Quantitation of Cytomegalovirus DNA and Characterization of Viral Gene Expression in Bronchoalveolar Cells of Infected Patients with and without Pneumonitis

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Cytomegalovirus (CMV) is often present in bronchoalveolar lavage (BAL) fluid of immunosuppressed patients without CMV pneumonitis. The amount of viral DNA within BAL cells of patients with definite CMV pneumonitis and of viral shedders was quantitated by polymerase chain reaction (PCR) and the extent of CMV gene expression within BAL cells was defined by reverse transcription–PCR. No viral DNA was detected in 6 viral shedders, and 12 had low copy numbers (mean, 72 copies/10^5 BAL cells; median, 20) compared with numbers in pneumonitis patients (267,580 and 57,000, respectively). When CMV intranuclear inclusions were absent within BAL cells of patients with pneumonitis, copy numbers (mean, 9362; median, 7110) were still significantly higher than among shedders. Expression of viral glycoprotein H mRNA was detected in BAL cells of all 11 pneumonitis patients tested but in 0 of 18 viral shedders. Thus, high-grade infection and viral replication within BAL cells are integral features of CMV pneumonitis but not viral shedding.

Cytomegalovirus (CMV) is a significant cause of morbidity and mortality in immunocompromised patients, and pneumonia is the most common manifestation of CMV visceral organ disease in solid organ and bone marrow transplant recipients [1–6]. The virus can be readily detected in bronchoalveolar lavage (BAL) fluid of patients with CMV pneumonia by conventional tissue culture techniques or by rapid shell vial centrifugation assays using monoclonal antibodies [7–10]. However, the virus is also frequently recovered from BAL fluid of immunosuppressed patients without pneumonia or with pneumonia caused by other pathogens [11–17]. For example, the presence of infectious CMV in BAL fluids of AIDS patients with pneumonitis does not appear to correlate with the presence or absence of CMV interstitial pneumonitis [18–21]. Similarly, lung transplant recipients frequently shed CMV in BAL fluid in the absence of pneumonitis [17]. In marrow transplant recipients, CMV is also frequently present in BAL fluid in patients without pneumonitis who undergo BAL for research purposes. In this context, however, there is a risk of ~70% that the patient will eventually develop CMV interstitial pneumonitis if antiviral therapy is not initiated [14]. Thus, the presence of CMV in BAL fluid has different significance depending on the type of host involved.

Little is known about the source of CMV detected by culture of BAL fluid of immunocompromised patients, primarily because open lung biopsies are now done only rarely. The virus could be produced by intact CMV-infected cells dislodged from within the lung by the BAL procedure, by lytically infected cells retained more deeply within the pulmonary parenchyma that release infectious virus directly into the BAL fluid, or both. In addition, recovery of CMV in culture from BAL fluid could reflect contamination during bronchoscopy by cell-free virus present in saliva.

In high-multiplicity infections of human diploid fibroblasts in vitro, CMV replication is regulated temporally with expression of three distinct classes of viral genes [22–25]. The immediate-early genes encode regulatory proteins and are transcribed within a few hours after the uncoating of virions. Subsequently, early genes, which encode primarily replicative enzymes and some structural proteins, are transcribed before viral DNA synthesis. Finally, late genes, which encode structural proteins, are transcribed after viral DNA replication. Thus, it is possible to determine whether at least some cells are partially or fully permissive for viral replication by detection of the appropriate viral mRNAs intracellularly.

To define the molecular pathogenesis of CMV pneumonitis, we chose to study the virologic events taking place within BAL cells of immunocompromised patients who have definite pneumonitis and in the cells of those who clearly do not have CMV pneumonitis despite positive viral cultures of BAL fluid.

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These studies are based on the assumption that further characterization of the molecular expression of CMV replication within cells of these patients will provide insights relevant to a better pathogenetic understanding of the disease and, eventually, to more definitive diagnostic methods. Our initial efforts have focused on precise quantitation of viral DNA and detection of specific viral transcripts within BAL cells of CMV culture–positive immunocompromised patients with and without CMV pneumonitis.

Materials and Methods

BAL cell samples. Fifty-one BAL cell samples from 48 immunocompromised adult patients who had bronchoscopy with lavage done as described previously [26] at the University of Minnesota Hospital and Clinics were studied. None of the patients was receiving antiviral therapy at the time of entry into the study. CMV was recovered from BAL fluid of every patient except negative controls. Among 15 patients classified as having definite CMV pneumonitis as defined below, 7 (46.7%) were solid organ transplant recipients, 4 (26.7%) were allogeneic marrow recipients, 3 (20%) had AIDS, and 1 (6.7%) had systemic lupus erythematosus. Among 18 virus shedders, 11 (61.1%) were solid organ transplant recipients, 2 (13.3%) were allogeneic marrow recipients, and 5 (27.8%) had AIDS.

Samples were considered culture-positive if CMV was recovered by either the rapid shell vial culture or in standard culture by the Clinical Virology Laboratory. BAL samples were held at 4°C after lavage and prepared for study within 20 h of collection after the necessary clinical tests had been done. Cells were sedimented by centrifugation of the lavage fluid at 1500 *g* for 5 min. One tube of sedimented cells was kept at −70°C for subsequent extraction of DNA, while another was resuspended in 0.5 mL of lysing solution containing 4 *M* guanidium isothiocyanate, 1% β-mercaptoethanol, 2% sarkosyl, and 50 m*M* TRIS-HCl (pH 7.6) and kept at −70°C until extraction of RNA as described below.

Definition of CMV pneumonitis and viral shedding. Definite CMV pneumonitis was considered to be present if the patient had radiographic evidence of interstitial pneumonitis and characteristic viral intranuclear inclusions were seen on cytologic examination of cells obtained by lavage or in tissue obtained by transbronchial biopsy or at autopsy. In the absence of CMV inclusions, the patient was classified as having probable CMV pneumonitis if clinical and radiographic findings were consistent, CMV was recovered from BAL fluid, no other pathogen or pathologic process was identified, and the patient subsequently responded to specific antiviral therapy. Patients were considered to be asymptomatic viral shedders when CMV BAL cultures were positive in the absence of clinical or radiographic findings of interstitial pneumonitis. Finally, immunocompromised CMV-seronegative subjects and CMV-seropositive subjects without CMV in BAL cells served as controls.

Extraction of cellular and viral nucleic acids from BAL cells. For extraction of DNA [5, 6], BAL cells were resuspended in 100 μL of lysis buffer (10 m*M* TRIS, pH 8; 25 m*M* EDTA; 100 m*M* NaCl; 0.5% SDS) and incubated with proteinase K (0.1 mg/mL) at 56°C for a minimum of 2 h. DNA was then obtained by standard phenol-chloroform extraction followed by ethanol precipitation at −20°C. The amount of cellular DNA contained in cells of each BAL sample was determined by spectrophotometry.

To extract RNA, BAL cells were lysed in 0.5 mL of solution containing 4 *M* guanidium isothiocyanate, 0.1 *M* 2-mercaptoethanol, and 0.5% sarcosyl in 25 m*M* sodium acetate buffer (pH 7.0). An aliquot of 0.25 mL was removed for analysis and the remainder of the sample was stored at −70°C. Glycogen (2 μg; Boehringer-Mannheim, Indianapolis) was added as carrier, and extraction of RNA was done according to the method of Chomczynski and Sacchi [27]. Total RNA (~1.5–3 μg) was precipitated from the aqueous phase by addition of an equal volume of isopropanol. To remove residual amounts of contaminating DNA, the pellet was resuspended in 0.1 mL of 40 m*M* TRIS-HCl (pH 8.0), 10 m*M* NaCl, 6 m*M* $MgCl_2$, and 0.01 m*M* dithiothreitol containing 10 U of RNase-free DNase I (Boehringer-Mannheim) and 40 U of RNasin (Promega, Madison, WI) and incubated at 37°C overnight. DNase I was inactivated by addition of 0.4 mL of the lysing solution (above), and RNA was again precipitated with isopropanol. The pellet was resuspended in 0.025 mL of 0.01 *M* dithiothreitol, and one-fourth of the sample was reserved for RNase digestion. RNasin (40 U) was added to the remaining RNA and the sample was stored at −70°C. RNase A (6 U) and RNase T1 (1 U) (both from Life Technologies GIBCO BRL, Gaithersburg, MD) were added to the reserved sample and incubated at 37°C overnight in TRIS-HCl buffer (pH 8.0) containing 1 m*M* EDTA. Enzymes were inactivated and nucleic acids precipitated as described above.

Quantitation of CMV DNA by competitive polymerase chain reaction (PCR). The quantitative-competitive (QC) PCR assay that we have used to quantitate the CMV glycoprotein H (gH) gene within cells has been described [28]. Briefly, serial dilutions of a linearized external standard plasmid (PSV-940) containing a 940-bp fragment of the CMV gH gene (position 148–1088) were amplified in the presence of 100 copies of an internal standard plasmid (PSV-848) with a 92-bp deletion in the sequence of the CMV gH gene from position 578 to 670 [29]. To generate a standard curve, serial dilutions of PSV-940 from 50,000 to 5 copies were coamplified in parallel with a fixed 100 copies of PSV-848 in the presence of 1 μg of cellular DNA obtained from BAL cells of CMV-seronegative persons. The 5′ primer A for amplification of the gH gene (table 1) was directly labeled at the 5′ end with 6-FAM amidite, a fluorescent dye containing 6-carboxy-fluorescein, using a DNA synthesizer (model 394; Applied Biosystems, Foster City, CA). Amplified DNA products were loaded on a 6% acrylamide–8 *M* urea gel for electrophoresis. When the amplified DNA fragments migrated into the laser scanning region, a photomultiplier tube detected the fluorescent light and converted it into an electrical signal. The signal was analyzed with Genescan 672 software (Applied Biosystems), which measured the size, height, and area of each specific DNA peak. For each QC-PCR assay, a standard curve was obtained by plotting the log of the ratio of amplified products (PSV-940/PSV-848) against the log of the PSV-940 copy number added to the reaction. The number of copies of the CMV gH gene within cells was then determined by interpolating the log of the ratio of amplified products (sample BAL DNA/PSV-848) from the standard curve. This QC-PCR assay reliably detects as few as 5 copies of the CMV gH gene coamplified with 100 copies of PSV-848 in the presence of 1.0 μg of cellular DNA corresponding to $\sim 1.0 \times 10^5$ BAL cells. Over the quantitative range of external standards studied (5–50,000 copies of CMV gH DNA),

Table 1. Primers and probes used to quantitate CMV DNA by quantitative-competitive polymerase chain reaction (QC-PCR) and to detect viral transcription within BAL cells.

Assay and designation	Description	Positions	Sequence (5′ to 3′)
gH QC-PCR			
A	5′ primer	512–533	GTATTCCATATGCCTCGATGTC
B	3′ primer	789–808	ATGTAGTCCCGAGGTGGTGT
C	Probe	704–728	GATCAATGGGCGGTGGCACGGTGGT
IE-1 RT-PCR			
D	5′ outer primer	23–39*	TCAGATCGCCTGGAGACG
E	3′ outer primer	1722–1751*	AGGTACAATGTAGTTCTCATACATGCTGTG
F	5′ inner primer	64–83*	ATAGAAGACACCGGGACCGA
G	3′ inner primer	1261–1280*	TCGGCCAACTCTGGAAACAG
H	Probe	1230–1249*	GGTTAACAGTCAGCTGAGTC
gH RT-PCR			
I	5′ outer primer	175–194	TCCTTCTCTCGGGTGTAAC
J	3′ outer primer	1617–1636	GAAGGCTGAAAGAAAAGA
K	5′ inner primer	203–222	CACCTGGATCACGCCGCTG
L	3′ inner primer	1575–1583	TAGGGCAAAGTCGGCGATC
M	Probe	565–584	GAACCAGGTAGATCTGACCG

NOTE. gH, glycoprotein H; IE-1, immediate-early gene 1; RT, reverse transcription.
* Per [30]; all others are per [29].

the results varied within each assay by 22% and between different assays by 40%. Samples yielding signals >50,000 copies of gH DNA were diluted and retested to fit in the linear range of the curve. To verify the specificity of the PCR products, Southern blot transfer and nonisotopic hybridization were done using probe C (table 1) labeled with digoxigenin according to the manufacturer's instructions (Genius Systems; Boehringer-Mannheim).

Detection of CMV immediate-early gene 1 (IE-1) and gH mRNAs. The IE-1 transcript of CMV was sought as an indicator of viral transcription that occurs before CMV DNA synthesis. Because the IE-1 gene contains three introns [22, 30], primers were designed to distinguish DNA from RNA on the basis of the presence or absence, respectively, of these introns. The mRNA of gH was studied as representative of the late CMV transcripts synthesized after viral DNA replication [29]. Since no introns are contained in the CMV gH gene, extensive controls were done in each experiment to readily differentiate viral genomic DNA from cDNA amplified products. Thus, each RNA sample was first digested with DNase before reverse transcription (RT)–PCR. All samples that were positive were reamplified omitting reverse transcriptase and subjected to RNase A and RNase T1 treatment to verify that the cDNA product had been derived from viral messenger RNA and was not genomic CMV DNA.

Primers and probes. With use of the sequence for CMV strain AD169 [30], primers to be used for detection of IE-1 mRNA were designed to distinguish DNA from mRNA on the basis of the presence or absence, respectively, of introns. Primer D (table 1) corresponds to bases 23–39 located in exon 1. The corresponding downstream primer, E, is the reverse complement of the base sequence 1722–1751 located in exon 4. The inner set of primers for the nested PCR included primer F, which corresponds to bases 64–83, also in exon 1, and primer G, the reverse complement of bases 1261–1280, located in exon 3. Probe H, used for hybridization studies, comprises bases 1230–1249, also located in exon 3. Oligonucleotide sequences of the primers and probe are given in table 1. The outer primers yield a product of 600 bp from mRNA, with a corresponding DNA product of 1729 bp; the inner primers yield a 257-bp product from mRNA and a corresponding product of 1217 bp from DNA.

The mRNA for CMV gH was studied as a representative of a late transcript [29]. Primer I corresponds to bases 175–194 and primer J corresponds to the reverse complement of bases 1617–1636 (table 1). The inner set of primers, K and L, correspond to bases 203–222 and the reverse complement of bases 1575–1593, respectively. The outer primers amplify a sequence of 1461 bases, while the inner primers amplify a sequence of 1390 bases. Probe M represents bases 565–584. Computer searches of GenBank were used to verify that oligonucleotide sequences were unique to CMV. All oligonucleotides were synthesized and purified by the University of Minnesota Microchemical Facility.

RT of CMV mRNA. CMV mRNA was transcribed to cDNA with 200 U of Maloney murine leukemia virus reverse transcriptase (Life Technologies) according to the manufacturer's instructions, using 2 μL of the total RNA extract as template. The 3′ primers (10 pmol) for the respective mRNA transcripts were primer E for IE-1 and primer J for gH (table 1).

Amplification of cDNA. CMV cDNA generated by RT was amplified by nested PCR [27, 31]. Each reaction contained 10 m*M* TRIS-HCl, pH 8.0, 50 m*M* potassium chloride, 1.7 m*M* magnesium chloride, 0.5 μM each appropriate primer, 200 μM each dNTP, and 2.5 U of *Thermus aquaticus* DNA polymerase (Perkin-Elmer Cetus, Foster City, CA) in a volume of 0.1 mL. Samples were overlaid with mineral oil and amplified in a DNA thermal cycler (Perkin-Elmer Cetus). For amplification of cDNA from IE-1 mRNA, the program was denaturation at 94°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 1.5 min. Using the outer primer pair, D and E, amplification was done for 25 cycles, and then 1 μL of the reaction mixture was transferred to a new

tube containing the inner primers, F and G, and amplified for 35 cycles, using the same conditions. For the outer gH primers, the program was denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min for 45 cycles, followed by a nested program of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, again for 45 cycles using 5 μL of the initial amplification mixture.

To verify that the RT-PCR procedure was working correctly, cellular β-actin mRNA was amplified using the primers described by Delfau et al. [32]. The amplification consisted of 45 cycles with denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and elongation at 72°C for 1 min. To prevent false-positive results due to carryover of amplified CMV cDNA, all sample processing, RT experiments, and PCR preparation were done in a separate room, free of amplified product. A second area was used for preparation of the nested PCR reaction. Multiple mock samples (tubes without cells) and RNA from leukocytes of CMV-seronegative volunteers were also extracted and subjected to the amplification procedures in each experiment. Aerosol barrier pipette tips were used for all steps. Other procedures described by Kwok and Higuchi [33] to prevent contamination were also used.

Analysis of amplified cDNA. A 15-μL aliquot of each sample was analyzed by electrophoresis in agarose gels and stained with ethidium bromide. The cDNA was then transferred to a nylon membrane (Duralon; Stratagene, LaJolla, CA), and Southern hybridization was done using the appropriate oligonucleotide probes (table 1). The oligonucleotide (20 pmol) was radiolabeled with [^{32}P]dCTP (3000 Ci/mol; DuPont, Newtown, CT) and terminal deoxynucleotide transferase (Life Technologies). Radiolabeled probe (~10^8 dpm) was added to each membrane. Hybridization and washing were done at 50°C for probes H and M, followed by autoradiography at −70°C for 24–72 h.

Positive control RNA. Synthetic RNA was transcribed from a pGEM plasmid containing the cDNA corresponding to the first 1730 b of the CMV IE-1 mRNA (strain AD169), using T7 RNA polymerase (Promega). The pGEM plasmid was constructed in our laboratory from a plasmid containing cDNA of the *Eco*RI J fragment of CMV, obtained from G. Miller (Children's Biomedical Research Institute, St. Paul, MN). Serial logarithmic dilutions of the mRNA in 2 μg of glycogen were prepared to assess the sensitivity of the RT-PCR assay. Total RNA extracted from a fibroblast culture infected with CMV strain AD169 and exhaustively digested with DNase I was used as a positive control for gH mRNA. Negative control (non-CMV) RNA was extracted from peripheral blood mononuclear cells of a CMV-seronegative donor.

Statistical analyses. The Mann-Whitney-Wilcoxon test was used to compare the mean CMV gH DNA copy number between groups of patients. Fisher's exact test was applied to compare the percentage of patients whose BAL cells contained CMV mRNA in groups of patients having definite, probable, or no CMV pneumonitis. All tests were two-sided and $P < .05$ was considered statistically significant. No adjustment was made for multiple testing.

Results

Quantitative analysis of the CMV gH gene. Serial dilutions of the external standard plasmid PSV-940 (5×10^0, 5×10^1, 1×10^2, 5×10^2, 1×10^3, 5×10^3, 5×10^4 copies) were coamplified with a fixed amount of the internal standard plasmid PSV-848 (1×10^2 copies) in the presence of 1 μg of cellular DNA obtained from CMV-seronegative persons to reproduce the in vivo conditions as closely as possible. Figure 1 shows an agarose gel of a typical QC-PCR experiment and the corresponding autoradiograph obtained after Southern blot transfer and nonisotopic hybridization, respectively. Figure 2 illustrates the fluorescent signals obtained after PCR amplification of DNA from BAL cells of an asymptomatic CMV shedder and a patient with cytology-proven CMV pneumonitis.

Qualitative analysis of CMV transcription. The sensitivity of the nested PCR for detection of CMV IE-1 RNA is illustrated in figure 3. By serially diluting the synthetic CMV IE-1 recombinant RNA plasmid, the lower limit of detection of the assay was ~18,000 copies using a single amplification of 40 cycles with the outer primer set and visualization in an agarose gel stained with ethidium bromide. In contrast, the nested RT-PCR assay increased the sensitivity by a factor of 1000 so that 18 copies could be reproducibly detected in the agarose gel and the corresponding Southern blot. The results of a typical experiment in which IE-1 RNA was detected in some of the BAL cell samples are shown in figure 4. A specific cDNA product of 257 bp (contrasting with the 1217-bp amplified fragment from IE-1 DNA) was present in 3 of the 8 BAL cell specimens analyzed in this experiment. Eighteen copies of the synthetic CMV IE-1 RNA positive control were readily detected in each experiment, and no signal was detected in aliquots of mock samples or from BAL cells of a CMV-seronegative person. β-actin RNA was also amplified for each CMV IE-1 RNA–negative BAL sample to verify the cellular integrity of the specimen.

Samples already examined for CMV IE-1 RNA and shown not to contain IE-1 DNA after treatment with DNase were then assayed for the presence of late gene (gH) transcripts. Detection of the specific 1390-bp CMV gH fragment from 2 BAL cell samples is shown in figure 5. Total RNA extracted from a cell culture infected with CMV for 7 days was also positive for gH RNA; in contrast, this transcript was not detected in fibroblasts infected with CMV for only 5 h. Absence of cDNA after RNase A and T1 digestion as well as after amplification without prior RT (not shown) was used to verify that the amplified product was generated from viral mRNA.

Quantitation of CMV DNA in BAL cell samples. The detection and quantitation of the CMV gH gene within BAL cells are summarized in table 2. No viral DNA was detected in BAL cells of the 14 immunocompromised control subjects with BAL cultures negative for CMV regardless of their CMV serologic status. Among the 18 asymptomatic CMV shedders, the CMV gH gene was detected in extremely low copy numbers (10–352 copies of viral DNA/10^5 BAL cells) in 12, and none was detected in 6 subjects. In striking contrast, the mean number of copies of the CMV gH gene in BAL cells of the 15 patients with definite CMV pneumonitis was 267,580 (range, 1332–

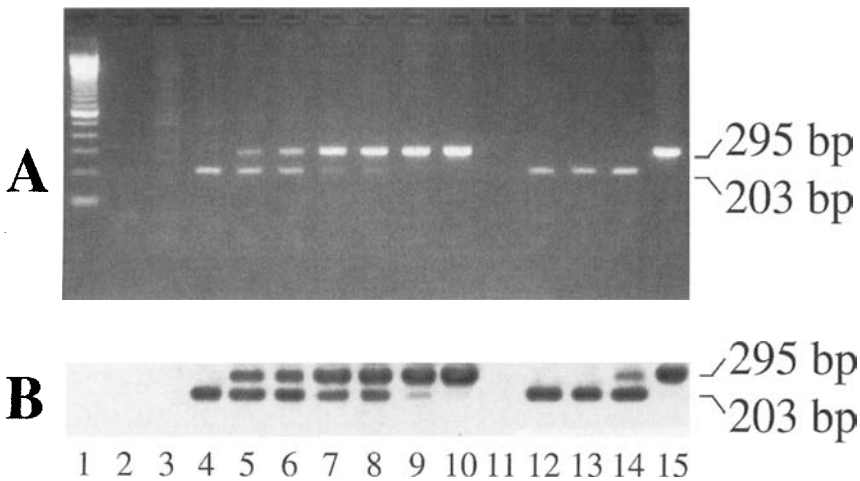

Figure 1. Quantitation of CMV glycoprotein H DNA in BAL cells of immunocompromised patients by quantitative-competitive polymerase chain reaction. **A**, Photograph of 2% agarose gel of typical experiment. Lanes: 1, molecular weight ladder; 2, blank control; 3, no amplification product from 1 μg of cellular DNA from BAL cells of CMV-seronegative subject; 4–10, 5, 50, 100, 500, 1000, 5000, and 50,000 copies of external standard plasmid (295-bp product) coamplified with 100 copies of internal standard plasmid (203-bp product) in presence of 1 μg of DNA from BAL cells of CMV-seronegative subjects; 11, blank control; 12–15, 1 μg of DNA from BAL cells of CMV-seronegative subject, BAL culture–negative CMV-seropositive subject, asymptomatic shedder, and subject with cytology-proven CMV pneumonitis, respectively, coamplified with 100 copies of internal standard plasmid. Detection of CMV DNA in BAL cells is indicated by 295-bp amplified product in lane 15. **B**, Autoradiograph of gel in **A** after Southern blot and nonisotopic hybridization using digoxigenin-labeled probe and chemiluminescent substrate.

1,596,208) ($P < .001$ vs. asymptomatic shedders). The BAL cells of all 15 patients were positive for CMV DNA. Here, 11 of 15 patients with definite CMV pneumonitis had viral inclusions within the BAL cells examined cytologically. Although it may not be surprising that these BAL samples contained large amounts of CMV DNA, it should be noted that in 6 patients only a single inclusion-bearing cell was detected in BAL cells. Four patients in whom CMV pneumonitis was diagnosed by biopsy of lung tissue or at autopsy (i.e., no CMV inclusions were detected within BAL cells) also had large amounts of viral DNA within their BAL cells (range, 3273–19,953 copies; mean, 9362; median, 7110), albeit ~40-fold less than the patients with BAL cell inclusions (range, 1332–1,596,208 copies; mean, 361,477; median, 161,674). It is also noteworthy that the 4 patients with probable CMV pneumonitis had large amounts of viral DNA in BAL cells despite the absence of inclusion-bearing cells (table 2).

CMV transcription within BAL cells. Detection of CMV IE-1 and gH mRNA within BAL cells of patients for whom a sufficient number of cells was available for RNA analysis are also summarized in table 2. As with CMV DNA, no CMV transcription was detected in BAL cells from subjects with BAL cultures negative for CMV regardless of their serologic status for the virus. The lower limit of detection for the assays of CMV mRNAs was ~18 copies/10^5 BAL cells, as previously shown. Among the asymptomatic CMV shedders, 7 of 18 BAL cell samples contained IE-1 mRNA but none contained CMV gH mRNA. In contrast, both CMV mRNA transcripts were detected in BAL cells of all 11 patients tested with definite or probable CMV pneumonitis ($P < .001$), strongly suggesting

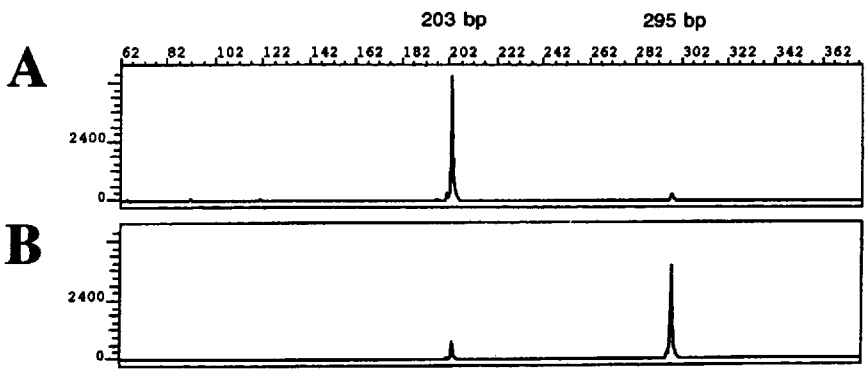

Figure 2. Genescan analysis of amplified CMV glycoprotein H (gH) DNA from BAL cells. **A**, Fluorescent amplified signals from BAL cells of lung transplant recipient with asymptomatic CMV shedding. Ratio of peak areas (CMV gH amplified 295-bp product/internal standard amplified 203-bp product) was 0.07. Log of this ratio was interpolated into experimental standard curve to determine no. of CMV gH copies/1 μg of cellular DNA: <5 copies. **B**, Signals obtained from BAL cells of bone marrow recipient with cytologically proven CMV pneumonitis. Ratio of peak areas (295 bp/203 bp) was 7.6, equivalent to 3273 copies of CMV gH gene.

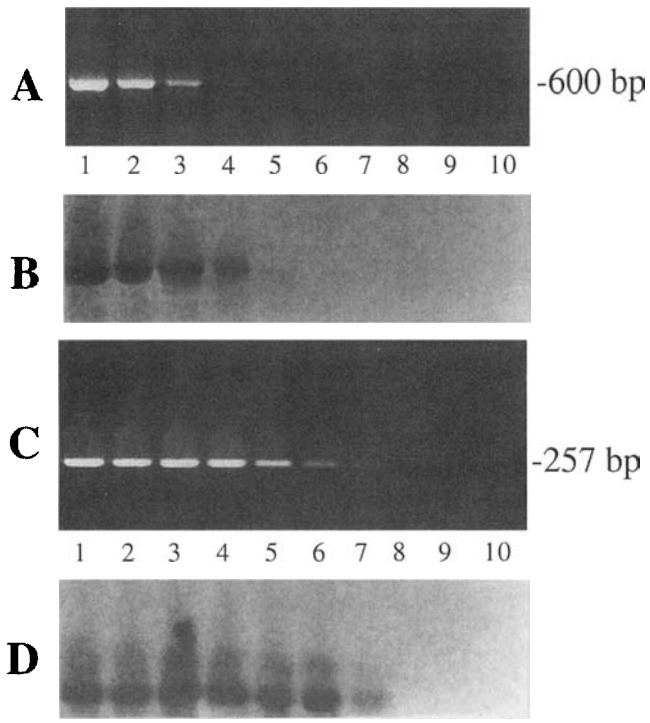

Figure 3. Sensitivity of reverse transcription–polymerase chain reaction (PCR) for detection of CMV immediate-early gene 1 (IE-1) RNA. **A**, Photograph of gel containing 600-bp product generated by 40 cycles of PCR using primers D and E (table 1). Lanes 1–9, aliquots of cDNA generated from 1.8×10^7 to 1.8×10^{-1} copies of IE-1 RNA; lane 10, control containing no RNA. Product derived from 1.8×10^4 copies of CMV IE-1 RNA is visible in lane 4. **B**, Autoradiograph of gel in **A** after Southern hybridization with specific ^{32}P-labeled CMV IE-1 probe. **C**, Photograph of gel containing 257-bp product generated by PCR using CMV IE-1 outer primers for 25 cycles, followed by nested PCR using IE-1 inner primers for 35 cycles. Product from 18 copies of CMV IE-1 RNA is visible in lane 7. **D**, Autoradiograph of gel in **C** after Southern hybridization with specific ^{32}P-labeled CMV IE-1 probe.

that CMV was replicating completely within BAL cells of these patients. Thus, detection of gH mRNA within the BAL cells as an indicator of viral replication was closely associated with the presence of high copy numbers of CMV DNA in patients with pneumonitis but not among viral shedders.

Discussion

In these experiments, we sought to determine whether there were significant differences in the virus-cell interactions occurring within BAL cells of patients with definite CMV pneumonitis and those without pneumonitis who were shedding the virus. No viral DNA was detected within BAL cells of persons with negative CMV cultures, regardless of their serologic status for the virus. Among asymptomatic shedders, no CMV DNA could be detected by the sensitive QC-PCR assay in the BAL cells of 6 of 18 subjects. The remaining 12 patients had between 10 and 352 copies of CMV DNA within 10^5 BAL cells probed. In contrast, BAL cells from patients with definite CMV pneumonitis contained a mean of 267,580 copies of viral DNA and a median of 57,000. These values are ~3700- and 2850-fold those detected in the BAL cells of asymptomatic shedders. Thus, compared with results in asymptomatic shedders, the amount of CMV DNA detected in BAL cells of patients with definite CMV pneumonitis was significantly greater by >3 orders of magnitude. However, the results are somewhat biased by the fact that the presence of CMV inclusion–bearing cells in BAL cells was one of the criteria used for diagnosis of definite CMV pneumonitis in some patients. The presence of viral inclusions in the cell nucleus is in itself evidence that CMV DNA has been replicated and that progeny virions have been produced in the cell [1]. However, it is important to note that viral inclusions were present only in a very small minority of BAL cells and that detection of a single cell with an intranuclear inclusion was sufficient for a diagnosis of CMV pneu-

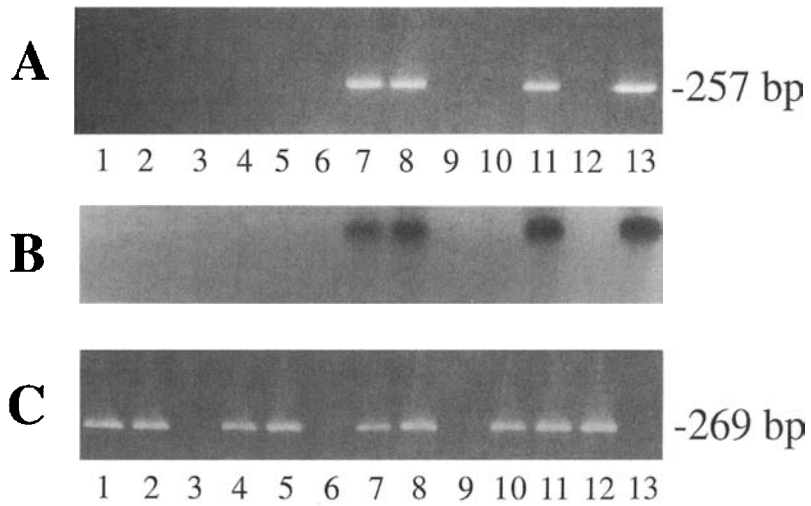

Figure 4. Detection of CMV immediate-early gene 1 (IE-1) RNA in BAL cells of immunocompromised patients. **A**, Photograph of gel from typical experiment. Lanes 1, 2, 4, 5, 7, 8, 10, 11: product from RNA extracted from BAL cells; lanes 3, 6, 9: mock samples to monitor for contamination; lane 12, RNA from BAL cells of CMV-seronegative donor (negative control); lane 13, product generated from 1.8×10 copies of synthetic CMV IE-1 RNA (positive control). **B**, Autoradiograph of gel in **A** after Southern hybridization with specific ^{32}P-labeled CMV IE-1 probe. **C**, Product of 269 bp visualized after analysis for β-actin RNA by reverse transcription–polymerase chain reaction to verify that all cell extracts contained RNA.

Table 2. Quantitation of CMV glycoprotein H (gH) gene and detection of CMV transcripts in bronchoalveolar lavage (BAL) cells of immunocompromised patients.

Category	No. of BAL samples	Presence of IE-1 mRNA	Presence of gH mRNA	gH copy no./μg of cell DNA		
				Mean	Median	Range
CMV-seronegative*	8	0/8	0/8	0	0	
CMV-seropositive only*	6	0/6	0/6	0	0	
Asymptomatic CMV shedders	18	7/18	0/18	72	20	0–352†
Definite CMV pneumonitis	15	7/7	7/7	267,580	57,000	1332–1,596,208
Probable CMV pneumonitis	4	4/4	4/4	16,128	11,992	1002–40,095

NOTE. IE-1, immediate early gene 1.
* BAL culture–negative for CMV.
† No viral DNA was detected in 6/18 samples.

monitis. More importantly, large amounts of viral DNA were also present in BAL cells of patients in whom the diagnosis of CMV pneumonitis was made by histopathologic examination of the lung in the absence of BAL cell inclusions (mean and median copy numbers of CMV DNA, 9362 and 7110, respectively). Similarly, the patients who met the criteria for a diagnosis of probable CMV pneumonitis had mean and median copy numbers of CMV DNA of 16,128, and 11,992, respectively, but did not have inclusions in their BAL cells. In the latter group, these values are ~250- to 600-fold greater than those detected in virus shedders. Thus, although the presence of a small number of CMV inclusions was clearly indicative of a very high-grade viral infection within BAL cells, the lack of inclusions in patients with definite or probable pneumonitis did not indicate a low-grade infection similar to that among asymptomatic shedders.

Other data indicate that there are important quantitative factors in the pathogenesis of CMV infection and disease, although the type of host studied may be an important variable. We have previously shown that AIDS patients and solid organ transplant recipients with CMV viremia and visceral organ disease due to CMV have significantly larger amounts of viral DNA in their neutrophils and mononuclear cells than do their counterparts with viremia who lack clinically apparent CMV visceral organ involvement [6]. Similarly, viremic patients with CMV visceral organ disease have significantly greater amounts of viral DNA in their leukocytes than viremic patients with retinitis. Among transplant recipients, invasive CMV disease is associated with relatively large numbers of infected blood leukocytes as detected by staining of viral antigens with monoclonal antibodies [34–37]. Similarly, virus titers are significantly higher in BAL fluid and cells of lung transplant recipients with biopsy-proven CMV pneumonitis than in virus shedders, although some degree of overlap was found [38]. On the other hand, neither the amount of CMV DNA in blood leukocytes nor the amount of infectious virus in BAL fluid of marrow transplant recipients correlates closely with the presence of CMV visceral organ disease or interstitial pneumonitis, respectively [6, 39].

In our studies, significant differences in the extent of viral transcription were also found in BAL cells of shedders compared with patients with definite or probable CMV pneumonitis. Among the shedders, the CMV IE-1 mRNA, which is transcribed in infected cells before viral DNA synthesis, was detected in 7 of 18. gH mRNA, which is transcribed only after viral DNA has replicated, was not detected in BAL cells of

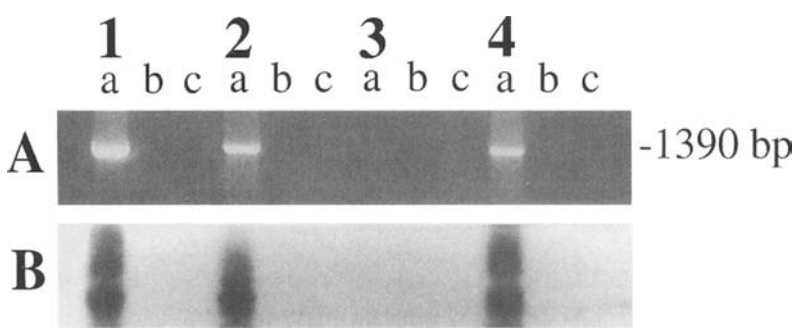

Figure 5. Detection of CMV glycoprotein H (gH) RNA in BAL cells of immunocompromised patients. **A**, Photograph of agarose gel containing products amplified from gH RNA. Samples 1 and 2, RNA extracted from BAL cells; samples 3 and 4, RNA extracted from fibroblasts infected with CMV AD169 for 5 h and 7 days, respectively. CMV gH mRNA should not be present at 5 h because it is produced late in CMV replicative cycle. For each sample: lane a, product from aliquot amplified with CMV gH outer and inner primers; lane b, product of aliquot digested with RNase A and RNase T1 before reverse transcription–nested polymerase chain reaction; lane c, negative control. **B**, Autoradiograph of gel in **A** after Southern hybridization with specific ^{32}P-labeled CMV gH probe.

any of the 18 shedders. On the other hand, both IE-1 and gH mRNA were present in BAL cells of all 11 patients with definite or probable CMV pneumonitis tested. These findings strongly suggest that replication of CMV within BAL cells is an integral pathogenetic feature of CMV pneumonitis whether or not intranuclear viral inclusions are present in the cells. CMV replication within BAL cells may also be the reason these cells contain such large amounts of viral DNA as detected by the QC-PCR assay. If this conclusion is correct, the question arises as to the source of infectious CMV detected in BAL cells of virus shedders. From our studies, no evidence was forthcoming that CMV found in BAL cells in this setting is produced and released by BAL cells themselves (i.e., little or no viral DNA was detected and no evidence of late viral transcription was found). One possibility is that CMV in BAL fluid of shedders is synthesized by lytically infected cells that do not remain intact during or after the lavage procedure or by fixed cells in the lung parenchyma that release CMV directly into the bronchoalveolar spaces. Another possible explanation is that infectious virus in BAL fluid of shedders reflects contamination during bronchoscopy by cell-free CMV present in saliva or upper respiratory tract secretions. In support of the latter hypothesis, the rate of recovery of CMV from saliva, induced sputum, and BAL fluid was comparable in one study of patients with HIV infection [20].

Our findings may also explain in part the results of Storch et al. [38] in a recently published study of BAL cells and fluid obtained from lung transplant recipients. These investigators found that CMV could be recovered frequently from both BAL cells and cell-free fluid of patients with and without biopsy evidence of CMV pneumonitis. However, recovery of the virus from cells within BAL fluid was much more closely correlated with the presence of CMV pneumonitis than was recovery of cell-free virus. In addition, virus titers from cells and supernatants were significantly higher in patients with histologically proven CMV pneumonitis.

The studies presented here have elucidated certain molecular virologic aspects of both CMV pneumonitis and virus shedding in the absence of CMV pneumonitis. In addition, there are important diagnostic implications for these findings. For example, detection of either large amounts of viral DNA or evidence of transcription of late viral genes in BAL cells may be shown to be diagnostic of CMV pneumonitis, thus identifying patients who would clearly benefit from antiviral therapy. Conversely, failure to detect these events in BAL cells might obviate the use of toxic and expensive therapeutic agents in virus shedders who would not be expected to benefit from treatment. However, studies of a much larger group of patients will be necessary to determine whether this is the case. In addition, studies of specific patient groups at risk for CMV pneumonitis will need to be done and compared because of host factors that may alter the pathogenesis of CMV disease as noted above. For example, quantitation of CMV DNA and detection of viral transcription in BAL cells may be shown to be subtly or significantly different when large numbers of marrow recipients are compared with lung transplant patients or patients with AIDS. Finally, further studies of the pathogenesis of CMV pneumonitis should be done to identify the specific cell type(s) in BAL fluid infected with CMV and to determine the percentage of cells permissive for viral replication.

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