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Ribavirin Prophylaxis and Therapy for Experimental Argentine Hemorrhagic Fever

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Junin virus-infected rhesus macaques received prophylactic and therapeutic ribavirin to assess the potential of this drug for treating humans with Argentine hemorrhagic fever. When ribavirin was administered intramuscularly at the time of experimental infection with the lethal P3790 strain of Junin virus, all animals were protected from clinical disease. A delay in the initiation of therapy until after the onset of illness resulted in improvement and resolution of systemic signs of disease; however, survivors subsequently developed a late-onset central nervous system infection which was fatal in two of three animals. Side effects of ribavirin included thrombocytosis and severe anemia, both of which resolved promptly on withdrawal of drug therapy. Results of this study suggest that ribavirin may prove useful in treating humans with Argentine hemorrhagic fever.

Argentine hemorrhagic fever (AHF) is a debilitating, rodent-borne zoonosis which afflicts hundreds to thousands of the inhabitants of northern Argentina each year. The causative agent, Junin virus, is a member of the *Arenaviridae* family. Infection with this virus is generally symptomatic in humans (24, 26), with death occurring in 15 to 30% of untreated individuals (11, 18). Human immune plasma has been shown to be effective in reducing mortality to <1% when administered within 8 days of the appearance of clinical illness (3, 12). However, a neurological syndrome with a late onset and obscure etiology occurs in about 10% of treated survivors (12). Difficulty with the maintenance of adequate plasma reserves together with the hazards associated with administration of human blood products dictate a need for alternative forms of therapy.

Guinea pigs (7, 17, 25), marmosets (5, 22), and rhesus macaques (6, 15, 16) have been shown to be useful models for studying issues related to the pathogenesis of AHF. Recently, ribavirin (1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide), a broad-spectrum antiviral compound with significant activity in vitro and in vivo against a variety of viruses that cause hemorrhagic fever syndromes in humans, was evaluated for its therapeutic efficacy in guinea pigs infected with Junin virus (12). In that study, mortality among animals infected with strain P3235 (previously called Romero [a Junin viral strain that is highly pathogenic for guinea pigs]) was not affected by ribavirin or a derivative, tributyl-ribavirin, although viral replication was delayed and mean time to death was prolonged. Among *Callithrix jacchus* (marmosets) infected with the prototype XJ strain of Junin virus and tested with ribavirin following the onset of viremia, the time to onset of disease as well as the mean time to death and survival were improved over those seen in untreated controls (23). Because rhesus macaques more accurately reflect the clinical and pathological features of human infection with Junin virus than do guinea pigs or marmosets (6, 15, 16), we felt that an assessment of the therapeutic and prophylactic potential of this drug in this well-characterized primate model system was warranted. The results of these

investigations suggest that ribavirin may be a useful adjunct to therapy in humans with AHF.

MATERIALS AND METHODS

Ribavirin. Ribavirin (1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide) was obtained from ICN Nucleic Acid Research Institute (Covina, Calif.). Powdered drug was dissolved in sterile water for injection, USP, to a concentration of 100 mg/ml and was sterilized by passage through a 22- μ m-pore-size filter. A diluent prepared in the same manner served as a placebo.

Virus. Derivation of the P3790 (referred to as Espindola in previous reports) strain of Junin virus has been described previously (15). In brief, the virus was recovered from the blood of a patient who died of "hemorrhagic" AHF. Following isolation in MRC-5 (human diploid lung) cells, the virus was passaged two additional times in the same cell line to provide a pool containing 6.13 log₁₀ PFU/ml for animal inoculation.

Infectivity assay. Materials for quantitative viral titration were assayed by plaquing the virus on Vero cells, as described previously (14).

Serology. A constant virus, serum dilution technique was used to detect neutralizing antibodies (16). The viral strain used in the neutralization test was XJ clone 3; in previous studies in our laboratory equivalent or higher antibody titers have been shown in standard neutralization tests when this attenuated strain of Junin virus is used rather than those seen when more virulent, homologous strains are used (R. Kenyon and K. McKee, unpublished observations). Endpoints were recorded as the highest dilutions that yielded $\geq 80\%$ reduction in plaque number.

Clinical hematology. Venous blood that was anticoagulated with EDTA in glass tubes was assayed by using an automated hematology analyzer (ELT-8/ds; Ortho Diagnostics, Inc., Westwood, Mass.). Parameters measured included total leukocyte count, hematocrit, hemoglobin, total erythrocyte count, platelet count, and differential estimate. All determinations in this system were based on cell size and internal complexity except hemoglobin, which was measured by a modified cyanomethemoglobin method.

Animal manipulations. All experimental animal manipula-

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TABLE 1. Ribavirin administration schedule and survival for Junin virus-infected rhesus macaques^a

Group	Therapy (schedule ^b)	Survival (no. survived/total no.)	Day of death
1 (placebo)	Placebo ^c (days 0 to 27)	0/4	21, 24, 25, 26
2 (prophylaxis)	Ribavirin (60 mg/kg per day on days 0 to 3; 30 mg/kg per day on days 4 to 7; 15 mg/kg per day on days 8 to 17)	4/4 ^d	
3 (therapy)	Ribavirin (60 mg/kg per day on day 6; 15 mg/kg per day on days 7 to 20 ^e)	1/4 ^f	8, 36, 43 ^g

^a All animals ($n = 4$ in each group) were inoculated i.m. with Junin virus ($4.29 \log_{10}$ PFU) on day 0.

^b Administered i.m. in divided doses at 12-h intervals.

^c Ribavirin diluent.

^d $P = 0.03$ versus group 1, Fisher exact test (two-tailed).

^e $P = 0.99$ versus group 1, Fisher exact test (two-tailed).

^f $P > 0.05$ versus group 1, Mann-Whitney U-test.

^g The mid-range dose was eliminated because of anemia in experimental subjects in group 2.

tions were performed under P-4-level containment conditions, wherein investigators conducted work while wearing flexible plastic suits fed by an external air supply (ILC Dover, Frederica, Del.). Twelve healthy adult male *Macaca mulatta* obtained from the primate colony of the U.S. Army Medical Research Institute of Infectious Diseases served as experimental subjects. Animals were housed individually in banks of four stainless steel cages with collapsible backs. Each cage bank was segregated from the others by means of a portable laminar flow containment cubicle (Bioclean; Hazelton Systems, Aberdeen, Md.). Their diet consisted of monkey chow (Ralston Purina Co., St. Louis, Mo.) and fruit; water was provided ad libitum.

Sedation was used to obtain blood and for physical examination. This was accomplished with ketamine hydrochloride (7 mg/kg per dose). Blood specimens were obtained from saphenous and, preterminally, femoral veins by using a 23-gauge butterfly needle. Venipuncture sites were cleansed with 70% ethanol prior to needle insertion.

For reasons of safety and practicality, animals were not sedated before they received twice daily ribavirin or placebo injections. For drug inoculation, animals were restrained by means of brief compression against the front of the cage. Intramuscular (i.m.) injections were administered as a single bolus dose on each occasion, with rotation of the extremities used to vary the inoculation sites.

Experimental design. Macaques were assigned to one of three treatment or placebo groups via a system of random numbers (Table 1). Each animal was inoculated i.m. on day 0 with 1.0 ml of the P3790 strain of Junin virus containing $4.29 \log_{10}$ PFU. Thirty minutes later, animals in group 1 received 1 ml of placebo i.m., while those in group 2 received appropriately diluted ribavirin. Macaques in group 3 were observed until day 6, when they received appropriately diluted ribavirin. Subsequent to the initial inoculations, each animal received ribavirin or placebo as appropriate for his group (Table 1) every 12 h. The total duration of therapy was 28 days for all groups.

Each animal was observed daily for signs of clinical illness or adverse drug effects. Objective disease parameters were monitored twice weekly for 5 weeks and then weekly for 4 weeks. These included physical examination, body weight, viremia, virus shedding from the oropharynx, neutralizing antibodies, and hematological indices. At selected intervals, lumbar punctures were performed on surviving animals at the time of clinical examination.

Throat swab, serum, and cerebrospinal fluid specimens for virus and antibody determinations were processed as described previously (15). Hematological studies were performed under P-4-level containment conditions inside a

specially designed, maximum containment, clinical pathology laboratory.

Complete necropsies were performed on all animals under P-4-level containment conditions. In preparation for the procedure, animals were heavily sedated with ketamine and then euthanized by exsanguination. Histopathological examination was performed as described in detail elsewhere (6).

RESULTS

Clinical and pathological findings. All animals in the group receiving placebo (group 1) died during the fourth week after infection (Table 1). The progression of clinical signs and physical findings was typical for infection caused by the P3790 strain of Junin virus in rhesus macaques (15, 16). Anorexia, lassitude, and weight loss beginning during the second week was followed within 7 to 10 days by a petechial and ecchymotic skin rash, facial erythema, bloody nasal discharge, conjunctivitis, and lip ulcerations. Profound cachexia with a loss of body weight to 25% of preinfection values was evident preterminally. At necropsy, gross and histological lesions consistent with those reported previously (6) were observed: cutaneous and mucosal hemorrhage, lymphocytic depletion of spleen and lymph nodes, depletion of bone marrow elements, and minimal to mild gliosis and perivascular inflammation within the central nervous system.

Those animals that received prophylactic ribavirin (group 2) developed mild anorexia and lassitude by the middle of the second week, along with a distinctive pallor. These findings persisted for 2 to 3 weeks and then gradually disappeared. Moderate weight loss occurred, averaging 10% of preinfection values; however, weight change did not differ significantly ($P < 0.05$) between prophylactic and placebo groups until 21 days postinfection. All animals in group 2 survived ($P = 0.03$ versus group 1). When euthanized at 6 months postinfection, three of four macaques were essentially free of pathological lesions. The fourth member of this cohort had mild, multifocal perivascular cuffing in the central nervous system.

Three of the four macaques in the therapeutic group (group 3) died. The median time to death was prolonged in comparison with macaques in group 1 (36 versus 24.5 days, respectively), but the difference did not achieve statistical significance ($P > 0.05$). The disease course in this cohort differed substantially from that seen in placebo-treated animals, however. One macaque in this therapeutic group died early. At the time of his demise, this animal was viremic ($2.35 \log_{10}$ PFU/ml of serum). Unfortunately, autolysis of tissues subsequent to a delay in processing precluded patho-

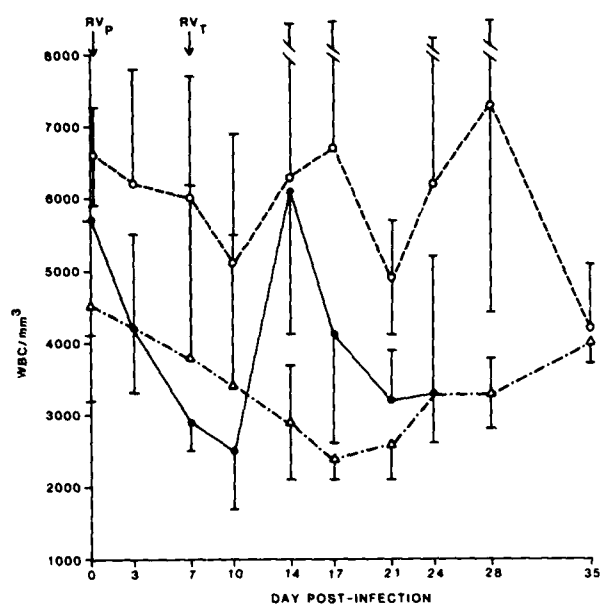


FIG. 1. Total peripheral leukocyte (WBC) counts in rhesus macaques infected with Junin virus. Each point represents the mean \pm 1 standard deviation. Symbols: ●, group 1 (placebo); ○, group 2 (prophylactic ribavirin); △, group 3 (therapeutic ribavirin). RV_P indicates the initiation of prophylactic ribavirin; RV_T indicates the initiation of therapeutic ribavirin.

logical assessment. Among the three remaining animals, all developed appetite loss and lassitude at the same time as those in group 1. Early hemorrhagic phenomena (bloody nasal discharge, facial rash, and conjunctivitis) were seen as well. These findings were transient, however, persisting for no more than 3 to 5 days. The animals then recovered clinically and remained reasonably healthy, aside from mild anorexia, until day 28. At that time, all three macaques developed severe ataxia, intention tremor, and aggression. Central nervous system signs progressed in two of the three animals to include paresis and loss of deep tendon reflexes. Two of the three animals died; ataxia persisted in the surviving macaque until after the ninth postinfection week. Among the two macaques that died, pathological lesions similar superficially to those in group 1 macaques were noted. Congestion and mild lymphocytic depletion of the spleen occurred, but lymph nodes were spared. The bone marrow was normal. Lesions in the central nervous system were more pronounced, with mild to moderate perivascular cuffing and gliosis. Inflammatory infiltrates within autonomic ganglia were noted as well. When the final survivor was necropsied at 6 months postinfection, pathological changes were found to be minimal. Central nervous system lesions included multifocal, minimal, nonsuppurative vascular cuffing and focal choroiditis.

Hematological findings. A variety of hematological parameters were followed serially in the three animal cohorts. In the placebo group, changes were similar to those reported previously for macaques that were infected with this strain of Junin virus (16); the total leukocyte count fell to a nadir of <50% of the base line at 7 to 10 days and then rebounded (Fig. 1), while platelet counts fell to $1 \times 10^5/mm^3$ or less (Fig. 2), and erythrocyte parameters (hematocrit, hemoglobin,

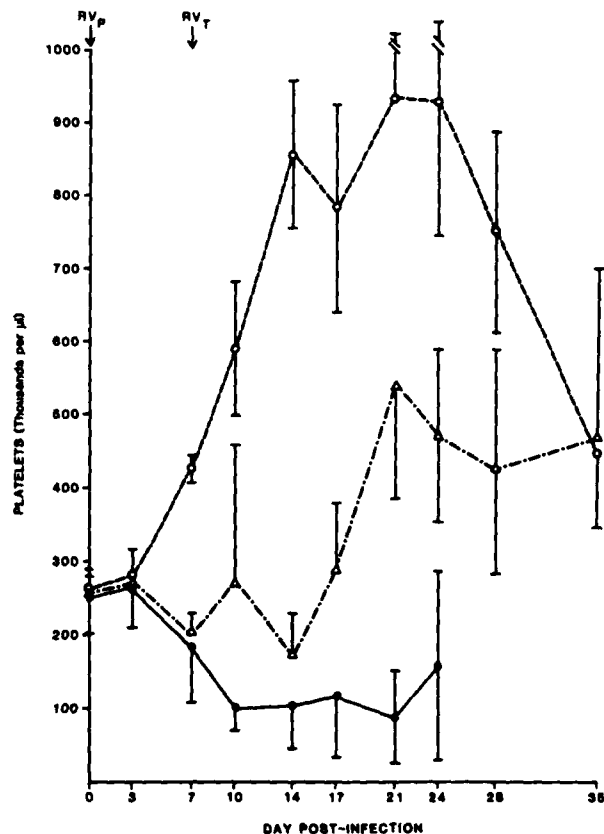


FIG. 2. Platelet counts in rhesus macaques infected with Junin virus. Each point represents the mean \pm 1 standard deviation. Symbols: ●, group 1 (placebo); ○, group 2 (prophylactic ribavirin); △, group 3 (therapeutic ribavirin). RV_P indicates the initiation of prophylactic ribavirin; RV_T indicates the initiation of therapeutic ribavirin.

and total erythrocytes) fell modestly until the time of death (Fig. 3).

Ribavirin administered prophylactically or therapeutically had little effect on leukocyte counts, except that the magnitude of depression was somewhat dampened (Fig. 1). A significant difference from animals that received placebo was observed only in prophylactically treated animals on day 21 ($P = 0.034$); no differences were detected at any other time point ($P > 0.05$).

We observed a striking effect of ribavirin on circulating platelet numbers (Fig. 2). Animals that received drug from the outset (prophylactic group) exhibited progressive thrombocytosis; peak platelet counts exceeded $1 \times 10^6/mm^3$ in some animals on days 21 and 24. These changes in platelet numbers differed significantly ($P < 0.05$) for the placebo group from day 7 onward. A similar pattern, although it was expressed less dramatically, was seen in animals that received ribavirin therapeutically (Fig. 2). Beginning 1 week after the initiation of drug therapy, platelet counts increased progressively, reaching a significant deviation from that in the placebo group ($P = 0.007$) by day 21. An influence of infection on platelet values was apparent as well; differences between prophylactic and therapeutic groups achieved significance ($P < 0.05$) at 7, 14, 17, and 24 days following infection.

The impact of ribavirin on erythrocytes was profound

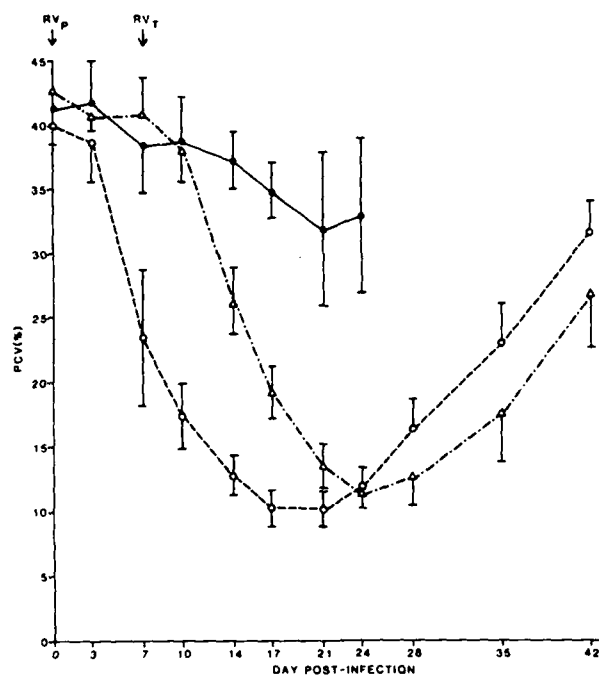


FIG. 3. Central hematocrit (packed cell volume [PCV]) values in rhesus macaques infected with Junin virus. Each point represents the mean \pm 1 standard deviation. Symbols: ●, group 1 (placebo); ○, group 2 (prophylactic ribavirin); △, group 3 (therapeutic ribavirin). RV_p indicates the initiation of prophylactic ribavirin; RV_t indicates the initiation of therapeutic ribavirin.

(Fig. 3). Within 1 week of drug administration, hematocrit values showed depression from base-line and placebo values ($P < 0.05$), which continued until drug use discontinued. Although the pattern of hematocrit change was displaced by an interval of 1 week in therapeutically treated animals (because of a corresponding delay in the initiation of therapy), no quantitative differences in effect were seen between groups that received ribavirin prophylactically or therapeutically. Within 3 to 4 days of drug cessation, values began to increase, progressively returning toward the base line. The additional erythrocyte parameters measured (hemoglobin and total erythrocytes) displayed similar behavior (data not shown).

Virus isolation and serology. Virus was recovered from all placebo-treated macaques. In general, viremia and throat swab patterns of virus isolation were similar to those reported previously in rhesus macaques infected with the P3790 strain of Junin virus (15, 16), with peak geometric mean titers reaching 3.49 and 3.46 \log_{10} PFU/ml on day 17 postinoculation in serum and throat swabs, respectively. Virus was recovered from most animals up to the time of death, with peak individual titers in serum reaching 5.88 \log_{10} PFU/ml and titers in throat swabs reaching 5.30 \log_{10} PFU/ml. In one exceptional animal, repeated attempts at isolating the virus from serum were unsuccessful. However, more than 3 \log_{10} PFU/ml was shed from his oropharynx.

No virus was recovered from the serum or oropharynx of any animal in the ribavirin prophylaxis group. Among the macaques that received the drug therapeutically, Junin virus (2.35 \log_{10} PFU/ml) was isolated at the time of therapy initiation in one animal (the macaque who subsequently died early) and in a second macaque 3 days later (1.88 \log_{10} PFU/

ml). Virus was not present in serum at any other time in this group of animals, nor was it recovered at any time from the oropharynx.

Virus isolation was attempted from cerebrospinal fluid on three occasions (days 17, 31, and 35 to 36 postinfection) from macaques that received ribavirin therapeutically. Junin virus was recovered only once, on day 35, at a titer of 2.51 \log_{10} PFU/ml. Central nervous system dysfunction was apparent in this macaque at the time that the lumbar puncture was performed; simultaneous attempts to isolate virus from serum yielded no virus.

With the exception of the single macaque who died 8 days following infection, all animals in the study developed neutralizing antibodies to Junin virus. Seroconversion occurred in most macaques in groups 1 and 3 by 24 days postinfection (range, 14 to 35 days), while antibodies were not detected in animals in group 2 before day 35. Aside from the time to initial antibody appearance, the kinetics of antibody development and geometric mean titers did not differ importantly among the three groups of macaques studied.

DISCUSSION

In previous studies the effectiveness of ribavirin has been demonstrated in treating infections caused by arenaviruses in experimental animals and humans (1, 20). Improvements in virological and other disease parameters in guinea pigs infected with Lassa (P. B. Jahrling, unpublished observations), Machupo (20), and Junin (10) viruses have stimulated more extensive assessments of ribavirin efficacy in nonhuman primate systems.

The rate of mortality in rhesus macaques infected with Lassa virus is about 60%; ribavirin administered prophylactically or therapeutically (50 mg of loading dose per kg, followed by 30 mg/kg per day) resulted in improved survival, delayed onset of viremia, and depression of peak viremia levels in comparison with untreated controls (8). Even when therapy was delayed until day 5 following infection, all treated animals survived. Similarly, cynomolgus monkeys treated by day 4 after Lassa virus infection universally survived (9). These impressive preclinical findings stimulated chemotherapeutic trials to be conducted in human patients with Lassa fever in Sierra Leone, where mortality reductions of >50% were seen in patients from high-risk subgroups defined by admission viremia and serum aspartate aminotransferase values (13).

AHF is clinically and pathologically more similar to Bolivian hemorrhagic fever than to Lassa fever in humans (18). Moreover, their etiologic agents (Junin and Machupo viruses, respectively) are more closely related (18). Treatment of rhesus macaques infected with Machupo virus by using several different prophylactic and therapeutic dose regimens of ribavirin and ribavirin triacetate resulted in a significant ($P < 0.001$) reduction in viremia and improved survival of the acute (hemorrhagic) phase of Bolivian hemorrhagic fever compared with placebo-treated controls (20). However, the late neurological syndrome seen in most untreated monkeys that survived the hemorrhagic phase was not prevented (20), and all treated animals eventually died.

In the present study, rhesus macaques that received ribavirin on a prophylactic schedule became infected following inoculation with Junin virus. Infectious virus could not be recovered from serum or oropharyngeal secretions, and animals exhibited no clinical or hematological evidence of disease. When drug therapy was begun after the onset of viremia and clinical signs (day 6 postinfection), rapid clear-

ance of viremia, together with resolution of clinical disease over 2 weeks, occurred. Within 2 weeks of the conclusion of treatment in this latter group, a neurological syndrome similar to that seen in ribavirin-treated monkeys with Bolivian hemorrhagic fever appeared. Two of three animals died, while the third progressed to clinical recovery.

Depression of erythrocyte parameters, together with thrombocytosis, developed in all ribavirin-treated macaques. The rapid fall in hematocrit that we observed among animals in the prophylactic group prompted modification of the planned dosing schedule for animals in group 3 (from 60 mg/kg for 5 days to 60 mg/kg for 1 day [Table 1]). The degree of anemia that was observed was striking (Fig. 3), but it resolved promptly and consistently on withdrawal of drug therapy. Rhesus macaques have been shown to be particularly sensitive to the erythrotoxic effects of ribavirin, to an extent greater than that seen in humans (2, 21). While the short-term impact on bone marrow in these animals can only be inferred from peripheral hematological studies, histopathological findings conducted up to 5 months following therapy revealed no long-term adverse effects.

Thrombocytosis is a recognized side effect of ribavirin administration in rhesus macaques (and, to a lesser extent, in humans) (2). The mechanism for this phenomenon is undefined; however, an increase in megakaryocyte number and ploidy in ribavirin-treated macaques suggests that there is a stimulus to production (T. M. Cosgriff, P. G. Canonico, L. Hodgson, D. Parrish, T. Chapman, J. W. Huggins, Z.-J. Gong; L.-B. Xiang, and C.-H. Hsiang, *Thromb. Haemost.* 58:1746, 1987 [abstract]). Results of aggregation studies reveal that these platelets function normally (2; Cosgriff et al., *Thromb. Haemost.* 58:1746, 1987 [abstract]). Assuming that depressed platelet function accompanies the thrombocytopenia which occurs in patients with AHF, as has been demonstrated in Machupo virus-infected macaques (P. B. Jahrling, R. W. Trotter, J. G. Barreva-Oro, H. W. Lupton, T. M. Cosgriff, R. W. Louis, D. B. Parrish, S. B. Smith, and C. J. Peters, *Int. Conf. Impact of Viral Diseases on the Development of Latin American Countries and the Caribbean Region*, 1988), one might hypothesize that there is a potentially beneficial effect on hemostasis resulting from the administration of ribavirin.

Ribavirin (30 mg/kg for 30 days) recently has been shown to improve the clinical outcome among neotropical marmosets infected with Junin virus (23). In these experiments, the survival rate was improved over that in untreated controls (28 versus 0%), and the mean time to death was prolonged (36 versus 18 days). As occurred among rhesus macaques that received ribavirin in the present study, progression of clinical disease in marmosets was prevented until after therapy was concluded, with late-appearing signs and symptoms related predominantly to central nervous system involvement. In contrast to our findings in rhesus macaques, however, viremia in serum was not significantly affected until after therapy was concluded.

The utility of rhesus macaques infected with low-passage, human-virulent strains of Junin virus as a realistic model for AHF in humans has been established (6, 15, 16). The occurrence of aggressive viral invasion of the central nervous system in this model constitutes its major inconsistency with infection in humans (6, 16). The efficacy of treatment appeared to be restricted here, as in the macaque model of Bolivian hemorrhagic fever and the marmoset model of AHF, to protection against the acute, hemorrhagic phase of infection. Failure to prevent (or ameliorate) the late neurological disease probably results from the inefficiency with

which ribavirin crosses the blood-brain barrier (4). As virus is isolated only occasionally from the central nervous system of humans with fatal AHF (J. I. Maiztegui, C. Rivero, S. C. Levis, A. J. de Damilano, N. J. Fernandez, and D. A. Enria, *III Congreso Argentina de Microbiología*, abstr. 6-191, 1982), the pathogenesis of this phase of disease in nonhuman primates may differ from that which occurs in humans (19). Evaluation of ribavirin in humans with AHF would therefore appear justified, with the expectation that those individuals in whom central nervous system disease has been established by direct viral invasion prior to the initiation of therapy will not likely be benefited.

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