Monoclonal Antibodies to Cytomegalovirus: Rapid Identification of Clinical Isolates and Preliminary Use in Diagnosis of Cytomegalovirus Pneumonia

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Two monoclonal antibodies which react specifically with cells infected by cytomegalovirus (CMV) are described. One antibody, 6-E3, reacts with a 72,000-dalton protein that appears early in infection and remains localized in the cell nucleus. The other antibody, 6-C5, reacts with an 80,000-dalton protein that appears late in infection and remains localized in cytoplasmic inclusion bodies. Both monoclonal antibodies react with conventional laboratory strains of CMV and can be used in immunofluorescence assays to identify clinical isolates of CMV in culture. Preliminary tests on lung tissues from patients with CMV pneumonia show that only antibody 6-C5 detects CMV infection in primary clinical specimens. A comparison of culture, histological, and immunological methods demonstrates that the monoclonal antibodies possess sufficient specificity and sensitivity to warrant their continued development as immunodiagnostic tools for the detection of CMV infection in both tissue culture and tissues obtained directly from patients.

Cytomegalovirus (CMV) plays a role in a wide spectrum of clinical disorders, from inapparent infection to severe congenital disease (17). In recent years, the morbidity and mortality due to CMV infection in organ transplant recipients has gained recognition (10, 15). In fact, CMV has become the most common infection that occurs after allogeneic bone marrow transplantation and is now an important determinant of the success or failure of the transplant procedure (8, 15). Patients receiving bone marrow transplants for hematological malignancy have a 19% incidence of CMV pneumonia, with a mortality rate of 90% (8, 15; J. D. Meyers, N. Flournoy, and E. D. Thomas, Rev. Infect. Dis., in press).

Recognition of CMV infection was initially dependent on identification of the specific cytopathology of infected cells: notably, their enlargement and formation of nuclear and cytoplasmic inclusions. Viral isolation in tissue culture has been an additional tool for identification of CMV (18). However, due to the slow growth of the virus, there are limitations in the utility of this method for measuring low-titer virus samples. In some instances, 4 to 6 weeks of culture are required before diagnostic cytopathic effects can be observed, and occasionally specimens containing a low amount of virus may be falsely negative.

Although efforts have been made to diagnose CMV infections by immunological methods (18), the lack of specific antibodies has been a significant problem. To eliminate problems associated with conventional antisera, we have developed two monoclonal antibodies for detecting CMV infection in tissue culture and in primary clinical specimens. As described here, these antibodies react with multiple strains of CMV: one antibody detects an early viral antigen, and the other detects a late viral antigen. Immunofluorescence (IF) studies with these antibodies demonstrate that they will provide powerful tools for the more rapid and specific diagnosis of CMV infection.

MATERIALS AND METHODS

Cells and viruses. Human embryonic fibroblasts (HEF) (Flow 5000; Flow Laboratories, Rockville, Md.) were cultured in Dulbecco modified Eagle medium (DME) with 10% fetal calf serum (FCS). Viruses used in this study included CMV strains AD169 (obtained from E. R. Alexander, University of Washington, Seattle), Towne (obtained from G. Haywood, Johns Hopkins Medical Center, Baltimore, Md.), and Davis (obtained from American Type Culture Collection, Rockville, Md.): herpes simplex type 1 (HSV-1) Macintyre strain (obtained from J. Stevens, UCLA); herpes simplex type 2 (HSV-2) strain 333; herpes varicella-zoster virus (VZV) strain 80-2 (obtained from...
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Mice. Inbred BALB/c mice were initially purchased from Jackson Laboratories (Bar Harbor, Me.) and later bred in our laboratory facility. These mice were used for immunization, as donors for thymocyte feeder cells in culture, and for the production of ascites.

Preparation of hybrid cell lines. The 8-azaguanine-resistant (HGPRT+) myeloma cell line MOPC21 NSI/1 (5) (provided by C. Milstein, MRC, Cambridge, England) was grown in RPMI 1640 containing 15% FCS. Spleen cells were obtained from BALB/c mice immunized with HEF cells infected with CMV strain AD169. For this purpose, HEF cells were infected with AD169 at an MOI of 1.0. The cells were harvested by scraping and centrifugation when the cytopathic effect appeared complete. A suspension of 10^6 infected cells in DME without FCS was injected into two 8-week-old BALB/c mice by intraperitoneal inoculation. Mice were given similar booster injections 1, 2, 8, 9, and 10 weeks later, and spleens were harvested 3 days after the last injection. The hybridization procedure was a modification of methods previously described (5, 9). For this purpose, a total of 10^7 NSI/1 myeloma and spleen cells were mixed in a round-bottom glass centrifuge tube at a ratio of 1:4 in 1 ml of 40% polyethylene glycol 1500 (Eastman, Inc.) and then centrifuged at 1,200 rpm for 12 min. The cells were washed in RPMI 1640 to remove the polyethylene glycol, mixed with an equal number of BALB/c thymocytes, and plated into 96-well microtest culture plates (Costar, Cambridge, Mass.) at a concentration of 10^4 cells per well in HAT medium (RPMI 1640 supplemented with 15% FCS, 10^{-5} M hypoxanthine, 4 \times 10^{-7} M aminopterin, and 1.6 \times 10^{-6} M thymidine). Cultures were routinely incubated at 37°C in 5% CO_2. Cells were fed (50% by volume) with HAT medium on days 3, 5, and 8, and hybrid cells were observed for 5 to 7 days after hybridization. After the cells reached 50% confluence (usually 8 to 11 days after the fusion), the culture fluids were assayed by antibody-binding (AB) assays for anti-CMV antibodies. Hybrid cells that produced antibodies of the appropriate specificity were then passaged at low density (five hybrids per well) on a feeder cell layer of thymocytes (8 \times 10^5 per well) (9).

Culture fluids from each of the passage 1 wells were assayed by AB tests, and cells that produced anti-CMV antibodies were cloned by endpoint dilution (one cell per three wells) on thymocyte feeder layers in microtest plates. Each of the wells was scored microscopically 5 to 7 days after seeding to eliminate those wells which contained more than one clone. Approximately 10 days later, when they had grown to sufficient size, the clones were transferred into one plate, and their culture fluids were again assayed for antibody activity.

Production of ascitic fluids. Clones were expanded by daily passage in RPMI 1640 with 15% FCS. To produce ascites, 10^6 hybrid cells were inoculated intraperitoneally into BALB/c mice. The mice used for this purpose had been pretreated 1 to 3 weeks before the inoculation of hybrid cells with a 0.5 ml intraperitoneal dose of Pristane (2,6,10,14-tetramethyl pentadecane; Aldrich Chemical Co., Milwaukee, Wis.) (11). Ascites from these hybridoma-bearing mice were analyzed for monoclonal immunoglobulins by electrophoresis on agarose membranes (Beckman Paragon System; procedure provided by manufacturer). Densitometry or visual inspection of Coomassie blue-stained membranes showed the presence of a monoclonal antibody “spike” in the gamma globulin region (7). Ascites containing appreciable amounts (> 5 mg/ml) of monoclonal antibody were pooled, divided into portions, and frozen for future use.

AB assays. Anti-CMV antibodies were detected by a binding assay with 125I-labeled protein A (IPA) from Staphylococcus aureus (4, 9). Culture fluids were tested for anti-CMV antibodies by a replicate plating technique against different herpesvirus antigens. For the preparation of antigen plates, HEF cells were infected in petri dishes with CMV strains AD169, Towne, and Davis or VZV at an approximate MOI of 1; when the cytopathic effect was fully developed, the cells were trypsinized and seeded into 96-well microtest plates at a concentration of 10^5 cells per well. HEF cells were also infected with either HSV-1 or HSV-2 at an MOI of 10; the infected cells were harvested 4 h later and then seeded in microtest plates at 10^5 cells per well. Uninfected HEF cells were seeded in microtest plates at 10^6 cells per well. The plates were then incubated overnight at 37°C, washed with DME without FCS, fixed with absolute methanol for 15 min, and air dried. On the day of the assay, the wells of the plates were blocked from further nonspecific protein absorption by a 2-h incubation with 50 μl of 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS).

The AB assay was performed in three steps. (i) A 50-μl amount of culture fluid was incubated in each of the wells of the antigen plates for 45 min at 37°C. Nonbound immunoglobulins were then removed from the wells by washing twice with PBS containing 1% BSA. (ii) IPA (10^5 cpm) in 50 μl of PBS was added to each well for 45 min at 37°C. The residual nonbound IPA was then removed by washing three times with PBS. (iii) The immune reactions were detected by 16-h autoradiography of the IPA-treated microtest plates on Kodak XR-5 film with enhancement by X-ray intensifying screens (14).

Immunoprecipitation analysis with anti-CMV antibodies. HEF cells were infected with CMV AD169 at an MOI of 1, and 70 to 72 h postinfection the medium was replaced with DME containing 10% FCS and one fifth the usual concentration of methionine. Two hours later, 25 μCi of [35S]methionine (New England Nuclear Corp., Boston, Mass.) per ml was added, and the cells were harvested after 4 h of incubation at 37°C. The infected cells were collected by centrifugation, and lysates were prepared for immunoprecipitation by suspending the cells in extraction buffer (PBS containing 0.1% sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate, and 1.0% Nonidet P-40 [pH 7.5]) at a concentration of 10^6 cells per ml. The cell suspension was then sonicated on ice with a probe sonicator for two 15-s pulses and centrifuged at 2,500 rpm for 10 min. The supernatant was collected and frozen at -70°C.
After thawing, the cell lysates were centrifuged at 25,000 rpm for 1 h to remove residual aggregates. To further reduce the background reactions commonly observed in immunoprecipitation procedures, the supernatants were pretreated by incubation for 1 h on ice with a 1:40 dilution of normal mouse serum and S. aureus protein A (5 mg/ml). The S. aureus protein A was removed by centrifugation, and the supernatants were then utilized in immunoprecipitation reactions. Each immunoprecipitation assay consisted of 100 µl of cell lysate and 100 µl of diluted asctic fluid (1:100 in PBS) incubated for 1 h on ice. Immunoprecipitates were collected by the addition of S. aureus protein A (5 mg) and a further incubation for 1 h on ice. The S. aureus protein A with bound antigen-antibody complexes was then washed five times in 0.1 M Tris-hydrochloride, 0.5 M LiCl, and 1.0% β-mercaptoethanol (pH 8.0) (20), and the immune complexes were solubilized in sample buffer (0.05 M Tris-hydrochloride, 2.0% SDS, 5% β-mercaptoethanol, and 0.005% bromophenol blue (pH 7.0)) at 100°C for 2 min and then analyzed by SDS-polyacrylamide gel electrophoresis in 9% slab gels (3, 6, 12). Autoradiography of the dried gels was enhanced by fluorography with Enhance (New England Nuclear Corp.) (1, 14).

**Western blot analysis of antibodies.** Anti-CMV antibodies were analyzed by reaction with CMV proteins that were transferred to nitrocellulose sheets by the technique of Western blotting (2). Briefly, HEF cells infected with CMV AD169 at an MOI of 1 were harvested 72 h postinfection. Cell lysates, prepared as described above, were solubilized in sample buffer and subjected to SDS-polyacrylamide gel electrophoresis in 9% slab gels. Proteins in the gel were then transferred by electrophoresis to a nitrocellulose sheet in a Canalco gel destainer at 6 to 8 V/cm for 16 to 22 h. After the electrophoretic transfer, the sheet was immersed in a solution containing 0.9% NaCl, 5.0% BSA, and 0.01 M Tris-hydrochloride (pH 7.4) (Tris-saline) and incubated at 40°C for 90 min on a rocking platform. The sheet was then transferred to a fresh solution of Tris-saline containing a 1:100 dilution of asctic fluid and incubated for an additional 90 min at room temperature on a rocking platform. The nitrocellulose was then washed by rocking for 10 min in a fresh solution of Tris-saline without BSA, for two additional 20-min incubation periods in two changes of Tris-saline containing 0.05% Nonidet P-40, and once again for 10 min in Tris-saline alone. The sheet was then immersed in fresh Tris-saline with 5% BSA containing 2 × 10^5 to 5 × 10^6 cpm of IPA per ml. Binding of IPA to antigen-antibody complexes was allowed to occur for 90 min with rocking at room temperature. The radioactive solution was then aspirated, and the nitrocellulose sheet was again washed according to the procedure described above. The sheet was then briefly blotted with paper towels, wrapped in plastic wrap, and developed by autoradiography on Kodak XR-2 film with an X-ray intensifying screen at −70°C.

**IF assays.** HEF cells in DME containing 10% FCS were seeded (40,000 cells in 50 µl) onto the wells of sterile Hendley-Essex 1F slides (C. A. Hendley [Essex] Ltd., Leighton, Essex, England) and incubated overnight. After attachment, the cells were infected with CMV strain AD169, Davis, or Towne or isolates from 15 patients with CMV infections. When 50% of the cells showed cytopathic effects, the slides were washed in PBS, fixed in absolute methanol for 15 min, and air dried. In addition, cells on Hendley-Essex slides were infected with cell-free CMV AD169 at an MOI of 1 and fixed at various times postinfection (2, 4, 8, 12, 24, 48, 72, and 108 h). For IF assays, 20 µl of diluted asctic fluid was applied to each well of the slide, incubated for 60 min at 37°C, and washed three times by immersion of the slide in PBS. A 20-µl amount of 1:20 diluted fluorescein-conjugated goat anti-mouse immunoglobulin (Liton-Bionetics) was then applied to each well and incubated for 60 min at 37°C. The slides were washed three times in PBS, and once in distilled water and then counterstained by 1 min of incubation in 0.02% Evans blue. Cover slips were mounted with a solution containing equal parts of glycerol and PBS, and slides were read with a Zeiss Optikon fluorescence microscope.

**Assays on lung tissues.** Lung biopsy and autopsy tissues were obtained from narrow transplant recipients at the Fred Hutchinson Cancer Research Center (11a). These included 5 biopsy specimens from patients undergoing open lung biopsy for early diagnosis of interstitial pneumonia, 12 autopsy specimens from patients with CMV pneumonia diagnosed by culture and histology, and 2 autopsy specimens from patients with idiopathic pneumonia. Controls included five normal lung specimens from victims of fatal traffic accidents.

For virus culture, lung tissues that had been frozen at −70°C were homogenized at 10% (wt/vol), and the resultant cell suspensions were inoculated in serial 10-fold dilutions onto monolayer cultures of human foreskin fibroblasts. Cultures were examined microscopically for 6 weeks and scored for the presence of typical CMV cytopathic effects. Lung tissues from the same patients were also fixed in Millonig buffered Formalin and stained by standard histological techniques (11a). Sections were viewed microscopically, and 100 fields at 240× magnification were evaluated for the presence of characteristic intranuclear CMV inclusions. Some cells displayed both intranuclear and intracytoplasmic inclusions. In addition, a third piece of each lung specimen was frozen in Optimal Cutting Temperature Compound (Miles Laboratories, Inc., Elkhart, Ind.), and 5- to 8-µm sections were cut and applied to slides. The slides were then fixed in methanol for 15 min, air dried, and utilized in IF assays.

**RESULTS**

**Isolation of hybrid cell lines producing monoclonal anti-CMV antibodies.** In AB assays, culture fluids from four wells (1% of total) showed selective reactivity for CMV-infected HEF cells. Of these, two hybrids, designated 6-E3 and 6-C5, established phenotypically stable lines. Cells from these lines were inoculated into Pristane-primed BALB/c mice to produce hybridomas.

Asctic fluids from mice bearing hybridomas were tested for specificity to CMV by AB assays against a panel of herpesvirus-infected cells. For this purpose, HEF cells were infected with one of three different laboratory strains of CMV (AD169, Towne, or Davis), a patient isolate...
The results of this test are shown in Fig. 1. Both monoclonal antibodies, 6-E3 and 6-C5, reacted to high titer (8,000 to 256,000) with all four of the CMV-infected cells. The same antibodies did not react with uninfected HEF or HEF infected with HSV-1, HSV-2, or VZV. Although each of the monoclonal antibodies reacted with an antigen common to all four strains of CMV, the strength of reaction varied among the different strains. Explanations for this variation in strength of reaction could include (i) qualitative differences in the avidities of the antibodies for slightly variant antigenic determinants in the viruses or (ii) quantitative differences in the antigenic content of each of the virus-infected cell preparations. Since efforts were not made to determine which of the factors was involved, this question remained unresolved.

Identification of viral proteins detected by monoclonal anti-CMV antibodies. Representative reactions of each of the monoclonal antibodies in an immunoprecipitation assay are shown in Fig. 2A. Monoclonal antibody 6-E3 precipitated a single protein of 72,000 daltons from the lysate of CMV-infected cells. This precipitation reaction was considered specific, since normal mouse serum failed to react with any proteins in the same cell lysate. Further evidence of specificity was observed in parallel tests (data not shown) in which antibody 6-E3 did not react with any proteins in [35S]methionine-labeled lysates of HEF cells infected with either HSV-1 or HSV-2. In these and in other immunoprecipitation assays, antibody 6-C5 consistently failed to precipitate proteins from the detergent lysate of CMV-infected cells. Consequently, another form of immunosassay was utilized to detect the homolog antigen for the 6-C5 antibody. As shown in Fig. 2B, the Western blot technique proved to be successful. In such assays, the 6-C5 antibody was found to react with a single protein of 80,000 daltons.

If assays on CMV-infected cells in culture. Monoclonal antibodies 6-E3 and 6-C5 were ex-
monoclonal antibodies to cytomegalovirus

amined by indirect IF assays for reactivity against CMV-infected HEF cells grown in tissue culture on microwell slides. Characteristic IF patterns observed with infected cells are shown in Fig. 3. Antibody 6-E3 reacted with an antigen that was restricted to the nucleus (Fig. 3B). Antibody 6-C5 reacted with an antigen that was distributed in the cytoplasm with a speckled and "donut" pattern and was suggestive of localization in intracytoplasmic inclusion bodies (Fig. 3C). Uninfected HEF cells were not stained with either antibody 6-E3 (Fig. 3A) or antibody 6-C5 (data not shown).

To characterize further the homolog antigens of antibodies 6-E3 and 6-C5, HEF cells infected with CMV were fixed in methanol at 2, 4, 8, 12, 24, 48, 72, and 108 h postinfection and then tested for the presence of antigens by IF assays. Nuclear staining with antibody 6-E3 was detected as early as 2 h postinfection; the restricted presence of this antigen in the nucleus remained throughout the 108-h incubation period after infection. IF staining with 6-E3 was therefore detected before the characteristic cytopathic effect, which appeared 24 to 48 h postinfection. In contrast, the cytoplasmic antigen detected by antibody 6-C5 was not detected until 48 h postinfection; the restricted presence of this antigen in cytoplasmic inclusion bodies remained throughout the 108-h incubation period after infection. Thus, it was concluded that 6-E3 antibody reacted with a protein that appeared early in infection, whereas 6-C5 antibody reacted with a protein that appeared late in infection.

**IF assays with culture isolates of CMV.** To test for utility in identification of viral isolates in tissue culture, antibodies 6-E3 and 6-C5 were tested for reactivity against 15 fresh isolates of CMV from marrow transplant patients. Both 6-E3 and 6-C5 stained HEF cells infected with all 15 strains of CMV, demonstrating the presence of common antigens in these virus-infected cells. In each case, the pattern of fluorescence was characteristic of that observed with the antibodies on laboratory strains of CMV (i.e., nuclear staining with 6-E3 and cytoplasmic staining with 6-C5).

On this basis, the two monoclonal antibodies appeared to be useful for the detection of virus in tissue culture. Since antibody 6-E3 reacted with a CMV-specific antigen that appeared early in infection (before the cytopathic effect), it should be useful for the rapid identification of CMV in cells inoculated with clinical specimens in tissue culture.

**IF assays on frozen sections of lung tissues.** Detection of CMV antigens in primary clinical isolates was performed on lung tissues obtained from marrow transplant recipients. For control purposes, lung tissues obtained from traffic accident victims were tested in a similar manner. Preliminary tests performed with lung tissues from several patients demonstrated that the two monoclonal antibodies differed in their activities, with only antibody 6-C5 showing appreciable detection of CMV-infected cells. Antibody E3 did not react with infected cells in the lung tissue tested. Whether this was due to the denaturation of the antigen during tissue processing or to the lack of expression of the antigen in infected lung tissue was not determined.

As a general observation, antibody 6-C5 stained a relatively small number of morphologically enlarged cells that were scattered throughout the specimen. In a typical tissue section (0.5 by 1.0 cm), approximately 3 to 20 antigen-positive cells were observed. These were distributed in the lung as either individual cells or in small clusters of less than five cells each. In one specimen, the expression of CMV was observed in larger numbers of cells (hundreds per section) consisting of cells of mixed morphology. In direct comparison, identification of CMV-infected cells by IF reactions with 6-C5 antibody was far more striking than identification by histological methods.

Representative IF reactions with antibody 6-C5 on frozen sections of lung tissue from a patient with culture-positive CMV pneumonia are shown in Fig. 4. In Fig. 4A, a lung section is shown at low magnification (×250). The sensitivity of the antibody reaction, coupled with the low background of the assay, allowed the detection of individual antigen-positive cells within the normal cellular architecture of the lung. Fig. 4B shows a high-magnification (×400) field of antigen-positive cells in a lung alveolus. The cells that stained with antibody 6-C5 in this field displayed the same speckled pattern that was observed in culture-infected cells, suggestive of intracytoplasmic inclusion bodies. The specificity of these reactions was assured by the findings that (i) 6-C5 antibody did not react with cells in the lung tissues of normal individuals (five autopsy samples obtained from fatal traffic accident victims), and (ii) lung tissues from patients with CMV pneumonia did not show nonspecific IF reactions when tested in parallel with normal mouse serum or a monoclonal antibody (3-G11) against HSV-1.

**Comparison of culture, histology, and IF assays in the detection of CMV infection in lung tissues.** Twenty-four different lung specimens were examined by three independent criteria for the presence of CMV. These included virus culture, histology for the detection of CMV-associated nuclear and cytoplasmic inclusion bodies, and IF assays with 6-C5 antibody for the detection of CMV antigens. Due to the retrospective nature of the study, each of the tests was performed at
different times on separate sections of the lung tissue of a single patient. Preliminary results showed a clear association between all of the parameters that were used to identify CMV infection. A summary of this analysis is presented in Table 1.

In 15 of the lung specimens obtained from bone marrow transplant recipients, CMV was detected by culture. The virus titers in these specimens varied from $10^4$ to $10^6$, with three of the specimens requiring blind passage of infected cells before characteristic CMV-induced cytopathic effects were observed. A general concordance was observed between the recovery of CMV in culture and the presence of CMV-associated inclusion bodies in histological specimens. In five lung specimens from presumably healthy donors (obtained from traffic accident fatalities), neither infectious CMV or CMV-associated inclusion bodies were observed.

The results of IF assays performed with 6-C5 antibody were also in accord with the culture methods. Of the five lung biopsy specimens from pneumonia patients, three contained CMV by culture. Two of these specimens had high titers of virus ($>10^6$), and one of the specimens required blind passage of the culture cells before infectious CMV was detected. In IF assays with frozen sections derived from these specimens, the two samples which contained high titers of virus by culture were found to contain numerous cells ($>20$ per section) that stained with antibody 6-C5. The single specimen which required blind passage to reveal infectious virus did not

FIG. 3. IF reactions with monoclonal antibodies 6-E3 and 6-C5. Uninfected and CMV-infected HEF cells were grown overnight in the wells of Hendley-Essex slides, fixed with methanol, and tested in IF assays with monoclonal antibodies. (A) IF reaction of uninfected HEF cells with antibody 6-E3 (1:1,000 dilution). (B) IF reaction of CMV-infected HEF cells with 6-E3 ascitic fluid (1:1,000 dilution). (C) IF reaction of CMV-infected HEF cells with 6-C5 ascitic fluid (1:1,000 dilution). Under the fluorescence microscope, all IF reactions appeared apple-green in color; depending on the exposure time or use of filters, the photographic reproductions demonstrated a yellow to blue-green fluorescence.

FIG. 4. IF reactions of monoclonal antibody 6-C5 on lung tissues. Frozen sections of lung tissue from a patient with culture-positive CMV pneumonia were fixed in methanol and stained in IF assays with 6-C5 ascitic fluid (1:500 dilution). (A) A lung section at $\times 240$. (B) A lung section showing an alveolus at $\times 400$. Under the fluorescence microscope, all IF reactions appeared apple-green in color; depending on the exposure time or use of filters, the photographic reproductions demonstrated a yellow to blue-green fluorescence.
TABLE 1. Comparative detection of CMV infection in lung tissues by culture, histology, and IF methodsa

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<td>CMV pneumonia</td>
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a Separate pieces of lung tissue from 24 specimens were (i) used to inoculate monolayers of human foreskin fibroblasts for the detection of cytopathic effect caused by infectious CMV, (ii) fixed in buffered Formalin and used in standard histological preparations to detect the presence of intranuclear and intracytoplasmic inclusions, and (iii) frozen in Optimal Cutting Temperature Compound with sections fixed on slides and stained by IF assay with antibody 6-C5. The diagnosis of CMV pneumonia required both positive culture for CMV from lung tissue and the presence of inclusion bodies by histology. The "normal" lung specimens were obtained from traffic accident victims.

b Of the 15 virus-containing specimens, 3 had low titers of virus, requiring blind serial passage of infected cells in vitro for the identification of cytopathic effect. These same specimens were scored negative by IF assay and histology, although further examination of numerous tissue sections revealed low numbers of inclusion-positive cells by histological analysis.

contain a sufficient number of antigen-positive cells to yield a positive diagnosis. The failure to detect antigen-positive cells in this lung specimen, however, appeared to be a result of quantitative effects, since IF assays performed with cells infected in vitro with virus derived from this specimen (obtained by blind passage) showed the characteristic staining patterns with antibodies 6-E3 and 6-C5.

These findings were further substantiated by histological analysis. As in the IF assays, the two specimens that showed high titers of virus in culture could be readily identified as having characteristic CMV inclusions, whereas the specimen with a low titer of virus was initially scored as inclusion negative. Upon detailed examination of this specimen, inclusion bodies, although in low numbers, were then found.

Examination of 19 other lung specimens (obtained at autopsy) showed a similar concordance among the results of culture, histological, and serological methods. Of the specimens, 9 failed to show the presence of CMV by any of the three methods, and of the 12 specimens from which CMV was cultured, 10 contained cells that were stained by the 6-C5 monoclonal antibody. As observed above, the culture-positive specimens which did not react with 6-C5 antibody contained low titers of infectious virus. These same specimens were initially classified as inclusion negative by histology, which, upon further detailed analysis, revealed low numbers of cells with characteristic inclusion bodies. IF assays performed with cells infected in vitro with virus derived from these specimens (obtained by blind passage) demonstrated the characteristic patterns of staining with antibodies 6-E3 and 6-C5.

DISCUSSION

We describe here the production of two monoclonal antibodies that react specifically with CMV-infected cells. Hybrid cells producing these antibodies were selected by the use of a replicate plating technique for the screening of large numbers of culture fluids against a panel of herpesvirus-infected cells. In this manner, it was possible to rapidly obtain information concerning the immunological specificities of each of the antibodies that were produced by several hundred different hybrid clones in culture.

Monoclonal antibodies selected for specific binding to CMV-infected cells were further characterized by immunoprecipitation analysis. One of the antibodies, 6-E3, efficiently precipitated a protein of 72,000 daltons from detergent lysates of [35S]methionine-labeled CMV-infected cells. The second antibody, 6-C5, did not demonstrate precipitating activity with the same cell lysates. This failure to detect precipitation with 6-C5 was not surprising, however, since our previous experience indicated that monoclonal antibodies varied in an unpredictable manner in their ability to precipitate proteins from detergent-solubilized cell lysates. The reasons for certain monoclonal antibodies having low precipitating activity could include the following: (i) antigenic determinants on the viral proteins were denatured by the solubilization procedure; (ii) the physical location of certain determinants in the viral proteins conferred steric constraints on the for-
mation of precipitates; and (iii) the reactions of antibodies of low avidity were not detectable by the precipitation methods that utilized vigorous washing procedures. Which of these factors was involved in the low precipitating activity of 6-C5 was not determined. Instead, after several efforts at modifying the precipitation procedure without success, we turned to a completely different form of immunoassay. In this case, separation of the viral proteins on SDS gels and subsequent use of the Western blot technique proved to be successful. By this means, it was demonstrated that 6-C5 antibody reacted with a single polypeptide of 80,000 daltons.

Further information concerning the homolog antigens of the monoclonal antibodies was obtained by IF tests. Monoclonal antibody 6-E3 reacted with a protein that appeared early after infection (within 2 h) and which remained localized to the cell nucleus throughout the infectious cycle. In contrast, monoclonal antibody 6-C5 reacted with a protein that appeared to localize in intracytoplasmic inclusion bodies. Identification by IF staining with 6-C5 antibody was far more striking than identification with conventional histological methods.

It should be noted that our ability to detect CMV antigens by IF in cells infected in culture confirmed the studies of others (13) which were performed with polyvalent human sera. There is, however, a distinct advantage to utilizing monoclonal antibodies for this purpose. Due to the unique specificity of the monoclonal reagents, it is possible to eliminate the problem of false positives which occur as a result of antibodies in human sera that are directed against viruses other than CMV. Furthermore, since murine immunoglobulins have a low avidity for the Fc receptor that is induced by CMV infection, there is the fortuitous elimination of another factor in the nonspecific IF staining that is observed with both human and rabbit polyvalent sera.

Antigenic heterogeneity among strains of CMV has been well documented (16, 17, 19). To develop monoclonal antibodies useful for diagnosis, it was necessary to select antibodies that cross-react with multiple strains of CMV. Antibodies 6-E3 and 6-C5 appear to satisfy this requirement, since both antibodies were found to react with each of the 3 common laboratory strains and 15 different patient isolates in culture. The detection within 2 to 3 h of CMV antigen in cells infected in culture (before the appearance of cytopathic effects) suggests that antibody 6-E3 will be of particular use for the rapid diagnosis of isolates in vitro.

In preliminary studies, we also detected CMV antigens in lung tissues from patients with culture-positive CMV pneumonia after marrow transplantation. The incidence of interstitial nonbacterial pneumonia after allogeneic bone marrow transplantation for hematological malignancy approaches 50%, with 68% mortality overall resulting from respiratory insufficiency (Meyers et al., in press). Nearly one-half of the patients dying of interstitial pneumonia have unambiguous evidence of disseminated CMV, with characteristic viral inclusions on histological examination of the lung, as well as virus isolation in tissue culture.

In 12 different specimens, monoclonal antibody 6-C5 stained antigen-positive cells which were scattered throughout the lung sections. Each of these specimens also demonstrated the unequivocal evidence of CMV by culture and histological methods. In most sections, only a small number (5 to 20) of individual, morphologically large cells were antigen positive. Each of these sections showed the characteristic speckled and donut pattern of cytoplasmic antigen that was also observed with virus-infected cells in vitro. These staining patterns with antibody 6-C5 indicate that the lung cells which express active CMV infection are few in number and are diffusely scattered. Considering the severity of CMV pneumonia, it was surprising that active CMV infection was detectable in only a few scattered cells throughout the lung. It is possible that the severity of the pneumonia in marrow transplant patients may reflect a component of irradiation damage as well as viral infection.

With further study, antibody 6-C5 should prove to be of considerable utility in understanding the unique pathogenesis of this pneumonia.

In a related study, lung tissues from bone marrow transplant patients have also been tested for the presence of CMV-specific RNA by the technique of in situ hybridization (E. Medeiros and J. McDougall, manuscript in preparation). With each of the lung specimens presented in Table 1, the results of in situ hybridization and IF assays with antibody 6-C5 were in concordance. In particular, tissue sections from one patient were initially stained with antibody 6-C5 and then later processed for in situ hybridization. In this experiment, there was complete concordance in the results of both methods; cells stained by monoclonal antibody 6-C5 also showed the accumulation of autoradiographic grains in situ hybridization.

In conclusion, we have produced two monoclonal antibodies that can rapidly and specifically diagnose clinical isolates in tissue culture. In addition, one antibody, 6-C5, has been used to directly diagnose CMV infection on primary clinical specimens in lung tissue. A prospective study comparing conventional virological assays and IF assays for the diagnosis of CMV pneumonia is in progress. These studies should allow the
determination of the sensitivity, specificity, and predictive value of the diagnostic IF assay.

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LITERATURE CITED