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Mucosal Immunization With *Salmonella typhimurium* Expressing Lassa Virus Nucleocapsid Protein Cross-protects Mice From Lethal Challenge With Lymphocytic Choriomeningitis Virus

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Abstract

Objectives—Lassa fever virus (LAS) is transmitted to man by rodent carriers and is fatal in a third of untreated cases. Our goal is to provide immune protection from Lassa fever by mucosal vaccination.

Study Design/Methods—Mice were vaccinated intragastrically with control vectors or with vectors (vaccinia or *Salmonella*) expressing LAS nucleocapsid protein (NP). Mice were challenged intracranially with a lethal dose of the related arenavirus, lymphocytic choriomeningitis virus (LCMV), as a measure of the vaccine's ability to elicit cross-protection.

Results—*Salmonella* and vaccinia vectors expressing LAS NP each protected a third of the mice from lethal challenge with LCMV. All mice vaccinated with a vector expressing LCMV NP were protected as expected.

Conclusions—The LAS recombinant *Salmonella* vector is comparable to the LAS recombinant vaccinia vector in its ability to cross-protect mice from lethal challenge. Nucleocapsid protein is an inadequate immunogen on its own, but provides sufficient cross-protection to make it a useful component of a broadly reactive arenavirus vaccine.

Keywords

Lassa virus; *Salmonella* vector; mucosal vaccine; arenaviruses

Lassa fever virus (LAS) is an emerging pathogen that is known to cause hemorrhagic fever, with significant human mortality in endemic regions of West Africa. Natural infections of carrier rodents and experimental infections of laboratory mice, either with LAS or with the prototypic arenavirus lymphocytic choriomeningitis virus (LCMV), are usually mild or asymptomatic.[1–3] However, intracranial inoculation of laboratory mice is lethal unless those mice have been exposed to virus or immunogen and have successfully developed protective immunity.[2,3] Thus, lethal challenge of the mouse is used herein to assess cell-mediated immunity and cross-protection, not as a model for pathogenesis of human Lassa fever.

Cell-mediated immunity plays a primary role in clearance of LAS[4,5] and LCMV[6] from infected mice and primates. The most frequently used vector in these animal models has been

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recombinant vaccinia virus expressing LAS or LCMV antigens NP or glycoprotein (GP), which can elicit cytotoxic T lymphocytes (CTL) and protective immunity. Recombinant vaccinia expressing LCMV NP, or epitopes derived from NP, protect mice of different haplotypes from lethal LCMV challenge.[7–9] In the primate model, however, a recombinant vaccinia expressing the LAS nucleoprotein (VV-LAS NP) offered little protection from a lethal Lassa virus challenge.[10,11] In contrast, guinea pigs immunized with vaccinia virus vectors expressing LAS NP or GP genes experienced more protection than did the primates.[12–14] Although these vaccine studies used parenteral delivery routes, our studies use the intragastric route for immunizing mice with vaccinia and *Salmonella* vectors in an effort to develop a mucosal vaccine.

The development of mucosal arenavirus vaccines is important because arenavirus infections can be acquired mucosally and mucosal vaccines can induce immunity in both mucosal inductive sites and in systemic compartments.[15,16] Arenaviruses, recombinant vaccinia viruses, and bacterial vectors expressing viral genes can all cross the gastric mucosa and elicit cell-mediated immune responses.[1,15–17] Orally delivered recombinant *Salmonella typhimurium* can induce both secretory immunoglobulin A (IgA) for the protection of mucosal surfaces and systemic immunity to pathogens invading murine or primate hosts.[18,19] We recently showed that *Salmonella* expressing LAS antigen, delivered intragastrically, can induce mucosal antibody against LAS NP and NP-specific CTL responses.[1] Although those results documented CTL cross-reactivity between LAS NP and LCMV NP, in the current study, we document cross-protection and compare mucosal immunization via the *Salmonella* and vaccinia vectors.

MATERIALS AND METHODS

Vaccine and Virus Stocks

We constructed an *aroA*-attenuated recombinant *Salmonella* (DS228), expressing the full-length LAS NP gene under the control of the groEL heat shock promoter and designated it Salm-LasNP.[1] As a negative control, we also constructed an *aroA*-attenuated *Salmonella* (DS116), designated Ctrl.Salm., which contains the groEL expression cassette without LAS NP.

Recombinant vaccinia virus expressing LAS NP (VV-LASNP), a gift from David Auperin, and recombinant vaccinia virus expressing LCMV NP (VV-LCMNP), a gift from Lindsay Whitton, were propagated as described previously.[13] As a control, NYCBH wild-type vaccinia virus (VV-WT) was obtained from Therion Biologics (Cambridge, MA, USA). Vaccinia virus titers were determined by plaque assay on Vero E6 cells.[13] Vaccinia and *Salmonella* vectors were used to express the LAS NP gene in mice without having to use the biosafety level-4 facilities required for LAS infections. A stock of the arenavirus, LCMV Armstrong 53b strain, was plaque-purified and stored at 10^7 to 10^8 plaque-forming units (PFU)/mL. Virus was titered by plaque assay on Vero E6 cells as described previously.[20]

Immunizations and Challenge

Groups of 7- to 9-week-old male BALB/c mice were used in these experiments. Mice were inoculated with recombinant *Salmonella* expressing LAS NP as described previously.[1] Briefly, groups of mice were deprived of water and food for 2 hours and inoculated by intragastric intubation with 5×10^9 bacteria suspended in 0.2 mL of sodium bicarbonate on day 0. Two to three mice per group were used to assess cell-mediated immunity, and 10 to 20 mice per group were generally used for lethal challenge studies. Mice were inoculated with DS228 (Salm-LasNP), or DS116 (Ctrl.Salm.), or were mock immunized with sodium bicarbonate. Some mice were also inoculated intragastrically with 0.2 mL of phosphate-buffered saline containing

10^6 PFU of vaccinia virus VV-LASNP, VV-LCMNP, or VV-WT. Twenty-one days after inoculation, mice were reinoculated in the same manner. To assess the efficacy of immunization 4 days after the second inoculation, mice were either euthanized to test splenocytes for cell-mediated immunity or were challenged intracerebrally with lethal doses of LCMV: $0.2–1 \times 10^3$ PFU of LCMV in 20 μL of cell-culture medium (RPMI-1640; Gibco BRL, Rockville, MD, USA).

Cytotoxic T Lymphocyte Assays

Four days after the second inoculation, splenocytes were removed and CTL responses were measured as described previously.[1] Briefly, unstimulated splenocytes from immunized mice were cocultured with target cells (H-2^d Clone 7 cells) that were either uninfected or infected with VV-LASNP or VV-WT at a multiplicity of infection of 1 PFU per cell for 16 hours or LCMV at an multiplicity of infection of 1 PFU per cell for 48 hours at 37°C. Standard 5-hour chromium-release assays were performed. Spontaneous lysis was always less than 20%.

T Cell Proliferation Assays

Splenocytes from mice that survived challenge were plated in triplicate at 2×10^5 per well in flat-bottomed 96-well tissue culture plates in complete RPMI medium, in 0.5 μg of recombinant LAS NP, or in 0.5 μg/mL of heat-inactivated LCMV. The recombinant nucleoprotein and heat-inactivated LCMV were prepared as described previously.[1,21] Plates were incubated in a humidified atmosphere of 5% carbon dioxide at 37°C. On day 6, all wells were pulsed with 1 μCi of tritiated thymidine (Sigma, St. Louis, MO, USA) and harvested 18 hours later using a cell harvester (PhD Cell Harvester; Cambridge Technologies, Cambridge, MA, USA). Tritiated cell extracts were deposited onto glass fiber filters, washed, and allowed to dry. Thymidine incorporation was measured by liquid scintillation counting. Counts per minute in triplicate samples were determined and reported as a stimulation index (SI): the mean number of counts per minute incorporated in the presence of antigen divided by the mean number of counts incorporated in the presence of medium alone.

Statistical Analysis

JMP software (SAS Institute Inc., Version 3.1, Cary, NC, USA) was used for statistical analysis of data. The exact two-sided test was used to determine the 95% confidence interval (95% CI). If the CI of two data sets are overlapping, there is no significant difference between the two; however, there is a significant difference when the CI of two data sets do not overlap.

RESULTS

Cell-mediated Immune Responses

Mice were immunized with LCMV or vectors expressing NP, and their splenocytes were assayed for cell-mediated responses: CTL activity (Table 1) and proliferative capacity (Table 2). Mice immunized with VV-LASNP or Salm-LasNP demonstrated CTL activity against LCMV-infected Clone 7 target cells, as well as against Clone 7 cells infected with VV-LASNP. Likewise, mice inoculated with LCMV demonstrated CTL activity against Clone 7 cells infected with VV-LASNP.

Lassa fever virus NP–specific proliferative responses were detected in mice immunized and surviving with either Salm-LasNP or VV-LASNP. Overall, we observed low but reproducible proliferative responses to LAS NP in splenocytes from mice immunized with the recombinant vectors (Table 2).

Cross-protective Immunity to Lymphocytic Choriomeningitis Virus Induced by Mucosal Immunization With Recombinant VV-NP and Salm-LasNP

We previously showed that CTL elicited by LAS epitopes cross-react with LCMV.[1] Here, we reaffirmed that cross-reactive cell-mediated immune responses can be detected, and we tested the ability of recombinant *S. typhimurium* to cross-protect mice from lethal LCMV challenge. Groups of mice were inoculated intragastrically with recombinant Salm-LasNP (DS228), or Ctrl.Salm. (DS116). Mice were also mock-vaccinated or inoculated intragastrically with VV-WT or VV-LASNP. Four days after the second inoculation, mice were challenged intracerebrally with LCMV to test for cross-protective immunization.

Immunization with recombinant bacteria expressing LAS NP (Salm-LasNP, DS228) cross-protected roughly a third of the mice challenged with LCMV (Table 3). Mice inoculated with Ctrl.Salm. or VV-WT or who were mock-immunized did not survive. In comparison, a third of the mice inoculated with VV-LAS NP and all mice inoculated with VV-LCM NP survived LCMV challenge. Although there is no significant difference between survival rates of VV-LAS NP and *Salmonella*-LasNP vaccinees, those two vaccinated groups were significantly protected in comparison to VV-WT vaccinees (note that 95% CIs were nonoverlapping, Table 3).

DISCUSSION

Mucosal infection with the arenavirus LCMV occurs after low-dose intragastric inoculation, despite the acid lability of the virus.[16–18] Additionally, our laboratory and others demonstrated that mucosal immune responses to LCMV could be detected after intragastric inoculation with LCMV or with recombinant *Salmonella* expressing viral epitopes.[1,22] Our experimental work with LCMV was corroborated by epidemiologic studies of LAS indicating that arenaviruses might infect after ingestion.[23] Recently, we reported that oral immunization of mice with Salm-LasNP elicited both mucosal antibodies and LAS NP–specific CTL responses, as well as cross-reactive responses, to LCMV.[1] In this study, we show that intragastric inoculation of mice with recombinant *Salmonella* expressing LAS NP can cross-protect from subsequent challenge with LCMV. Thus, we present a case in which cross-reaction resembled cross-protection, and, significantly, these results were achieved using mucosal inoculation.

The mucosal immune system is functionally distinct from the peripheral immune system.[24,25] Vaccines delivered directly to the mucosa elicit distinct lymphocyte effector cells bearing integrins that mediate homing to gut-associated lymphoid tissue.[24,25] Thus, mucosal vaccines can potentially surpass parenteral vaccines in providing both local and systemic immunity and, thereby, broader protection from multiple routes of infection.[24,25] In our experiments, the route of immunization had no significant influence on the degree of protection because we used an intracerebral rather than a mucosal challenge. The immunizations reported by La Posta et al.[26] were parenteral, whereas our immunizations were intragastric, but both had the ability to cross-protect from intracerebral challenge: they immunized with vaccinia expressing LAS GP and protected most of their mice. A significant digression in their work was that protection was not corroborated by immunization with peptides, and protection seemed to rely on CD4- rather than CD8-mediated responses.[26] In our work, intragastric immunization with VV-LCMNP protected all mice from lethal LCMV challenge (Table 3), similar to the protection of BALB/c mice after intra-peritoneal delivery of VV-LCMNP.[8] Taken together, these findings suggest that the mucosal and parenteral routes of delivery with the vaccinia and *Salmonella* vectors are similarly effective against intracerebral challenge.

Lassa virus is a biosafety level-4 agent, so our studies were designed to use biosafety level-2 vectors, recombinant vaccinia, or *Salmonella* to express LAS NP, and biosafety level-3 conditions (LCMV challenge in mice) to assess the cross-protection of mucosal vaccines

against Lassa fever. We were constrained to use intracerebral challenge to obtain survival data because, in this model, LCMV infection is only lethal if the mice are inoculated intracerebrally. Lethality after intracerebral challenge is caused by the influx of virus-specific lymphocytes and their release of cytokines[27,28] rather than the cytopathic effects of the virus. It is well known that inoculation of mice with LCMV or a vaccine delivering LCMV gene products can protect mice from lethal intracerebral challenge. Adoptive transfer experiments established that protection is mediated by MHC-restricted virus-specific T lymphocytes.[29] Thus, protection in this experimental system is a measure of a memory cell-mediated immune response that somehow mitigates the infiltration of virus-specific lymphocytes after an intracerebral challenge.

The MHC background of the host and the viral epitopes presented in this background influence the cross-reactivity of cell-mediated immunity and, ultimately, the protective capacity of the immunogen. The LCMV NP and GP epitopes known to be protective in various murine strains have 60% to 70% sequence identity to the analogous regions of LAS NP and GP. Also, the sequence variation of LAS-like viruses in Africa can range from 10% to 27%.[30,31] Here, we document proliferative responses of Salm-LasNP–immunized mice to purified recombinant LAS NP and to heat-inactivated LCMV. Low but reproducible cross-reactive proliferative responses to LAS GP synthetic peptides were reported by La Posta et al.[26] after delivering VV-LASGP intraperitoneally to H-2^k mice. Furthermore, they showed that immunization with a LAS GP–specific T-cell clone could protect some mice from LCMV lethal challenge (20–200 PFU intracerebrally). As expected, only mice of the H-2^k background were protected,[26] as GP epitopes are the dominant protective epitopes in that haplotype; NP epitopes are protective in the H-2^d background that we used.

Vaccinia is a commonly used delivery vector and compares well with *Salmonella* in delivering immunogens by the intragastric route. Although all of the mice in the control groups died, a third of the mice immunized intragastrically with recombinant Salm-LasNP or with VV-LASNP were protected from lethal intracerebral challenge with LCMV. The partial protection is caused by the inadequacy of the NP immunogen and not to the vector for several reasons: 1) different vectors elicit the same amount of protection, 2) immunogens identical to the challenge strain elicit complete protection, and 3) another immunogen (GP) is known to protect a higher percentage of animals when compared with NP.[11]

CONCLUSION

This study supports previous work showing that *Salmonella typhimurium* expressing viral antigens from herpes simplex virus-1, simian immunodeficiency virus, and LCMV can induce protective immune responses in mice and in primates.[19,22,32] Recombinant *Salmonella* expressing LAS NP would be a reasonable component of a mucosal vaccine against Lassa virus infection. Mucosal delivery using vaccinia or *Salmonella* vectors will confer broad but modest protection. “Broad” refers to a breadth of cross-protective ability as LCMV and Lassa have only 60% sequence homology. “Modest” refers to the fact that only a third of the mice given a lethal LCMV challenge are protected by the vaccine. This study and the others mentioned herein lead us to suggest an optimal formulation for a LAS vaccine: a mucosal vector, like *Salmonella*, expressing both the NP and GP antigens of LAS.

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Table 1

Cross-reactive CTL From BALB/c Mice Inoculated With LCMV or Vectors Expressing LAS NP[‡]

Immunization[*]	E:T	Percent Specific Lysis of Infected Clone 7 Cells[†]			
		Uninfected	VV-WT	VV-LASNP	LCMV
Salm-LasNP	12.5	0.6 ± 0.4	0.5 ± 0.9	4.0 ± 1.6	7.6 ± 0.9
	25	2.1 ± 0.4	3.3 ± 0.3	9.6 ± 0.8	13.8 ± 1.0
	50	2.3 ± 0.4	3.3 ± 0.2	14.8 ± 0.4	17.1 ± 0.7
Ctrl. Salm.	12.5	1.9 ± 1.0	1.4 ± 1.1	1.3 ± 1.2	2.4 ± 0.4
	25	1.0 ± 1.1	1.5 ± 1.4	1.2 ± 0.7	2.6 ± 0.6
	50	1.3 ± 1.0	1.9 ± 0.2	0.5 ± 0.9	4.0 ± 0.9
VV-LASNP	12.5	2.2 ± 1.0	1.3 ± 1.1	6.4 ± 1.2	7.5 ± 0.4
	25	1.9 ± 1.1	3.6 ± 1.4	9.3 ± 0.7	11.3 ± 0.6
	50	2.8 ± 1.0	3.8 ± 0.2	14.5 ± 0.9	16.5 ± 0.9
LCMV	12.5	2.3 ± 0.3	4.3 ± 0.3	10.2 ± 1.0	35.9 ± 3.2
	25	1.9 ± 0.1	3.9 ± 0.6	14.0 ± 0.7	46.0 ± 4.9
	50	3.4 ± 0.6	4.9 ± 0.5	16.5 ± 1.7	69.3 ± 1.8

[*] BALB/c mice, two to three per group, were immunized by intragastric intubation and sacrificed 4 days after their second inoculation to assess splenocyte CTL activity.

[†] Percent specific lysis of uninfected or infected Clone 7 target cells is presented as the average of determinations from two to three individual mice.

[‡] These data are very similar but not identical to data presented in our previous publication[1]; they were done with new groups of mice to obtain parallel proliferation and challenge data.

E:T indicates effector:target cell ratio.

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Table 2

Proliferative Responses of Splenocytes From Immunized and Surviving Mice

Immunization*	SI to Immunogen†	
	LCMV (0.5 μg/mL)	LAS NP (0.5 μg/mL)
VV-LASNP		
Mouse 1	6.0	7.2
Mouse 2	29.3	16.2
Mouse 3	24.9	8.4
Salm-LasNP		
Mouse 1	23.0	18.2
Mouse 2	21.4	8.5

*Splenocytes were taken from mice immunized and surviving as described in the Methods section and Table 1 and were assessed for ability to proliferate to LAS or LCMV antigens.

†SI were calculated as described in the Methods section, and indicate uptake of ^{3}H-thymidine after 6 days of antigen stimulation. In this case, antigens were recombinant LASNP (0.5 μg/mL) or heat-inactivated LCMV (0.5 μg/mL). Each line represents data from a single mouse. Splenocytes from mice inoculated with Ctrl.Salm. or mock-infected had no proliferative responses (ie, SI values of <5.0).

Table 3

Mucosal Vaccination With Vectors Expressing LAS NP Cross-protects Mice From Lethal LCMV Challenge

Immunization*†	No. of Survivors/Total (% Survivors)		95% CI‡
Ctrl.Salm.	0/10	(0%)	0–31%
Salm-LasNP	5/14	(37%)	25–49%
Mock	0/10	(0%)	0–31%
VV-WT	0/20	(0%)	0–18%
VV-LASNP	4/12	(33%)	24–42%
VV-LCMNP	20/20	(100%)	82–100%

*BALB/c mice were inoculated by intragastric intubation with 5×10^9 bacteria either Ctrl.Salm. (DS116) or Salm-LasNP (DS228), or with 1×10^6 PFU of VV-recombinant, and boosted with the same recombinant vector 21 days after the primary inoculation. “Mock” indicates that mice were inoculated intragastrically with only bicarbonate solution.

†Mice were challenged i.c. with 2×10^2 to 10^3 PFU of LCMV Armstrong at day 24, and mortality was recorded for 15 days.

‡The method used to determine the 95% confidence interval (95% CI) was the exact two-sided test. Because of CI overlaps, there is no significant difference between the 37% survival of the *Salmonella* vaccinees and the 33% survival of the vaccinia vaccinees. In contrast, there is significant difference between the control (VV-WT, 0% survival) and the vaccinees given LAS NP constructs (33–37% survival). There is also a significant difference between the VV-LCMNP vaccinees with 100% survival and the VV-LASNP vaccinees with 33% survival.