Experimental infection of cynomolgus and African green monkeys with human herpesvirus 6

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Cynomolgus and African green monkeys were inoculated with human herpesvirus 6 (HHV-6). An antibody response was first observed 10 days and 5 days after inoculation of cynomolgus monkeys and African green monkeys, respectively, and was detectable for the duration of the experiment (33 days). HHV-6 DNA was first detected by the polymerase chain reaction in mononuclear cells of one cynomolgus monkey and one African green monkey 10 days after virus inoculation, and in a total of three of four cynomolgus monkeys (75%) and four of five African green monkeys (80%) later after inoculation. Furthermore, HHV-6 DNA was detected in the lymph nodes and spleen of monkeys killed 33 days after virus inoculation. A rash was observed on the trunk of one African green monkey 13 days after virus inoculation, otherwise the infection was asymptomatic. When mononuclear cells from both groups of monkeys were cultured in medium containing concanavalin A and interleukin 2, and infected with HHV-6 in vitro, virus replication was observed. The data suggest that HHV-6 infects these species of monkey and that this system could be useful as an animal model of HHV-6 infection.

Introduction

Human herpesvirus 6 (HHV-6) is a newly recognized human herpesvirus that has been isolated from patients with a variety of lymphoproliferative disorders, including AIDS (Salahuddin et al., 1986; Tedder et al., 1987; Downing et al., 1987; Lopez et al., 1988). Recently, we have reported that HHV-6 is the causative agent of exanthem subitum, a common disease of infants which is associated with fever and rash (Yamanishi et al., 1988). Once HHV-6 infects humans, the virus persists in a latent form (Kondo et al., 1991), but can be reactivated in immunosuppressed states such as lymphoproliferative disorders (Salahuddin et al., 1986) and after organ transplantation (Okuno et al., 1989).

The cellular tropism of this virus is limited, there is no report of infection in animals, and the mechanisms of virus pathogenicity, latency and reactivation are unknown, indicating the importance of developing an experimental animal model for HHV-6. We have previously shown the existence of HHV-6 or an HHV-6-related virus in Old and New World monkeys by using a serological survey (Higashi et al., 1989). Here we report that an antibody response is observed in monkeys experimentally inoculated with HHV-6, and that HHV-6 DNA can be detected in such monkeys by using the polymerase chain reaction (PCR).

Methods

Cells and viruses. Umbilical cord blood mononuclear (CBMN) cells were cultured at 37 °C in complete medium [RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS), 0.1 units (U)/ml recombinant human interleukin 2 (IL-2; kindly supplied by Takeda Chemical Industries) and 5 μg/ml phytohaemagglutinin (Honen Oil)]. Cells were infected with HHV-6 (HST strain), which was isolated in our laboratory from a patient with exanthem subitum, and were cultured as described previously (Yamanishi et al., 1988). When c.p.e. was observed, approximately 3 to 4 days after infection, cells were collected by centrifugation (K-8OH; Kubota) at 3000 r.p.m. at 4 °C, and the supernatant was stored at −80 °C until use. The titre of HHV-6 in umbilical CBMN cells was determined and expressed as TCID₅₀ (Asada et al., 1989).

Animals. Four adult cynomolgus monkeys (Macaca fascicularis; no. 2741, 2742, 2743 and 2744) and five adult African green monkeys (Cercopithecus aethiops; no. 2756, 2757, 2758, 2759 and 2760) were used. The PCR amplification products were 776 bp in length. Japan were used for the experiment. Before inoculation, each animal was determined to be clinically healthy by physical examination.

Virus inoculation. Monkeys were inoculated subcutaneously (no. 2741, 2742, 2746 and 2757) or intravenously (no. 2743, 2744, 2758, 2759 and 2760) with 10⁵ TCID₅₀ HHV-6, and peripheral blood was collected at various times. Plasma was used for antibody detection and DNA was extracted from mononuclear cells as described previously for PCR (Kondo et al., 1990).

Collection of specimens from monkeys. Blood samples (approximately 5 ml) from monkeys were collected into heparinized tubes before and after virus inoculation, and mononuclear cells were separated by Ficoll-
Lymph nodes and spleens were also collected from monkeys 33 days after virus inoculation and minced into approximately 1 mm³ pieces. The mononuclear cells, lymph nodes and spleens were incubated for 3 h at 37 °C in 500 µl NET buffer (150 mM-NaCl, 15 mM-Tris-HCl, 1 mM-EDTA) with 0.1% SDS and 1.0 mg/ml proteinase K (Boehringer Mannheim). The mixture was then extracted five times with equal volumes of phenol–chloroform–isoamyl alcohol (25:24:1). The DNA was precipitated by adding ethanol, washed with 80% ethanol and solubilized in distilled water at 65 °C overnight. The DNA concentration was determined by measuring the ratio of absorbance at 260 nm to that at 280 nm. Samples of about 100 ng of DNA were used for the PCR.

**Antibody detection.** The HST strain of HHV-6 was inoculated onto MT-4 cells, an established T cell line (Myoshi et al., 1982), which were cultured in RPMI 1640 medium supplemented with 10% FBS. When some cells showed a c.p.e, with characteristic balloon-like syncytia, the cells were mounted on spotted glass slides and fixed in acetone at −20 °C for 10 min. Uninfected cells were also prepared on spotted glass slides in the same way. In the indirect immunofluorescence (IF) test, approximately 30% to 50% of the infected cells stained with antibody-positive human serum, whereas uninfected cells were not stained. Sera, serially diluted twofold, were placed onto spotted slides which were incubated for 1 h at 37 °C and then washed twice with PBS. The slides were treated with fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG serum (Dako) and incubated for another 30 min at 37 °C. They were then washed with PBS and observed with a fluorescence microscope after the addition of buffered glycerine. Antibody titres are expressed as the highest serum dilution yielding detectable immunofluorescence.

**Virus recovery from inoculated monkeys.** To recover virus from monkeys inoculated with HHV-6, mononuclear cells were isolated at the same times as cells isolated for DNA detection, as described above, and cultured with human umbilical cord blood cells in complete medium for 4 weeks. Cells were fixed and stained in the IF test using a monoclonal antibody to HHV-6 (Okuno et al., 1990).

**Oligomer synthesis.** Primers were from parts of the SalI fragment (approximately 6 kbp) of the HST strain with the sequences 5'-GTGTTTCCATTGTACTGAAACCGGT 3' and 5' TAAACATCAATGCGTTGCATACAGT 3'; these are located in the region encoding a major capsid protein (Lawrence et al., 1990). These oligomers were synthesized in a DNA synthesizer (Applied Biosystems). The PCR amplification products were 776 bp in length.

**PCR.** DNA was amplified in a total volume of 50 µl of a reaction mixture consisting of 50 mM-KCl, 10 mM-Tris–HCl pH 8.3, 1.5 mM-MgCl₂, 0.01% (w/v) gelatin, 200 µM each of dATP, dGTP, dTTP and dCTP, and 2.5 U Taq polymerase (Perkin-Elmer Cetus). The sample was first denatured at 94 °C for 10 min and then subjected to 30 amplification cycles, each consisting of annealing at 62 °C for 2 min, extension at 72 °C for 5 min and denaturation at 90 °C for 1 min. The primers were each used at a concentration of 1.0 µM. The amplification reaction was carried out in a thermal cycler (Hybaid). Positive and negative control samples were used for each cycle to check the efficiency and for contamination of the PCR. A second run of 30 cycles was done for each sample to increase the copy number.

**Detection of amplified product.** The amplified product was detected by direct gel analysis and by Southern blot hybridization with a cloned DNA probe (a part of the SalI fragment described above).

For direct gel analysis, 10 µl of the reaction mixture was subjected to electrophoresis on a 1.2% agarose gel and DNA was located by u.v. fluorescence after staining with ethidium bromide; M, markers were included in each gel. A band of 776 bp was seen when samples were amplified. For Southern blot analysis, DNA was separated by electrophoresis and transferred to a nylon filter membrane (Hybond N+, Amersham) after treatment with 0.4 M-NaOH for 3 h (alkali blotting and fixation). The filter was neutralized with 2 × SSPE (0.3 mM-NaCl, 20 mM-NaH₂PO₄ pH 7.4, 2 mM-disodium EDTA) for a few minutes. The DNA samples were then hybridized for 12 h with a homologous 32P-labelled cloned probe (2·0 × 10⁶ c.p.m./ml) in hybridization fluid [6 × SSPE, 3% skim milk (Difco), 1% SDS]. The filter was then washed twice with 2 × SSPE, 0.1% SDS for 10 min each at room temperature, once with 2 × SSPE, 0.1% SDS for 15 min at 65 °C, and then twice with 0.2 × SSPE, 0.1% SDS for 20 min each at 65 °C. Bound probe was detected by autoradiography at −70 °C for 8 h with intensifying screens.

**Virus infection of monkey mononuclear cells.** Monkey mononuclear cells were collected as described above. Cells were cultured in RPMI 1640, 10% FBS, 25 µg/ml concanavalin A (Con A) (Boehringer Mannheim) and 0·1 U recombinant IL-2 for 3 days before virus inoculation. Stimulated cells were infected with HHV-6 at a multiplicity of 0·1 TCID₅₀/cell, collected and stained in the IF test using a monoclonal antibody to HHV-6 (Okuno et al., 1990). Culture fluid was also harvested for titration of virus.

**Results**

**Clinical symptoms**

To investigate the replication of HHV-6 in monkeys, we inoculated four cynomolgus and five African green monkeys with HHV-6. In general, body temperature was little affected (data not shown). When monkeys were examined for clinical symptoms on a daily basis, a macular erythematous skin rash on the trunk of one of the five African green monkeys (no. 2756) was observed 13 days after virus inoculation, lasting for only 1 day. Other monkeys remained well throughout the course of the study and all were killed 33 days post-inoculation.

**Antibody response to HHV-6**

Serum samples from monkeys were tested for antibody to HHV-6 by the IF test before and after virus inoculation. Whereas cynomolgus monkeys had no or a very low antibody titre (ranging from < 1:10 to 1:10) against HHV-6, low titre antibody to HHV-6 antigen was detectable in African green monkeys before virus inoculation (1:10 to 1:40). Antibody titres increased markedly in all the cynomolgus monkeys 10 to 15 days after virus inoculation, ranging from 1:160 to 1:2560, and reached a maximum level 2 to 3 weeks after inoculation (Fig. 1a). Antibody titres started to increase in African green monkeys earlier than in cynomolgus monkeys, and they also reached a maximum in 2 to 3 weeks (Fig. 1b).
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Virus recovery from peripheral blood and detection of HHV-6 DNA in peripheral blood mononuclear cells

Virus isolation from mononuclear cells of monkeys inoculated with HHV-6 was not successful at any time.

No HHV-6 DNA was detected by PCR followed by Southern blot analysis in control blood samples collected before virus inoculation (Table 1). HHV-6 DNA could be detected by PCR in some samples taken after inoculation (no. 2742, 2744, 2756 and 2760) after 30 cycles of amplification, but became detectable in the other samples after two rounds of 30 cycles. In all, HHV-6 DNA was detected in three of four cynomolgus and in four of five African green monkeys at various times (Table 1). There was no significant correlation between the antibody titres to HHV-6 and the detection of viral DNA. Furthermore, no difference was found in the detection of DNA after inoculation of virus by different routes.

Detection of HHV-6 DNA in lymph nodes and spleen

An attempt was made to detect HHV-6 DNA by PCR in the lymph nodes and spleen of monkeys inoculated with HHV-6 and killed 33 days later. HHV-6 DNA was detected in the inguinal lymph nodes of two African green monkeys (no. 2756 and 2760) and in the spleen of one cynomolgus monkey (no. 2743), as shown in Fig. 2.

Replication of HHV-6 in mononuclear cells of monkeys in vitro

Mononuclear cells from the peripheral blood of two cynomolgus and two African green monkeys were collected and infected with HHV-6. Cells were cultured in medium containing Con A and IL-2. Although no detectable cell-free virus could be found in culture fluid,
viral antigens were detected in cells infected with HHV-6 at 4 days after infection, as shown in Fig. 3.

Discussion

In this report, we have shown that cynomolgus and African green monkeys experimentally inoculated with HHV-6 develop a serum antibody response to the virus and harbour HHV-6 DNA in peripheral blood mononuclear cells and in some organs, including the lymph nodes and spleen. During the course of studies to isolate the causative agent of exanthem subitum, Kempe et al. (1950) demonstrated the transmission of a fever-producing agent to infants and monkeys inoculated with serum taken from a patient on the 3rd day of the febrile period of exanthem subitum. They reported that the monkeys developed a 3-day febrile illness 5 days after challenge, characterized by a spiking temperature curve with a subsequent drop in temperature to subnormal levels with no other symptoms. In another study, 14 children were inoculated with the infecting agent of exanthem subitum by intramuscular injection of blood from typical cases in the exanthematous stage; three of these recipients developed typical symptoms, with fever beginning 6 to 9 days post-inoculation, followed by a fall in temperature between 9 and 12 days with simultaneous exanthem (Hellstrom & Vahlquist, 1951). In the present study, although it is not clear that HHV-6 induced an erythematous reaction, a rash did appear in one African green monkey 13 days post-infection, but no increase in body temperature was detected.

As shown in Fig. 1, antibody to HHV-6 was detectable in African green monkeys before virus inoculation, although the titre was low (1:10 to 1:40). Antibody titres in these monkeys increased earlier after virus inoculation than those in cynomolgus monkeys, suggesting a booster effect in the antibody response. The antibody response in one monkey with an antibody titre of 1:40 before virus inoculation was poorer than that in others. From these results, it seems that the African green monkeys may have had antibody to HHV-6 or an HHV-6-related virus before virus inoculation.

A PCR has been developed to amplify HHV-6 DNA sequences specifically (Buchbinder et al., 1988; Kondo et al., 1990). In our study, HHV-6 DNA was not detectable in samples collected before virus inoculation, but was amplified from peripheral blood mononuclear cells of three of the four cynomolgus and four of the five African green monkeys experimentally infected. The appearance of viral DNA generally occurred 10 days after virus inoculation, and the antibody titre to HHV-6 showed a parallel increase; this is consistent with virus replication in these animals, although no virus was recovered at any time. The fact that no virus could be isolated from blood samples from these monkeys might suggest that they had been latently infected. No significant differences in the rate of detection of DNA in cynomolgus and African green monkeys were seen, although the antibody response appeared earlier in African green monkeys. We have reported that monkeys, including cynomolgus and African green monkeys, may be infected with HHV-6 or an HHV-6-related virus, as judged by serum testing (Higashi et al., 1989). However, it has been reported that HHV-6 can only infect chimpanzee lymphocytes (Lusso et al., 1990). Although it is not clear at present, this discrepancy may be due to the different virus strains used; HHV-6 might be classified into two subclasses (Aubin et al., 1991; Schirmer et al., 1991).

HHV-6 might replicate in some monkeys and remain in a latent state in cynomolgus and African green monkeys. Non-human primates have a host response to infection closely resembling that of humans, so they could serve as good animal models for the evaluation of
HHV-6 and may be helpful in developing drugs or vaccines for protection against virus infection.

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References


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