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CONCISE COMMUNICATIONS

Detection of Cytomegalovirus in Urine by Nonisotopic DNA Hybridization (2-85-0011)

Cytomegalovirus (CMV) is an opportunistic pathogen that is responsible for significant morbidity and mortality in newborns and immunocompromised patients. Approximately 1% of all newborns in the United States are congenitally infected, and identification of these infants would define a population at risk for developmental and hearing abnormalities [1]. Hepatitis, pneumonitis, and chorioretinitis due to CMV are also devastating illnesses in recipients of bone marrow, renal, and cardiac transplants and in patients with AIDS [2–5]. A rapid, inexpensive test for active CMV infection would identify patients in need of prompt therapeutic intervention.

The reference procedure for identification of CMV is tissue culture. Because of its expense, however, tissue culture is not a practical method for routine screening of newborns. Also, because up to six weeks may be necessary to identify CMV in tissue culture, it may not be rapid enough to allow prompt therapeutic intervention in the immunocompromised patient.

Isotopic DNA hybridization assays for rapid detection of CMV in urine and buffy coat specimens have adequate sensitivity and specificity to identify infected newborns and immunocompromised patients [6, 7]. The use of radioisotopes, however, has several disadvantages, such as the need to use the short-lived radioisotope ^{32}P and the time and expense required for autoradiography. These disadvantages have made clinical application impractical for the majority of laboratories.

Biotin-labeled DNA probes, linked through avidin to an enzyme such as horseradish peroxidase or alkaline phosphatase, can be used to detect DNA bound to nitrocellulose. Biotin-labeled DNA and RNA probes have the advantage of a long shelf life and are reusable in hybridization assays. We have developed a nonisotopic DNA hybridization assay capable of detecting as little as 10 pg of isolated CMV DNA. This report shows an application of this assay for the rapid detection of CMV in clinical urine specimens.

Materials and Methods

Patients and clinical specimens. Random urine specimens (1–5 ml) were obtained from hospitalized patients with a variety of clinical problems, including heart disease, malignancy, chronic pulmonary disease, CMV mononucleosis, and AIDS, as well as from recipients of organ transplants. Urine specimens from neonates with congenital or transfusion-acquired CMV infection and from healthy children and adults who were asymptomatically shedding CMV were also evaluated.

Tissue culture. Random urine specimens for isolation of virus were transported, pretreated, inoculated, and maintained according to standard tissue culture techniques [8]. CMV was identified by production of its characteristic CPE on human foreskin fibroblasts and by failure to grow on HEp-2 cell lines. Urine samples were stored at −70 C until DNA hybridization was performed.

Calculation of the $TCID_{50}$ was by the method of Reed and Muench [8].

Biotinylation of alkaline phosphatase. Biotinyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester (Calbiochem, San Diego, Calif) dissolved in dry dimethylformamide (20 g/liter) was added to alkaline phosphatase (AP) in NMZT buffer (3 mol of NaCl/liter, 1 mmol of $MgCl_2$/liter, 0.1 mmol of $ZnCl_2$/liter, 30 mmol of triethanolamine/liter; pH 7.6) to give a final ratio of 1:5 (wt/wt) or 10 μl/mg of enzyme. The mixture was gently shaken at 4 C for 2 hr. The biotinylated AP was then dialyzed against NMZT buffer. Biotinylated AP was found to be stable for more than six months when stored at 4 C.

CMV-DNA probe. Cloned *Bam*HI fragment E (14.3 kilobases), representing <10% of the DNA of the Towne strain of CMV, was used as a DNA probe in this study. The cloned fragment was designated PRL-5 (the clone was provided by Drs. Gary Haywood and R. LaFemina, Johns Hopkins University, Baltimore). Propagation of the *Escherichia coli* host and amplification of the plasmid pBR322 was done according to standard microbiological procedures [9]. The CMV-specific sequences were isolated from the vector by repetitive preparative agarose gel electrophoresis.

The probe was tested against human genomic DNA prepared from laboratory volunteers and from human hepatomas for possible reactivity with human genomic sequences. No positive reactions were noted under the stringency of our assay conditions.

Biotinylation of CMV-DNA probe. Bio-11-dUTP, a biotin-labeled dUTP analogue, was incorporated into the probe by using a standard nick-translation method [9]. Reagents were purchased from Enzo Biochem (New York). The biotinylated probe is stable indefinitely.

Hybridization assay. Urine samples, stored frozen at −70 C, were thawed and centrifuged at 75,000 *g* for 75 min to collect extracellular virus present due to cell lysis. The supernatant was removed, and DNA was extracted

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from the sediment by standard phenol–nucleic acid extraction procedures [9]. The DNA samples were spotted onto nitrocellulose filters and detection of hybridization and enzyme performed according to previously published methods [10]. Briefly, detection of enzyme involves extensive washing of the nitrocellulose membrane, followed by incubation of the membrane in a solution containing avidin–biotinylated AP complexes, more extensive washing, and finally, incubation of the membrane in a substrate solution. The substrates, 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium, are converted to an insoluble product by AP and precipitate in the paper at the site of enzyme action. The intensity of the color developed is proportional to the enzyme activity bound, within the linear range of the system.

Results

Evaluation of nonisotopic label. Horseradish peroxidase was initially evaluated as a nonisotopic label for detecting CMV DNA. Because this label required at least 40 pg of target DNA for a positive reaction and used a potentially carcinogenic substrate, however, it was judged to be undesirable for detecting CMV in routine clinical specimens.

To evaluate the potential sensitivity of the AP-labeled probe and the degree of cross-reactivity that might be observed with *E. coli* DNA, we blotted various quantities of the vector containing the insert (PRL-5) onto nitrocellulose. In addition, known quantities of the vector alone (pBR322) and salmon testes DNA, a nonspecific DNA, were applied to the same blot. The blotted DNA was then hybridized with the purified probe and developed with the enzyme system described. The resulting pattern was scanned by using reflectance densitometry (figure 1). The reactivity of the purified probe with salmon testes DNA and pBR322 was low and did not increase with concentrations up to 10 ng of DNA per spot. The reaction observed with 10 ng of pBR322 per spot was found to be equivalent to that with ~2–5 pg of PRL-5. The reaction of the probe with increasing concentrations of CMV-specific sequences was found to be linear up to 80 pg per spot.

The sensitivity of AP as a nonisotopic label for detecting CMV DNA in urine samples was evaluated by adding known quantitities of CMV grown in tissue culture to urine samples that were then coded and analyzed as unknowns. Grading of the colorimetric reaction was done blindly by two independent investigators. Figure 2, A shows results of the analysis of the urine samples containing known quantitites of CMV and of several patient samples. An increase in precipitated substrate (color intensity) was noted for increases in viral titer (see figure legend for details). If the graded scores were plotted against viral titer, the range of linear response for visual evaluation was from 1.75 to 3.45 $\log_{10}$ $TCID_{50}$/0.2 ml.

The AP-labeled probe was compared with the same probe labeled with ^{32}P. If autoradiography was carried out for 1.5 hr (the same time as for the enzyme reaction), the relative sensitivity of the two labels was similar. If the film was exposed for longer times, the ^{32}P-labeled probe was ~1 log more sensitive.

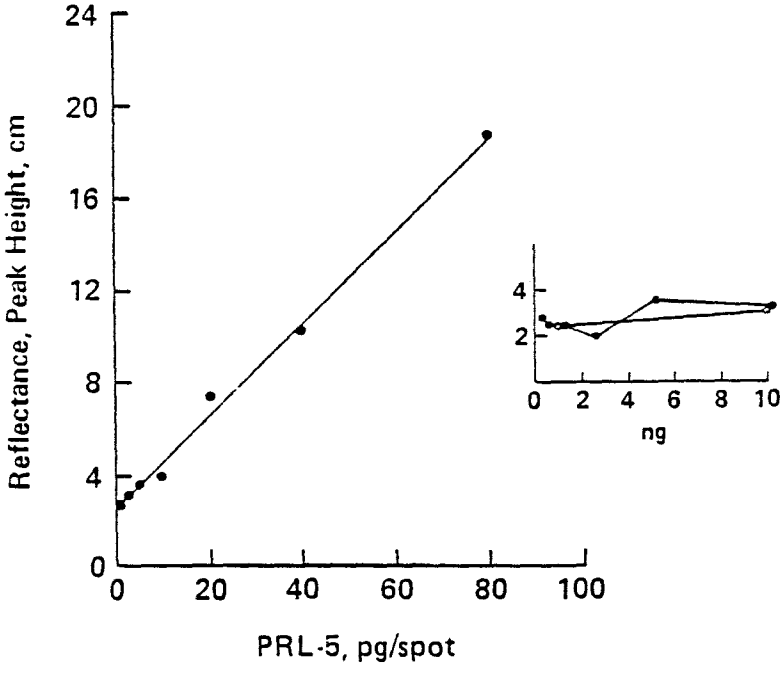

Figure 1. Reflectance density of enzyme reaction produced by reaction of purified *Bam*HI probe with PRL-5, pBR322 (●), and salmon testes DNA (○). The vertical scales for the main graph (PRL-5) and the insert (pBR322 and salmon testes DNA) are identical.

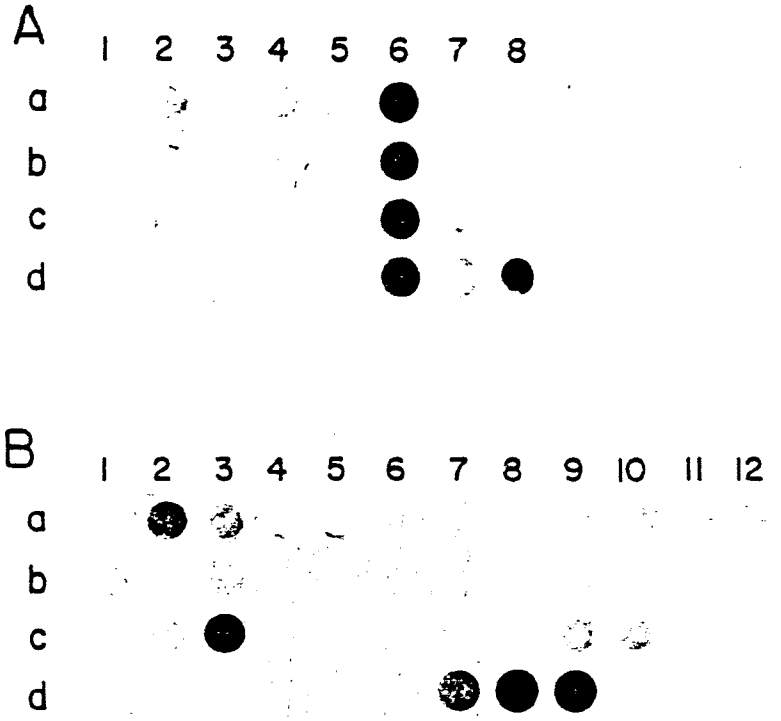

Figure 2. (*A*) Dot blot of urine samples with known quantities of CMV. Extracted urine samples were blotted and then hybridized with purified probe before development with the enzyme system. No CMV was added to the urine samples in spots *a3*, *d4*, and *c4*. Viral titers in other spots were as follows: 1. 45 $\log_{10}$ $TCID_{50}$/0.2 ml (*a1*, *b1*, *d2*), 1.75 (*b3*, *c3*, *d3*), 2.45 (*a1*, *b2*, *c2*), 2.75 (*a4*, *b4*, *d5*), and 3.45 (*b6*, *c6*, *d6*). Spot *a6* represents the reaction observed with a urine sample from an AIDS patient with CMV retinitis. (*B*) Results of nonisotopic blots from 39 urine samples representing the following variety of clinical conditions: congenital CMV infection (*c3*, *d8*, *d9*), acquired CMV infection in adults (*a2*, *a3*, *c9*), AIDS patient with CMV infection (*b8*), and a bone marrow transplant recipient with CMV pneumonia (*b6*).

Evaluation of urine samples. Eighty clinical urine samples were evaluated by tissue culture and by nonisotopic DNA hybridization using the AP-labeled probe. A representative blot shows results from 39 clinical urine samples and illustrates the relative intensity of reaction observed in a variety of clinical situations (figure 2, B).

When compared with tissue culture, the DNA hybridization assay was successful in detecting CMV DNA in urine from patients with congenital infection (21 of 21), patients with AIDS or CMV retinitis (5 of 5), transplant recipients with CMV pneumonia (2 of 3), and patients with acquired CMV infection (19 of 22). In one case the DNA assay was positive while tissue culture was negative. An additional sample of urine was not available in this case to determine whether this false-positive result was due to noninfectious viral particles or perhaps to a urinary tract infection with *E. coli*. The nonisotopic DNA hybridization assay had a sensitivity of 92.2%, with a predictive value of 97.9% for a positive result, when compared with tissue culture.

Discussion

A nonisotopic DNA hybridization assay, using an AP-labeled probe, was found to be a reasonably sensitive and convenient assay for detecting human CMV in clinical urine samples. The nonisotopic assay eliminates the need for ^{32}P labeling of DNA probes to detect probe reaction with target DNA. This means that the handling and disposal of radioactive materials is no longer necessary. In our experience, the biotinylated probes are stable indefinitely and are reusable. We have used hybridization mixtures containing the biotinylated probes up to six times without detectable loss of sensitivity. AP and avidin are also stable for more than a year when stored at 4 C. Consequently, these reagents are easily used, even in a routine laboratory setting.

Some authors have reported the use of direct urine analysis without extraction when ^{32}P-labeled probes were used. Our experience with nonisotopic probes has shown that direct analysis without extraction results in a significant number of false-negative reactions and in some false-positive reactions due to pigments and other interferences that carry over in the urine sediment. Because digestion and extraction of DNA from urine require ~36 hr, a more rapid sample preparation method will be required to make the nonisotopic DNA hybridization assay more efficient.

The nonisotopic AP-DNA hybridization assay consistently detected CMV DNA in urine samples containing viral titers $\geq$1.75 $\log_{10}$ $TCID_{50}$/0.2 ml. Newborns congenitally infected with CMV frequently have viral titers >4 $\log_{10}$ $TCID_{50}$/0.2 ml in their urine [11]. A widespread screening program using a rapid, inexpensive assay, such as the one described here, would allow early detection of infected newborns, optimal management of sequelae such as hearing and neurological defects, and therapeutic intervention once antiviral agents become available.

Immunosuppressed patients also excrete CMV in their

urine in sufficiently high titers (2.0–4.5 $\log_{10}$ $TCID_{50}$/0.2 ml) to allow detection by this nonisotopic hybridization assay [6, 7]. With use of this assay, these patients could be monitored for evidence of excretion of virus; this would allow antiviral therapy to be promptly instituted when clinically indicated. Furthermore, since there was good correlation between quantitative tissue culture and subjective grading of the colorimetric reaction on nitrocellulose filters, the effect of therapy on excretion of virus could also be monitored.

Although not as sensitive as tissue culture, the nonisotopic assay also detected most of the cases of acquired CMV infection in our patient population. These included two cases of adult CMV mononucleosis and 17 of 22 cases of perinatally acquired CMV.

A practical and rapid DNA assay for CMV excretion would also simplify infection control measures. The patient-to-patient nosocomial transmission of CMV has been documented, and patient-to-personnel transmission, though undocumented, remains a possibility [12]. Identification of patients excreting CMV would alert personnel to use protective measures, such as scrupulous handwashing, when caring for these patients.

In summary, the successful use of nonisotopic, enzymatically labeled probes, such as the AP assay system described here, has many potential clinical applications and brings this technology a step closer to widespread clinical use.

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