Pathogenesis of Machupo virus infection in primates *

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Experimental Machupo virus infection of rhesus and cynomolgus monkeys produced a severe illness consisting of an initial clinical phase and a later neurological phase. Cumulative mortality during the two phases was 80 % and 95 % respectively. Attempts to alter the pathogenesis with decomplementation or immunosuppression resulted in earlier deaths of the monkeys.

In our initial studies of experimental infection with Machupo virus, the causative agent of Bolivian haemorrhagic fever (BHF), in the rhesus monkey (*Macaca mulatta*), we found this animal to be highly susceptible and the clinical course to be similar to that of severe human illness with BHF (M. D. Kastello et al., unpublished data). We have subsequently extended our observations and measurements to a large number of cage-confined rhesus monkeys and cynomolgus monkeys (*M. fascicularis*) and this report summarizes their responses. It also describes our efforts to alter the pathogenesis of the disease.

MATERIALS AND METHODS

Monkeys

Clinical observations of monkeys infected with Machupo virus were made daily. Our data are based on 43 young rhesus monkeys weighing 2.5–4 kg, 4 mature rhesus monkeys, 5–8 kg in weight, and 7 cynomolgus monkeys weighing 2–3.5 kg. All the young rhesus monkeys in this study were untreated, virus-inoculated controls from other experimental studies with Machupo virus. The monkeys were healthy and clinically normal, and none had a known exposure to any arenaviruses prior to Machupo virus inoculation.

Virus, virus assay, and virus inoculations

All monkeys were inoculated subcutaneously with approximately 1000 plaque-forming units (PFU) of either the third or fourth suckling hamster brain passage of the Carvallo strain of Machupo virus (1).

Virus was assayed by enumeration of PFU in Vero cell cultures (a continuous line of African green monkey kidney cells) as described previously (2).

Serology

Plaque reduction, serum dilution, neutralizing antibody tests were done by the method of Earley et al. (3), except that we used Vero cells with the same overlay medium as in the virus assay.

RESULTS

Clinical responses of monkeys to Machupo virus infection

Rhesus monkeys showed a distinct sequence of clinical signs, usually culminating in death about 19 days after virus inoculation. All the rhesus monkeys became clinically ill by day 6–7, with conjunctivitis, depression, anorexia, and fever, when measured. Conjunctivitis was observed in all monkeys throughout the illness. Depression and anorexia became progressively more severe with time, and most rhesus monkeys ceased eating entirely by day 14 and became moribund 1 or 2 days before death. Dehydration occurred in all monkeys and became maximal on about day 18–19. At the onset of clinical signs most monkeys became constipated; later diarrhoea occurred sporadically in a majority of monkeys. Clonic spasms, consisting of intermittent clonic muscular contractions, occurred in less than half the monkeys studied, and were seen as early as day 12–14. We do not know whether they were of

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neurological origin. Nasal discharge, sometimes haemorrhagic, was seen in approximately half the monkeys; it became severe in a fraction of the monkeys at about the time of death. An erythematous facial rash was very common in the rhesus monkeys and an abdominal rash was also observed, but less frequently. All clinical signs were more pronounced in the rhesus than in the cynomolgus monkeys. Typically, rhesus monkeys became increasingly depressed, anorectic, and dehydrated before death, whereas the cynomolgus monkeys often died unexpectedly without exhibiting prior clinical responses as severe as in *M. mulatta*.

Approximately 20% of the monkeys began to show clinical improvement after day 20–21. They gradually became more active, appetites improved, and dehydration diminished. Beginning about days 26–30 and as late as days 36-40 most of the surviving monkeys developed pronounced neurological signs, including severe intention tremors, nystagmus, incoordination, paresis, and coma. Of the 54 monkeys in the three groups shown in Table 1, all became ill during the initial clinical period, and 10 showed improvement after day 20–21. Seven of these monkeys died during the late encephalitic phase. The 3 survivors, 1 cynomolgus and 2 young rhesus monkeys, did not become severely ill during the neurological phase of the illness. Two of the 3 survivors were killed on postinoculation days 42 and 43 for a complete necropsy. Upon histopathological examination they showed moderate to severe encephalitis with vasculitis, as have all other survivors of

Table 1. Deaths, late encephalitis, and survivors among monkeys inoculated with Machupo virus

Monkeys (weight)	No.	Survivors		Mean day of death±SD[c]
		Initial illness[a]	Late encephalitis[b]	
Rhesus (2.5-4 kg)	43	0.14	0.05	19.3±5.6
Rhesus (5-8 kg)	4	0.50	0	30.5±9.4
Cynomolgus (2-3.5 kg)	7	0.29	0.14	17.0±6.6
All monkeys	54	0.19	0.06	19.9±6.7

[a] Fraction of total that survived the initial illness and exhibited clinical improvement prior to onset of late encephalitis.

[b] Fraction of total that survived both initial disease and late encephalitis.

[c] Mean day of death and standard deviation of all monkeys that died following the inoculation of 10^3 PFU of Machupo virus on day 0.

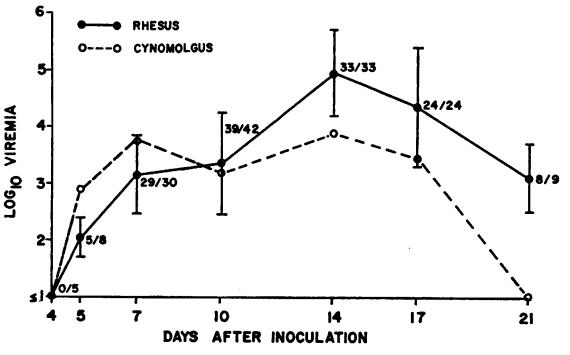

Fig. 1. Mean viraemia levels in rhesus and cynomolgus monkeys following the inoculation of 1000 PFU of Machupo virus on day 0. Brackets indicate 1 standard deviation (shown for rhesus only); the fractions show number of rhesus monkeys viraemic over the total number tested on that day.

experimental Machupo virus infection, regardless of clinical signs or treatment, that have been examined after infection (C. H. McLeod, personal communication, 1975). Although the number of mature rhesus monkeys studied was limited, they were more likely to survive the initial clinical disease than the younger monkeys (Table 1). Two of the 4 mature rhesus monkeys improved clinically following the initial signs of illness but subsequently died with severe encephalitic signs on days 34 and 42 after virus inoculation. The mature rhesus monkeys died significantly later than either the young rhesus ($P < 0.05$) or the cynomolgus monkeys ($P < 0.01$). The cynomolgus monkeys did not differ appreciably from the young rhesus group in terms of survival or day of death.

Serological responses to Machupo virus infection

The pattern of serological responses fell into 3 categories. The majority of monkeys died while still viraemic and developed no detectable neutralizing antibody prior to death. In contrast the 7 monkeys that survived the initial clinical signs and subsequently died during the encephalitic phase of the disease invariably developed neutralizing antibody titres of 1 : 32 or higher between days 21 and 28. The 3 monkeys that survived both phases of illness developed neutralizing antibody by days 14–17.

Viraemia

The sera of most of the rhesus and cynomolgus monkeys were assayed for virus on days 7, 10, 14,

Table 2. Mean viraemia and day of death for decomplemented, Machupo-virus-infected monkeys [a]

Treatment	Viraemia [b] by day±SE			Mean day of death±SE
	7	10	13	
CVF	4.0±0.43	3.1±1.12	5.9±0.42[c]	15.3±1.5
HSA controls	3.4±0.39	3.8±0.45	4.6±0.14	26 ±8.1

[a] Six monkeys were inoculated with 1000 PFU of Machupo virus on day 0 and 3 were each subsequently treated with either CVF or HSA as described in the text.

[b] Geometric mean virus titre, $\log_{10}$ PFU/ml of serum.

[c] Mean virus titre differed significantly from control on the same day ($P<0.05$).

and 17, and a smaller number were assayed on days 4, 5, and 21. The results (Fig. 1) showed peak levels of circulating virus in the rhesus monkeys on days 14 and 17. Viraemia in the cynomolgus monkeys did not differ significantly from that in the rhesus monkeys. None of the 5 monkeys assayed was detectably viraemic on day 4, but 5 out of 8 were by day 5, and assays through day 21 showed that a large proportion of monkeys were viraemic over a 14-day period. The 3 survivors exhibited low or intermittent viraemias, and their maximum titres never exceeded 4×10^3 PFU/ml of serum.

Attempts to alter clinical and viraemia responses by decomplementation

To test the possibility that complement might in some way contribute to pathogenesis, we measured the effect of decomplementation on viraemia and time to death. Each of 3 monkeys 2.5–3 kg in weight received 1000 units of freshly reconstituted cobra venom factor [a] (CVF) by intravenous injection on days 10 and 13, whereas 3 control monkeys received a similar volume of 0.01% human serum albumin (HSA). It was our intention to give a third dose of CVF on day 15 or 16, but because 2 treated monkeys died on days 13 and 15, only 1 monkey received the third injection of CVF. The results (Table 2) show that a significantly higher peak viraemia occurred on day 13 in the decomplemented monkeys. Although the time to death for treated monkeys in this study was not significantly longer than for the HSA controls, there was a trend toward earlier deaths in the group that received CVF.

[a] Supplied by Cordis Corporation, Miami, FL 33137, USA.

Table 3. Onset of viraemia and mean day of death in Machupo-virus-infected monkeys treated with cyclophosphamide

Cyclophosphamide given on days:	No. monkeys	Viraemic/survivors		Mean day of death±SE
		Day 5	Day 7	
None	2	2/2	2/2	15.5
–3, –2, –1	3	3/3	2/2	14.0±3.5
3, 2, 1	3	1/3	1/1	7.7±0.7

Immunosuppression

To assess the role of the immune response in the pathogenesis of Machupo virus, we administered cyclophosphamide intravenously, 20 mg/kg, to monkeys for 3 days, either prior to or after the inoculation of Machupo virus on day 0, as shown in Table 3. The data show that the treatment with cyclophosphamide for 3 days prior to Machupo virus infection had little effect on either the day of onset of viraemia or on the time to death of the monkeys. Treatment with the drug during the 3 days following virus inoculation appeared to shorten the time to death, despite the lack of statistical significance, and it may have delayed the onset of viraemia.

We have also attempted similar cyclophosphamide therapy on 3 monkeys with severe late neurological signs. There was no apparent effect, and the monkeys died.

DISCUSSION

The clinical observations on the monkeys reported herein extend our previous studies (M. D. Kastello et al., unpublished data) of rhesus monkeys as a model of BHF. In this relatively large number of Machupo-virus-infected monkeys of the genus *Macaca*, we observed a severe and highly fatal illness occurring in 2 distinct phases. Mortality in the young monkeys occurred principally during the initial phase of illness, and the clinical signs paralleled those described in highly fatal outbreaks of BHF in humans (2). The occurrence of late encephalitis, although not a feature of human illness with the virus, was common among the monkeys surviving the initial clinical signs, and may provide a model for postinfectious encephalitis. Our attempts to re-

cover virus from such monkeys during this phase of illness have yielded variable results, possibly influenced by the presence of circulating antibody, often in high titre, in the monkeys during the encephalitic phase of the disease.

In the monkeys surviving the acute infection, viraemia persisted until approximately the time of appearance of neutralizing antibody in the circulation, between days 21 and 28. This differed from the reports of human illness, in which, assuming a 10–14 day incubation period, neutralizing antibody did not appear until 4–6 weeks after initial virus exposure (4).

No firm conclusions regarding mechanisms of pathogenesis may be drawn from our data on the effects of decomplementation and immunosuppression. The number of monkeys was relatively small and in view of the increasingly short supply of monkeys it is possible that the experiments may never be repeated or extended. It is our opinion, however, given the nature of the severe arenavirus diseases, that the data should be included in the literature. Data from other arenavirus models (5) suggest that the immune response or the complement system might play a role in human arenavirus pathogenesis. The one conclusion that seems apparent from both our decomplementation and our immunosuppression results is that neither procedure was of benefit to the monkey in surviving infection with Machupo virus. During the initial phase of the illness the effects were deleterious, and decomplementation and immunosuppression as attempted herein would not be recommended for therapy. It is suggested that further immunosuppression studies might be tried during the neurological phase.

RÉSUMÉ

PATHOGENÈSE DE L'INFECTION PAR L'ARÉNOVIRUS DE MACHUPO CHEZ LES PRIMATES

Les auteurs rendent compte des observations cliniques, virologiques et sérologique faites sur les singes auxquels on avait injecté au laboratoire l'arénovirus de Machupo. A des singes *Macaca mulatta* (rhésus) et *Macaca fasicularis* (cynomolgus), on avait inoculé approximativement 1000 unités formatrices de plages. Les jeunes rhésus, d'un poids de 2,5-4 kg, sont tous tombés malades (fièvre, anorexie, dépression, conjonctivite) environ 6-7 jours après inoculation du virus de Machupo. Ces signes cliniques et d'autres (diarrhée, éruptions faciales et abdominales, saignements du nez, spasmes ou convulsions cloniques et déshydratation) se sont habituellement aggravées jusqu'à la mort. La mort est survenue entre 10 et 40 jours après l'inoculation de virus, en moyenne après 19,3 jours. Dix des 54 singes ont manifesté une amélioration clinique après 21 jours, ont survécu jusqu'au 30^{e} jour voire davantage et 7 d'entre eux ont présenté des signes neurologiques graves et sont morts. Des virémies ont commencé à se manifester entre le 5^{e} et le 7^{e}, le point culminant étant atteint entre le 14^{e} et le 17^{e} jours. Trois singes qui ont surmonté l'infection ont présenté, entre le 14^{e} et le 17^{e} jour, des anticorps neutralisants, tandis que chez tous les autres singes qui ont survécu jusqu'au 28^{e} jour les anticorps sont apparus entre le 21^{e} et le 28^{e} jour. Les tentatives faites pour modifier l'évolution de la maladie chez les singes ont montré que la « décomplémentation » par le facteur de venin de cobra accroissait la virémie et peut-être avançait quelque peu l'heure de la mort. Le traitement au cyclophosphamide après inoculation du virus semble avoir retardé légèrement la virémie et avancé le moment de la mort. Ni l'un ni l'autre des traitements n'avait un intérêt thérapeutique chez les primates.

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DISCUSSION

WALKER: Was there any evidence of the presence of circulating immune complexes? Were you able to detect immunofluorescent staining of vessels in the brain or kidneys, or was there any other histopathological evidence of vasculitis?

EDDY: We did not do any of those things. We treated the viraemic serum, both early and late in the viraemia, with anti-monkey globulin in an attempt to find out whether or not there was antibody complexed with the virus. In no case did this decrease the apparent viraemia.

HOTCHIN: We have found recently that early neutralizing antibody for LCM virus is apparently complement dependent. If we add complement to persistently infected hamster serum, the titre drops approximately five-fold. We have a little evidence that in persistently infected hamsters complement is grossly depleted. Do you know whether this happens in human arenaviral disease? I am wondering whether complement might even be therapeutic sometimes.

EDDY: We have done a number of C3 assays in monkeys but have not seen any significant change in complement levels.

MIMS: Did you fractionate any of the blood constituents and do separate virus assays—on leucocytes as opposed to serum? Secondly, did you do brain titrations at the time of the neurological disease?

EDDY: All the virus we found was in the serum. Three months ago I would have said that we do find lots of virus in the brain in the late neurological phase, but that would have been based on titrations in two monkeys. Since then we have done brain titrations on four additional monkeys and have been unable to recover any virus.

NATHANSON: Have you used the FA test to measure the levels of antigen in the brain?

EDDY: We have not done that.

BOND: Yesterday Dr Webb discussed briefly her use of a New World marmoset for studies of viraemia and neutralizing antibody production in Machupo virus infection. You have been using the rhesus model, and I understand there is quite a shortage of rhesus monkeys. Could the marmoset be used in your pathogenesis or vaccine studies, and if so, could this kind of research be done in the countries where the marmoset exists naturally in Latin America?

EDDY: The marmoset is not an entirely satisfactory model because of intercurrent problems of parasites and spontaneous deaths. We have never assessed any other New World monkey.

K. JOHNSON: I should like to make it clear that, to the best of my knowledge, the very interesting late neurological disease seen in the rhesus monkey has never occurred in human infections with Machupo virus, nor am I aware that it has with the related Junin virus. There is a so-called neurological phase to the acute disease, which is seen in both the Bolivian and the Argentinian disease, but I have never seen, at least in man, a late neurological illness.

MAIZTEGUI: I would agree. Personally I have never seen a case like that. A few case reports describe mild neurological disorders at about 2½ to 3 weeks, but a severe type of encephalitis has not been noted.

NATHANSON: You mentioned that vasculitis may be a problem in the brain. What about the rest of the animal? Is there evidence of a generalized vasculitis or is it strictly confined to the central nervous system?

EDDY: Perivascular cuffs without any striking necrosis are present throughout the monkey, even in such out of the way places as the parathyroid glands.

NATHANSON: This is evidently quite different from a "conventional" viral encephalitis, which is not accompanied by general vascular changes. Perhaps it is a somewhat different syndrome.