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Cytotoxic T Cells Specific for a Single Peptide on the M2 Protein of Respiratory Syncytial Virus Are the Sole Mediators of Resistance Induced by Immunization with M2 Encoded by a Recombinant Vaccinia Virus

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We have studied the immunobiology of respiratory syncytial virus (RSV), a major cause of respiratory tract morbidity in children. As part of these studies, it was previously found that immunization of BALB/c (*H-2^d*) mice with a recombinant vaccinia virus (rVV) which encoded the M2 protein of RSV provided complete protection against infection with RSV. This protection was transient and associated with M2-specific CD8$^+$ T-cell (T_{CD8^+}) responses. In this study, we used two approaches to demonstrate that expression of an *H-2K^d*-restricted nonameric peptide (Ser Tyr Ile Gly Ser Ile Asn Asn Ile) corresponding to M2 residues 82 to 90 is necessary and sufficient to induce protective T_{CD8^+} responses. First, infection of mice with an rVV which encoded the peptide $M2_{Met82-90}$ induced levels of primary pulmonary T_{CD8^+} and resistance to RSV challenge equivalent to that induced by infection with an rVV which expressed the complete M2 protein. Second, elimination of peptide binding to *K^d* by the replacement of Tyr with Arg at amino acid position 83 of the full-length protein completely abrogated the ability of an rVV-expressing full-length M2 to induce either M2-specific T_{CD8^+} responses or resistance to RSV infection. These findings demonstrate that the $M2_{82-90}$ peptide is the sole determinant of immunity induced in BALB/c mice by the M2 protein and that a remarkably high level of transient resistance to infection with pulmonary virus is associated with T_{CD8^+} responses to a single determinant.

CD8$^+$ T cells (T_{CD8^+}) play a crucial role in the immune response to some viruses. T_{CD8^+} recognize major histocompatibility complex class I molecules which bear peptides of 8 to 10 residues in a prominent groove formed by the α1 and α2 domains (1, 19). Most class I binding peptides are derived from a cytosolic pool of proteins (22, 25), possibly through the action of large multicatalytic proteases known as proteasomes (13). Peptides are conveyed from the cytosol to the secretory pathway by transporters associated with antigen processing, major histocompatibility complex-encoded members of the ATP binding cassette superfamily of membrane transporters (5, 14, 20, 24). Newly synthesized class I molecules are retained in the early secretory pathway until they bind peptide, which triggers the final steps in folding, resulting in the release of class I molecules to the cell surface, where the peptide is displayed for perusal by T_{CD8^+} (8, 17). Alternatively, under experimental conditions, synthetic peptides added to extracellular fluids can bind to sufficient numbers of class I molecules expressed on the plasma membrane to sensitize cells for lysis by T_{CD8^+} (23).

Sequencing peptides derived from purified class I molecules reveals that most peptides that bind to a given allomorph possess identical or conserved residues at one or two positions (12). Such residues, known as anchor residues, are usually required (but are not sufficient) for binding to a given class I allomorph. In the case of *H-2 K^d*, the anchor residues are a Tyr at position 2 and an Ile, Leu, or Val at position 9 or 10 (numbering is from the NH_2 terminus) (12, 19). The definition of class I binding motifs has allowed for the rapid screening of peptides to deduce the antigenic determinants present on proteins known to elicit T_{CD8^+} responses to a given allomorph. We have previously shown that infection of BALB/c (*H-2^d*) mice with a recombinant vaccinia virus (rVV) which expresses the M2 protein of respiratory syncytial virus (RSV) induces resistance to RSV infection mediated largely by T_{CD8^+} (9). Synthetic peptides corresponding to two of the four peptides with *K^d* binding motifs in M2 sensitized target cells for lysis by M2-specific T_{CD8^+} (10). One of the peptides, representing residues 82 to 90, sensitized cells at nanomolar concentrations, while the other, representing residues 71 to 79, required 1,000-fold-greater concentrations to achieve the same degree of sensitization. On the basis of cold-target inhibition assays with peptide-pulsed target cells, it was clear that $M2_{71-79}$ peptide was recognized in a cross-reactive manner. These studies did not unequivocally resolve which peptide was naturally processed from M2, nor did they address the possibility of additional protective determinants in M2 recognized by *D^d*- or *L^d*-restricted T_{CD8^+} or T_{CD4^+}.

In this paper, we explore these questions by using rVVs which contain minigenes encoding an initiating Met followed by residues 82 to 90 or 71 to 79 or a full-length gene encoding M2 with Tyr-83, the anchor residue of $M2_{82-90}$ peptide, mutated to Arg. Mice infected with these rVVs were examined for M2-specific T_{CD8^+} responses and their ability to withstand pulmonary challenge with RSV.

The A2 strain of RSV subgroup A was grown and titrated in HEp-2 cells as previously described (15, 18). Influenza virus A/Puerto Rico/8/34 (A/PR/8/34) was grown in 10-day-old embryonated chicken eggs, and the virus titer was determined by infectivity assay in MDCK tissue culture as previously de-

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scribed (10). rVVs which contain the RSV M2 proteins (rVV_{M2}) and the parainfluenza type 3 hemagglutinin-neuraminidase protein (rVV_{HN}) have been described elsewhere (9, 21), as has $rVV_{Met147-155}$, which expresses the influenza nucleoprotein K^d-restricted peptide *M*TYQRTRALV (2). rVVs which expressed the $M2_{71-79}$ and $M2_{82-90}$ nonameric peptides were made by inserting the synthetic double-stranded oligonucleotides (shown as the plus strand) 5′-GTCGACCCACC
M E Y A
ATGGAGTATGC
L G V V G V
TCTTGGTGTAGTTGGAGTGTGATAGGTACCGCGG
M S Y I G
CCGC-3′ and 5′-GTCGACCACC
ATGAGTTATATAGGA
S I N N I
TCAATAAACAATATATGATAGGTACCGCGGCCGC-3′
encoding peptides $M2_{71-79}$ and $M2_{82-90}$, respectively, into the *SalI* and *NotI* sites of a version of pSC11 (3) that had been modified to contain a multiple cloning site downstream of the 7.5 promoter (6). In the oligonucleotide sequence shown above, the flanking *SalI* (left) and *NotI* (right) sites are double underlined and the minigene and its encoded amino acids are single underlined. The nucleotide sequences of the minigenes were confirmed by sequencing plasmid DNA. They were then inserted into vaccinia virus by homologous recombination as described previously (3). To produce $rVV_{M2(Y\rightarrow R)}$, the Tyr at position 83 was replaced with an Arg in the complete M2 open reading frame by site-directed mutagenesis with the positive-sense mutagenic oligonucleotide 5′-GTGCTAGAGAGTAGA ATGGATCAATA-3′, representing nucleotides 244 to 269 in the M2 protein. The procedure outlined for the Muta-gene phagemid in vitro mutagenesis kit (Bio-Rad Laboratories, Richmond, Calif.) was followed by using M2 cDNA cloned in $pGem3Zf^+$ plasmid (Promega, Madison, Wis.). The nucleotide sequence of the mutagenized DNA of the M2 gene was confirmed to differ from the wild-type M2 gene only at nucleotides 256 to 258, where AGA (underlined) replaced TAT to predict an amino acid substitution of Arg for Tyr. This mutated M2 gene from $pGem3Zf^+$ was excised with *Bam*HI, filled in, and cloned in pSC11. It was then inserted into vaccinia virus via homologous recombination as described previously (3). Lysates obtained from metabolically labeled ([^{35}S]methionine) HEp-2 cells infected with $rVV_{M2(Y\rightarrow R)}$ were analyzed by radioimmunoprecipitation with polyclonal rabbit anti-RSV serum and found to express an M2 protein that comigrated with that in rVV_{M2}-infected cells and was of similar abundance (data not shown).

Six- to ten-week-old BALB/c (*H-2*^{d}) female mice were obtained from the Frederick Cancer Research and Development Center of the National Cancer Institute (Frederick, Md.). Mice were anesthetized with methoxyfluorane and inoculated intranasally with a 10^6-PFU dose of RSV or rVV in 0.05 ml. Mice immunized intravenously were inoculated with 10^6 PFU of rVV in a 0.2-ml inoculum 6 days prior to assay.

Target cells used in cytotoxicity assays were P815 (*H-2*^{d}) mastocytoma cells, a BALB/c (*H-2*^{d}) fibroblast line, or BCH4 cells (7), a BALB/c fibroblast line persistently infected with the Long strain of RSV (highly related to the A2 strain). These cell lines were grown in Iscoves modified Dulbecco's medium supplemented with 5% fetal bovine serum. BALB/c fibroblasts and P815 cells used as targets were infected with RSV (10 PFU per cell) or A/PR/8/34 (50 PFU per cell) for 18 h prior to assay. Peptide-pulsed P815 cells were prepared for use as targets by incubation with 50 μl of the indicated peptide (10^{-5} M). Peptides used were synthesized by the Biological Research Branch of the National Institute of Allergy and Infectious Diseases, Bethesda, Md., on an ABI peptide synthesizer (model 430-A) and peptide sequences were confirmed with a Beckman 6300 analyzer.

TABLE 1. Immunization of mice with rVV which encodes a minigene of the M2-specific K^d binding motif induces primary splenic T_{CD8^+}

rVV (virus specificity)	Effector/target ratio	% Specific lysis of *H-2*^{d} targets by splenic lymphocytes[a]	
		RSV infected[b]	A/PR/8/34 infected[c]
rVV_{71-79} (RSV)	100	10	0
	50	9	0
	25	5	0
	12.5	3	0
$rVV_{Met82-90}$ (RSV)	100	35	2
	50	30	5
	25	14	0
	12.5	7	0
$rVV_{Met147-155}$ (influenza virus)	100	9	38
	50	8	30
	25	5	23
	12.5	0	19
rVV_{M2} (RSV)	100	50	3
	50	37	1
	25	27	0
	12.5	17	0
rVV_{HN} (control)	100	8	0
	50	6	0
	25	7	0
	12.5	2	0

[a] Primary T_{CD8^+} responses were determined by using pooled splenic effector cells derived from three mice infected with rVV 6 days previously.
[b] Uninfected BALB/c fibroblasts had <5% lysis at all effector/target ratios (data not shown).
[c] Uninfected P815 cells had <5% lysis at all effector/target ratios (data not shown).

Cytotoxicity assays were performed as described previously (9). Briefly, target cells were labeled with $Na^{51}CrO_4$ for 1 h at 37°C, washed twice, and dispensed into 96-well microtiter plates at 10^4 cells per well with various effector cell ratios. After 4 h of incubation at 37°C, the amount of ^{51}Cr released was determined by gamma counting. Percent specific lysis was calculated as follows: 100 × [(experimental counts per minute [cpm] − spontaneous cpm)/(maximal cpm − spontaneous cpm)]. Effector cells were splenocytes obtained from mice 6 days after intravenous immunization with rVV or cells isolated from the lungs of mice 6 days after intranasal inoculation.

Lungs were removed 4 days after intranasal RSV challenge, and tissue suspensions were titered as previously described (9). Virus titrations were performed on HEp-2 cells, and titers were expressed as mean $\log_{10}$ PFU ± standard error of the mean per gram of lung homogenate (10% wt/vol). Lung specimens from which virus was not recovered were assigned a titer of ≤1.7 since this was the lowest level of detectable virus.

In order to evaluate the immunogenicity of the $M2_{71-79}$ and $M2_{82-90}$ nonameric peptides, mice were infected intravenously with rVV which expressed the minigene from residues 71 to 79 or 82 to 90 (designated $rVV_{Met71-79}$ and $rVV_{Met82-90}$, respectively). An rVV which expressed a K^d-restricted T_{CD8^+} determinant of the nucleoprotein of A/PR/8/34 as a minigene ($rVV_{Met147-155}$) was used as a control (2). The T_{CD8^+} responses of splenocytes induced by these rVVs were compared with

TABLE 2. Correlation of the resistance to RSV challenge in mice immunized with rVV which encodes M2-specific minigenes with the induction of primary pulmonary T_{CD8^+}

rVV (virus specificity)	Effector/target ratio	% Specific lysis of *H-2*^{d} targets by pulmonary lymphocytes[a]		RSV titer in lungs (mean $\log_{10}$ PFU/g ± SEM)
		RSV infected[b]	A/PR/8/34 infected[c]	
$rVV_{71\text{-}79}$ (RSV)	75	10	0	4.0 ± 0.13
	25	6	0	
	8	2	0	
$rVV_{Met82\text{-}90}$ (RSV)	75	59	0	≤1.7
	25	42	0	
	8	25	0	
$rVV_{Met147\text{-}155}$ (influenza virus)	75	6	19	4.0 ± 0.10
	25	5	13	
	8	2	8	
rVV_{M2} (RSV)	75	72	0	≤1.7
	25	60	0	
	8	34	0	
rVV_{HN} (control)	75	3	0	4.8 ± 0.02
	25	1	0	
	8	1	0	
RSV	75	87	0	≤1.7
	25	77	0	
	8	53	0	

[a] BALB/c mice received rVV or RSV ($10^{6.0}$ PFU/0.05 ml) intranasally. Primary T_{CD8^+} activity was determined by using pooled lung effectors from six mice harvested 6 days after infection. Another group of five mice similarly infected was challenged on day 6 with RSV (A2) ($10^{6.0}$ PFU/0.05 ml) intranasally, and lungs were harvested 4 days later. Virus titration was performed on individual lung homogenates.

[b] Uninfected BALB/c fibroblasts had <5% lysis at all effector/target ratios (data not shown).

[c] Uninfected P815 cells had <5% lysis at all effector/target ratios (data not shown).

TABLE 3. Failure of immunization of BALB/c mice with rVV which encodes a mutated M2 gene to induce RSV-specific primary pulmonary T_{CD8^+} cytotoxic activity and resistance to replication of RSV in lungs

Virus used to immunize mice	Effector/target ratio	% Specific lysis of *H-2*^{d} targets by pulmonary lymphocytes[a]			RSV titer in lungs (mean $\log_{10}$ PFU/g ± SEM)
		RSV infected[b]	P815 pulsed with[c]:		
			$M2_{82\text{-}90}$	$M2_{71\text{-}79}$	
$rVV_{M2(Y\rightarrow R)}$ (RSV)	100	21	2	1	3.5 ± 0.20
	50	7	2	1	
	25	7	1	0	
	12.5	4	0	0	
rVV_{M2} (RSV)	100	66	78	42	≤1.7
	50	48	39	19	
	25	26	23	4	
	12.5	13	11	1	
rVV_{HN} (control)	100	14	1	0	3.4 ± 0.17
	50	9	1	0	
	25	8	1	0	
	12.5	4	1	0	
RSV	100	70	NT	NT	≤1.7
	50	65			
	25	56			
	12.5	34			

[a] See Table 2, footnote *a*.

[b] Uninfected BALB/c fibroblasts had <5% lysis at all effector/target ratios (data not shown).

[c] P815 cells similarly treated with the control nucleoprotein-specific peptide (residues 147 to 155) of A/PR/8/34 were not lysed by any of the effectors (data not shown). NT, not tested.

those from animals immunized with rVV_{M2} or with control virus rVV_{HN} (21). As shown in Table 1, primary splenic effector T_{CD8^+} obtained from groups of three mice infected with $rVV_{Met82\text{-}90}$, but not from those infected with $rVV_{Met71\text{-}79}$, efficiently lysed BCH4 fibroblasts persistently infected with RSV. The magnitude of this T_{CD8^+} response was comparable to that induced by infection with rVV_{M2}. The T_{CD8^+} induced by $rVV_{Met82\text{-}90}$ were RSV specific since P815 cells infected with A/PR/8/34 were not lysed. Conversely, T_{CD8^+} from $rVV_{Met147\text{-}155}$-infected mice efficiently lysed targets infected with influenza virus but not those infected with RSV. These results demonstrate that T_{CD8^+} effectors from mice infected with $rVV_{Met82\text{-}90}$ recognized RSV-infected targets in a virus-specific manner.

Previously, we found that primary pulmonary T_{CD8^+} activity induced by infection with rVV_{M2} correlated with resistance to RSV challenge (9). In these studies, we extended these observations to address whether expression of the $M2_{82\text{-}90}$ peptide alone was sufficient to confer resistance to RSV challenge. Groups of 12 mice were immunized intranasally with $10^{6.0}$ PFU of rVV per animal (Table 2); 6 days later, at the peak of pulmonary anti-RSV T_{CD8^+} activity (9), six mice were sacrificed for the isolation of pulmonary T_{CD8^+} and the other six were challenged intranasally with RSV to measure resistance to RSV replication. Resistance to RSV challenge was determined by assaying virus titers in lungs 4 days after challenge. As shown in Table 2, the lack of primary T_{CD8^+} activity following infection with $rVV_{Met71\text{-}79}$ was associated with a lack of resistance to RSV challenge. The virus titers in lungs from this group were comparable to those from control rVV_{HN}-immunized mice. rVV_{HN} has previously been shown to induce homotypic protection in experimental animals (21). In contrast, mice immunized with $rVV_{Met82\text{-}90}$ had a high level of RSV-specific T_{CD8^+} and were highly resistant to replication of RSV in lungs. The level of resistance to virus challenge was comparable to that observed for mice previously infected with rVV_{M2} or RSV. These results demonstrate that T_{CD8^+} effectors induced by $M2_{Met82\text{-}90}$ synthesized endogenously from a minigene inserted into vaccinia virus mediate resistance to virus challenge. This observation unequivocally identifies this peptide as a major functional T_{CD8^+} determinant on the M2 protein.

If the T_{CD8^+} specific for the $M2_{82\text{-}90}$ determinant were the sole mediators of resistance to RSV challenge, then amino acid substitution for the Tyr anchor residue at position 83 should inhibit binding to *H-2K*^{d} and thereby abrogate both the induction of T_{CD8^+} activity and resistance to challenge. In order to test this hypothesis, a full-length M2 rVV with an Arg substitution for the Tyr at position 83, $rVV_{M2(Y\rightarrow R)}$, was produced. Mice were intranasally infected with $rVV_{M2(Y\rightarrow R)}$, rVV_{M2}, RSV (A2), or control rVV_{HN}. Primary pulmonary T_{CD8^+} activity was tested with BCH4 cells and P815 cells sensitized with either RSV $M2_{82\text{-}90}$ or $M2_{71\text{-}79}$ peptide (Table 3). Resistance to RSV infection was determined as described above. As shown in Table 3, substitution for the Tyr at position 83 completely abrogated the T_{CD8^+} response as well as the resistance to RSV replication induced by infection with rVV_{M2}. In contrast, as previously shown (10), infection with rVV_{M2} or RSV (A2) induced RSV-specific and peptide ($M2_{82\text{-}90}$)-specific T_{CD8^+} activity in lung tissues on day 6, which correlated with an

approximately 100-fold reduction in the titer of virus. These results indicate that the $M2_{82\text{-}90}$ T_{CD8^+} determinant is essential for resistance induced by infection with rVV_{M2}.

These findings have two important implications. First, identification of the $M2_{82\text{-}90}$ T_{CD8^+} determinant as the sole determinant of immunity induced by RSV M2 protein demonstrates that T_{CD8^+} induced by immunization with minimal determinant rVV vectors can provide a high level of resistance to virus replication in lungs. It is important to note, however, that such resistance is transient, as it is no longer detected 45 days after immunization (9). Moreover, the efficacy of T_{CD8^+} in reducing the replication of viruses in lungs varies greatly among respiratory viruses. An rVV which expressed a K^d-restricted influenza A virus nucleoprotein peptide readily induced primary pulmonary T_{CD8^+}, but these T_{CD8^+}, unlike RSV M2-specific T_{CD8^+}, did not mediate resistance to infection (11). Our finding of protection with $M2_{82\text{-}90}$ more closely resembles the resistance induced by rVVs which encode oligopeptides containing T_{CD8^+} determinants derived from cytomegalovirus and lymphocytic choriomeningitis virus (4, 16).

The second important point of this paper is the usefulness of mutagenesis of putative T_{CD8^+} determinants in full-length viral proteins to define the relative contribution of the T_{CD8^+} determinant to overall resistance to viral challenge induced by a particular protein. Importantly, this method of mutagenesis of T_{CD8^+} determinants in full-length proteins extends the previous methodologies used to define the biological importance of T_{CD8^+} determinants in two ways. First, peptide labeling of target cells to identify immunogenic T_{CD8^+} determinants does not unequivocally identify immunogenic T_{CD8^+} determinants in proteins since nonimmunogenic, nonfunctional determinants can be recognized by T_{CD8^+} in a cross-reactive manner (10). In contrast, mutagenesis of T_{CD8^+} determinants which abrogates antigenic activity can unequivocally identify such determinants. Second, although vaccinia virus-minigene constructs can identify protective T_{CD8^+} determinants, they cannot assess the relative contribution that a T_{CD8^+} T-cell determinant makes to the immunity induced by the full-length protein. It is likely that mutagenesis of T_{CD8^+} determinants in full-length proteins will be used frequently to define the importance of the determinant in the induction of immunity.

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