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Quantification of Cytomegalovirus in Bronchoalveolar Lavage Fluid after Allogeneic Marrow Transplantation by Centrifugation Culture

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A technique to quantify cytomegalovirus (CMV) by centrifugation culture of bronchoalveolar lavage fluid from marrow transplant recipients was developed. This technique was used to assess the CMV response to antiviral treatment and the relationship between viral load, asymptomatic excretion versus symptomatic infection, and prognosis. Relative to tube cell culture, centrifugation culture of bronchoalveolar lavage fluid was more sensitive than direct fluorescent-antibody staining. It was also a rapid, replicable method for detecting and measuring the amount of CMV. There was no significant difference between viral load at diagnosis and after 9 days of treatment with ganciclovir and intravenous immunoglobulin. Viral load was not predictive of outcome, and there was no difference in amount of virus between patients with asymptomatic CMV excretion and those with CMV pneumonia. The amount of CMV may not be as important as other factors (e.g., host immune response) in the pathogenesis of CMV pneumonia.

After allogeneic marrow transplantation, cytomegalovirus (CMV) pneumonia has been a frequent infectious cause of death. Treatment with ganciclovir alone did not improve the survival rate beyond the 15% observed for untreated patients (16). An improved survival rate of 50 to 70% was reported with combined intravenous (i.v.) immunoglobulin (Ig) and ganciclovir treatment (2, 12).

Quantitative culturing of lung tissue has been the standard method for assessing the amount of virus present in patients with CMV pneumonia (7, 11, 14). In situ DNA hybridization and immunofluorescent cell counts on lung tissue have correlated well with quantitative culture (7). Bronchoalveolar lavage (BAL) is now used more commonly than lung biopsy to obtain specimens for the diagnosis (1) of CMV pneumonia in marrow transplant recipients. Centrifugation culture of BAL cells including staining with a fluorescent monoclonal antibody specific for immediate-early antigen is a rapid, sensitive, and specific test for the early detection of CMV (9), equal to viral culture of lung biopsy material for these patients (1). Although this technique shows presence or absence of CMV, a quantitative test would aid in clarifying whether viral load is significant in the pathogenesis of CMV pneumonia.

The purpose of this study was to develop a sensitive and rapid technique for quantifying CMV in BAL fluid. This was then used to determine whether there was an appreciable difference in viral load between CMV disease and asymptomatic excretion and whether the amount of virus present at diagnosis was prognostic for outcome and to evaluate the efficacy of antiviral treatment. The amount of CMV in BAL fluid, as determined by centrifugation culture, was compared with that determined by direct fluorescent-antibody (DFA) staining of BAL cells. Rapidity of growth in tube cell culture is also a crude indicator of the amount of virus in the inoculum (10). Thus, time to positive tube cell culture was also recorded for each sample.

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MATERIALS AND METHODS

Patients. Between August 1990 and June 1991, consecutive patients undergoing allogeneic marrow transplant for hematologic malignancy or aplastic anemia who had a diagnostic bronchoscopy for new pulmonary infiltrates were included in this study. Two patients without pulmonary infiltrates, but known to be excreting CMV, who underwent bronchoscopy were also included. Conditioning and graft-versus-host disease prophylaxis were used as previously described (18). Quantification of CMV in BAL fluid was performed for patients who were CMV antibody positive, whose donor was CMV antibody positive, or who were excreting CMV and thus were at risk for CMV pneumonia.

CMV pneumonia was defined as a syndrome of clinical features of pneumonia with new or changing pulmonary infiltrates seen by chest radiography and positive culture of CMV from BAL fluid, a positive DFA test of BAL cells, or positive cytologic diagnosis (presence of typical CMV inclusion bodies in BAL cells) in the absence of evidence of other infectious agents. If other infectious agents (bacteria, *Pneumocystis carinii*, fungi, or other viruses) were detected, the patient was regarded as having a concomitant pulmonary infection. All patients diagnosed with CMV pneumonia received ganciclovir (5 mg/kg of body weight twice a day for 2 weeks) and i.v. Ig (Cutter Biological, Miles Laboratories, Inc., Berkeley, Calif.) (500 mg/kg on alternate days for 2 weeks and then once weekly for 5 weeks). A follow-up bronchoscopy was performed 9 days after diagnosis and institution of ganciclovir treatment.

BAL. A flexible fiberoptic bronchoscope was wedged in the area of maximal radiographic abnormality or, in the absence of infiltrate, in the right middle lobe. Lavage with four serial 50-ml aliquots of sterile 0.9% NaCl was performed (1). Specimens were examined for bacteria, *P. carinii*, fungi, and other viruses.

Virologic techniques. (i) DFA stain. Cytospins were prepared after centrifugation of the BAL fluid specimen at 200 × *g* and separation of the supernatant. The cells were washed with and resuspended in 12 ml of RPMI 1640 (GICCO, Grand Island, N.Y.) with 10% fetal bovine serum added; 100 μl was used to make each of two cytospins. These cells were centrifuged onto glass poly-L-lysine-coated slides at 500 × *g* in a cytocentrifuge (Cytospin-2; Shandon Southern, Sewickly, Pa.), fixed in cold acetone, and air dried, and 0.05 ml of fluorescein isothiocyanate-conjugated CMV reagent (Bartels, Seattle, Wash.) was added to each. This reagent is a pool of three monoclonal antibodies specific for early and late CMV antigens. These antibodies detect both nuclear and cytoplasmic antigens (4). The slides were then incubated at 37°C, washed with phosphate-buffered saline and distilled water, dried, and examined for fluorescence. The total number of cells was counted by using a grid microscope eyepiece, and the number of positive cells was expressed as a percentage of the total.

(ii) Centrifugation cultures. To maximize detection of CMV in BAL fluid, standard procedure with centrifugation culture in our diagnostic laboratory has been to resuspend a concentrate of BAL cells in a fixed volume of lavage fluid because the supernatant may contain some virions (13). For quantitative culture in the present study, 10^6 BAL cells were resuspended in 1 ml of BAL supernatant, and then serial 10-fold dilutions to 10^{-6} were made with RPMI 1640 with 10% fetal bovine serum.

Shell vials were prepared, and the suspension of BAL cells and supernatant was inoculated, centrifuged, and incubated as described previously (5). Briefly, 0.3 ml from each dilution was inoculated in duplicate into 1-dram (1 fluidram = 3.697 ml) shell vials (Fisher Scientific Co., Seattle, Wash.) containing coverslip cultures of MRC-5 cells (Viromed Laboratories, Minneapolis, Minn.). After 24 h of incubation, culture media were changed to remove BAL cells in the media. Before staining, the monolayers were washed with phosphate-buffered saline and Tween 20 and then agitated for 3 min to remove cellular debris and any adherent BAL cells. The monolayers were then stained with the monoclonal antibody 2H2.4 (Dupont Diagnostics, Wilmington, Del.) specific for CMV immediate-early antigen. Two vials were also inoculated with the AD169 laboratory strain as positive controls. At 40 h, all vials were stained. The titer was defined as the reciprocal of the final dilution (10^0- to 10^6-fold) in which CMV was identified. A dilution of 10^0 was the initial standard solution of 10^6 cells per ml. Where the titer differed between duplicate dilutions, the higher titer was recorded.

(iii) Conventional tube cell culture. From each undiluted BAL sample, 0.25 ml was inoculated into human diploid foreskin fibroblast cultures and processed according to established methods (5). Cultures were read twice weekly for 1 week and then weekly for another 3 weeks.

Statistical methods. The sensitivity of a test was defined as the number of samples in which CMV was detected by both the test and the tube cell culture divided by the number positive by the tube cell culture alone. Tube cell culture was used as the reference test for CMV detection. Correlation of titers and percentage of cells positive by DFA was tested by Spearman's rank correlation, change in titers with ganciclovir treatment was tested by a two-sided sign test, and association of titer and mortality was tested by a two-sided Fisher's exact test.

RESULTS

Patient specimens. Quantitative cultures of 22 BAL samples from 17 patients were performed. Twenty of these samples were from 15 patients with CMV pneumonia; 9 patients had diagnostic bronchoscopy only, 5 had both a diagnostic and a follow-up bronchoscopy, and 1 had a follow-up bronchoscopy only. The remaining two samples were from two patients with no radiographic evidence of pneumonia.

Comparison of centrifugation culture, DFA, and standard tube cell culture. (i) Centrifugation culture. Centrifugation culture detected CMV in 21 of 22 BAL samples. One was negative by centrifugation culture but positive by tube cell culture and DFA. Titers for duplicate dilutions were within 1 dilution for all but one sample, for which simultaneous titers were 10^4 and 10^1. There was no correlation between centrifugation culture titer and percentage of cells positive by DFA.

(ii) Tube cell culture. Of the 22 samples, 2 were toxic to the monolayer and 3 were contaminated. These were excluded for calculation of sensitivity. Of the remaining 17 samples, 3 were positive by 1 week, 13 were positive by 2 weeks, and 1 from a patient with CMV pneumonia was not positive until 3 weeks. Of these 17, 16 were also positive by centrifugation culture (sensitivity of 94% compared with tube cell culture). In tube cell cultures, the samples with the fastest growth (positive at 1 week) did not have a higher titer by centrifugation culture or a higher percentage of cells positive by DFA than samples which grew more slowly (positive at 2 to 3 weeks).

(iii) DFA. DFA was positive for 14 of 22 samples. DFA was negative for samples from three patients who died from CMV pneumonia (centrifugation culture titers of 10^6, 10^3, and 10^1) and four samples from three patients who had CMV pneumonia yet survived (titers of 10^3, 10^2, 10^2, and 10^0) and one patient who had no radiographic evidence of pneumonia (titer of 10^3). Sensitivity was 59% compared with that of the tube cell culture.

Ganciclovir treatment. Five patients with CMV pneumonia had both diagnostic and follow-up BAL after 9 days of treatment with ganciclovir. Compared with the titer at diagnosis, the follow-up titer fell by 1 dilution in three patients and by more than 1 dilution in only one of the five patients. While there was a trend for the titer to fall after ganciclovir treatment, this was not significant ($P = 0.125$). However, all five survived this episode of CMV pneumonia. Another three patients had been receiving ganciclovir (5 mg/kg twice a day) prior to initial bronchoscopy—one for esophagitis (for 8 days), one for CMV excretion (for 3 days), and another for a presumed diagnosis of CMV pneumonia (for 1 day). All three patients had positive centrifugation cultures, with titers of 10^0, 10^6, and 10^6, respectively, despite ganciclovir therapy. One other patient who had quantitation performed only on a follow-up BAL after 9 days of ganciclovir treatment had a titer of 10^5.

Correlation of titer at diagnosis with clinical status and outcome. Titers ranged from 0 to 10^6 in patients with CMV pneumonia. Samples from the two patients with asymptomatic CMV excretion in BAL fluid had higher titers than some samples from patients with CMV pneumonia. Seven of 17 patients died, and all 7 had CMV pneumonia. The median titers in both survivors and nonsurvivors were identical (10^3). There was no correlation between a high titer ($>10^5$) at diagnosis in any of the 17 patients and death ($P = 0.25$, by two-sided Fisher's exact test). Furthermore, of the 14 pa-

tients with CMV pneumonia who had diagnostic BAL titers determined, there was no correlation between high titer and death ($P = 0.08$, by two-sided Fisher's exact test). However, more nonsurvivors (four of seven) had concomitant pulmonary infections than survivors (only one of eight). Of the five cases of concomitant pulmonary infections, two were diagnosed at the time of BAL (respiratory syncytial virus, *Mycobacterium tuberculosis*), and another three were diagnosed at autopsy (*Pseudomonas maltophilia*, *Aspergillus flavus*, respiratory syncytial virus).

DISCUSSION

This study demonstrated that centrifugation culture was a reliable and sensitive method for measuring CMV in BAL fluid. It was more sensitive than DFA and as sensitive as tube cell culture. The advantages of centrifugation culture over tube cell culture were that centrifugation culture was more rapid than tube cell culture and was not subject to contamination or toxicity. The absence of contamination in centrifugation cultures may be related to their relatively short incubation time. Centrifugation culture also detected CMV in all nine patients receiving ganciclovir at the time of BAL, whereas DFA detected CMV in six patients and tube cell culture detected CMV in seven patients.

High centrifugation culture titers did not always correlate with a high percentage of cells positive by DFA. This disparity may arise because DFA detects CMV only within cells, while centrifugation culture can detect both intracellular virus and free virus within the BAL supernatant. It is generally accepted that CMV is highly cell associated (10). One would thus expect little difference in CMV titer between methods using cells alone and those using cells plus supernatant to inoculate cultures. Previous studies of nonquantitative centrifugation culture have used BAL cells for inoculation (1, 4, 9), although one study has used BAL supernatant (13). However, subsequent to this study, the number of positive foci of CMV in centrifugation cultures inoculated with BAL supernatant alone was compared with that in centrifugation cultures inoculated with BAL supernatant plus cells for seven samples. In six samples, supernatant alone produced only slightly fewer positive foci than cells plus supernatant. In one sample, the number of positive foci for BAL cells plus supernatant was 100-fold greater than the number of foci for supernatant alone. Because supernatant can contain significant amounts of cell-free virus, future studies will be directed at comparing titers in centrifugation cultures inoculated with BAL cells with those of cultures inoculated with BAL cells with those of cultures inoculated with supernatant.

The lack of correlation between titer and time to positive standard tube cell culture may have been related to several factors, including infrequent readings of tube cell cultures after the first week, the inoculation of tube cell cultures with raw BAL fluid without standardizing the number of cells in it, and perhaps inherent growth differences among viral strains. If centrifugation culture is accepted as a reference test of quantitation, DFA was not as good an indicator of the amount of virus and had a lower sensitivity for the detection of CMV.

This study showed no apparent correlation between virus quantity and disease, antiviral treatment, and outcome. Our work supports previous studies with murine models of CMV infection (14, 15). Particularly surprising was the observation that the amount of virus present in BAL samples after treatment with ganciclovir and i.v. Ig for 9 days fell by more than 1 dilution in only one patient. Unpublished observations from our center show that 80% of marrow transplant recipients with CMV pneumonia still have virus detectable in BAL fluid after 9 days of treatment with ganciclovir and i.v. Ig. However, quantitative cultures were not performed, and thus it was unknown whether treatment had reduced the amount of CMV. In a recent report on the treatment of asymptomatic CMV excretion in BAL fluid in marrow transplant recipients with 12 days of ganciclovir and i.v. Ig treatment, all 10 patients who had follow-up BAL still had CMV present (13).

An earlier study of CMV pneumonia in 10 patients treated with ganciclovir alone found a mean decrease of over 99.99% (or 4 log units) in virus titer by conventional tube cell culture between diagnostic and follow-up lung biopsies (16). Follow-up biopsies occurred after a mean of 17 days of treatment (range, 4 to 20 days). There are two possible explanations for the apparent disparity between the results of that study and those of the present report. Repeat biopsies were performed later in the previous study; however, four patients had biopsies at less than 9 days which also showed significant reductions in the amount of virus (16). Another possibility is that patients in the present study also received Ig, which may block the ability of T lymphocytes to recognize viral antigens on the surface of infected cells (6). This may reduce CMV clearance from the lung.

Since asymptomatic CMV excretion can occur after marrow transplantation (8, 13), controversy as to the role of CMV in interstitial pneumonia exists. One aim of this study was to determine whether titers were higher in patients with CMV pneumonia than in patients with asymptomatic excretion, thereby providing a means for distinguishing the two. Although the number of patients in the present study was small, this was found not to be the case, because higher titers were found in the two asymptomatic patients excreting CMV than in some patients with pneumonia. One study which quantified CMV in BAL fluid distinguished CMV pneumonia from excretion by the percentage of cells positive by indirect immunofluorescence (3). DFA studies did not distinguish asymptomatic excretors in our study.

Viral load at diagnosis did not correlate with outcome. Although all seven patients who died had CMV pneumonia, four of the seven had concomitant respiratory infections which may have contributed to the lack of correlation between titer and death. Clinical improvement was not accompanied by a reduction in the amount of virus in BAL fluid, and there was not a significant difference between the amount of CMV present in excretion and in diseased patients. This supports the theory that other factors (e.g., the immune response) may be more critical determinants of outcome than CMV infection per se (6, 14, 15).

In conclusion, quantitative centrifugation culture was a sensitive, rapid, and replicable method of measuring CMV in BAL and was not subject to contamination or toxicity as was tube cell culture. Treatment of CMV pneumonia with ganciclovir and i.v. Ig did not result in a significant fall in virus titer. High titers did not correlate with clinical severity or outcome of CMV pneumonia and were also found in asymptomatic patients excreting CMV. This study suggests that the pulmonary specific immune and nonspecific inflammatory responses to CMV may be more important pathogenic factors than viral load in the lung after marrow transplantation.

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