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Pathogenesis of Hantaan Virus Infection in Suckling Mice

Clinical, Virologic and Serologic Observations

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ABSTRACT

Hemorrhagic fever with renal syndrome (HFRS) is a debilitating disease of humans caused by Hantaan virus (HV), the prototype member of a newly proposed genus of *Bunyaviridae*. Studies of HV pathogenesis have been limited by the absence of a well defined model for a virus-induced disease state. In an attempt to devise a model for HV pathogenesis in laboratory rodents, newborn outbred suckling ICR mice were shown to be uniformly susceptible to lethal infection with non-mouse adapted HV by intracerebral (IC), intraperitoneal (IP), intramuscular (IM), and subcutaneous (SC) inoculation routes. Clinical coures, mean time to death, and fatal outcome were age-dependent. With an inoculum of 10 LD_{50}, mortality was 100% in mice infected within 72h of birth, but declined to 50% by 7 days. By 2-2.5 weeks, animals developed complete resistance to clinical disease. Virus was consistently detected in serum by day 6 post-infection in IC- and IP- inoculated animals, and reached peak levels of 10^8 PFU/ml by day 8. Mice infected IM and SC showed delays in onset of viremia, but achieved similar titers. Immunofluorescent antibody appeared by 17-18 days, and neutralizing antibody by 15 days, in all experimental groups. Two of 8 inbred mouse strains were identified as resistant to clinical disease : SJL/J and A/J. Manipulation of this model will allow investigation of natural rodent pathogenesis with HV, as well as offer insight into disease mechanisms and therapy of HFRS.

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INTRODUCTION

Mainland China, the Korean peninsula, Japan, and the Soviet Far East are recognized endemic foci of a severe and often fatal disease of humans characterized by fever, renal disease, and hemorrhage[12]. This disorder, hemorrhagic fever with renal syndrome (HFRS), or Muroid virus nephropathy[4], has been known over the years by a large number of synonyms, and has been linked epidemiologically and serologically to similar diseases in northern Europe (nephropathia epidemica) and the Balkans[3, 4, 12]. HFRS was initially brought to the attention of Western scientists following

its ravages of United Nations forces during the Korean War, and has since since elicited considerable effort on the part of numerous investigators attempting to define its etiology and pathogenesis.

Efforts to identify a causative agent for H FRS were unproductive until 1976, when Lee and Lee[8,9], demonstrated a specific antigen for Korean hemorrhagic fever in the lungs of the striped field mouse, *Apodemus agrarius*. Subsequent propagation in cell culture of antigenically indistinguishable Hantaan virus (H V)[2] led to an explosion of information on its biochemical[6,16], morphologic[11,19], and virologic [15,16] properties. However, concurrent studies of HV pathogenesis have lagged behind, hampered by difficulties associated with colonization of natural rodent reservoir species, as well as the lack of a model for a virus-induced disease state.

In nature, HV infection in reservoir species of the rodent family *Muroidea* results in a chronic, asymptomatic carrier state analogous to that seen with *Arenaviridae*. Similarly, experimental inoculation of colonized laboratory rats leads to clinically inapparant infection, albeit accompanied by transient serum viremia and persistent viral antigen in tissues (P. W. Lee, personal communication), Recently, lethal infection using a cloned strain of HV in suckling mice has been described[5,17]. In these reports, uniformly fatal disease was produced only after several mouse adaptation passages with the virus. We now report successful induction of lethal disease using non-mouse adapted HV in outbred suckling ICR mice by a variety of inoculation routes. The accompanying studies of disease pathogenesis promote improved understanding of the HV- host biological relationship.

MATERIALS AND METHODS

Virus: The 76-118 strain of Hantaan virus at the third A549 cell culture passage was provided by Dr. G.R. French[2]. This isolate, originally adapted to *in vitro* culture following 5 passages in *Apodemus agrarius* lung, was passaged twice more in the E-6 clone of VERO cells (ATCC C1008, CRL 1586) to achieve a titer of 1.3×10^7PFU/ml. For animal inoculation ten -fold dilutions of this virus stock were made in Hanks balanced salt solution (HBSS) containing 2% heat-inactivated fetal calf serum and 1% penicillin-streptomycin (100u/ml penicillin, 100 μg/ml streptomycin), and stored in multiple aliquots −70℃ until used. Seed pools were determined by culture and serology to be free from reovirus contamination (J. Dalrymple, personal communication).

Animals: All pathogenesis studies were performed using outbred ICR mice (Harlan Sprague-Dawley Co., Walkersville, MD.). Pregnant mice were observed daily and suckling mice obtained at precisely timed intervals following birth. Inbred strains of mice were obtained from Jackson Laboratories, Bar Harbor, ME. Experimental manipulations with inbred mice were performed using sucklings less than 24 hours of age.

Infectivity Assay: Infectious virus in serum specimens was assayed by counting plaques on E6 VERO cell monolayers inoculated with 10 -fold serial dilutions in 12-well (22mm diameter) plastic culture plates (Costar, Cambridge, Mass). Following a 1h adsorption at 37℃, 2.5 ml of overlay medium consisting of EMEM (reinforced Eagles minimum essential medium) with 10% heat-inactivated fetal calf serum, 4 % L-glutamine (200mM, 100X concentration, Gibco Labs, Grand Island, N.Y.), 1% non-essential amino acids (10mM of each amino acid, M.A. Bioproducts, Walkersville, Md.), 1% penicillin-streptomycin, and 0.6% agarose(FM C, Marine Colloidal Div., Rockland, ME.)was

added to each well. After a 7 day incubation at 37℃ in 5% CO_2, a second overlay containing 5% neutral red (1 : 300, Gibco Labs, Grand Island, N.Y.) was added, and visible plaques enumerated 3-4 days later.

Serology: Immunofluorescent antibodies were detected by a modification of the method of Peters et al[14]. Test sera were inoculated on acetone-fixed spot slides coated with HV (76-118 strain)- infected E6 VERO cells. Specimens were then stained for indirect immunofluoresecnce with fluorescein isothiocyanate—labeled goat anti-mouse IgG. Slides were examined at a magnification of 250X using a Carl Zeiss-standard microscope with epifluorescence and FITC filter system.

Neutralizing antibodies were assayed using a constant virus (10-20 plaques/well), serum dilution technique on E6 VERO cells. Serial 2-fold dilutions of serum samples heat—inactivated at 56℃ for 30 min were incubated over night at 4℃ with an equal volume of virus suspension, then added to 12-well tissue culture plates for plaque assay as described above. Endpoints were recorded as the highest dilution of serum yielding ≧80% plaque reduction.

Serum Chemistries: Measurements of serum biochemical values were performed using a Multistat centrifugal analyzer (Instrumentation Laboratories, Lexington, Mass). Assay systems employing kinetic (rate)-type reactions with commercially available kits were adapted for use on the Multistat, and tests performed under maximum biological containment (P-4 level) conditions.

Experimental Conditions : Pregnant ICR mice were maintained in individual filter-top cages until delivery of sucklings. Uninfected and infected litters were segregated in different rooms throughout the experimental period. Suckling mice less than 24h of age, or at increasing intervals up to 10 days, were inoculated by intracerebral (IC), intraperitoneal (IP), intramuscular (IM), or subcutaneous (SC) routes with 0.02ml of virus suspension. Mice similarly inoculated with diluent HBSS served as controls. Animals were observed daily for signs of clinical disease, and body weights determined at 2-day intervals until death. Blood samples were obtained aseptically by bilateral cervical artery bisection, and the sera diluted in diluent HBSS for viremia studies, in 0.01M phosphate buffered saline (pH= 7.2) for antibody studies, or used undiluted for biochemical assays. Fatalities occurring within 2 days of inoculation were considered traumatic, and not included in subsequent data analyses.

All manipulations using infected animals were performed under P-3 containment conditions at the laboratories of the US Army Medical Research Institute for Infectious Diseases, Ft. Detrick, MD. As an additional precaution, because of the known biological hazard of aerosolized Hantaan-like viruses[1, 7, 18], facemask respirators, shoe covers, gowns, and gloves were worn by all personnel entering the infected animal room.

Statistical Methods: Analyses of differences among blood biochemistry values were performed using 2-way ANOVA (analysis of variance) and Duncan's Multiple Range general linear models procedures. To compute differences among LD_{50} values, probit analysis and subsequent comparison by the method of Least Significant Differences were performed. Differences in mean times to death by inoculation route were examined by 1-way ANOV A and Student-Neuman-Keuls tests. All statistical calculations were performed at the Computer Sciences Div., USAMRIID, using SAS statistical programs (SAS Institute, Cary,N.C.)

RESULTS

Table 1. Comparative LD_{50} and mean time to death in HV-infected suckling mice

Inoculation route	LD_{50}		MTC[c]
	Log_{10} Dilution	PFU	
IC	−5.22	8.3×10^{1}	20.0+/−0.8
IP	−4.92	1.6×10^{2}	20.1+/−0.9
IM	−4.67	3.0×10^{2a}	21.2+/−1.1
SC	−4.32	6.6×10^{2b}	23.1+/−0.8

All inocula were 0.02ml/animal; mouse age≦24h; 20 animals/inoculation route used for each determination [a]$p<.01$ vs IC, [b]$p<.001$ vs IC; $p<.01$ vs IP, [c]Mean time to death +/−SEM at HV LD_{50}.

Table 2. Age susceptibility of suckling mice infected with HV

Age	Motrality (%)	MTD(days)[a]
<24h	20/20 (100%)	19.3+/−0.5
24−48h	20/20 (100%)	19.6+/−0.4
48−72h	20/20 (100%)	19.6+/−0.3
72−96h	15/20 (75%)	22.5+/−0.7
5 days	14/20 (70%)	22.4+/−0.8
7 days	10/20 (50%)	23.0+/−0.9
10days	8/20 (40%)	24.4+/−0.9
Diluent control	0/20 (0%)	.....

All mice inoculated IC with 830 PFU/ml (10 LD_{50} for suckling mice <24h of age) in 0.02 ml HV suspension.
[a]mean times to death +/− SEM.

LD_{50} Determination: Preliminary experiments in suckling mice less than 24h old demonstrated uniform lethality following IC inoculation with 10^2 PFU/animal of 76-118 strain HV. To confirm and extend these findings, serial 10-fold dilutions of virus seed were used to determine susceptibility by a variety of inoculation routes. IC, IP, IM, and SC infection of newborns less than 24h old were performed. Daily observations of clinical status were made for up to 35 days, and LD_{50} values established by probit analysis. Sensitivity to virus inoculum and disease course were basically similar, regardless of inoculation route (Table 1). Mice infected IC and IP displayed somewhat greater susceptibility and shorter duration of disease, while animals inoculated IM and SC showed a slightly enhanced resistance and delay in mean time to death. The differences in disease susceptibility were reflected in significant ($p<0.1$) differences in LD_{50} between IC and IM, IC and SC, and IP and SC routes (Table 1). However, differences in mean times to death at or near the LD_{50} level were not significantly different ($p>.05$) among the same groups(Table 1).

Table 3. Susceptibility of inbred mouse strains to HV

Mouse starin[a]	Inoculation routes					
	IC		IP		IM	
	N	% Mortality	N	% Mortality	N	% Mortality
C3H/HEJ (k)	7	100%	6	100%	4	100%
C57B1/6J (b)	6	100%	4	100%	3	60%
DBA/2J (d)	4	100%	5	100%	4	100%
SWR/J (q)	6	100%	5	100%	7	100%
Balb/CJ (d)	6	100%	9	100%	7	100%
AKR(k)	6	100%	10	90%	7	100%
SJL (s)	6	67%	9	33%	4	10 %
A/J (a)	4	25%	4	25%	ND	.....

All mice inoculated with 0.02ml of 10 ICR strain LD_{50} of HV at≦24h of age
[a]H-2 haplotype in parentheses

Age Susceptibility: The influence of postnatal age on susceptibility to HV was determined using 7 groups of animals ranging in age from 24h to 10 days, inoculated IC with 830 PFU/ml of virus suspension (10 LD_{50} for mice < 24h old) (Table 2). A lethal outcome was uniformly seen in mice inoculated when less than 72h old. With increasing age, mice became more resistant, with a concurrent prolongation in time to death. By 2-2.5 weeks, animals had developed complete resistance to fatal disease(data not shown).

Clinical Features: Mice less than 24h old, infected with 10 LD_{50} of virus, showed a characteristic progression of disease findings. Beginning day 10-11 following IC, IP, or IM Inoculation, a transient state of hyperactivity which lasted for 2-3 days was observed in all animals. A simultaneous failure to gain weight was noted, which evolved over the remainder of the disease course to frank weight loss. By day 13-14, mice began to show a ruffled appearance to their coats, together with a hunched posture and progressively diminishing mobility. Paralysis of both hind limbs appeared by 14-15 days, and deaths occurred beginning days 16-17. At the time of their demise, mice were typically dehydrated and runted, with body weights less than 50% that of control animals.

In day-old mice infected SC, an identical sequence of clinical events was observed. However, all findings were delayed by 2-3 days when compared with other inoculation routes. Older mice infected by all routes demonstrated similar disease patterns, moderated somewhat in severity and lethal outcome (Table2). Surviving animals that became ill showed varying degrees of all findings noted above, but recovered by 30 days post-inoculation without apparent sequellae. Age-matched, diluent -inoculated controls remained healthy throughout the experimental course. With increasing age at inoculation, mice became progressively more resistant to clinical disease. By 2-2.5 weeks, no identifiable illness was recognized.

Viremia and Antibody Response: Duration of viremia was studied using 24h suckling mice inoculated with 830 PFU/ml of HV suspension (10 LD_{50} when given IC) by IC, IP, IM, and SC routes. Virus was first detected by plaque assay on day 6 following inoculation in serum from mice infected IC and IP (Fig. 1). Appearance of viremia was delayed by 1 day in animals inoculated IM and SC. Peak titers of 10^5 PFU/ml were observed on day 8 in IC and IP-infected mice, while IM and SC-inoculated animals reached peak viremia levels by 1 and 6 days following maximum titers in IP and IC-infected animals.

Assay of serum antibody was performed in animals infected with 1 LD_{50} of HV. Subsequent experiments in age-matched mice infected with 10 LD_{50} revealed similar kinetics over the initial 3 week period of measurement (da ta not shown). Coincident with disappearance of plaque-detectable virus in serum, immunofluorescent antibody appeared by day 14 in IC and IP-infected mice, and by days 16 and 18 in IM and SC-infected animals(Fig. 2). Titers in all groups rose rapidly over a 2 week period, reaching similar levels(1 : 1024) by day 30. Thereafter, titers continued to rise slowly until day 70(last day of measurement) in surviving animas.

In contrast to the response measured by immunofluorescence, appearance of neutralizing antibody did not vary substantially by inoculation route. Following its appearance by day 15 in all groups, titers rose gradually to highest values by 70 days, detectable at serum dilutions similar to those seen by immunofluorescence(Fig. 3).

Serum Chemistry Determinations: A batte-

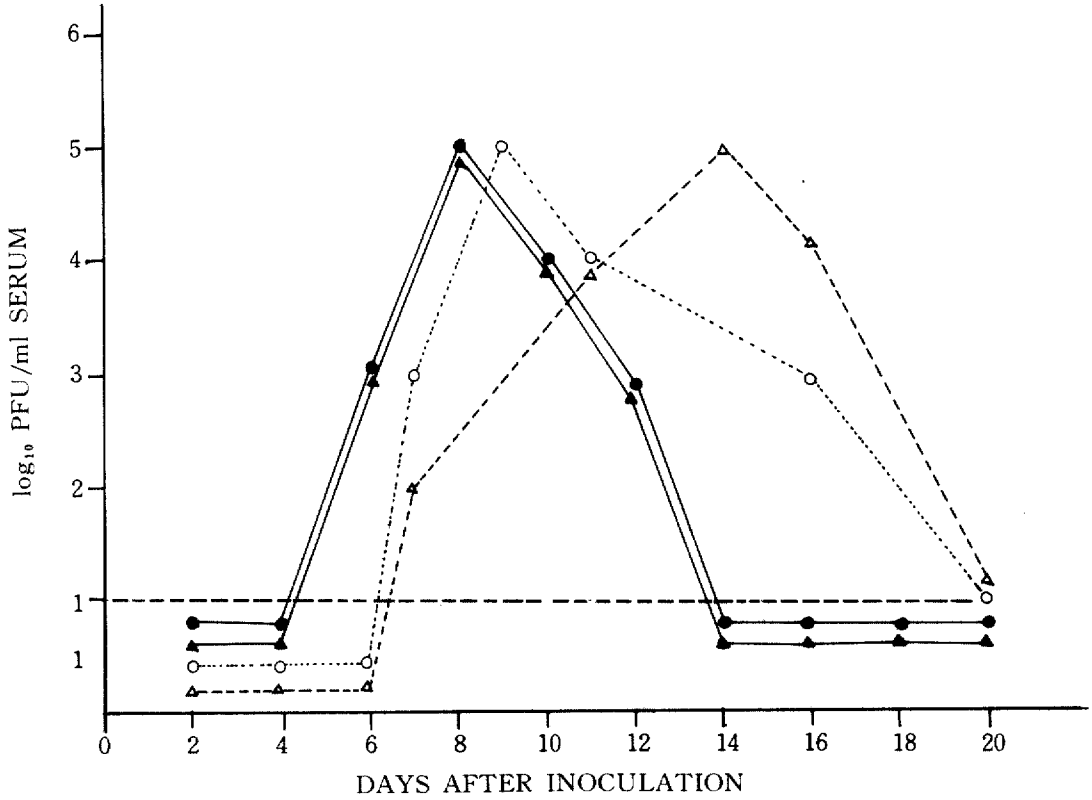

Fig. 1. Serum viremia in HV-infected suckling mice. Virus titers were obtained by direct plaque assay of serum, as described in the text. Each point represents a pooled specimen from 2 animals infected with 10 LD_{50} of HV. Titers of all points below dashed horizontal line were less than 1 $\log_{10}$ PFU/ml serum: IC-inoculated (●——●); IP-inoculated (▲——▲); IM-inoculated (○——○); SC-inoculated (△——△).

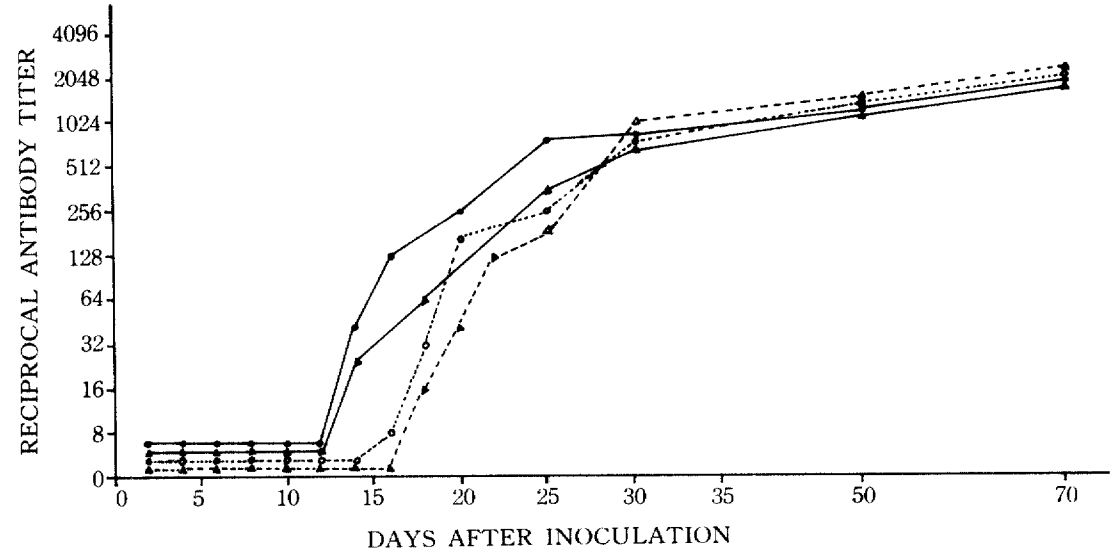

Fig. 2. Immunofluorescent antibody response in suckling mice infected with HV. Determinations were made on sera pooled from 2 individuals infected with 1 LD_{50} of HV. Antibodies were detected by indirect immunofluorescence on 76-118 strain HV spot slides: IC-inoculated (●——●); IP-inoculated (▲——▲); IM-inoculated (○——○); SC-inoculated (△——△).

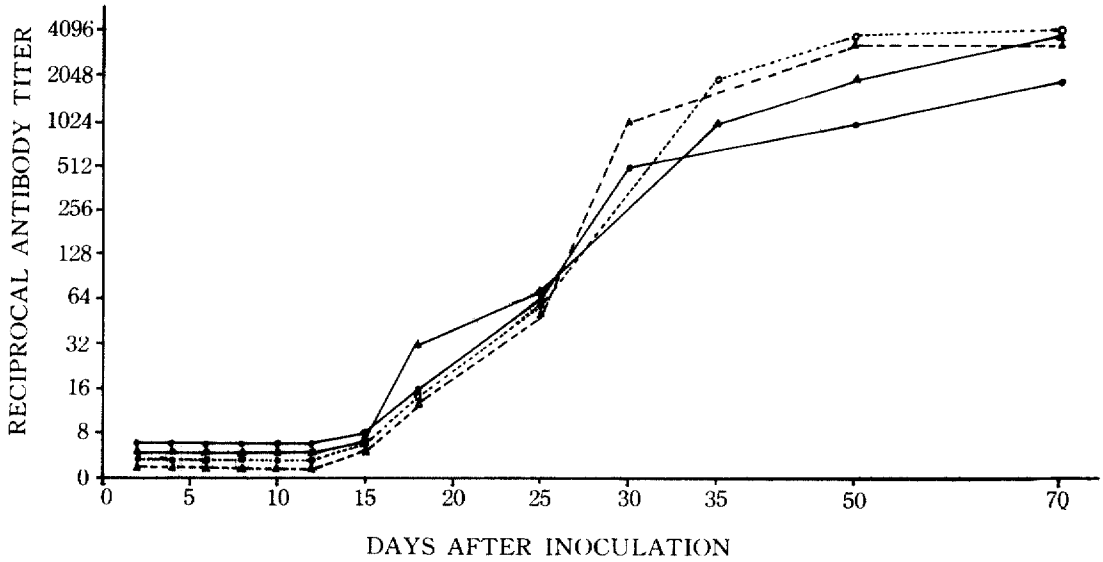

Fig. 3. Neutralizing antibody response in suckling mice infected with HV. Determinations were made on sera pooled from 2 individuals infected with 1 LD_{50} of HV. Eighty percent reduction in 76-118 strain HV plaques was used to determine endpoint titer: IC-inoculated (●——●); IP-inoculated (▲——▲); IM-inoculated (○——○); SC-inoculated (△——△).

ry of biochemical tests was selected to screen for functional abnormalities of renal, hepatic, and musculoskeletal systems. Blood utea nitrogen (BUN) and creatinine, AST (aspartate áminotransferase), ALT (alanine aminotransferase), alkaline phosphatase, LDH-L (lactate dehydrogenase), and CPK (creatine phosphokinase) were assayed at frequent intervals in day-old mice from 1 to 20 days following inoculation with 10 LD_{50} of virus (Fig. 4). Considerable variation was observed in several tests during the initial 2 days of measurement. These changes probably reflect inoculation trauma, and were considered inconsequential. Despite these and other fluctuations in absolute values in the tests selected. however, differences from control animals were not significant ($p>.05$) at any time during the observation period. Furthermore, these fluctuations did not reflect clinically significant deviations from normal values. Therefore, no consistent evidence for dysfunction among any of the organ systems tested was obtained.

Studies in Inbred Mouse Strains: The influence of genetic background on susceptibility to lethal infection was studied in litters of inbred C3H/HEJ, C57B1/6J, DBA/2J, SWR/J, Balb/CJ, AKR/J, SJL/J, and A/J mice infected by IC, IP, and IM routes(Table 3). All strains tested except SJL/J and A/J appeared highly sensitive to HV, with mortality similar to that seen in outbred ICR animals. Expression of clinical disease in susceptible inbred animals paralleled that seen in ICR mice, while in resistant strains, mice became ill and lost weight, then recovered in a fashion analogous to that seen in older outbreds.

DISCUSSION

Current undnerstanding of HFRS pathogenesis derives predominantly from empirical study of affected patients. Experimental systems to examine specific disease parameters have proven difficult to devise. While a lethal rodent model is not likely to mimic with accuracy the human clinical syndrome, its development, characterization, and exploitation should non-

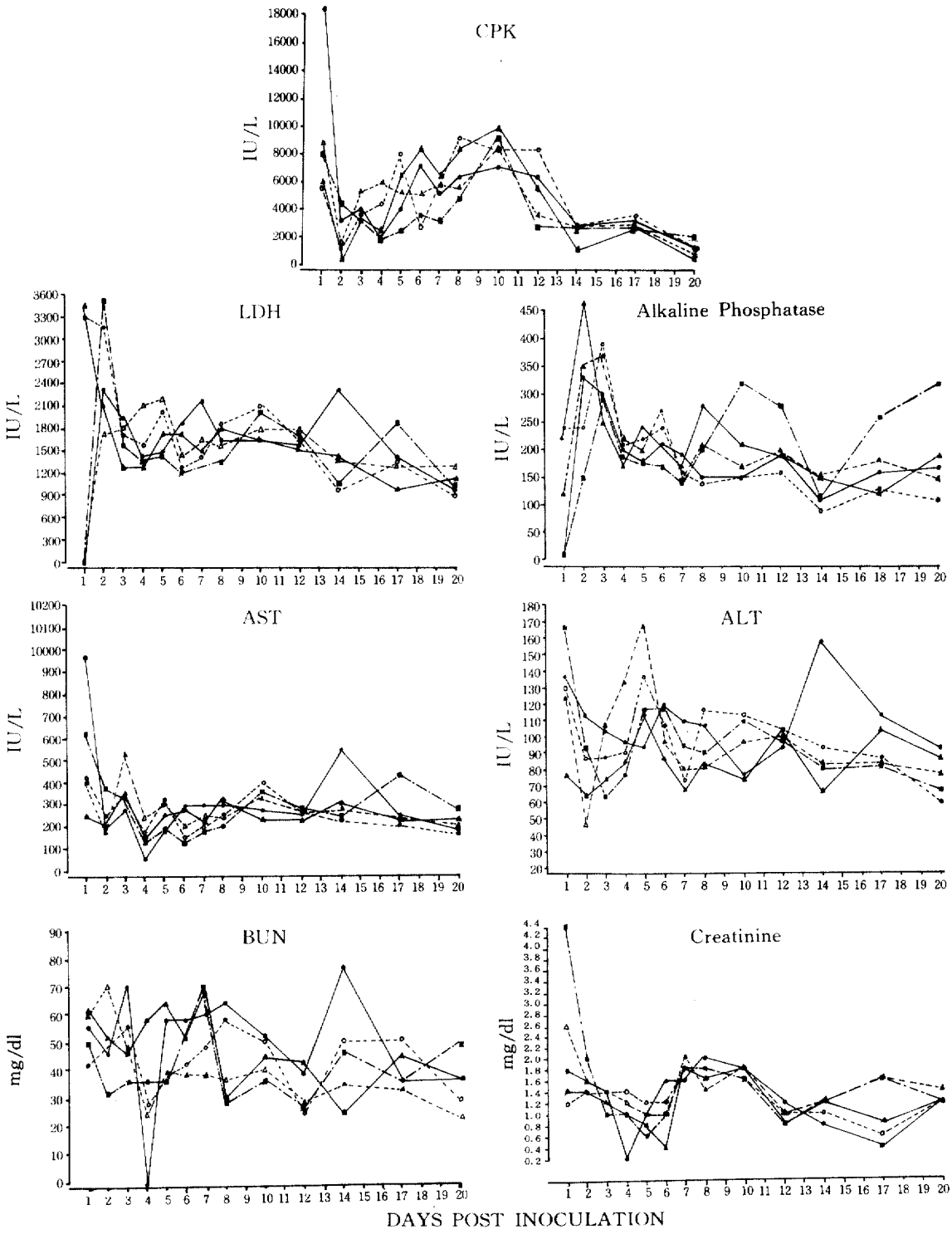

Fig. 4. Serum biochemistry determinations in HV-infected suckling mice. Each value was obtained from a pool of 2 mouse sera. Animals were infected IP with 10 LD_{50} of HV. Analyses were performed with an automated centrifugal analyzer designed for micro-volume assay: IC-inoculated (●——●); IP-inoculated (▲——▲); IM-inoculated (○——○); SC-inoculated (△——△); Diluent control (■——■).

etheless provide valuable insight into the mechanisms and therapy of this disease. In the present study, we have described such a model for HV using a non-mouse adapted virus strain selected only by routine passage through cell culture. Outbred suckling mice less than 3 days of age were uniformly susceptible to lethal infection with this HV strain. With increasing post-natal age, animals developed progressive resistance to fatal disease, mean times to death increased, and severity of clinical signs diminished. By young adulthood, clinically inapparent infection reminiscent of the natural reservoir state resulted.

The sequence of clinical, virologic, and serologic events observed in newborn mice infected by each of 4 parenteral routes was characteristic. Preliminary experiments in animals infected intranasally revealed similar findings (G.R. Kim, K. McKee, unpublished observations). Progressive wasting and runting, accompanied terminally by signs of central nervous system involvement, resulted uniformly in death by 3 weeks. Serum viremia appeared consistently by 6 days in IC-and IP-infected animals, peaked by 8-10 days, then fell to undetectable levels by 2 weeks. Antibody was detected by 12-15 days, and continued to rise until the time of death. The observed disease was not accompanied by consistent biochemical evidence of renal, hepatic, or musculoskeletal dysfunction, suggesting that failure of vital visceral organs was not instrumental in a fatal outcome. These findings were corroborated further by histopathologic observations (G.R. Kim, D.E. Green, K.T. McKee, manuscript in preparation), in which progressive bilaterial cellular inflitration and necrosis of the midbrain was seen. These results, in concert with clinical illness patterns, implicate disease of the central nervous system as critical in the demise of animals in this model.

Mice infected SC exhibited a delay of 1-3 days in onset of clinical signs, with a consequent slight prolongation in mean time to death. Similarly, a 2-4 day postponement in detectable serum viremia and onset of immunofluorescent antibody response was seen in animals inoculated both SC and IM. These findings suggest that local or regional replication of virus may play a role in disease pathogenesis in this system.

The appearance of immunofluorescent and neutralizing antibody coincident with disappearance of circulating virus and onset of clinical findings raises the possibility of immunologically-mediated disease. This speculation was supported further by histopathologic and immunohistochemical studies (G.R. Kim, D.E. Green, K.T. McKee, manuscript in preparation), wherein visceral and central nervous system cellular infiltrates, together with renal glomerular deposition of immunoglobulins, have been observed. We have not yet attempted to detect circulating immune complexes or soluble factors resulting from complement activation. However, since certain pathologic features of human HFRS strongly suggest that the consequences of immune complex formation play a central role in disease pathogenesis[12, 13, 20], intensive study of this aspect of HV disease in the mouse model is warranted.

Host genetics appear to play a role in disease susceptibility of suckling mice. Results of a limited survey of 8 inbred mouse strains suggested that animals from 6 of 8 backgrounds were as sensitive as outbred mice to lethal disease. However, 2 inbred strains, SJL/J and A/J, were identified as resistant by IC and IP inoculation routes. Subsequent experiments have confirmed this resistance pattern for these 2 strains (G.R. Kim and K.T. McKee, unpublished data). Although there is no obvious correlation with major histocompatability ha-

plotype, this possibility and the mechanisms underlying resistance are currently under study.

In previous reports, consistent production of lethal disease with HV was accomplished only after adaptation of the virus to suckling mice by serial organ passage[5, 17]. The consequences of *in vitro* clonal selection and mouse adaptation remain to be elucidated, but it is reasonable to question whether such selection procedures alter certain aspects of infection, such as virulence, oragn tropism, or pathogenesis. Since many of the observations from the current study are at variance with those reported with mouse-adapted HV, it would appear that the virus strain selection procedures described in these earlier reports do, in fact, affect this model system. However, the relative merits of disease induction via cloned or uncloned HV must be considered with the realization that, regardless of virus strain used, available rodent models will be useful primarily to explore some of the principles of pathogenesis with the Hantaan group of agents, rather than as a realistic model of human HFRS.

Despite the recognized limitations of this system, definition of HV disease features in suckling mice provides important insight into the natural rodent pathogenicity of this virus. The ease and consistency with which parameters of disease can be measured and manipulated furnish a framework for further examination of HFRS-related pathogenesis and therapy. Studies are currently underway to assess the utility of this system for testing antiviral therapeutic regimens.

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