Molecularly Engineered Vaccine Which Expresses an Immunodominant T-Cell Epitope Induces Cytotoxic T Lymphocytes That Confer Protection from Lethal Virus Infection†

LINDA S. KLAVINSKIS, J. LINDSAY WHITTON, AND MICHAEL B. A. OLDSTONE*

*Department of Immunology, Scripps Clinic and Research Foundation,
10666 North Torrey Pines Road, La Jolla, California 92037*

Received 19 April 1989/Accepted 5 July 1989

Identification of a single viral T-cell epitope, associated with >95% of the virus-specific cytotoxic T-lymphocyte (CTL) activity in BALB/c (*H-2^d*) mice (J. L. Whitton, A. Tishon, H. Lewicki, J. Gebhard, T. Cook, M. Salvato, E. Joly, and M. B. A. Oldstone, *J. Virol.* 63:4303-4310, 1989), permitted us to design a CTL vaccine and test its ability to protect against a lethal virus challenge. Here we show that a single immunization with a recombinant vaccinia virus-lymphocytic choriomeningitis virus (LCMV) vaccine (VVNPaa1-201) expressing the immunodominant epitope completely protected *H-2^d* mice from lethal infection with LCMV but did not protect *H-2^b* mice. Furthermore, we show that the success or failure of immunization was determined entirely by the host class I major histocompatibility glycoproteins. The difference in outcome between mice of these two haplotypes was consistent with the presence or absence in the immunizing sequences of an epitope for CTL recognition and is correlated with the induction of LCMV-specific *H-2*-restricted CTL in *H-2^d* mice. Protection is not conferred by a humoral immune response, since LCMV-specific antibodies were not detectable in sera from VVNPaa1-201-immunized mice. In addition, passive transfer of sera from vaccinated mice did not confer protection upon naive recipients challenged with LCMV. Hence, the molecular dissection of viral proteins can uncover immunodominant CTL epitope(s) that can be engineered into vaccines that elicit CTL. A single CTL epitope can protect against a lethal virus infection, but the efficacy of the vaccine varies in a major histocompatibility complex-dependent manner.

T-cell-mediated immunity, particularly involving class I major histocompatibility complex (MHC)-restricted cytotoxic T lymphocytes (CTL), plays an important role in resistance to several viral infections (10, 11, 19, 21, 27). The use of transfected cell lines and recombinant viruses expressing individual viral genes has demonstrated that class I-restricted CTL can be elicited to multiple gene products of a virus, such as influenza virus (4, 5, 16). The hierarchy and dominance of T-cell responses elicited to particular viral genes and their role in controlling the infection remain unclear. However, recent data suggest that the T-cell repertoire of class I- as well as class II-restricted responses is focused to a limited number of discrete immunodominant epitopes of a protein (6, 29, 32). This finding has led to the suggestion that there may be only a limited number of antigenic sites available for class I-restricted recognition and that this is further influenced by the direct binding of the determinant to the MHC, as demonstrated for the interaction of class II MHC gene products and peptides (3, 8).

In the accompanying report (34), molecular genetics and biochemical analysis were employed to identify the lymphocytic choriomeningitis virus (LCMV) recognition molecules for CTL generated by *H-2^d* mice. CTL recognition and activity mapped almost exclusively to the amino portion of the viral nucleoprotein (NP). The epitope NPaa122-127, as defined by studies with peptides, was restricted by *L^d* and comprised >95% of the *H-2^d*-restricted CTL response. In this study, we examine whether a vaccine containing this immunodominant epitope can be constructed first to induce

CTL responses and second to protect against an ordinarily lethal viral challenge.

MATERIALS AND METHODS

Mice. BALB/c/byj (*H-2K^dI^dD^d*), B10.A(18R) (*H-2K^bI^bD^d*), and C57BL/6 (*H-2K^bI^bD^b*) mice were obtained from the breeding colony at the Research Institute of Scripps Clinic. All mice were used at between 5 and 8 weeks of age.

Cells and viruses. BALB Cl.7 (*H-2^d*) fibroblasts and Vero and 143TK⁻ cells were grown in minimal essential medium supplemented with 10% heat-inactivated fetal calf serum, 1 mM L-glutamine, 10⁴ U of penicillin per ml, and 10 mg of streptomycin per ml. MC57 cells (*H-2^b*) were cultured in RPMI 1640 medium with the supplements listed above.

The origin, cloning, plaque purification, and recognition by CTL of the LCMV strain Armstrong (ARM) CA1371 clone 53b used in this study have been described previously (1, 14, 32, 33). Virus was plaque purified three times on Vero cells, and thereafter stock virus was prepared by a single passage in BHK-21 cells. The construction and recognition by CTL of the recombinant vaccinia viruses (VV) VVNPaa1-558 (expressing full-length LCMV NP, amino acids 1 to 558), VVNPaa1-201 (expressing a truncated NP, amino acids 1 to 201), and VVSC11 (which lacks any LCMV sequences) have been described elsewhere (33, 34). Stocks of recombinant VV were prepared by infecting 143TK⁻ cells in media containing bromodeoxyuridine as described by Whitton et al. (33).

Vaccination and challenge. Mice were immunized with a single intraperitoneal (i.p.) inoculation of 2 × 10⁷ PFU of recombinant VV (VVNPaa1-558, VVNPaa1-201, or VVSC11) or with 2 × 10⁵ PFU of LCMV ARM in 0.2 ml. At 5 weeks postinfection, mice were bled by the retro-orbital

* Corresponding author.

† Publication no. 5749-IMM of the Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, Calif.

plexus for serological studies and challenged at 6 weeks under anesthesia by intracranial (i.c.) inoculation with 50 μ l of phosphate-buffered saline (PBS) containing 10 PFU of LCMV ARM (20 50% lethal doses). Mice were monitored for morbidity and mortality for a minimum of 14 days following virus challenge.

Generation of primary and secondary cytotoxic effector populations. Primary LCMV-specific CTL were generated by priming mice in vivo with 2×10^5 PFU of LCMV ARM. Anti-VV CTL were similarly induced by i.p. inoculation with 2×10^7 PFU of VVSC11. Spleens were isolated on day 7; single-cell suspensions of splenocytes (free from erythrocytes) were prepared in complete RPMI 1640 medium and tested for cytotoxicity as described previously (9).

Secondary cytotoxic effector cells were generated by immunizing BALB/c mice i.p. with 2×10^5 PFU of LCMV ARM or with 2×10^7 PFU of VVNpaa1-558, VVNpaa1-201, or VVSC11. Six weeks later, spleens were removed and restimulated in vitro with syngeneic LCMV ARM-infected peritoneal exudate macrophages. In brief, peritoneal exudate cells were induced by injecting 3 ml of 3% (wt/vol) thioglycolate (Difco Laboratories, Detroit, Mich.) i.p. 3 days before harvesting. For restimulation, 2×10^5 macrophages, irradiated with 2,000 rads and infected 48 h previously with LCMV ARM (multiplicity of infection of 3), were incubated with 4×10^6 spleen cells per ml of RPMI 1640 medium containing 10% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, penicillin, and streptomycin. After 5 days at 37°C, effector cells were harvested and tested by the ^{51}Cr release assay.

^{51}Cr Chromium release cytotoxicity assay. A standard 6-h ^{51}Cr (Amersham Corp., Arlington Heights, Ill.) release assay was used as described by Byrne et al. (9). Target cells were infected with LCMV ARM at a multiplicity of infection of 1 48 h before the assay or with recombinant VV 8 h before the assay at a multiplicity of infection of 3. Secondary in vitro-restimulated spleen cells were incubated with target cells at effector-to-target cell ratios of 100:1 to 6.25:1. The percent specific lysis was calculated as $100 \times (\text{counts per minute released by CTL} - \text{counts per minute of spontaneous release}) / (\text{counts per minute of total release} - \text{counts per minute of spontaneous release})$.

Antibody titration. LCMV-specific antibody was measured by a solid-phase enzyme-linked immunosorbent assay (ELISA) using purified virus as the antigen, as described by Ahmed et al. (2). Briefly, microdilution plates (Linbro Titer-tek; Flow Laboratories, Inc., McLean, Va.) were coated with 200 ng of purified viral antigen per well in 0.1 ml of PBS. Following overnight incubation, free binding sites were blocked with 0.15 ml of PBS supplemented with 10% (wt/vol) bovine serum albumin–0.2% (vol/vol) Tween 20 for 1 h. Antigen-coated plates were incubated for 90 min with four-fold serial dilutions of serum, washed three times with PBS containing 0.05% (vol/vol) Tween 20, and reincubated (90 min) with horseradish peroxidase-labeled affinity-purified goat anti-mouse immunoglobulin G (IgG) and IgM (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Plates were washed three times as described above, and 0.1 ml of substrate was added (0.1 M sodium citrate buffer [pH 5] containing 3 mM hydrogen peroxide and 2.2 mM *O*-phenylenediamine). The reaction was stopped by addition of 1 N HCl, and the optical density at 492 nm was read with a photometer (Titertek Multiskan). The LCMV-specific antibody titer is expressed as the reciprocal of the highest dilution showing an optical density at 492 nm reading >2

standard deviation from the mean optical density at 492 nm reading of serum samples from nonimmune mice.

VV-specific antibody was measured by ELISA as indicated above, using 200 ng of recombinant VV per ml (which lacks LCMV-specific sequences) as the antigen. VV was semipurified by a modification of the method of Joklik (17), by centrifugation at 25,000 rpm for 2 h through 36% sucrose in 10 mM Tris (pH 9). The virus pellet was diluted in PBS and sonicated to disrupt clumps, and the protein concentration was determined by the method of Lowry et al. (20).

Virus neutralization. The LCMV-specific neutralizing-antibody titer was determined by the plaque reduction assay, using Vero cell monolayers in six-well dishes (Costar, Cambridge, Mass.). Sera were serially diluted in minimal essential medium, and 0.3 ml of each dilution was incubated for 1 h at 37°C with an equal volume containing 200 PFU of LCMV ARM. Residual virus was quantitated by plaque assay (14). A hyperimmune guinea pig serum with a known LCMV-specific neutralizing titer was included in each assay.

Passive transfer of serum. Sera were pooled from 20 BALB/c byj mice immunized 6 weeks previously with 2×10^7 PFU of VVNpaa1-201. The total IgG concentration was determined as 2.3 mg/ml by radial immunodiffusion (ICN ImmunoBiologicals, Lisle, Ill.). Nonimmune BALB/c byj mice (four per group) were injected intravenously with 0.5 ml of serum containing either 1.15 or 3.45 mg of total IgG at 24 and 4 h before virus challenge by i.c. inoculation with 10 PFU of LCMV ARM in 50 μ l of PBS. Mice vaccinated with LCMV ARM or VVNpaa1-201 6 weeks previously and challenged by infection with LCMV ARM were included as positive controls. Animals were monitored for mortality for 14 days after lethal virus infection.

RESULTS

Vaccination with a recombinant VV-expressing LCMV NP protects $H-2^d$ and $H-2^b$ mice from lethal infection with LCMV. A single injection with a VVNpaa1-558 vaccine (expressing the full-length LCMV NP sequence) protected both $H-2^b$ and $H-2^d$ mice from lethal i.c. infection with LCMV (Fig. 1) in three separate experiments. However, mice vaccinated with recombinant VVSC11, which lacks LCMV sequences, and

FIG. 1. Immunization of $H-2^d$ mice with a single inoculation of a recombinant VV expressing a truncated LCMV VVNpaa1-201 (containing the epitope for CTL recognition) protects against lethal LCMV infection. BALB/c ($H-2^d$) and C57BL/6 ($H-2^b$) mice were primed by i.p. injection with 2×10^5 PFU of LCMV ARM or with 2×10^7 PFU of VVNpaa1-558, VVNpaa1-201, or VVSC11. Six weeks later, these mice were challenged intracerebrally with 10 PFU of LCMV-ARM (20 50% lethal doses). Symbol: *, number of mice protected of total immunized.

TABLE 1. Vaccination of *H-2^b* recombinant mice

Immunization ^a	No. of mice	% Protection ^b		
		<i>K^bI^bD^d</i>	<i>K^bI^bD^b</i>	<i>K^bI^dD^d</i>
None	5	0	0	0
LCMV ARM	5	100	100	100
VVNPaa1-201	10	100	0	100

^a B10.A(18R) (*K^bI^bD^d*), C57BL/6 (*K^bI^bD^b*), and BALB/c/byj (*K^dI^dD^d*) mice were immunized by a single i.p. inoculation with 2×10^5 PFU of LCMV ARM or with 2×10^7 PFU of VVNPaa1-201. Six weeks later, mice were challenged intracerebrally with 10 PFU of LCMV ARM (20 50% lethal doses).

^b Recorded 14 days after lethal challenge with LCMV ARM.

nonimmune mice died from acute LCMV infection within 7 to 9 days (Fig. 1). Mice immunized with VVNPaa1-558 showed some evidence of illness 5 to 6 days later, suggesting an accelerated cell-mediated immune response to virus replication, but these animals recovered by day 8 and remained healthy for the 90 days of study. Brain tissues and sera isolated from VVNPaa1-558-immunized mice at 14, 30, and 90 days after virus challenge and examined in vitro by infectivity assay showed no evidence of infectious LCMV. This indicated that the protection resulting from vaccination was associated with a systemic immune response that eliminated the virus. There was no evidence that immunity was incomplete and that a persistent LCMV infection was established.

Vaccination with a truncated LCMV NP protects *H-2^d* but not *H-2^b* mice from lethal LCMV infection. The full-length NP, amino acid residues 1 to 558, contains at least one CTL epitope for mice of both the *H-2^b* and *H-2^d* haplotypes (33), although the *H-2^b* epitope maps to residues 322 to 558 at the carboxy end of the molecule (Whitton et al., unpublished observations) and the *H-2^d* epitope maps to residues 122 to 127 at the amino end (34). To ascertain whether protection was dependent on the presence of such an epitope, both *H-2^b* and *H-2^d* mice were inoculated with VVNPaa1-201, which contains a recognition epitope only for *H-2^d* (18a). A single inoculation of this truncated NP (VVNPaa1-201) provided complete protection from lethal virus infection in *H-2^d* (survivors, 18 of 18) but not in *H-2^b* mice (Fig. 1).

Successful vaccination with VVNPaa1-201 requires the *H-2^d* glycoprotein. Experiments in which the *L^d* gene was used to transfect non-*H-2^d* cells that were either sensitized with an LCMV ARM NPaa122-127 peptide or infected with LCMV defined the CTL recognition epitope as restricted to the *L* locus of the *H-2^d* haplotype (34). To further investigate the importance of class I MHC glycoproteins in the protection conferred by vaccination with VVNPaa1-201, *H-2^b* mice or *H-2^b* recombinant mice expressing *L^d* [B10.A(18R)] were inoculated with VVNPaa1-201, which contains a recognition epitope restricted by *L^d*. A single inoculation of VVNPaa1-201 failed to protect C57BL/6 mice (*H-2^b*), and they uniformly died from LCMV infection within 8 days of i.c. inoculation. In contrast, 100% of B10.A(18R) mice, which are congenic with the C57BL/6 strain except at the *L* locus, were completely protected after a single vaccination with VVNPaa1-201 (Table 1).

VVNPaa1-558 induces LCMV-specific CTL. We next wished to establish whether the degree of protection conferred by vaccination was related to the induction of LCMV-specific CTL. VVNPaa1-558 protected both *H-2^b* and *H-2^d* mice from challenge. Secondary in vitro LCMV-specific CTL activity was demonstrable for both *H-2^b* and *H-2^d* mice primed in vivo with VVNPaa1-558 and restimulated 6 weeks later with LCMV in vitro (Table 2). This cytolytic activity was LCMV specific, since target cells infected with VV lacking LCMV sequences were not lysed above background levels (Table 2), although these cells were productively infected and were efficiently lysed by day 7 primary anti-VV CTL (data not shown). Additionally, the cytotoxic activity was *H-2* restricted, since spleen cells from mice immunized with VVNPaa1-558 did not lyse allogeneic target cells after in vitro restimulation (Table 2). Finally, this CTL activity was not a primary response generated in vitro by stimulation with LCMV-infected syngeneic cells, since we could find no evidence of lysis by nonimmune spleen cells (Table 2). Thus, in *H-2^b* and *H-2^d* mice, induction of LCMV-specific CTL by recombinant VV expressing full-length NP mirrored CTL induction during LCMV infection, and the vaccinated mice were fully protected from LCMV challenge.

TABLE 2. LCMV-specific CTL response after immunization with recombinant VV^a

Mouse splenocytes	Primary in vivo treatment	E:T ^b	CTL response (% ⁵¹ Cr released) ^c							
			<i>H-2^d</i> mice infected with:				<i>H-2^b</i> mice infected with:			
			LCMV ARM	Nothing	VVNPaa1-558	VVSC11	LCMV ARM	Nothing	VVNPaa1-558	VVSC11
<i>H-2^d</i>	LCMV ARM	50:1	79	0	76	5	18	ND	ND	ND
		25:1	77	0	74	3	11			
	VVNPaa1-558	50:1	47	0	47	19	7	ND	ND	ND
		25:1	44	0	42	15	3			
	VVSC11	50:1	0	0	33	34	3	ND	ND	ND
		25:1	0	0	23	25	1			
<i>H-2^b</i>	LCMV ARM	50:1	ND	ND	ND	ND	64	0	46	ND
		25:1	11				50	0	30	0
	VVNPaa1-558	50:1	0	ND	ND	ND	34	7	34	13
		25:1	1				20	0	16	4
	VVSC11	50:1	0	ND	ND	ND	2	6	17	19
		25:1	0				0	1	15	11
	None	50:1	0	ND	ND	ND	1	5	6	7
		25:1	0				0	0	0	3

^a Secondary CTL cultures were established from *H-2^d* and *H-2^b* mice primed in vivo with LCMV ARM, VVNPaa1-558, or VVSC11 and restimulated in vitro with LCMV ARM-infected syngeneic peritoneal exudate cells, as described in Materials and Methods. CTL were harvested after 5 days and tested by ⁵¹Cr release assay, as described in the text.

^b E:T, Effector-to-target cell ratio.

^c The significant percent specific ⁵¹Cr release is indicated (boxed data). ND, Not determined.

FIG. 2. Immunization with VVNPaa1-201 primes *H-2^d* mice for a secondary in vitro LCMV-specific CTL response. Six weeks after primary immunization in vivo with 2×10^5 PFU of LCMV ARM or with 2×10^7 PFU of VVNPaa1-201 or VVSC11, BALB/c splenocytes were restimulated in vitro with LCMV ARM, and effector cells were harvested after 5 days as described in Materials and Methods. Cytotoxicity was measured in a ⁵¹Cr release assay by testing CTL at a variety of effector/target cell (E:T) ratios. (Top left) Recognition of LCMV ARM-infected *H-2^d* targets. (Remaining panels) Recognition of *H-2^d* cells infected with VVNPaa1-201, uninfected *H-2^d* cells, and *H-2^b* cells infected with LCMV ARM.

VVNPaa1-201 induces LCMV-specific *H-2^d*-restricted CTL. The observation that (i) amino acid residues 1 to 201 (VVNPaa1-201) contain an immunodominant epitope for CTL recognition (34) in mice of the *H-2^d* haplotype and (ii) that *H-2^d* but not *H-2^b* mice immunized with this epitope are protected from lethal challenge with LCMV suggested that priming with VVNPaa1-201 induced LCMV-specific CTL. A single immunization with VVNPaa1-201 primed *H-2^d* spleen cells in vivo for secondary LCMV-specific class I-restricted CTL following restimulation in vitro with LCMV (Fig. 2). This response was specific, as shown by the failure of spleen cells primed in vivo with VVSC11 to lyse LCMV-infected target cells after restimulation in vitro with LCMV (Fig. 2).

VVNPaa1-201-immunized mice do not develop LCMV-neutralizing antibody. We next determined whether humoral responses played a role in protection from LCMV in vaccinated mice. By virus neutralization assay, no antibody activity was demonstrable in sera from mice vaccinated with VVNPaa1-201 or with VVNPaa1-558 (results not shown). Inclusion of positive control sera with known LCMV-specific neutralizing activities indicated that this result did not reflect the sensitivity of the assay. It should be noted that there is no prior evidence for neutralizing antibody directed to the NP of LCMV (25).

The absence of neutralizing activity in vitro, however, does not exclude protective effects of antiserum in vivo, where infected cells with surface-bound immunoglobulin could be eliminated by cellular mechanisms. To further characterize the antibody responses, sera were analyzed by ELISA, using purified LCMV as the target antigen. *H-2^d* mice immunized with LCMV ARM or with VVNPaa1-558 did in fact develop an LCMV-specific antibody response, with endpoint titers of 163,840 and 10,240, respectively (Fig. 3). In contrast, little or no LCMV-specific antibody was detectable by ELISA in sera from mice vaccinated with a truncated LCMV NP (Fig. 3). These mice did, however,

FIG. 3. Immunization with a truncated LCMV VVNPaa1-201 does not induce detectable LCMV-specific antibody in *H-2^d* mice. BALB/c mice were immunized i.p. with 2×10^5 PFU of LCMV ARM or with 2×10^7 PFU of VVNPaa1-558 or VVNPaa1-201. At 5 weeks postimmunization, LCMV-specific antibody titers were determined by ELISA using purified LCMV, as described in Materials and Methods. Each point represents the mean $\pm$ 1 standard deviation of the mean for five mice (for LCMV ARM, there were only four mice per group).

develop anti-VV antibody titers comparable with those of animals primed with VVSC11 or VVNPaa1-558 (results not shown) and were subsequently protected when challenged with LCMV. Thus, mice inoculated with VVNP1-201, although fully protected, mount no LCMV-specific antibody response.

To determine whether levels of LCMV-specific antibody too low for detection by ELISA could confer the protection observed in VVNPaa1-201-primed animals, we treated naive mice with pooled sera from VVNPaa1-201-primed donors and monitored recipients for protection from i.c. challenge with LCMV. None of four naive mice treated with two injections of either 1.15 or 3.45 mg of total IgG from VVNPaa1-201-primed mice were protected from virus challenge. In contrast, all four recipients vaccinated with either LCMV ARM or VVNPaa1-201 were completely protected from the lethal virus infection (results not shown).

DISCUSSION

LCMV is a natural pathogen of mice, and the outcome of acute infection is determined primarily by the activity of virus-specific CTL (7, 12, 35). We have used LCMV infection as a model to show that one can prevent lethal virus disease by vaccinating with a selected CTL recognition epitope. Using as a vaccine a recombinant VV that expresses a truncated LCMV NP, we demonstrate that protection from LCMV challenge depends on the presence or absence of a CTL recognition epitope in mice of the *H-2^d* and *H-2^b* haplotypes and that this protection is mediated by the induction of LCMV-specific CTL. Humoral responses were not associated with the protection. With respect to the broad aspects of vaccine design, these results illustrate the advantage of identifying immunodominant viral epitopes, the role of memory CTL induced following vaccination, and, in terms of vaccine specificity, that the *L^d*-restricted CTL epitope is clearly the site of immune defense against LCMV in the *H-2^d* mice tested in this study.

We first demonstrated that vaccination of both *H-2^b* and *H-2^d* mice with recombinant VV expressing the entire LCMV NP (amino acid residues 1 to 558) conferred com-

plete protection from a lethal i.c. challenge with LCMV ARM (Fig. 1). Since this full-length NP contains at least one CTL epitope for each of these haplotypes (34), subsequent experiments were designed to define the minimal region of the NP necessary for complete protection. Such studies demonstrated that a single immunization with an amino-terminal truncated NP expressing residues 1 to 201 provided complete protection from lethal virus challenge in *H-2^d* but not in *H-2^b* mice (Fig. 1). Since the epitope for CTL recognition in *H-2^d* mice has been mapped to amino acid residues 122 to 127 in the accompanying report (34), our results indicate that the immune status of these vaccinated recipients correlates with the CTL recognition epitopes defined for their haplotypes. However, as well as illustrating the potential utility of CTL vaccines, our data also highlight the potential limitations of using subunit vaccines. Since different MHC molecules select different regions of viruses for presentation to the immune system, the administration of subunit vaccines may fail to confer protection on certain members of a population (33). We now prove the reality of this risk, as *H-2^b* mice were not protected by the vaccine; this susceptibility maps to the MHC, as *H-2^b* congenic mice expressing the *L^d* gene were fully protected by vaccination.

Protection of *H-2^d* mice is most likely associated with induction of class I MHC-restricted memory CTL to the NPaa122-127 epitope. Inoculation with the truncated NP primed *H-2^d* mice for memory CTL specific for the *L^d*-restricted epitope expressed by both LCMV ARM- and VVNPaa1-201-sensitized target cells. Hence, endogenous synthesis of a truncated NP resulted in successful presentation of the NPaa122-127 epitope in vivo, strongly supporting the emerging hypothesis that class I-restricted CTL are induced by (22; F. R. Carbone and M. J. Bevan, J. Exp. Med., in press) and recognize denatured or degraded peptide fragments (16, 30, 32).

Considerable effort has been directed toward identifying the minimal viral sequence requirements for directing CTL recognition (13, 24, 30); yet understanding the sequence constraints for inducing T cells to biologically relevant epitopes is imperative in our quest to manipulate these requirements in the design of protective molecules. Our experiments have begun to approach this issue. A 6-amino-acid epitope, NPaa122-127, has been identified as the minimal requirement for CTL recognition (34). Yet this minimal sequence by itself may not be sufficient to induce CTL. To address this issue of minimal sequence requirements for T-cell induction, a library of truncated minigenes and extended peptides spanning amino acids 1 to 210 is currently being constructed. In preliminary data, a 15-amino-acid peptide, NPaa116-129, that includes and extends from the immunodominant NPaa122-127 recognition site does not elicit CTL in vivo (Klavinskis and Oldstone, unpublished observations). Experiments similar to those presented here and elsewhere (15; Carbone and Bevan, in press) should chemically define the importance and dependence of neighboring or distant residues which may influence the induction of CTL responses to endogenously synthesized epitopes and should provide insights into manipulating these requirements with recombinant materials.

Accumulating data examining T-cell recognition of influenza virus, human immunodeficiency virus, and LCMV indicate that while CTL responses to multiple gene products occur (23, 26, 29-32), only an extremely limited number of these responses are immunodominant (6, 28, 32) and hence of biological significance. Thus, only a restricted number of antigenic sites on a molecule are recognized by class I-

restricted CTL which are likely to be of biological importance (immunodominant) (6, 32). Recognition of these sites is determined by the ability of these molecules to bind to the MHC (6, 32). Identification of these immunodominant epitopes may define the minimal viral sequences required to achieve protective immunity. From our present data, inclusion of only one such epitope is required to protect *H-2^d* mice from lethal infection with LCMV. Using a similar strategy, Jonjić et al. (18) have shown that a single gene product can protect mice against murine cytomegalovirus disease. Hence, once immunodominant domains are mapped, it should be possible to construct vaccines with multiple immunodominant CTL epitopes in order to accommodate polymorphism of the MHC proteins in outbred populations, such as humans, thus minimizing the chance that an individual will be unable to respond to the vaccines (as seen here in *H-2^b* mice). Finally, a significant advantage of a CTL vaccine may be its ability to induce protection that is cross-reactive among serologically distinct virus strains. In this regard, knowledge currently being obtained through mapping of CTL and antibody epitopes of LCMV ARM as well as LCMV Pasteur and LCMV WE coupled with the *H-2^d*-restricted VVNPaa1-201 vaccine model described here will be useful in deciding CTL cross-reactivity and protection in vivo.

These studies establish the protective potential of CTL induced by vaccination. We are pursuing our analyses of such CTL vaccines because they may have two critical advantages over procedures which induce only humoral responses. First, certain viruses appear to be able to evade humoral responses by remaining highly cell associated or by rapidly evolving in the host (e.g., human immunodeficiency virus). These agents may find it more difficult to evade primed CTL. Second, CTL often cross-react between serotypically distinct virus strains and may thus confer a broader protection than antibodies.

ACKNOWLEDGMENTS

The excellent technical assistance of Robert Geckeler is gratefully acknowledged. We thank A. Nash for critically reviewing the manuscript and Gay Schilling and Phyllis Minick for secretarial and editorial assistance.

This work was supported in part by Public Health Service grants AI09484, NS12428, and AG04342 from the National Institutes of Health. L.S.K. was supported by a Fulbright travel fellowship and by a grant from the Burroughs Wellcome Foundation.

LITERATURE CITED

1. Ahmed, R., J. A. Byrne, and M. B. A. Oldstone. 1984. Virus specificity of cytotoxic T lymphocytes generated during acute lymphocytic choriomeningitis virus infection: role of the *H-2* region in determining cross-reactivity for different lymphocytic choriomeningitis virus strains. J. Virol. 51:34-41.
2. Ahmed, R., A. Salmi, L. D. Butler, J. M. Chiller, and M. B. A. Oldstone. 1984. Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. Role in suppression of cytotoxic T lymphocyte response and viral persistence. J. Exp. Med. 160:521-540.
3. Babbitt, B. P., P. M. Allen, G. Matsueda, E. Haber, and E. R. Unanue. 1985. Binding of immunogenic peptides to Ia histocompatibility molecules. Nature (London) 317:359-361.
4. Bennink, J. R., J. W. Yewdell, G. L. Smith, and B. Moss. 1987. Anti-influenza virus cytotoxic T lymphocytes recognize the three viral polymerases and a nonstructural protein: responsiveness to individual viral antigens is major histocompatibility complex controlled. J. Virol. 61:1098-1102.
5. Braciale, T. J., V. L. Braciale, T. J. Henkel, J. Sambrook, and M. J. Gething. 1984. On the role of the transmembrane anchor

- sequence of influenza hemagglutinin in target cell recognition by class I MHC-restricted, hemagglutinin-specific cytolytic T lymphocytes. *J. Exp. Med.* **159**:341–354.
6. Braciale, T. J., M. T. Sweetser, L. A. Morrison, D. J. Kittlesen, and V. L. Braciale. 1989. Class I major histocompatibility complex-restricted cytolytic T lymphocytes recognize a limited number of sites on the influenza hemagglutinin. *Proc. Natl. Acad. Sci. USA* **86**:277–281.
 7. Buchmeier, M. J., R. M. Welsh, F. J. Dutko, and M. B. A. Oldstone. 1980. The virology and immunobiology of lymphocytic choriomeningitis virus infections. *Adv. Immunol.* **30**: 275–331.
 8. Buss, S., A. Sette, S. M. Colon, C. Miles, and H. M. Grey. 1987. The relation between major histocompatibility complex (MHC) restriction and the capacity of Ia to bind immunogenic peptides. *Science* **235**:1353–1357.
 9. Byrne, J. A., R. Ahmed, and M. B. A. Oldstone. 1984. Biology of cloned cytotoxic T lymphocytes specific for lymphocytic choriomeningitis virus. I. Generation and recognition of virus strains and H-2^b mutants. *J. Immunol.* **133**:433–439.
 10. Byrne, J. A., and M. B. A. Oldstone. 1984. Biology of cloned cytotoxic T lymphocytes specific for lymphocytic choriomeningitis virus: clearance of virus *in vivo*. *J. Virol.* **51**:682–686.
 11. Cannon, M. J., J. Stott, G. Taylor, and B. A. Askonas. 1987. Clearance of persistent respiratory syncytial virus infections in immunodeficient mice following transfer of primed T cells. *Immunology* **62**:133–138.
 12. Cerny, A., A. W. Huegin, S. Sutter, H. Bazin, H. H. Hengartner, and R. M. Zinkernagel. 1986. Immunity to lymphocytic choriomeningitis virus in B cell-depleted mice: evidence for B cell and antibody independent protection by memory T cells. *Eur. J. Immunol.* **16**:913–917.
 13. Del Val, M., H. Volkmer, J. B. Rothbard, S. Jonjić, M. Messerle, J. Schickedanz, M. J. Reddehase, and U. Koszinowski. 1988. Molecular basis for cytolytic T-lymphocyte recognition of the murine cytomegalovirus immediate-early protein pp89. *J. Virol.* **62**:3965–3972.
 14. Dutko, F. J., and M. B. A. Oldstone. 1983. Genomic and biological variation among commonly used lymphocytic choriomeningitis virus strains. *J. Gen. Virol.* **64**:1689–1698.
 15. Gammon, G., N. Shastri, J. Cogswall, S. Wilbur, S. Sadegh-Nasseri, V. Krzych, A. Miller, and E. Sercarz. 1987. The choice of T-cell epitopes utilized on a protein antigen depends on multiple factors distant from, as well as at the determinant site. *Immunol. Rev.* **98**:53–73.
 16. Gotch, F. M., J. Rothbard, K. Howland, A. R. M. Townsend, and A. J. McMichael. 1987. Cytotoxic T lymphocytes recognize a fragment of influenza virus matrix protein in association with HLA-A2. *Nature (London)* **26**:881–882.
 17. Joklik, W. K. 1962. The purification of four strains of poxvirus. *Virology* **18**:9–18.
 18. Jonjić, S., M. Del Val, G. M. Keil, M. J. Reddehase, and U. H. Koszinowski. 1988. A nonstructural viral protein expressed by a recombinant vaccinia virus protects against lethal cytomegalovirus infection. *J. Virol.* **62**:1653–1658.
 - 18a. Klavinskis, L. S., M. B. A. Oldstone, and J. L. Whitton. 1989. Designing vaccines to induce cytotoxic T lymphocytes: protection from lethal viral infection, p. 485–489. *In* F. Brown, R. M. Chanock, H. S. Ginsberg, and R. A. Lerner (ed.), *Vaccines 89: modern approaches to new vaccines including prevention of AIDS*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 19. Lin, Y. L., and B. A. Askonas. 1981. Biological properties of an influenza A virus-specific killer T cell clones. Inhibitor of virus replication *in vivo* and induction of delayed-type hypersensitivity reactions. *J. Exp. Med.* **154**:225–234.
 20. Lowry, O. H., N. J. Roseborough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275.
 21. Lukacher, A. E., V. L. Braciale, and T. J. Braciale. 1984. *In vivo* effector functions of influenza virus-specific cytotoxic T lymphocyte clones is highly specific. *J. Exp. Med.* **160**:814–826.
 22. Moore, M. W., F. R. Carbone, and M. J. Bevan. 1988. Introduction of soluble protein into the class-I pathway of antigen processing and presentation. *Cell* **54**:777–785.
 23. Nixon, D. F., A. R. M. Townsend, J. G. Elvin, C. R. Rizza, J. Gallwey, and A. J. McMichael. 1988. HIV-1 gag-specific cytotoxic T lymphocytes defined with recombinant vaccinia virus and synthetic peptides. *Nature (London)* **336**:484–487.
 24. Oldstone, M. B. A., J. L. Whitton, H. Lewicki, and A. Tishon. 1988. Fine dissection of a nine amino acid glycoprotein epitope, a major determinant recognized by lymphocytic choriomeningitis virus-specific class I-restricted H-2D^b cytotoxic T lymphocytes. *J. Exp. Med.* **168**:559–570.
 25. Parekh, B. S., and M. J. Buchmeier. 1986. Proteins of lymphocytic choriomeningitis virus: antigenic topography of the viral glycoproteins. *Virology* **153**:168–178.
 26. Plata, F., B. Autran, L. P. Martins, S. Wain-Hobson, M. Raphaël, C. Mayaud, M. Denis, J.-M. Guillon, and P. Debre. 1987. AIDS virus-specific cytotoxic T lymphocytes in lung disorders. *Nature (London)* **328**:348–351.
 27. Reddehase, M. J., W. Mutter, K. Münch, H.-J. Bühring, and U. H. Koszinowski. 1987. CD8-positive T lymphocytes specific for murine cytomegalovirus immediate-early antigens mediate protective immunity. *J. Virol.* **61**:3102–3108.
 28. Takahashi, H., J. Cohen, A. Hosmalin, K. B. Ceasa, R. Houghton, J. L. Cornette, C. DeLisi, B. Moss, R. N. Germain, and J. A. Berzofsky. 1988. An immunodominant epitope of the human immunodeficiency virus envelope glycoprotein gp160 recognized by class-I major histocompatibility complex molecule-restricted murine cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA* **85**:3105–3109.
 29. Taylor, P. M., J. Davey, K. Howland, J. B. Rothbard, and B. A. Askonas. 1987. Class-I MHC molecules rather than other mouse genes dictate influenza epitope recognition by cytotoxic T cells. *Immunogenetics* **26**:267–272.
 30. Townsend, A. R. M., J. Rothbard, F. M. Gotch, G. Badhadur, D. Wraith, and A. J. McMichael. 1986. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short peptides. *Cell* **44**:959–968.
 31. Walker, B. D., C. Flexner, T. J. Paradis, T. C. Fuller, M. S. Hirsch, R. Schooley, and B. Moss. 1988. HIV-1 reverse transcriptase is a target for cytotoxic T lymphocytes in infected individuals. *Science* **240**:64–68.
 32. Whitton, J. L., J. R. Gebhard, H. Lewicki, A. Tishon, and M. B. A. Oldstone. 1988. Molecular definition of a major cytotoxic T-lymphocyte epitope in the glycoprotein of lymphocytic choriomeningitis virus. *J. Virol.* **62**:687–695.
 33. Whitton, J. L., P. J. Southern, and M. B. A. Oldstone. 1988. Analyses of the cytotoxic T lymphocyte responses to glycoprotein and nucleoprotein components of lymphocytic choriomeningitis virus. *Virology* **162**:321–327.
 34. Whitton, J. L., A. Tishon, H. Lewicki, J. Gebhard, T. Cook, M. Salvato, E. Joly, and M. B. A. Oldstone. 1989. Molecular analyses of a five-amino-acid cytotoxic T-lymphocyte (CTL) epitope: an immunodominant region which induces nonreciprocal CTL cross-reactivity. *J. Virol.* **63**:4303–4310.
 35. Zinkernagel, R. M., and P. C. Doherty. 1979. MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determine T-cell restriction-specificity, function, and responsiveness. *Adv. Immunol.* **27**: 51–178.