recognition and in vivo CTL induction requires CTL frequency analysis. Potential formation of novel hybrid epitopes, documented for class II peptides (20), was not detected. This approach offers the advantage of selectively inducing heterogeneous CTL to epitopes from different proteins of a single pathogen and to epitopes from distinct virus strains, thereby minimizing escape via antigenic drift. A single report demonstrates the efficacy of this approach in conferring protective immunity to lymphocytic choriomeningitis virus by immunization with chimeric peptides comprising linked epitopes restricted to MHC molecules of different haplotypes (27). Our data show that this strategy can be extended to include immunodominant epitopes presented by different MHC molecules within one haplotype.

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