

# Flanking Sequences Influence the Presentation of an Endogenously Synthesized Peptide to Cytotoxic T Lymphocytes

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## Summary

Cytotoxic T lymphocytes (CTL) recognize class I major histocompatibility complex molecules complexed to peptides of eight to nine residues generated from cytosolic proteins. We find that CTL recognize, *in vitro* and *in vivo*, cells synthesizing a 10-residue peptide consisting of an initiating methionine followed by nine residues corresponding to a naturally processed determinant from influenza virus nucleoprotein (NP) (residues 147–155). Addition of two COOH-terminal residues corresponding to NP residues 157 and 158 severely reduced presentation of the endogenously produced peptide to CTL *in vitro* and *in vivo*. Extension of NH<sub>2</sub> and COOH terminal flanking residues to include residues corresponding to NP residues 137–146 and 159–168 failed to increase the antigenicity of this peptide. Its presentation was greatly enhanced, however, by further extending the NH<sub>2</sub> and COOH termini to include all of the additional residues of NP. These findings indicate first, that a naturally processed viral ligand (with an NH<sub>2</sub>-terminal Met) of a class I molecule contains sufficient information to access intracellular class I molecules, and second, that flanking residues can influence the processing and presentation of antigens to CTL.

CTL recognize peptides of 8–10 residues complexed with MHC class I molecules (1–3). Synthetic peptides provided exogenously (4) bind to class I molecules at the plasma membrane (5–7). Proteins synthesized endogenously are processed within the cell, and the resultant peptides appear to bind class I molecules in an early exocytic compartment (6–9).

Little is known about the process by which antigenic fragments are generated and delivered to class I molecules. While evidence suggests that some proteolysis occurs in the cytosol (10–14), it is uncertain whether the final cleavage steps occur within the cytosol, or after determinants have entered the exocytic compartment. The specificity of proteolysis is also poorly appreciated. Although a protein might have many peptides able to bind class I molecules with high affinity, only a fraction might be liberated by proteolysis in quantities sufficient to load enough class I molecules to activate T cells. Some flanking sequences might hinder production of antigenic peptides, while others might render them sensitive to digestion into smaller, nonantigenic fragments.

Knowledge is also limited regarding the transport of peptides to the exocytic compartment (possibly the endoplasmic reticulum), where association with class I molecules is presumed to occur. Recent reports describe genes within the MHC encoding members of the “ABC” transporter protein

family (15–18) that might perform this function. The peptide specificity of any such transporter molecule would be expected to be one of the critical factors in determining the pool of determinants that class I molecules are able to bind and transport to the cell surface for immune surveillance. It is uncertain whether the putative peptide pumps are able to transport minimal peptide determinants with high affinity for class I molecules, or whether flanking sequences are required for efficient transport. It is also possible that flanking sequences could inhibit transport of class I binding peptides. In the present study we have examined whether cells are able to properly process small antigenic peptides expressed in the cytosol, and have tested the effects of various flanking sequences on the processing of these peptides.

## Materials and Methods

**Synthetic Peptides.** Peptides (provided by Dr. John Coligan of the Biological Resources Branch, National Institute of Allergy and Infectious Diseases [NIAID], Bethesda, MD) were synthesized on peptide synthesizers (9050; Milligen/Biosearch, Burlington, MA; and 430; ABI, Foster City, CA). After HPLC purification, sequences were confirmed using an ABI 477A peptide sequencer, and the masses were confirmed by fast atom bombardment (M-Scan Inc., West Chester, PA).

**Viruses.** The A/Puerto Rico/8/34 (PR8)<sup>1</sup> influenza virus infectious stock was generated by growth of virus in the allantoic cavity of embryonated hen eggs. Virus concentration in hemagglutinating units was determined by chicken red cell agglutination (19). The PR8 NP, PR8 hemagglutinin (HA), and basic polymerase 2 (PB2) vaccinia virus (Vac) recombinants have been described (20, 21). Vac recombinants were generated as described previously (22) with the following modifications. Minigenes were made by PCR of the PR8 influenza virus NP cDNA (kindly provided by Dr. Peter Palese, Mt. Sinai School of Medicine, New York, NY) using synthetic oligonucleotide primers containing restriction endonuclease sites (Sall in the upstream primer and NotI in the downstream primer) to enable directional ligation, the ATG initiation codon preceded by the sequence CCACC for efficient translation (23) in the upstream primer, and a double stop codon (TAGTGA) in the downstream primer. In each primer, nonannealing bases were flanked by 10 bases of fully annealing sequence. PCR products were digested in Sall and NotI and inserted behind the Vac early-late P<sub>7.5</sub> promoter in a modified version of the pSC11 plasmid in which the restriction sites Sall and NotI were substituted for the SmaI site. The resulting constructs were then recombined into the wild-type vaccinia virus genome. The NP/R<sup>-</sup> gene was constructed as follows. PCR was used to generate two fragments coding for NP residues 1–159 and 149–498. Primers were designed to eliminate the Arg<sub>156</sub> codon in both fragments, to effect a conservative T to C change in the Ala<sub>153</sub> codon, thereby generating a unique ApaI site in both fragments, and to allow cloning into the modified pSC11 vector described above. After treatment of the 1–159 fragment with Sall and ApaI, and the 149–498 fragment with ApaI and NotI, the two fragments were inserted into the modified pSC11 plasmid. Sequencing of the gene (using the Sequenase kit from IBI, Inc., New Haven, CT) confirmed the loss of the Arg<sub>156</sub> codon, and also indicated a point mutation resulting in a Tyr<sub>313</sub> to His<sub>313</sub> substitution. The M147–158/R<sup>-</sup> and 137–168/R<sup>-</sup> minigenes were made by PCR using as template the NP/R<sup>-</sup> gene and the same strategy described for generation of the M147–155 minigene.

**Northern Blotting.** 10<sup>8</sup> P815 cells were infected with various Vac recombinants under the same conditions used in the CTL assays (described below). After a 5-h infection, cells were pelleted, washed with Dulbecco's PBS, and total RNA was extracted using RNeasy B (Cinna/Biotech Laboratories International, Inc., Friendswood, TX). RNA pellets were dissolved in 0.2 ml H<sub>2</sub>O, aliquoted, and either left untreated, or incubated with RNase-free DNase or DNase-free RNase (Boehringer Mannheim Biochemicals, Indianapolis, IN) with 10 mM CaCl<sub>2</sub> at 0.1 and 0.05 μg/ml, respectively, for 30 min at 37°C. Samples were then diluted 10-fold and adjusted to 7.5× SSC, 4.9 M formaldehyde, and 0.1-ml volumes applied to nitrocellulose (BA-S 85 type; Schleicher & Schuell, Inc., Keene, NH) mounted in a Minifold II slot blot apparatus (Schleicher & Schuell, Inc.). The blot was dried under vacuum and probed using <sup>32</sup>P end-labeled oligonucleotides for the presence of RNA bearing the 147–155 sequence (27 bases in length) or a sequence encoding the Vac complement binding protein early gene product (28 bases in length), generously provided by Dr. Stuart Isaacs (NIAID).

**CTL Assay.** P815 cells, serving as APC in the assays, were maintained in RPMI 1640 with 7.5% FCS at 37°C in an air/CO<sub>2</sub> (94%/6%) atmosphere. APC were infected for 1 h at 37°C with Vac recombinants at 10 PFU/cell at a concentration of 10<sup>7</sup> cells/ml in balanced salt solution with 0.1% BSA. Generally 2 × 10<sup>6</sup> APC

were used for each infection. Afterwards, 10 ml IMDM supplemented with 7.5% FCS was added, and the P815 cells were incubated an additional 3 h at 37°C with rotation. Cells were pelleted, suspended at 5 × 10<sup>7</sup> cells/ml in IMDM with 50–100 μCi of Na<sup>51</sup>CrO<sub>4</sub> (Amersham Corp., Arlington, Heights, IL), and incubated for 1 h at 37°C. APC were then washed three times with DPBS, suspended in IMDM, and combined with CTL populations (described below) in round-bottomed plates at 10<sup>4</sup> APCs/well. The final volume per well was 0.2 ml. APC and CTL were cocultured for 4 h at 37°C before supernatants were collected (0.1-ml volumes) and counted in a gamma detector (5010 Cobra; Packard Instrument Co., Inc., Downers Grove, IL). The data are presented as percent specific <sup>51</sup>Cr, defined as follows: 100 × [(experimental cpm – spontaneous cpm)/(total cpm – spontaneous cpm)].

**Generation of CTL.** CTL populations were generated as described (20). Briefly, BALB/cByJ mice (The Jackson Laboratory, Bar Harbor, ME) were injected intravenously with 10<sup>7</sup> PFU of Vac recombinants in 0.5 ml of DPBS. After at least 2 wk, splenocytes were harvested and cocultured with splenocytes from naive mice that had been infected with PR8 or a Vac recombinant. Cultures were harvested after 6 or 7 d of incubation at 37°C and used in the CTL assays as described above.

## Results

**Presentation of the Endogenous Peptide.** Recent work has shown that mouse H-2K<sup>d</sup> molecules derived from influenza virus-infected cells are complexed to a major species of antigenically active peptide derived from the viral nucleoprotein (NP), consisting of residues 147–155 (TYQRTRALV) (3). To examine the antigenicity of this peptide when originating in the cytosol of APC, a minigene (12) encoding the 147–155 determinant was inserted into the Vac genome (termed M147–155 Vac). Although it was necessary to add an NH<sub>2</sub>-terminal Met to initiate translation, it is likely that Met is efficiently removed posttranslationally by Met-amino peptidase, since the penultimate residue is Thr (24). The antigenicity of the peptide synthesized endogenously was first examined by testing the lysis of Vac-infected APC by NP-specific CTL. Table 1 A shows that cells infected with the Vac recombinant-encoding peptide M147–155 were lysed by NP-specific CTL as efficiently as cells infected with a Vac recombinant-expressing full-length NP. The specificity of recognition was demonstrated by the low levels of lysis obtained using CTL specific for another viral gene product (HA) recognized in association with K<sup>d</sup> (25) (Table 1 A).

It was important to demonstrate that the peptide was acting as a true endogenous antigen and not sensitizing APC by an exogenous route. Cells were treated with brefeldin A (BFA), an agent that blocks the presentation of antigens processed from the cytosol while not affecting presentation of exogenously added peptides (6, 7). BFA completely inhibited presentation of the M147–155 Vac-produced peptide, while having no effect upon presentation of exogenous peptide (Table 1 A, BFA). This is not simply due to inhibition of peptide biosynthesis, since reversal of the BFA blockade in the presence of protein synthesis inhibitors to prevent additional peptide biosynthesis completely restored presentation (BFA Reverse). This strongly suggests that the endogenously synthesized peptide associates with K<sup>d</sup> in an intracellular

<sup>1</sup> Abbreviations used in this paper: BFA, brefeldin A; HA, hemagglutinin; NP, nucleoprotein; PR8, A/Puerto Rico/8/34; Vac, vaccinia virus.

**Table 1.** Ability of Minigene Products to Sensitize APC for Lysis by NP-specific CTL

P815 APC infected/treated with:	Percent specific <sup>51</sup> Cr release by CTL specific for:*							
	A/PR8 influenza		NP		HA		Vaccinia virus	
<b>A</b>								
M147-155 Vac	55	53	80	62	11	8	75	60
M147-155 Vac BFA	0	0	3	2	0	0	0	0
M147-155 Vac BFA reverse	52	51	73	66	4	6	76	62
147-158/R <sup>-</sup> peptide	33	31	50	35	0	0	0	0
147-158/R <sup>-</sup> peptide BFA	27	27	49	35	0	0	0	0
NP Vac	51	53	66	58	16	11	86	69
HA Vac	50	42	4	3	55	46	76	63
<b>B</b>								
M147-158/R <sup>-</sup> Vac	7	6	7	4	9	1	84	72
M147-158 Vac	44	41	60	51	13	9	78	61
<b>C</b>								
137-168/R <sup>-</sup> Vac	12	8	3	1	7	1	71	55
NP/R <sup>-</sup> Vac	43	39	56	53	13	6	67	47

\* Values for PR8-, NP-, and HA-specific <sup>51</sup>Cr release are from CTL/APC ratios 30:1 and 10:1; for Vac-specific <sup>51</sup>Cr release, values shown are from ratios 3.3:1 and 1.1:1.

compartment. It also demonstrates that the effect of BFA on antigen presentation occurs independently of any effect it might have on intracellular proteolysis.

M147-155 Vac was further tested for its ability to prime splenocytes for secondary in vitro NP-specific responses to PR8. The NP-specific CTL response generated was similar

in magnitude to that observed with PR8-restimulated splenocytes derived from mice primed with NP-Vac (Table 2, Exp. A). Thus, the endogenously synthesized peptide was efficiently presented to T cells in vivo.

*Flanking Sequences Can Negatively Influence Presentation of the 147-155 Determinant.* To examine the effects of flanking

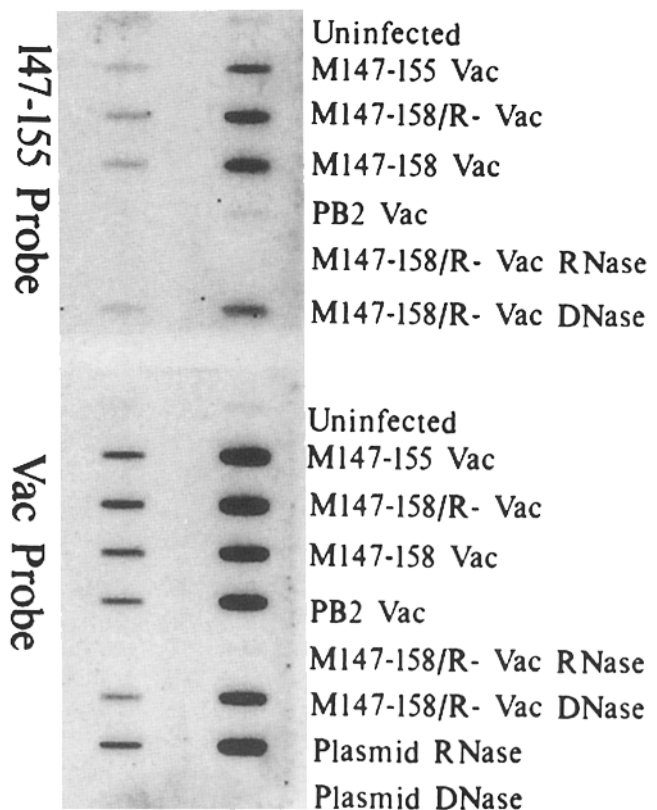
**Table 2.** Ability of the Endogenously Produced Peptides to Prime Mice for a NP-specific Response

Exp.	Priming	Restimulation	Percent specific <sup>51</sup> Cr release by APC treated with:*							
			NP Vac		147-158/R <sup>-</sup> peptide		NP/R <sup>-</sup> Vac		HA Vac	
A	M147-155 Vac	PR8	57	44	43	32	59	47	3	0
	NP Vac	PR8	66	58	49	35	56	53	4	3
	HA Vac	PR8	16	11	0	0	13	6	55	46
B	M147-158/R <sup>-</sup> Vac	PR8	22	15	11	5			20	9
		HA Vac	82	67	0	0			77	64
	M147-158 Vac	PR8	56	47	55	34			31	16
		NP Vac	58	39	63	35			9	3
		HA Vac	14	6	0	0			58	38
		PR8	24	9	0	0			48	28

\* For CTL populations generated by priming with a recombinant Vac and stimulating with a recombinant Vac, values shown are from CTL/APC ratios 3.3:1 and 1.1:1; all other values are from CTL/APC ratios 30:1 and 10:1.

sequences on the presentation of a CTL determinant, various minigenes encoding the 147–155 determinant within larger polypeptides were inserted into the Vac genome. Work by others has shown that the synthetic peptide representing amino acids 147–158 (TYQRTRALVRTG) is much less potent in sensitizing APC for lysis by NP-specific CTL than the 147–155 peptide (26). Remarkably, elimination from the 147–158 peptide of the Arg at position 156 (TYQRTRALVTG) (147–158/R<sup>-</sup>) was observed to enhance significantly the potency of the synthetic peptide (26). Minigenes coding for the larger 147–158 and 147–158/R<sup>-</sup> peptides, again with initiating Met residues, were inserted into the Vac genome (termed M147–158 Vac and M147–158/R<sup>-</sup> Vac, respectively). Each virus was tested for its ability to sensitize APC for lysis by NP-specific CTL, and to prime mice for a NP-specific response. M147–158 Vac was effective in both respects (Tables 1 B and 2, Exp. B). M147–158/R<sup>-</sup> Vac, however, did not sensitize APC for lysis by NP-specific APC and was minimally active in priming mice for a NP-specific response (Table 1 B and Table 2, Exp. B). Adequate infection of APCs by M147–158/R<sup>-</sup> Vac was verified by the high degree of lysis by Vac-specific CTL (Table 1 A). Adequate priming of mice with the recombinant was demonstrated by high anti-Vac CTL activity after *in vitro* restimulation with a recombinant Vac but not PR8 (Table 2, Exp. B). Northern blot analysis demonstrated that levels of minigene mRNA were similar in cells infected with all three recombinants (Fig. 1). Sequencing of genomic DNA from the recombinants confirmed that each virus contained the proper gene in the correct orientation. A second M147–158/R<sup>-</sup> Vac constructed using an alternative strategy (synthetic oligonucleotides utilized in place of PCR products) gave similar results in sensitizing and priming (not shown). Thus, the poor antigenicity of M147–158/R<sup>-</sup> Vac is likely to reflect inefficient processing of the peptide and not other factors.

**Flanking Sequences Can Positively Influence the Presentation of an Endogenous Peptide.** To investigate whether the 147–158/R<sup>-</sup> sequence could be processed from intact NP for presentation to CTL, we produced a Vac recombinant encoding the full-length NP with Arg<sub>156</sub> deleted (NP/R<sup>-</sup> Vac) (see Materials and Methods). APC infected with NP/R<sup>-</sup> Vac were efficiently lysed by NP-specific CTL (Table 1 C). The antigenic determinant within the NP/R<sup>-</sup> molecule was confirmed to be 147–155 since APC infected with NP/R<sup>-</sup> Vac were lysed by CTL generated by priming with M147–155 Vac (Table 2, Exp. A). These findings indicate that the negative effect of flanking residues on presentation of a T cell determinant can be overcome by extension of the flanking residues. This was further explored by construction of a Vac recombinant expressing the 147–158/R<sup>-</sup> core with 10 amino acids corresponding to the NP sequence added to the NH<sub>2</sub> and COOH termini (137–168/R<sup>-</sup> Vac). The resulting peptide (137–168/R<sup>-</sup>) is a precise segment of NP/R<sup>-</sup> as Met is the natural residue at position 137. Cells infected with this recombinant were no better recognized by NP-specific CTL than those infected with M147–158/R<sup>-</sup> Vac (Table 1). Thus, restoration of the immediate amino acid residues sur-



**Figure 1.** Equal amounts of mRNA encoding the 147–155 NP sequence are generated by the various Vac recombinants. Total RNA was extracted from uninfected P815 cells or cells infected for 5 h with Vac recombinants expressing NP peptides or an unrelated influenza virus gene product (PB2). RNA was bound to nitrocellulose and probed for the presence of sequences encoding the 147–155 NP segment (*top*) or a Vac gene product (*bottom*). The left-hand column is a 10-fold dilution of the material applied to the right-hand column. In the case of the M147–158/R<sup>-</sup> Vac infection, the identity of hybridizing sequences as RNA is demonstrated by sensitivity to RNase and resistance to DNase. The specificity of the nucleases was confirmed by digestion of plasmid DNA.

rounding the 147–158/R<sup>-</sup> peptide in NP/R<sup>-</sup> was not sufficient to rescue presentation of the 147–155 determinant.

## Discussion

Others have shown that endogenously synthesized peptides containing from 9 to 13 flanking residues are efficiently presented to CTL (12–14). Our findings indicate that the naturally processed determinant itself (with an initiating Met) contains sufficient information to be trafficked to the appropriate exocytic compartment. This is consistent with the idea that the ultimate determinant of eight to nine residues is produced in the cytosol. It is also possible that the processing of 147–155 from full-length NP (and presumably NP/R<sup>-</sup>) results from the generation of larger peptides in the cytosol that are trimmed after transport (27). However, there are clearly limits to the kinds of larger peptides that would be active in this model, since M147–158/R<sup>-</sup> and 137–168/R<sup>-</sup> were not recognized.

It has been reported that cells can be sensitized for CTL recognition by synthetic peptides but not by endogenously synthesized proteins containing the peptide sequence (28). In this case, however, it was uncertain whether this was due to deficiencies in antigen processing, or in the ability of any form of the peptide processed from the protein to associate with class I molecules in an intracellular environment. This limitation does not apply to our study since we identify a peptide that associates with class I molecules and show that addition of flanking sequences can destroy its antigenicity. Thus, we can conclude that the antigenicity of a peptide capable of binding class I molecules can be influenced by extra-antigenic sequences. Related observations were made by Townsend et al. (30) who found that a blockade in antigen presentation associated with Vac virus infection, first described by Coupar et al. (29), could be surmounted by altering extra-determinant residues in a manner resulting in enhanced degradation of the protein. It is unlikely that a similar phenomenon is responsible for our observations, since in additional experiments (unpublished) we observed no correlation between presentation of 147-155 and the stability of its carrier protein or peptides (both NP and NP/R<sup>-</sup> were stable while all of the minigene products appeared to be rapidly degraded, based on our failure to detect them in competition RIAs using antisera raised to the 147-158 or 147-158R<sup>-</sup> peptides). Del Val et al. (31) recently reported that an antigenic determinant from a murine cytomegalovirus protein was not presented when placed into certain regions of a foreign protein. This effect did not appear to be related to the overall stability of the protein and occurred in cells expressing the gene products either as a result of DNA-mediated transfection or recombinant Vac infection. Thus, extra-antigenic residues can influence presentation of CTL determinants independently of a Vac infection.

Further experiments are required to determine the mechanism by which flanking sequences influence the presentation of the 147-155 determinant. Any one or combination of the following could be involved in the negative effects of flanking sequences, any of which could be influenced by Vac infection: (a) sequestration of the peptides on peptide binding proteins in the cytosol or exocytic compartment; (b) inability of peptide intermediates to be transported from the cytosol to the exocytic compartment; (c) inability of peptide intermediates to associate with accessory molecules that might function to deliver peptides to class I molecules in the exocytic compartment; or (d) inability of cellular proteases to generate the antigenic peptide from the protein or longer peptide.

Possibly the most striking finding in the present study is that addition of two amino acids to the 147-155 determinant abolishes its presentation as an endogenous antigen. The observation that this peptide is extremely efficient at sensitizing target cells when provided as an exogenous antigen might suggest that the endogenous peptide is not properly transported to class I molecules from the cytosol. It was recently found, however, that the ability of exogenous 147-158/R<sup>-</sup> to sensitize target cells for CTL lysis depends on a protease present in FCS that converts the peptide to the highly efficient 147-155 peptide (L. A. Sherman, personal communication). Thus, there is no compelling reason to suspect that the M147-158/R<sup>-</sup> peptide is not transported from the cytosol. Rather, the only firm conclusion that can be made at present is that neither P815 cells nor APC *in vivo* possess a similar cytosolic proteolytic activity.

It has recently been reported that "contaminants" consisting of eight to nine residues can account for essentially all of the antigenic activity in preparations of synthetic peptides longer than eight or nine residues (32). Thus, it is likely that the binding of the 147-158 peptide to K<sup>d</sup> relative to 147-155 is even less than the 1% value suggested by peptide titration experiments (26). In this case, the presentation of the M147-158 peptide would almost certainly require trimming of the COOH-terminal residues and perhaps the NH<sub>2</sub>-terminal Met as well. That the endogenous M147-158/R<sup>-</sup> peptide is presented at such a low efficiency suggests that the absence of Arg<sub>156</sub> might negatively influence the activity of the trimming enzymes.

The ability of cells to process NP/R<sup>-</sup> efficiently for association with K<sup>d</sup> indicates that the effect of negatively acting flanking sequences can be overcome by additional flanking sequences. Restoration of the structure of NP is not, however, essential for the processing of the 147-155 determinant in the context of 147-158/R<sup>-</sup>, since addition of flanking residues unrelated to NP at the COOH terminus greatly increases the antigenicity of the 147-158/R<sup>-</sup> peptide without enhancing its apparent stability (unpublished results). This latter observation is consistent with reports that placement of other CTL determinants into new protein contexts can have little effect on their presentation (33, 34).

Finally, our findings indicate that minimal determinants expressed by infectious recombinant vaccines are able to induce CTL efficiently. This complements previous findings that immunization with synthetic peptides can induce CTL (35). As flanking sequences can negatively affect the immunogenicity of minimal determinants, recombinant vaccines might best utilize the naturally processed peptides.

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