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Infection of *Macaca radiata* with viruses of the tick-borne encephalitis group

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Our studies confirmed the susceptibility of *Macaca radiata* (bonnet macaques) to Kyasanur Forest disease (KFD) and enabled us to demonstrate KFD virus-specific gastrointestinal and lymphoid lesions. Significant histopathological changes occurred in the small and large intestine, spleen and lymph nodes; and viral antigens were found in these same organs by immunohistochemistry. Viral antigen-positive cells were always associated with histological evidence of necrosis, which suggests that cell death occurred directly from viral replication or secondarily from attack by immune mechanisms. In contrast, *M. radiata* infected with Omsk virus did not show any signs of clinical disease, and no virus could be isolated from tissues or blood at the end of the experiment. However, *M. radiata* infected with Russian spring-summer encephalitis (RSSE) developed clinical signs in the central nervous system; and, in one monkey, RSSE virus was isolated from the brain, and viral antigen was localized in neurons. Our data indicate that *M. radiata* is an excellent model to study human disease caused by KFD virus and could serve as a model for human disease caused by other, related strains of this group of viruses.

Key words: *Macaca radiata*; tick-borne encephalitis; pathogenesis; Kyasanur Forest disease (KFD); Russian spring-summer encephalitis.

Introduction

The tick-borne, or Russian spring-summer, encephalitis (TBE) complex of flaviviruses is a geographically diverse, but antigenically related group of at least eight distinct agents of infection, many of which are of considerable virological and medical interest.[1] Several of these viruses, including Kyasanur Forest disease (KFD), Russian spring-summer encephalitis (RSSE), central European encephalitis (CEE) and Omsk hemorrhagic fever (OHF), cause significant morbidity and, depending on the viral strain, occasional mortality in humans.[2] In general, the clinical syndrome induced by KFD and OHF is best characterized as a viral hemorrhagic fever. In KFD, the clinical pattern is characterized initially by visceral disease with subsequent encephalitis. In contrast, RSSE and CEE viruses typically cause febrile illness, with the central nervous

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system (CNS) as the principal target organ. With TBE, case fatality rates historically ranged as high as 5–10%, and RSSE survivors can suffer long-term neurological sequellae.

One of the more serious constraints for study of TBE pathogenesis and for vaccine development has been the absence of realistic animal models that mimic human disease. Mice have been the animal species most often used for studies of flavivirus behavior in mammals, but the resulting illness developed in these animals bears little resemblance to human infection.[1] Both guinea-pigs and several macaque species become viremic after peripheral inoculation of TBE complex viruses;[3–5] however, clinical illness is mild to absent in these animals. Although clinical, virological and pathological evidence of encephalitis has been produced in rhesus macaques by intranasal inoculation of CEE virus,[5] it is likely that infection by this route subverts normal disease pathogenesis by allowing passage of the virus directly across the cribiform plate and into the CNS. Observations made in the natural environment showed the susceptibility of *Presbytis entellus* (langur) and *Macaca radiata* (bonnet macaque) to lethal infections with KFD virus.[6] Subsequent experimental studies documented the exquisite susceptibility *M. radiata* to KFD and enabled the disease to be more fully characterized.[4,7,8] Because of the close antigenic relationships among TBE complex flaviviruses, we hypothesized that the susceptibility of *M. radiata* to KFD might extend to other pathogens of this group of viruses. In this report, we describe the clinical and pathological findings in this primate species after inoculation with KFD, OHF and RSSE viruses.

Results

Clinical findings

Seven macaques were inoculated with KFD virus, two with OHF virus and two with RSSE virus. The results are summarized in Table 1. All seven of the macaques inoculated with KFD exhibited diarrhea by day 4 after infection and were moderately dehydrated by the time they died (days 5–11). None of the animals lost weight before death, presumably because of the fulminant nature of this disease (data not shown). There were no hemorrhages observed clinically or at necropsy. Both macaques inoculated with RSSE virus were clinically normal until day 14 after infection, when intention tremors occurred in both animals. One monkey was more severely affected and showed a head tilt to the left. This individual was killed on day 15. The remaining macaque improved and remained well until the end of the observation period (60 days after infection), when she was killed. The two macaques inoculated with OHF virus remained healthy throughout the 60-day observation.

Viremia levels, recovery of virus from the oropharynx and, at death, virus in the cerebrospinal fluid (CSF) and urine of KFD virus-infected macaques are shown in Fig. 1. In all these animals, there were measurable viremias by day 1 or 2, which persisted

Table 1 Illness and death in *M. radiata* inculated with viruses of the TBE complex

Virus	No. macaques	Clinical disease	Mortality	Days to death
KFD	7	7/7	7/7	6, 6, 7, 7, 7, 8, 11
RSSE	2	2/2	1[a]/2	15 (?)
OHF	2	0/2	0/2	

[a] Killed when moribund.

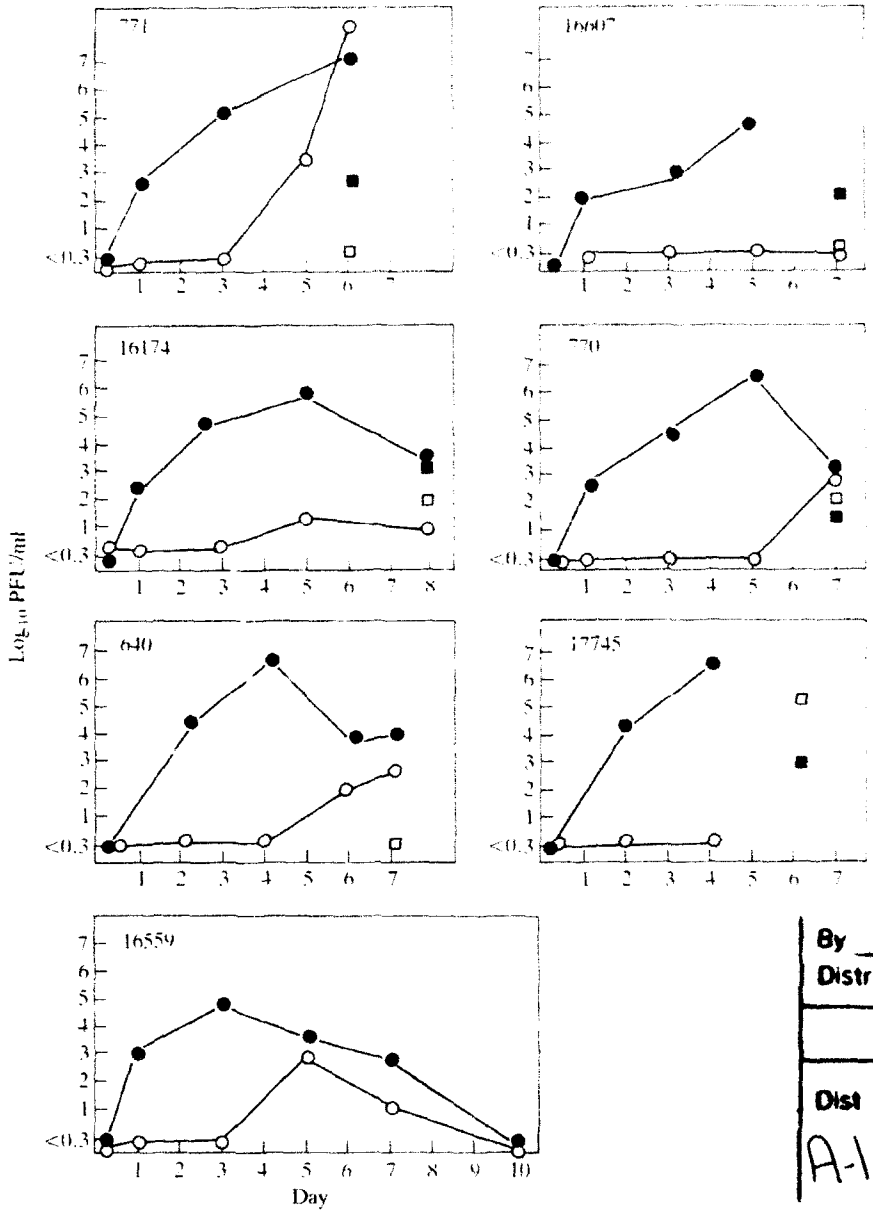

Fig. 1. Virus recovered from serum, CSF, pharyngeal washes and urine from KFD virus-infected *M radiata*. ●, Viremia; ○, pharyngeal wash; ■, urine; □, CSF.

until day 6 or 7 (or death); none were observed after day 8. Virus recovery from oropharyngeal swabs was inconsistent. Although the time of virus shedding in the oropharynx coincided with the time of viremia, viral titers in the serum were always higher than those from the throat.

None of the animals inoculated with OHF virus became viremic, nor did any shed virus in their pharyngeal secretions.

Organ samples were cultured to detect virus in the seven macaques that died from KFD (Table 2). The highest viral titers occurred in lymph nodes, liver, spleen and large

Table 2 Viral titers[a] of KFD virus in tissues from infected *M. radiata*

Tissue	Macaque nos. (day of death)						
	770 (7)	771 (6)	16559 (11)	16174 (8)	16607 (7)	640 (7)	17745 (6)
Cerebrum	2.5	<1.5	<1.5	2.3	2.2	2.3	2.7
Cerebellum	2.4	6.6	<1.5	2.9	4.3	1.9	4.9
Olfactory lobe	1.7	2.6	<1.5	3.3	2.7	<1.5	3.1
Mesenteric lymph node	<1.5	2.7	6.0	<1.5	6.7	7.5	N.D.
Spleen	3.0	6.3	4.9	N.D.	6.6	7.7	6.7
Stomach	3.2	3.0	4.9	2.3	6.1	N.D.	N.D.
Small intestine	4.5	4.5	6.0	3.5	5.7	N.D.	N.D.
Large intestine	6.5	4.4	6.2	2.3	7.1	4.9	N.D.
Adrenal	3.5	4.8	<1.5	1.9	4.0	3.5	5.5
Lung	4.5	2.7	0.9	4.0	4.0	4.1	5.1
Pancreas	<1.5	<1.5	<1.5	<1.5	<1.5	N.D.	N.D.
Liver	4.6	4.5	2.7	5.4	3.9	5.9	6.0
Kidney	3.7	5.4	<1.5	3.7	4.5	4.1	6.3
Heart	N.D.	5.0	<1.5	N.D.	N.D.	N.D.	N.D.
Ovary	2.5	N.D.	2.5	4.5	3.7	3.3	4.6
Serum	3.6	7.2	<0.3	3.7	N.D.	4.0	N.D.

[a] Log 10 PFU/g.

and small intestines. Except for one macaque (771) with a high viral titer in the cerebellum, viral titers were low in the CNS. No virus was detected by plaque assay in any of the organs from the two macaques inoculated with OHF virus or with RSSE. However, when a blind passage of brain tissue from the RSSE virus-infected macaque killed on day 15 was made intracerebrally in suckling mice, the mice died on days 5–7 with high viral titers in their brains. When we recovered virus from the suckling mouse brains, we identified it as RSSE virus by neutralization with specific antiserum (American Type Culture Collection, Rockville, MD), data not shown.

Clinical pathology

Hematological changes, including lymphopenia and anemia, were observed only in KFD virus-infected animals. Relative lymphocyte counts were significantly reduced by days 3–5 after infection (mean of $36.6 \pm 1.7\%$ on day 0 to $10.1 \pm 6.5\%$ on days 3–5). Non-regenerative anemia was also evident by day 3 after infection and persisted until death. Myeloid/erythroid ratios were elevated above normal in all but one macaque which died earliest (day 6). Stainable iron was identified in histology sections of bone marrow.

Blood samples collected from all KFD virus-infected macaques were elevated in levels of alanine transaminase (ALT), aspartate aminotransaminase (AST) but not in alkaline phosphatase (ALP) (Fig. 2). There was little change in the ALT and AST levels in macaques infected with RSSE virus (Fig. 3) (ALP not shown). A single macaque infected with OHF virus had a transient rise in ALP as well as ALT and AST (Fig. 4).

Pathology

There were no significant gross lesions attributable to viral infection with members of the TBE complex. Major histopathology changes as a result of KFD viral infection were limited to lymphoid organs and the gastrointestinal (GI) mucosa, while those changes as a result of RSSE viral infection were confined to the CNS.

Among KFD virus-infected macaques, peripheral and visceral lymph nodes, spleens, and all mucosal lymphoid tissues showed moderate to severe follicular involution and

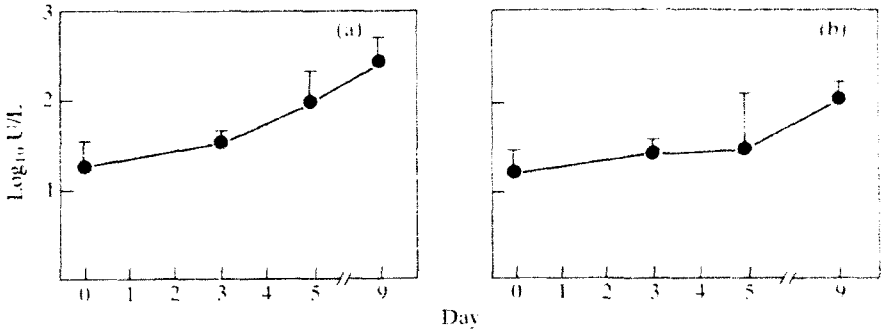

Fig. 2. (a) AST and (b) ALT values in KFD virus-infected *M. radiata*. Bars represent the standard error of the means.

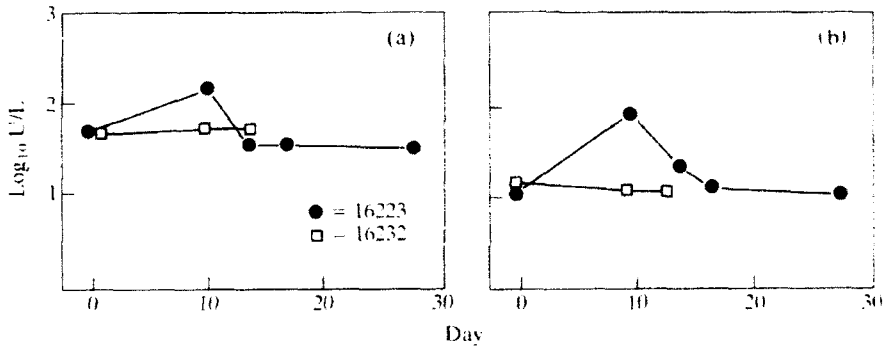

Fig. 3. (a) AST and (b) ALT values in RSSE virus-infected *M. radiata*. ●, 16223; □, 16232.

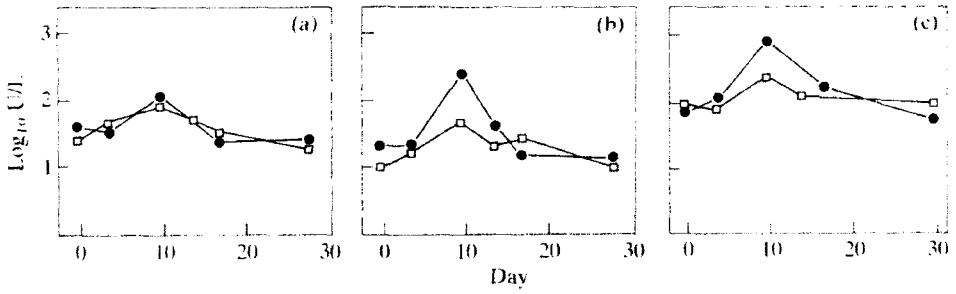

Fig. 4. (a) AST, (b) ALT and (c) ALP values in OHF virus-infected *M. radiata*.

a variable depletion of lymphocytes within the T-cell-dependent zones. Follicles were smaller and often lacked visible germinal centers. Lymphocytic necrosis did not accompany T-cell loss, except in several depleted splenic periarteriolar sheaths. T-cell zones typically contained moderate numbers of large lymphoblasts.

The GI tract was affected segmentally in KFD virus-infected macaques. There was mucosal erosion characterized by reduced luminal epithelial surface area in the stomach and large intestine, and villus blunting and fusion in the small intestine. Crypt loss was observed occasionally in severely affected regions. A diffuse mild to moderate lymphohistiocytic infiltrate was present in the lamina propria and often was

accompanied by karyorrhectic debris with adjacent, intact, crypt epithelium. All KFD virus-infected macaques had moderate to marked, diffuse, hepatic fatty changes.

In all regions of the brains and spinal cords of severely affected RSSE virus-infected animals, there were lesions primarily with the grey matter characterized by variably sized areas of neuronal necrosis, neuronophagia, satelliosis, glial nodules, scattered neutrophilic foci and angiocentric lymphocytic aggregates. The meninges frequently contained multiple perivascular aggregates of lymphocytes and neutrophils. The meningeal inflammation appeared most prominent within the sulci of the cerebral cortices. Lymphoid tissues appeared to be unaffected when examined by light microscopy.

Tissues from the remaining macaque inoculated with RSSE virus, and the two macaques inoculated with OHF virus, did not contain any significant histological lesions.

Tissue localization of antigen

Immunohistochemistry with polyclonal antibodies was used to identify viral antigen-positive cells within virus-infected tissues. Target cells in KFD virus-inoculated animals included lymphocytes and large lymphoblasts in lymphoid tissues (Fig. 5); macrophages, plasma cells and lymphocytes within the GI lamina propria (Fig. 6); and epithelial cells of the GI mucosa, predominantly those lining crypts (Fig. 6).

Fig. 5. Spleen, KFD virus-infected *M. radiata*. This section demonstrates lymphocytes and lymphoblasts (arrow heads) staining intensely positive (brown precipitate) with KFD virus antiserum in the periarteriolar lymphoid sheath area, a T-cell-dependent zone. Immunoperoxidase (see Materials and methods). ABC, hematoxylin, ×100.

Fig. 6. Ileum, KFD virus-infected *M. radiata*. Lymphoid cells within the lamina propria (arrow head), and epithelial cells (arrows) with the crypts and covering the luminal surface stain positive for KFD viral antigens. Immunoperoxidase (see Materials and methods). Hematoxylin, ×50.

Viral antigen-positive lymphocytes were usually located in the T-cell-depleted zones of lymphoid tissues.

Target cells in the one RSSE-inoculated animal with pathological changes were limited to neurons throughout the CNS (Fig. 7). Occasionally, there were free viral antigens, detected in the neural parenchyma, which were associated with inflammatory nodules, neural necrosis and loss. In contrast to those of the KFD virus-infected animals, the lymphoid tissues of this RSSE virus-infected macaque did not contain any viral antigens. None of the tissues from the remaining RSSE virus-infected macaque contained viral antigens.

We did not perform any immunohistochemistry with tissues from the OHF virus-infected macaques.

Serology

Antibody responses in virus-infected macaques were measured by an ELISA for IgG against viruses of the TBE complex. Seroconversion in the one surviving RSSE virus-infected animal and in one of the OHF virus-infected animals occurred between days 17 and 21, and between days 14 and 17 for the other OHF virus-infected macaque (Fig. 8). None of the KFD virus-infected macaques developed a specific IgG response before their death (data not shown).

Discussion

Human disease caused by infection with viruses of the TBE complex is unevenly distributed over much of the former USSR, regions of central and eastern Europe, the

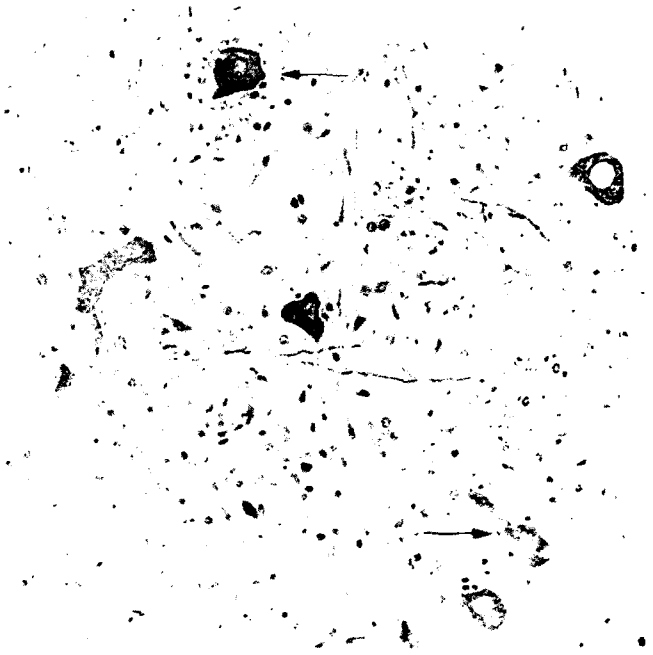

Fig. 7. Cervical spinal cord, RSSE virus-infected *M. radiata*. Neuronal cell bodies as well as axonal and dendritic processes stain viral antigen-positive neurons (arrows). Immunoperoxidase (see Materials and methods). ABC, hematoxylin, ×50.

United Kingdom, Scandinavia and India. Although the true incidence of these diseases is unknown, there is serological evidence of many cases, but only a small percentage of these people develop neurological sequelae.[9,10] In Austria, more than 100 hospitalized cases of TBE are recorded each year, and the disease is considered to be a major health problem.[11] Although KFD, the tropical member of the complex, is usually not a CNS disease, it undoubtedly causes the greatest incidence of human disease. More than 1000 cases of KFD are recorded each year in India, and at least 2% of these cases are fatal.[12] A formalin-inactivated, zonally purified, chick embryo cell culture-grown TBE vaccine, licensed in Europe, offers immunoprophylaxis against the TBE complex viruses; however, little is known about the extent of cross-protection with KFD virus. Although adult mice are susceptible to TBE viral strains, pathogenesis in these animals does not closely resemble the disease in humans. Some early studies showed that *M. radiata* became ill and died with KFD,[8] but the pathogenesis of the disease was not well described. There are no other reports of TBE viruses tested in this animal model. A non-human primate model for these viruses is needed, however, not only to elucidate the pathogenesis, but also to test the efficacy of the current vaccine and any new-generation vaccines, as they become available. Although studies have been published on flavivirus cross-neutralizations, these data have been notoriously useless in predicting cross-protection.[13]

Our studies of the pathogenesis of KFD in *M. radiata* differed somewhat from previous observations made by others. Although erythrophagocytosis in peripheral blood, liver and spleen was reported in studies of KFD in *M. radiata*,[8] we did not observe these effects in any of the seven macaques we inoculated with KFD virus. However, exsanguination and vascular perfusion at necropsy may have eliminated circulating erythrophagocytic cells. Viral antigen-positive cells were always associated with histological evidence of necrosis, which suggests that cell death occurred directly from viral replication or secondarily from attack by immune mechanisms. Antigen-

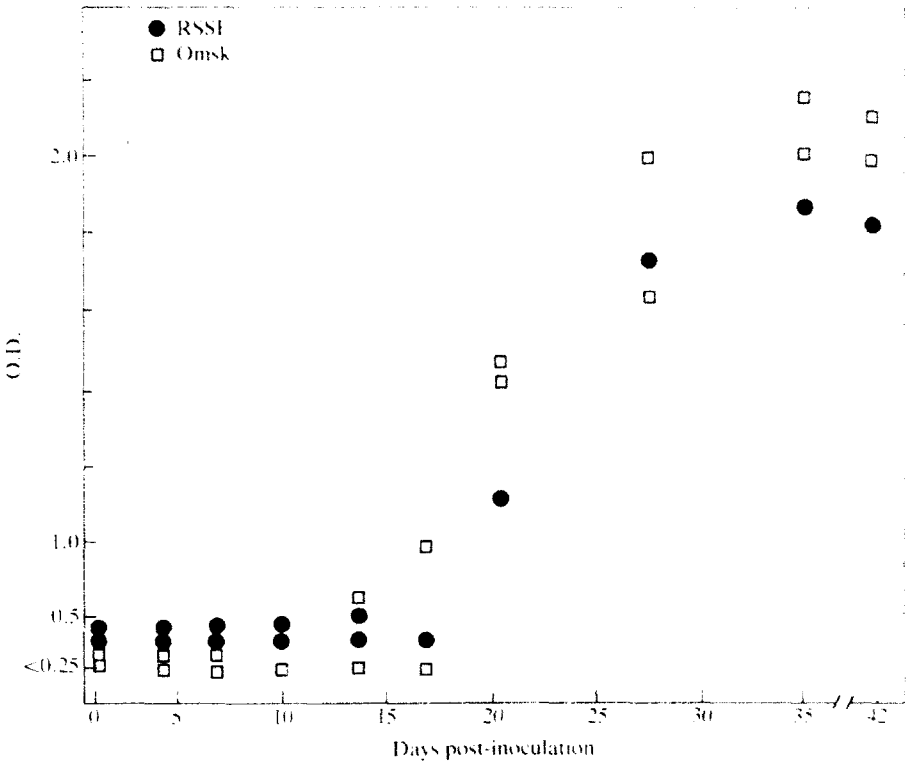

Fig. 8. IgG ELISA antibody responses of *M. radiata* after inoculation with RSSE or OHF viruses. ●, RSSE. □, Omsk.

positive cells were not always evident in tissues from which virus was detected. For example, viral titers in the liver were similar to those measured in the GI tract, but we could detect no viral antigens by immunohistochemistry procedures or by electron microscopy. This finding may reflect the presence of free virions in the vasculature as opposed to viral replication within the cells of that organ. The fact that antigen-positive cells were always associated with histological evidence of necrosis sharply contrasts with our observations with arenaviruses.[14] In guinea-pigs infected with Pichinde virus, in rhesus macaques infected with Junin virus and in cynomolgus monkeys infected with Lassa virus, there was extensive viral infections of various target organs, but only minor histopathology.[14]

Reduced numbers of peripheral blood lymphocytes in humans and primates infected with KFD virus were also reported by other investigators.[4,8] The large numbers of antigen-positive lymphocytes and lymphoblasts in depleted, T-cell-dependent zones of lymphatic tissues suggest that the T-lymphocyte is a primary target of KFD viral replication.

In macaques infected with RSSE virus, serum transaminases were transiently elevated in both animals by 10 days after infection, with evidence of CNS involvement by day 14, which suggests that the virus may replicate innocuously early in the viscera, then later in the CNS (day 14). There were no systemic changes in these animals. In humans infected with RSSE virus, significant illness occurs only after the virus is transported to and replicates in the CNS. Our observations of RSSE virus-infected *M. radiata* appear to be consistent with this pattern.

Macaques infected with OHF virus appeared healthy throughout the 60-day experiment. However, serum transaminases were transiently elevated and animals clearly seroconverted, which indicate that viral replication occurred.

Our data suggest that the GI tract, the lymphoreticular system and possibly the liver are the initial sites of replication for TBE complex viruses. After an early, visceral phase of disease, the virus may kill the host (KFD), may be eliminated (OHF), or the virus may cross the blood–brain barrier, replicate in neurons and cause CNS disease (RSSE). Because these patterns are similar to those in humans, *M. radiata* appear to be a good animal model for the study of disease caused by the TBE complex of viruses. This primate species is no longer readily available in the United States because of export restrictions by India, but these macaques are readily available elsewhere. An increased availability of *M. radiata* for medical research would allow further studies of TBE viruses.

Materials and methods

Viruses. We made attempts to use low-passage viral isolates for this study. We used sucking mouse brain (SMB), passage 9, of KFD virus strain #1639. For RSSE virus, we used SMB passage 9 (Sophy strain); and for OHF virus, we used SMB, passage 6 (Belangul strain). Viral titers were determined by plaque assay on Vero cells, as previously described.[7]

Experimental design. Adult *M. radiata*, weighing 4.5–6.5 kg, were inoculated intramuscularly with $5 \cdot 10^5$ plaque-forming units (PFU) of KFD virus, $1.7 \cdot 10^7$ PFU of RSSE virus or $5 \cdot 10^7$ PFU of OHF virus. Animals were maintained in individual cages on a diet of Monkey Chow (Ralston Purina Co., St Louis, MO), fruit and water *ad libitum*. Behavior and general clinical status were assessed on unanesthetized macaques at least once daily; more detailed clinical observations were made on sedated animals (ketamine hydrochloride, 7 mg/kg) two or three times a week for 3 weeks, and then weekly until day 60. At these times, blood was collected from the saphenous or femoral veins by venipuncture with a 23-G needle. Oropharyngeal swabs were obtained with a cotton-tipped applicator, which was immersed immediately in Hank's balanced salt solution supplemented with 2% heat-inactivated (56 C for 30 min) fetal bovine serum. These solutions were titrated by plaque assay in Vero cells, as described previously. Terminally ill macaques were killed by exsanguination while under deep anesthesia. Complete necropsies followed whole-body perfusion first with phosphate-buffered saline (PBS), then by 4% paraformaldehyde/0.5% glutaraldehyde.

(Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals, and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, NIH publication 86-23, 1985 edition, as promulgated by the Committee on Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.)

Tissue preparations. Tissue samples were taken for virus quantitation and light microscopy. Samples were aseptically dissected from the body, suspended in L-15 medium, and triturated in tissue grinders to produce a 10% (w/v) suspension. Tissue samples for microscopy were fixed by immersion in 4% paraformaldehyde/0.5% glutaraldehyde and paraffin-embedded sections were prepared and stained with hematoxylin and eosin. We paid particular attention to tissues of the GI tract and prepared several samples from all regions. Other paraffin sections were processed for immunohistochemistry.

Immunohistochemistry. KFD and RSSE viral antigens were visualized in paraffin-embedded sections by using a modified avidin-biotin-peroxidase complex method, as previously described.[13] Briefly, 5 μm sections were mounted on Fischer Superfrost-Plus (Fischer Scientific, Pittsburgh, PA), deparaffinized and hydrated. Sections were then immersed in a 0.05% solution of protease VIII (subtilysin, Sigma Chemical Co., St Louis, MO), pH 7.8, 37 C, for 3 min and washed in PBS, pH 7.4. Mouse ascites fluids (kindly supplied by Dr R. Shope, Yale University) served as the primary antibody and were diluted 1:600 for KFD virus and 1:500 for RSSE virus.

Antibodies in ascites fluid do not react with normal macaque tissues. Slides containing tissue sections were incubated with the appropriate ascites dilution at room temperature for 1 h. Slides were then rinsed in PBS and incubated with biotinylated horse anti-mouse IgG, then with the avidin biotin complex (Vectastain, Vector Laboratories, Burlingame, CA). Diaminobenzedine was the substrate for the peroxidase. Sections were counterstained with hematoxylin. Normal mouse serum was substituted for the primary antibody to serve as the assay control.

Serological assays. Virus-specific antibodies against TBE viruses were detected and measured using an enzyme-linked immunosorbent assay (ELISA). Plates (96 well) were coated with Vero cell-grown HYPR strain of central European TBE virus and reacted with anti-human serum conjugated to horseradish peroxidase (Accurate Chemical and Scientific Corp., Westbury, NY). Substrate [2,2'-azino-di(3-ethylbenzthiazoline sulfonate)] (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added, and plates were read at 410 nm.

Clinical chemistry. Sera obtained from macaques were frozen and stored at −70 C. After all samples from the experiment were collected, they were simultaneously analyzed (Kodak Ektachrome 700XR Chemistry Autoanalyzer, Rochester, NY) for glucose, urea nitrogen, creatinine, sodium, potassium, chloride, carbon dioxide, amylase, total protein, AST, ALT, LDH, ALP and total bilirubin. There were sufficient sera to complete these assays for all macaques except three of the KFD virus-infected animals.

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