Distinguishing Baboon Cytomegalovirus from Human Cytomegalovirus: Importance for Xenotransplantation

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The severe shortage of human organs for transplantation is the driving force behind xenotransplantation. The escalating demand for transplantation coupled with the increasing shortage of human organ donors is the driving force behind xenotransplantation. Baboon cytomegalovirus (BCMV) is endemic in baboon populations and therefore is a potential cause of donor-associated disease after xenotransplantation. Human fibroblasts were permissive for BCMV, isolates exhibited cytopathology characteristic of HCMV, and herpesvirus-like virions were observed by electron microscopy. BCMV and HCMV could be distinguished by restriction fragment length polymorphism patterns and by polymerase chain reaction with primers targeting the BCMV major immediate early gene promoter. These methods can be used to evaluate BCMV pathogenicity in laboratory and clinical xenotransplant trials.

Allotransplantation has become an effective treatment for end-stage organ disorders involving the kidney, liver, heart, or lung. The increasing shortage of human organ donors is the driving force behind xenotransplantation, using animal tissue for human transplantation. In addition, the apparent resistance of some animals to specific human pathogens (e.g., hepatitis B virus [1] and human immunodeficiency virus type 1 [2]) has led to an increasing number of experimental protocols in which animal organs or bone marrow are transplanted into humans [3-5].

Currently, the animals considered the best potential sources of organs and tissues for xenotransplantation into humans are the baboon and pig [6-8]. While immunologic barriers leading to rejection of the animal organs are substantial with either animal, they appear to be more easily overcome when transplantation is undertaken between more closely related species [8]. Organs from nonhuman primates, which are phylogenetically closer to humans, are rejected less vigorously than organs from animals, such as the pig, that are more distantly related. Consequently, recent experiments with xenotransplantation have been done using baboon livers [3, 4] and baboon bone marrow [9]. Increasing public debate has focused on the safety of these procedures. However, investigation into the infectious risk associated with the use of baboon organs or bone marrow has been limited [10-14]. A major obstacle has been the lack of available techniques to distinguish nonhuman primate viruses from analogous human viruses.

Human cytomegalovirus (HCMV) remains the most common and important donor-associated infection after allotransplantation [15-19]. The greatest risk factor for development of disease due to HCMV, in this population, is transplantation of an organ from a seropositive donor to a seronegative recipient [15-20]. Even patients who are HCMV-seropositive before transplant are at risk for reactivation or reinfection with other strains [15]. Consequently, the analogous baboon cytomegalovirus (BCMV) may be a serious pathogen after xenotransplantation. Evaluation of adult baboon sera measuring antibody directed against HCMV found a high prevalence of seropositivity [10-12]. Accordingly, serology will not discriminate between infection with BCMV and HCMV. Methods to distinguish BCMV from HCMV are necessary to evaluate the pathogenicity of BCMV in a xenogeneic environment. We have, therefore, investigated the ability of BCMV to grow on human tissues and have developed a sensitive polymerase chain reaction (PCR)-based method with which to distinguish HCMV from BCMV.

Methods

Animal subjects. Baboons (Papio spp.) were bred and raised in captivity in the United States and ranged in age from 1 day to...
16 years. Viral cultures were obtained from healthy animals as part of a surveillance protocol and from immunosuppressed animals for diagnostic purposes when they developed fever or other signs of illness. Barrier methods were used to protect caretakers and avoid cross-contamination of baboons with human viruses [21].

**Viral culture.** Blood, throat swabs, urine, tissue, and bone marrow were inoculated onto monolayers of human foreskin fibroblasts (HFF) and Vero cells (Baxter Diagnostics, Bartels Division, Issaquah, WA), rhesus monkey kidney cells (Viromed Laboratories, Minneapolis), and A549 and MRC5 (BioWhittaker, Walkersville, MD) cells as previously described [22]. Cultures were incubated at 37°C and examined under light microscopy for evidence of cytopathic effect (CPE). Isolates of virus were frozen at −80°C. If no CPE was detected at 21 days, trypsinization and blind passage were done. Specimens with cytopathic changes consistent with CMV [23] were scraped and frozen at −80°C for further analysis.

**CMV rapid spin culture.** Rapid spin culture for HCMV (shell vial assays) was evaluated by standard techniques with minor modifications [24, 25]. Briefly, clinical specimens were seeded onto 3.7-mL vials containing MRC-5 human lung fibroblast cells on coverslips (BioWhittaker). After inoculation, the vials were centrifuged at 2000 g for 45 min at 36°C. Vials were inoculated for 18 h at 36°C. Coverslips were fixed with acetone and stained with a monoclonal antibody directed against the HCMV p72 major immediate early (MIE) antigen (MAB810; Chemicon, Temecula, CA). Coverslips were then incubated with affinity-purified fluorescein isothiocyanate (FITC)–labeled goat anti-mouse IgG (AP124F; Chemicon) and visualized under a fluorescence microscope. Positive and negative HCMV controls were included with each experiment.

**CMV pp65 antigenemia.** HCMV antigenemia studies (directed against HCMV pp65 antigen) were done directly on buffy coat specimens as described [26, 27]. In brief, white blood cells were obtained by dextran sedimentation of heparinized whole blood from individual animals and diluted to 1 × 10⁶ cells/mL. Three cytospin preparations were prepared for each specimen, with 2 × 10⁵ cells (0.2 mL) /slide. Slides were formalin-fixed, permeabilized, and stained with an anti-pp65 monoclonal antibody (1C3 clone; Argene Biosoft, Varilhes, France). Slides were then incubated with FITC-labeled goat anti-mouse F(ab’)² fragment (Cappel, Durham, NC) and examined under a fluorescence microscope. Positive and negative controls were included with each experiment.

**Electron microscopy.** Representative isolates suggestive of BCMV growth on HFF were subjected to electron microscopy by the dideoxy chain-termination method [29]. A portion of this DNA sequencing and sequence comparisons.

**Southern blot hybridization.** DNA fragments in the gel were denatured and transferred to nylon membranes with a blotting unit (Trans Vac TE 80; Hoefer Scientific, San Francisco) in the presence of 10× standard saline citrate (SSC) [34]. The gel-purified DNA insert, from plasmid pDJA7, representing the BCMV MIE gene locus, was labeled with [32P]dATP by random primer labeling. Hybridization was done [35] at 65°C for 18 h with a final wash in 0.1× SSC and 0.1% SDS at 65°C for 30 min, followed by autoradiography at −70°C.

**Results**

**Conventional viral cultures for detection of BCMV.** Ninety-one specimens from 21 baboons were inoculated for viral culture. Microscopic evidence of CPE similar to that found with HCMV was observed in 25 specimens (27%) (figure 1A). Virus isolates continue to develop CPE after 1–4 passages of supernatant or trypsinized cells to fresh fibroblasts. The median

**DNA sequencing and sequence comparisons.** Plasmid pDJA7, encoding the full-length BCMV MIE gene, is a 10.2-kb BglII fragment that was cloned into the vector pSP73 (Promega, Madison, WI). This fragment was cloned from a genomic digest of total DNA, extracted from BCMV-infected HFF cells, that was shown to hybridize to the rhesus CMV MIE gene (Alcendor DJ, Hayward GS, unpublished data). The purified DNA insert was sheared and subcloned into M13 phage, and DNA fragments were sequenced by the dideoxy chain-termination method [29]. A portion of this sequence representing the BCMV MIE gene promoter-enhancer region was used to design oligonucleotide primers for the specific amplification of a 173-bp DNA product [30–32]. Comparative analysis of sequences between BCMV and HCMV was done using Generows sequence analysis software (Intelligenetics, Mountain View, CA) [33].

**PCR.** The final mixture for PCR amplification included 200–400 ng of total DNA from infected HFF cells, 1× PCR buffer, 0.2 μM dNTPs, 1.5 mM MgCl₂, 40 pmol of each oligonucleotide primer (5’-ATTGACGTCAATAGGGACCA-3’ [BBCMV001] and 3’-TAGTACATTGGCAGTACTCC-5’ [BBCMV002]), and 2.5 U of Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) in a 100-μL reaction volume. Amplifications were done in a thermal cycler (Pheat3; Tecne, Princeton, NJ). Cycling conditions consisted of initial denaturation at 95°C for 5 min, a subsequent denaturation at 95°C for 1 min, annealing at 52°C for 30 s, and extension at 72°C for 2 min for 40 cycles. Fifteen microliters of each PCR reaction underwent electrophoresis in a 1.5% agarose gel. Molecular weight markers were included for DNA fragment size reference.

**Detection of CMV in Xenotransplantation.** Viral cultures of baboon CMV were performed as described above. Viral DNA was extracted with phenol-chloroform and precipitated in 2.5 M ammonium acetate with ethanol [28]. Total DNA from virus infected cells was resuspended in TE (10 mM TRIS, 1 mM EDTA, pH 7.6) buffer and quantitated by absorbance spectrophotometry at 260 nm. DNA was then digested with the Sall restriction enzyme (Boehringer Mannheim, Mannheim, Germany), and fragments underwent electrophoresis in a 0.8% agarose gel at 22 V for 18 h. The gel was stained with ethidium bromide, and DNA fragments were visualized under UV light.

**Restriction fragment length polymorphism (RFLP) analysis.** Southern hybridization of the DNA fragments obtained from virus infected cells was performed with a 2.1-kb BglII fragment from plasmid pDJA7, encoding the full-length BCMV MIE gene, and A325C. Vials were incubated for 18 h with a final buffer and dehydrated with a graded series of ethanol and propylene oxide. Samples were embedded in EMBed 812/Ardaldite 502 wash in 0.1 standard saline citrate (SSC) [34]. The gel-purified DNA insert, from plasmid pDJA7, representing the BCMV MIE gene locus, was labeled with [32P]dATP by random primer labeling. Hybridization was done [35] at 65°C for 18 h with a final wash in 0.1× SSC and 0.1% SDS at 65°C for 30 min, followed by autoradiography at −70°C.

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time for evidence of CPE in primary culture of the HFF or MRC5 cells was 12 days (range, 6–27). Specimens growing BCMV were collected from swabs of the oropharynx (13/40), mouth lesions (3/3), conjunctiva (1/1), urine (1/2), and buffy coats (2/24). Tissues from autopsies were inoculated from 7 animals; BCMV was isolated from lungs (2/4), lymph nodes (1/3), kidney (1/3), and spleen (1/4) but not from liver (0/3) or stomach ulceration (0/1).

Electron microscopy of specimens. An electron micrograph of virus isolates obtained from the oropharynx of a baboon and grown on HFF cells is shown in figure 1B. The cytoplasm of a human fibroblast is shown, with numerous lysosomes containing budded herpes-like particles. Some complete virions containing membrane envelope, as well as nuclear dense core particles characteristic of the herpesvirus family, can be seen.

Anti-HCMV monoclonal antibody staining. Monoclonal antibodies directed against the HCMV p72 MIE antigen recognized some BCMV on rapid spin cultures. One (2%) of 48 specimens inoculated onto rapid spin cultures demonstrated positive staining, with a pattern that was indistinguishable from that of control HCMV isolates. In addition, cytopathology was positive on conventional culture of this specimen. Four BCMV isolates, obtained from throat, urine, and lung cultures, gave inconsistent results when stained with a p72 monoclonal antibody. Fifteen buffy coat specimens obtained from baboons were negative on direct staining with monoclonal antibody.
speciﬁc for HCMV matrix protein pp65; only 2 of the 15 specimens developed CPE by conventional culture.

RFLP. RFLP profiles of the DNA extracted from a laboratory strain of HCMV, chimpanzee CMV, and BCMV are shown in figure 2. Notable differences are seen between the fragment profiles after \( \text{SalI} \) digestion of BCMV compared with the CMV isolates from human and chimpanzee. A second isolate of BCMV from a different baboon was compared with the first isolate of BCMV, with only minimal differences in restriction fragment profiles (data not shown).

CMV PCR. PCR-based detection was done using primers derived from the MIE gene promoter-enhancer region, which were determined to be unique to BCMV when compared with homologous regions of the HCMV genome (figure 3). Sequences of the MIE promoter regions of BCMV, rhesus monkey CMV, and Colburn strain CMV were also compared [28, 36]. Amplification with the BCMV-speciﬁc primers produced a 173-bp product from 2 isolates of BCMV. No product was detected after ampliﬁcation of DNA extracted from CMV from other primates, including human, chimpanzee, and African green monkeys, Colburn and SA6 (ﬁgure 4A). Southern blot hybridization is shown in ﬁgure 4B, done on the PCR products from ﬁgure 4A using \(^{32}\)P-labeled BCMV MIE gene as a probe. Hybridization occurred only with ampliﬁed DNA from the 2 BCMV isolates. To determine whether these PCR primers would amplify DNA from BCMV isolates of other baboons, DNA was separately extracted from 7 isolates obtained from 5 different animals; 3 isolates were from a single animal. The 173-bp product was seen with all of these specimens but not from the laboratory strains of HCMV (AD169, Towne) or from a low-passage primary HCMV isolate from a clinical specimen (ﬁgure 5). In addition, these primers did not amplify DNA extracted from 3 other primary HCMV clinical isolates or from other human herpesviruses, including herpes simplex virus types 1 and 2 and varicella-zoster virus (data not shown). PCR-based assays using previously described HCMV-speciﬁc primers [37] failed to support ampliﬁcation of the isolates of BCMV, whereas ampliﬁcation was found with DNA extracted from control isolates of HCMV (data not shown).

Discussion

Animal viruses, which can be latent in organs or the accompanying hematopoietic cells, may have potential to be transmitted to humans through xenotransplantation. To address issues of whether BCMV can be transmitted to humans, investigations such as the current study are necessary to ﬁrst develop methods that can detect BCMV and differentiate it from HCMV. Earlier studies have indicated as high as 98% seropositivity to BCMV in mature animals [10], making it unlikely that seronegative animals will be readily available for xenotransplant procedures. Serologic studies show cross-reactivity between BCMV and HCMV [10–12]. Additionally, serologic markers, even if unique, may be inadequate in severely immunocompromised xenotransplant recipients, who may have limited abilities to mount an immunologic response. The current investigation indicates that BCMV and HCMV cannot be reliably distinguished morphologically when grown on standard HFF cultures nor when observed with electron microscopy. Inconsistent results were also found when using immunofluorescent detection methods for HCMV antigens, making these methods of detection inadequate. None of the baboon buffy coat specimens stained positive with an anti-HCMV pp65 monoclonal antibody, which is routinely used in our laboratory for HCMV antigenemia testing. However, BCMV was isolated from only 2 of these specimens on direct tissue culture. Viremia in humans is usually only detected after primary infection or with
Figure 3. Sequence comparison of CMV promoter-enhancer regions. Portion of nucleotide sequence from major immediate-early (MIE) promoter enhancer region of HCMV strain AD169 and BCMV strain M58-94 were compared and analyzed (position relative to start site of transcription). Oligonucleotide primers used for polymerase chain reaction amplification are underlined. Homologous nucleotide bases are indicated by asterisk; dashes show gaps required for best-fit comparison.

Substantial immunosuppression. Acyclovir prophylaxis was used in the immunosuppressed animals from whom cultures were obtained and thereby may have decreased the risk of BCMV shedding.

This study demonstrates that BCMV and HCMV can be readily distinguished on the basis of DNA differences through RFLP analysis and PCR. The RFLP patterns suggest that HCMV and chimpanzee CMV are more similar to each other.

Figure 4. Specific amplification of BCMV sequences. A, Ethidium bromide–stained gel of polymerase chain reaction (PCR) amplification of viral DNA from CMV-infected human foreskin fibroblast cells using BCMV-specific primers BCMV001 and BCMV002. Lane 1, PhiX174 phage DNA digested with HaeIII; 2, blank; 3, HCMV laboratory strain AD169; 4, HCMV laboratory strain Towne; 5, chimpanzee CMV isolate; 6, BCMV from throat swab; 7, BCMV from swab of mouth lesion; 8, African green monkey (AGM) CMV strain Colburn; 9, AGM-like CMV isolate. B, Southern blot hybridization pattern for PCR products of ethidium-stained gel in A, transferred to nylon membrane and hybridized to 32P-labeled BCMV major immediate-early gene probe. Arrows indicate position of 173-bp PCR product (A) and its corresponding hybridization pattern (B).
than either are to BCMV; sequence data from several loci support this (data not shown). Recognizable differences between BCMV and HCMV are critical to monitor transmission of BCMV for xenotransplant procedures. Similar to our strategy, Hilliard et al. [38] used a PCR-based technique to distinguish BCMV from HCMV. Their report evaluated BCMV isolated from urine samples of baboons and from the parotid gland of 1 baboon. Interestingly, CPE developed only after 7 passages on Vero cells. This compares to a median of 12 days on HFF cells in the current study, which is similar to growth characteristics of HCMV [23]. The faster growth in our study is likely due to the cells chosen for virus isolation.

The similar growth characteristics of BCMV and HCMV in human tissue culture noted in this study, along with the ability to passage virus, demonstrates replication competence in vitro and suggests the potential for in vivo infectivity. Recognition of transmission of herpesviruses from animals to humans is uncommon. Typically, these viruses are considered species-restricted. In a few instances, however, herpesviruses have been found to cross the species barrier. Transmission of the α-herpesvirus, herpes simiae (B virus), of macaques to humans is a dramatic example of a herpesvirus that is characteristically benign in its normal host but causes disease in a new species [39]. This virus is analogous to human herpes simplex virus and causes self-limited recurrent oral or genital lesions in its natural host. Accidental introduction into a human host through a monkey bite or scratch leads to an ascending myelitis that is almost always fatal when untreated [39]. Human-to-human transmission of B virus has been documented from an infected person to the spouse [39]. Thus, this simian α-herpesvirus is adaptable to the human species. Pseudorabies, the α-herpesvirus of swine, also leads to fatal disease when species lines are broached, as happens with experimental or accidental transmission to dogs, sheep, or cows [40]. Anecdotal evidence exists for nonfatal pseudorabies infection in humans [41]. The ability of a benign herpesvirus to cause more severe disease in another species and to be transmissible within the new species creates public health concerns for infection and secondary transmission with a baboon (or swine) herpesvirus after xenotransplantation [14].

Less is known about transmission of β-herpesviruses, such as CMV, across species barriers into humans. However, non-human primate (African green monkey) CMV has been implicated as the cause of neurologic disease in 2 humans [42–45]. The CMV strain, Colburn, isolated from the brain biopsy of an encephalopathic young boy was found to have substantial homology with a simian CMV (strain GR2757) by reassociation kinetic analyses, suggesting cross-species infection [36, 42]. In a separate case, Martin and colleagues [44, 45] reported that a virus compatible with African green monkey–like CMV was repeatedly isolated from the peripheral blood of a woman with chronic fatigue syndrome. Experimental inoculation of Colburn strain CMV showed that it was also able to establish primary and persistent infection in marmosets (a third primate species) [46]. These reports highlight the possibility of transmission of animal viruses to humans.
through xenotransplantation. It is therefore critical to develop reproducible laboratory methods, as described here, for the detection of animal viruses within the human environment. With these tools, the true risk of disease from xenotransplantation can begin to be evaluated.

References


