A Macaque Model for Hantavirus Infection

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Cynomolgus macaques *(Macaca fascicularis)* were experimentally infected with Puumala virus (strain Hällnäs), which causes nephropathia epidemica in humans in western Europe. During the first week after intratracheal inoculation, the monkeys exhibited signs of lethargy followed by mild proteinuria and microhematuria. Histopathologic changes during the first 7 weeks after infection were largely confined to abnormalities in medullary tubular cells of the kidneys, which coincided with the demonstration of viral antigen and viral RNA. The development of different classes of virus-specific plasma antibodies to the respective viral antigens were similar to those observed in humans with nephropathia epidemica. This first description of a nonhuman primate model for hantavirus infection shows that the cynomolgus macaque provides a suitable model with which to study the pathogenesis of Puumala virus infections and to evaluate new diagnostic methods, immunization strategies, and therapies.

Nephropathia epidemica (NE) is a form of hemorrhagic fever with renal syndrome (HFRS) in humans, which occurs in Europe and is caused by infection with Puumala virus (PUUV) belonging to the genus *Hantavirus* of the Bunyaviridae family. Several hantavirus subtypes have been described [1, 2], including Hantaan-like, Seoul-like, and PUUV-like viruses. Recently, another subtype, named Muerto Canyon virus, was shown to cause hantavirus pulmonary syndrome in humans in the United States [3]. The spectrum of clinical symptoms caused by infections with the different hantavirus subtypes varies from severe to mild, depending on the viral subtype involved. PUUV infections in humans are usually accompanied by an influenza-like illness at the onset, followed by transient acute renal failure. Since many infections run a mild course, the true incidence of NE is underestimated in most European countries. Fewer than 1% of infections with PUUV-like viruses are fatal. This contrasts sharply with Hantaan-like virus and Muerto Canyon virus infections, which are fatal in ~10% and 60% of cases, respectively [4].

The primary pathophysiologic findings of HFRS caused by hantavirus infection are largely the result of an increased vascular permeability, and in vitro viral replication has been demonstrated in human vascular endothelial cells [5]. Histopathologic abnormalities have been demonstrated in tubular epithelium cells of hantavirus antigen–positive kidneys. In general, histologic lesions in kidney biopsies of NE patients vary according to the stage of the disease and are usually relatively mild and unspecific [6]. The most common histopathologic changes in the kidney are flattened tubular epithelium and dilated lumina, occasionally filled with desquamated cells [6, 7]. In addition to direct damage due to cytopathic changes in vascular endothelial cells and kidneys, immune-mediated mechanisms are implicated in the pathogenesis of hantavirus infections [8, 9].

The different hantavirus subtypes have different reservoir rodent host species from which they are transmitted to humans, predominantly via aerosols. In the rodent reservoir species, hantaviruses cause persistent and usually subclinical infections [10]. The main reservoir host species of PUUV, the red bank vole *(Clethrionomys glareolus),* becomes infected persistently and subclinically. Because of the apparently different pathogeneses of hantavirus infections in rodents and humans, rodent models are considered of limited value for studying the pathogenesis of human hantavirus infections. However, for practical reasons, predominantly hantavirus rodent models have been explored [11–13]. Although nonhuman primates may develop hantavirus infection similar to that in humans, little or no attention has been paid to the use of nonhuman primates for this purpose.

Here we describe the experimental infection of cynomolgus macaques *(Macaca fascicularis)* with PUUV, which results in a mild form of acute renal infection similar to mild forms of NE in humans. This monkey model will allow in-depth studies of the pathogenesis of primate hantavirus infections and the evaluation of new diagnostic methods, immunization strategies, and therapeutic approaches.

Materials and Methods

Virus preparation. PUUV strain Hällnäs B1 was passaged in Vero E6 cells in 150-cm^2 bottles using standard techniques [14].

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Fourteen days after infection of the cells with an MOI of $\sim 10^3$ $TCID_{50}$/mL, they were washed with PBS and harvested by three cycles of freezing and thawing. Aliquots of 1 mL of the infected cell lysate were stored at −70°C. To determine virus infectivity titers, 10-fold dilutions of this preparation were made in cell culture medium and mixed with 4×10^5 Vero E6 cells in 0.5 mL. After incubation for 1 h at 37°C, the cells were washed and cultured in slide flasks (Nunc, Roskilde, Denmark) for 8 days at 37°C. Infected cell cultures were identified by an indirect fluorescence assay (IFA) with a specific rabbit anti-PUUV antibody as described [14]. Infectivity titers were calculated and expressed as $TCID_{50}$.

Infection of cynomolgus macaques with PUUV. Cynomolgus macaques were kept in pressurized biologic safety glove boxes. Animals (1 male, 7 female; age range, 2–10 years; weight, 2.5–7.0 kg) were inoculated intratracheally with 10^6 $TCID_{50}$ of PUUV in 5 mL of PBS by use of a flexible canula. After inoculation, monkeys were monitored daily for behavioral changes and clinical signs. Before manipulations, the monkeys were anesthetized intramuscularly with 10 mg/kg ketamine hydrochloride, and heparinized blood samples were collected for virus isolation, serology, blood chemistry, and hybridization analysis. In the first experiment, 2 monkeys (nos. 7 and 8) were infected, and the various laboratory parameters (serology, serum creatinine, and virus isolation) were monitored at various time points (figure 1) before they were euthanatized at week 17 and 30, respectively. In a second experiment, the 6 other animals were euthanatized at predetermined time points. Euthanatized animals underwent complete necropsy; blood samples were collected for the various laboratory parameters and tissues were processed for histopathologic examination. For practical reasons, urine samples could not be collected from all animals; limited amounts of urine could be collected by needle aspiration from urinary bladders of 4 monkeys after euthanasia.

Clinical chemistry. Immediately after collection, sodium citrate blood samples were sent to our clinical laboratory, where blood was analyzed for potassium, albumin, serum alanine aminotransferase, and serum creatinine levels by use of an autoanalyzer. Interleukin-2 was measured in plasma of the respective monkeys with a commercially available ELISA kit (CLB, Amsterdam). Urine sediment was immediately measured after urine was collected. Proteinuria was measured by urine dipsticks.

Histochemistry and immunostaining. For histopathologic examination of the organs, specimens were fixed in 4% buffered formaldehyde, paraffin-embedded, cut in sections 4–5 μm thick, stained with hematoxylin-eosin, and scored: −, no abnormalities; +, histopathologic abnormalities.

Immunohistochemical staining using a three-step method was done with monoclonal antibodies directed against group-specific epitopes of the viral glycoprotein G2 (8E10, 11E10, 3D7) and the nucleoprotein (GB04, AA07) [15, 16]. All were of the IgG1 isotype. The specificities of the glycoprotein-specific monoclonal antibodies in immunostaining have been demonstrated [17]. Monoclonal antibody to rabies virus glycoprotein of the same isotype (2-22C5) was used as control. Paraffin sections for immunostaining were mounted on 3-aminopropyl-3-etoxisilane–coated slides and cleared, and endogenous peroxidase activity was blocked in methanol with H_2O_2 (0.3% vol/vol). They were then washed in distilled water and processed for immunostaining. Monoclonal antibodies and conjugates were titrated for the optimal working dilution in PBS supplemented with bovine serum albumin (1% wt/vol), normal monkey serum (2% vol/vol), and normal swine serum (2% vol/vol). Slides were incubated with monoclonal antibodies for 60 min at room temperature, washed three times for 10 min with PBS + Tween 20 (0.5% vol/vol), and incubated with rabbit anti-mouse IgG–horseradish peroxidase conjugate (DAKO, Glostrup, Denmark) and swine anti-rabbit IgG–horseradish peroxidase conjugate (DAKO). Binding of the respective antibodies to the antigens was visualized with a nickel-enhanced diaminobenzidine peroxidase reaction to obtain black deposits. For the positive and negative controls, Vero E6 cells infected with PUUV and uninfected cell pellets were embedded in paraffin and processed in the same way as the monkey tissues.

Immunoperoxidase staining was scored on an arbitrary scale: −, negative; +, positive; ++, strongly positive.

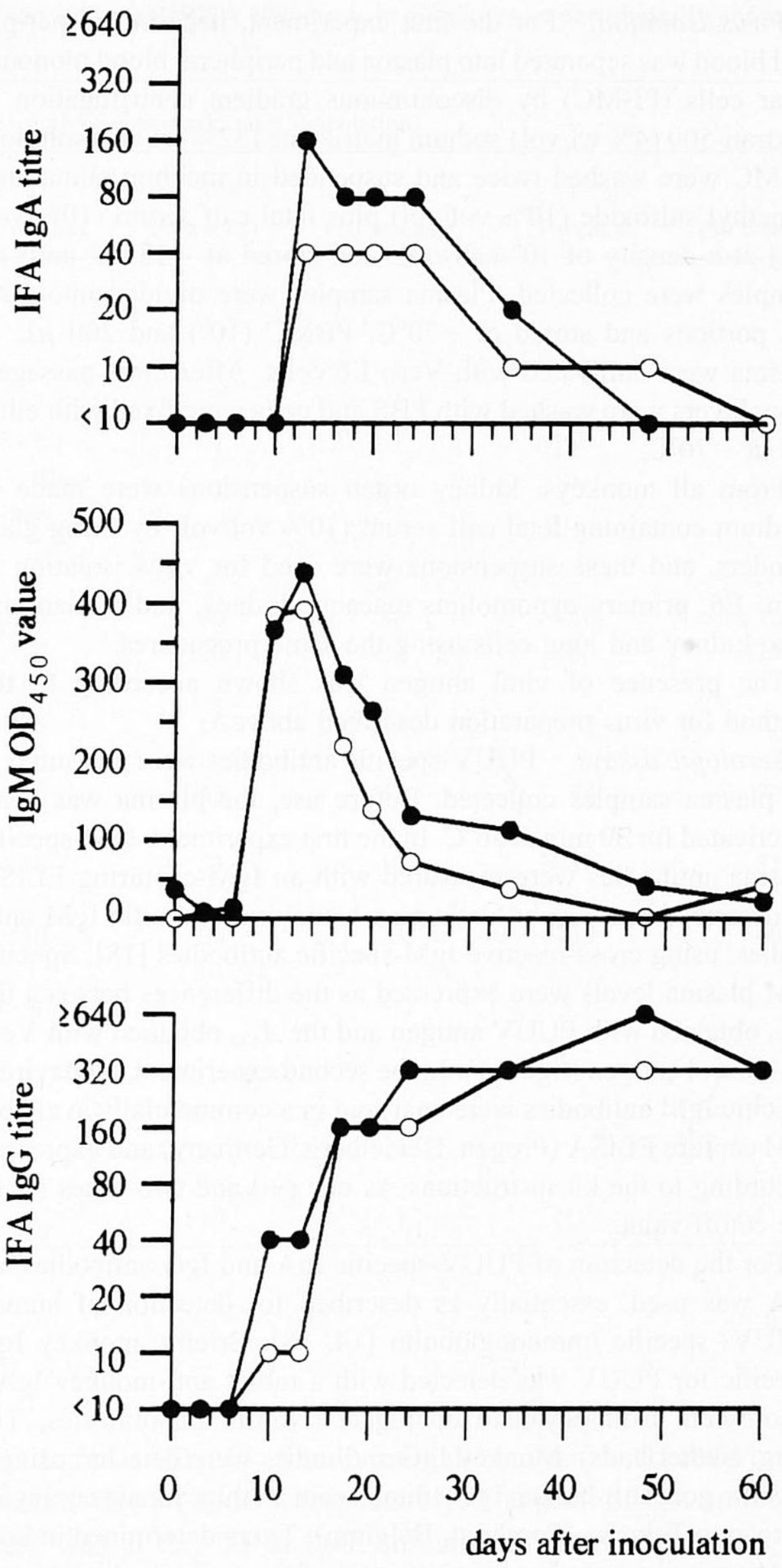


Figure 1. Development of Puumala virus–specific plasma IgA, IgM, and IgG antibodies in 2 monkeys (○, monkey no. 7; ●, monkey no. 8) infected intratracheally.

Virus isolation. For the first experiment, heparinized peripheral blood was separated into plasma and peripheral blood mononuclear cells (PBMC) by discontinuous gradient centrifugation in dextran-500 (4% wt/vol)/sodium metrizoate (32% wt/vol) solution. PBMC were washed twice and suspended in medium containing dimethyl sulfoxide (10% vol/vol) plus fetal calf serum (10% vol/vol) at a density of 10^6 cells/mL and stored at −135°C until all samples were collected. Plasma samples were divided into 200-μL portions and stored at −70°C. PBMC (10^6) and 200 μL of plasma were cultivated with Vero E6 cells. After three passages, monolayers were washed with PBS and cells were fixed with ethanol at −70°C.

From all monkeys, kidney organ suspensions were made in medium containing fetal calf serum (10% vol/vol) by using glass grinders, and these suspensions were used for virus isolation in Vero E6, primary cynomolgus macaque kidney, and human embryo kidney and lung cells using the same procedures.

The presence of viral antigen was shown according to the method for virus preparation described above.

Serologic assays. PUUV-specific antibodies were measured in all plasma samples collected. Before use, the plasma was heat-inactivated for 30 min at 56°C. In the first experiment, IgM-specific plasma antibodies were measured with an IgM-capturing ELISA as described previously for human hantavirus–specific IgM antibodies, using cross-reactive IgM-specific antibodies [18]. Specific IgM plasma levels were expressed as the differences between the A_{450} obtained with PUUV antigen and the A_{450} obtained with Vero E6 control antigen (figure 1). In the second experiment, hantavirus-specific IgM antibodies were analyzed in a commercially available IgM capture ELISA (Progen, Heidelberg, Germany) and expressed according to the kit instructions, as one (+) and two times (++) the cutoff value.

For the detection of PUUV-specific IgA and IgG antibodies, an IFA was used, essentially as described for detection of human PUUV–specific immunoglobulin [14, 18]. Briefly, monkey IgA specific for PUUV was detected with a rabbit anti-monkey IgA–fluorescein isothiocyanate conjugate (Nordic Laboratories, Tilburg, Netherlands). Monkey IgG antibodies were detected using a specific goat anti-human IgG–fluorescein isothiocyanate conjugate (Organon Teknika, Turnhout, Belgium). Titers determined in both IFAs were expressed as the reciprocal of the antibody dilution still giving a clear characteristic dot-like immunofluorescence pattern. For measurement of the antibody response against the structural proteins of PUUV, plasma samples were tested in a recently described inhibition ELISA [18]. The IgG response against the viral nucleoprotein was also analyzed in a commercially available IgG ELISA (Progen) and expressed in units per milliliter according to the kit instructions.

RNA extraction and hybridization. Kidney tissue for hybridization was stored at −80°C until analysis. Total RNA was extracted from kidney tissue by a modification of the guanidinium method. Kidney tissue (~0.5 g [wt/vol]) was homogenized in Eppendorf cups with 500 μL of solution D (4 *M* guanidine thiocyanate, 25 m*M* sodium citrate [pH 7.0], sarcosyl [0.5% wt/vol], 100 m*M* β-mercaptoethanol, and 20 μg of poly A/mL) using a disposable grinder. PUUV-infected and uninfected cell pellets were treated the same way and used as positive and negative controls. The homogenate was extracted with phenol-chloroform–isoamyl alcohol (25:24:1) and chloroform–isoamyl alcohol (24:1), respectively. Total RNA was precipitated with 600 μL of 2-propanol and 35 μL of 3 *M* sodium acetate (pH 5.2) at −20°C for 18 h. Precipitates were pelleted at 10,000 *g* at 4°C and washed once with 500 μL of cold ethanol (80% vol/vol). Air-dried pellets were resuspended in 50 μL of diethylpolycarbonate-treated H_2O. Total RNA (5 μg) was spotted on Hybond N+ membranes (Amersham Laboratories, Amersham, UK) and cross-linked by UV irradiation for 2 min. Hybridization was done with ^{32}P-labeled M (AATTTAAATGCAGGTTGGACAG) [19]– and S (GCACCAGATCGGTGCCCCCC) [20]–specific oligonucleotide probes. The intensity of the hybridization signals was scored on an arbitrary scale: −, negative; (+), weak; +, positive; ++, strongly positive.

Results

Development of clinical signs. After infection, all 8 monkeys lost their appetite and became clearly apathetic from day 2 to 6 after infection. Four developed skin rash between day 9 and 11, which was most prominent at nonpigmented skin areas such as their faces. During the remaining period, no clinical signs were observed. Serum chemistry analyses for all monkeys yielded normal values throughout the study, including serum potassium, serum creatinine, and albumin levels. Plasma alanine aminotransferase levels were measured in monkeys 1–5 at the day of euthanasia, and an increase was found only in monkey 3 (table 1). Plasma interleukin-2 levels at the time of euthanasia were increased in monkeys 1 and 2 (euthanatized at day 6 and 11, respectively) and not in monkeys 3–8 (euthanatized at week 2, 3, 7, 10, 17, and 30, respectively) (table 1). For the 4 monkeys from which urine could be collected on the day of euthanasia, urinalysis was done. Slight proteinuria was seen in monkeys 3 (euthanatized at 2 weeks) and 4 (euthanatized at 3 weeks), with protein levels of 1.0 and 0.3 g/L, respectively.

Microhematuria was demonstrated in monkeys 2 (euthanatized at 11 days) and 3 (euthanatized at 2 weeks), with 5–10 and 50–150 red blood cells/μL, respectively (table 1).

Pathologic findings. Gross postmortem examination on the day of euthanasia demonstrated no abnormalities in brains, aorta, kidneys, liver, spleen, heart, lung, urinary bladder, and multiple locations in the gastrointestinal tract. Histopathologic examination of these organs showed only abnormalities in the tubules of the kidneys, limited to the medullary epithelium and consisting of pycnotic nuclei and desquamated cells in monkeys 1–5 at the time of euthanasia (table 1). In monkey 3, euthanatized at 2 weeks, desquamated epithelial cells present in the tubuli were most prominent. The tissues of monkeys 6–8 (euthanatized at week 10, 17, and 30, respectively) showed no lesions related to the infection. No histologic changes in glomeruli and cortex histology were observed in any of the monkeys.

Virus isolation. Virus isolation procedures with all plasma and PBMC samples collected at various time points (as shown in figure 1) from monkeys 7 and 8 in the first experiment and with kidney suspensions from all monkeys were unsuccessful when three subsequent passages were done. Cell substrates

Table 1. Results of clinical chemistry, serology, histopathology, and antigen and viral RNA detection in monkeys experimentally infected with Puumala virus at different times after inoculation.

Parameter	Monkey no. (time of euthanasia after inoculation)							
	1 (6 days)	2 (11 days)	3 (2 weeks)	4 (3 weeks)	5 (7 weeks)	6 (10 weeks)	7 (17 weeks)	8 (30 weeks)
Clinical chemistry								
Alanine aminotransferase (× preinfection value)	1	1	8	1	1	1	1	1
Interleukin-2 (× preinfection value)	6	11	1	1.5	1	1	NT	NT
Proteinuria (g/L)	Neg	Neg	1.0	0.3				
Hematuria (red blood cells/μL)	Neg	10	50–150	Neg				
Serology								
IgM (ELISA cutoff value)*	−	++	++	−	+	−	−	−
IgA (IFA titer)	<10	40	320	<10	80	80	<10	<10
IgG (IFA titer)	<10	<10	10	<10	320	320	160	320
IgG to nucleoprotein (ELISA U/mL)	<25	<25	<25	<25	66	70	27	47
Nucleoprotein (inhibition ELISA %)	<10	<10	<10	<10	62	47	76	73
Glycoprotein G1 (inhibition ELISA %)	<10	<10	<10	<10	53	10	<10	31
Glycoprotein G2 (inhibition ELISA %)	<10	<10	<10	<10	20	23	<10	60
Other								
Histopathologic changes*	+	+	+	+	+	−	−	−
Antigen detection*								
Glycoprotein G2 (immunoperoxidase)	−	+	++	++	+	+	−	−
Nucleoprotein (immunoperoxidase)	−	+	++	+	+	−	−	−
Dot-spot hybridization*	(+)	++	++	+	(+)	(+)	NT	(+)

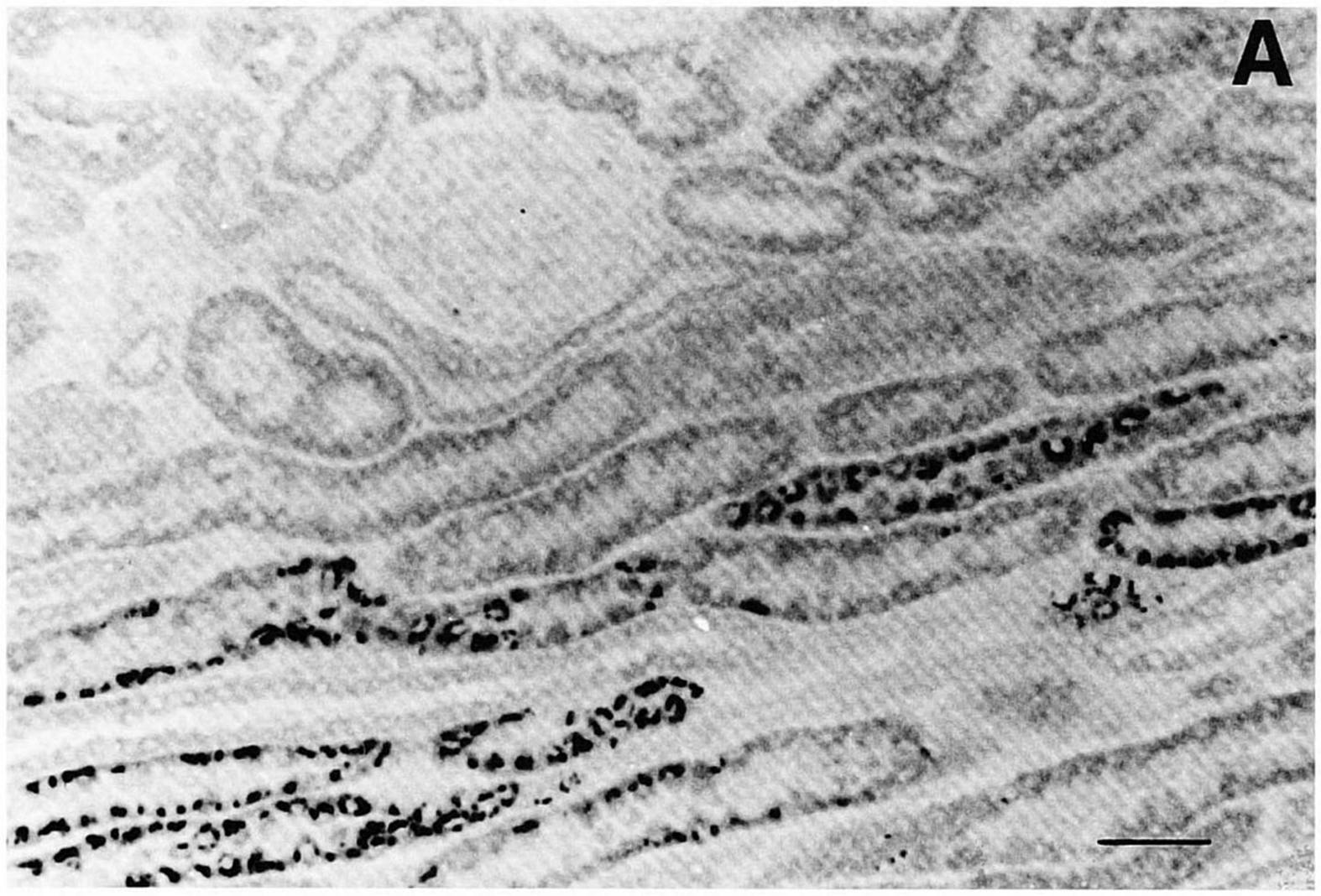
NOTE. NT, not tested.
* Arbitrarily scored (see Methods).

used in these experiments included Vero E6, primary cynomolgus macaque kidney, and human embryo kidney and lung cells, which all were permissive for infection with the stock PUUV used for the intratracheal inoculation in the first passage (not shown).

Immunoperoxidase staining for viral proteins in monkey kidneys. By immunoperoxidase techniques, PUUV-specific glyco- and nucleoproteins were detected in kidney sections collected from day 11 to week 10 after infection. Both viral antigens were located in the cytoplasm of renal tubular epithelial cells in the cortical and medullary areas of the kidneys (figure 2, table 1). Two weeks after infection, the presence of viral antigens in the tubuli was most pronounced. The distribution of antigen-positive tubules was largely focal and mainly located in the area between cortex and medulla. No nonspecific staining was observed with isotype-matched control antibodies.

Development of PUUV-specific IgM, IgA, and IgG plasma antibodies. In the first experiment, both animals (nos. 7 and 8) infected with PUUV were tested for the development of IgM, IgA, and IgG-specific plasma antibodies at regular intervals (figure 1). The peak of PUUV-specific IgM antibodies was found at day 13 in both animals and reached background levels at day 34 and 48, respectively. The peak of IgA antibody titers was at day 13 in both animals and started declining at day 24 to disappear at day 48 and 60, respectively. IgG antibodies were not detected before day 10 and reached a plateau between days 24 and 60.

In the second experiment, the 6 other monkeys were tested for PUUV-specific antibody development at the day of euthanasia. In monkey 4 (euthanatized at 3 weeks), no PUUV-specific plasma antibodies were found. In the other 5 monkeys, the presence of PUUV-specific IgA, IgM, and IgG plasma antibodies coincided with the patterns found in the 2 monkeys of the first experiment (figure 1, table 1). IgG plasma antibody levels measured with a commercially available ELISA against an *Escherichia coli* expression product of the nucleoprotein of PUUV were <25 units/mL in monkeys 1–4, were relatively high in monkeys 5 and 6 (plasma antibody levels of 66–70 units/mL), and were slightly lower in monkeys 7 and 8 (plasma antibody levels of 27–47 units/mL) (table 1). Antibodies against the viral glycoproteins G1 and G2 and the nucleoprotein were not detected with the inhibition ELISA in monkeys 1–



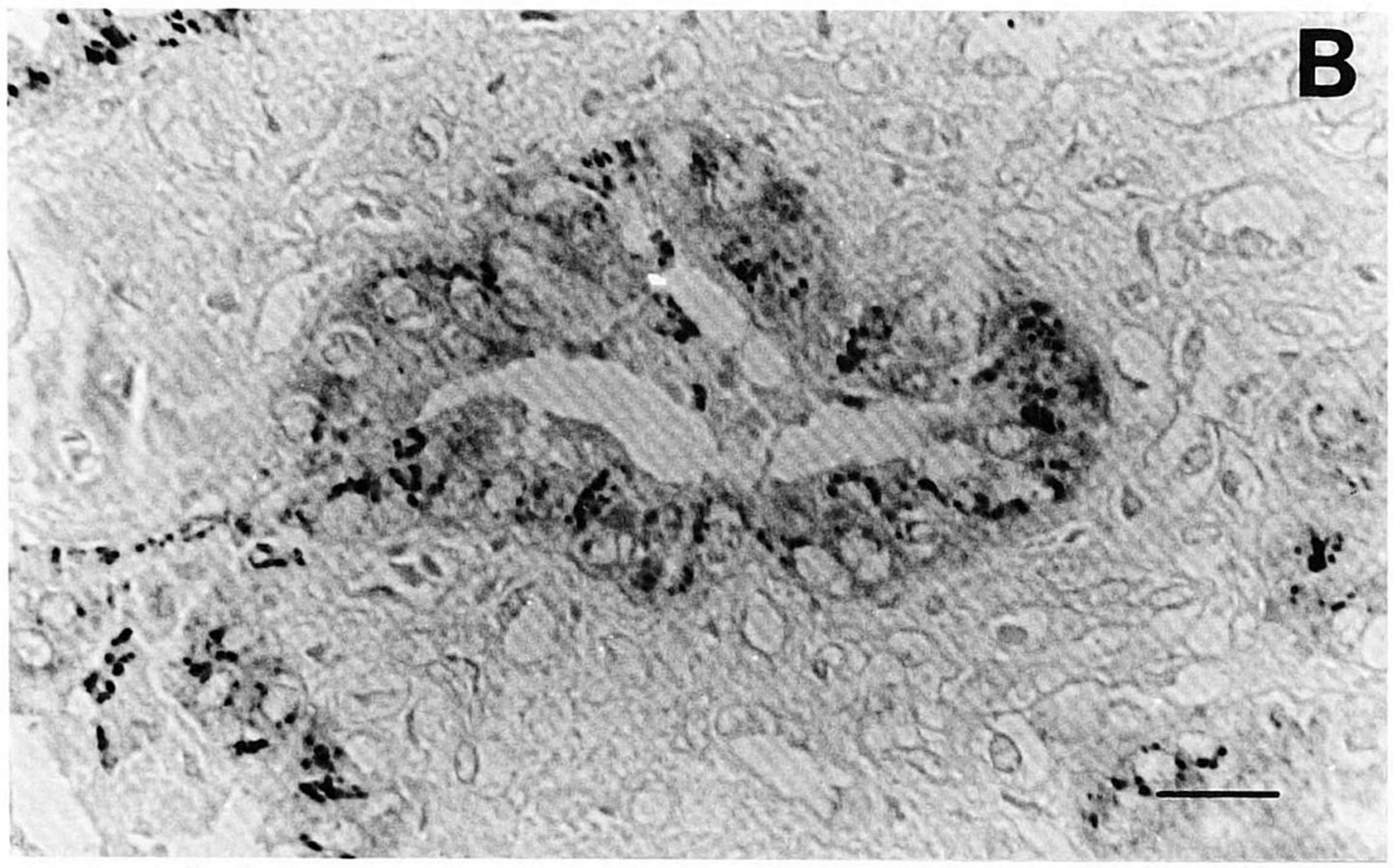


Figure 2. Puumala virus–specific positive immunoperoxidase staining in kidney tissue from monkey no. 3. **A,** Epithelium of straight tubules of cortical area; convoluted tubules and glomerulus are negative (bar = 100 μm). **B,** Collecting tubule of medulla (bar = 25 μm).

3. Plasma antibodies against G1 and G2 were detected in the plasma of monkeys 5, 6, and 8 in the inhibition ELISA, with inhibition levels of 10%–53%, but not in monkey 7 (table 1). In the plasma from monkeys 5–8, antibodies against the nucleoprotein were detected in the inhibition ELISA, with inhibition levels of 62%–73%.

Determination of virus-specific RNA. The hybridization assay done with RNA extracted from kidney cells collected at different intervals after initial infection using oligonucleotide-specific probes for the M-RNA and S-RNA segments are shown in table 1 and figure 3. Positive results were obtained with samples from all tested monkeys; monkey 7 could not be tested for logistical reasons. The highest amounts of viral RNA were detected in monkeys 2 and 3, euthanatized at day 11 and week 2, respectively (table 1, figure 3).

Discussion

In the present study, we have shown that the cynomolgus macaque is susceptible to intratracheal infection with PUUV, which apparently causes a mild NE without clear functional abnormalities. We used the intratracheal route in these experiments, since the most common mode of hantavirus infections in humans is probably through aerosols via the respiratory tract [10]. The clinical symptoms observed were similar to those found in humans with NE, with a transient proteinuria. The skin rash was not observed in all of the infected monkeys, possibly because of pigmentation of the skin. A rise in plasma interleukin-2 levels is often also observed in humans with hantavirus infections in concordance with the degree of illness [21]. Other symptoms, such as general malaise, apathy, flank

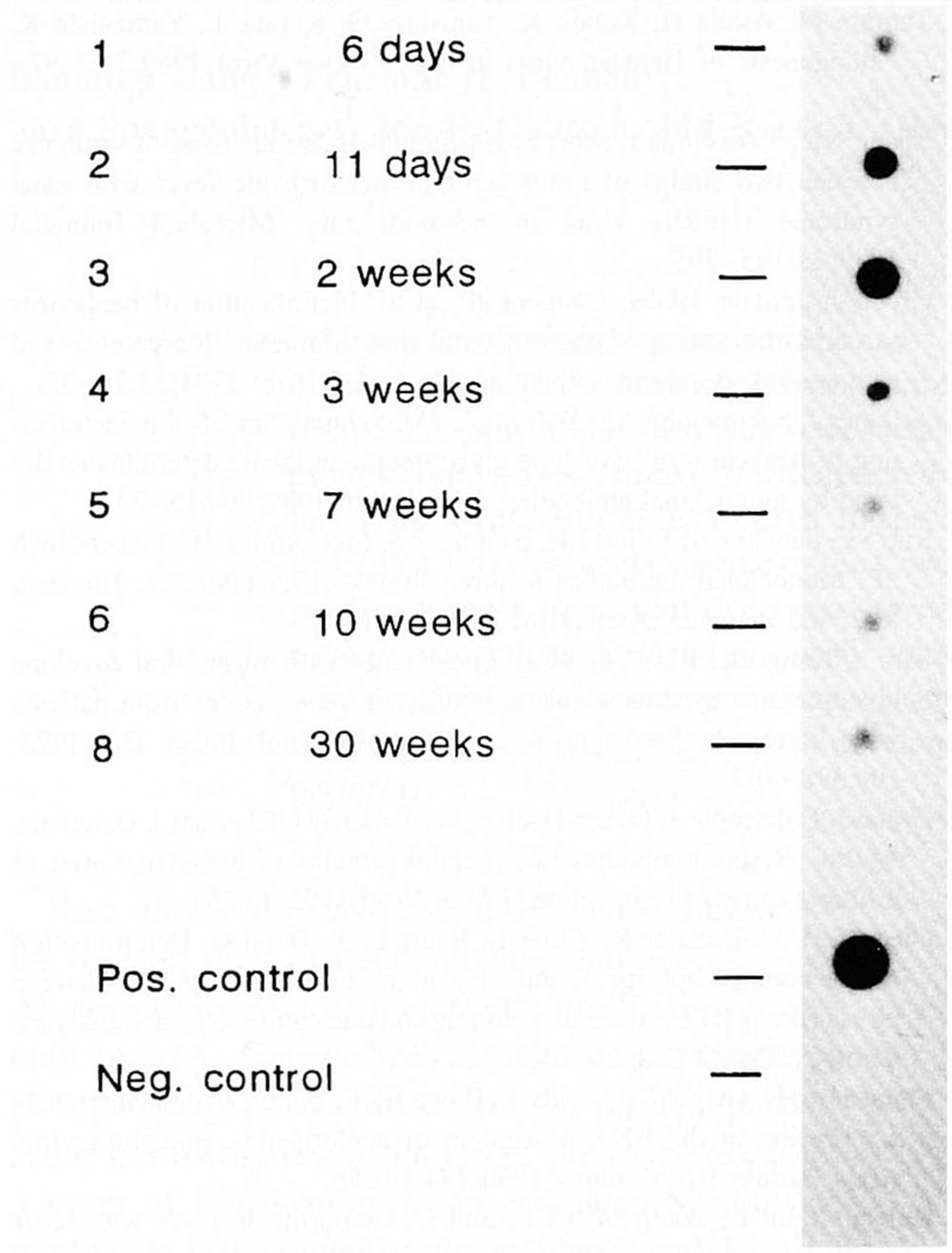


Figure 3. Dot-spot hybridization signal of Puumala virus–specific RNA extracted from kidney cells of experimentally infected monkeys at different times after inoculation.

pain, or fever, are more difficult to assess in monkeys. Changes in blood chemistry values were not found, except for a rise in the liver enzyme alanine aminotransferase in monkey no. 3, which did not show histopathologic abnormalities of the liver when it was euthanatized 2 weeks after inoculation. The anesthesia and changing housing conditions may have influenced these parameters [22].

Epidemiologic studies in areas in which PUUV is endemic have shown that the prevalence of hantavirus-specific serum antibodies is much higher than the actual numbers of individuals with a history of NE would suggest [23–25]. This indicates that also in humans, PUUV infections usually run a milder course than the severe cases of NE that require hospitalization. Thus, the observed clinical signs of PUUV infection in cynomolgus macaques may be quite similar to the relatively mild symptoms usually observed in humans. With the exception of medullar interstitial hemorrhages noticed in ~60% of the kidney biopsies of NE patients, histopathologic abnormalities in the tubules are usually mild and unspecific [6]. Histopathologic changes in the monkeys, which appeared to be related to transient abnormalities in the renal tubular epithelium, correlate with findings in humans with NE [6, 7]. The histopathologic lesions in the monkeys clearly coincided with the presence of viral antigen as well as RNA in the kidneys (table 1). The distribution of viral antigen in the kidneys was focal and restricted to the tubular epithelium. This finding, together with the typical intracellular localization of the antigen, resulted in a pattern similar to that observed in humans with HFRS [17]. This suggests that during a period of ~7 weeks after infection, viral replication takes place in the kidneys. Direct involvement of tubular epithelial cells was also suggested by others studying kidney biopsies of patients with Hantaan virus infections [7, 17]. The virtual absence of infiltration of lymphoid cells or any other signs of inflammatory reactions at this site during this period would be unexpected but has also been documented in humans with NE [7, 26]. This indicates that the direct effects of infection of tubular epithelium, rather than a local inflammatory reaction, are the primary cause of the acute renal symptoms. It is puzzling why isolation of the virus from the kidneys of these animals during these experiments remained unsuccessful after three passages, although the cell substrates used were susceptible to infection with the PUUV preparation used for infection of monkeys (not shown). Others have shown that after nine passages of human specimens on Vero E6 cells, no PUUV-specific antigen could be detected by indirect IFA [27]. These and our findings indicate the difficulties of isolating PUUV from human and primate specimens, which contrasts sharply with the relative ease with which Hantaan virus can be isolated from individuals with HFRS [28]. This may indicate that little infectious virus is formed in the kidneys or that the presence of virus-neutralizing antibodies interferes with replication of the virus in vitro.

The outline of the experiment, in which PUUV-infected monkeys were subjected to regular blood sampling or were euthanatized at regular intervals, allowed us to study the kinetics of several parameters, such as the presence of histologic changes, viral antigen, RNA, and antibody development. The kinetics of virus-specific IgM, IgA, and IgG plasma antibodies coincided well with previous observations in humans at different stages of the infection [18, 29]. The kinetics of the plasma antibody responses of the monkeys against the individual structural proteins is also largely similar to our findings in humans with a PUUV-like infection [18]. The kinetics of the antibody responses against the nucleoprotein and the G2 proteins were almost identical to those observed in humans. Antibody responses to G1 protein, which are quite marked in the acute phase of the infection in humans, appeared to be low in the infected monkeys studied (table 1). In monkey no. 4, which was euthanatized 3 weeks after infection, no virus-specific plasma antibodies were detected, although viral antigen and RNA were demonstrated in kidneys. This shows that as in humans [30], some individual animals may have very poor or slow antibody responses, complicating the diagnosis of the infection, which at present is almost exclusively based on serology.

Taken together, our data show that PUUV infection of cynomolgus macaques is a suitable model with which to study the pathogenesis of NE in humans. For practical reasons, studies

on the pathogenesis of the early stages of the infection, in which the most alarming clinical phenomena are found, have been limited in humans. This monkey model may be valuable for studying the changes that lead to the acute symptoms of anuria followed by polyuria early after infection with PUUV. Monkey infection studies with other hantavirus subtypes, causing more severe renal or respiratory disease in humans, may also prove to be valuable in understanding the pathogenesis and variability of their clinical presentations. Finally, the model described will allow the evaluation of new diagnostic methods and of the efficacy and safety of candidate vaccines and therapies for PUUV and possibly other hantavirus infections.

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