Dot Immunoperoxidase Assay Using Monoclonal Antibody for Direct Detection of Cytomegalovirus in Urine Samples

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A rapid, simple dot immunoperoxidase assay for the direct detection of cytomegalovirus in clinical urine samples was developed. The assay was performed on nitrocellulose paper dotted with urine pellets free of cellular debris. Cytomegalovirus was detected with a monoclonal antibody to the capsid antigen, and the complex was visualized by immunoperoxidase staining. Positive reactions appeared as well-defined dark blue spots. Of the 87 urine samples examined, 10 proved positive in the dot immunoperoxidase assay, and 77 proved negative. The results agreed completely with the detection of cytomegalovirus-induced antigens in cell cultures inoculated with clinical specimens.

Human cytomegalovirus (CMV) is a ubiquitous member of the Herpesviridae family. Although most CMV infections occur asymptomatically or with mild symptoms, human CMV can cause serious illnesses, including congenital malformations, mononucleosis syndrome following blood transfusion, and serious, sometimes fatal complications in immunocompromised hosts. The wide range of CMV infections emphasizes the need for diagnostic techniques which can rapidly and reliably detect active CMV infections.

The diagnosis of active CMV infections can mainly be achieved by (i) serological methods, (ii) viral isolation or detection of CMV-induced antigens in cell cultures inoculated with clinical specimens, and (iii) hybridization techniques able to detect CMV DNA in clinical samples.

To a large extent, serological assays represent the most common method for the laboratory diagnosis of CMV infections (11, 17, 18). In fact, serum samples can easily be obtained, and several immunological assays are commercially available. With serological tests, however, it is only possible to obtain indirect evidence of CMV infections, and for the diagnosis of active or recent CMV infections, accurate analyses of antibody patterns against CMV-induced antigens must be performed (12). The techniques of viral isolation and detection of virus-specific antigens, on the other hand, require cell cultures and depend on a number of variables that can influence CMV recovery and are thus technically demanding and time-consuming. Recently, several authors (1, 5, 6) improved the techniques of virus identification in cell cultures for the rapid diagnosis of active CMV infections. The methods combine the enhancement of the infectivity of clinical samples by centrifugation onto cell cultures and the specificity and sensitivity of a monoclonal antibody for the identification of CMV-induced antigens. Furthermore, the rapid diagnosis of CMV has recently been achieved by detecting CMV genomes in urine samples by DNA-DNA hybridization, but the major disadvantage of this technique is the need to have reproducible, well-characterized, labeled probes (3).

All these considerations have prompted us to develop a simple, rapid diagnostic assay for the direct detection of CMV infections. Since the use of nitrocellulose as a solid phase for immunoassays (2, 7, 8, 16) has previously been described, we have explored the possibility of developing a dot immunoassay on nitrocellulose paper for the direct detection of CMV in clinical samples.

MATERIALS AND METHODS

Urine specimens. Samples (55 ml) of urine were collected from 87 immunocompromised patients. Each urine sample was divided into two portions; one 5-ml urine sample was used for CMV antigen detection in cell cultures, and the other 50-ml one was used to develop the dot immunoperoxidase assay (DIPA).

CMV antigen detection in cell cultures. Five milliliters of each fresh 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 Leighton tubes. Inoculated cell cultures were fixed at 7 days postinfection and stained in an indirect immunofluorescence assay (IAPHA) by using a monoclonal antibody against the major antigenic component (66.8 kilodaltons) of the CMV capsid (Du Pont de Nemours Italiana) (13, 14). The number of positive foci present on a cover slip in a Leighton tube was counted under a light microscope. All tests were performed in triplicate and read in a blind fashion.

DIPA. (i) Antigen dot preparation. Fifty milliliters of each urine sample was centrifuged at 2,000 × g for 15 min. The pellet was discarded, and the supernatant was centrifuged at 50,000 × g for 30 min. The pellet was suspended in 10 μl of sodium dodecyl sulfate (SDS) sample buffer (2.3% SDS, 5% β-mercaptoethanol, 0.015 M Tris hydrochloride [pH 6.8], 10% glycerol) and serially diluted in the same buffer up to a 1/80 dilution. Two microliters of each dilution was dotted onto a strip of a white, opaque nitrocellulose card and air dried. The nitrocellulose strips with air-dried antigen dots were processed for blocking treatment.

(ii) Blocking treatments. The antigen dots were coated with 3% bovine serum albumin in 0.15 M phosphate-buffered saline (PBS; pH 7.4) for 30 min at room temperature. Further incubation was performed with 3% gelatin-PBS for 30 min at room temperature. This procedure adequately blocked all nonspecific protein-binding sites.

(iii) Antibody incubation. The blocking solution was removed, and the antigen-dotted strips were incubated at room temperature for 3 h with a monoclonal antibody against CMV-induced late antigens (Du Pont de Nemours Italiana) at the optimal dilution of 1/20 in PBS.
(iv) **Washing.** The monoclonal antibody was removed, and three washings of 5 min each in PBS were performed with gentle shaking.

(v) **Conjugate incubation.** A second incubation with peroxidase-conjugated goat anti-mouse immunoglobulin G (Dako, Denmark) at the optimal dilution of 1/50 in PBS was done for 2 h at room temperature. Subsequently, three washings were performed as described above, and a rapid fourth washing was performed in distilled water. The optimal working dilution of each immune reagent was determined by preliminary block titration.

(vi) **Enzyme substrate.** A solution of 10 ml of 4-chloro-1-naphthol (Bio-Rad Laboratories) in methanol (3 mg/ml) was added immediately before use to a solution of 10 ml of PBS containing 6 μl of 30% hydrogen peroxide. The antigen-dotted strips were incubated with the enzyme substrate for 5 min at room temperature. After incubation, well-defined, dark blue spots were noted for positive antigens. The strips were then washed in distilled water, air dried, and stored for a permanent record.

**Controls.** (i) **Positive controls.** The supernatant of human embryo fibroblast cells infected with the Towne strain of human CMV was collected 3 days after 100% cytopathic effects appeared. Five milliliters of the supernatant was added to 45 ml of a CMV-negative urine sample. Five milliliters of the 50-ml positive control samples was treated as described for CMV antigen detection in cell cultures, and the remaining 45 ml was treated as described for DIPA.

(ii) **Negative controls.** As negative controls, five CMV-negative urine samples were collected from five individuals who were negative for both CMV isolation and CMV-induced antigens in cell cultures. The CMV-negative urine samples were processed for CMV antigen detection in cell cultures and for DIPA as described above. Moreover, the SDS sample buffer used to suspend the pellets was introduced as a negative control dot on each strip of nitrocellulose.

Positive and negative controls were included in every test. All tests were performed in duplicate and read in a blind fashion.

**RESULTS**

A total of 87 urine samples were examined for the presence of CMV by DIPA and by the detection of CMV-induced antigens in cell cultures. Of the 87 urine samples tested, 10 proved positive in both assays, while 77 were negative; no urine sample gave discordant results in the two assays.

Of the 10 urine samples positive in DIPA, 4 were positive when tested undiluted, 4 were positive at a dilution of 1/10, and 2 were positive at a dilution of 1/20 (Table 1). The DIPA titer was read as the highest dilution of antigen which showed well-defined, dark blue dots (Fig. 1).

The same urine samples that were positive in DIPA were also positive for the detection of CMV-induced antigens in infected human embryo fibroblast cells fixed at 7 days postinfection (Table 1). The number of foci positive in IAPHA varied from 3 to 27 in the different urine samples tested. The four urine specimens which proved positive in DIPA when tested undiluted had 3, 6, 3, and 4 positive foci in IAPHA, the four urine specimens which proved positive in DIPA at a dilution of 1/10 had 4, 5, 6, and 5 positive foci in IAPHA, and the two specimens which proved positive in DIPA at a dilution of 1/20 had 8 and 27 CMV-antigen-positive foci in cell cultures.

As a positive control, the Towne strain of CMV was added to a CMV-negative urine sample and processed both for the detection of CMV-induced antigens in cell cultures and for DIPA. The positive control induced the appearance of 48 foci of CMV-positive cells when 0.2 ml was inoculated into human embryo fibroblast cells. In DIPA, the positive control proved positive up to a dilution of 1/1,280 and was therefore used in each experimental run at a dilution of 1/640.

To assess the reproducibility of the assay, we retested three urine samples positive for both DIPA and CMV antigen detection in cell cultures by DIPA in three different runs and obtained the same results. To test the specificity of our assay, we assayed three urine samples positive for CMV by DIPA with monoclonal antibodies to herpes simplex virus type 2 and Epstein-Barr virus and found them to be negative. Moreover, BK virus and adenovirus type 7 were each added to 50 ml of a CMV-negative urine sample and tested by DIPA with a monoclonal antibody to CMV LA. All the assays proved negative.

**DISCUSSION**

In this paper we describe DIPA for rapid, direct detection of CMV from clinical urine samples. The assay was performed on nitrocellulose paper dotted with urine pellets. CMV from the urine samples was detected with a monoclonal antibody directed against the major component of the CMV capsid, and the complex was visualized by immunoperoxidase staining. In our assay, cellular debris-free urine pellets were treated with SDS sample buffer to expose the antigenic sites present in the virions. In fact, it has been demonstrated that the β2 microglobulin present in urine
samples prevents the detection of CMV by the enzyme-linked immunosorbent assay (10) by binding to the virus and masking its antigenic determinants.

In preliminary experiments to optimize our technique, the urine pellets were either suspended in PBS and boiled or suspended in sample buffer containing the detergent Nonidet P-40. All these treatments resulted in a decreased assay sensitivity when compared with that obtained with the SDS sample buffer we used. Moreover, the presence of Nonidet P-40 in the sample buffer caused the dots to spread on the nitrocellulose paper (data not shown).

In addition, antigen fixation proved unnecessary in our assay, even though some workers have demonstrated that parasite antigens need optimal fixation to avoid false-positive reactions (15). Furthermore, since it has been demonstrated that in dot immunoassays incubation times can vary as a function of serum dilutions (16), we previously tested several incubation times for both monoclonal antibodies and enzyme-labeled antisera. At working dilutions of monoclonal antibodies and labeled antisera, 3 h of incubation with monoclonal antibodies and 2 h with labeled antisera were the minimum times required to produce optimal results. A further decrease in incubation times resulted in a decreased sensitivity of the assay.

The washing buffer used in our assay did not contain Tween 20 which, however, is commonly used in immunoblotting assays, since it has recently been demonstrated that Tween 20 can remove some proteins and antibodies from nitrocellulose paper (9).

With optimized working conditions, our assay was able to detect CMV in urine specimens, yielding positive results in about 8 h. The results obtained with our assay for 87 urine samples completely agreed with the results obtained by the detection of viral antigens in cell cultures. The variability in the number of CMV-antigen-positive foci induced by urine samples in cell cultures as compared with the titer obtained with DIPA may depend on the capacity of DIPA to detect noninfective viral particles in clinical specimens. In fact, it is well known that CMV virions in urine samples can easily be inactivated. The DIPA that we describe was completely flexible with regard to the number of specimens that could be tested in each run. Moreover, the results obtained could be interpreted visually without any absorbance measurement and could be stored as a permanent record. All the reagents in the assay were commercially available, thus minimizing technical time. Our findings suggest that the DIPA that we describe fulfills the criteria of a practical, rapid, reliable assay for detecting CMV in clinical samples and may be a useful tool for the rapid diagnosis of active CMV infections.

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LITERATURE CITED


