Rapid Detection of Cytomegalovirus DNA in Urine Samples with a Dot Blot Hybridization Immunoenzymatic Assay

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A dot blot hybridization immunoenzymatic assay for the rapid detection of cytomegalovirus DNA in urine samples was developed by using a digoxigenin-labeled probe which was immunoenzymatically visualized by antidigoxigenin Fab fragments labeled with alkaline phosphatase. A total of 516 urine samples from different groups of subjects were analyzed, and the hybridization assay was able to yield results within 24 h. The results obtained were compared with results for detection of cytomegalovirus antigens in infected cell cultures.

Hybridization techniques for cytomegalovirus (CMV) DNA have been suggested as a promising tool for the specific and reliable diagnosis of CMV infection (2, 3, 6, 7, 12, 13). We recently reported in situ detection of CMV DNA both in cultured cells infected with laboratory-isolated CMV strains (5) and in biopsies of acquired immunodeficiency syndrome patients with concomitant CMV infection (9a). In those studies, we used a CMV DNA probe obtained in our laboratory by using fragments belonging to the repeated sequences of the Towne CMV genome. The probe was constructed by incorporating dUTP labeled with digoxigenin; the in situ-hybridized CMV DNA probe was immunocytochemically visualized by using antidigoxigenin Fab fragments labeled with alkaline phosphatase. The specificity and sensitivity of the in situ hybridization assay for CMV DNA with our probe prompted us to develop a rapid, specific, and reliable dot blot hybridization assay to detect CMV DNA directly in urine samples from different groups of patients. The results obtained in our hybridization immunoenzymatic assay were compared with the results obtained by the detection of CMV antigens in infected cell cultures using a monoclonal antibody against the CMV-induced 68-kilodalton immediate early antigen.

Clinical samples. A total of 516 urine specimens were collected from organ transplant recipients, leukemic patients, acquired immunodeficiency syndrome patients, children aged between 3 and 6 years, and our laboratory staff.

CMV antigen detection in cell cultures. A 5-ml volume of each urine sample was centrifuged at 2,000 × g for 15 min, and 0.2 ml of the supernatant was inoculated into human embryo fibroblasts grown on cover slips in Nunc plates (Nunc). Inoculated cells were fixed at 5 days postinfection and stained in an indirect immunofluorescent assay (11) by using a monoclonal antibody (Techno Genetics) against the 68-kilodalton CMV-induced nuclear protein (10).

DNA hybridization. A 15-ml volume of each urine sample was centrifuged at 2,000 × g for 15 min to remove bacteria and cellular debris. The pellet was discarded, and the supernatant was centrifuged at 50,000 × g for 1 h. The pellet was suspended in 100 μl of TE buffer (Tris, 10 mM; EDTA, 1 mM; pH 7.6) and was spotted by using a Bio-Dot Apparatus (Bio-Rad Laboratories) on the nylon membrane. In each run, a sample of Towne CMV was spotted as the positive control. The nylon membrane was air dried and treated with UV light for 5 min. Specimens on the membranes were alkalized denatured with 0.1 M NaOH–1 M NaCl for 10 min and neutralized with 0.1 M Tris hydrochloride (pH 7.4) containing 1 M NaCl for 10 min. The membranes were prehybridized with 50 mM Tris hydrochloride-1 M EDTA containing 1 mg of pronase (Merck) per ml and incubated for 60 min at 37°C. Pronase was removed with three 5-min rinses in 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate [pH 7]). The Towne CMV XbaI D fragment, which belongs to repeated regions of the CMV genome, was used to construct the CMV DNA probe. The fragment was cloned in plasmid pACYC184 and amplified in Escherichia coli HB101 (14). The CMV D fragment was excised from the pACYC184 vector, separated by electrophoresis in 0.6% agarose gel, and recovered from the gel by using the method of Maniatis et al. (9). Probe labeling was done by incorporating digoxigenin-labeled dUTP (Boehringer GmbH), using the random-primed DNA labeling method developed by Feinberg and Vogelstein (4). In brief, 1 μg of heat-denatured CMV D fragment DNA was incubated for 30 min at 37°C with deoxynucleoside triphosphate labeling mixture (1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, and 0.35 mM digoxigenin-labeled dUTP) in 20 μl of labeling solution containing 10 μg of random hexanucleotides per ml as primers and 2 U of Klenow enzyme (Boehringer). The labeling solution consisted of 50 μl of 1 N 2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (Sigma Chemical Co.) added to 50 μl of 250 mM Tris hydrochloride–25 mM MgCl2–50 mM 2-mercaptoethanol (pH 8) plus 15 μl of 1 M Tris–1 mM EDTA (pH 7.5). The reaction was stopped by adding 0.2 M EDTA (pH 8), and the labeled DNA was precipitated with ethanol.

The CMV DNA probe could be stored at −20°C for at least 4 months with no decrease in its activity.

Nylon membranes with dotted specimens were sealed in a polypropylene bag with (per cm2) 200 μl of a prehybridization mixture containing 4× SET (20× SET is 3 M NaCl, 0.4 M Tris hydrochloride [pH 7.8], and 20 mM EDTA). 5× Denhardt solution (50× Denhardt solution is 1% Ficoll, 1% polyvinylpyrrolidone, and 1% bovine serum albumin in distilled H2O), 100 μg of denatured calf thymus DNA per ml, and 0.5% sodium dodecyl sulfate. The membranes were incubated at 65°C for 1 h. The prehybridization mixture was removed and replaced with (per cm2) 100 μl of hybridization

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mixture containing 4× SET, 5× Denhardt solution, 50% formamide, 0.5% sodium dodecyl sulfate, 100 μg of denatured calf thymus DNA per ml, and 50 ng of denatured CMV DNA probe per ml. The membranes were then incubated in water with shaking at 42°C for 16 to 18 h. The membranes were washed after hybridization under stringent conditions (1). After a brief wash in 100 mM Tris hydrochloride buffer (pH 7.5) with 150 mM NaCl, the membranes were incubated at room temperature for 30 min with antidigoxigenin Fab fragments conjugated to alkaline phosphatase. The membranes were then equilibrated for 2 min with equilibration buffer (100 mM Tris hydrochloride, 100 mM NaCl, 50 mM MgCl2 [pH 9.5]), after which the alkaline phosphatase substrate was added. The alkaline phosphatase substrate consisted of 45 μl of solution A (75 mg of Nitro Blue Tetrazolium per ml in 70% dimethylformamide) and 35 μl of solution B (75 mg of 5-bromo-4-chloro-3-indolyl phosphate per ml and toluidine salt [Sigma] in dimethylformamide) plus 10 ml of equilibration buffer per 100 cm2. The development of a dark blue positive reaction was allowed to proceed for 2 h at room temperature.

**Specificity and sensitivity of the probe.** Our digoxigenin-labeled CMV DNA probe was first hybridized with dotted homologous DNA, and 0.1 to 1 pg of the homologous DNA was detectable after the immunoenzymatic treatment. The same sensitivity of detection was obtained in a recent work of ours with a digoxigenin-labeled parvovirus B19 DNA (1); in that work, we compared the sensitivity of the digoxigenin-labeled B19 probe with the sensitivity of the same probe labeled with 32P; in both assays, 0.1 pg of hybridized DNA was detectable. Moreover, Kimp ton et al. (8) recently compared the sensitivities of biotin-, 32P-, and digoxigenin-labeled AD169 CMV DNA probes and found that while biotin-labeled probes could detect 10 to 50 pg of homologous DNA, 32P- and digoxigenin-labeled probes detected 0.1 pg of DNA.

The labeled CMV DNA probe was also hybridized with the pACYC184 vector, and no hybridization signal was present. To avoid any cross-reactivity with bacterial DNA present in urine samples, our CMV DNA probe was hybridized with DNA of 24 strains of bacteria representing the major causes of bacterial urinary tract infections, and no immunoenzymatic signals were observed.

**Detection of CMV in urine samples.** Among the 516 urine samples examined by DNA hybridization and for detection of CMV antigens in cell cultures, concordant results were observable with 488 samples (94.57%) and discordant results were seen with 28 samples (5.43%). Of the 488 samples which gave concordant results, 51 samples proved to be positive and 437 proved to be negative in both tests. Sensitivity of hybridization versus antigen detection was 85% (51 of 60), while specificity was 95.8% (437 of 456). The predictive value of a positive test result was 72.8% (51 of 70), while the predictive value of a negative test result was 97.9% (437 of 446).

Of the 28 urine samples which gave discordant results in the two assays, 9 samples proved to be positive only for the detection of CMV antigens in cell cultures, while 19 proved to be positive only in the DNA hybridization test. Of the nine urine samples that proved to be positive only for detection of CMV antigen in cell cultures, seven showed only between one and four CMV antigen-positive cells per slide, and in these cases the viral titer was so low that it was probably below the detection limit of the hybridization assay. The other two had 53 and 128 positive cells, respectively, per slide. Of the 19 urine samples that proved to be positive only in the DNA hybridization test with our digoxigenin-labeled probe, 5 were from acquired immunodeficiency syndrome patients, 9 were from transplant recipients, 3 were from young children, and 2 were from students in our laboratory. For the five acquired immunodeficiency syndrome patients and the nine transplant recipients, serological tests demonstrated high titers of antibody to CMV-induced late, early, and immediate early antigens, and in seven of them immunoglobulin M to CMV-induced late antigens was present. Sera were not available from the three children; one of them had a mild fever, while the other two were asymptomatic. The two students in our laboratory were completely asymptomatic, but their sera showed high titers for immunoglobulin G to CMV-induced late, early, and immediate early antigens, and immunoglobulin M to CMV late antigens was present, with titers of 1/10 and 1/20. Moreover, with these two subjects we examined a urine pellet by negative staining with electron microscopy. Viral particles were clearly detectable in both cases.

Our results show that the dot blot hybridization immunoenzymatic assay with the digoxigenin-labeled CMV DNA probe is a rapid and specific method for the detection of CMV in clinical samples. In certain instances, it may indeed be more sensitive than detection of CMV antigen in cell cultures. Our DNA hybridization assay does not require cell cultures, and the CMV probe can be quickly prepared; it can be used over long periods, given its chemical stability, and safely handled in any laboratory equipped to perform routine immunoenzymatic reactions.

In conclusion, our findings suggest that the dot blot immunoenzymatic assay herein described has the prerequisites of a rapid, sensitive, and practical assay to detect CMV DNA in clinical samples.

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