A Recombinant Minigene Vaccine Containing a Nonameric Cytotoxic-T-Lymphocyte Epitope Confers Limited Protection against *Listeria monocytogenes* Infection†

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We have previously shown that vaccines expressing virus-derived cytotoxic-T-lymphocyte (CTL) epitopes as short minigenes can confer effective protection against virus challenges, and here we extend these studies to the bacterium *Listeria monocytogenes*. Host defense against this important human pathogen appears largely T cell mediated, and a nonamer CTL epitope from the listeriolysin O (LLO) protein has been identified in BALB/c mice. We have synthesized this nonamer as a minigene, expressed it in a recombinant vaccinia virus (VV-list), and used this to immunize mice. Memory CTLs cultured from VV-list-immunized mice specifically lyse target cells pulsed with a nonamer peptide identified at LLO amino acid residues 91 to 99. Four weeks postimmunization, mice were challenged with *L. monocytogenes*. By day 6 following challenge with a sublethal dose of *L. monocytogenes*, mice immunized with VV-list showed a ~2,000- to 6,000-fold reduction in bacterial CFU in the spleen and liver. At this time point, with control mice, bacteria were readily detectable by Gram stain of the liver but were undetectable in the VV-list-immunized animals. Additionally, when a normally lethal dose of bacteria was given, death was delayed in VV-list-immunized animals. This study has demonstrated that a single immunization with a recombinant vaccinia virus bearing only nine amino acids from a bacterial pathogen can induce specific CTLs able to confer partial protection against bacterial challenge.

Listeria monocytogenes is an important human pathogen; clinical infection remains rare, but when it occurs, it has a mortality rate of approximately 25% (10), sufficient to render this organism one of the commonest food-borne causes of bacterial lethal infection in the United States. Immunity to *L. monocytogenes* has long been known to be cell mediated. Clearance of primary infection requires activation of macrophages by cytokines produced by T and NK cells (27, 30). Several studies have shown that macrophages, neutrophils, and natural killer cells are responsible for host defense in the early stage of infection (1, 8), leading to the idea that specific T-cell responses are developed later, to finally eradicate bacteria, and function mainly to protect against disease caused by secondary infection (22, 24). Previous studies on immunity to listerial antigens have found that listeriolysin O (LLO) is crucial for induction of protective immunity. LLO, a hemolysin, is the major virulence factor (14) whose function is to lyse the phagosome membrane, enabling bacteria to enter and multiply in the host cytoplasm (13). This protein is a major target for CD8⁺ T cells (2, 5), and an H2-K^d-restricted epitope has been identified at LLO amino acid residues 91 to 99 (LLO 91–99) (34). Adoptive transfer of a CD8⁺ cytotoxic-T-lymphocyte (CTL) clone specific for this epitope was shown to protect naive mice from bacterial challenge (18).

We have previously shown (25, 26, 45) that expression of a single CTL epitope can protect against subsequent challenge with a normally lethal dose of virus. Furthermore, CTL epitopes can be linked in a “string of beads” to broaden the protective efficacy of a vaccine (43). We wished to determine

whether this approach to vaccination was applicable also to the control of bacterial infection, and the presence of a precisely defined CTL epitope, of known biological importance, led us to select listeria as the test microbe.

In this report, we describe the construction of a recombinant vaccinia virus containing the nonamer CTL epitope LLO (LLO 91–99) in the form of a minigene. The encoded epitope is recognized by CTLs of appropriate specificity, and a single inoculation of the recombinant vaccinia induces anti-LLO CTLs. CTL induction results in protection against listerial challenge by several criteria, including accelerated clearance from the liver and spleen and delay of lethal outcome.

MATERIALS AND METHODS

Mice. Male and female BALB/cByJ mice were obtained from the animal facility of the Scripps Research Institute. Mice were used when 8 to 9 weeks old.

Bacteria. *L. monocytogenes* ATCC 43251 was a kind gift from J. T. Harty of the University of Iowa. A single colony from a master Trypticase soy agar plate was inoculated in Trypticase soy broth and grown at 37°C on a shaker. The culture was harvested at the log phase of growth (3.5×10^8 CFU/ml), and aliquots were stored at –70°C until used.

Vaccinia constructs. Complementary oligonucleotides encoding the defined nonamer CTL epitope of LLO from *L. monocytogenes* (34) were synthesized at the Core Facility of The Scripps Research Institute, hybridized, and cloned into the vaccinia virus transfer plasmid pVVT2, which is similar to pSC11 (6) but has additional enzyme sites to facilitate cloning (42a). While exact minigene epitopes (9 residues flanked by ATG and stop codons) are often presented, we have previous experience of some exact minigene epitopes failing to be presented (42a). However, our previous experience with minigenes has shown that their attachment to the amino acid triplet VTS invariably allows processing and presentation (42, 43); for this reason, this triplet was included in the minigene oligonucleotide. The sequence of the designed minigene (ATG GGG TAT AAA GAC GGC AAC GAA TAT ATC GTA ACT AGT TAA) was confirmed by DNA sequencing (Sequenase version 2; United States Biochemical, Cleveland, Ohio), and 5 µg of DNA was transfected into vaccinia virus-infected cells. Recombinant viruses were isolated as described previously (44) and subjected to PCR-based DNA sequencing (39) with the fmol DNA sequencing system (Promega Corporation, Madison, Wis.) to ensure the correct incorporation of the minigene. This recombinant virus was named VV-list.

In vitro cytotoxicity assay. The standard method for assay of in vitro cytotox-

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icity was used as described previously (32). Target cells were produced either by (i) incubation with or without peptide LLO 91–99 or (ii) infection (at a multiplicity of infection of 3) with VV-list or VV-SC11. Cells were labeled with ^{51}Cr for 1.5 h at 37°C. After washing, 10^4 target cells were plated in each well of a flat-bottom 96-well plate, and effectors were added in triplicate at the effector/target cell ratios described for the individual experiments. Five hours later, 100 μl of supernatant from each well was harvested and counted. The specific lysis was calculated as follows: [(sample release – spontaneous release) $\times$ 100]/(total release – spontaneous release).

Secondary in vitro stimulation of LLO-specific memory CTLs. Mice were immunized with either VV-SC11 (a control vaccinia virus recombinant differing from VV-list only in the absence of the LLO 91–99 sequence) or VV-list, and 6 weeks later, they were sacrificed, their spleens were harvested, and a single cell suspension was produced by use of a loose glass homogenizer. After lysing the erythrocytes with 0.83% NH_4Cl , the cell suspension was washed three times in medium. The cells thus prepared from each mouse were then added to stimulator cells in 12 wells of 24-well plates. The stimulators were syngeneic spleen cells incubated with or without peptide LLO 91–99 for 1.5 h at 37°C and then subjected to irradiation (2,000 rads). Consequently, splenocytes from each mouse were grown in six wells containing peptide LLO 91–99-coated stimulators and six wells containing stimulators without peptide (approximately 2×10^7 cells per well). The growth medium was AIM medium (Gibco BRL, Bethesda, Md.) containing 10% fetal calf serum and 20% MLA.144 cell supernatant (as a source of interleukin 2). Seven days later, each individual well was scored for LLO-specific activity. Cells from each individual well were harvested and counted, and half of each population was divided among triplicate wells for the in vitro cytotoxicity assay, which was carried out as outlined above. Approximately 8.6×10^5 effector cells were added to each well of the cytotoxicity assay (effector-to-target cell ratio, 43:1).

Determination of LD_{50} for *L. monocytogenes*. Groups of BALB/cByJ mice were challenged with *L. monocytogenes* at doses ranging from 10^4 to 10^8 CFU and observed daily. Deaths were recorded. All deaths occurred within 8 days of infection. The 50% lethal dose (LD_{50}) was calculated by the method of Reed and Muench (36) and found to be 2.5×10^7 CFU for this mouse strain.

Evaluation of in vivo protection against sublethal bacterial challenge. Two identical experiments were carried out. In each experiment, three groups of mice (12 mice per group) were immunized by the intraperitoneal route with one of the following: (i) 0.1 LD_{50} of *L. monocytogenes*, (ii) 2×10^7 PFU of VV-list, or (iii) 2×10^7 PFU of VV-SC11. Four weeks later, mice were challenged with a sublethal dose ($\sim \text{LD}_{50}$) of *L. monocytogenes* and were bled and sacrificed at days 2, 4, 6, and 8 after challenge (three mice at each time point, in each experiment). The spleens and livers were taken, weighed, and processed as described below.

Determination of *L. monocytogenes* titer in the liver and spleen. Known weights of liver and spleen from each mouse were homogenized in a known volume of saline with a glass homogenizer. The homogenates were serially diluted and plated on Trypticase soy agars (18). Colonies were counted after 48 h of incubation at 37°C.

Histology. Samples of brain, liver, and spleen were immersed in Bouin's fixative and paraffin embedded, and 3- μm sections were cut and either were stained with hematoxylin and eosin or were Gram stained by Brown and Bann's method for detecting *L. monocytogenes* in the liver and spleen. Slides were photographed with a Nikon microscope.

Evaluation of in vivo protection against lethal dose bacterial challenge. Mice were immunized as for the sublethal challenge (12 mice per group for VV-list and VV-SC11 immunization; 8 mice per group for *L. monocytogenes* immunization) and 4 weeks later were challenged with $\sim 8 \text{ LD}_{50}$ of virulent *L. monocytogenes*. Mice were observed daily for 14 days to determine the survival rate.

Sequencing of the LLO 91–99 epitope from *L. monocytogenes* growing in immunized mice. To attempt to enrich for bacteria carrying a mutated form of the CTL epitope, the liver homogenate taken from VV-list-immunized mice on day 6 or 8 after listerial challenge was injected intraperitoneally into mice immunized 4 weeks previously with VV-list. On day 6 after challenge, the spleens and livers were taken, homogenized, and plated in serial dilution on Trypticase soy agar plates. After 48 h at 37°C, single colonies were picked and lysed, and PCR was performed (12). Primer 1 (TCG AAA AGA AAC ACG CGG) and primer 2 (CAC AAC TTG AAT GTC TGC) flank the LLO 91–99 epitope in the *L. monocytogenes* genome and represent bases 208 to 225 and 404 to 386, respectively (31). The thermophilic DNA polymerase used was Deep Vent DNA polymerase (New England Biolabs, Inc., Cambridge, Mass.).

RESULTS

Construction of a recombinant vaccinia virus expressing the *L. monocytogenes* 91–99 epitope. The cloning scheme for the LLO 91–99 epitope from *L. monocytogenes* is shown in Fig. 1. The constructed plasmid was transfected into cells along with wild-type vaccinia virus, and a recombinant virus was isolated. This virus was PCR sequenced to confirm correct cloning. To determine if the final recombinant (VV-list) was able to cor-

FIG. 1. Construction of VV-list. The oligonucleotides shown, encoding LLO 91–99 (bases 122 to 148 of the listerial *hly* gene), were cloned into the vaccinia virus transfer plasmid pVVT2 (42a) under the control of the p7.5 promoter as shown. The cloned plasmid was then transfected into cells infected with vaccinia virus (VV-SC11), and TK^- recombinant viruses were selected by β -galactosidase activity. The correct incorporation of the minigene was determined by PCR sequencing of VV-list DNA. MCS, multiple cloning site.

rectly present the encoded *L. monocytogenes* epitope, the macrophage cell line (J774; *H-2^d*) was infected either with VV-list or a control vaccinia recombinant lacking the epitope (VV-SC11), and an in vitro cytotoxicity assay was carried out by using as effector cells a CD8^+ CTL clone (L12.3) specific to this epitope (33). As shown in Fig. 2B, VV-list-infected targets are recognized and lysed by this CTL clone. The level of lysis is comparable to, or better than, that of J774 target cells pulsed with peptide (LLO 91–99) (Fig. 2A). This demonstrates that the minigene-encoded epitope is presented by VV-list.

Induction of specific CTL. Having shown that VV-list expressed the epitope in recognizable form, we attempted to evaluate the capacity of this recombinant virus to induce CTLs specific for this bacterial epitope. First we sought evidence of antilisterial CTLs in the acute response induced by VV-list (i.e., in primary CTLs). Mice were immunized with VV-list or VV-SC11 and sacrificed 7 days later, and their splenocytes were prepared as described in Materials and Methods. Following incubation with appropriate target cells, no antilisterial activity could be demonstrated (data not shown). The inability to detect such primary CTL activity is unsurprising; in our previous studies using recombinant vaccinia viruses, we were unable to detect any primary CTLs directed against the foreign protein. To increase the resolving power of our assay, we

FIG. 2. VV-list expresses the LLO 91-99 minigene in a form recognizable to T cells. An in vitro cytotoxicity assay was carried out with L12.3 cells, a CD8⁺ T-cell clone specific for the LLO 91-99 sequence, as the effector cells. J774 cells (*H-2^d*) were pulsed with peptide LLO 91-99 (A) or infected with either VV-list or VV-SC11 (B). Following a 5-h incubation, specific lysis was determined as described in Materials and Methods. E/T, effector to target cell ratio.

subjected the splenocytes to secondary in vitro stimulation with syngeneic cells and interleukin 2 in the absence or presence of peptide LLO 91-99. Following 7 days of in vitro stimulation, specific CTLs were found in four of six wells of splenocytes from VV-list-immunized mice when either peptide-stimulated (Fig. 3C) or peptide-free (Fig. 3A), while none were found when splenocytes originated from VV-SC11-immunized mice (Fig. 3B and D). The *P* values shown in Fig. 3 demonstrate the level of significance of the lysis of peptide-coated and peptide-free target cells by each effector group and show that for VV-list, there is LLO 91-99 peptide-specific lysis (Fig. 3A and C), while for VV-SC11, no peptide-specific lysis is demonstrable (Fig. 3B and D). The presence of lytic activity in the VV-list-immune wells in the absence of peptide stimulation (Fig. 3A) is explicable, since the presence of syngeneic cells and interleukin 2 can render antigen-specific restimulation inessential. That the lysis seen with VV-list effectors is not artifactual is demonstrated by the absence of such activity in effector cells from VV-SC11-immune mice (Fig. 3B and D). We have also evaluated the statistical significance of the differences between VV-list and VV-SC11 effectors when assayed on peptide-coated target cells. Lysis by VV-list effectors was

significantly greater than lysis by VV-SC11 effectors both in the absence of peptide stimulation (Fig. 3A versus B, *P* < 0.006) and in its presence (Fig. 3C versus D, *P* < 0.02). Thus, VV-list induces CTL responses specific for the LLO 91-99 sequence, and these cells are detectable following secondary in vitro stimulation.

VV-list immunization limits replication and pathogenicity of *L. monocytogenes*. Having shown that VV-list induced detectable CTLs, we wished to determine the biologic effect of this vaccine in combating bacterial challenge. BALB/cByJ mice were immunized with 2×10^7 PFU of VV-list. Positive and negative controls were mice immunized with a sublethal dose of *L. monocytogenes* (0.1 LD₅₀) or with VV-SC11 (2×10^7 PFU), respectively. Four weeks postimmunization, protection was measured by two assays. First, mice were challenged with a sublethal dose of bacteria, and their ability to control bacterial multiplication was determined. Second, mice were challenged with a dose of bacteria sufficient to kill nonimmune animals (~ 8 LD₅₀), and the survival rate of immunized animals was determined.

VV-list-immunized mice appear capable of limiting multiplication of *L. monocytogenes*. At four time points after sublethal bacterial challenge, three mice from each group were sacrificed and the CFU from the spleens and livers were counted. Data for the spleen and liver are presented as log₁₀ CFU in Fig. 4A and B, respectively. Similar patterns were found for both organs. By day 4, mice immunized with VV-list have approximately 70-fold less CFU in the spleen than mice immunized with VV-SC11; in the liver, the difference is only 5-fold and is of questionable significance. Clearer differences emerge by day 6 postinfection. VV-list vaccinees have some 6,000-fold less listeriae in the spleen than do recipients of the control vaccine (Fig. 4A), and hepatic microbial load is 2,300-fold diminished in minigene recipients (Fig. 4B). When compared with mice immunized with intact *L. monocytogenes*, VV-list appears equally successful up to and including the day 6 time point. These results provide evidence that a recombinant containing a single CTL epitope is as effective as intact *L. monocytogenes* in controlling the organism replication on days 4 and 6 after infection. By day 8 following challenge, mice immunized with intact live *L. monocytogenes* have cleared the subsequent challenge organisms from the spleen, although some organisms remain detectable in the liver. In contrast, VV-list-immune animals retain detectable *L. monocytogenes* in the spleen at this time point, although at levels 1 to 2 logs lower than those in control animals. An identical second experiment was carried out, and parallel results were obtained.

Diminished histopathological changes in VV-list-immunized mice. Infection with a pathogenic strain of *L. monocytogenes* resulted in characteristic histologic changes in the liver and spleen of infected mice. Tissues from immunized and challenged mice were analyzed (Fig. 5). Six days following *L. monocytogenes* challenge of nonimmune mice, inflammatory foci were clearly seen in the liver (Fig. 5A, panel 1); these foci were relatively large and numerous. A similar appearance was seen in VV-SC11-immunized mice (Fig. 5A, panel 2), while in mice immunized 4 weeks prechallenge with live *L. monocytogenes*, foci were sparse and very small (Fig. 5A, panel 3). Mice immunized with VV-list (Fig. 5A, panel 4) had fewer and smaller inflammatory foci even than the listeria-immunized hosts. Furthermore, Gram stain of a 3- μ m section from the liver of a VV-SC11-immune animal showed multiple instances of *L. monocytogenes* scattered throughout the tissue (Fig. 5B, panel 1); in contrast, the organism is undetectable in sections from a VV-list-immunized host (Fig. 5B, panel 2), reflecting the reduction in CFU in this organ (Fig. 4B). Finally, splenic

FIG. 3. VV-list immunization induces CTLs specific for LLO 91–99. Mice were immunized with VV-list (A and C) or VV-SC11 (B and D), and 6 weeks later spleens were harvested and cells were prepared and counted. For both groups, equal cell numbers (approximately 2.5×10^8 cells) were divided among 12 wells of a 24-well plate, and for each group of 12 wells, 6 were incubated with peptide LLO 91–99 (C and D) and 6 were incubated without it (A and B). After 7 days of stimulation, half of the cells from each well were taken and CTL assays were performed in triplicate wells with either nonpulsed or peptide LLO 91–99-pulsed J774 targets. Data are shown for each original well of the 24-well plate, for effector cells incubated with either peptide-coated targets (hatched bars) or peptide-free targets (open bars). Within each panel is shown the *P* value (*t* test) obtained by comparison of lysis of the two target cell types (no peptide and peptide).

architecture was severely disturbed in a VV-SC11-immunized host (Fig. 5C, panel 1), while it was well maintained in a VV-list-immune host (Fig. 5C, panel 2).

Death following lethal-dose challenge with *L. monocytogenes* is slightly delayed in mice immunized with VV-list. Having demonstrated an effect of VV-list immunization in controlling sublethal challenge, we next evaluated the effect of the vaccine on the outcome of a normally lethal challenge. Four weeks postimmunization, when challenged with ~ 8 LD₅₀ of *L. monocytogenes*, 87.5% of mice immunized with *L. monocytogenes* survived (Fig. 6), demonstrating the efficacy of vaccination against this lethal disease. VV-list immunization did not alter the final outcome to a statistically significant extent (25% survival [3 of 12 mice]; identical to that of VV-SC11 group), but death was somewhat delayed in VV-list-immunized animals. On day 4, 92% (11 of 12) of VV-list-immunized mice survived, compared with 75% (9 of 12) of VV-SC11 recipients. On day 6, the same time point at which maximal effects were seen in liver and spleen colony counts following sublethal challenge, 87.5% (7 of 8) of *L. monocytogenes*-immunized mice survived, compared with 58% (7 of 12) of VV-list-immunized mice and 25% (3 of 12) of mice immunized with VV-SC11. The probabilities that the survival pattern on day 6 resulted from chance is 0.01 when *L. monocytogenes* and VV-SC11 are compared and 0.09 when VV-list and VV-SC11 are compared. Thus, the efficacy of *L. monocytogenes* vaccine is clear, with a 99% confidence limit. The efficacy of VV-list is less certain, but there is a >90% likelihood that the difference seen at day 6 is signifi-

cant. By day 7, there was no difference in survival between VV-list- and VV-SC11-immunized animals.

CTL escape probably does not account for the apparent resurgence in *L. monocytogenes* multiplication in VV-list-immune animals. The beneficial effects seen at day 6 in VV-list-immune mice appear incomplete or temporary, since by day 8 *L. monocytogenes* levels in these vaccinees appear higher than those in the *L. monocytogenes*-vaccinated animals. We considered it possible that, in VV-list-immunized mice, *L. monocytogenes* which were mutated in the LLO 91–99 epitope might evolve; such bacteria would be able to evade the attention of the CTLs induced by VV-list. Similar evolution would be less likely in the *L. monocytogenes*-immunized mice, because many additional *L. monocytogenes*-specific CTLs might be induced; in that case, the selective advantage of bacteria mutated in the LLO 91–99 epitope would be less marked. The liver homogenate taken at day 6 from VV-list-immune *L. monocytogenes*-challenged mice was plated, 10 colonies were picked and subjected to PCR amplification around the LLO 91–99 sequence on the *L. monocytogenes* genome, and the DNA sequence of this region of the bacterial genome was obtained (data not shown). In all cases, the LLO 91–99 epitope was unchanged. To attempt to enrich for any mutants, this liver homogenate was passed in a new group of VV-list-immune mice and, at day 6, liver homogenates were made and plated and colonies were isolated. Again, 10 colonies were subjected to PCR amplification and DNA sequencing. In all cases, the LLO 91–99 epitope was unaltered, indicating that CTL escape mutants, if present,

FIG. 4. VV-list immunization leads to accelerated clearance of *L. monocytogenes*. Mice were immunized with intact listeriae (solid bars), VV-list (shaded bars), or VV-SC11 (open bars) as described in Materials and Methods (12 mice per group). One month after immunization, mice were challenged with ~ 1 LD₅₀ of listeriae. At each of the four time points shown, three mice of each group were sacrificed, and the spleens (A) and livers (B) were harvested. Serial dilutions of organ homogenates were plated on Trypticase soy agar plates, and CFU were counted 48 h later. Each bar represents the average of log₁₀ CFU from three mice.

do not constitute a major factor in the apparent resurgence of *L. monocytogenes* multiplication in the VV-list-immune mice.

DISCUSSION

CD8⁺ T cells are vital in protection against listerial challenge (9, 15, 29). As in other microbial infections, responses to primary and secondary *L. monocytogenes* infections are mounted by different components of the immune armamentarium. In the nonimmune host (primary exposure), gamma interferon (produced mainly by CD4⁺ T cells) is important, and eradication of this molecule renders the mouse much more susceptible to disease. Consistent with this is the observation that CD4⁺ T-cell responses to primary infection are largely Th-1 class, perhaps driven down this path by $\gamma\delta$ T cells (11). In contrast, diminished CD8⁺ T-cell function has minimal effect on primary infection, but in the immune host (secondary exposure), CD8⁺ T cells are vital and exert their antilisterial effect in a perforin-dependent manner (22, 23), most likely by direct lysis of infected cells.

In this study, we demonstrate the efficacy of a single dose of a recombinant viral vaccine encoding nine amino acids from *L.*

monocytogenes. The encoded epitope is presented by major histocompatibility complex class I for perusal by the immune system and induces antilisterial CTL responses in vivo. To evaluate the biological relevance of this response, we employed three criteria of vaccine efficacy, which are presented in Fig. 4 to 6. VV-list-immunized mice cleared bacteria more rapidly from the liver and spleen and showed prolonged survival. However, the results appear biphasic; up to and including day 6 postinfection, immunity induced by VV-list appears almost as efficacious as that induced by whole listeria, but at the later time point (day 8), efficacy of the minigene vaccine appears diminished. For clarity, these two phases may be considered separately.

The equivalence of VV-list and *L. monocytogenes* immunization, to day 6, might appear at first sight very surprising, since the intact bacterium contains many more proteins and hence many more potential immunogens than does VV-list, which contains a single listerial CTL epitope. However, studies have shown that LLO is an important target molecule for CD8⁺ T cells (2, 5). This does not reflect the absence, from the host major histocompatibility complex of alleles capable of binding sequences from other listerial proteins; indeed, the size of the bacterial genome would render this extremely unlikely. Instead the phenomenon most probably results from the failure of most listerial proteins to enter the cytosol and, hence, the major histocompatibility complex class I antigen presentation pathway. Thus, bacteria lacking LLO not only are avirulent but most also fail to confer protective immunity (3). Since LLO is such a important CTL target, the efficacy of VV-list is understandable. Other workers have demonstrated the efficacy of the LLO 91–99 epitope, administered as a liposome-peptide complex (28) or expressed from a salmonella recombinant as a minigene embedded in the open reading frame of the salmonella flagellin protein (40). In the former case, two peptide immunizations (primary and booster, separated by a 14-day interval) resulted in enhanced survival following a lethal-dose challenge; these findings are analogous to those shown in Fig. 6, which resulted from a single immunization with VV-list. After immunization with the salmonella vector bearing the minigene, the ability to control listerial multiplication was evaluated at a single time point, 3 days following listerial challenge; a small (6- to 10-fold) but statistically significant reduction in CFU was seen in the spleen and liver. In our studies, 1 day later (day 4 time point) (Fig. 4), we found similar reductions (5- to 70-fold reductions in titer), with much more marked effects (up to 6,000-fold reductions) observable by day 6. This time point was not evaluated for the salmonella-based vaccine (40), and thus a comparison cannot be made. Taken together, these data suggest that vaccines containing only an LLO 91–99 minigene can exert significant biological effects in controlling subsequent challenge.

The second phase, in which VV-list immunity appears to falter, resulting in elevated bacterial titers (Fig. 4) and death (Fig. 6) compared with those of *L. monocytogenes*-immunized mice, is open to several possible interpretations. First, we demonstrate that 6 weeks following immunization, antilisterial memory CTLs are present in mice immunized with VV-list. However, it is possible that VV-list-immune animals have lower frequencies of anti-listerial CTLs and/or have CTLs of lower avidities than mice immunized with intact listeriae. We have previously shown that minigene epitopes, recognized in vitro, may differ from their native counterparts in their protective capacities in vivo (25). Second, despite the apparent dominance of LLO in determining immunity, antilisterial immunity may be conferred by CTL responses to other determinants either from LLO or other listerial proteins. Under appropriate

A **B**

FIG. 5. Histological evidence for efficacy of VV-list immunization. The same organs described in the legend to Fig. 4 were evaluated histologically. All panels represent the day 6 time point postchallenge. (A) Liver samples stained with hematoxylin and eosin. Samples are from nonimmune mice (panel 1) and mice immunized with VV-SC11 (panel 2), intact listeriae (panel 3), and VV-list (panel 4). (B) Liver samples stained by Brown and Brenn's method of Gram staining. Panels: 1, mice immunized with VV-SC11 (many dark-blue gram-positive *L. monocytogenes* organisms are present and are easily visible in color; to facilitate identification in these gray-scale reproductions, some of the organisms are indicated by arrows); 2, mice immunized with VV-list (no listeriae were visible in any fields; a representative field is shown). (C) Spleen samples from mice immunized with VV-SC11 (panel 1) or VV-list (panel 2), stained with hematoxylin and eosin.

circumstances, other listerial proteins can induce protective CTLs. For example, some LLO⁻ mutants can induce both antilisterial cytotoxicity and protection against wild-type challenge; the two may be related, since the immunity is conferred by splenocytes (4). In addition, the secreted p60 protein can

induce CD8⁺ CTLs in *H-2^d* mice (33) and can protect against challenge (19). A third possible explanation for the benefit of *L. monocytogenes* immunization compared with VV-list immunization may reflect other, non-CD8⁺ immune responses. Although CD8⁺ T-cell responses are considered vital in protec-

C

FIG. 5—Continued.

tion, $CD4^+$ T cells also can transfer immunity (35), although this appears to be gamma interferon dependent (20). VV-list would not be expected to induce listeria-specific $CD4^+$ -based immunity, while *L. monocytogenes* undoubtedly could do so. Fourth, the reappearance of bacteria 8 days postchallenge in VV-list-immune animals together with the known importance of the LLO 91–99 sequence led us to consider the possibility that CTL escape mutants had been favorably selected in the bacterial population. Escape mutants in a virus CTL epitope have been identified in mice immunized with minigenes in a manner similar to that described herein (41). We therefore isolated individual colonies from spleens harvested 6 days postinfection and subjected 10 such colonies to PCR amplification and sequencing around the LLO 91–99 sequence. All 10 bacteria had wild-type sequences. Even after attempting in vivo enrichment of such mutants, all epitope sequences and flanking residues were wild type. Thus, we have no evidence that bacterial CTL escape mutants contribute to the relapse in disease.

Although our studies clearly confirm the importance of $CD8^+$ T cells in antilisterial immunity, the mechanism by which these cells lead to bacterial eradication is less certain. Gamma interferon appears only marginally important in CTL-mediated protection against listeriae (20, 29), while perforin is vital. Is each microbe-infested cell lysed by CTLs or can other mechanisms be suggested? We have analyzed, by immunohistochemistry, the inflammatory cells seen in the livers of infected mice; in VV-list-immunized animals, this analysis was done 4 days postinfection, since at day 6 the inflammatory foci

were too sparse and small to allow such evaluation (Fig. 5A). We found an abundance of $Mac1^+$ cells but few or no $CD8^+$ cells (data not shown). These data are similar to other findings from our laboratory in which development of inflammatory

FIG. 6. Prolonged survival of mice immunized with VV-list. Mice were immunized as described in Materials and Methods and the legend to Fig. 4. Groups included 12 mice (immunized with VV-list [$\diamond$] or VV-SC11 [$\circ$]) or 8 mice (immunized with *L. monocytogenes* [$\square$]). Following intraperitoneal challenge with ~ 8 LD₅₀ *L. monocytogenes*, mice were observed daily for 14 days. No deaths occurred after this time point.

foci in the myocardium of virus-infected mice requires CTLs but the foci themselves contain many macrophages and only relatively small numbers of CTLs (21). In both cases, we would argue that only a small amount of antigen-specific CTL-mediated cell lysis is required to act as a trigger, inciting a much more marked inflammatory response in which macrophages are major players, and effect microbial clearance in a non-antigen-specific manner.

L. monocytogenes has been proposed as a possible vaccine vehicle (16, 17, 37, 38). This organism shares the major advantage of all bacterial vectors when compared with their viral counterparts, i.e., an immensely greater capacity for foreign DNA. In addition, as discussed above, listeriae induce CTLs, mainly to proteins secreted into the cytosol. Furthermore, well-attenuated variants have been isolated from contaminated food sources, and it has been suggested that such variants may represent natural vaccines endemic in the population (7); if true, this would represent an impressive natural demonstration of the potential safety of this vaccine vehicle. Our studies reiterate the importance of LLO as a protective immunogen and suggest that any *Listeria*-based vector system should, if possible, contain this protein, perhaps in a variant form which reduces microbial virulence but retains immunogenicity.

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