Direct Detection of Cytomegalovirus from Bronchoalveolar Lavage Samples by Using a Rapid In Situ DNA Hybridization Assay

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An in situ DNA hybridization assay was compared with centrifugation culture for rapid detection of cytomegalovirus (CMV) from bronchoalveolar lavage (BAL) samples. Eighty BAL samples were inoculated into both centrifugation culture and standard culture. Cytospin preparations of the BAL samples were studied in a 75-min in situ DNA hybridization assay using the PathoGene CMV kit (Enzo Biochem, Inc., New York, N.Y.). Of the 80 samples, 39 (49%) were positive for CMV; 37 of 39 (95%) were positive by centrifugation culture, 34 of 39 (87%) were positive in standard culture, 24 of 39 (62%) were positive by in situ hybridization, and 20 of 39 (56%) were positive by histologic and/or immunofluorescence techniques. The in situ hybridization assay detected 23 of the 37 samples positive in centrifugation culture, for a sensitivity of 62% and a specificity of 98%. We conclude that the in situ hybridization assay is a specific and more rapid test than centrifugation culture and standard culture for diagnosis of CMV pulmonary infection. For the clinical laboratory, however, current hybridization methods are not sufficiently sensitive to replace centrifugation culture for detection of CMV in BAL specimens.

In the immunocompromised host, rapid diagnosis of cytomegalovirus (CMV) disease is often critical for patient management, particularly with the availability of investigational antiviral agents that show promise for the treatment of CMV disease (1, 3, 4, 13, 15). Recently, the use of bronchoalveolar lavage (BAL) has been shown to be highly sensitive and specific for the diagnosis of CMV pneumonia in bone marrow transplant patients (2, 5, 6, 12, 17). Rapid identification of CMV in such specimens by the clinical laboratory is desirable to facilitate patient management.

We investigated the use of a rapid in situ DNA hybridization kit for the direct detection of CMV in BAL specimens obtained from bone marrow transplant recipients. Results of the hybridization assay were compared with cell culture techniques as well as with histopathologic staining techniques.

MATERIALS AND METHODS

Specimens. A total of 80 BAL specimens were obtained from 75 bone marrow transplant recipients by flexible fiber optic bronchoscopy (2). The cells and fluid recovered from BAL were pooled and subsequently processed for the identification of bacterial, fungal, and viral agents.

Cytospin preparation. Cytospin preparations of the cells recovered by BAL were made on glass slides pretreated with poly-D-lysine by centrifugation at $500 \times g$ in a cytocentrifuge (cytospin-2; Shandon Southern, Sewickley, Pa.). The cell count in the specimen was first adjusted to 2×10^6 to 4×10^6 cells per ml (2). Seven cytospin preparations were made per sample. The preparations were evaluated for CMV-infected cells by in situ hybridization as well as immunologically and histologically.

Cell culture techniques. Locally produced human diploid foreskin fibroblast (HF) and A549 cells (American Type Culture Collection, Rockville, Md.) were maintained with Eagle minimal essential medium supplemented with fetal

bovine serum and antibiotics. Cells were subcultured weekly into tubes (16 by 125 mm) and maintained on minimal essential medium until use. Primary monkey kidney (MK) cells were obtained weekly in tubes (16 by 125 mm) from ViroMed Laboratories (Minneapolis, Minn.).

MRC-5 cells used to prepare shell vials for centrifugation culture were originally obtained from ViroMed Laboratories in 175-cm² flasks and subcultured weekly with minimal essential medium into 1-dram (~4-ml) shell vials containing a 12-mm round cover slip (9).

Specimen inoculation. BAL cell suspensions were inoculated at a volume of 0.25 ml into two standard culture tubes of HF cells, into one tube each of A549 and MK cells, and into three MRC-5 shell vials for centrifugation culture. HF cells were examined for up to 28 days for evidence of CMV cytopathic effect. The A549 and MK tubes were discarded at day 14 if they were considered negative for other viral agents. MRC-5 vials were centrifuged at $700 \times g$ for 40 min at 34°C, after which 1.0 ml of minimal essential medium with 2% fetal bovine serum was added back to each vial and incubated at 36°C for 16 and 40 h (8).

In situ DNA hybridization. A rapid in situ DNA hybridization was performed on BAL cytospin preparations by using the CMV PathoGene kit (Enzo Biochem, Inc., New York, N.Y.) (7). The hybridization procedure was as follows. The BAL cytospin preparations were fixed in cold acetone for 10 min, after which 1 drop of DNA probe solution was placed on the slide and covered with a cover slip. Slides were heated in a 92°C water bath for 4 min and then incubated at room temperature for 10 min. The slides were then treated for 10 min with 4 drops of probe wash solution at room temperature. They were then rinsed in wash buffer (provided with the kit) for 10 to 15 s, after which 3 drops of probe detection complex was added to the slides and the slides were incubated at room temperature for 15 min. Slides were again washed as described above, after which each slide was treated with 4 drops of a chromogen substrate solution containing aminoethylcarbazole-H₂O₂-acetate, incubated at

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FIG. 1. CMV BAL cytospin preparation by in situ hybridization.

room temperature for 10 to 15 min, and then rinsed in distilled water. The slides were cover slipped with distilled water and viewed under a light microscope. Dark red inclusionlike deposits in the BAL cells indicated a positive DNA hybridization reaction (Fig. 1). One or more BAL cells matching the above description (as illustrated by the figure) were considered positive for CMV.

Staining procedure for centrifugation cultures. Indirect immunofluorescence (IF) staining for the detection of CMV in centrifugation culture was performed in the shell vials by using either an immediate-early CMV monoclonal antibody reagent (Dupont Co., North Billerica, Mass.) or an immediate-early/early CMV monoclonal antibody reagent mixture (Syva Co., Palo Alto, Calif.) (7-9). At 16 h after specimen inoculation, two cover slips were fixed in cold acetone and stained for CMV antigens. After fixation, 0.15 ml of monoclonal antibody reagent was added to each vial and incubated for 30 min at 36°C. The cell monolayers were then washed, and 0.15 ml of a goat anti-mouse fluorescein isothiocyanate-labeled conjugate was added to each vial and incubated at 36°C for 30 min. Cover slips were washed and counterstained with 0.02% Evans blue and mounted on a glass slide with cells downward in a glycerol-based mounting medium (Syva Co.). The monolayers were viewed at a magnification of $\times 250$ on an epifluorescence Zeiss microscope. The additional cover slip was processed as described above at 40 h after inoculation, if necessary.

Histopathology. Cytospin cell preparations were stained with Papanicolaou and Wright-Giemsa stains and examined for the presence of intranuclear or intracytoplasmic CMV inclusions. Additionally, cytospins were stained by indirect IF by using a murine monoclonal antibody (antibody 6-C5;

Syva Co.) reactive with a late CMV antigen for detection of CMV-infected cells (10, 16).

Statistical analysis. Results of centrifugation culture, standard culture, and in situ hybridization were compared by using McNemar's chi-square test.

RESULTS

Of 80 specimens tested, a total of 39 (49%) were positive for CMV. Of the 39, 37 (95%) were positive in centrifugation culture, 34 (87%) were positive in standard culture, 24 (62%) were positive by in situ hybridization, and 20 (56%) were positive by histologic and/or IF techniques. This last figure excludes three cytospin preparations which were considered inadequate for CMV detection by histopathologic techniques. In situ hybridization detected 23 of the 37 (62%) samples positive in centrifugation culture. In addition, two BAL samples from different patients that were negative for CMV were positive for other viral agents. One was positive for adenovirus, and the other was positive for parainfluenza virus type 3.

Among the 14 specimens which were positive in centrifugation culture but hybridization negative, 11 were also positive in standard culture and 3 were positive by histopathologic techniques. One specimen was hybridization positive but negative in both centrifugation and standard cultures. This sample is considered a true positive on the basis of the fact that autopsy lung tissue obtained 7 days post-BAL was CMV positive by centrifugation, standard culture, and histology. Compared with centrifugation culture, the in situ hybridization assay had a sensitivity of 62% and a specificity

TABLE 1. Correlation of in situ DNA hybridization results and centrifugation culture results for detection of CMV from BAL samples

In situ DNA hybridization result	No. of centrifugation culture results ^a	
	Positive	Negative
Positive	23	1
Negative	14 ^b	42

^a Sensitivity, 62%; specificity, 98%; positive predictive value, 96%; negative predictive value, 75%.

^b Eleven of 14 positive in standard culture; 3 of 14 positive by histopathologic techniques.

of 98%, with a positive predictive value of 96% and a negative predictive value of 75% (Table 1).

Table 2 shows the correlation of all four diagnostic techniques. Overall, the hybridization assay had sensitivities of 62 and 85% compared with standard culture and histopathologic techniques with specificities of 93 and 89%, respectively.

There was no significant difference in the detection of CMV by centrifugation culture and standard culture other than time to detection. Standard culture was positive for 33 of 37 specimens (89%) positive in centrifugation culture; 2 of the 4 negative specimens were contaminated. Only one specimen was positive in standard but not centrifugation culture (33 of 34 [97%]). However, there were significant differences between the two culture methods and the in situ hybridization assay. CMV was detected more frequently in both centrifugation culture ($P = 0.001$) and standard culture ($P = 0.002$) than it was by the hybridization assay. Only five specimens could not be evaluated by all four techniques. As mentioned above, two specimens were contaminated in standard culture and three were inadequate for full immunohistopathologic examination.

DISCUSSION

In situ hybridization has been used for direct detection of CMV in Formalin-fixed lung tissues and, more recently, in BAL samples (11, 14). Hilborne et al. (11) described overnight hybridization of BAL cytospin preparations using a

TABLE 2. Detection of CMV from BAL specimens by centrifugation culture, standard culture, in situ DNA hybridization, and histopathologic techniques

No. of BAL specimens positive for CMV	Detection ^a by:			
	Centrifugation culture	Standard culture	In situ hybridization	Immunohistopathologic techniques
16	+	+	+	+
10 ^b	+	+	—	—
5 ^c	+	+	+	—
2	+	+	—	+
1	—	+	—	—
1	+	— ^d	+	+
1	+	— ^d	+	—
1	+	—	—	+
1	—	—	+	—
1	+	—	—	—

^a +, Detected; —, not detected.

^b One specimen, histology not done and immunofluorescence inadequate.

^c Two specimens, histology negative and immunofluorescence inadequate.

^d Specimens contaminated in standard culture.

high concentration of a biotinylated CMV DNA probe also obtained from Enzo. With cell culture used as the standard, a sensitivity of 90% (28 of 31 specimens) and a specificity of 63% were observed. This is in contrast to the present study, in which a sensitivity of 62% was observed, compared with both standard and centrifugation culture with specificities of 94 and 98%, respectively. Both studies used the same DNA probe but in different assay formats. The present study used a 45-min assay procedure, compared with the overnight procedure of Hilborne et al., resulting in lower sensitivity but better specificity. Additionally, the present study was limited to bone marrow transplant recipients, whereas 43 of the 50 immunocompromised patients had the acquired immune deficiency syndrome in the study of Hilborne et al. While both patient populations had a high incidence of CMV pulmonary infection, acquired immune deficiency syndrome patients may have a higher virus load in BAL cells than marrow transplant patients. Thus, the difference in patient population examined may contribute to the sensitivity and specificity of this technique (2, 3, 5, 6).

The major advantage of this in situ hybridization assay is that results can be available in 2 h or less after the laboratory receives the specimen. However, this result can also be achieved by indirect IF, using single or pooled murine monoclonal antibodies to CMV, with approximately equal sensitivity (56%) and specificity (90%) (2). Additionally, in this study we occasionally encountered high background staining in the hybridization assay in the cytospin preparations, and the assay required greater technical expertise to interpret than was required with centrifugation culture.

It has been shown previously that both standard histologic techniques and indirect IF are faster (2 to 4 h) but less sensitive than either culture system used in this study for detection of CMV in BAL samples (2, 5, 6). Centrifugation culture currently remains the most sensitive method for the detection of CMV in BAL specimens in the diagnostic virology laboratory (2, 7).

We conclude that the Enzo CMV PathoGene in situ DNA hybridization assay is as specific as and more rapid than centrifugation culture for the detection of CMV from BAL specimens. It could be used concurrently with centrifugation culture as well as standard culture for diagnosis of pulmonary CMV infection. However, its sensitivity is approximately equal to that of direct IF staining, which is technically simpler and less expensive. Current hybridization methods are not sufficiently sensitive to replace the centrifugation culture assay for the rapid detection of CMV in BAL specimens.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants CA 18025, CA 26966, and CA 15704 from the National Cancer Institute and HL 36444 (formerly CA 30924) from the National Heart, Lung, and Blood Institute.

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