JOURNAL OF VIROLOGY, Jan. 1993, p. 348–352
0022-538X/93/010348-05$02.00/0

Vol. 67, No. 1

A "String-of-Beads" Vaccine, Comprising Linked Minigenes, Confers Protection from Lethal-Dose Virus Challenge†

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Received 10 July 1992/Accepted 29 September 1992

We have previously demonstrated that induction of antiviral cytotoxic T lymphocytes (CTL), in the absence of antiviral antibodies, can confer protection against a lethal-dose virus challenge. Here we extend those findings as follows. First, three discrete viral CTL epitopes expressed from minigenes encoding peptides as short as 12 amino acids can be recognized when expressed from recombinant vaccinia virus; second, concentrating on two of the three epitopes, we show that these vaccinia virus recombinants can confer protection in a major histocompatibility complex (MHC)-restricted manner; third, the minigenes can be fused to generate a "string of beads," and the close proximity of the two epitopes within one oligopeptide does not disrupt recognition of either epitope; fourth, this string-of-beads vaccine, in contrast to the single epitope vaccines, can protect on both MHC backgrounds; and, fifth, CTL to different epitopes may act synergistically, as protection is improved when the vaccine contains more than one CTL epitope for a given MHC background.

Adaptive immunity to virus infection can be conferred in either of two ways: passive, usually by transfer of immune serum, or active, by exposure to antigen. The latter method, most commonly by using as antigen live, attenuated viruses, has yielded outstanding results: smallpox has been eradicated, and the incidence of polio, measles, mumps, and rubella, among others, has declined dramatically, at least in the developed countries. Nevertheless, virus infection remains a major and, particularly since the emergence of human immunodeficiency virus, an increasing cause of human morbidity and mortality. Thus, a detailed understanding of effective vaccine responses is essential, to minimize vaccine failures and to develop new-generatation vaccines to cope with novel challenges.

The antigen-specific immune response can be divided into two arms: antibody and T lymphocyte. Antibodies recognize free antigen, while T cells recognize antigen in the form of processed peptide, bound by and presented in the groove of a host glycoprotein encoded by the major histocompatibility complex (MHC). T cells submit to subdivision into two functional groups, characterized by surface marker phenotype and by the MHC class with which they interact (for a review, see reference 2). In general, cytotoxic T lymphocytes (CTL) carry the CD8 molecule and interact with (and kill) cells expressing antigen presented by class I MHC molecules, while helper T cells, which provide help for antibody production, bear the CD4 molecule and interact with cells expressing antigen plus class II MHC molecules. Class I MHC proteins are expressed on almost all somatic cells, neurons being an exception (16), and present peptides of 9 to 10 amino acids (6, 7, 14, 28) which usually are generated from a protein made within the cell. Thus, following virus infection, fragments from even the earliest virus proteins (which generally are not cell surface proteins) can be picked up by class I molecules and displayed at the cell surface. In this way almost any somatic cell can signal that it is infected very soon postinfection. In contrast, recognition by antibody of a virus-infected cell requires expression of virus protein on the cell membrane, often a late event just preceding virus release.

Hence, when controlling primary virus infection, the potential benefit to the host of the early recognition afforded by the class I MHC-CD8$^+$ CTL interaction is clear; studies in many animal model systems have demonstrated the critical role played by CTL in eradication of primary virus infections (for a review, see reference 22). However, the role of CTL in acquired immunity (i.e., postimmunization) is less well appreciated. Antibodies are often considered the major protective components induced by vaccination; certainly, virus-specific antibody responses are induced by all of the vaccines in current use and are easily detected by routine laboratory assays. However, "experiments of nature" suggest that T cells alone play a critical role not only in controlling primary virus infection but also in protecting against disease from subsequent reexposure. For instance, congenitally agammaglobulinemic children cope well with almost all viral infections; upon measles virus challenge, they get typical disease, clear the virus, and are resistant to disease upon reexposure to measles virus, although at no time do they produce detectable antiviral antibody (9).

We have begun to dissect the importance of this antiviral acquired CTL immunity by using as a model system the arenavirus lymphocytic choriomeningitis virus (LCMV) and have shown that induction of an LCMV-specific CTL response (induced in the absence of a detectable antiviral antibody response) can protect mice from subsequent lethal-dose LCMV challenge (20, 21, 34). These findings have been confirmed in other laboratories (12, 29). This CTL immunity can be conferred by a single inoculation of a recombinant vaccinia virus expressing an LCMV protein. The effect is dependent on the MHC haplotype of the mouse and requires a class I molecule capable of binding and presenting an antigenic peptide from the LCMV protein (21). However, a recombinant vaccine expressing a single LCMV protein could protect only some mouse strains, and other strains, whose class I MHC molecules were unable to present peptides from the encoded LCMV protein, remained unpro-

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tected and succumbed to lethal-dose LCMV challenge. This problem could be overcome by expressing all viral proteins in the recombinant vector. However, for viral pathogens with large genomes, another problem would ensue; viral vectors have a limited capacity for foreign sequences, which would be rapidly exceeded if multiple complete genes were to be incorporated. In part to solve this problem, we have attempted to compress the foreign sequences by first identifying the epitopes recognized on several MHC backgrounds and by then expressing these as short open reading frames, or "minigenes," in isolation from their immunologically irrelevant protein backbone. Using expression vectors encoding minigenes designed to express peptides as short as 15 to 20 amino acids, we (35) and others (10) have previously shown that short polypeptide products can be synthesized and presented on the cell surface in a manner recognizable to T cells. The ability of these molecules to induce CTL, and the biological activity of such cells, was not analyzed. Here we extend these observations to show that first, three discrete LCMV epitopes expressed from minigenes encoding peptides as short as 12 amino acids can be recognized when expressed from recombinant vaccinia virus; second, concentrating on two of the three epitopes, we show that these vaccinia virus recombinants can confer protection in an MHC-restricted manner; third, the minigenes can be fused to generate a "string of beads," and the close proximity of the two epitopes within one oligopeptide does not disrupt recognition of either epitope; fourth, this string-of-beads vaccine, in contrast to the single epitope vaccines, can protect on both MHC backgrounds; and, fifth, CTL to different epitopes may act synergistically, as protection is improved when the vaccine contains more than one CTL epitope for a given MHC background.

MATERIALS AND METHODS

Mouse strains used. Mouse strains [C57BL/6 ($H2^{bb}$) and BALB/c ($H2^{dd}$)] were obtained from the breeding colony at Scripps Research Institute. Mice were used at 6 to 12 weeks of age.

Cell lines and viruses. BALB C17 ($H2^{d}$) and MC57 ($H2^{b}$) cell lines are maintained in continuous culture in the laboratory. Both lines are maintained in RPMI supplemented with 7% fetal calf serum, L-glutamine and penicillin-streptomycin. Viruses used were LCMV (Armstrong strain) and recombinant vaccinia viruses as outlined below.

Construction of recombinant vaccinia viruses. Recombinant vaccinia viruses carrying minigenes were constructed as follows: minigene sequences were designed and synthesized as complementary synthetic oligonucleotides, and the double-stranded DNA was cloned into a vaccinia virus transfer vector and subsequently introduced into the virus by homologous recombination, as described previously (36). In all cases, the recombinant plasmids were sequenced to ensure the correct sequence and orientation of the minigenes. Furthermore, to ensure that recombination had occurred faithfully, the appropriate region of the recombinant vaccinia virus DNA was amplified by polymerase chain reaction (PCR), and the resulting DNA was sequenced.

Cloning procedures and DNA sequencing. All cloning procedures were carried out according to the manufacturer's recommendations, and DNA sequencing was done using the dideoxy chain termination method with double-stranded template DNA and Sequenase version 2 (U.S. Biochemical Corporation, Cleveland, Ohio).

In vitro cytotoxicity assays. These assays were carried out as previously described (36). Effector cells were either day 7 primary splenocytes or were CTL clones of previously defined specificity. Target cells were infected appropriately with virus, labeled with ^{51}Cr, washed, and incubated for 5 h with effector cells at the indicated effector-to-target ratio. Supernate was harvested, and specific Cr release was calculated by using the following formula: [(sample release − spontaneous release) × 100]/(total release − spontaneous release).

In vivo protection studies. Mice (6 to 12 weeks of age) were inoculated with a single intraperitoneal (i.p.) dose of 2×10^7 PFU of recombinant vaccinia virus, a single dose of LCMV Armstrong (2×10^5 PFU i.p.) as a positive control, or with 200 μl of medium i.p. as a negative control. Six weeks later, the animals were challenged with a potentially lethal dose (20 50% lethal doses) of LCMV administered intracranially. Mice were observed daily, and all recorded deaths occurred between days 6 and 12 following lethal-dose LCMV challenge.

A

Recombinant & Presenting MHC	DNA sequence & epitope peptide sequence
MG3 GP1/D^b *	AGGATG**AAGGCTGTCTACAATTTTGCCACCTGTGGG**GTAACTAGTTAA **M K A V Y N F A T C G** V T S -
MG4 NP/L^d	ACCATG**GAAAGGCCCCCAGGCTTCAGGGGTATATATGGGGAACTTAAC**AGTAACTAGTTAA **M E R P Q A S G V Y M G N L T** V T S -
MG7 GP2/D^b	GCCATG**AGTGGGGTGGAGAATCCAGGTGGTTATTGCCTG**TAA **M S G V E N P G G Y C L** -
MG34 GP1/D^b + NP/L^d	CCCATG**AAGGCTGTCTACAATTTTGCCACCTGTGGG**GGGAGGACCATG**GAAAGGCCCCAG** **M K A V Y N F A T C G G R T M E R P Q** **GCTTCAGGGGTATATATGGGGAACTTAAC**AGTAACTAGTTAA **A S G V Y M G N L T** V T S -

B

Numbering for 9-residue motif		1 2 3 4 5 6 7 8 9
Db consensus motif (6), with proposed functions in next column	Anchor Strong . .	N M M I K L I L E F P Q V V
LCMV GP1 in MG3	matches with	**M K A V Y N F A T C G V T S**
LCMV GP2 in MG7	motif are in **bold**	**M S G V E N P G G Y C L**

FIG. 1. The DNA sequences of the minigenes and the encoded amino acids. (A) DNA and encoded peptide sequences of the four constructs described in the text. The LCMV sequences are shown in boldface, and non-LCMV sequences are in normal type. (B) Consensus nonameric peptide motif for the D^b class I MHC molecule, the proposed role of each residue, and the location of matching residues (shown in boldface) in the two LCMV epitopes presented by D^b.

RESULTS

Construction of recombinant vaccinia viruses encoding minigenes. Figure 1 shows the DNA and amino acid sequences of the minigenes which were studied. The minigenes from the two mature glycoproteins GP1 and GP2 of LCMV (MG3 and MG7, respectively) are presented by the D^b molecule while that from the LCMV nucleoprotein (NP) (MG4) is presented by the L^d molecule. Finally, two of the minigenes (MG3 and MG4) were linked in tandem (connected by three nonviral amino acids) in construct VVMG34. All DNA sequences were confirmed both in the vaccinia virus transfer plasmid and directly from a polymerase chain reaction product of vaccinia virus DNA. In all cases, the ATG shown is the first one in the RNA transcript. Also shown in Fig. 1 are the peptide consensus motif for binding and presentation by the D^b molecule (6, 27) and the relative alignments of the two LCMV minigenes presented by this molecule (no such motif has been obtained for the L^d molecule).

Recognition of minigenes by primary CTL and by CTL clones. VVMG3, VVMG4, VVMG7, and VVMG34 were used to infect $H2^b$ or $H2^d$ target cells which were subsequently incubated with the appropriate effector cells, as shown in Table 1. As can be seen, VVMG3 is recognized by

TABLE 1. Recognition of single epitopes and a string-of-beads construct on two MHC backgrounds[a]

Effectors	E:T[c]	% Specific Cr release[b] from following target cells:													
		$H2^{bb}$ (MC57)							$H2^{dd}$ (BALB C17)						
		UN	LCM	SC11	MG3	MG4	MG7	MG34	UN	LCM	SC11	MG3	MG4	MG7	MG34
$H2^{bb}$	50	6	**40**	3	**49**	6	**11**	**42**							
Splenocytes	25	4	**30**	1	**37**	5	**6**	**30**							
$H2^{dd}$	50								0	**68**	0	1	**65**	0	**60**
Splenocytes	25								1	**61**	0	0	**54**	0	**50**
αGP1/D^b	5	0	**40**	0	**37**		0	**35**							
αGP2/D^b															
	5	0	**40**	0	0		**60**	0							
	1	0	**16**	0	0			0							
αNP/L^d	5							0	**38**	0	0	**30**	0	**35**	

[a] In vitro cytotoxicity assays were carried out as described in Materials and Methods. Effector cells were primary splenocytes (taken 7 days post-LCMV infection) from $H2^{bb}$ or $H2^{dd}$ mice or cloned cell lines of known epitope specificity and MHC restriction.
[b] Levels above background are in boldface.
[c] E:T, effector-to-target cell ratio.

$H2^b$ primary CTL and by $H2^b$ CTL clones specific for the GP1 epitope but is not recognized by any $H2^d$-derived CTL. VVMG7 is recognized by $H2^b$ primary spenocytes (at a low level, characteristic of this epitope [35]) and directs a high level of lysis by an anti-GP2 CTL clone. Conversely, VVMG4 is recognized by $H2^d$ primary CTL and $H2^d$ anti-NP CTL clones but not by $H2^b$ cells. Thus, each of these individual minigenes produces a short peptide capable of being correctly processed and presented by class I MHC. When the $H2^b$/GP1 and $H2^d$/NP epitopes are linked together in VVMG34, the recombinant virus is recognized both by $H2^b$ and $H2^d$ primary CTL and by the appropriate CTL clones (Table 1). Thus, the close physical linkage of these two epitopes does not prevent their correct processing and presentation by different class I MHC molecules.

Minigenes: protection studies and a string-of-beads vaccine. VVMG3, VVMG4, and VVMG34 along with the control virus VVSC11 were used in vaccination studies. Which of these agents would protect various mice of various MHC haplotypes? As can be seen from Fig. 2, VVMG3 containing the $H2^b$ GP1 epitope protected 85% of $H2^b$ mice. Thus, this minigene vaccine, which is recognizable by CTL, is capable also of inducing protective responses. Note also that this recombinant virus conferred some protection (38%) on $H2^d$ mice. This was unexpected, as primary $H2^d$ CTL and over 30 $H2^d$ CTL clones which we derived all were directed towards the single NP epitope and did not recognize $H2^d$ target cells infected with VVMG3. A single $H2^d$ CTL clone which recognized the virus glycoprotein (unpublished data; clone no longer available) was obtained, and initial mapping showed recognition of a CTL epitope on the $H2^d$ background which lay between GP residues 1 to 60. Thus, it appears likely that this epitope is present in VVMG3; we have previously reported epitopes shared between several MHC backgrounds (24). VVMG4, as expected, protects $H2^d$ mice (94% survival) but not $H2^b$ mice (no survivors). VVMG34 protects 100% of $H2^{dd}$ mice and 87% of $H2^{bb}$. Therefore, two CTL epitopes in close proximity do not prevent each other from inducing appropriate responses.

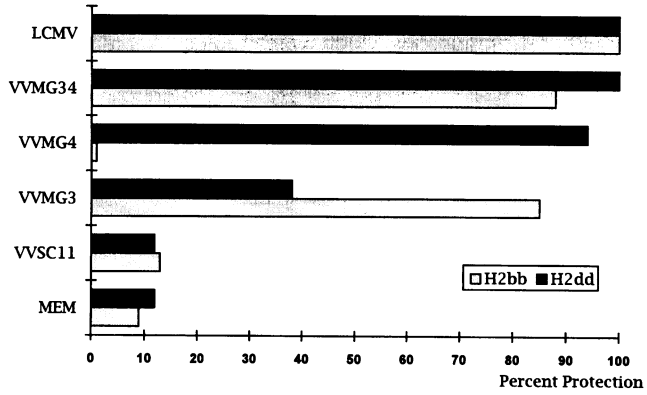

FIG. 2. Protection against lethal-dose LCMV challenge. Mice (BALB/c $H2^{dd}$ or C57BL/6 $H2^{bb}$) were vaccinated as described in the text, with the materials shown on the y axis, and 6 weeks later were challenged with a normally lethal intracranial dose of LCMV. Mice were observed daily for 21 days. All deaths occurred within 12 days of challenge.

DISCUSSION

Here we confirm and extend previous observations by using minigenes to study antiviral CTL responses. We make three novel observations. First, CTL epitopes as short as 12 amino acids (VVMG7, 11 LCMV residues linked to a Met initiator) can, when encoded in recombinant vaccinia viruses as minigenes, be recognized by virus-specific CTL. Second, a minigene encoding 14 residues (10 viral and 4 nonviral) can confer protection against lethal-dose viral challenge when administered as a single dose of recombinant vaccinia virus. By using VVMG3 or VVMG4, good protection is conferred upon the appropriate MHC haplotypes, but less or no protection is seen in mice of other MHC backgrounds. Third, multiple short minigenes can be arranged in tandem; to improve vaccine coverage and efficacy, we expressed in VVMG34 the two minigenes in tandem, in a string-of-beads arrangement. Despite their close proximity in this construct, both epitopes were recognized by appropriate CTL, and a protective response was elicited in both $H2^{bb}$ and $H2^{dd}$ mice. All of these findings have implications for both the development of vaccines and the processing and presentation of MHC class I restricted epitopes.

The use of live, attenuated viral vaccines has contributed greatly to the diminution or eradication of several viral diseases. Our aim is to dissect the components of protective immunity induced by immunization and to design new vaccines which are both safe and effective, protecting most or all individuals from disease. Although the virus-specific antibody response is considered critical in immunization—antibody responses are often used as a surrogate measure of vaccine-induced immunity—we and others have shown that the induction of antiviral CTL, in the absence of antiviral antibody induction, can protect against challenge with a normally lethal dose of virus (12, 17, 19–21, 26, 30). The epitopes encoded in the recombinant vaccinia viruses described here do not induce detectable LCMV-specific antibody responses. Most previous studies used recombinant vaccinia viruses encoding full-length viral proteins or relatively large subfragments thereof, although in one study a short CTL epitope of murine cytomegalovirus was embedded in a much larger protein, the surface antigen of hepatitis B virus, and this molecule was shown to present the epitope in a biologically relevant manner (4). However, in order to protect an outbred population, such as humans, a vaccine must induce responses on most or all MHC backgrounds and, as shown herein, one defect in subunit vaccination is the risk of vaccine failure due to nonresponder vaccinees. Both of the single minigene recombinants are susceptible to vaccine failure; when equal numbers of $H2^{bb}$ and $H2^{dd}$ mice are vaccinated, VVMG3 protects 61% of the mixed population and VVMG4 protects only 47%. Vaccines must, therefore, contain sufficient immunogenic information to induce protective responses on most or all haplotypes; if lengthy proteins had to be used, this would necessitate cloning of lengthy DNA fragments into the expression vector. Thus, the relatively small capacity of live viral vectors for foreign DNA may argue against their use as a delivery system for immunogenic sequences; even vaccinia virus may have an upper limit of 25 kb. In part for this reason, others have assessed bacterial delivery systems, which have a much larger potential capacity. However, by using this minigene approach, it would be possible to encode around 50 immunogenic fragments in an average-size protein (50,000 Da, 1,500 bp). In the present study, vaccine failure was diminished by use of the string-of-beads vaccine VVMG34. The benefit of this recombinant to an equally mixed population, when compared with that of recombinants containing a single epitope, is clear; 93.5% of vaccinees are protected. Thus, renewed consideration can be given to live delivery systems such as viral vectors and to immunization with replication-incompetent plasmid DNA (31, 37). The results in Fig. 2 also suggest that any protective effects of individual epitopes may be synergistic. For example, both VVMG3 and VVMG4 protect a certain proportion of $H2^{dd}$ mice (38 and 94%, respectively), and their combination confers 100% protection. Moreover, VVMG3 protects 85% of $H2^{bb}$ mice, while VVMG4 confers no protection; the combined vaccine VVMG34 confers a level of protection virtually identical to that conferred by VVMG3 alone (88 and 85%, respectively). Thus, two benefits may accrue from such combinatorial immunization. First, protective responses can be elicited on several MHC backgrounds, and second, on a single background the biological effectiveness of the response may be enhanced. We have concentrated on live vaccines because most studies to date have shown that the immunity resulting from this is more solid, and of greater duration, than that induced by killed vaccines (15, 32). The alternative approach to CTL induction uses soluble polypeptides, either in the form of ISCOMs (23) or as short synthetic peptides. In the LCMV system, peptides can be injected (with incomplete Freund's adjuvant) to induce an antiviral response (1), and subsequent analysis (following peptide injection with or without adjuvant) showed protection against viral challenge (30). Similar results have been observed by using Sendai virus (18). The precise requirements for peptide-mediated induction remain unclear, although recent data suggest that such peptides contain an epitope recognized by $CD4^+$ T cells (classically, helper cells), in addition to the epitope recognized by $CD8^+$ T cells (8).

The mechanism by which immunogenic peptide fragments are presented on class I molecules on the cell surface is the subject of intense investigation. What effects do the amino acid sequences within and around the minimal epitope have on processes which occur prior to binding to the class I molecules, for instance, in the generation of immunogenic peptides and/or their transport into the endoplasmic reticulum? Evidence that at least some level of sequence specificity is present at the level of transport comes from the rat RT1a system, in which polymorphism in a putative peptide transporter leads to a different spectrum of peptides being bound by identical class I molecules (25). Furthermore, it has been suggested that certain residues flanking the peptide comprising the CTL epitope may inhibit its eventual presentation. Studies of minigene constructs of influenza virus epitopes revealed such an effect (5), and in one case, the protective efficacy of an epitope embedded in a larger protein was diminished by the flanking residues (3). In contrast, others have found little or no effect of flanking sequences (11), which is mirrored by our results. First, our observation that an epitope of only 12 amino acids can be processed and presented normally suggests that extensive flanking residues are not required for processing and presentation. Second, despite the close proximity of these two CTL epitopes, both are recognized on the appropriate MHC backgrounds, and a protective response is elicited on both $H2^b$ and $H2^d$ backgrounds. Thus, the flanking sequences in this case have little or no effect on antigen presentation or on the biological efficacy of this vaccine. Finally, it is unclear whether the peptides generated from minigenes undergo further processing within the cell prior, or indeed subsequent, to their association with class I molecules. It is known that epitopes present on signal peptides that enter the endoplasmic reticulum by routes other than the class I processing pathway appear to undergo no further proteolytic cleavage (13, 33). We are currently investigating the further processing of peptides that enter the endoplasmic reticulum through the normal pathway by expressing the minigenes described here within cell lines deficient in processing or transport machinery and by altering the minigene sequences by site-specific mutagenesis.

ACKNOWLEDGMENTS

We are grateful to T. Calhoun for skilled secretarial assistance.

This work was supported by USPHS grants AI-27028 and AG-04342 to J.L.W. and AI-09484 and NS-12428 to M.B.A.O. J.L.W. is a Harry Weaver Scholar of the National Multiple Sclerosis Society.

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