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Vaccination To Prevent Persistent Viral Infection†

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Persistent virus infections are increasingly being recognized as a significant cause of human morbidity and mortality. To establish persistence, a virus must establish infection and evade eradication by the host immune response, in particular by cytotoxic T lymphocytes (CTL). We have studied a virus that establishes persistence in part by suppressing the CTL response of the infected host. The virus persists in many cell types, including lymphocytes and macrophages. We show that prior immunization with a vaccine designed to induce CTL (in the absence of antiviral antibody) confers complete protection against subsequent establishment of persistence in all tissues analyzed. The vaccine can be designed to express as few as 10 amino acids of a viral protein that comprise the CTL epitope. Further, two CTL epitopes for two discrete MHC haplotypes can be successfully used in a single vaccine that protects both strains of mice. Hence, a "string of CTL epitopes" (beads) concept for vaccination is feasible. Finally, the CTL vaccine provided protection against the establishment of persistence by an immunosuppressive virus.

The recognition that viruses can persist for life in their hosts and as a consequence cause chronic diseases (2, 10, 26) is an important advance of modern virology. One hallmark of persistent infection by DNA or RNA viruses is their ability to exist within selected cells, especially lymphocytes and/or macrophages, of the immune system (reviewed reference 13). Indeed, with several such persistent or latent viruses, lymphocytes and macrophages serve not only as a source for harboring the viral genome but also as transporters that carry viruses to multiple sites (8, 20) and as potential factories where viruses can reactivate, thereby reinitiating infection (8, 9, 20, 21).

The incidence of many acute viral diseases (smallpox, polio, measles, mumps, etc.) has fallen dramatically as a result of vaccination. However, while such vaccinations protect against disease, they do not necessarily prevent infection. For example, poliovirus can still infect, and replicate in, the gut tissues of vaccinated individuals; however, the primed immune response is sufficiently rapid and effective to prevent extensive virus production and viral spread to nervous tissue. This viral replication in immunized hosts would present a problem when one is dealing with persistent or latent viruses, since it provides the virus a window of opportunity in which to establish persistence and evade the host immune response. Therefore, vaccine strategies to protect against viruses that persist or cause latency need to differ from those used to combat acute viral diseases (14, 22). Protection against persistent or latent viruses may require effective prevention of infection in the immunized host, and any infection which does occur must be recognized at a early stage and eradicated. We have focused on the second requirement, by evaluating the role of cytotoxic T lymphocytes (CTL) in countering persistent viral infection. Although neutralizing antibody produced by vaccines to combat infection is effective against cell-free virus in body fluids, it is generally inefficient against cell-associated virus of persistent infection. CTL attack infected cells whose surfaces express viral antigens (peptide) bound to major histocompatibility complex (MHC) glycoproteins (16, 27, 31). This attack system is highly efficient in that 10^2 or fewer viral molecules are required for T-cell activation and lysis, compared with 10^6 or more viral molecules needed for antiviral antibody- and complement-mediated lysis (6, 24, 25). Additionally, because CTL can recognize nonstructural early viral antigens before structural late antigens are made (reviewed reference 16), infected cells can be killed before the virus can assemble and release infectious progeny or when minimal amounts of early proteins are being produced, thus preventing virus spread.

The CTL response in an individual is frequently limited to recognition of a few discrete peptide sequences on a viral protein, reflecting the limited numbers of viral peptides which bind with sufficient affinity to the MHC class I molecules. Using molecular genetic and biochemical analyses, we have mapped, on several MHC backgrounds, the epitopes on lymphocytic choriomeningitis virus (LCMV) Armstrong strain (Arm) with which CTL interact. In the context of restriction by MHC class I molecules for the $H\text{-}2^d$ haplotype, >96% of the bulk or clonal response CTL recognize LCMV nucleoprotein (NP) amino acids (aa) 119 to 127 (restricted to the L^d allele) (29). In the $H\text{-}2^b$ environment (all restricted by the D^b allele), CTL recognize NP aa 396 to 404 (30) and glycoprotein (GP) aa 33 to 41 (12) and 278 to 286 (19). Identification of these sequences enabled us to develop strategies to manipulate this interaction.

Here, we report successful vaccination against persistent viral infection. Immunization with a vaccine designed to induce CTL, in the absence of antiviral antibody, confers protection against subsequent establishment of persistence by an immunosuppressive virus. Replication of virus was prevented in all tissues as well as cells comprising the immune system, i.e., macrophages and lymphocytes. The vaccine could be designed to express as few as 10 amino acids that form the viral peptide functioning as a CTL epitope. Two different CTL epitopes for two distinct MHC

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TABLE 1. Prevention by CTL vaccine of persistent infection[a]

Mouse strain	MHC *KIDL* haplotype	LCMV NP aa expressed	Vaccine effectiveness[b]	
			Virus in mice[c]	Mean PFU/ml of serum
C57BL/6	*bbb*	1–201	30/30	1.4×10^5
	bbb	None	14/14	2.4×10^5
	bbb	1–558	0/8	0
BALB/WEHI	*dddd*	1–201	0/10	0
	dddd	None	5/5	1.7×10^5
SWR/J	*qqqq*	1–201	0/24	0
	qqqq	None	8/8	2.7×10^5
C3H.Q	*qqqq*	1–201	0/6	0
	qqqq	None	2/2	8.2×10^4
PL/J[d]	*uuu*	1–201	0/5	0

[a] Mice received a single inoculation i.p. of 2×10^7 PFU of a recombinant VV vaccine expressing LCMV NP aa 1 to 201 or LCMV NP aa 1 to 558. Six weeks later, mice were challenged i.v. with 2×10^6 PFU of LCMV Arm cl 13.
[b] Determined 28 days after LCMV Arm cl 13 challenge.
[c] Number of mice with virus in sera/total number of mice in each group.
[d] PL/J mice inoculated only i.v. with 2×10^6 PFU of LCMV Arm cl 13 routinely died of a $CD8^+$-dependent CTL-mediated immunopathologic disease (26a).

haplotypes were successfully used in one vaccine, validating the string-of-beads concept for vaccinating an outbred population such as humans.

Virus, construction of vaccines, vaccination, and challenge. The origin, cloning, and plaque purification of LCMV Arm clone (cl) 13 and LCMV Arm cl 53b have been described elsewhere (1, 7, 23). Virus was quantitated by standard plaque assay on Vero cells (7). Cells containing infectious virus were quantitated by a standard infectious center procedure (3). Construction and use of a recombinant vaccinia virus (VV) vaccine expressing the full-length NP of LCMV Arm (VV NP aa 1 to 558) Cl 53b or the first 201 amino acids (VV NP aa 1 to 201) have been published elsewhere (12, 18, 29). By using a similar approach, recombinant VV vaccines that expressed the M3 peptide ([M]KAVYNFATCG[VTS]; the GP1 CTL epitope that protects *H-2*^{b} mice) (12), MG4 peptide ([M]ERPQASGVYMGNLT[VTS]; the NP CTL epitope that protects *H-2*^{d}, *H-2*^{q}, and *H-2*^{u} mice) (18, 29), or MG3 plus MG4 ([M]KAVYNFATCG[GRTM]ERPQASG VYMGNLT[VTS]) were synthesized. The amino acids in brackets were added to provide a start for translation (M), spacers (VTS) preceding a stop codon, or linkers (GRTM) to allow expression of both epitopes in a single peptide.

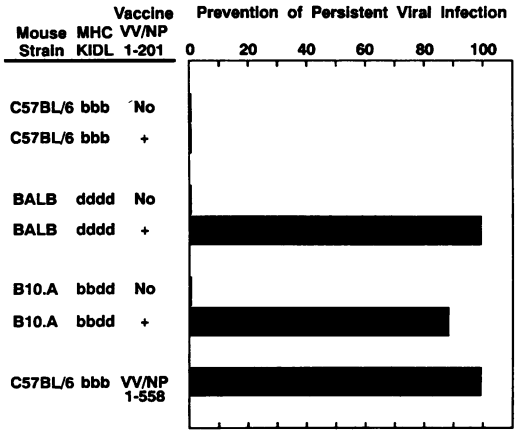

FIG. 1. Involvement of D^dL^d class I MHC restriction in preventing persistent viral infection of BALB and B10.A 18r mice. Mice (groups of eight or more) received a single inoculation of 2×10^7 PFU of a recombinant VV vaccine expressing LCMV NP aa 1 to 201 or LCMV NP aa 1 to 558 i.p. Six weeks later, mice were challenged with 2×10^6 PFU of LCMV Arm cl 13 i.v. Their blood sampled 30 days after cl 13 challenge was tested by standard plaque assay on Vero cells. Prevention of persistent viral infection (number of mice not having virus/total number inoculated) was associated with the absence of virus PFU in the blood.

BALB/WEHI or BALB/c/byj (*H-2K*^{d}*I*^{d}*D*^{d}*L*^{d}), C57BL/6 (*H-2K*^{b}*I*^{b}*D*^{b}), C3H.Q (*H-2K*^{q}*I*^{q}*D*^{q}), PL/J (*H-2K*^{u}*I*^{u}*D*^{u}), SWR/J (*H-2K*^{q}*I*^{q}*D*^{q}), and B10.A 18r (*H-2K*^{b}*I*^{b}*D*^{d}*L*^{d}) mice were obtained from the vivarium of The Scripps Research Institute. Various experiments used six to eight mice per group unless noted otherwise. Mice at 6 to 8 weeks of age, of either sex, were immunized with a single intraperitoneal (i.p.) inoculation of 2×10^7 PFU of recombinant VV (VV NP aa 1 to 558, VV NP aa 1 to 201, VV GP aa 33 to 42, VV NP aa 117 to 130, or VV GP aa 34 to 42 plus NP aa 117 to 130 or VV SC11 (which is devoid of LCMV sequences) in 0.2 ml. Six to eight weeks later, mice received 2×10^6 PFU of LCMV Arm cl 13 intravenously (i.v.). This inoculation routinely led to persistent viral infection in otherwise untreated mice (1, 3, 17).

CTL generation and detection. 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 VV SC11. 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 (12, 29).

Secondary cytotoxic effector cells were generated by immunizing mice i.p. with 2×10^5 PFU of LCMV Arm or with 2×10^7 PFU of VV recombinants. Six weeks later, spleens were removed, freed of erythrocytes, and restimulated in vitro with syngeneic LCMV Arm-infected peritoneal exudate macrophages as described previously (12). For restimulation, 2×10^5 macrophages, irradiated with 2,000 rads for 30 min and infected 48 h previously with LCMV Arm (multiplicity of infection of 1), were incubated with 4×10^6 spleen cells per ml of RPMI 1640 medium supplemented with calf serum (12, 29). After 5 days at 37°C, effector cells were harvested and tested by the ^{51}Cr release assay.

A standard 6-h ^{51}Cr release assay was used as described previously (3, 12, 19, 29). Target cells were infected with

TABLE 2. Prevention by CTL vaccine of establishment of persistent viral infection[a]

Experimental condition	Mouse no.	Day 23, PFU/ml of blood	Day 107, no. of infectious centers/10^6 PBMN	Day 195, in situ hybridization[b]		PCR		
				PBMN	Spleen	Spleen	Liver	Brain
Vaccinated	SWR/J 36	0	0	0/1,512	0/1,500	Nil	Nil	Nil
Nonvaccinated	SWR/J 6	5×10^5	103	ND	49/959	+	+	+
Vaccinated	SWR/J 38	0	0	1/2,800	0/2,500	Nil	Nil	Nil
Nonvaccinated	SWR/J 7	6×10^5	110	60/1,600	60/630	+	+	+
Vaccinated	SWR/J 40	0	0	0/1,280	1/2,500	Nil	Nil	Nil
Nonvaccinated	SWR/J 41	9×10^5	200	220/1,800	36/1,296	+	+	+

[a] Mice received a single inoculation i.p. of 2×10^7 PFU of a recombinant VV vaccine expressing LCMV NP aa 1 to 201. Six weeks later, mice were challenged i.v. with 2×10^6 PFU of LCMV Arm cl 13. Days reflect days on which assays were performed after cl 13 challenge. PBMN, peripheral blood mononuclear cells. Data were recorded for six mice. For similar results with other vaccinated mice, see Table 1.

[b] Number of cells containing viral nucleic acid sequences/total number of cells counted. ND, not determined. For details of PFU, infectious center, and in situ assays, see Materials and Methods.

LCMV Arm at a multiplicity of infection of 1 48 h before the assay or with recombinant VV 16 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 ratios of 50:1 to 6.25:1. The percent specific lysis was calculated as 100 × (counts per minute released by CTL − counts per minute of spontaneous release)/(counts per minute of total release − counts per minute of spontaneous release).

Nucleic acid analysis. In situ hybridization of splenic or peripheral blood mononuclear cells by using [^{35}S]cDNA to LCMV NP was done according to our published methods (17).

Northern (RNA) blot analysis and polymerase chain reaction (PCR) priming were performed as described previously (5). Briefly, RNA was harvested from tissues by the method of Chomczynski and Sacchi (4). RNA was immobilized on a nylon membrane and probed with LCMV Arm NP (5). For PCR, LCMV NP RNA was amplified by Perkin-Elmer's protocol and 30 cycles of 95°C for denaturation and 58°C for annealing. The PCR fragments were sized with Bethesda Research Laboratories' 100-bp ladder. The PCR product was resolved on a 2% agarose gel and visualized with ethidium bromide. Oligonucleotides used were 21-mers, primer A (CAGTTATAGGTGCTCTTCCGC [complementary to nucleotides 1994 to 1974 of LCMV NP]) (5) and primer B (AGATCTGGGAGCCTTGCTTTG [nucleotides 1706 to 1726 of LCMV NP]), which amplify a 289-bp fragment.

One injection of a VV-LCMV vaccine containing NP aa 1 to 201 totally protected *H-2*^{d} mice from developing persistent infection (Table 1). The NP epitope aa 116 to 127 is also a sequence recognized by CTL in *H-2*^{q} and *H-2*^{u} mice (18), and animals of these haplotypes were similarly protected by a vaccine containing that sequence (Table 1). Further, NP aa 1 to 201 do not generate either neutralizing or enzyme-linked immunosorbent assay (ELISA) binding antibody; thus, protection by this vaccine is dependent on CTL activity (12, 18, 29, 30). Indeed, both vaccine preparations induced MHC-restricted LCMV-specific CTL, as demonstrated by generation of memory CTL in vitro (data not shown) (17, 19). However, in *H-2*^{b} mice, which do not possess a CTL epitope contained within this vaccine (NP aa 1 to 201), vaccination failed to prevent persistent infection, although vaccination with full-length NP (aa 1 to 558) confers complete protection upon these mice (Table 1). *H-2*^{b} mice have a CTL epitope at NP aa 396 to 404 (30). Protection maps with the class I MHC molecules which present the epitopes (Fig. 1) and the generation of anti-LCMV CTL. Interestingly, PL/J mice, when inoculated with cl 13 at doses of 1×10^5 to 2×10^6 PFU i.v., unexpectedly died 6 to 8 days following viral inoculation. Studies to be reported elsewhere (26a) show that death was due to CD8$^+$ LCMV-specific CTL.

Earlier in vitro studies using non-*H-2*^{d} cells transfected with the *L*^{d} gene (29) documented that the NP epitope aa 119 to 127 was restricted by *L*^{d}. To determine whether the *K* or *DL* end of the MHC locus was required for protection against viral persistence in vivo, groups of C57BL/6 (*H-2K*^{b}*I*^{b}*D*^{b}), BALB/CDJ (*H-2K*^{d}*I*^{d}*D*^{d}*L*^{d}), and B10.A 18r (*H-2K*^{b}*I*^{b}*D*^{d}*L*^{d}) mice (8 to 15 per group) were vaccinated with VV NP 1 to 201 and challenged 6 weeks later with cl 13. As seen in Fig. 1, B10.A 18r mice and BALB mice were alike in that both were protected but were different from C57BL/6

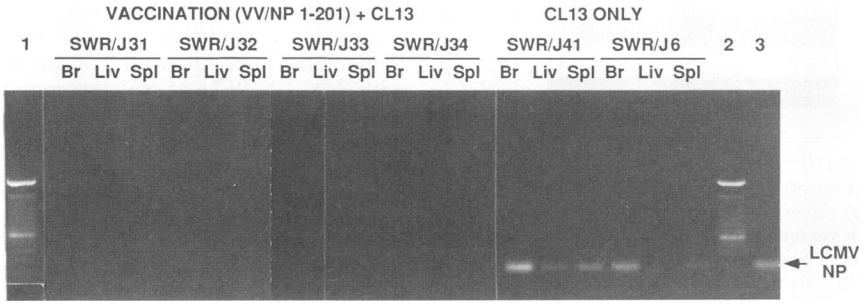

FIG. 2. PCR analysis showing that mice receiving the recombinant VV-LCMV vaccine containing NP aa 1 to 201 6 weeks prior to cl 13 challenge failed to show viral nucleic acid sequences in the brain (Br), liver (Liv), or spleen (Spl). Sixty days after cl 13 challenge, mice were sacrificed and RNA was harvested from tissue. LCMV NP RNA was amplified by PCR as described in Materials and Methods.

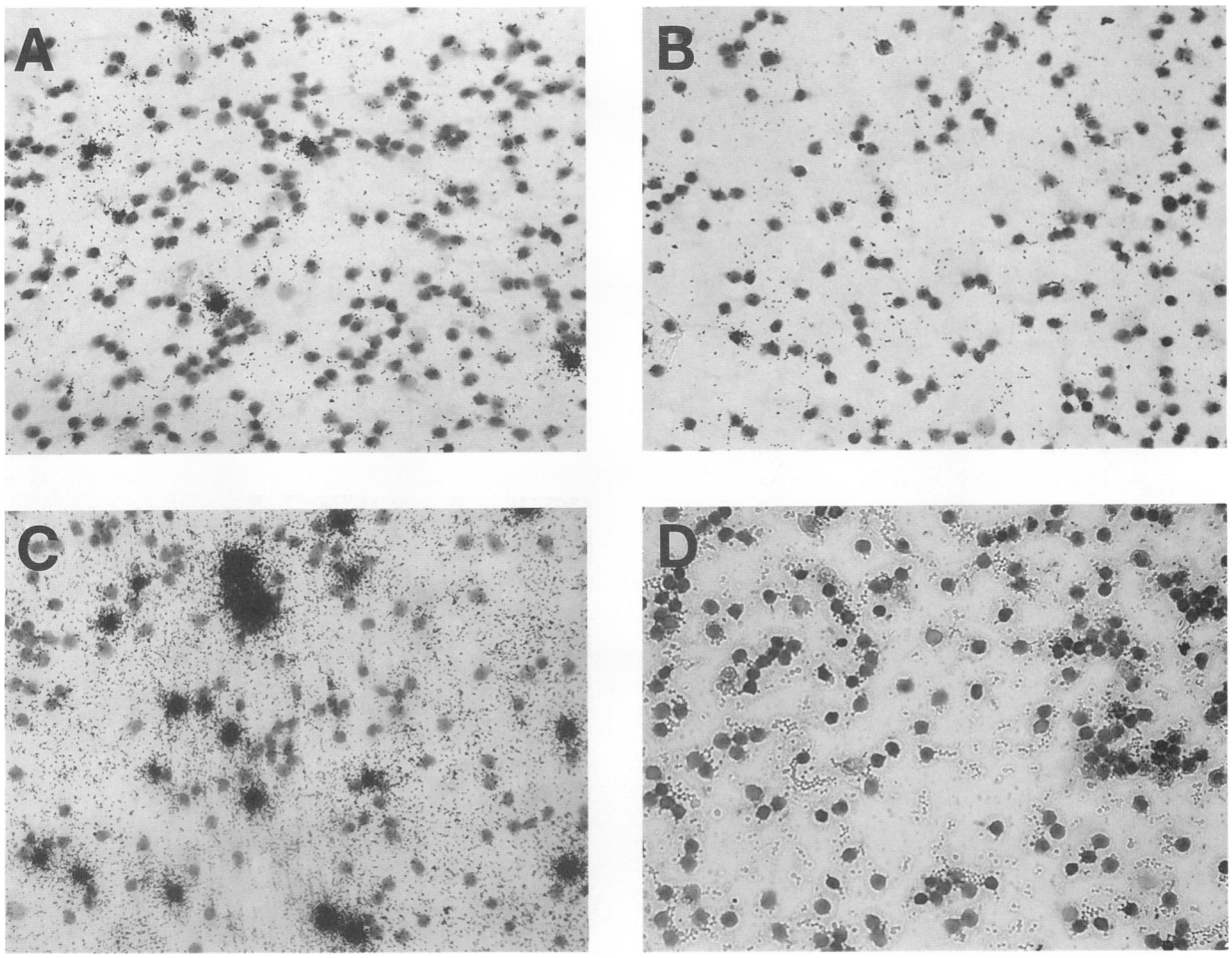

FIG. 3. In situ hybridization of splenic lymphoid cells (A and B) and peripheral blood mononuclear cells (C and D), using [^{35}S]cDNA to LCMV NP (see Materials and Methods). (A and C) SWR/J mouse 7, which did not receive the recombinant VV-LCMV NP vaccine containing aa 1 to 201; (B and D) SWR/J mouse 38, which did receive the vaccine (see Table 2). Six weeks later, both mice received 2×10^6 PFU of cl 13 i.v. Analysis of viral nucleic acid sequences in lymphoid cells was performed 195 days after the cl 13 challenge.

mice, which developed persistent infection. Hence, in vivo protection with VV NP aa 1 to 201 was associated with the *DL* end of the MHC class I complex.

Intravenous inoculation of 2×10^6 PFU of cl 13, a variant of LCMV Arm (1, 3, 17), into 6-week-old or older C57BL/6 ($H\text{-}2^b$), BALB/WEHI or BALB/CbyJ ($H\text{-}2^d$), B10.A 18r ($H\text{-}2^{bd}$), SWR/J ($H\text{-}2^q$), or C3H.q ($H\text{-}2^q$) mice suppresses their virus-specific CTL response and causes persistent infection. Persistence is demonstrated by recovery from blood and tissues of $1 \times 10^{3.5}$ to 1×10^6 PFU of virus and by detection of viral nucleic acid sequences in tissues from these mice throughout most of their lives (Tables 1 and 2). The NP epitope aa 116 to 127 is a shared CTL epitope for BALB ($H\text{-}2^d$), SWR/J ($H\text{-}2^q$), and PL/J ($H\text{-}2^u$) mice (18); after a single i.p. inoculation of 2×10^7 PFU of recombinant vaccine containing cDNA of LCMV encoding NP aa 1 to 201 (12) and challenge 6 weeks later with 2×10^6 PFU of cl 13 i.v., all mice of these strains were protected from persistent viral infection (Table 1). In contrast, this vaccine failed to protect C57BL/6 mice, which do not bind this sequence but rather possess a CTL epitope at LCMV NP aa 396 to 404 (30). As seen in Table 1, when this sequence was included in the recombinant vaccine expressing NP 1 to 558, C57BL/6 mice were protected from persistent infection when challenged with LCMV cl 13.

Above we demonstrate the absence, over a short term, of virus in vaccinated hosts. However, the formal possibility exists that virus is latent and undetected but may emerge at a later date. Therefore, individually vaccinated mice and unvaccinated controls were monitored for 1.5 years after viral inoculation. As observed in Table 2 and Fig. 2 to 4, the vast majority of vaccinated mice contained no viral nucleic acid sequences. Equally important was the absence of infectious virus, as determined by infectious center analysis, or of viral nucleic acid sequences, as assayed by PCR and in situ hybridization in lymphocytes and/or monocytes of vaccinated mice (Table 2; Fig. 2 to 4).

In a final series of experiments, recombinant VV vaccines were constructed to include oligonucleotides that encoded LCMV NP aa 119 to 127 or LCMV GP aa 33 to 42. A methionine was added to the beginning of each sequence (NP 117 ERPQASGVYMGNL and GP 33 KAVYNFATCG) followed by stop codons. All six C57BL/6 mice given a single inoculation of recombinant VV expressing only LCMV GP MKAVYNFATCG were completely protected from persistent infection, since their blood contained no viral PFU,

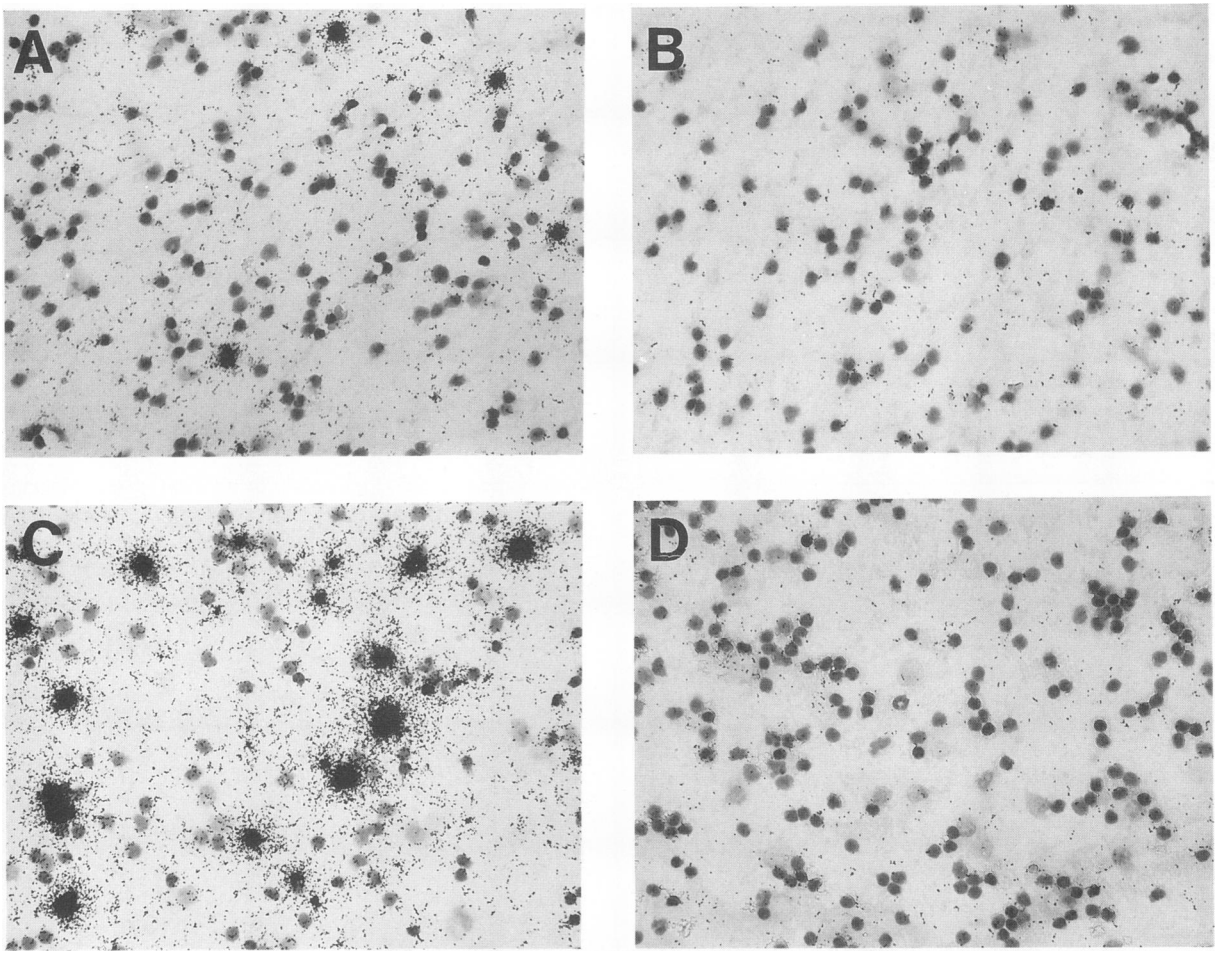

FIG. 4. In situ hybridization of splenic lymphoid cells (A and B) and peripheral blood mononuclear cells (C and D), using [^{35}S]cDNA to LCMV NP. (A and C) BALB/CbyJ mouse 12, which did not receive the recombinant VV-LCMV vaccine containing NP aa 1 to 201 vaccine; (B and D) BALB/CbyJ mouse 40, which did receive the vaccine. Six weeks later, both mice received 2×10^6 PFU of cl 13 i.v. Analysis of viral nucleic acid sequences in lymphoid cells was performed 195 days after the cl 13 challenge.

their peripheral blood mononuclear cells did not score as infectious centers, and their tissues were free from viral nucleic acid sequences (Fig. 5). Further, antibodies to LCMV were not generated by using either ELISA or immunofluorescence assays (12). In contrast, age- and sex-matched C57BL/6 mice that either received no vaccine or received recombinant VV vaccine expressing only LCMV NP aa 117 to 129 all showed infectious virus in their blood ranging from 2×10^4 to 8×10^4 PFU/ml of serum. SWR/J mice receiving a VV recombinant expressing LCMV NP aa 117 to 129 were protected from developing persistent infection resulting from a later challenge with 2×10^6 PFU of LCMV Arm cl 13 administered i.v. When a single VV recombinant containing oligonucleotides encoding KAVYN FATCG and ERPQASGVYMGNL was used, both $H\text{-}2^b$ and $H\text{-}2^d$ mice were protected from developing persistent infection after LCMV Arm cl 13 challenge (Fig. 5).

In summary, the immune system functions to recognize foreign materials and then attack and destroy them. Thus, the immune response against virus or virus-infected cells is a balance between protective immunity and immunopathologic injury. Consequently, care must be taken in design, dosage, and timing of vaccination to ensure wide and effective protection without harmful side effects (11, 15). In this report, we document the construction and use of a vaccine that prevented the establishment of persistent viral infection. Importantly, the CTL vaccine protected against a virus that possessed the ability to persist and cause immunosuppression. Interestingly, the vaccine construct contained LCMV amino acid sequences NP aa 1 to 201 or GP aa 1 to 51, which generated CTL responses in $H\text{-}2^d$, $H\text{-}2^q$, $H\text{-}2^u$, or $H\text{-}2^b$ mice, respectively. Vaccination into the appropriate *H-2* haplotype prevented the establishment of persistent infection, including infection of host lymphocytes or monocytes. This vaccine could be modified to express as few as 10 amino acids of viral sequence and still provide complete protection. Incorporation of two disparate sequences that elicit CTL responses in different haplotypes provided protection for both haplotypes. Similar observations of the utility of the string-of-beads concept were recently seen for control of acute viral infection (28). Since, for several MHC haplotypes, similar viral sequences promote CTL generation and recognition, vaccines containing these shared subunits may provide highly effective protection for a number of MHC backgrounds. This observation coupled with the use of short sequences (10 amino acids) in an effective vaccine indicates

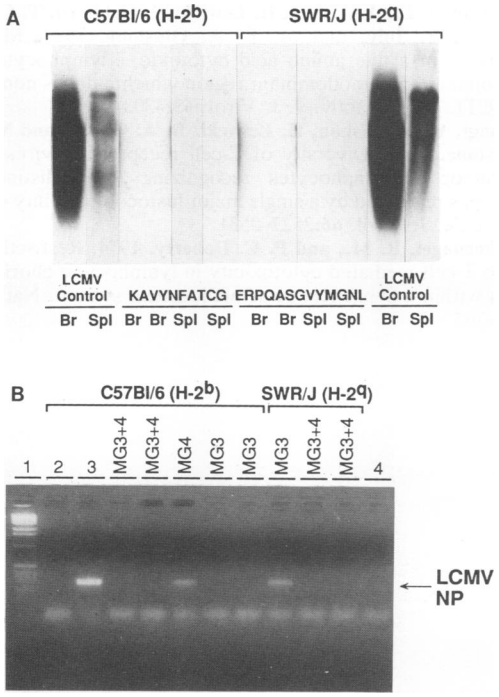

FIG. 5. Evidence that vaccination with recombinant VV that contains oligonucleotides which encoding peptides corresponding to CTL epitopes protects mice from persistent viral infection. C57BL/6 (*H-2*^{b}) or SWR/J (*H-2*^{q}) mice received a single inoculation of 2×10^7 PFU of a recombinant VV vaccine expressing MG3 peptide, MG4 peptide, or MG3 plus MG4 (for descriptions, see text). Control mice received either no vaccination or VV alone (not expressing MG3 or MG4). Six weeks later, all mice received 2×10^6 PFU of cl 13 i.v. (A) Northern blot of two vaccinated C57BL/6 and SWR/J mice that were representative of the four to six mice studied from each group. The blot was hybridized to an LCMV NP probe. Br, brain; Spl, spleen. (B) PCR analysis in other vaccinated mice (groups of four each). Lane 1 contains 1-kilobase markers; lanes 2, 3, and 4 are from a C57BL/6 mouse that did not receive the vaccine but was challenged with 2×10^6 PFU of cl 13. In lane 2, the PCR is run in the absence of reverse transcriptase but with RNA extracted from the brain of a cl 13-infected mouse; in lane 3, the reverse transcriptase is present. In lane 4, the PCR is run with water substituting for the RNA. The expected viral NP amplified fragment is shown as a 289-bp product.

the feasibility of inserting multiple sets of such sequences from the same and different viral proteins that bind specifically to a wide variety of MHC glycoproteins and hence offer vaccine protection to an outbred population such as humans.

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