Published in final edited form as:
J Med Virol. 2004 March ; 72(3): 424–435. doi:10.1002/jmv.20000.

Mucosal Arenavirus Infection of Primates Can Protect Them From Lethal Hemorrhagic Fever

Juan D. Rodas, **Igor S. Lukashevich**, **Juan C. Zapata**, **Cristiana Cairo**, **Ilia Tikhonov**, **Mahmoud Djavani**, **C. David Pauza**, and **Maria S. Salvato***
Institute of Human Virology, University of Maryland Biotechnology Institute, Baltimore, Maryland

Abstract

Arenaviruses are transmitted from rodents to human beings by blood or mucosal exposure. The most devastating arenavirus in terms of human disease is Lassa fever virus, causing up to 300,000 annual infections in West Africa. We used a model for Lassa fever in which Rhesus macaques were infected with a related virus, lymphocytic choriomeningitis virus (LCMV). Our goals were to determine the outcome of infection after mucosal inoculation and later lethal challenge, to characterize protective immune responses, and to test cross-protection between a virulent (LCMV-WE) and an avirulent (LCMV-ARM) strain of virus. Although intravenous infections in the monkey model were uniformly lethal, intragastric infections recapitulated the spectrum of clinical outcomes seen in human exposure to Lassa fever virus: death, recovery from disease, and most often, subclinical infection. Plaque neutralization, ELISA, lymphocyte proliferation, and chromium-release assays were used to monitor humoral and cellular immune responses. Cross protection between the two strains was observed. The three out of seven monkeys that experienced protection were also the three with the strongest cell-mediated immunity.

Keywords

arenavirus; mucosal infection; primates; hemorrhagic fever; immunity

INTRODUCTION

Arenaviruses are rodent-borne pathogens that can cause lethal disease in guinea pigs, primates, and human beings [Oldstone, 2002]. Numerous murine studies of viral persistence and cell-mediated immunity feature the prototype arenavirus, lymphocytic choriomeningitis virus (LCMV) [Zinkernagel and Doherty, 1974; Ahmed et al., 1984; Salvato et al., 1991; Oldstone, 2002]. In contrast to murine LCMV infections, LCMV infection of Rhesus macaques can resemble Lassa hemorrhagic fever in human beings [Jahrling et al., 1980; Peters et al., 1987; Lukashevich et al., 2002, 2003]. This study describes the consequences of intragastric inoculation of Rhesus macaques with LCMV.

Mucosal exposure accounts for a significant proportion of natural Lassa fever virus infections. Every year Lassa fever virus (LAS) infects approximately 300,000 human beings with 30% morbidity and 16% mortality [Jahrling et al., 1985; McCormick et al., 1986a, 1987; McCormick, 1990; McCormick and Fisher-Hoch, 2002]. The natural reservoir for LAS is the rat *Mastomys natalensis*, and the acquisition, preparation, and consumption of rodent meat is a major risk factor for rodent-to-human transmission [ter Meulen et al., 1996]. Another

*Correspondence to: Maria S. Salvato, Institute of Human Virology, University of Maryland Biotechnology Institute, 725 West Lombard St., Baltimore, MD 21201. E-mail: salvato@umbi.umd.edu.

indication that ingestion of arenaviruses can lead to disease is that some hepatitis outbreaks in zoo-kept tamarinds and marmosets were traced to feeding with LCMV-infected mice [Montali et al., 1993, 1995]. Our experimental studies showed that mucosal inoculation leads to attenuated LCMV infection in mice [Rai et al., 1996, 1997] and in macaques [Lukashevich et al., 2002, 2003] in comparison with same-dose inoculations by the intravenous route.

The WE strain of LCMV used in the present study is hepatotropic in mice, guinea pigs, and primates [Riviere et al., 1985; Zinkernagel et al., 1986; Lukashevich et al., 2002, 2003]. When inoculated intravenously, LCMV-WE, induces a lethal hemorrhagic fever in macaques that resembles human hemorrhagic fever [Jahrling et al., 1980; Peters et al., 1987; Lukashevich et al., 2002, 2003]. The Armstrong (ARM) strain of LCMV does not cause overt disease, even after intravenous inoculation [Danes et al., 1963; Peter et al., 1987; Lukashevich et al., 2002].

Our goals in this study were to establish a model for mucosal infection of non-human primates using the LCMV-WE strain, and to determine whether mucosal inoculation protected against a subsequent lethal virus challenge. Mucosal inoculation of Rhesus macaques with LCMV-WE induced the range of outcomes seen in humans naturally infected with Lassa fever virus, from subclinical to fatal, and varied in its ability to elicit protective immunity. Cell-mediated immunity was recorded in four of five mucosally inoculated monkeys; the fifth animal died before immune responses could be assessed. Two of five mucosally inoculated monkeys were protected from lethal challenge: one protected animal was inoculated with LCMV-WE, and one protected animal was inoculated with LCMV-ARM, that is known to confer cross protection after intravenous infection [Peters et al., 1987].

MATERIALS AND METHODS

Virus Stocks and Cell Culture

Large stocks of serum-free LCMV were produced in Vero E6 cells and stored at 10^7 to 10^8 plaque-forming units (pfu)/ml for use in all monkey inoculations. The LCMV-WE and LCMV-ARM strains have been well characterized [Salvato and Shimomaye, 1989; Djavani et al., 1998], and their use in monkeys has been described [Lukashevich et al., 2002]. Viral stocks used for proliferation assays were heat-inactivated at 56°C for 30 min. Stocks of vaccinia virus wild type (VVwt) and vaccinia viruses recombinant for LCMV glycoprotein (GP) and nucleocapsid protein (NP) genes, VV-GP_{LCMV} and VV-NP_{LCMV}, were described elsewhere [Whitton et al., 1988]. Vaccinia viruses were titered as described [Mackett et al., 1984]. All cell cultures were incubated in humidified chambers at 37°C with 5% CO_2.

Rhesus Macaque Inoculations and Challenges

Seven healthy rhesus monkeys were housed in a BSL-2/3 facility. They were all 2–5 year old females with normal weights and activity levels. Four monkeys were inoculated by gavage (i.g.) with the LCMV-WE strain: two with 10^7 pfu (Rh-ig7a; Rh-ig7b) and two others with 10^8 pfu (Rh-ig8a; Rh-ig8b). One monkey (Rh-iv3b) received a standard lethal challenge of 10^3 pfu LCMV-WE intravenously (i.v.) as a positive control for the virulence of the virus stock. Two more Rhesus macaques, Rh-ig8 (ARM) and Rh-iv3 (ARM), were used to assess the cross-protective ability of LCMV-ARM after i.g. (10^8 pfu) or i.v. (10^3 pfu) inoculation. Animals were first anesthetized (ketamine, 20 mg/kg) and then either given 1 ml of virus in PBS by intragastric gavage, or given 0.5 ml of virus in PBS via the left saphenous vein. Animals were observed daily. Body temperatures (rectal thermometer) and weights (digital balance) were taken before every bleeding. For the first lethal challenge, all monkeys that survived the primary inoculation were anesthetized as previously described, and inoculated intravenously with 10^3 pfu of LCMV-WE. A second lethal challenge was administered to the three animals that

survived the first inoculation, and all three survived. Table I has a comprehensive summary of animals, routes of inoculation, virus doses, and outcomes.

Blood Collection

Blood samples were collected at biweekly intervals and submitted to the clinical laboratory for complete blood counts and standard blood chemistry. Peripheral blood mononuclear cells (PBMC) were obtained from anticoagulant-treated blood and centrifuged over Ficoll-Paque plus (Amersham Biosciences) as described elsewhere [Hinds et al., 1997]. Some remarkable changes in blood chemistry are noted in Table II.

Detection of Infectious Virus in Plasma

LCMV-WE and ARM were detected in plasma by plaque assay as described [Lukashevich et al., 2002]. In brief, ten-fold dilutions of plasma samples were added to monolayers of Vero cells (about 2.5×10^5/well) in six-well plates, and incubated for 1 hr at 37°C. After incubation, wells were overlayed with1% agarosein MEM +2% FBS and incubated again at 37°C. Five days later, the wells were treated with formaldehyde for 30 min. Then the agarose overlay was removed and monolayers were stained with crystal violet, washed with tap water, dried, and visible plaques were counted to estimate the pfu/ml of virus in plasma.

RT-PCR for Detection of Viral RNA

RNA was extracted from PBMC or tissue samples using Trizol reagent (Invitrogen, Carlsbad, CA). Isolated RNA was treated with DNAse (Promega, Madison, WI) 1 U/μg, at 37°C for 30 min and purified again with the RNAeasy Mini Kit (Qiagen, Valencia, CA). RNA was converted to cDNA with the avian myeloblastosis virus reverse transcriptase (5 U, RT, Promega) using random hexamers (Invitrogen) for 1 hr at 42°C. The cDNA was subsequently amplified using standard PCR conditions as described [Lukashevich et al., 2002].

Analysis of Lymphocyte Subsets and Activation-Induced Cell Death (AICD)

Flow cytometry was used to determine changes in white blood cell subsets after infection. PBMC were paraformaldehyde-fixed and antibody-stained to identify $CD3^+$, $CD4^+$, $CD8^+$, and $CD20^+$ lymphocyte subsets. FITC-conjugated monoclonal antibody against CD4 or CD8 (Antigenix America, Franklin Square, NY), or FITC-conjugated anti-CD3 or CD20 (Becton-Dickinson, Mountain View, CA), were used with relevant isotype controls as described [Pauza et al., 1997; Lukashevich et al., 2002]. Samples were analyzed on a FACScan flow cytometer (Becton-Dickinson), and data were processed using FlowJo 2.7.8 software (Three star, 1997–1998). Absolute counts were determined by multiplying the percentage for each subset by the absolute lymphocyte count obtained from clinical hematology data.

Activation-induced cell death (AICD) was measured as the frequency of apoptosis induced in different cell subsets after PHA stimulation and was detected by staining with 7-amino actinomycin D as described [Lecoeur et al., 1997]. Briefly, 2×10^6 PBMC/well in a 6-well plate were cultured for 24 hr in RPMI with 10% FBS with or without 1 μg/ml of PHA (Sigma). After the incubation, PBMC were labeled for surface markers and then incubated for 20 min at 4°C in PBS that contained 20 μg/ml of 7-AAD (Sigma, St. Louis, MO). Samples were washed in PBS +2% FBS containing 20 μg/ml of non-fluorescent actinomycin D (AD, Sigma) and fixed in the same buffer containing 1% paraformaldehyde. Samples were analysed 15 min later (10,000 events/sample) using the FL-3 channel to detect 7-AAD staining.

Antibody Titers and Plaque-Reduction Neutralization (PRN) Assays

Antibody titers were measured by ELISA as described previously [Lukashevich et al., 2003]. Neutralizing antibodies were measured by a plaque reduction neutralization assay (PRN). This

assay used the same protocol as the plaque assays except that each plasma dilution was pre-incubated with a constant number of viral pfu for 1 hr at 37°C. The neutralization titer is the highest dilution of plasma sample able to reduce the number of plaques to 50% of the positive control. To determine whether plaque reduction could be enhanced by complement, we followed a published technique [Meyer et al., 2002]. Once the virus–plasma mixtures were incubated for 1 hr at 37°C, a 1/20 dilution of titered guinea pig complement was added and samples were incubated for an additional 45 min at 37°C. This mixture was added to cell monolayers and incubated for 1 hr more at 37°C before the semisolid agar was added. The assay was analyzed as described for the plaque reduction assay.

Cytotoxic T Lymphocyte (CTL) Assays

CTL assays followed published protocols [Trivedi et al., 1996; Yin et al., 1999] with slight modifications. Briefly, effector cells were peripheral blood mono-nuclear cells (PBMC) that had been cryopreserved in RPMI 1640 (Gibco, Grand Island, NY) containing 10% dimethyl sulfoxide (tissue culture grade: Sigma, St. Louis, MO) and 20% fetal bovine serum (FBS, Gibco, Grand Island, NY). The PBMC used as effectors were comprised of lymphocytes (70–90%; $CD2^+$) and monocytes (10–30%; $CD2^-$). PBMC for these monkeys were 21–26% $CD4^+$, 24–62% $CD8^+$, and 9–28% $CD20^+$. In preparation for the CTL assays, effector cells were thawed, washed three times with RPMI 1640 +10% FBS, and cultured at 37°C for 3 days at 2×10^6 cells per ml in RPMI 1640 with 20% FBS, penicillin/streptomycin (100 U/ml and 100 μg/ml respectively, Gibco), L-glutamine (2 mM, Gibco), Concanavalin A (ConA, 1 μg/ml, Sigma), and interleukin-2 (IL-2, 20 U/ml, Roche Diagnostics, Mannheim, Germany). After this, cells were washed and cultured again without the Con A, adding new IL-2 every other day for 7 days. Targets were autologous B-lymphoblastoid lines made by transformation with *Herpes papio* (a gift from N. Letvin, Harvard Medical School) and clones of transformed cells were obtained by limiting dilution as we described [Yin et al., 1999]. Target cells were cultured for 6 hr at 37°C either uninfected or infected with 3 moi of VVwt, VV-NP, or VV-GP. After incubation, target cells were washed with RPMI +10% FBS and labeled for 2 hr with [^{51}Cr] Na_2CrO_4 (Amersham Pharmacia Biotec, Irvine, CA), 200 μCi/2×10^6 cells. Then cells were washed three times and resuspended in RPMI +10% FBS at 2×10^6 cells/ml. Hundred microlitres of these cells or 2×10^4 cells, were plated per well in a round-bottomed 96 well plate in triplicate for each effector:target ratio. Three ratios were used in this assay, 50:1, 25:1, and 12.5:1. Spontaneous lysis varied from 10 to 20% and specific release was calculated as follows: [(experimental lysis–spontaneous lysis)/(maximal lysis–sponta-spontaneous lysis)] × 100. Specific lysis values above mean and standard deviation of background lysis using uninfected targets or VV-infected targets were considered positive.

Lymphocyte Proliferation Assay

Fresh PBMC were adjusted to 10^6 cells per ml in RPMI + 10% FBS. The proliferation assay [Djavani et al., 2001], was performed with the following modifications: 100 μl of cells/well were plated in triplicate for each of the different stimuli using round-bottomed 96 well plates. Each well received an additional 100 μl of RPMI for negative controls, an additional 100 μl of RPMI + PHA (10 μg/ml, Sigma, St. Louis, MO) for proliferation to mitogen, or an additional 100 μl of RPMI + LCMV antigen (WE or ARM) that was 3×10^5 pfu prior to inactivation. Plates were set up in duplicate to be collected 3 and 6 days after incubation. Each well was pulsed with 1 μCi of [^{3}H] thymidine (NEN Life Science Products, Boston, MA) and supernatants were harvested 12 hr later using a 96-well glass fiber filtermat on an automatic cell harvester (Tomtec harvester 96). Thymidine incorporation was measured by liquid scintillation counting using an automatic microbeta Wallac plate reader (Trilux, 1450 Microbeta). Counts pe minute (cpm) were recorded and the stimulation index (SI) was determined as the mean number of ^{3}H cpm incorporated in the presence of antigen divided by

the mean cpm incorporated in the presence of medium alone. Anything less than an SI of 5 was considered background.

RESULTS

Outcomes in Monkeys Infected Intragastrically With LCMV

Of four monkeys inoculated i.g. with LCMV-WE, two showed no signs of disease, and two developed disease, (Table I). We previously reported on i.g.-infected monkeys that displayed no disease signs but contained viral nucleic acids [Lukashevich et al., 2002]. Of the two "healthy" monkeys in this study, one displayed elevated liver enzymes (Table II, Rh-ig8b, 2 weeks after infection) and both Rh-ig7a and Rh-ig8b developed LCMV-specific CTL responses that were present in at least 12 assays each, using at least three different blood draws each, before lethal challenge (see CMI results). Of the two diseased animals, one had a rapidly fatal disease course similar to Rhesus macaques inoculated intravenously [Lukashevich et al., 2002], while the other was previously described as having a transient viremia and a transient elevation of liver enzymes [Lukashevich et al., 2003]. We add here that the transiently ill animal, Rh-ig7b, experienced a 25% weight loss and a burst of circulating neutrophils followed by a burst of circulating monocytes during its illness (Fig. 1).

Intragastric inoculation with LCMV-WE did not provide uniform protection against a lethal challenge with the same virus. Of four animals inoculated i.g. with WE, two showed no signs of disease and failed to resist challenge and one died before being challenged (Table I). Only the animal that recovered from acute WE disease was protected against a subsequent homologous challenge. Two additional animals inoculated with LCMV-ARM, Rh-ig8, and Rh-iv3, were both protected against a heterologous challenge with LCMV-WE, and only one of these, Rh-iv3, showed neutrophilia. In this study, neither acute viremia nor neutrophilia was correlated with protection against lethal challenge.

Our previous studies showed a reduction in platelet numbers after i.v. inoculation with 10^3 and 10^6 pfu of LCMV-WE [Lukashevich et al., 2002], but no changes after i.g. inoculation with 10^6 pfu of the same strain. In human cases of Lassa, platelet numbers are only moderately depressed but their function is almost completely abolished by an unknown circulating inhibitor [Fisher-Hoch, 1993]. No significant platelet changes were noticed before and after non-lethal i.g. inoculation of monkeys with LCMV. Lethal i.g. infections were similar to lethal i.v. infections with regards to reduced platelet numbers.

It is known that elevated levels of aspartate aminotransferase (AST) >150 IU/L are associated with lethal disease in Lassa fever [McCormick et al., 1986b]. As we described earlier [Lukashevich et al., 2003], a monkey that recovered from disease reached a peak of 184 IU/L 2 weeks after infection whereas the monkeys that died reached AST levels of around 1,000. All monkeys that survived lethal challenge in this study, Rh-ig7b (WE), Rh-iv3 (ARM), and Rh-ig8 (ARM), did not deviate from normal AST, alanine aminotransferase (ALT), or γ-glutamyltransferase (GGT) levels after challenge, whereas those animals that succumbed developed AST and ALT levels around 1,000. Although normal GGT level is 40–80 IU/L, GGT levels were 160–300 IU/L at necropsy for lethal cases. It is notable that two out of the three lethal-challenge survivors (ARM inoculated monkeys) experienced an increased GGT of 100 IU/L at 8–12 weeks after the lethal challenge (Table II).

Of the seven monkeys listed in Table I, plasma viremia was only detectable in monkeys that experienced disease: Rh-ig7b (WE), Rh-ig8a (WE), and Rh-iv3b (WE). RT-PCR of PBMC confirmed the presence of viral RNA for these same three monkeys but not for any others. Infection was indicated for the four non-viremic monkeys by their immune responses (all four monkeys), resistance to challenge (two monkeys), or blood chemistries (one monkey).

Flow Cytometry of Lymphocyte Subsets After LCMV-ARM Inoculations

We tried to detect alterations in lymphocyte subsets after infection with LCMV-ARM (i.v. or i.g.) and after challenge with the WE strain. The absolute number of $CD3^+$ cells went up substantially in the intravenously inoculated monkey by 2 weeks after infection (Fig. 2A). This increase corresponds to a significant increase in the number of $CD8^+$ T cells. However, the opposite pattern was seen in the i.g.-inoculated animal. Samples were always run in triplicate in order to obtain a standard deviation. By the time of challenge (Fig. 2) neither of the two animals exhibited large changes in the absolute number of cell subsets, although the number of $CD3^+$ was slightly lower in both before the challenge, seemingly a consequence of the decrease in $CD8^+$ T cells. We also noted a $CD8^+$ T cell increase (Fig. 2A,B) after a second challenge. In that case, not only absolute $CD8^+$ but also absolute $CD4^+$ numbers increased substantially.

Neutralizing Antibodies do not Explain Survival of Monkeys Exposed to Lethal Challenge With LCMV-WE

The PRN assay was carried out for all the monkeys but only the three monkeys that survived the challenge are shown. Only two of the three monkeys that survived lethal challenge with LCMV-WE, had neutralizing antibodies as detected by PRN assays (Fig. 3). The addition of complement to the assay only slightly enhanced the neutralizing capability of the plasma samples in vitro.

Cell-Mediated Immunity Is Correlated With Protection From Lethal Challenge

CTL assays were performed to detect anamnestic responses for one lethal-challenge survivor and two non-surviving monkeys. Target cells for chromium-release assays were autologous B cell lines and effector cells were cryopreserved PBMC stimulated with Con A and IL-2 (see Materials and Methods). Monkey Rh-ig7b that survived the lethal i.v. challenge with LCMV-WE, had a strong, specific CTL response against targets infected with the recombinant vaccinia viruses, VV-NP or VV-GP. Two monkeys that did not survive challenge, Rh-ig7a and Rh-ig8b, had relatively poor but LCMV-specific CTL responses (Fig. 4).

Monkey Rh-ig7b showed high CTL responses to GP at 8 weeks after infection, 4 weeks after the first challenge, and 1 week after the second challenge; whereas the strongest CTL responses to NP were observed at 8 weeks after infection, 2 weeks after the first challenge, and 1 week after the second challenge. Nonspecific cytotoxicity against the uninfected and the vaccinia virus-infected cells was usually under 10% specific lysis. Although the assays were performed at effector: target ratios of 50:1, 25:1, and 12.5:1, only results from the 50:1 and 25:1 ratios are shown (Fig. 4) because these ratios gave the strongest responses. Low but reproducible LCMV-specific cytotoxic activities were also observed in two other WE-inoculated monkeys (Rh-ig7a and Rh-ig8b) that did not survive the challenge. In the case of Rh-ig7a, specific lysis against GP and NP peaked at 10 weeks after infection and for Rh-ig8b, these responses did not begin until week 12 after infection. Responses in these monkeys were never more than 21% specific lysis above background levels and were apparently not enough to save them from lethal challenge.

Lymphocyte proliferation assays were performed for three challenge-surviving and one non-surviving monkey. Fresh PBMC were cultured in vitro to determine their proliferative capacity in the presence of media, PHA, or LCMV antigen (Fig. 5). Proliferation was determined by tritium incorporation. We observed that PBMC from Rh-ig7b (WE), Rh-iv3 (ARM), and Rh-ig8 (ARM) proliferated against autologous antigens, LCMV-ARM for the ARM-infected monkeys and LCMV-WE for the WE-infected monkeys. Results were similar for proliferation against heterologous antigens (WE-infected to ARM and vice versa) indicating that some of the helper epitopes are similar on the two viruses. Proliferative responses of circulating PBMC

peak at 2–4 weeks after inoculation, and then again at 4 weeks after challenge. The SI was highest in Rh-iv3 (ARM) that had been inoculated intravenously unlike the other two surviving monkeys. Proliferative responses for two non-surviving monkeys (Rh-ig7a and Rh-ig8b) were monitored for the day of challenge and they were both below SI = 5 (not shown). Proliferation data required fresh PBMC and could not be obtained from cryopreserved blood because few antigen-presenting cells survived and responses to mitogens were low. In general, the SI for PHA-treated PBMC was between 100 and 1,000, peaking at day 3 (not shown) and SI for antigen was 5–100 peaking at day 6 (Fig. 5A).

Activation-Induced Cell Death (AICD) Fails to Explain Decreased Proliferative Capacity of PBMC After Injection of Antigen

Proliferative capacities of PBMC decreased immediately after the first and second i.v. challenges with LCMV-WE for the three survivors (Fig. 5). There are at least two explanations for this drop in proliferative capacity after an antigenic boost: (1) T_H cells were being depleted from the circulation by being sequestered to virus-infected tissues, and (2) viral antigens made lymphocytes susceptible to AICD as seen in AIDS [Wallace et al., 1999]. To explore this latter possibility for Rh-ig8 (ARM) and Rh-iv3 (ARM) we performed a flow cytometric analysis of different lymphocyte subsets after activation with PHA (Fig. 6). PBMC were incubated with or without 1 μg/ml of PHA, and then were stained with 7-AAD as an early marker for apoptosis [Wallace et al., 1999]. In general, early PBMC samples had higher dramatic increases in cell death as a result of PHA activation (Fig. 5). In conclusion there was no AICD associated with our antigen inoculations that could be compared to the phenomenon observed in the rhesus/AIDS model.

DISCUSSION

Murine studies in our laboratory showed that LCMV could infect and disseminate via the i.g. route and that i.g. infection could protect mice from lethal intacerebral (ic) challenge [Rai et al., 1996, 1997]. In later studies, we used the macaque model to describe the disease course of a virulent LCMV infection after i.g. inoculation [Lukashevich et al., 2002]. We found that 10^3 pfu i.v. was a reproducibly lethal dose, and that 10^7–10^8 pfu by the i.g. route could sometimes be lethal. This dose is similar to the virus ingested by consuming a single infected rodent spleen, for example [Rai et al., 1997]. In addition to the acute disease cases, we were able to study one case in which a monkey recovered from disease and two cases in which monkeys were infected with an avirulent strain and became immune to disease. It was surprising that protection after mucosal infection was observed so rarely (only two out of five monkeys were protected). In natural settings repeated mucosal exposures probably improve chances for developing protective immunity.

Of five mucosally inoculated monkeys, only two became viremic and a third became seropositive (though not viremic). Evidence that the remaining two were infected came primarily from CTL assays in which LCMV-specific lytic responses were measured: for monkey Rh-ig7a, specific lysis exceeded background on four separate occasions before challenge, and for monkey Rh-ig8b specific lysis exceeded background on two occasions before challenge responses could begin. Ample precedent exists for arenavirus infections that are only detectable by cell-mediated immunity. For example, mice given low i.g. doses of LCMV lacked virological or serological evidence of infection yet resisted intracerebral challenge with LCMV [Rai et al., 1996], and this resistance is due to virus-specific cell-mediated immunity [Doherty and Zinkernagel, 1975]. In LCMV transgenic mice, CTL responses could be detected in the absence of detectable gene expression [Oldstone et al., 1991]. In AIDS, there are numerous examples of mucosally infected subjects with no detectable virus or antibody but with virus-specific cell-mediated immunity [Clerici et al., 1992; Salvato

et al., 1993; Trivedi et al., 1996]. Our experiences with cell-mediated immunity in SIV- or LCMV-infected monkeys convinced us that all seven of the animals reported here were infected by the initial inoculation.

Elevated levels of liver enzymes, particularly aminotransferases, correlate well with disease progression in both monkeys and in Lassa fever patients [McCormick et al., 1986b; Fisher-Hoch and McCormick, 1987; Fisher-Hoch, 1993]. In this study, all three animals that experienced disease after inoculation had high aminotransferases, but only one of the four animals that had no disease experienced abnormal aminotransferase levels. In our previous publications [Lukashevich et al., 2003], we suggest that increases in IL-6 and soluble TNF-receptors are markers of infection and potentially useful in prognosis, but these markers did not reach abnormal levels in any animals that did not experience disease [Lukashevich, unpublished].

Three out of seven monkeys survived all challenges and, of these three, two experienced neutrophilia at 4 weeks after the primary infection. Neutrophilia in our monkey experiments was associated with a delayed disease or a subclinical infection, and was not essential for surviving lethal challenge. The neutrophil-attracting chemokine, IL-8, was suppressed during in vitro studies with Lassa fever virus, but was not suppressed in parallel cultures with the avirulent Mopeia virus [Lukashevich et al., 1999]. In monkeys [Lukashevich et al., 2003] and in human beings [Mahanty et al., 2001] high levels of IL-8 during infection are associated with a better prognosis. This is consistent with the interpretation that the neutrophil burst we observed in two monkeys could have contributed positively to their survival.

Flow cytometry of lymphocyte subsets showed few remarkable fluctuations. In previous studies we noted lymphocytosis upon death for one monkey and at week 2 for a subclinically infected animal [Lukashevich et al., 2002]. Changes in circulating populations of white blood cells are most likely driven by high levels of viral antigen. We noted a rise in $CD3^+$ cells due to a rise in $CD8^+$ cells 2 weeks after infection of Rh-iv3 (ARM) but a fall in the same cells during the same period in Rh-ig8 (ARM). In human EBV infections, in cases of inflammation, and in acute murine LCMV infection, increases in total $CD8^+$ cells can be linked to clonal expansions of virus-specific T cells [Even et al., 1995; Silins et al., 1998; Sourdive et al., 1998]. It is possible the expansion of $CD8^+$ cells observed in Rh-iv3 was driven by antigen and linked to its protection from lethal challenge. No similar expansion was observed for another challenge-protected animal that had been mucosally infected, but it is likely that expansion in that animal only occurred amongst mucosal immunocytes, such as intraepithelial lymphocytes and mesenteric lymph node lymphocytes, and these were not sampled.

Since only 30% of LAS-infected individuals ever experience acute disease [McCormick et al., 1986a,b], an important question has been “How did the other 70% escape disease?” Our effort to analyze immune responses in surviving monkeys addresses this question. In our study, three (of five) monkeys survived lethal challenge, and these were also the monkeys with the highest cell-mediated immunity (CMI). Lassa vaccine studies and surveys of Lassa patients provided circumstantial evidence that CMI is required for protection from lethal challenge or for recovery. First, LAS patients, convalescing primates, and guinea pigs often lacked neutralizing antibody responses [Jahrling et al., 1980, 1982; Fisher-Hoch et al., 2000, 2001]. Neutralizing antibodies often appear late in infection and the transfer of “immune”plasma is rarely effective for clearing virus, cross-protection, or recovery from disease in animal or human trials [Jahrling and Peters, 1984; McCormick et al., 1986a]. Second, gamma-irradiated preparations of purified LAS virions elicited antibodies in monkeys to all three major structural proteins, N, G1, and G2, but did not protect against LAS challenge [McCormick et al., 1992]. Third, infectious virus vaccines that are known to elicit CMI (such as LAS-expressing alphavirus replicons, vaccinia recombinants, *Salmonella* recombinants, or Mopeia) elicit protective immunity in animals

[Fisher-Hoch et al., 1989, 2000; Auperin, 1993; Pushko et al., 1997; Djavani et al., 2000]. Fourth, adoptive transfer of effector spleen cells can mediate recovery and cross-protection from LAS infection in a mouse model [Peters et al., 1987; Lukashevich, 1992]. Finally, it has been demonstrated that LAS-seropositive persons from an endemic area have very strong $CD4^+$ T cell responses against LAS NP [ter Meulen et al., 2000]. Thus, by the accumulation of circumstantial evidence and by extrapolation from the mouse model, CMI is considered essential for survival or recovery from hemorrhagic fever.

Humoral responses also have a role in protection from arenaviral hemorrhagic fevers. Neutralizing activity as measured by PRN does not reflect antibody-dependent cellular cytotoxicity, antibody-mediated antigen uptake by phagocytes, or antibody-mediated antigen presentation, all of which modulate virus infection in vivo [Villinger et al., 2003]. The adoptive transfer studies in the mouse were contradicted by studies in a more relevant guinea pig model, in which splenocyte transfer resulted in death for 5 of 5 challenged animals whereas the transfer of immune plasma protected 4 of 5 challenged animals [Peters et al., 1987]. Neutralizing plasma from monkeys with titers above 4 logs could protect Lassa-infected cynomolgous macaques, and were especially effective when given with ribavirin [Jahrling and Peters, 1984; Jahrling et al., 1984]. Convalescent plasma from Lassa patients has also been shown to be protective in guinea pigs when the titer of neutralizing antibodies exceeds 2 logs, however this level is difficult to obtain from human plasma [Jahrling, 1983]. Our experience with immune responses in the rhesus/AIDS model and now in the rhesus/LCMV model indicates that a good CMI is always attended by a good humoral response, and that neutralizing antibodies are rarely correlated with protection. In the absence of costly immune depletion studies and adoptive transfer studies as have been performed in the rhesus/AIDS model [Schmitz et al., 1999, 2003], it is impossible to determine whether one or both responses are critical for immunity. It seems plausible that the humoral response alone cannot protect, that high antibody responses are indicative of good helper and CMI responses, and that CMI is most critical for survival.

Proliferative responses to LCMV antigen were observed in all three challenge-surviving monkeys but not in monkeys that succumbed to lethal challenge. It was curious that proliferative capacity frequently decreased following inoculation with more virus; so we tested the hypothesis that decreases in SI were due to AICD as observed in AIDS [Wallace et al., 1999]. This was not the case, however, since lymphocyte subsets were just as susceptible to cell death before and after activation with PHA (Fig. 6). There is an alternative hypothesis that we did not pursue in this study, but which finds support in murine studies. Klenerman et al. [2002], found that after infection, virus-specific memory T cells were sequestered primarily in solid organs such as kidney, liver, lungs, and spleen, and less than 20% of these T cells remained in the circulation. This finding raises the possibility that inoculations with virus can drive the majority of the proliferative response out of the circulation and into infected organs.

The significance of this study is primarily in characterizing the diversity of outcomes arising from a narrowly defined set of inoculi. Such outcomes run the gamut of what would be expected in nature after infection with hemorrhagic fever virus. The most fertile area of research for the future will be to define the earliest responses to infection that can be used to predict progression to the various outcomes.

Acknowledgements

This work was supported by National Institutes of Health (NIH) grants RR138980 and AI5252367 (to ISL) and by NIH grants AI53620 and AI53619 (to MSS). We are grateful for excellent animal care facilities and pathology support from Dr. Joseph Bryant and Dr. Harry Davis.

Grant sponsor: NIH; Grant numbers: RR138980, AI5252367, AI53620, AI53619.

Abbreviations

moi
: multiplicity of infection

pfu
: plaque-forming unit

IU/L
: international units per liter (referring to aminotransferase activity)

References

Ahmed R, Salmi A, Butler LD, Chiller JM, Oldstone MB. Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. Role in suppression of cytotoxic T lymphocyte response and viral persistence. J Exp Med 1984;16:521–540. [PubMed: 6332167]

Auperin, DD. Construction and evaluation of recombinant virus vaccines for Lassa fever. In: Salvato, MS., editor. The Arenaviridae. New York: Plenum Press; 1993. p. 259-280.

Clerici M, Giorgi JV, Chou CC, Gudeman VK, Zack JA, Gupta P, Ho HN, Nishanian PG, Berzofsky JA, Shearer GM. Cell-mediated immune response to human immunodeficiency virus (HIV) type 1 in seronegative homosexual men with recent sexual exposure to HIV-1. J Infect Dis 1992;165:1012–1019. [PubMed: 1533867]

Danes L, Benda R, Fuchsova M. Experimentalni inhalacni nakaza opic druhu macacus cynomolgus a macacus rhesus virem lymphocitarni choriomeningitidy (kmenem WE). Bratisl Lek Listy 1963;43:71–79. [PubMed: 14065052](in Czech.)

Djavani M, Lukashevich IS, Salvato MS. Sequence comparison of the large genomic RNA segments of two strains of lymphocytic choriomeningitis virus differing in pathogenic potential for guinea pigs. Virus Genes 1998;12(2):149–153.

Djavani M, Cheng Y, Xia L, Lukashevich IS, Pauza CD, Salvato MS. Murine immune responses to mucosally delivered *Salmonella* expressing Lassa fever virus nucleoprotein. Vaccine 2000;18:1543–1554. [PubMed: 10618553]

Djavani M, Yin C, Lukashevich IS, Rodas J, Rai SK, Salvato MS. Mucosal immunization with *S. typhimurium* expressing Lassa virus NP cross-protects mice from lethal challenge with LCMV. J Hum Virol 2001;4:103–108. [PubMed: 11437313]

Doherty PC, Zinkernagel RM. Capacity of sensitized thymus-derived lymphocytes to induce fatal lymphocytic choriomeningitis is restricted by the H-2 locus. J Immunol 1975;114:30–33. [PubMed: 1078677]

Even J, Lim A, Puisieux I, Ferradini L, Dietrich PY, Toubert A, Hercend T, Triebel F, Pannetier C, Kourilsky P. T-cell repertoires in healthy and diseased human tissues analysed by T-cell receptor beta-chain CDR3 size determination: Evidence for oligoclonal expansions in tumours and inflammatory diseases. Res Immunol 1995;146(2):65–80. [PubMed: 7481075]

Fisher-Hoch, SP. Arenavirus pathophysiology. In: Salvato, MS., editor. The Arenaviridae. New York, NY: Plenum Press; 1993. p. 299-323.

Fisher-Hoch SP, McCormick JB. Pathophysiology and treatment of Lassa fever. Curr Top Microbiol Immunol 1987;134:231–239. [PubMed: 3581898]

Fisher-Hoch SP, McCormick JB. Towards a human Lassa fever vaccine. Rev Med Virol 2001;11:331–341. [PubMed: 11590670]

Fisher-Hoch SP, McCormick JB, Auperin D, Brown BG, Castor M, Perez G, Ruo S, Conaty A, Brammer L, Bauer S. Protection of rhesus monkeys from fatal Lassa fever by vaccination with a recombinant vaccinia virus containing the Lassa virus glycoprotein gene. Proc Natl Acad Sci USA 1989;86:317–321. [PubMed: 2911575]

Fisher-Hoch SP, Hutwagner L, Brown B, McCormick JB. Effective vaccine for lassa fever. J Virol 2000;74:6777–6783. [PubMed: 10888616]

Hinds PW, Yin C, Salvato MS, Pauza CD. Pertussis toxin-induced leukocytosis in Rhesus macaques. J Med Primatol 1997;25:1–7.

Jahrling PB. Protection of Lassa virus-infected guinea pigs with Lassa-immune plasma of guinea pig, primate, and human origin. J Med Virol 1983;12:93–102. [PubMed: 6619814]

Jahrling PB, Peters CJ. Passive antibody therapy of Lassa fever in cynomolgus monkeys: Importance of neuralizing antibody and Lassa virus strains. Infect Immunol 1984;44:528–533. [PubMed: 6715049]

Jahrling PB, Hesse RA, Eddy GA, Johnson KM, Callis RT, Stephen EL. Lassa virus infection of rhesus monkeys: Pathogenesis and treatment with ribavirin. J Infect Dis 1980;141:580–589. [PubMed: 6768812]

Jahrling PB, Smith S, Hesse RA, Rhoderick JB. Pathogenesis of Lassa virus infection in guinea pigs. Infect Immunol 1982;37:771–778. [PubMed: 6749685]

Jahrling PB, Peters CJ, Stephen EL. Enhanced treatment of Lassa fever by immune plasma combined with ribavirin in cynomolgus monkeys. J Infect Dis 1984;149:420–427. [PubMed: 6715898]

Jahrling PB, Frame JD, Smith SB, Monson MH. Endemic Lassa fever in Liberia. III. Characterization of Lassa virus isolates. Trans R Soc Trop Med Hyg 1985;79:374–379. [PubMed: 3898483]

Klenerman P, Cerundolo V, Dunbar PR. Tracking T cells with tetramers: New tales from new tools. Nat Rev Immunol 2002;2:263–272. [PubMed: 12001997]

Lecoeur H, Ledru E, Prevost MC, Gougeon ML. Strategies for phenotyping apoptotic peripheral human lymphocytes comparing ISNT, annexin-V, and 7-AAD cytofluorometric staining methods. J Immunol Methods 1997;209:111–123. [PubMed: 9461328]

Lukashevich IS. Generation of reassortants between African arenaviruses. Virology 1992;188:600–605. [PubMed: 1585636]

Lukashevich IS, Maryankova R, Vladyko AS, Nashkevich N, Koleda S, Djavani M, Horejsh D, Voitenok NN, Salvato MS. Lassa and Mopeia virus replication in human monocytes/macrophages and in endothelial cells: Different effects on IL-8 and TNF-alpha gene expression. J Med Virol 1999;59:552–560. [PubMed: 10534741]

Lukashevich IS, Djavani M, Rodas JD, Zapata JC, Usborne A, Emerson C, Mitchen J, Jahrling PB, Salvato MS. Hemorrhagic fever occurs after intravenous, but not after intragastric, inoculation of Rhesus macaques with lymphocytic choriomeningitis virus. J Med Virol 2002;67:171–186. [PubMed: 11992578]

Lukashevich IS, Tikhonov I, Rodas JD, Zapata JC, Yang Y, Djavani M, Salvato MS. Arenavirus-mediated liver pathology: Acute lymphocytic choriomeningitis virus infection of Rhesus macaques is characterized by high-level interleukin-6 expression and hepatocyte proliferation. J Virol 2003;77:1727–1737. [PubMed: 12525606]

Mackett M, Smith GL, Moss B. General method for production and selection of infectious vaccinia virus recombinants expressing foreign genes. J Virol 1984;49:857–864. [PubMed: 6321770]

Mahanty S, Bausch DG, Thomas RL, Goba A, Bah A, Peters CJ, Rollin PE. Low levels of interleukin-8 and interferon-inducible protein-10 in serum are associated with fatal infections in acute Lassa fever. J Infect Dis 2001;183:1713–1721. [PubMed: 11372023]

McCormick, J. Arenaviruses. In: Fields, BN.; Knipe, DM.; Howley, PM., editors. Field's virology. 2. New York: Lippincott-Raven Press; 1990. p. 1245-1267.(Ch. 44)

McCormick JB, Fisher-Hoch SP. Lassa fever. Curr Top Med Microbiol Immunol 2002;262:75–109.

McCormick JB, King IJ, Webb PA. Lassa fever: Effective therapy with ribavirin. N Engl J Med 1986a; 314:202–226.

McCormick JB, Walker DH, King IJ, Webb PA, Elliott LH, Whitfield SG, Johnson KM. Lassa virus hepatitis: A study of fatal Lassa fever in humans. Am J Trop Med Hyg 1986b;35:401–407. [PubMed: 3953952]

McCormick JB, Webb PA, Krebs JW, Johnson KM, Smith ES. A prospective study of the epidemiology and ecology of Lassa fever. J Infect Dis 1987;155:437–442. [PubMed: 3805771]

McCormick JB, Mitchell SW, Kiley MP, Ruo M, Fisher-Hoch S. Inactivated Lassa virus elicits a non-protective immune response in rhesus monkeys. J Med Virol 1992;37:1–7. [PubMed: 1619397]

Meyer K, Basu A, Przyiecki CT, Lagging LM, Bisceglie AM, Conley AJ, Ray R. Complement-mediated enhancement of antibody function for neutralization of pseudotype virus containing hepatitis C virus E2 chimeric glycoprotein. J Virol 2002;76:2150–2158. [PubMed: 11836392]

Montali RJ, Scanga CA, Pernikoff D, Wessner DR, Ward R, Holmes KV. A common source outbreak of callitrichid hepatitis in captive tamarins and marmosets. J Infect Dis 1993;167:946–950. [PubMed: 8450260]

Montali RJ, Connolly BM, Armstrong DL, Scanga CA, Holmes KV. Pathology and immunohistochemistry of callitrichid hepatitis, an emerging disease of captive New World primates caused by lymphocytic choriomeningitis virus. Am J Pathol 1995;147:1441–1449. [PubMed: 7485406]

Oldstone MB. Biology and pathogenesis of lymphocytic choriomeningitis virus infection. Curr Top Microbiol Immunol 2002;263:83–117. [PubMed: 11987822]

Oldstone MB, Nerenberg M, Southern P, Price J, Lewicki H. Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: Role of anti-self (virus) immune response. Cell 1991;19:319–331. [PubMed: 1901765]

Pauza CD, Hinds PW II, Yin C, McKechnie TS, Hinds SB, Salvato MS. The lymphocytosis promoting agent pertussis toxin affects virus burden and lymphocyte distribution in the SIV-infected rhesus macaque. AIDS Res Hum Retroviruses 1997;13:87–95. [PubMed: 8989431]

Peters CJ, Jahrling PB, Lui CT, Kenyon RH, McKee KT, Barrera-Oro JG. Experimental studies of arenaviral hemorrhagic fevers. Curr Top Microbiol Immunol 1987;134:5–68. [PubMed: 3034512]

Pushko P, Parker M, Ludwig GV, Davis NL, Johnston RE, Smith JF. Replicon-helper systems from attenuated Venezuelan equine encephalitis virus: Expression of heterologous genes in vitro and immunization against heterologous pathogens in vivo. Virology 1997;239:389–401. [PubMed: 9434729]

Rai SK, Cheung DS, Wu MS, Warner TF, Salvato MS. Murine infection with lymphocytic choriomeningitis virus following gastric inoculation. J Virol 1996;70:7213–7218. [PubMed: 8794369]

Rai SK, Micales BK, Wu MS, Cheung DS, Pugh TD, Lyons GE, Salvato MS. Timed appearance of lymphocytic choriomeningitis virus after gastric inoculation of mice. Am J Pathol 1997;151:633–639. [PubMed: 9250174]

Riviere Y, Ahmed R, Southern PJ, Buchmeier MJ, Oldstone MBA. Genetic mapping of lymphocytic choriomeningitis virus pathogenicity: Virulence in guinea pigs is associated with the L RNA segment. J Virol 1985;55:704–708. [PubMed: 4020963]

Salvato MS, Shimomaye EM. The completed sequence of lymphocytic choriomeningitis virus reveals a unique RNA structure and a gene for a zinc finger protein. Virology 1989;173:1–10. [PubMed: 2510401]

Salvato M, Borrow P, Shimomaye E, Oldstone MB. Molecular basis of viral persistence: A single amino acid change in the glycoprotein of lymphocytic choriomeningitis virus is associated with suppression of the antiviral cytotoxic T-lymphocyte response and establishment of persistence. J Virol 1991;65:1863–1869. [PubMed: 1840619]

Salvato M, Emau P, Malkovsky M, Schultz K, MacKenzie D, Johnson E, Pauza CD. Cellular immune responses in Rhesus macaques with low dose SIV infection. J Med Primatol 1993;23:125–130. [PubMed: 7966226]

Schmitz JE, Kuroda MJ, Santra S, Sasseville VG, Simon MA, Lifton MA, Racz P, Tenner-Racz K, Dalesandro M, Scallon BJ, Ghrayeb J, Forman MA, Montefiori DC, Rieber EP, Letvin NL, Reimann KA. Control of viremia in Simian immunodeficiency virus infection by $CD8^+$ lymphocytes. Science 1999;283:857–860. [PubMed: 9933172]

Schmitz JE, Kuroda MJ, Santra S, Simon MA, Lifton MA, Lin W, Khunkhun R, Piatak M, Lifson JD, Grosschupff G, Gelman RS, Racz P, Tenner-Racz K, Mansfield KA, Letvin NL, Montefiori DC, Reimann KA. Effect of humoral immune responses on controlling viremia during primary infection of rhesus monkeys with simian immunodeficiency virus. J Virol 2003;77:2165–2173. [PubMed: 12525651]

Silins SL, Cross SM, Krauer KG, Moss DJ, Schmidt CW, Misko IS. A functional link for major TCR expansions in healthy adults caused by persistent Epstein–Barr virus infection. J Clin Invest 1998;102:1551–1558. [PubMed: 9788968]

Sourdive DJ, Murali-Krishna K, Altman JD, Zajac AJ, Whitmire JK, Pannetier C, Kourilsky P, Evavold B, Sette A, Ahmed R. Conserved T cell receptor repertoire in primary and memory CD8 T cell responses to an acute viral infection. J Exp Med 1998;188:71–82. [PubMed: 9653085]

ter Meulen J, Lukashevich I, Sidibe K, Inapogui A, Marx M, Dorlemann A, Yansane ML, Koulemou K, Chang-Claude J, Schmitz H. Hunting of peridomestic rodents and consumption of their meat as possible risk factors for rodent-to-human transmission of Lassa virus in the Republic of Guinea. Am J Trop Med Hyg 1996;55:661–666. [PubMed: 9025695]

ter Meulen J, Badusche M, Kuhnt K, Doetze A, Satoguina J, Marti T, Loeliger C, Koulemou K, Koivogui L, Schmitz H, Fleischer B, Hoerauf A. Characterization of human $CD4^+$ T-cell clones recognizing conserved and variable epitopes of the Lassa virus nucleoprotein. J Virol 2000;74:2186–2192. [PubMed: 10666248]

Trivedi P, Horejsh D, Hinds SB, Hinds PW II, Wu MS, Salvato MS, Pauza CD. Intrarectal transmission of Simian immunodeficiency virus in Rhesus macaques: Selective amplification and host responses to transient or persistent viremia. J Virol 1996;70:6876–6883. [PubMed: 8794330]

Villinger F, Mayne AE, Bostik P, Mori K, Jensen PE, Ahmed R, Ansari AA. Evidence for antibody-mediated enhancement of simian immunodeficiency virus (SIV) Gag antigen processing and cross presentation in SIV-infected Rhesus macaques. J Virol 2003;77:10–24. [PubMed: 12477806]

Wallace M, Waterman PM, Mitchen JL, Djavani M, Brown C, Trivedi P, Horejsh D, Dykhuizen M, Kitabwalla M, Pauza CD. Lymphocyte activation during acute Simian/human immunodeficiency virus SHIV(89.6PD) infection in macaques. J Virol 1999;73:10236–10244. [PubMed: 10559340]

Whitton JL, Southern PJ, Oldstone MBA. Analysis of the cytotoxic T lymphocyte responses to glycoprotein and nucleoprotein components of lymphocytic choriomeningitis virus. Virology 1988;162:321–327. [PubMed: 3257596]

Wolford ST, Schroer RA, Gohs FX, Gallo PP, Brodeck M, Falk HB, Ruhren R. Reference range data base for serum chemistry and hematology values in laboratory animals. J Toxicol Environ Health 1986;18:161–88. [PubMed: 3712484]

Wunderlich, J.; Schearer, G. Unit 3: In vitro assays for lymphocyte function. In: Colligan, JE.; Kruisbeek, AM.; Margulies, DH.; Shevach, EM.; Strober, W., editors. Current Protocols in Immunology. 1. New York: John Wiley and Sons, Inc; 1994. Section 3.11

Yin C, Wu MS, Pauza CD, Salvato MS. High major histocompatibility complex-unrestricted lysis of simian immunodeficiency virus envelope-expressing cells predisposes macaques to rapid AIDS progression. J Virol 1999;73:3692–701. [PubMed: 10196261]

Zinkernagel RM, Doherty PC. Restriction of in vitro T cell-mediated cytotoxicity in LCMV with syngenic or semiallogenic system. Nature 1974;248:701–702. [PubMed: 4133807]

Zinkernagel RM, Haenseler E, Leist T, Cerny A, Hengartner H, Altage A. T cell-mediated hepatitis in mice infected with lymphocytic choriomeningitis virus. Liver cell destruction by H-2 class-I-restricted. J Exp Med 1986;162:2125–2141. [PubMed: 3877779]

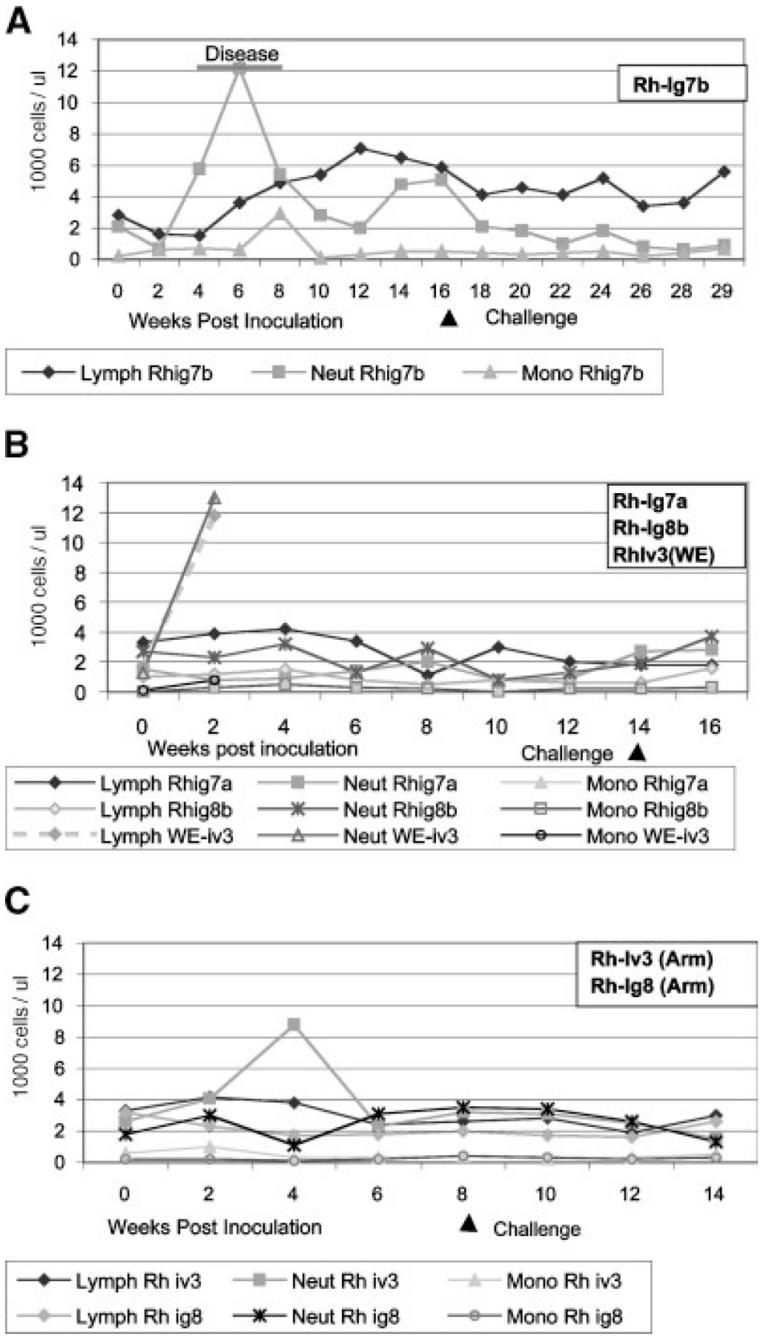

Fig. 1.

Hematological data. Blood cell counts were obtained over the course of our study for two monkeys that failed to survive lethal challenge and for three monkeys that survived. **A**: Monkey Rh-ig7b had been i.g. inoculated with 10^7 pfu of LCMV-WE and subsequently survived challenge with 10^3 pfu LCMV-WE i.v. Note the rise in neutrophils during disease and the monocytosis at the end of disease. **B**: Monkey Rh-ig7a, initially inoculated with 10^7 pfu WE and Rh-ig8b inoculated with 10^8 pfu WE did not survive the lethal challenge (10^3 pfu of LCMV-WE). A sharp rise in lymphocytes and neutrophils is depicted for Rh-iv3 (WE) that succumbed after the first inoculation. One additional monkey, Rh-8a (not shown) also succumbed immediately after the first inoculation (10^8 pfu WE) and experienced a similar rise

in lymphocytes and neutrophils. **C**: Monkey Rh-iv3 inoculated i.v. with 10^3 pfu ARM, and monkey Rh-ig8 inoculated with 10^8 pfu ARM both survived lethal challenge. Note the spike in neutrophils at week 4 for Rh-iv3. Neither monkey experienced disease.

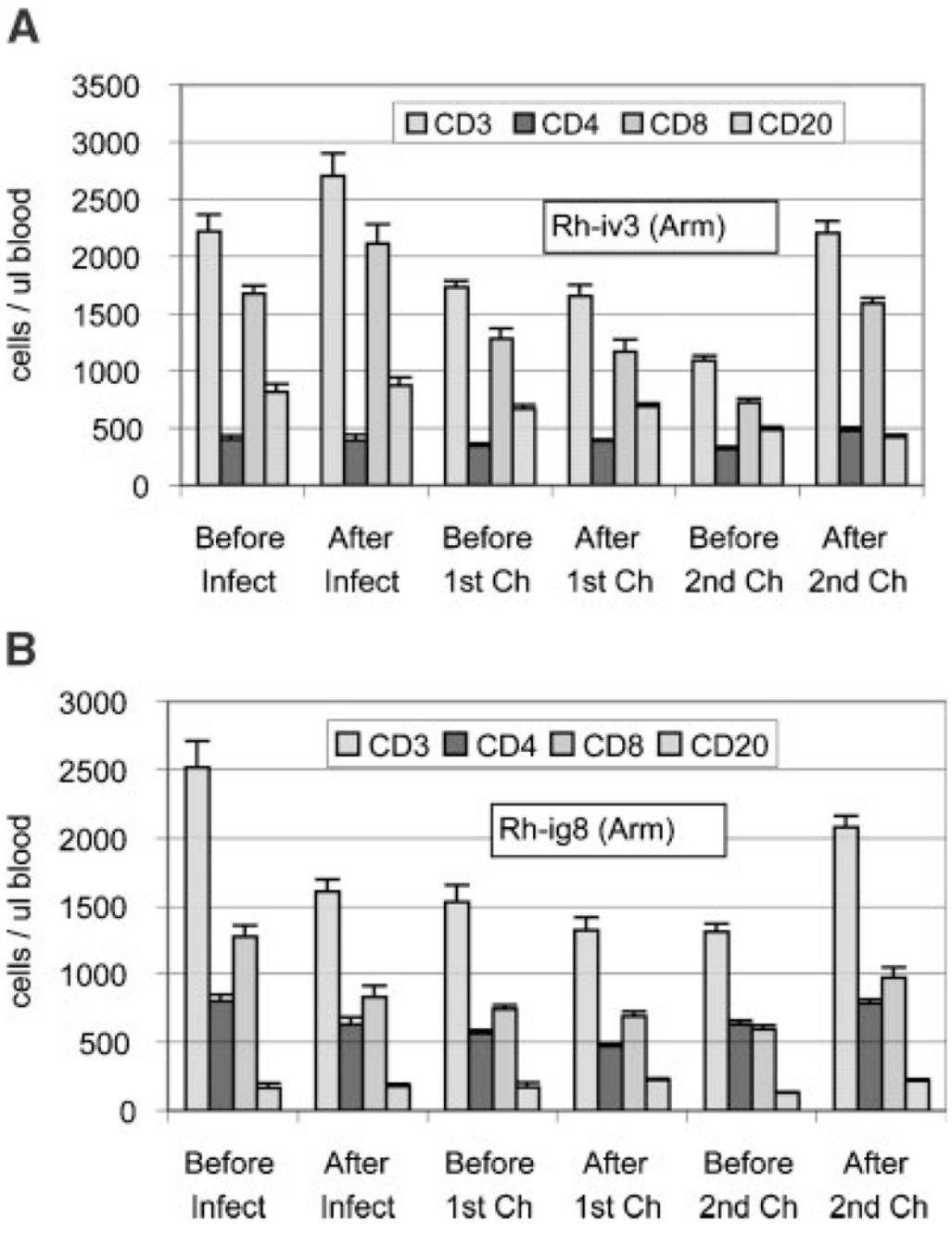

Fig. 2.

Flow cytometry. Only the LCMV-ARM-infected animals were analyzed for fluctuations in lymphocyte subsets since LCMV-WE-infected animals were analyzed previously [Lukashevich et al., 2002]. $CD3^+$ cells are roughly the sum of $CD4^+$ and $CD8^+$ T lymphocytes, and $CD20^+$ are B lymphocytes. The search for particular changes was only extended to samples taken 2 weeks before or 2 weeks after the inoculation, and 2 weeks before and after the 1st and 2nd challenge.

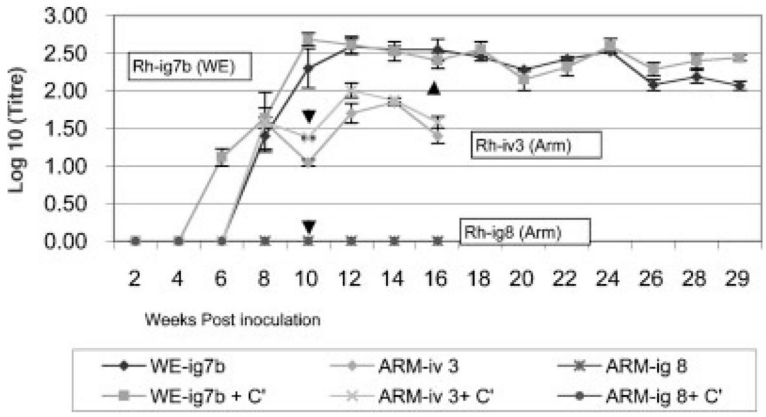

Fig. 3.

Plaque-reduction neutralization assay (PRN). This assay was carried out for all the monkeys included in this study, but only the three monkeys that survived the challenge are shown to illustrate the contribution of humoral immune responses in protecting against a lethal challenge. Note that complement-assisted PRN is similar to, but more strongly neutralizing than, complement un-assisted PRN. Whereas monkey Rh-ig8 (ARM) never had any neutralizing titer, the other two animals had good neutralizing titers. The antibody titer is expressed as the $\log_{10}$ of the plasma dilution that inhibited 50% of the plaques compared to the viral control. The time of challenge is depicted by an arrowhead (▲). No other animals showed any neutralizing titers.

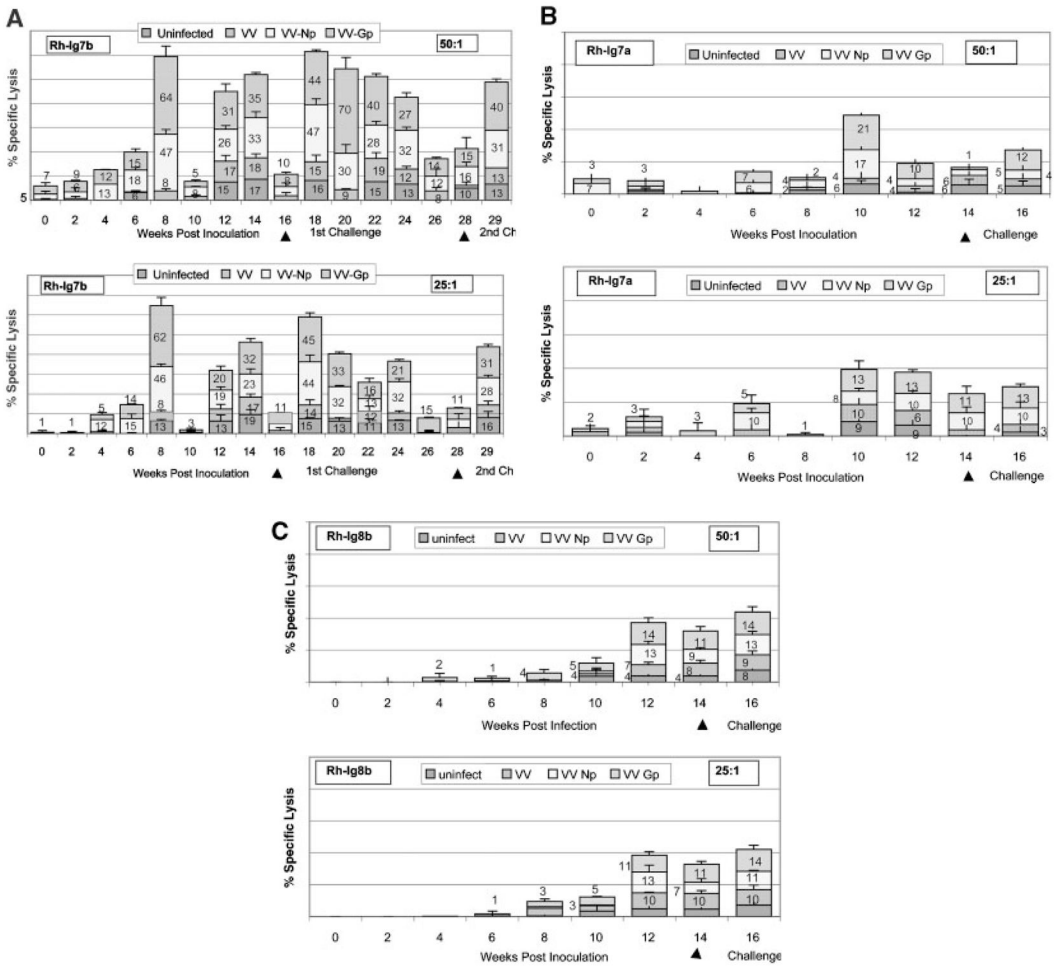

Fig. 4. Chromium-release CTL assays

The percent specific lysis was calculated as described in the Materials and Methods section and is depicted as a number on each bar associated with the different antigenic stimuli. CTL activity over the course of infection is shown for challenge-surviving animal Rh-ig7b (**A**), and for two animals that succumbed, Rh-ig7a (**B**) and Rh-ig8b (**C**). Only the results for the 50:1 and 25:1 effector:target ratio are shown.

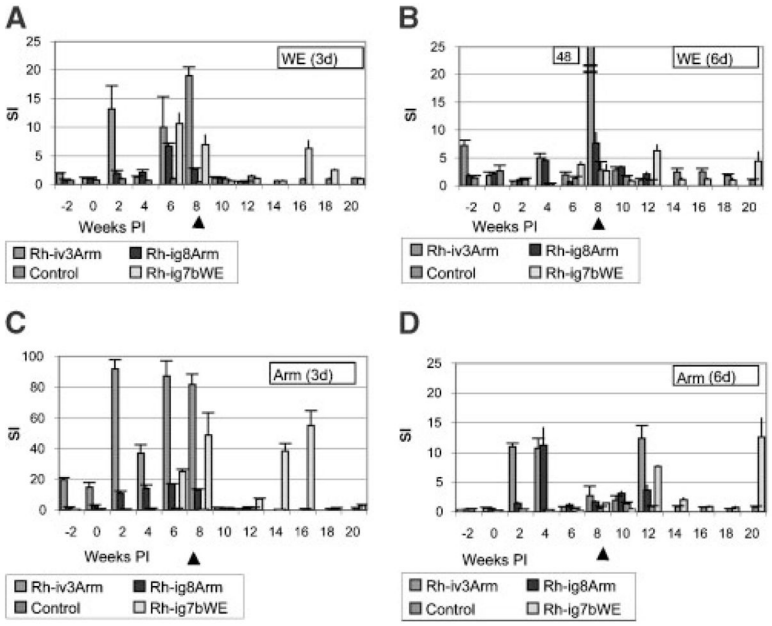

Fig. 5. Lymphocyte proliferation assay for rhesus macaques that survived lethal challenge

Fresh PBMC were incubated with antigenic stimuli during the course of infection. The upper right corner of each panel indicates the antigenic stimulus: LCMV-WE for 3 days (**A**), LCMV-WE for 6 days (**B**), LCMV-ARM for 3 days (**C**) and LCMV-ARM for 6 days (**D**). PBMC tested were from monkeys Rh-ig7b (WE-infected), Rh-iv3 (ARM-infected) or Rh-ig8 (ARM-infected). Stimulation index (SI) is the thymidine incorporation in the presence of test antigen divided by the thymidine incorporation in the presence of medium alone. Non-surviving monkeys only exhibited background levels and are not included in this figure. The time of challenge is depicted by an arrowhead (▲).

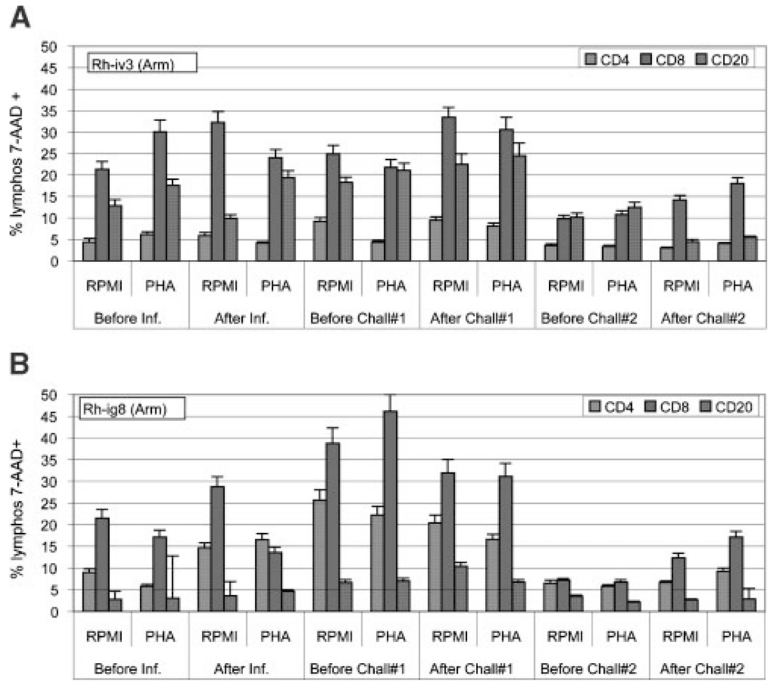

Fig. 6. No activation-induced cell death (AICD)

Apoptosis was experimentally determined based on the progressive loss of membrane permeability through the incorporation of 7-aminoactinomycin D. The extent of apoptosis after PHA-stimulation of PBMC was examined to explain the apparent in vitro proliferation inhibition observed in the samples taken after each inoculation with virus. CD4+, CD8+, and CD20+ subsets were examined from samples cryo-preserved 2 weeks before infection, 2 weeks after infection, 2 weeks before the first challenge, 2 weeks after the first challenge, 2 weeks before the second challenge, or two weeks after the second challenge. **Panel A** shows the % of 7AAD+ cells from Rh-iv3 (LCMV-ARM-infected) and **panel B** shows the % of 7AAD+ cells from Rh-ig8 (LCMV-ARM-infected). There are no increases in PHA-stimulated cell death to explain the dissapearance of proliferative responses after antigenic challenges.

TABLE I

Outcome After Inoculation and Challenge of Rhesus macaques

Monkey	Virus[a] (dose, route)	Viremia (pfu/ml)	Outcome after infection	Challenge[b] (days p.i.)	Outcome	Antibody response		CMI[e]	
						ELISA[c]	PRN[d]	Prolif.	CTL (LU)[f]
Rh-ig7a	WE (10^7 pfu, i.g.)	Neg.	No disease	98	Died d14	<2	<1	−	2
Rh-ig7b	WE (10^7 pfu, i.g.)	8.8×10^4	Disease/[g] recovered	112	Survived	8.7	2.5	+	27
Rh-ig8a	WE (10^8 pfu, i.g.)	8.0×10^6	Died d10	N/A	−	<2	<1	ND	–[h]
Rh-ig8b	WE (10^8 pfu, i.g.)	Neg.	No disease	98	Died d13	<2	<1	−	1
Rh-iv3	Arm (10^3 pfu, i.v.)	Neg.	No disease	56	Survived	5.9	1.9	+++	ND
Rh-ig8	Arm (10^8 pfu, i.g.)	Neg.	No disease	56	Survived	5.8	<1	+	ND
Rh-iv3b[i]	WE (10^3 pfu, i.v.)	2×10^7	Died d14	N/A	−	ND[j]	ND	ND	ND

[a] Viremia was determined by plaque assay of plasma.

[b] All the animals that survived the primary inoculation were given a lethal challenge with 10^3 pfu LCMV WE i.v.

[c] IgG levels are expressed as the inverse of the $\log_{10}$.

[d] Neutralizing antibody titers are expressed as the inverse of the $\log_{10}$ dilution that blocked 50% of the plaques.

[e] Cell-mediated immunity (CMI) was measured by lymphocyte proliferation assays (prolif.) and cytotoxic T lymphocyte (CTL) assay.

[f] LU is lytic units per 10^7 effector cells as described by Wunderlich and Schearer (64).

[g] Monkey Rh-ig7b became sick 4 weeks after inoculation and recovered 8 weeks after inoculation. Virus was isolated from plasma and PBMC only during the prodromal period.

[h] Only two time points were assessed: one before inoculation and one upon death. Both showed no CTL response above background.

[i] This monkey was only introduced into the experiment as a control for our viral stock during the primary inoculation.

[j] ND means not done.

TABLE II

Blood Chemistries

Monkey	Weeks after inoculation	AST[a]	ALT	GGT
Rh-ig7a	0	25	69	68
	2	33	53	54
	4	35	48	60
	* (nothing abnormal for weeks)			
	14 (challenge)	28	49	59
	16 (morbid/death)	**553**	**1,004**	**187**
Rh-ig7b	0	17	52	74
	2	38	42	47
	4	**184**	**398**	**90**
	6	38	34	**125**
	8	40	36	**94**
	10	16	30	65
	* (nothing abnormal for weeks)			
	16 (challenge)	30	36	56
	* (nothing abnormal for weeks)			
	26	41	93	57
	28	25	73	62
	29 (healthy/sacrificed)	29	65	—
Rh-ig8a	0	17	58	72
	1.5 (morbid/death)	**6,062**	**2,429**	**216**
Rh-ig8b	0	66	**91**	**78**
	2	42	**102**	**124**
	4	26	54	85
	* (nothing abnormal for weeks)			
	14 (challenge)	34	**92**	79
	16 (morbid/death)	**1,302**	**1,312**	**217**
Rh-iv3 (ARM)	0	44	44	62
	2	36	48	51
	4	47	47	56
	6	35	53	54
	8 (challenge)	46	58	50
	10	59	83	49
	12 (2nd challege)	51	87	**96**
	13 (healthy/sacrificed)	45	74	—
Rh-ig8 (ARM)	0	35	39	70
	2	23	38	67
	4	12	43	75
	6	20	48	82
	8 (challenge)	23	43	**99**
	10	22	55	**104**
	12	16	50	**104**
	13 (healthy/sacrificed)	15	39	—
Rh-iv3b	0	28	121	164
	14 (morbid/death)	**1,694**	**1,499**	**305**

[a]Data are in international units per liter (IU/L). Aminotransferases (AST, ALT) and gamma-glutamyltransferase (GGT) reference range data are 18–82 (AST), 22–87 (ALT), and 28–86 (GGT) IU/L [Wolford et al., 1986]. Bolded data are those outside the normal reference range.