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Passive Antibody Therapy of Lassa Fever in Cynomolgus Monkeys: Importance of Neutralizing Antibody and Lassa Virus Strain

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Lassa virus-infected cynomolgus monkeys were passively immunized with immune plasma of primate or human origin to gain insight into criteria for plasma selection and administration to human Lassa fever patients. Protective efficacy was correlated with neutralizing antibody concentrations, expressed as a $\log_{10}$ neutralization index (LNI). Convalescent Lassa-immune monkey plasma was titrated for protective efficacy in monkeys by intravenous inoculation with dilutions of plasma on the day of subcutaneous Lassa virus inoculation (day 0) and again on days 3 and 6. Monkeys that received undiluted plasma (LNI = 4.1) (1 ml/kg per treatment) survived a lethal viral dose, whereas those given a 1:3 dilution (LNI = 2.6) of this same plasma (1 ml/kg per treatment) died. Protection was restored when the volume of the 1:3 plasma dilution was increased to 3 ml/kg per treatment. Plasma diluted 1:9 or more (LNI = 1.5 or less) delayed onset and suppressed the magnitude of viremia but failed to confer protection at 3 ml/kg per treatment. Immunological enhancement, defined as increased viremia or accelerated death, did not occur following inadequate treatment. Human convalescent plasma also protected recipient monkeys; reductions in mortality and viremia were accurately predicted by the LNI of the plasma. Plasma of Liberian origin neutralized a Liberian Lassa strain more effectively than a Sierra Leone strain in vitro (LNI = 2.8 and 1.6, respectively) and protected monkeys more effectively against the Liberian strain. Geographic origin is thus a factor in the selection of optimal plasma for treatment of human Lassa fever, since geographically matched plasma is more likely to contain adequate LNI titers against homologous Lassa virus strains. Early infusion of high-LNI plasma appears to be critical for treatment success.

Lassa fever, a severe and often fatal human disease endemic to regions of West Africa, is frequently treated by infusion of plasma obtained from another patient who has recovered from the disease. Plasma is usually selected on the basis of the immune response to Lassa virus measured in the indirect fluorescent antibody (IFA) or complement fixation tests (1, 14, 16–18). Early anecdotal reports of dramatic success attributed to passive immunization (14, 16) contributed to a general acceptance of this procedure for treatment of Lassa fever. Yet treatment success is far from certain (1, 11); in one instance (18), plasma administration preceded precipitous clinical deterioration. Systematic evaluation of passive immunization for Lassa fever in human populations is complicated by numerous variables, including quantity, quality, and timing of immune plasma infusion; and reports to date have not routinely correlated virological or serological parameters with treatment success or failure. These variables are more easily controlled and assessed in a uniformly responding animal model. Using guinea pigs, we established that protective efficacy of passively administered plasma was more accurately predicted by the neutralizing antibody titer than by the IFA test (7). The present report extends protective efficacy testing to cynomolgus monkeys, treated prophylactically with primate or human Lassa-immune plasma. Protection, as measured by increased survival and reduction in viremia, was correlated with neutralizing antibody titer and volume of immune plasma infused.

In conducting the research described in this report, the investigators adhered to the Guide for the Care and Use of Laboratory Animals, as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The animal facilities at Fort Detrick are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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MATERIALS AND METHODS

Preparation of virus stocks and virus assays. Two strains of Lassa virus were employed. The prototype, strain Josiah, was isolated from the serum of a severely ill patient in Sierra Leone (19). A second, strain Macenta, was isolated in our laboratory from the serum of a patient who died in Liberia. Both strains were isolated and passaged at low multiplicity in monolayer cultures of Vero cells (an African green monkey kidney cell line) grown to confluency in 75-cm^2 flasks. Strain Josiah was used at the fourth passage; this virus stock contained 2.5×10^7 PFU/ml and was used in a previous study of Lassa virus pathogenesis in rhesus monkeys (8). Strain Macenta was used at the third Vero cell passage, contained 1.9×10^7 PFU/ml, and has not been employed in previous studies. All virus stocks and infectious material were stored at −70°C until assayed for infectivity. All of the infectious Lassa virus assays were performed by counting PFU on confluent monolayers of Vero cells as described previously (8). All manipulations of infectious material were conducted within the maximal biological containment (P-4) facilities at the U.S. Army Medical Research Institute of Infectious Diseases.

Inoculation and treatment of monkeys. Male cynomolgus monkeys (*Macaca fascicularis*) weighing 3.5 to 5.6 kg were caged individually. Monkeys were sedated by intramuscular inoculation of ketamine-hydrochloride (0.1 ml/kg) before manipulation. Virus suspensions were inoculated subcutaneously (s.c.) in 0.5-ml volumes and diluted in Eagle minimal essential medium to final concentrations as stated in the text.

Monkeys treated with immune plasma or plasma dilutions were inoculated via the saphenous veins over a 10 to 15 min period with volumes based on monkey weight. Plasma treatment was initiated on the day of virus inoculation (day 0) and repeated on days 3 and 6. In one experiment, additional plasma infusions were performed on days 9 and 12. Blood was collected from monkeys for virus and antibody studies by femoral venipuncture with Vacutainer tubes without anticoagulant. Monkeys were observed daily and provided with standard monkey chow and water ad lib. Routinely, samples were taken on day 0 and were repeated on days 3, 6, and 9 and at 3- to 4-day intervals thereafter. In selected cases, blood for virus assay was also drawn 2 h after plasma infusion. Infections were permitted to follow their natural clinical course without therapeutic intervention other than scheduled infusions of immune plasma. Complete necropsies were performed on all monkeys that died (results to be reported separately). Data presented for 20 control monkeys infected with Lassa, strain Josiah, are pooled from 8 monkeys infected for the present experiment and 14 monkeys infected previously as reported (9).

Lassa-immune plasma. Heparinized plasma from two Lassa-immune monkeys was pooled from bleedings obtained at 1-week intervals from 180 to 240 days after infection. Human Lassa-immune plasma was obtained from a donor who had contracted Lassa fever in Liberia 2 to 3 years previously. This donor had donated more than 60 units of plasma for potential use in treating Lassa fever patients. Nonimmune human plasma was donated by a laboratory worker, and nonimmune monkey plasma was pooled from a group of eight rhesus monkeys from the Institute primate colony. Nonimmune plasma was used as a control for some plasma treatment studies and as diluent for the high-titered monkey plasma used in the efficacy study detailed in Table 1.

Antibody assays. The IFA test was performed by using acetone-fixed Vero cells infected with Josiah strain Lassa virus, as described previously (6). Neutralizing antibody titers were measured in a plaque reduction test by using Vero cells and a constant serum dilution (1:10), varying-virus format, as detailed previously (10). Routinely, the Josiah strain was used as challenge virus unless the Macenta strain was substituted, as detailed in the text.

RESULTS

Cynomolgus monkeys inoculated with Lassa virus were protected from death by infusions of high-titered Lassa-immune monkey plasma initiated on day 0 and repeated on days 3 and 6 (Table 1). Whereas 19 of 20 untreated, infected control monkeys died, only 1 of 8 monkeys treated with 1 ml/kg of undiluted plasma died. However, both in vitro neutralizing antibody activity and protective efficacy were rapidly lost on dilution (Table 1). All three monkeys treated with 1 ml/kg of a 1:3 dilution of immune monkey plasma died, although the disease course and days to death were somewhat delayed. The total quantity of plasma, not the plasma dilution, appeared to be the important variable, since monkeys infused with 3 (as opposed to 1) ml/kg of a 1:3 dilution ($\log_{10}$ neutralization index [LNI] = 2.6) of plasma were uniformly protected. However, monkeys treated with higher dilutions of plasma (LNI = 1.5 and lower) all died.

TABLE 1. Protective efficacy of Lassa-immune monkey plasma for cynomolgus monkeys[a]

Plasma dilution	Plasma infused i.v.[b]			Days of inoculation	Results in monkeys	
	LNI	IFA titer	Dose (ml/kg)		No. dead/total	Day(s) of death
Undiluted	4.1	1,280	1	0, 3, 6	1/8	16
1:3	2.6	320	1	0, 3, 6	3/3	18, 22, 25
1:3	2.6	320	3	0, 3, 6	0/4	
1:9	1.5	80	3	0, 3, 6	3/3	15, 16, 16
1:9	1.5	80	3	0, 3, 6, 9, 12	3/3	14, 15, 21
1:27	0.5	20	3	0, 3, 6	3/3	11, 15, 17
Untreated					19/20	14.3 ± 1.8[c]

[a] Inoculated day 0 with Lassa virus, strain Josiah, 6.1 $\log_{10}$ PFU, s.c.
[b] i.v., Intravenously.
[c] Mean ± standard error.

Viremia measurements for these monkeys treated either successfully or not (as listed in Table 1) are displayed in comparison with untreated control viremias (Fig. 1A and B). Untreated monkeys were viremic on the first day sampled (day 3). In contrast, monkeys given undiluted plasma on days 0, 3, and 6 were not viremic on day 3. By day 6 they had developed minimal viremias (mean = 1.9 $\log_{10}$ PFU/ml) before plasma infusion (Fig. 1A). Two hours after undiluted plasma infusion, day-6 viremias were again undetectable, but by day 9 they had reached a moderate titer of 3.5 $\log_{10}$ PFU/ml. After day 9, viremias gradually receded. All seven surviving monkeys were still viremic on day 24 but not on day 35. In contrast, monkeys treated with the highest dilution of plasma tested, 1:27 (LNI = 0.5), developed viremias which were slightly delayed but similar in magnitude to those of untreated controls (Fig. 1A). Despite the fact that these monkeys died within the expected time frame, their viremias were undetectable on day 3 and were significantly lower than control titers even before plasma infusion on day 6. After infusion on day 6, these viremias were reduced to minimally detectable levels, but on day 9 viremias were essentially the same as those for untreated controls. Peak viremia in this group did not exceed that of controls; thus, there was no evidence that inadequate plasma treatment resulted in immunological enhancement, defined as increased viremia due to under-neutralization and increased severity of other disease processes.

Further comparisons in viremia curves among treatment groups are displayed in Fig. 1B. Monkeys treated successfully with a total dose of 1 ml/kg of plasma developed similar viremias whether the dose was administered as undiluted plasma or as 3 ml/kg of plasma diluted 1:3. Maximum titers did not exceed 3 $\log_{10}$ PFU/ml but were maintained at this level past day 21. In contrast, viremias for the 1:9 plasma dilution (LNI = 1.5) recipients eventually approached, but did not exceed, viremias for untreated controls. However, on day 3 viremias were undetectable, and on day 6 they were reduced to undetectable titers after plasma was infused. This observation suggested that additional plasma infusions might reduce the viremia at later times and ultimately result in survival. To test this hypothesis, another group of three monkeys was treated with plasma diluted 1:9 as before on days 0, 3, and 6, but continued on days 9 and 12 (Table 1 and Fig. 2). All treated monkeys died. As before, viremias were reduced to undetectable titers when measured 2 h after infusion on day 6 and rebounded to titers similar to controls by day 9. Plasma infusion on days 9 and 12 resulted in progressively less reduction in viremia. On day 9, preinfusion titers of 4.2 $\log_{10}$ PFU/ml were reduced to 2.6 $\log_{10}$ PFU/ml at 2 h after infusion, but they were not reduced to undetectable levels. On day 12, preinfusion viremias approached 5 $\log_{10}$ PFU/ml and were barely reduced by plasma infusion. Thus multiple infusions of low-titered plasma did

not adequately substitute for early infusions of higher titered plasma, as measured by survival and sustained viremia suppression.

These plasma treatment studies in monkeys were next extended to test the protective efficacy of human plasma representative of multiple units collected from a single patient who had contracted Lassa fever several years earlier in Liberia. Since this plasma collection comprised a large portion of the total Lassa-immune plasma available to us for treatment of human Lassa fever patients, demonstration of protective efficacy was desirable. This plasma neutralized 1.6 $\log_{10}$ PFU of Lassa strain Josiah (the prototype strain from Sierra Leone) and 2.8 $\log_{10}$ PFU of Lassa strain Macenta (recently isolated from a patient in Liberia). As

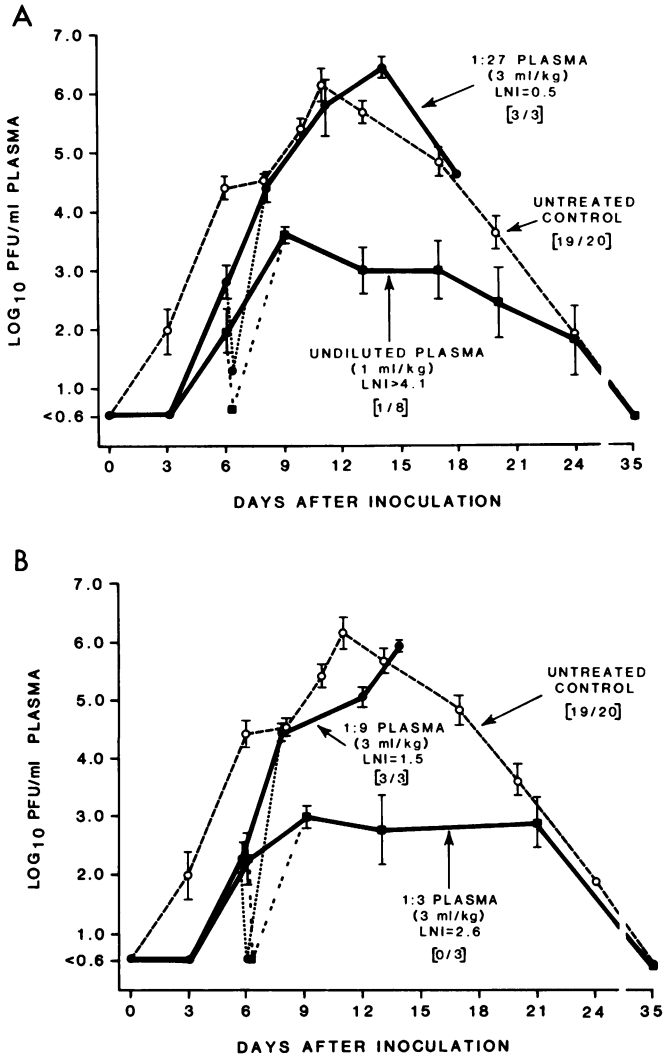

FIG. 1. Lassa viremias and mortalities (dead/total) in cynomolgus monkeys inoculated with Lassa virus ($10^{6.1}$ PFU, s.c.), strain Josiah, and treated with immune monkey plasma or plasma dilutions intravenously on days 0, 3, and 6. Points are geometric means (± standard error) for all monkeys in each group. (A) Monkeys treated with undiluted immune plasma (1 ml/kg) or plasma diluted 1:27 (3 ml/kg) versus untreated controls. (B) Monkeys treated with plasma diluted 1:3 (3 ml/kg) or 1:9 (3 ml/kg) versus untreated controls. Dashed lines accentuate the effect of plasma infusion measured 2 h after the preinfusion bleeding sample.

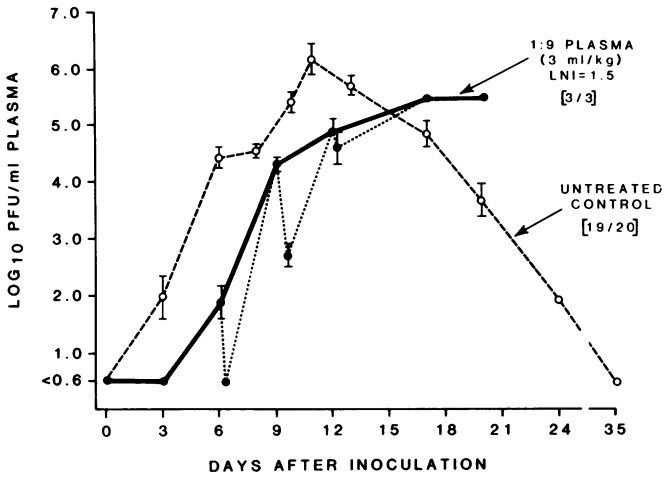

FIG. 2. Lassa viremias and mortalities (dead/total) in cynomolgus monkeys inoculated with Lassa virus ($10^{6.1}$ PFU, s.c.) strain Josiah and treated with immune monkey plasma diluted 1:9 (3 ml/kg) on days 0, 3, 6, 9, and 12. Points are geometric means (± standard error) for all monkeys in each group. Dashed lines accentuate the effect of plasma infusion measured 2 h after the preinfusion bleeding sample.

predicted from the LNI values, this Lassa-immune plasma conferred more effective protection to monkeys infected with the homologous, Liberian strain (Table 2). Comparison of viremias among groups aids interpretation of the results (see Fig. 3 and 4).

All monkeys inoculated with Lassa virus, strain Josiah, and treated with either low (3 ml/kg) or high (12 ml/kg) doses of the Lassa-immune plasma on days 0, 3, and 6 died within the expected time frame. Low-dose plasma infusion had a minimal effect on Josiah viremia, whereas high-dose infusions were associated with a complete suppression of viremia until after day 6 (Fig. 3). However, by day 10, viremias for this group approached those for control monkeys treated with normal plasma. In contrast, viremias and mortality after Macenta strain infection were favorably altered by the Lassa-immune plasma infusion (Fig. 4). For the low-dose (3 ml/kg) treatment group, viremias were undetectable on day 3; on day 6, viremias were suppressed before infusion and

TABLE 2. Protective efficacy of Lassa-convalescent human plasma for cynomolgus monkeys

Lassa virus challenge strain[a] (origin)	Human plasma[b]			Results in monkeys	
	Type	LNI	Dose (ml/kg)	No. dead/ total	Day(s) of death
Josiah (Sierra Leone)	Immune	1.6	3	4/4	8, 11, 13, 19
			12	4/4	14, 17, 17, 18
	Normal	0.0	12	4/4	12, 12, 17, 18
Macenta (Liberia/Guinea)	Immune	2.8	3	1/4	16
			12	0/4	
	No treatment			4/4	11, 13, 13, 15

[a] 6.1 and 6.0 $\log_{10}$ PFU of Lassa strains Josiah and Macenta, respectively, inoculated s.c., day 0.

[b] Plasma was inoculated intravenously at the dose specified on day 0 and was repeated on days 3 and 6. Neutralizing antibody (LNI) titers were measured against the challenge virus strain. IFA titer of the plasma was 1,280 versus both Lassa virus strains.

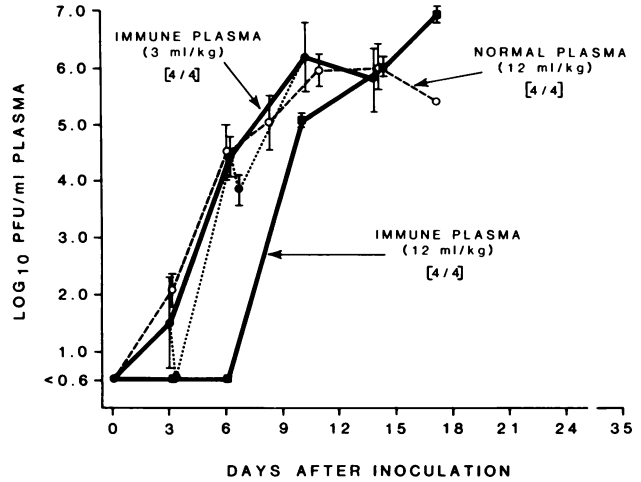

FIG. 3. Lassa viremias and mortalities (dead/total) in cynomolgus monkeys inoculated with Lassa virus ($10^{6.1}$ PFU, s.c.), strain Josiah (Sierra Leone origin) and treated with human Lassa-immune plasma (Liberian origin) infused at 3 or 12 ml/kg on days 0, 3, and 6 versus controls treated with nonimmune human plasma (12 ml/kg). Points are geometric means (± standard error). Dashed lines accentuate the effect of plasma infusion measured 2 h after the preinfusion bleeding sample.

reduced to undetectable titers after plasma infusion. Viremias remained suppressed relative to untreated Macenta control viremias throughout. An even more dramatic decrease in viremia accompanied high-dose (12 ml/kg) Lassa-immune plasma treatment. All surviving monkeys were still viremic on day 24 but had cleared their viremias by day 35.

To determine whether passive or active IFA responses were valuable in predicting the outcome of disease, IFA titers were measured sequentially in all monkeys treated with high-dose (12 ml/kg) Lassa-immune plasma, in comparison with infected controls. All treated monkeys had passive IFA titers of 10 to 40 after infusion on day 0, and they maintained these titers both before and after repeat plasma infusions on days 3 and 6. In comparison, lethally infected, untreated control monkeys infected with either Josiah or Macenta strains developed no detectable IFA response through day 6, but by day 10 they had IFA titers of 20 to 80, which increased to 160 or more in those controls surviving to day 13. In plasma recipients, passive and active IFA titers could not be distinguished on day 10, but total IFA titers ranged from 10 to 20 on day 10 and increased gradually to 20 to 80 on day 13 and to 40 to more than 160 on day 17. Thus, the active IFA response was only slightly delayed in plasma recipients relative to controls, and neither the active nor passive IFA titers were closely correlated with the eventual outcome of the disease.

DISCUSSION

This study clearly demonstrates the beneficial effects of prophylactically administered immune plasma in reducing the acute mortality of Lassa virus infection in cynomolgus monkeys. This study also helps to define some of the problems potentially associated with plasma therapy of human Lassa fever and suggests refinements which could improve treatment success.

Undiluted, convalescent monkey plasma conferred protection to seven of eight recipient monkeys. The in vitro neutralizing antibody titer of the plasma (LNI = 4.1) is characteristic of late convalescent plasma obtained from experimentally infected monkeys; however, the majority of human convalescent plasmas subjected to this standardized neutralization test have LNI titers of less than 2 (P. B. Jahrling, manuscript in preparation). To test the protective efficacy of even lower-titered plasma, more typical of unselected human plasma, two experiments were conducted with similar results. First, dilutions of monkey plasma were tested. Both the LNI and protective efficacy were rapidly reduced by dilution. Undiluted plasma at a dose of 1 mg/kg conferred protection, whereas the 1:3 plasma dilution (LNI = 2.6) did not. When the dosage of the 1:3 plasma dilution was increased to 3 ml/kg so that the total volume of immune plasma remained constant, protection was restored. Since human convalescent plasma is frequently of low titer, it was important to ascertain if increased protection might be similarly obtained by early infusion of increased volumes of plasma with marginally protective titers. This possibility was supported by the second experiment, in which human convalescent plasma, infused at the high dose of 12 ml/kg, suppressed viremia in monkeys more effectively than a low dose of 3 ml/kg.

Monkey plasma diluted 1:9 or more (LNI = 1.5 or less) failed to confer protection but did delay onset and suppressed magnitude of viremia. Even the 1:27 plasma dilution, with marginally detectable LNI and IFA titers (0.5 and 20, respectively), delayed viremia slightly and did not result in a viremia greater than controls. For other virus infections, primarily flaviviruses, under-neutralization frequently leads to immunological enhancement with increased viremias and increased severity of disease (5). This did not occur in the present studies or in a similar study involving two groups of four cynomolgus monkeys treated unsuccessfully with low-titer (LNI = 0.8 and 1.1) Lassa-immune human plasma (P. B. Jahrling, unpublished observation). Monkeys treated with concentrations of antibody adequate to protect against deaths from acute Lassa fever survived 180 days or more, with no occurrence of late sequelae. This contingency was of

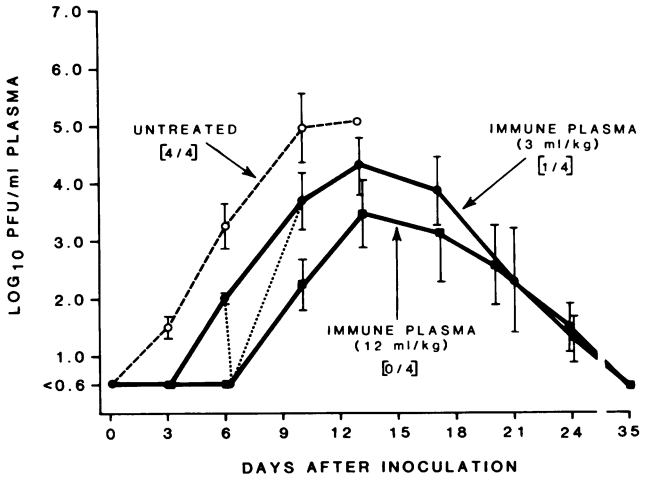

FIG. 4. Lassa viremias and mortalities (dead/total) in cynomolgus monkeys inoculated with Lassa virus ($10^{6.0}$ PFU) strain Macenta (Liberian origin) and treated with human Lassa-immune plasma (Liberian origin) at 3 or 12 ml/kg on days 0, 3, and 6 versus untreated controls. Points are geometric means (± standard error). Dashed lines accentuate the effect of plasma infusion measured 2 h after the preinfusion bleeding sample.

concern because in the treatment of two related arenavirus infections, Machupo virus in rhesus monkeys (4) and Junin virus in humans (15), late neurological sequelae referable to plasma or globulin treatment and thought to be immunologically mediated have been documented. Although the contingency of producing immunological enhancement or late sequelae by plasma therapy of Lassa fever seems remote, one anecdotal case report suggests that plasma therapy could be deleterious (18).

In an attempt to improve treatment success by using plasma with LNI typical of human convalescent plasma, treatment with monkey plasma diluted 1:9 (LNI = 1.5) was extended past days 0, 3, and 6 to include days 9 and 12. This approach failed. Viremias following plasma infusion days 9 and 12 were not dramatically reduced, and survival was not prolonged. Multiple infusions of low-titered plasma did not adequately substitute for early infusion of high-LNI plasma.

Acquisition of high-titered immune material thus appears essential to successful treatment. Identification and subsequent plasmapheresis of the occasional convalescent human with high LNI plasma is an important objective. Geographic matching of Lassa strain and plasma origin may also improve treatment success (e.g., Table 2), but even most geographically matched plasma will titer less than LNI = 2 against "homologous" Lassa virus. Another approach to improving the neutralizing activity of immune material for therapy is the fractionation and concentration of immunoglobulin G suitable for intravenous inoculation from convalescent plasma, by using procedures similar to those successfully employed to prepare immunoglobulin G for treatment of cytomegalovirus infections in human kidney transplant recipients (2, 3). Still another source of high-titered Lassa-immune plasma would be experimentally infected laboratory animals such as rhesus monkeys, which can be protected from death by antiviral drugs such as ribavirin (8) or immunized with attenuated Lassa virus strains (12). Plasma thus generated could be despeciated by using techniques currently accepted for the production of botulinal antitoxin in horses (13). Eventually, human monoclonal antibodies of appropriate specificity may become available in therapeutically useful quantities.

The mechanism by which Lassa immune plasma confers protection is unknown. In tissues obtained terminally from inadequately treated monkeys, concentrations of infectious virus and fluorescent viral antigens were similar to the distributions seen in untreated control monkeys (P. B. Jahrling, submitted for publication). Sequential sacrifice studies to examine tissue concentrations of viral infectivity and antigens in treated versus control infected monkeys might clarify which organs are spared in treated animals. However, sequential sacrifice studies are feasible only in smaller laboratory animal models such as strain 13 guinea pigs (10), which we have used in preliminary plasma efficacy studies (7) with results similar to those reported here for monkeys.

In the intact monkey, neutralization of viremia immediately after plasma infusion is greater than would be predicted by the LNI. For example, in Fig. 1, the 1:27 plasma dilution (LNI = 0.5) in vitro reduced the preinfusion viremia of 2.8 $\log_{10}$ PFU/ml to 1.2 $\log_{10}$ PFU/ml, despite a further dilution of the immune plasma by the blood volume of the recipient. The reason for plasma treatment failures despite complete suppression of viremia beyond day 6 is also of interest (see Fig. 3). Viremia rebounds rapidly after cessation of immune plasma treatment, suggesting that infectious virus replication continues in target tissues even in the presence of immune plasma. Perhaps this replicating virus is susceptible to inhibition by an antiviral drug, which would fit with our observation that combined therapy with immune plasma plus ribavirin is more effective than either treatment modality alone (9).

A limitation of the present study is that timing of plasma intervention relative to infection was not examined. It is probable that effective treatment initiated several days after infection will require higher titered plasma and after a certain period of time may not be feasible at all. With the exception of laboratory exposure, Lassa fever patients rarely seek treatment until the 7- to 10-day incubation period has elapsed. Although published data suggested that high-titered plasma conferred protection to monkeys initially treated on day 4, plasma used alone on day 7 was ineffective (9). Effective treatment of human Lassa fever may require both plasma and ribavirin. The data in the present report suggest that plasma used prophylactically should be selected on the basis of high LNI, and it is probable that the neutralizing antibody test will also prove to be appropriate for selecting optimal plasma for therapeutic use as well.

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