Evaluation of a Microtiter Plate Fluorescent-Antibody Assay for Rapid Detection of Human Cytomegalovirus Infection

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Received 23 August 1991/Accepted 2 December 1991

The use of monoclonal antibody (MAb) p63-27, which is reactive with the major immediate-early human cytomegalovirus (HCMV) protein pp72, was explored for the rapid diagnosis of HCMV viruria. The rapid assay detected all but 1 of 19 specimens identified by standard virus isolation methods from 1,676 newborn urine specimens, achieving a sensitivity of 94.5% and a specificity of 100%. The monoclonal antibody recognized 260 randomly obtained clinical isolates of HCMV, indicating the suitability of this reagent for use in screening assays. The sensitivity of the microtiter plate method declined rapidly for specimen from older infants and children with congenital CMV infection and virus-infected children attending a day-care center and was judged to be unacceptable for screening populations in this age group.

Human cytomegalovirus (HCMV) infections are often asymptomatic, but severe disease may occur in congenitally infected infants (4, 9, 14), immunocompromised hosts such as bone marrow and solid organ transplant recipients (6, 11, 12, 18), and individuals with human immunodeficiency virus type 1 infection (5). Standard tube culture virus isolation methods have been accepted as the most sensitive and specific techniques available for the detection of HCMV, although 4 weeks or more may be required for the characteristic cytopathic effect (CPE) to appear (13, 15). Recently, rapid methods that use cell cultures followed by detection of HCMV-specific antigens by immunologic techniques have been developed (1, 3, 7, 8, 16, 17). Several reports have documented the enhancement, by use of centrifugation, of the shell vial technique for the detection of HCMV (7, 8, 17). However, the value of these rapid methods has not been evaluated for use in infants and children with congenital HCMV infection, especially in the screening of large populations for viruria.

We have described in a previous report (2) the murine monoclonal antibody p63-27, which has been shown to recognize the major immediate-early (MIE) protein pp72 of HCMV. Because of its strong reactivity against this HCMV-encoded protein, we explored the use of this reagent for the rapid diagnosis of HCMV viruria in several different patient populations. This assay was based on the pre-CPE expression of the MIE protein in a monolayer of permissive human foreskin fibroblast cells and its subsequent detection in an immunofluorescence assay with antibody p63-27.

Urine specimens were collected from infants born at the hospitals of the University of Alabama at Birmingham between September 1987 and October 1988. In addition, urine from infants and children with congenital CMV infection attending a perinatal infectious diseases clinic and children from a day-care center were also screened by this assay. The urine specimens were processed and the standard tube culture with human foreskin fibroblasts was performed as described previously (15).

Rapid microtiter plate immunofluorescence assay. Flat-bottom 96-well microtiter plates were seeded with 2×10^5 to

5×10^5 human foreskin fibroblasts per well (in vitro passages 6 to 10) 3 days prior to use. The urine specimens were processed between 6 h and 3 days after collection, a 0.1-ml sample was inoculated into each of two wells of the microtiter plates, and then 0.1 ml of medium 199 (GIBCO, Long Island, N.Y.) supplemented with 2% fetal calf serum was added. Microtiter plates were centrifuged at $180 \times g$ for 45 min, and the specimen was aspirated and replaced with fresh medium. Plates were then incubated at 37°C in CO₂ for 16 to 24 h. Control wells included two wells inoculated with 100 infectious units of HCMV strain AD169 and two wells containing medium only. Following the overnight incubation, the medium was removed from the wells by aspiration, the plates were washed once with Dulbecco's phosphate-buffered saline, and the monolayer was fixed in absolute ethanol for 10 min at 37°C. Following removal of ethanol, plates were immediately rehydrated with Tris-buffered saline (TBS; pH 7.4). The wells were then reacted (60 min, 37°C) with 60 μ l of a cell culture supernatant containing monoclonal antibody p63-27. After the wells were washed twice with TBS, 35 μ l of fluorescein-conjugated goat anti-mouse immunoglobulin G (Tago, Burlingame, Calif.) diluted 1/40 in medium 199 with 2% fetal calf serum was added and the mixture was incubated at 37°C for 60 min. The wells were then washed twice with TBS and counterstained with 0.02% Evans blue, and 0.2 ml of TBS containing 70% glycerol was added. The plate was viewed with a Leitz epifluorescence microscope (model Diavert) with a stage adapter for microtiter plates, and the fluorescent nuclei were counted. The presence of one or more fluorescent nuclei was considered a positive result. Standard tube culture isolation of HCMV was carried out as described previously (15).

In an approximate 1-year period, 1,676 urine specimens from newborn infants were tested for HCMV by the conventional tube culture method and the rapid microtiter plate assay. Standard tube culture identified 19 positive specimens (1.13%), a rate consistent with the previously reported incidence of HCMV viruria in this population (14). The rapid microtiter plate method detected all but one of these positive specimens (Table 1). There were no false-positive results by the rapid microtiter plate assay. Furthermore, the one specimen not detected by the rapid microtiter plate assay eventually yielded a strain of HCMV that was recognized by the

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TABLE 1. Comparison of rapid microtiter plate assay with the conventional tube culture method in screening of newborns and children attending a perinatal infectious diseases clinic and a day-care center for the detection of HCMV viruria

Age (mo)	No. of specimens				Sensitivity (%) ^a
	Tube culture method		Rapid microplate assay		
	Positive	Negative	Positive	Negative	
Newborn	19	1,657	18	1,658	94.5
2	27	2	18	11	67.0
3-6	10	7	6	11	60.0
7-12	22	96	10	18	45.5
13-24	11	10	5	16	45.5
25-60	72	86	25	133	34.7
>60	9	39	2	46	22.2
Total	151	250	66	235	43.7

^a Sensitivity was calculated by defining the tube culture positives as the true-positive result.

monoclonal antibody. It is of interest that this single false-negative specimen was misidentified prior to inclusion of the centrifugation step in the assay and the use of absolute ethanol as a fixative. Both of these modifications were introduced during the second month of the study. Thus, the microtiter plate assay had a sensitivity of 94.5% and a specificity of 100% compared with conventional virus isolation for the detection of HCMV viruria.

A major concern surrounding the use of a single monoclonal antibody in a screening assay for HCMV is the possibility of viral strain variation, which would lead to false-negative results. The reactivity of antibody p63-27 against 260 clinical isolates of HCMV was examined. These isolates were obtained from the diagnostic virology laboratory at the University of Alabama at Birmingham and were tested for antibody p63-27 reactivity after routine tube culture isolation. All viruses were reactive with this single monoclonal antibody, indicating the conservation of this antigenic site in a large number of randomly obtained HCMV strains. We have not identified an HCMV strain which is nonreactive with antibody p63-27 (data not shown). Finally, in the initial phase of the study we found comparable reactivities in terms of the intensity of fluorescence with p63-27 and a commercially available MIE-specific monoclonal antibody (Syva Company, Palo Alto, Calif.).

To determine whether the rapid microtiter plate assay for HCMV viruria could be used to detect virus excretion in older infants and children, we compared the sensitivity and specificity of the rapid assay with those of the conventional tube culture method for specimens from children with congenital HCMV infection attending a perinatal infectious disease clinic and children in a day-care center. Although the rapid microtiter test remained quite specific for HCMV, its sensitivity was considerably reduced compared with that of the conventional tube culture method. Of 151 positive specimens from infants and children greater than 2 months of age, only 66 (43.7%) were identified as positive by the rapid assay (Table 1). This population included both congenitally infected infants as well as infants infected perinatally and during attendance at day-care centers. Although we did not analyze these populations separately, as the population as a whole became older, the sensitivity of the rapid assay declined (Table 1). This decline in sensitivity parallels the decline in virus excretion as a function of age in congenitally

and perinatally HCMV-infected infants (10). Overall, these results suggested that, in its present format, the rapid microplate assay is unsuitable for screening infants outside of the newborn period, because its sensitivity fell to 43.7%, whereas its specificity remained 100% (Table 1).

To investigate the reasons for the reduced sensitivity of the rapid assay in older infants and children, we examined the sensitivity of the rapid assay as a function of the time interval between inoculation and a visible CPE in the tube culture and the number of infectious foci present in the tube culture. The mean length of time required for a visible CPE in culture was shorter (7 days) when the rapid microtiter plate assay was positive, whereas it was 15 days when only the conventional tube culture method was positive. Of the 46 specimens which were positive both by culture and by rapid assay, 38 had a detectable CPE within 7 days of inoculation, whereas only 2 and 51 tube culture-positive and rapid microtiter plate assay-negative specimens, respectively, exhibited a CPE within 7 days. In addition, the mean number of infectious foci per milliliter of urine in the standard tube culture was 19.5 (range, 1 to 25) when the microtiter plate assay was also positive, whereas it was 2.91 (range, 1 to 10) when only the tube culture method was positive. Because the standard tube culture in our laboratory uses an inoculum of 0.4 ml of urine, compared with 0.2 ml in the microplate assay, the false-negative results in the rapid method may have resulted from a sampling error in the original inoculum. This possibility seems likely when urine samples contain less than 10 infectious units per ml. Finally, all virus isolates from tube culture-positive and rapid microplate assay-negative specimens were shown to be recognized by antibody p63-27 (data not shown). Thus, it appears that the rapid microtiter plate assay is suitable only for screening urine samples with significant quantities of HCMV.

In conclusion, the microtiter plate immunofluorescence assay with monoclonal antibody p63-27 is rapid, simple to perform, and inexpensive (mainly because larger numbers of specimens can be tested on a single 96-well microtiter plate). Furthermore, the majority of urine specimens assayed in this study were collected and stored for several days at 4°C, suggesting the applicability of this assay to large-scale screening of newborn urine specimens for HCMV infection. There was no obvious difference in the sensitivities of the test for specimens that were assayed shortly after collection or that were held at 4°C for up to 3 days. The data presented here also document the possibility that a single HCMV-specific monoclonal antibody could be used for screening assays because of its reactivity with a large number of randomly obtained HCMV viral strains.

This work was supported in part by grants from the National Institutes of Health and the National Institute of Child Health and Human Development (HD10699), a Clinical Research Center grant (RR-32), and a grant from the Deafness Research Foundation. S. B. Boppana is a recipient of the Dixon Fellowship award.

We thank Dana Pinson for preparation of the manuscript.

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