JOURNAL OF VIROLOGY, June 1994, p. 3486–3490
0022-538X/94/$04.00+0

Vol. 68, No. 6

Protection against Lethal Lymphocytic Choriomeningitis Virus (LCMV) Infection by Immunization of Mice with an Influenza Virus Containing an LCMV Epitope Recognized by Cytotoxic T Lymphocytes

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Received 7 December 1993/Accepted 1 March 1994

The reverse genetics system has made it possible to modify the influenza virus genome. By this method, we were able to assess influenza virus as a vaccine vector for protecting BALB/c mice against otherwise lethal lymphocytic choriomeningitis virus (LCMV) infection. A single dose of influenza virus [A/WSN/33 (H1N1)] bearing a cytotoxic T-lymphocyte-specific epitope of the LCMV nucleoprotein (residues 116 to 127) in the neuraminidase stalk protected mice against LCMV challenge for at least 4 months. The immunity was mediated by cytotoxic T lymphocytes and was haplotype specific, indicating that the observed protective response was solely a consequence of prior priming with the *H-2*^{d} LCMV nucleoprotein epitope expressed in the recombinant influenza virus. We also found that as many as 58 amino acids could be inserted into the neuraminidase stalk without loss of viral function. These findings demonstrate the potential of influenza virus as a vaccine vector, with the neuraminidase stalk as a repository for foreign epitopes.

Vaccination offers an efficient and cost-effective means to prevent infectious diseases. Although inactivated vaccines are safe, they often fail to stimulate adequate local immune or primary cytotoxic T-lymphocyte (CTL) responses. Live vaccines, by contrast, induce strong systemic and local humoral as well as cell-mediated immune responses. However, the genetic instability of live vaccines and their interference with naturally occurring viruses have prevented their routine use.

The CTL response is a central component of the host response to many viruses (10), including influenza virus (28, 29, 44) and lymphocytic choriomeningitis virus (LCMV) (5, 24). Indeed, Oldstone et al. (38) showed that a recombinant vaccinia virus expressing a CTL-specific epitope of LCMV nucleoprotein, residues 116 to 127, protects inoculated mice of three different haplotypes (*H-2*^{d}, *H-2*^{q}, and *H-2*^{u}) from lethal challenge with the virus.

The reverse genetics system established by Enami and colleagues to rescue genes derived from cDNA into influenza A viruses (14, 15, 31) has made it possible to modify the influenza virus genome (13). Using this method, we (6) and Luo et al. (30) have shown that the neuraminidase (NA) stalk can accommodate as many as 41 amino acid insertions. Here, we report the use of reverse genetics to generate mutant influenza viruses expressing a CTL-specific epitope of the LCMV nucleoprotein in the NA stalk and their immunizing effects against lethal LCMV challenge in mice.

MATERIALS AND METHODS

Viruses and cells. The A/WSN/33 (H1N1) (WSN) influenza virus was obtained from Thomas Chambers (University of Kentucky, Lexington, Ky.). Masahiro Ueda (The Institute of Public Health, Tokyo, Japan) provided a helper virus [WSN-HK (H1N2)] that contained the NA gene from A/Hong Kong/1/68 (H3N2) and all other genes from WSN (43), which was used to rescue the WSN NA gene. The CA 1371 strain of LCMV (12) was obtained from Peter J. Southern at the University of Minnesota Medical School. A recombinant vaccinia virus expressing the LCMV nucleoprotein (49) was obtained from J. L. Whitton at The Scripps Research Institute, and virus stocks were grown and titrated in Vero cells.

The Madin-Darby bovine kidney (MDBK) and Vero cell lines were cultured in Eagle's minimal essential medium containing 10% fetal calf serum. Madin-Darby canine kidney (MDCK) cells were cultured under the same conditions as MDBK cells, except that 5% calf serum was used.

Reverse genetics. Construction of a pT3WSN(NA15) plasmid, which was used to generate transfectant WSN(NA15) virus carrying the NA gene derived from cloned cDNA, has been described previously (13). Plasmids for the generation of SALCM (pT3SALCM) and SRLCM (pT3SRLCM) viruses were constructed from pT3WSN(NA15) by inserting or replacing nucleotides in the region encoding the NA stalk, by oligonucleotide-directed mutagenesis (23) (see Fig. 1).

The reverse genetics procedure was performed as described previously (13). Transfectant viruses were plaque purified five times in MDBK cells, and the altered nucleotide sequences were confirmed by direct sequencing of purified viral RNA.

Mice. The BALB/cJ (*H-2*^{d}) and C57BL/6J (B6 [*H-2*^{b}]) female mice used in these experiments were purchased from Jackson Laboratories, Bar Harbor, Maine. They were held under specific-pathogen-free conditions throughout the study, excluding their experimentally induced infections. Prior to their challenge with LCMV, the mice primed with the influenza viruses expressing the LCMV nucleoprotein epitope were maintained in an experimental room that did not contain LCMV-infected mice. The mice were approximately 8 weeks

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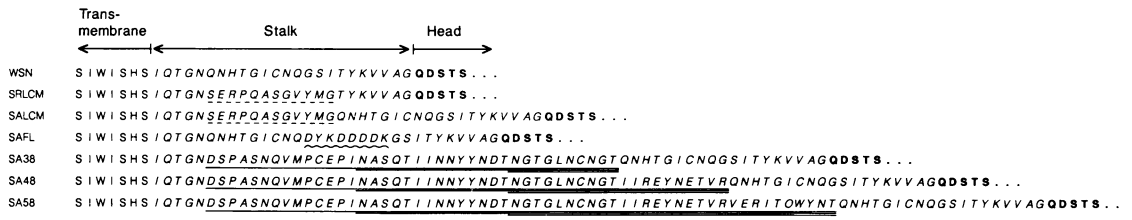

FIG. 1. Amino acid sequences of the NA stalk mutants from residues 31 to 66 of WSN NA. The stalk region is italicized, the transmembrane region is presented in roman type, and the head region is shown in boldface type. The LCMV nucleoprotein epitope sequence is indicated by a dotted line, and the FLAG sequence is indicated by a wavy line. Portions of the A/Tokyo/67 (H2N2), A/tern/Australia/G70C/75 (H11N9), and A/equine/Kentucky/1/81 (H3N8) stalks are indicated by single, double, and triple underlines, respectively.

old at the time of initial infection; age-matched controls were used throughout the study.

Determination of the MLD_{50}. Six-week-old female BALB/cJ mice, anesthetized with methoxyfluorane, were infected intranasally with 50 μl of virus at different dilutions (three mice per dilution) and observed for 21 days to determine the virus dose lethal to 50% of mice (MLD_{50}).

Protection of mice against LCMV challenge. Mice were infected with either wild-type WSN(NA15) or mutant influenza virus intranasally (3×10^2 PFU in 30 μl) and intraperitoneally (2×10^6 PFU in 500 μl) and were then intracerebrally challenged with the CA 1371 strain of LCMV (10^3 PFU). Mice were observed for 10 days for disease signs and death.

Inflammation and effector T cells. The severity of the T-cell-mediated inflammatory process characteristic of LCMV infection was measured by counting cells in cerebrospinal fluid samples taken from anesthetized (Avertin [2,2,2 tribromoethanol]), exsanguinated mice (11). Virus-specific CTLs were recovered (3) by bronchoalveolar lavage from mice infected intranasally with the influenza virus expressing the LCMV nucleoprotein (SALCM). Single-cell suspensions of spleen, cervical lymph node, and mediastinal lymph node cells were also assayed for virus-specific CTL activity (32).

CTL assays. Virus-specific effector function was measured with LB15 ($H\text{-}2^{bxd}$ [F_1]) target cells in a standard 6-h assay. The results are expressed as the mean percentages of specific ^{51}Cr released for replicates of three wells (32). The targets were labeled with Na ^{51}Cr and were then infected with a multiplicity of infection of the nucleoprotein-expressing or wild-type vaccinia viruses of 50 for 4 to 6 h prior to plating (5×10^4 cells in 100 μl) in U-bottom 96-well culture plates.

RESULTS

Generation of transfectant viruses with an LCMV nucleoprotein epitope in the NA stalk. We investigated the immunizing effect of an LCMV nucleoprotein epitope (residues 116 to 127) using two influenza viruses that contained the epitope in the NA stalk. In the virus designated SRLCM, a portion of the NA stalk was replaced with the LCMV epitope, while in the other, which was designated SALCM, the epitope was inserted into the stalk, lengthening it by 12 amino acids compared with the wild type and the SRLCM mutant (Fig. 1). The efficiency of generating SALCM and SRLCM transfectant viruses was similar to that for production of the wild-type WSN(NA15) virus (6); 10^2 to 10^3 PFU were present in the transfectant supernatant. Both mutant viruses were as virulent as the wild-type virus in mice, with an MLD_{50} of approximately 10^3 PFU.

Protection of mice immunized with recombinant influenza viruses bearing an LCMV epitope. Exposure to either the SALCM or the SRLCM mutant, but not the wild-type WSN(NA15) influenza virus, protected $H\text{-}2^d$ BALB/c mice against intracerebral challenge with LCMV for at least 4 months (Table 1). The age- and sex-matched $H\text{-}2^b$ B6 mice were susceptible to virus infection at all of the test intervals, establishing that immunization with the $H\text{-}2^d$ LCMV nucleoprotein epitope, which is expressed by both the SALCM and the SRLCM viruses, was solely responsible for the prevention of lethal infection. These findings suggest that the LCMV nucleoprotein epitope in the NA stalk of an influenza virus is expressed on virus-infected cells in the lungs and is recognized by LCMV-specific memory T cells.

An influenza virus with an LCMV epitope primes for a CTL response. To determine if the protection of mice described above is due to the priming of a specific CTL response by the recombinant virus, we infected BALB/c mice intranasally with a single dose of either WSN(NA15) or SALCM influenza virus and then challenged them intracerebrally with LCMV 10 weeks later. The cerebrospinal fluid cell counts in the WSN(NA15)-primed mice on day 5 (Table 2 [3.1 ± 0.4 $\log_{10}$ cells per μl]) were typical of the antigen-nonspecific inflammatory process seen at this stage of infection in unprimed BALB/c mice (2). Similarly, the lytic activity of cervical lymph node and spleen cells from mice inoculated with WSN(NA15) was characteristic of the natural killer cell response that is well known for LCMV infection (46); moreover, target cells infected with vaccinia virus tend to be highly susceptible to nonspecific effector mechanisms (4).

The cerebrospinal fluid samples from mice that had been primed with SALCM virus contained, on average, 50 times

TABLE 1. Protection against intracerebral challenge with LCMV

Days after priming[a]	Priming virus	% Mortality[b]	
		BALB/c ($H\text{-}2^d$)	B6 ($H\text{-}2^b$)
11	SALCM	0	100
	SRLCM	0	100
	WSN(NA15)	100	100
33	SALCM	0	100
	SRLCM	0	100
	WSN(NA15)	100	100
142	SALCM	20	100
	SRLCM	0	100
	WSN(NA15)	100	100

[a] Anesthetized female mice were given 30 μl of virus (3×10^2 PFU) intranasally and 500 μl of virus (2×10^6 PFU) intraperitoneally.

[b] Groups of five or six mice were injected intracerebrally with 1,000 PFU of LCMV; survival was assessed 11 days later.

TABLE 2. Secondary T-cell response at 5 days after intracerebral challenge

Priming virus[a]	LCMV (intracerebral)	Log_{10} cells/μl of cerebrospinal fluid	% Specific ^{51}Cr release (100:1)[b]			
			Cervical lymph node		Spleen	
			NP	WT	NP	WT
LCMV	−	1.3, 1.3	1	0	3	0
WSN(NA15)	+	3.1 ± 0.4	23	22	38	44
SALCM	+	4.8 ± 0.3[c]	38	5	25	10

[a] BALB/c mice were primed intravenously with 30,000 PFU of LCMV or intranasally with 30 μl (3×10^2 PFU) of WSN(NA15) or SALCM 10 weeks before intracerebral challenge with 1,000 PFU of LCMV. There were two control mice (both primed with LCMV) and five mice in each of the experimental groups.

[b] The LB15 ($H\text{-}2^{bxd}$ [F_1]) target cells were infected with the vaccinia virus-LCMV nucleoprotein construct (NP) or with wild-type (WT) vaccinia virus and were exposed to the immune cells in a 6-h ^{51}Cr release assay.

[c] Significantly different ($P < 0.01$) from the preceding group by Wilcoxon rank analysis.

more cells than the WSN(NA15) controls (Table 2 [4.8 ± 0.3 versus 3.1 ± 0.4 log_{10} cells per μl]). The cervical lymph nodes from these mice also showed strong LCMV-specific CTL activity (38% ^{51}Cr release), with only a little lysis of the target cells (5% ^{51}Cr release) infected by the wild-type vaccinia virus. This pattern of immune responsiveness was less apparent in spleen cells (25 versus 10% ^{51}Cr release), reflecting that the cervical lymph nodes seem to act as the regional lymph nodes for the central nervous system (7, 32).

A secondary CTL response by an influenza virus with an LCMV epitope. We then asked whether the SALCM virus induces a secondary CTL response in mice immunized with LCMV. In mice not immunized with LCMV, intranasal infection with SALCM, but not with WSN(NA15), resulted in a primary CTL response to the LCMV nucleoprotein, which could be detected in cells recovered by bronchoalveolar lavage at 8 but not at 6 days after exposure (Table 3). Again, antigen-nonspecific effector function (4, 46) was evident for day 6 samples from the immunologically naive mice given SALCM (20 and 19% ^{51}Cr release). Evidence of potent secondary CTL activity was found on both day 6 (63% ^{51}Cr release) and day 8 (47% ^{51}Cr release) for LCMV-primed mice that were challenged intranasally with SALCM (Table 3). CTL effectors were not detected in the regional mediastinal lymph nodes, which is typical for the influenza virus-specific response (3).

TABLE 3. LCMV primes for a secondary CTL response following intranasal challenge with SALCM

Challenge virus[a]	LCMV primed[a]	% Specific ^{51}Cr release by bronchoalveolar lavage cells (10:1)[b]			
		Day 6		Day 8	
		NP	WT	NP	WT
WSN(NA15)	−	21	25	8	10
	+	36	24	9	4
SALCM	−	20	19	23	6
	+	63	8	47	2

[a] BALB/c mice were primed intravenously with 30,000 PFU of LCMV 8 weeks before intranasal challenge with WSN(NA15) or SALCM (3×10^2 PFU). Bronchoalveolar lavage cells were obtained 6 or 8 days later and were adsorbed on plastic to remove most of the macrophages. The CTL assay was performed as described in footnote *b* to Table 2; mediastinal lymph node cells assayed (100:1) at the same time caused a maximum of 6% ^{51}Cr release from either target.

[b] NP, vaccinia virus-LCMV nucleoprotein construct; WT, wild-type vaccinia virus.

How many amino acids can be inserted into the NA stalk? The preceding results show that influenza virus with a foreign epitope in the NA stalk can protect mice against lethal viral infection. The future of influenza virus as a vaccine vector may well depend on whether multiple epitopes can be incorporated into a single virus. Therefore, we examined how many amino acid insertions can be tolerated in the NA stalk. We have previously shown that insertion of as many as 28 amino acids into the NA stalk does not impair NA function but rather increases the level of virus replication in eggs (6). Hence, we attempted to generate viruses that contained 38 (SA38), 48 (SA48), and 58 (SA58) additional amino acids (Fig. 1). These insertions consisted of 10 (SA38), 20 (SA48), and 30 (SA58) amino acids from the N8 NA stalk, as well as of 14 and 14 amino acids from the N2 and from the N9 NA stalk, respectively. We also attempted to create a mutant virus, SAFL, by inserting a B-cell epitope (FLAG, to which monoclonal antibodies are commercially available [41]) into the stalk. All of the mutant viruses were generated with the same efficiency as the wild-type WSN(NA15) virus, i.e., more than 10^3 PFU/ml.

All mutant viruses replicated efficiently in both eggs ($>10^7$ egg infectious $dose_{50}$) and MDCK cells ($>10^7$ PFU/ml). In mice, all of the viruses except SAFL were attenuated by comparison with the wild-type virus; the MLD_{50}s of SA38, SA48, and SA58 (all more than 5×10^4 PFU; no mice died at the highest virus concentration tested) were at least 100-fold higher than that of the WSN(NA15) virus (3×10^2 PFU). These findings indicate that insertion of more than 38 amino acids into the NA stalk attenuates virus without altering its replication in tissue culture and eggs.

DISCUSSION

In this paper, we show that a single inoculation of influenza viruses containing a T-cell-specific epitope of LCMV nucleoprotein (38) in the NA stalk can stimulate a protective immune response against subsequent challenge with LCMV in mice. Recently, Li et al. (26) reported protection against lethal *Plasmodium yoelii* infection in mice immunized with both an influenza virus carrying a *P. yoelii* T-cell epitope in the hemagglutinin and a vaccinia virus that expressed the same epitope. They also induced neutralizing antibodies and specific CTLs to human immunodeficiency virus type 1 epitopes by inoculating animals with chimeric influenza viruses carrying these epitopes (25). These findings, together with observations in the present study, suggest the possibility that influenza viruses could be used as a vector for vaccines protective against a variety of infectious diseases.

We demonstrate that as many as 58 amino acids can be inserted into the NA stalk without appreciably affecting the function of this molecule. Because the long amino acid sequences inserted into the NA are derived from the stalks of other NA subtypes, one could argue that amino acids must meet certain structural requirements to be successfully incorporated into this region. However, we have also generated viruses by inserting nonstalk amino acid sequences into the NA stalk (SRLCM, SALCM, and SAFL in this study as well as a virus with a herpes virus protein sequence [37a]). This suggests that the NA stalk does not impose rigid structural constraints on the insertion of amino acid sequences, making this portion an ideal region for inserting foreign epitopes. In recent reports (25, 27), foreign epitopes were inserted into the globular head

portion of the hemagglutinin molecule. Thus, both surface glycoproteins of influenza virus can be used for incorporating foreign epitopes. However, whether larger foreign sequences can be stably maintained in the influenza virus genes or as an additional gene segment remains to be investigated.

CTLs, which recognize proteolytic fragments of viral proteins presented at the cell surface by class I major histocompatibility complex molecules (45), play a critical role in the host's recovery from viral infection as well as in protection against subsequent reexposure (8, 10, 20, 22). Knowledge of the structural features that influence the immunogenicity of epitopes from endogenously synthesized proteins is vital to the design of vaccines containing CTL-specific epitopes. The variability of such epitopes with changes in major histocompatibility complex alleles (1, 45, 48) complicates preparation of CTL-based vaccines for outbred species, including humans. Whitton et al. (47) recently suggested that one might surmount this problem by expressing multiple haplotype-specific CTL epitopes in a single vaccinia virus construct. This approach is highly feasible with influenza virus, since the NA stalk could accommodate an expanded number of epitopes.

The $CD8^+$ CTL response to the influenza virus NA is minimal (50), since such effectors could be demonstrated only with an extensive immunizing protocol. However, incorporation of the immunogenic LCMV peptide into the influenza virus NA results in the development of both primary and memory CTL activity. It thus seems likely that the poor response to the native influenza virus NA is due not to the transit, processing, or temporal-generation characteristics of the NA glycoprotein. The alternative is that insertion of the foreign sequence into the NA stalk may have changed the degradation and processing characteristics of the NA molecule.

Poxviruses, especially vaccinia virus, have been studied extensively as potential vaccine vectors. They permit stable integration of multiple genes that confer protection against many different pathogens (17, 19, 21, 34, 36, 39, 42). However, the routine use of vaccinia virus in humans has been impeded by safety issues, particularly in immunocompromised persons. Human adenoviruses merit consideration as an alternative vector, since they infect animals orally and can induce an immune response against various foreign viral glycoproteins in model systems (9, 35, 40). Polioviruses have also been investigated as a vector for expression of foreign epitopes (16, 18, 37). A major disadvantage of these vaccine vectors is the immune response to the vectors themselves, which prohibits repetitive use in the same individual. By contrast, the availability of a spectrum of hemagglutinin and NA subtypes among influenza viruses as well as a large number of antigenic variants within the subtypes would permit repeated immunization of a single person. Moreover, influenza viruses have the advantage of stimulating vigorous humoral and cell-mediated responses. Finally, cold-adapted influenza viruses, which have been under clinical trials as live vaccines, have proved to be both safe and effective (33). Thus, live, attenuated influenza virus is an excellent candidate for use as a vaccine vector.

ACKNOWLEDGMENTS

We thank Krisna Wells for excellent technical assistance, P. J. Southern for the Armstrong LCMV, L. J. Whitton for the vaccinia virus-LCMV-NP construct, Thomas Chambers for WSN virus, Masahiro Ueda for WSN-HK virus, Clayton Naeve and the St. Jude Children's Research Hospital Molecular Resource Center for computer graphic analysis and preparation of oligonucleotides, Patricia Eddy and the St. Jude Children's Research Hospital Molecular Biology Computer Facility for computer support, and John Gilbert for scientific editing.

This work was supported by Public Health Service research grants AI-29599 and AI-29579 from the National Institute of Allergy and Infectious Diseases, Cancer Center Support (CORE) grant CA-21765, and the American Lebanese Syrian Associated Charities.

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