JOURNAL OF VIROLOGY, Nov. 2005, p. 13934–13942 Vol. 79, No. 22
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.22.13934–13942.2005

A Live Attenuated Vaccine for Lassa Fever Made by Reassortment of Lassa and Mopeia Viruses

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Received 2 August 2005/Accepted 18 August 2005

Lassa virus (LASV) and Mopeia virus (MOPV) are closely related Old World arenaviruses that can exchange genomic segments (reassort) during coinfection. Clone ML29, selected from a library of MOPV/LASV (MOP/LAS) reassortants, encodes the major antigens (nucleocapsid and glycoprotein) of LASV and the RNA polymerase and zinc-binding protein of MOPV. Replication of ML29 was attenuated in guinea pigs and nonhuman primates. In murine adoptive-transfer experiments, as little as 150 PFU of ML29 induced protective cell-mediated immunity. All strain 13 guinea pigs vaccinated with clone ML29 survived at least 70 days after LASV challenge without either disease signs or histological lesions. Rhesus macaques inoculated with clone ML29 developed primary virus-specific T cells capable of secreting gamma interferon in response to homologous MOP/LAS and heterologous MOPV and lymphocytic choriomeningitis virus. Detailed examination of two rhesus macaques infected with this MOPV/LAS reassortant revealed no histological lesions or disease signs. Thus, ML29 is a promising attenuated vaccine candidate for Lassa fever.

Lassa virus (LASV), an Old World member of the *Arenaviridae* (49), is a complex of closely related arenaviruses transmitted from rats (*Mastomys* spp.) to humans by direct contact and/or by mucosal exposure. LASV is the causative agent of Lassa fever (LF), which is characterized by fever, muscle aches, sore throat, nausea, vomiting, and chest and abdominal pain (34). Approximately 15 to 20% of hospitalized patients die from the illness; however, approximately 80% of human infections are mild or asymptomatic, and 1 to 2% of infections result in death. In 29% of patients, acute LF is accompanied by a sensorineural hearing deficit, which accounts for a permanent hearing loss in 17.6% of survivors. The sizeable disease burden and the possibility that LASV can be used as an agent of biological warfare make a strong case for vaccine development (3, 16).

In addition to LASV and the prototype lymphocytic choriomeningitis virus (LCMV), the Old World group of arenaviruses includes three other related viruses, Mopeia (MOPV), Mobala, and Ippy (49). These viruses are nonpathogenic for experimental animals and are able to induce protective immunity against LASV (40). LASV has a bisegmented, single-stranded RNA genome (33), and each segment contains two genes in ambisense orientation. The L RNA encodes a large protein (L, or RdRp) and a small zinc-binding (Z) protein (9, 26). The S RNA encodes the major structural proteins, nucleoprotein (NP) and glycoprotein precursor (GPC), cleaved into GP1 and GP2 (2, 7). Previously, we have shown that coinfection of cells with LASV and MOPV resulted in the generation of infectious reassortants (27, 28).

Here, we describe a clone that has been isolated from a MOPV/LASV (MOP/LAS) reassortant; ML29 contains the L RNA from MOPV and the S RNA segment from LASV. In laboratory studies with LCMV, it has been shown that virulence for guinea pigs is determined by the L RNA segment (10, 42, 43), and it is likely that the attenuation of ML29 is due to its MOPV-derived L segment. Natural reassortment between MOPV and LASV in African areas of endemicity has not been studied, though there are several examples of Bunyaviruses reassorting in the wild (5, 18–20, 25, 44). Influenza pandemics are believed, conventionally, to be derived solely from reassortment events in which wild viruses of humans acquire a new hemagglutinin gene of avian origin (21). Recently, it has been shown that natural reassortment is also involved in evolution of some hemorrhagic fever viruses from the *Bunyaviridae*, such as Rift Valley fever virus, Crimean-Congo hemorrhagic fever virus, and Garissa virus, the etiological agent of hemorrhagic fever in East Africa (18, 20, 46).

Currently, reassortant technology is widely used for generation of influenza and rotavirus vaccines. MedImmune has developed a cold-adapted influenza vaccine by making "6:2 reassortants" in which two genes encoding major viral surface antigens (hemagglutinin and NA) contain sequences found in the current endemic strains, and the remaining six genes derive from the cold-adapted master stock (FluMist). This will be the first infectious influenza vaccine marketed in the United States (1, 39). Improved reassortant human-rhesus rotavirus vaccines are likely to be licensed soon in the United States (6). The main objective of the present paper is to evaluate the immunogenicity and vaccine efficacy of the MOP/LAS reassortant clone ML29 in vaccination experiments in small-animal models and to characterize ML29 infection of nonhuman primates.

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TABLE 1. Nucleotide and amino acid differences between MOPV, LASV, and attenuated MOP/LAS reassortant (clone ML29)

RNA	Nucleotide position	Gene	Base	Amino acid	ML29 mutation[a]
MOPV L	6	5′ NCR[b]	A		C
(7,271 nt)	8	5′ NCR	U		G
	810	L	C	Asp2136	U, Asn
	1423	L	G		A
	3319	L	U		C
	3519	L	U	Arg1233	C, Gly
	3664	L	U		C
	4002	L	G		A
	4177	L	U		C
	4665	L	A	Tyr851	U, Asn
	7266	3′ NCR	U		G
	7264	3′ NCR	A		C
LASV S	896	GPC	A	Lys272	G, Glu
(3,402 nt)	1366	GPC	U		C
	1842	NP	G		A
	1849	NP	G	Ala485	U, Asp
	2785	NP	U	Asn173	C, Ser
	3328	3′ NCR	G		A

[a] Nonconservative amino acid substitutions are boxed.
[b] NCR, noncoding region.

(This work was presented in part at the 53rd Annual Meeting of the American Society of Tropical Medicine and Hygiene, Miami Beach, Fla.)

MATERIALS AND METHODS

Viruses and cell cultures. LASV (strain Josiah) was received from the CDC (Atlanta, GA), MOPV (clone An20410) was provided by G. van der Groen (Institute of Tropical Medicine, Antwerp, Belgium), and LCMV strain WE (LCMV-WE) was received from P. Jahrling (The U.S. Army Medical Research Institute of Infectious Diseases, USAMRIID, Fort Detrick, Frederick, MD). Clone ML29 was selected from the primary MOPV/LASV library (27, 28) and triple plaque purified. The viruses were grown on Vero E6 cells cultured in Dulbecco's modified minimum Eagle's medium (GIBCO-BRL) with 2% fetal calf serum (FCS; GIBCO-BRL), 1% penicillin-streptomycin, and L-glutamine (2 mM) at 37°C in 5% CO_2. Cells and virus stocks were free of mycoplasma contamination.

RNA preparation, reverse transcription (RT)-PCR, and sequencing. For detection of viral RNA in the blood of experimental animals, viral RNA was extracted from 140 μl of plasma or serum by using a QIAamp viral RNA minispin protocol (QIAGEN; catalog no. 52904). RNA from tissues was extracted from samples submerged in RNAlater using an RNeasy minikit (QIAGEN; catalog no. 75142). RNA was converted into cDNA and amplified with LASV-GPC (8) and β-actin primers. The sensitivity of semiquantitative SYBR PCR with 36E2 and 80F2 primers was between 100 and 1,000 copies/ml of plasma. For sequence analysis, viral RNA was extracted from the culture medium of infected Vero E6 cells. The L segments of both the wild-type (MOPV An20410) and MOP/LAS (clone 29) were reverse transcribed and amplified in three overlapping parts based on primers derived from the LASV L RNA sequences (9, 26, 52). The entire genome sequence of MOP/LAS was generated from overlapping cDNA clones and primary PCR products and reconfirmed by primer walking. Nucleotide and amino acid differences between parental MOPV L RNA, LASV S RNA, and MOP/LAS RNA genome segments are listed in Table 1.

Adoptive-transfer experiments. Adoptive transfer of immune splenocytes was performed in CBA mice as previously described (27). Briefly, 4-week-old mice were inoculated intraperitoneally (i.p.) with the ML29 reassortant or with saline solution (negative control), and splenocytes were prepared from euthanized animals. The splenocytes were intravenously (i.v.) inoculated into recipient mice at different time points after lethal intracranial (i.c.) LASV inoculation (10^3 PFU), and the animals were observed for 14 days. In some experiments, immune splenocytes were treated with anti-T, anti-B serum or complement for 1 h at 37°C before inoculation into recipient mice. Animals from two groups (those treated with ML29-immune splenocytes and control animals) were euthanized at 1-day intervals for 1 week after challenge, and 10% (wt/vol) tissue samples were prepared for plaque titration (27).

Challenge experiments. Strain 13 guinea pigs (350 to 500 g; female) were purchased from USAMRIID (Fort Detrick, Frederick, MD). After a 30-day quarantine period, the animals were transferred into the biosafety level 4 (BSL4) facility and individually housed in air-filtered cages. Ten animals were inoculated subcutaneously (s.c.) with the ML29 clone (10^3 PFU in 0.5 ml phosphate-buffered saline [PBS]), and 10 guinea pigs received the same dose of MOPV. Eight animals were used as negative controls (0.5 ml PBS). The animals were bled, and temperatures and weights were monitored at the indicated time intervals. At day 30 after vaccination, the animals were s.c. challenged with 10^3 PFU of LASV (Josiah) and followed for 70 days (with weekly bleeding and monitoring of temperature, weight, and blood chemistry). Liver enzymes (aspartate aminotransferase [AST], alanine aminotransferase [ALT], and alkaline phosphatase [AlkPh]) were measured in plasma using the Premiere Plus photometer system (Stanbio Laboratory, Inc., San Antonio, TX). Vaccinated animals were euthanized on day 70 after challenge, and tissues were removed, fixed in 10% neutral formalin for the preparation of standard histological sections, and stained with hematoxylin-eosin. All animals from the control group met euthanasia criteria (fever, weakness, labored breathing, and >25% loss in weight) and were sacrificed during the 11 to 14 days after LASV infection.

Rhesus macaque infection and immune responses. Two adult rhesus macaques (*Macaca mulata*) weighing 2 to 3 kg were obtained from the IHV animal facility (UMBI, Baltimore, MD). The animals were s.c. injected with 10^3 PFU of ML29. Blood samples were taken weekly and submitted to the clinical laboratory for complete blood counts and standard 20-assay chemistry panels. Peripheral blood mononuclear cell (PBMC) isolation was performed as previously described (30). At days 14 and 28, the monkeys were euthanized and total blood and tissues (lung, spleen, mesenteric lymph nodes [MLN], liver, stomach, ileum, kidney, heart, cerebrum, and cerebellum) were collected. A portion of each tissue was submerged in MEM with 10% FCS (for plaque titration) and in RNAlater (for RNA isolation). The remaining tissue portions were fixed in 10% neutral formalin for the preparation of standard histological sections and stained with hematoxylin-eosin.

An immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) was performed as previously described (29). ML29 antigen was prepared from serum-free culture medium of ML29-infected Vero E6 cells by ultracentrifugation in a Beckman Optima L90K (SW28; 27,000 rpm; 3 h). The virus was suspended in carbonate-bicarbonate buffer (pH 9.6), and 100 μl of viral antigen was used to cover the wells of microtitration plates (overnight at 4°C). Sera from LASV-infected individuals were used as positive controls. Neutralizing antibodies were determined by plaque reduction neutralization assay using constant doses of virus, Vero E6 cell monolayers, and serial dilutions of plasma (29).

The monkey gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assay (U-CyTech B.V., Utrecht University, Utrecht, The Netherlands) was performed according to the manufacturer's recommendations. Briefly, 2×10^6 cells in 0.5 ml of RPMI 1640 (Invitrogen) with 5% FCS, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 25 mM HEPES buffer were stimulated by coincubation with 2×10^6 PFU of MOP/LAS, MOPV, or LCMV-WE (overnight at 37°C). After the stimulation, the cells were washed and resuspended in the same medium, and 0.3×10^6 to 0.4×10^6 cells per well were added to ELISPOT 96-well plates precoated with mouse monoclonal antibodies specific for monkey IFN-γ. The plates were incubated at 37°C for 5 h, and the cells were washed away. Biotinylated anti-mouse antibodies were then added, and the plates were incubated for 1 h at 37°C. The plates were washed and incubated with anti-biotin antibody labeled with gold particles. The spot-forming cells (SFC) secreting IFN-γ were developed with activator solution and counted using an Immunospot 3.2 Analyzer (C.T.L. Cellular Technology, Ltd.).

Biosafety. LASV is a category A biothreat agent (3) (CDC, Select Agent Program). Experiments with this virus were performed within a BSL4 facility at the Southwest Foundation for Biomedical Research, San Antonio, TX, certified by CDC. The MOP/LAS (ML29) reassortant was not considered a select agent, and a letter to that effect was obtained from the CDC Select Agent Program. All experimental work on the MOP/LAS vaccine candidate was initiated after compliance with institutional and NIH/National Institute of Allergy and Infectious Diseases requirements.

The LASV S segment constitutes only a third of the entire ML29 genome (Table 1) and does not carry direct virulence determinants. The ML29 virus is not pathogenic in guinea pigs or monkeys (see below) and is likely to be nonvirulent for human beings, as is its parent virus, MOPV (40). MOPV is classified as a BSL3 agent in the United States due to its relatedness to LASV (14), but it is classified as level 2 in Europe due to its attenuated phenotype. Based on

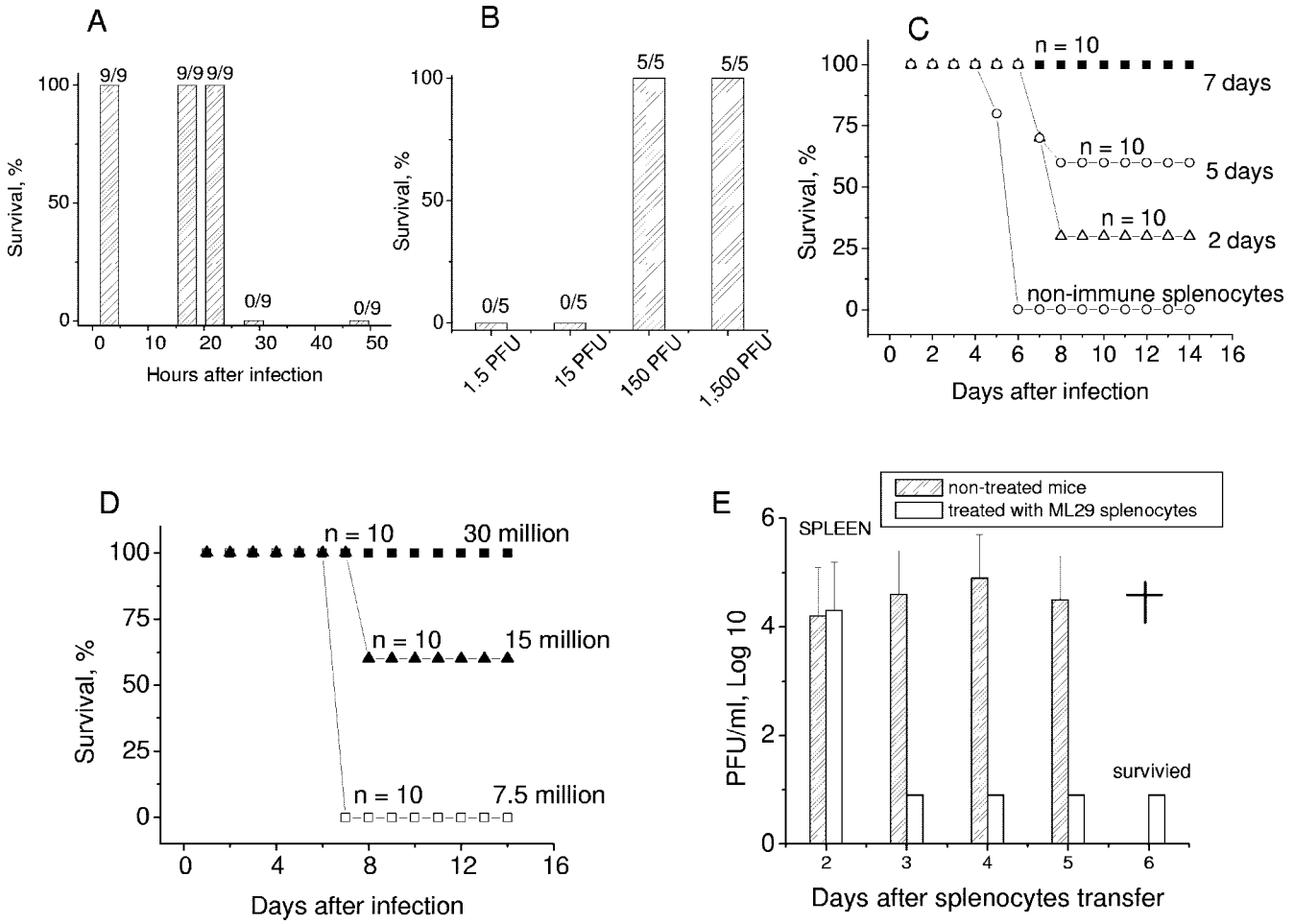

FIG. 1. Protective activity of splenocytes from ML29-vaccinated mice. (A) CBA mice were i.p. inoculated with 1,000 PFU of ML29, and at day 7, erythrocyte-free spleen cells were prepared. The recipient mice were i.c. challenged with 1,000 PFU of LASV (time zero), and at different times after challenge, the mice received 30×10^6 immune splenocytes i.v. (B) CBA mice were i.p. inoculated with ML29 at doses varying from 1.5 to 1,500 per animal. At day 7, immune splenocytes were i.v. injected into recipient mice at 3 h after lethal challenge with LASV. (C and D) ML29-immune splenocytes were collected at different time points after vaccination (C) and used at different doses (D) to treat LASV-challenged animals as described above. (E) Tissues from treated and nontreated CBA mice were collected at different time points after challenge and homogenized to prepare 10% (wt/vol) suspensions. Infectious LASV was determined in homogenates by plaque assay. Tissues from four animals were used at each time point. All treated animals survived; nontreated animals died (†) on day 6 after LASV challenge.

Centers for Disease Control and Prevention/Biosafety in Microbiological and Biochemical Laboratories recommendations, rhesus macaques were inoculated with ML29 in a BSL3 facility (UMBI, Baltimore, MD). All animal studies were conducted in compliance with federal guidelines (37a).

Data analysis. Statistical analyses and graphing were performed using the Origin 6.0 package (Microcal Software, Inc., Northampton, MA).

Nucleotide sequence accession numbers. The MOPV An20410 and the entire MOP/LAS (clone ML29) genome sequences were deposited in GenBank (accession no. AY772167 and AY772168).

RESULTS

ML29-immune splenocytes protect mice in a dose-dependent manner and effectively clear Lassa virus from tissues. Adoptive transfer of immune splenocytes is a specific and sensitive tool for assessing cell-mediated immune responses in arenavirus-infected animals (27, 35, 40). To address timing and dose issues of adoptive immunization, CBA mice were vaccinated with the ML29 reassortant and immune splenocytes were transferred into recipient mice at different times after lethal LASV infection. As seen in Fig. 1A, immunotherapy was effective only if splenocytes were injected during the first 24 h after LASV challenge. Treatment of immune splenocytes with anti-T-lymphocyte (but not with anti-B-lymphocyte) serum abolished the therapeutic activity of ML29 splenocytes (not shown). An additional experiment allowed us to determine a minimal vaccination dose. Four groups of CBA mice were vaccinated with 1.5, 15, 150, and 1,500 PFU of ML29, and their splenocytes were inoculated into LASV-challenged recipient mice. The immunizations with 1.5 and 15 PFU did not produce protective splenocytes. The ML29 immunization with 150 PFU and higher produced immune splenocytes that completely protected the animals against a fatal LASV challenge (Fig. 1B). Protective T lymphocytes were detected in the spleen as early as 2 days after vaccination. The cells collected on days 5 and 7 protected 60 and 100% of mice, respectively (Fig. 1C). Protection was dose dependent and required 30×10^6 splenocytes to protect all recipient mice against fatal LASV challenge (Fig. 1D).

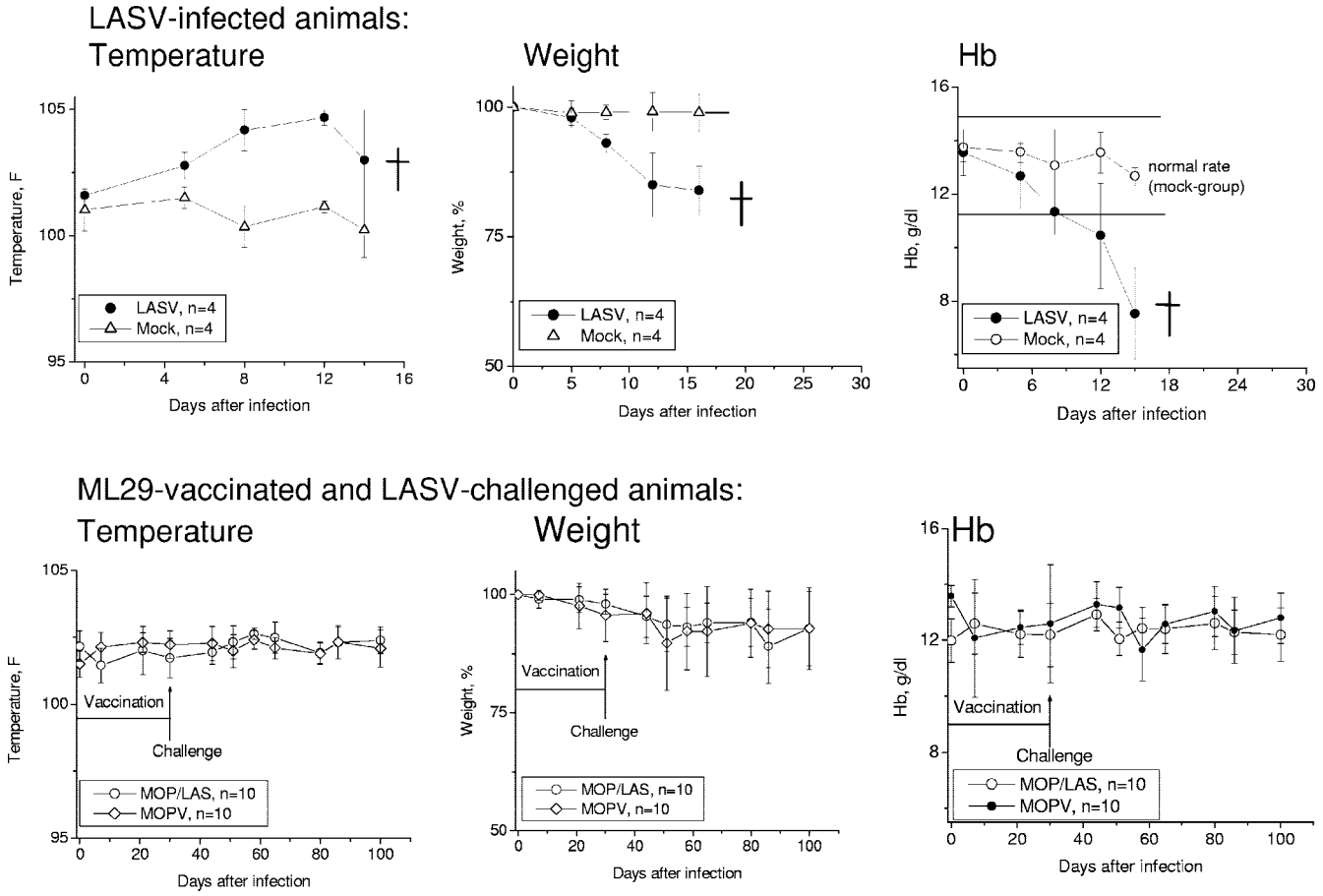

FIG. 2. Temperatures, weight measurements, and hemoglobulin levels in plasma of strain 13 guinea pigs. The top three panels show measurements from four mock-infected (PBS-inoculated) and LASV-challenged animals, all of which succumbed (†) within 2 weeks. The lower three panels show measurements from 10 ML29-vaccinated and 10 MOPV-vaccinated guinea pigs, all of which survived LASV challenge for at least 70 days. The error bars indicate standard deviations from the data points.

LASV infection of CBA mice resulted in rapid accumulation of the virus in tissues and the death of animals on days 6 to 7 after i.c. inoculation. Injection of the ML29-immune splenocytes effectively eliminated the virus from tissues of challenged animals. At day 3 after immune lymphocyte transfer, the virus was undetectable in the spleen (<1.2 $\log_{10}$ PFU/ml) (Fig. 1E) and other tested tissues (brain, liver, and kidney) of recipient mice. The effective virus clearance seems to be the main mechanism of protection of mice treated with ML29-specific T lymphocytes.

A single injection of the ML29 reassortant completely protects strain 13 guinea pigs from fatal Lassa fever. The LASV infection of strain 13 guinea pigs provides a small-animal model resembling human LF (23, 40, 41). Acute LASV infection resulted in fever, weight loss, and death of animals within 2 weeks after infection. The animals are very sensitive to the virus, since only 0.3 PFU kills 50% of strain 13 guinea pigs (23). Analysis of blood samples revealed mild leucopenia, decrease of hemoglobin concentration, and elevation of liver aminotransferases and alkaline phosphatase in plasma. In contrast, infection of strain 13 guinea pigs with MOPV or with the ML29 reassortant was not lethal for the animals and did not induce clinical or biochemical signs of the disease (Fig. 2 and 3). At day 30 after MOPV or ML29 vaccination, guinea pigs were challenged with LASV (10^3 PFU), and the animals were monitored for 70 days after challenge. All animals survived after challenge and had no clinical manifestations. All measured parameters were in normal ranges in ML29-vaccinated guinea pigs. In MOPV-vaccinated animals, a transient elevation of AST and AlkPh in plasma was observed at week 3 after challenge (Fig. 3).

Pathohistological studies did not reveal lesions in tissues of vaccinated and challenged animals. As seen in Fig. 4, acute LASV infection of strain 13 guinea pigs resulted in development of intestinal pneumonia with septal and alveolar edema. Histological findings in the liver included foci of severe hepatocytic vacuolization and lipidosis. In vaccinated animals, LASV infection did not induce alterations in target tissues. The lungs and livers of vaccinated animals looked essentially like normal tissues. There were also no lesions in other major organs.

The ML29 reassortant replicates poorly in monkey tissues but induces humoral and cellular immune responses. Rhesus and cynomolgus monkeys have often provided the most appropriate models for evaluation of vaccine efficacy (15, 17, 22, 40). As a first step in preclinical evaluation, we assessed the safety

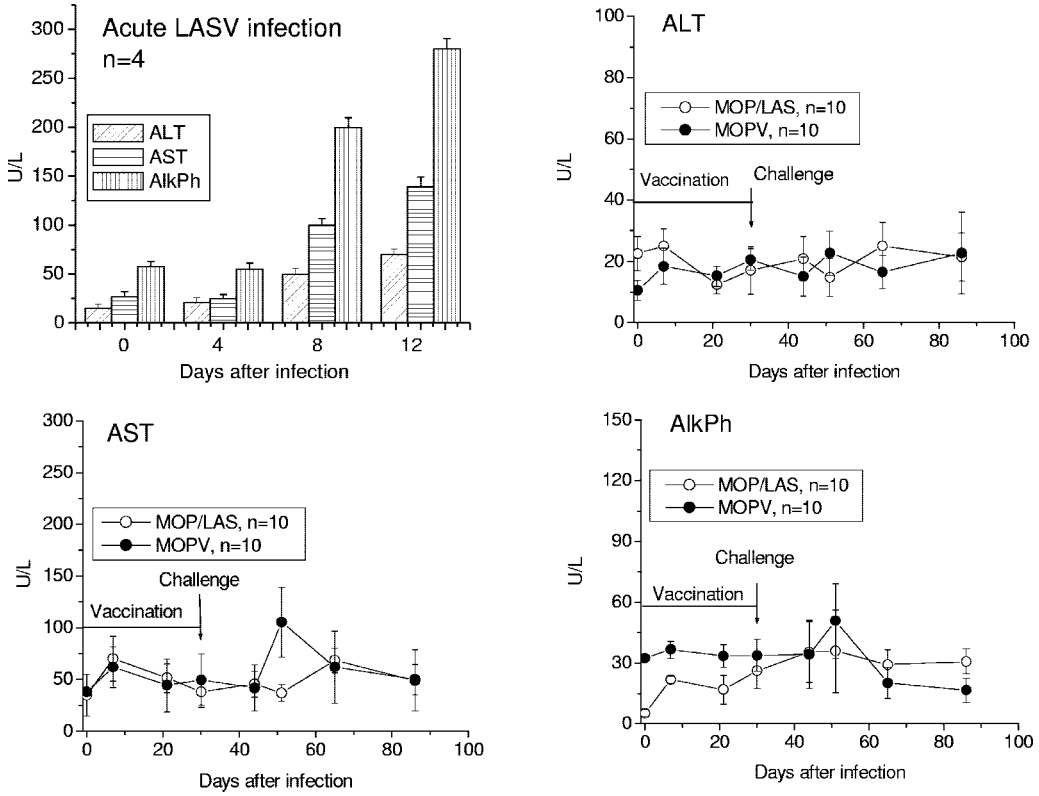

FIG. 3. Liver enzymes in ML29-vaccinated animals challenged with LASV. ALT, AST, and AlkPh were monitored in control and vaccinated animals as a sign of LF. The top left panel shows the elevation of these markers within 8 to 12 days after LASV challenge for the animals that were not vaccinated and died from LF. The remaining three panels show levels of liver enzymes in 10 guinea pigs vaccinated with ML29 (MOP/LAS) compared to 10 guinea pigs vaccinated with MOPV. The error bars indicate standard deviations from the data points.

and immunogenicity of ML29 in a pilot study outside of a BSL4 facility. Two rhesus macaques were vaccinated with 10^3 PFU of the ML29 reassortant, bled weekly, and euthanized at days 14 and 28 to track the ML29 distribution in tissues and to measure immune responses.

Vaccinated animals had no clinical manifestations during the observation period. All measurable hematological and chemical parameters were in the normal ranges, and gross appearance at necropsy was unremarkable. The ML29 virus was not detectable in plasma and tissue samples as judged by plaque assay. The virus was recovered only from the spleen after cocultivation with Vero E6 cells. A similar cocultivation procedure failed to recover virus from other tested tissues (lung, liver, MLN, kidney, and brain). Detection of viral RNA

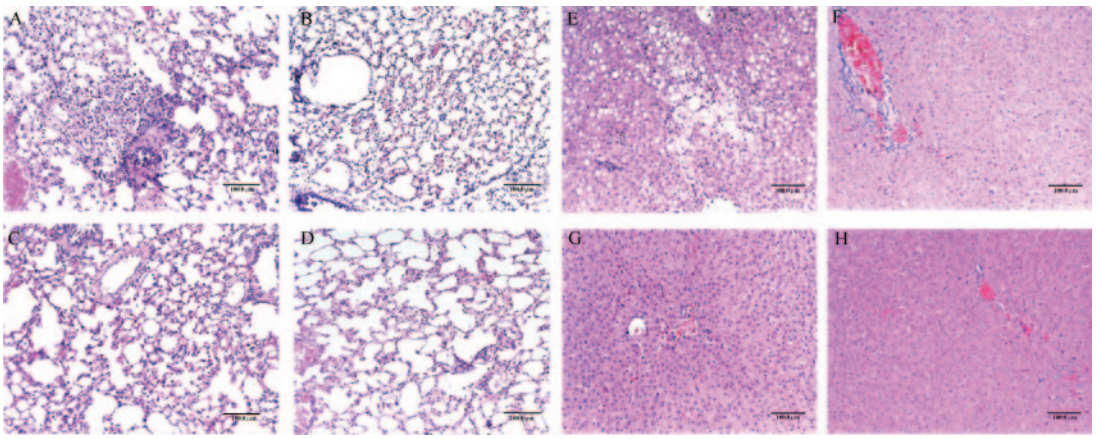

FIG. 4. Histology of lung (A to D) and liver (E to H), hematoxylin and eosin staining. (A and E) LASV-infected animals. (B and F) MOP/LAS-vaccinated and LASV-challenged guinea pigs. (C and G) MOPV-vaccinated and LASV-challenged animals. (D and H) Tissues of normal guinea pigs.

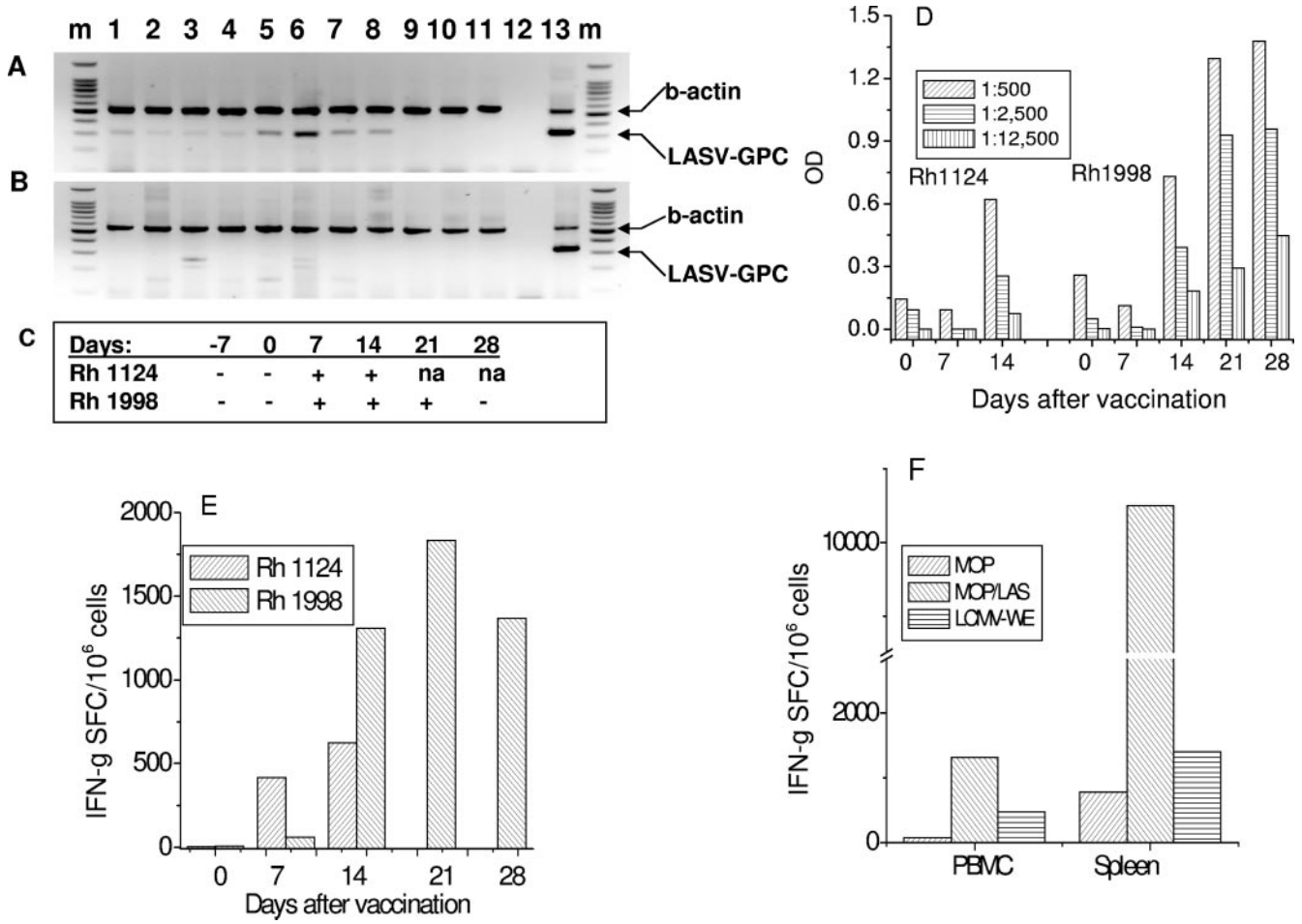

FIG. 5. Infection of rhesus macaques with ML29 reassortant. (A and B) Detection of ML29 sequences in tissues. Rhesus macaques were vaccinated with ML29 and sacrificed on day 14 (Rh 1124) (A) and day 28 (Rh 1998) (B). RNA samples were isolated, converted into cDNA, and amplified with LASV 36E2 and 80F2 primers (8). Lanes: 1, lung; 2, stomach; 3, liver; 4, kidney; 5, MLN; 6, spleen; 7, ileum; 8, heart; 9, cerebrum; 10, cerebellum; 11, hippocampus; 12, negative PCR control; 13, positive PCR control (RNA from LASV-infected Vero E6 cells); m, 100-bp DNA ladder. The arrows indicate the positions of β-actin (540 bp) and LASV (335 bp) amplicons. (C) Viremia in vaccinated animals detected by RT-PCR. +, RT-PCR positive; −, RT-PCR negative; n/a, not applicable. (D) IgG ELISA. Serum samples collected weekly were assayed with ML29 antigen as described in Materials and Methods. (E) T cells from peripheral blood secreting IFN-γ in response to in vitro stimulation with ML29. (F) IFN-γ-secreting T lymphocytes in spleen (28 days after vaccination) in response to specific ML29 and closely related antigens, MOPV and LCMV WE.

by RT-PCR with LASV GPC-derived primers revealed positive signals in plasma samples collected on days 7, 14, and 21 but not on day 28 (Fig. 5). On day 14, an RT-PCR assay detected viral RNA molecules in all analyzed tissues (except the brain). By day 28, all analyzed samples were PCR negative.

Infection with the ML29 reassortant induced antibody responses detected by IgG ELISA at week 2 after immunization (Fig. 5D), with an endpoint dilution titer of 1:62,500 on day 28. Neutralizing antibodies were undetectable (<1:20) in plasma (not shown). The ML29-specific T-lymphocyte responses were measured in PBMC and spleen by ELISPOT as antigen-specific SFC secreting IFN-γ after stimulation with viral antigens. As seen in Fig. 5, ML29-specific T cells were detected on day 7 (450 SFC/10^6 for Rh 1124). On day 14, Rh 1998 had higher numbers of IFN-γ-secreting cells (1,300 SFC/10^6 cells), peaking on day 21 (1,800 SFC/10^6 cells) and slightly decreasing at the end of the experiment. When PBMC and splenocytes were stimulated in vitro with genetically related viruses, LCMV-WE or MOPV, IFN-γ secretion was also observed. In peripheral PBMC, the numbers of SFC in response to MOPV slightly exceeded background (66 SFC/10^6 cells). Response to LCMV-WE was stronger at 470 SFC/10^6 cells. On day 28 after vaccination, 770 SFC/10^6 cells and 2,550 SFC/10^6 cells were detected in splenocytes after stimulation with MOPV and LCMV-WE, respectively. These data indicate that among the ML29-specific cells, up to 13% of T cells cross-reacted in response to closely related antigens.

DISCUSSION

In contrast to the discouraging search for human immunodeficiency virus/AIDS vaccines (37), research on acute hemorrhagic fever viruses has rapidly identified protective immunogens. For example, in a comprehensive study using 44

nonhuman primates, the recombinant vaccinia viruses expressing LASV NP, GPC, GP1, GP2, and combinations of these proteins showed that all animals vaccinated with a single glycoprotein, GP1 or GP2, died. However, vaccinia virus expressing LASV GPC protected 88% of primates from LF after a single s.c. inoculation, and 90% of the animals were protected after vaccination with a recombinant vaccine expressing both LASV S RNA-encoded products, NP and GPC. Importantly, in both cases, vaccination protected against lethal disease but not against infection, and viremia in animals vaccinated with GPC plus NP was 3 log units lower that in GPC-vaccinated monkeys (14, 15). A recent vesicular stomatitis virus recombinant expressing LASV GPC was also successful in protecting four of four monkeys from LASV challenge by induction of a nonsterilizing cellular immune response (17). In spite of these encouraging results, the vaccinia virus platform is no longer tenable because of potential side effects, particularly in immunosuppressed individuals in Africa (14), and such safety concerns have not yet been addressed for the vesicular stomatitis virus-based vaccine (17). Our results with the *Salmonella*/LASV NP construct (11) and data from a DNA vaccine expressing LASV NP in mice (45) and from alphavirus replicons expressing LASV GPC and NP in guinea pigs (41) clearly showed that NP epitopes are important for cross-protective activity. LASV-infected individuals in the area of endemicity had very strong memory $CD4^+$ T-cell responses against the LASV NP, which were partly strain specific and partly cross-reactive with other LASV strains (51).

Analysis of reassortants between pathogenic (WE) and nonpathogenic (ARM) strains of LCMV strongly indicates that the L RNA of WE is important for high levels of virus replication in vivo, and it is associated with fatal acute disease (43). Studies with other LCMV isolates indicated that complex phenotypes, such as viral persistence, sorted with the L RNA; however, this mapping was disputed (48) by evidence that the phenotype is dependent on both genome segments. It was shown that interaction with cell receptors and induction of cytotoxic T lymphocytes are associated with the S RNA-encoded products, GP and NP (11, 24, 45, 48, 53). Reassortment analysis of attenuated and virulent strains of Pichinde virus revealed involvement of both the L and S RNA segments in virulence for guinea pigs (54). We have completed the nucleotide sequences of the L RNA segments of WE and ARM and have found the most divergent regions in the N-terminal parts of the L and Z proteins, making these regions most likely to account for differences in pathogenic potential (10, 50). Interestingly, LASV isolates from long-term-infected Vero cell cultures had little detectable L RNA in proportion to S RNA, replicated poorly in cell cultures, and were not lethal for mice (31, 32). It seems that the attenuated phenotype of the MOP/LAS chimera is associated with the MOPV L RNA and/or with the L RNA-encoded products, RdRp and Z protein. The mutations that were introduced in the ML29 genome during selection (Table 1) could additionally contribute to the attenuated phenotype, but this contribution remains to be elucidated. As has been shown for hantaviruses (12, 38) and other members of the *Bunyaviridae*, the L segment and the L protein can carry mutations associated with host range restriction and attenuation (13).

The existence of natural reassortment raises the concern that vaccination with an attenuated reassortant vaccine in the presence of the virulent strain will somehow accelerate disease spread. Experiments with Rift Valley fever virus showed that reassortants between the wild-type virus and a live attenuated MP-12 reassortant vaccine generated only attenuated phenotypes with protective activity against the disease (47). These findings suggest that reversion to virulence is unlikely, and further indicate that genetic reassortment with wild-type viruses during a vaccination process in areas of endemicity would also be expected to yield attenuated variants and to reduce the overall incidence of disease (46, 47).

It has been shown that protection of experimental animals, including nonhuman primates, against LASV is associated with strong cellular immune responses in the absence of measurable neutralizing antibodies (16, 34, 41). We demonstrated in murine adoptive-transfer experiments that as little as 150 PFU of ML29 induced protective cell-mediated immunity and that protection was dose dependent on ML29 splenocytes (Fig. 1). Although mice do not accurately model human LF, they provide an economical assay for vaccine potency in terms of capacity to elicit cell-mediated immunity.

In contrast to mice, LASV inoculation of strain 13 guinea pigs is uniformly lethal and resembles human LF (23). A single vaccination with ML29 completely protected guinea pigs against LASV challenge. We found no signs of the disease, liver enzyme abnormalities, or tissue alterations during at least 70 days after challenge (Fig. 2 to 4). Although MOPV vaccination was also protective, MOPV-vaccinated animals experienced a transient elevation of AST and AlkPh in plasma after LASV challenge (Fig. 3), indicating that protection may not be as complete as it is with ML29.

Inoculation of rhesus macaques with the ML29 reassortant allowed us to track vaccine distribution in tissues and to measure LASV-specific immune responses. The ML29-vaccinated animals were afebrile throughout the experiment and had no clinical manifestations. Hematological and chemical parameters were in the normal ranges, as was gross appearance at necropsy. Detailed histological examination of rhesus macaques infected with the ML29 reassortant revealed no tissue lesions. The ML29 virus replicated poorly in monkeys and was not detectable in the plasma and tissues by conventional infectious plaque assay. The only organ from which the virus was recovered was the spleen. RT/PCR with LASV GPC-derived primers was transiently positive with RNA plasma and tissue samples. These data indicate that ML29 vaccination of rhesus macaques results in a short, inapparent, self-limited infection.

IgG ELISA antibodies against LASV antigens were found at week 2 after vaccination and neutralizing antibodies were not detectable during the observation period. The ML29-specific IFN-γ SFC were detected in blood of vaccinated animals at day 7, peaking on day 21 and slightly decreasing at the end of the experiment. On day 28, ML29-specific SFC represented approximately 1% of total spleen cells. Importantly, 13% of the ML29-specific spleen cells secreted IFN-γ in response to MOPV and LCMV.

Both NP- and GP-derived epitopes are important for induction of cross-reactive T-cell immunity (36, 51). LASV-infected individuals from areas of endemicity had very strong memory $CD4^+$ T-cell responses against NP and GP antigens derived from the Josiah strain of LASV, and these responses were

partly strain specific and partly cross-reactive with LASV strains from other phylogenetic clades. Because LASV strains vary as much as 7.4 to 10.5% in their NP and GPC amino acid sequences (4), the cross-protection is crucial for a successful vaccine candidate. Such a candidate should include both major antigens involved in protective immune responses, NP and GP. After a single-shot application, the ideal vaccine should be capable of inducing long-term protection against all LASV isolates circulating in West Africa. The ML29 reassortant encoding LASV NP and GP proteins meets all these criteria and has an important advantage in comparison with vaccine candidates expressing only LASV GP or NP genes (15, 17, 41, 45).

ACKNOWLEDGMENTS

This work was supported by grants R21-AI52367 and RO1-A52367 (to I.S.L.) and grants R21-AI53619 and R21-AI059247 (to M.S.S.) from the National Institutes of Health and by the Regional Center for Excellence in Biodefense and Emerging Infectious Diseases Research (to J.P.).

We thank H. Schmitz (Institute for Tropical Medicine, Hamburg, Germany) for LASV-specific sera.

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