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Identification of cytomegalovirus infection in acquired immunodeficiency syndrome

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Summary. Cytomegalovirus (CMV) infection was observed in 10 of 12 autopsy cases of acquired immunodeficiency syndrome (AIDS) and appears to be the commonest life-threatening viral infection in AIDS. In all 10 cases, adrenal glands were affected with CMV and adrenal medullary necrosis was present in 6 cases. Lungs were affected with CMV in 7 cases with disseminated infection and positive CMV culture. In situ hybridization of tissue sections with CMV-specific DNA provided positive staining for CMV in inclusions as well as other infected cells without obvious inclusions. Human diploid lung fibroblasts were infected with isolated CMV in culture, yielding positive CMV identification within 5 days by in situ hybridization before specific cytopathic changes appeared in the fibroblasts. The early and specific detection of CMV is possible by in situ hybridization with cultured fibroblasts.

Key words: Cytomegalovirus – AIDS – Adrenals – Immunohistochemical staining – In situ hybridization

Introduction

Cytomegalovirus (CMV) is ubiquitous and infects most humans at some point during their life-time (Talpers and Liu 1986). In the United States, approximately 50% of the population are reported to have positive CMV serology by the age of 35 years (Talpers and Liu 1986). An increased incidence of both primary and reactivated latent CMV infections are frequent complications of long-term immunosuppression (Masih et al. 1988). Overwhelming CMV viremia and its associated pulmonary and adrenal infections may contribute to the death of acquired immunodeficiency syndrome (AIDS) patients (Glasgow et al. 1985; Macher et al. 1983; Niedt and Schimella 1985). In autopsy cases of AIDS patients, disseminated CMV infections were reported in 77% (Niedt and Schimella 1985) to 90% (Macher et al. 1983) and CMV infections appear to be the most common devastating viral infection in AIDS (Welch et al. 1984). CMV infection is not only common in AIDS patients but frequently, observed in clinically deteriorating AIDS-related complex (Fiala et al. 1986). We have observed 10 cases of CMV infections out of 12 autopsy cases of AIDS in the Greater Kansas City area from 1985 to 1987, which were studied systematically by immunohistochemical staining and in situ hybridization with a commercially available, biotinylated, CMV-specific DNA probe. The objectives of the current study were: (1) to identify organ distribution of CMV infections in autopsy AIDS cases; (2) to compare identification of CMV infections by the immunoperoxidase method and in situ hybridization; and (3) to compare identification of CMV infections in infected human fibroblasts in culture by the immunoperoxidase method and in situ hybridization.

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Materials and methods

All 12 men in this study were homosexual and were diagnosed as AIDS by use of the clinical criteria for the diagnosis for AIDS of the Centers for Disease Control. These include secondary infectious diseases and cancers (Poon et al. 1983) and laboratory findings, including positive antibody to human immunodeficiency virus by enzyme immunoassay and Western blotting (Tomita and Chiga 1988). Among 12 cases, 9 cases had histological evidence of CMV infection in routine H&E sections. Complete autopsy was performed in 11 cases; in 1 case the brain was excluded. Ages at death ranged from 26 to 56 years with a median of 34.5 years. Autopsy reports and histological sections were reviewed in all 12 cases and CMV infections were initially diagnosed by the presence of inclusions in routine H&E sections. The diagnoses were further confirmed by immunohistochemical staining with rabbit anti-CMV (Balachandran et al. 1987) and in situ hybridization using commercially available, biotinylated human CMV-specific DNA probe (Masih et al. 1988). The hybridization cocktail (CMV Pathogene kit, EBP-872, Enzo Biochem, New York) contains a mixture of two CMV-DNA sequences of 25.2 and 17.2 kb cloned into the *Bam*3 restriction cleavage site of the plasmid pBR and biotinylated nucleotide, Bio-dUTP. The sections on the poly-L-lysine (0.1% v/

w) coated glass slides were deparaffinized and were treated with freshly prepared protease solution (0.2 mg/ml, Sigma Chemical, St. Louis, Mo.) in 50 mM Tris-HCl buffer (pH 7.4) at room temperature for 15 min. Then, a drop of this hybridization cocktail was placed on each section and covered with a coverglass. Both probe and cellular DNA were denatured together in a 95° C convection oven for 5 min. Hybridization was allowed to proceed at 37° C for 1 h. After the hybridization, the coverglass was removed and the sections were washed with post-hybridization wash solution and wash buffer. The procedure for detection of the biotinylated DNA consisted of sequential treatment with an avidin-horseradish peroxidase complex, wash buffer, Tris-HCl buffer containing diaminobenzidine tetrahydrochloride (DBA, Sigma) and hydrogen peroxide, and final rinsing with distilled water. Sections were lightly counterstained with hematoxylin and were coverslipped with Paramount.

Diploid fibroblasts from human fetal lung were cultured in flasks (15 ml) and were infected with the CMV virus, which was previously isolated and identified by cytopathic changes in the same tissue culture system (Ray et al. 1974). Before and after, CMV were identified by distinctive cytopathic effects on the fibroblasts (Ray et al. 1974). Cultured cells were washed with phosphate buffered saline (PBS), centrifuged and smears were made on poly-L-lysine coated glass slides, which were fixed with acetone and were used for routine H&E, immunohistochemical staining and in situ hybridization with CMV-DNA probe. For transmission electron microscopy, cells were centrifuged and were fixed in 2% glutaraldehyde in phosphate buffer (pH 7.4). For immunoelectron microscopy and in situ hybridization, cells were fixed in periodate-lysine-paraformaldehyde solution for 1 h (McLean and Nakane 1974). Cell blocks were obtained by centrifugation and were washed with 10%, 15% and 20% sucrose solution in PBS for 30 min each, then were frozen in solid carbon dioxide-acetone. Frozen sections (5–7 μm) were cut and placed on poly-L-lysine coated glass slides. Sections were air dried and used for immunoelectron microscopy with rabbit anti-CMV in the same way as in light microscopic immunohistochemistry. After processing with DBA and hydrogen peroxide, sections were washed and post-fixed with 1% osmium tetroxide for 20 min, then were subjected to the process of dehydration and flat embedding in Epon. For electron microscopic in situ hybridization, sections on the glass slides were treated with protease solution for 15 min at room temperature. The excess protease was removed and the tissue was washed twice in Tris-HCl buffer containing 2 mg/ml glycine to inhibit continuous digestion by protease. The sections were then processed with hybridization cocktail, followed by staining with DBA and hydrogen peroxide, post-fixation with 1% osmium tetroxide and were processed in the same way as in immunoelectron microscopy.

Results

Autopsy study

In a total of 12 AIDS cases, 9 revealed CMV infections of adrenal gland by routine H&E sections. Six of these cases contained medullary necrosis, and 3 contained inflammatory infiltrates without necrosis (Table 1). Premortem and post-mortem blood and lung culture provided positive CMV in 7 cases and negative cultures were obtained in 3 cases involving adrenal gland only, or adrenal gland and esophagus (cases 1, 3 and 4). Adrenal glands were not submitted for viral culture in this study and all 6 cases of CMV infection of lung yielded positive CMV culture (Table 1). In CMV infection of adrenal medulla, there were numerous medullary cells with intranuclear and intracytoplasmic inclusions (Fig. 1). Cortical CMV infection was observed in 1 case by in situ hybridization, in which inclusions were not observed by routine H&E sections (case 4, Fig. 2). The adrenal cortical infection was continuous from CMV infection of the medulla (cases 1, 2, 5–9). The pulmonary lesions of CMV infection were mostly interstitial and some intra-alveolar inflammation (Fig. 3). In infected lesions, numerous CMV inclusions were observed showing a granular appearance (Fig. 3). These were positively stained by in situ hybridization in the nucleus as well as in the cytoplasm. Immunohistochemical staining was relatively weak but positive in the nucleus (Fig. 3). Colonic CMV infection was observed in an ulcerated lesion where endothelium of blood vessels and fibroblasts were positive for CMV with or without characteristic inclusions (Fig. 4).

Table 1. Summary of CMV infections in AIDS patients

Case no.	Age years/sex	Organ involvement	Culture
1	26/M	Adrenals (c, mn)	− in blood
2	28/M	Adrenals (c, m), lungs	+ in lung
3	29/M	Adrenals (mn)	− in blood
4	32/M	Adrenals (c), esophagus	− in blood
5	32/M	Adrenals (c, mn), lungs, stomach, colon, pancreas, parathyroids, kidneys, brain	+ in blood
6	34/M	Adrenals (c, m), lungs, spleen, liver, lymph nodes, kidneys, brain	+ in lung
7	35/M	Adrenals (c, mn), lungs, pancreas, prostate	+ in lung
8	35/M	Adrenals (c, nm), lungs, spleen, liver	+ in lung
9	38/M	Adrenals (c, m), lungs, colon, spleen, kidneys, brain	+ in lung
10	56/M	Adrenals (mn), lungs	+ in blood, lung

c = Cortex; m = medulla without necrosis; mn = medulla with necrosis

Tissue culture study

The smear preparations of cultured human fetal fibroblasts revealed occasional large and many slighly larger nuclei (Fig. 5). Immunohistochemical staining revealed relatively weak positive staining in the nuclei (Fig. 5). In situ hybridization clearly demonstrated strongly positive staining in both the nucleus and cytoplasm as well within 5 days after CMV infection (Fig. 5). By transmission electron microscopy, numerous viral particles were observed in the disintegrated nucleus and lesser numbers in the cytoplasm (Fig. 6) whereas adjacent intact cells contained much less viral particles in the cytoplasm. Immunoelectron micrographs revealed strong positive staining in the nucleus and relatively weak staining at the membrane capsid of the intracytoplasmic viral particles (Fig. 7). In situ hybridization showed strong staining in both the nucleus and dense cores of intracytoplasmic viral particles (Fig. 8).

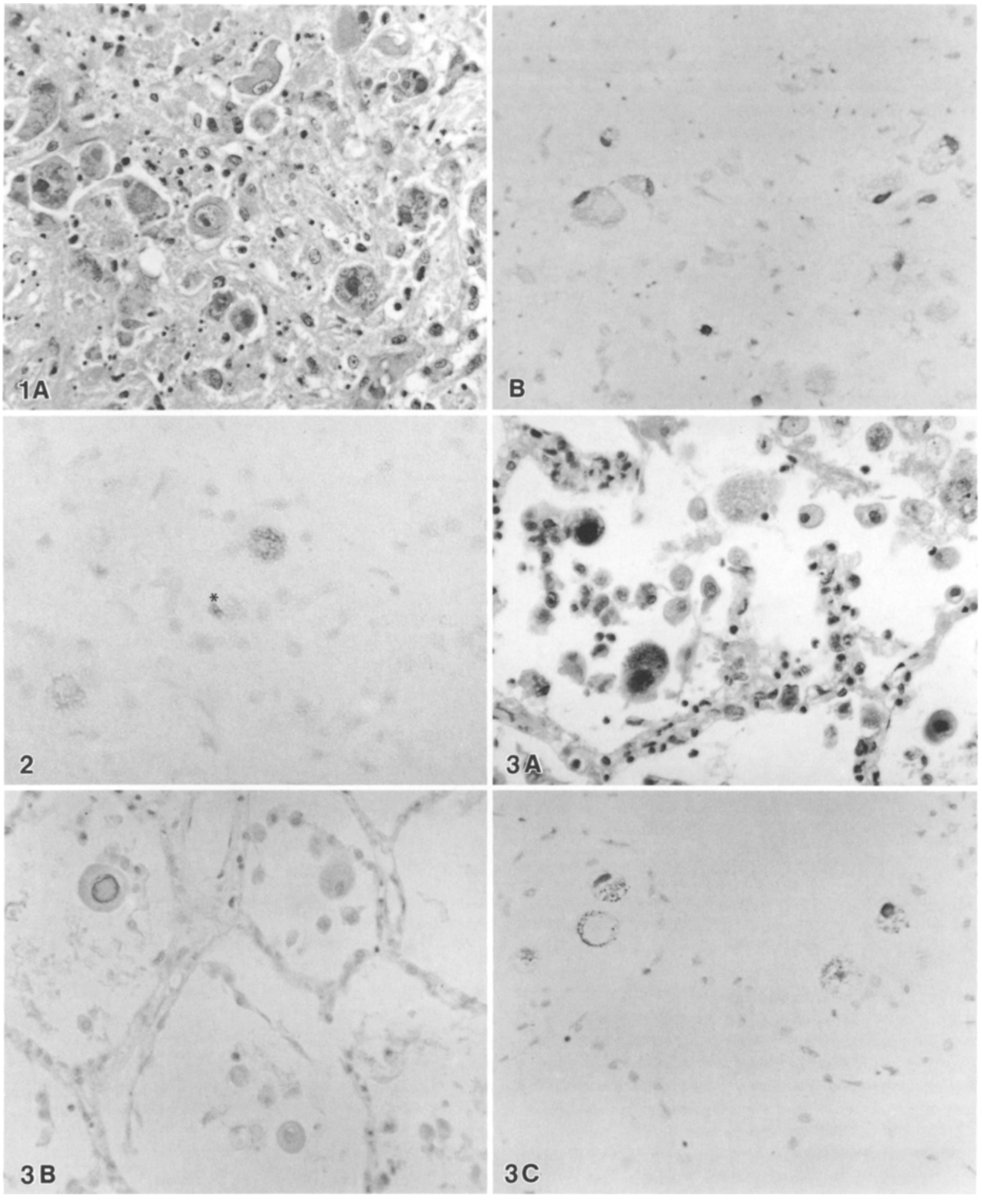
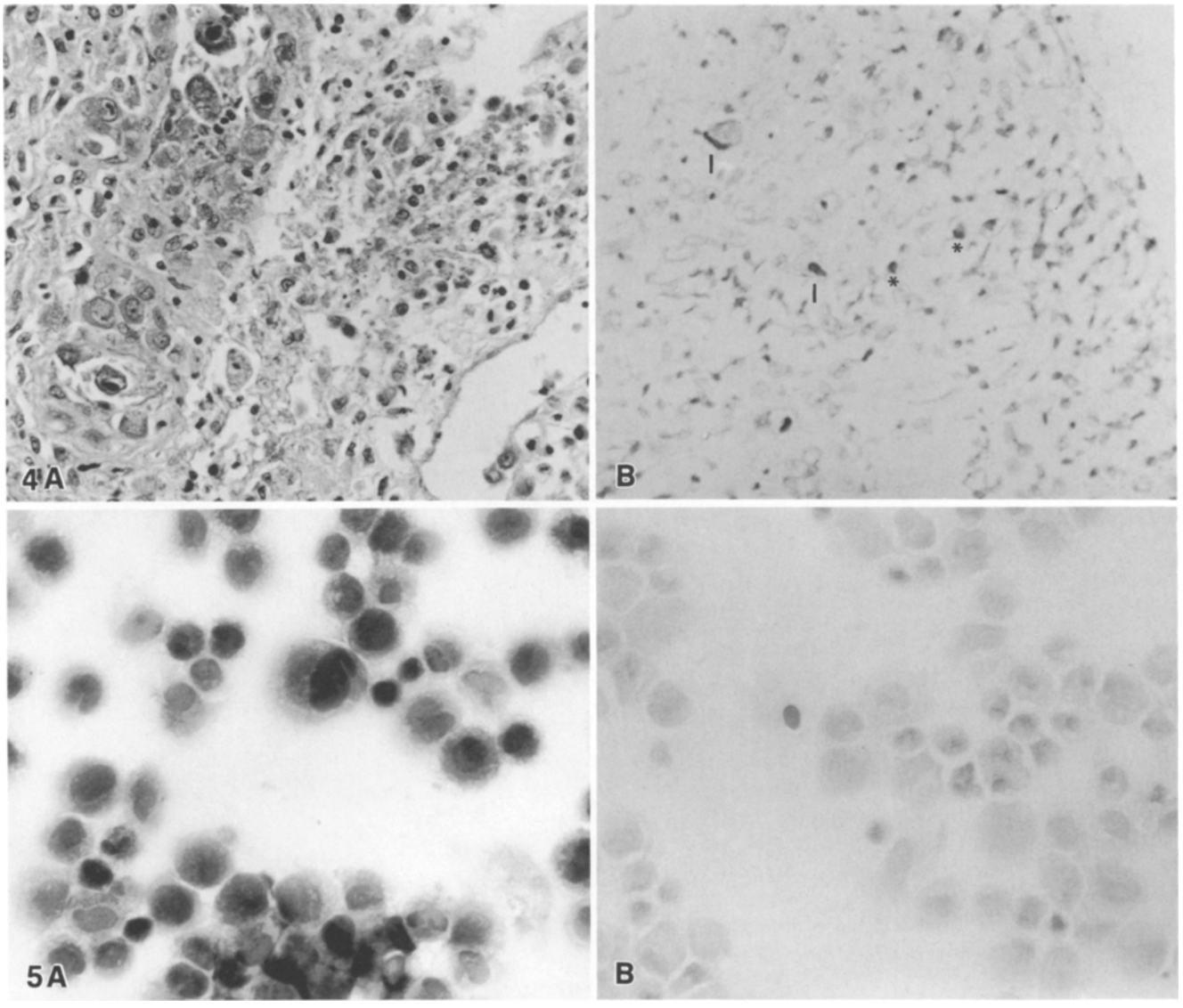


Fig. 1 A, B. Adrenal medulla, case 5.
Adrenal medulla (**A, B**) reveals numerous inclusions in the necrotic stroma containing numerous neutrophils **A** in situ hybridization depicts positive staining both in inclusions and smaller cells without inclusions. **A** H&E, ×340. **B** In situ hybridization, ×340

Fig. 2. Adrenal cortex, case 4.
In the zona fasciculata, there are three positively stained cells; two are diffusely and one is focally stained in the cytoplasm (*). In situ hybridization, ×340

Fig. 3 A–C. Lung, case 5.
Lung sections reveal interstitial and intra-alveolar infiltrates, containing exudate and inclusions (**A**). By immunohistochemistry, enlarged nuclei are positively stained (**B**), whereas by in situ hybridization, strong positive staining is present in the nucleus as well as in the cytoplasm, the latter revealing granular appearance (**C**). **A** H&E, ×340; **B** immunohistochemistry, ×340; **C** in situ hybridization, ×340



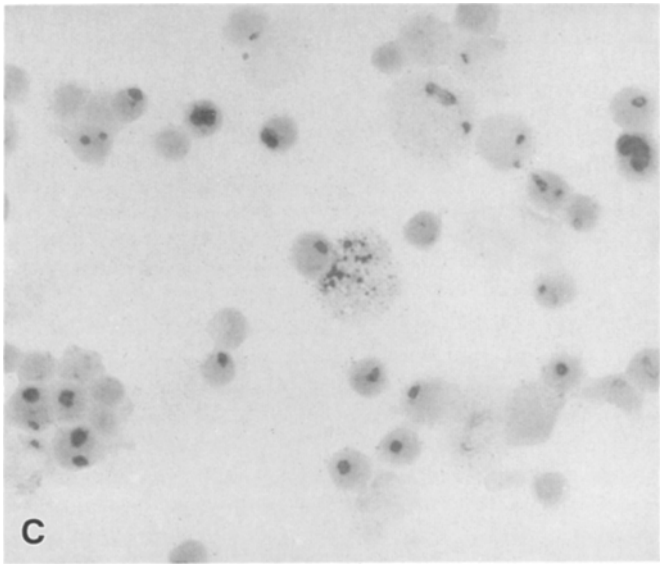


Fig. 4A, B. Colon, case 9.
In a lesion adjacent to mucosal ulceration, endothelial cells of blood vessels contain numerous inclusions (**A**). In situ hybridization reveals positively stained cells in inclusions (*I*) as well as in interstitial cells (*). **A** H&E; **B** in situ hybridization, × 340

Fig. 5A–C. Cultured fetal pulmonary fibroblasts.
At 5 days after CMV infection, there are occasional cells with large nuclei and numerous, slightly enlarged nuclei (**A**). By immunohistochemistry, many of the nuclei are weakly positive (**B**), whereas by in situ hybridization, there is obvious strong staining in nucleus and in cytoplasm (**C**). **A** H&E; **B** immunohistochemistry; **C** in situ hybridization, × 340

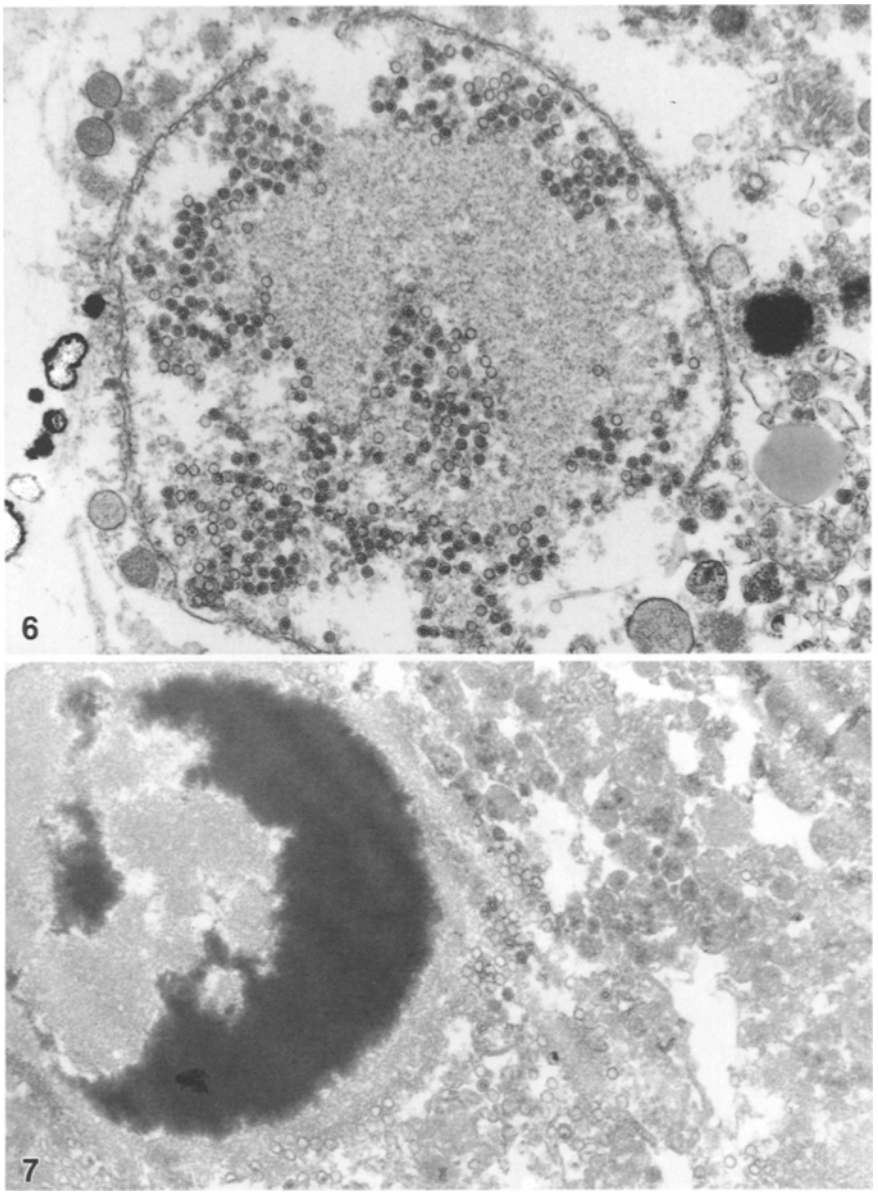


Fig. 6. Cultured fetal pulmonary fibroblasts.
Electron micrographs depict numerous viral particles with or without dense cores in the nucleus of disintegrated cells. Smaller numbers of viral particles are also present in the cytoplasm of disintegrated cells. ×24000

Fig. 7. Cultured fetal pulmonary fibroblasts. Immunoelectron micrographs depict strong positive staining in the nucleus and relatively weak positive staining at the membrane capsid of the intracytoplasmic viral particles. No uranium or lead staining. ×30000

Discussion

In 12 AIDS autopsy cases, 10 (83%) revealed CMV infection at the terminal stage and this incidence corresponds fairly well to the previously reported 77% Niedt and Schimella 1985) to 90% (Myerson et al. 1984a) in autopsied AIDS cases. Lung is a common organ for CMV infection in 7 of 10 cases, from focal to diffuse interstitial pneumonitis, containing numerous characteristic intranuclear and intracytoplasmic inclusion. The latter were best demonstrated by in situ hybridization with CMV-DNA probe. In this study, the most common organ for CMV infection was adrenal gland, which was involved in all 10 cases, 3 of them were without pulmonary involvement by CMV (Table 1). Previous AIDS autopsy studies reported necrotizing adrenalitis in 9 of 65 cases (Guarda et al. 1984; Macher et al. 1983; Tapper et al. 1984; Welch et al. 1984), whereas in this study 6 of 10 cases showed necrotizing adrenalitis (Table 1).

Glasgow et al. (1985) emphasized adrenal cortical necrosis by CMV infection associated with greater adrenal medullary necrosis. Cortical CMV infection appeared histologically to be an extension of the medullary infection, since early cortical infection was in the zona reticularis, continuous with older and larger medullary lesion of fibrosis and frequent necrosis. Viral culture of lung tissue was always positive in the cases of histologically proven CMV involvement of lung, whereas blood culture for CMV was negative in 2 cases with isolated adrenal involvement (cases 1 and 3) and in 1 case of adrenal and esophageal involvement (case 4). In these 3 cases, viral culture of the adrenal gland was always positive for CMV. Thus, adrenal gland is the best organ to look for CMV infection even when there are no apparent

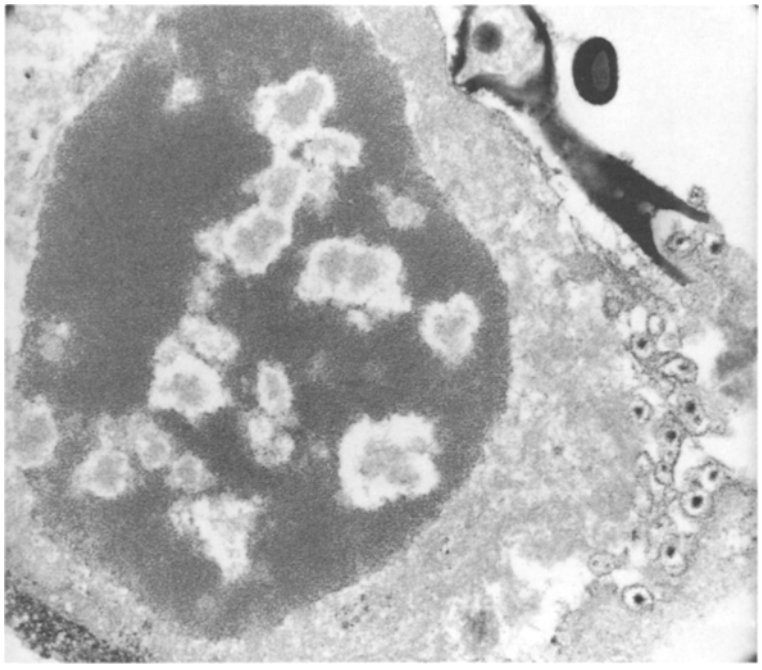


Fig. 8. Cultured fetal pulmonary fibroblasts. In situ hybridization depicts strong positive staining both in nucleus and dense cores of intracytoplasmic viral particles. No uranium or lead staining. ×48000

gross foci of medullary necrosis. Medullary CMV infection can be a small microscopic focus as seen in case 3, warranting careful cross-sectioning of the entire adrenal gland and submission of any suspicious tissue slices for microscopic examination.

In this study, microscopic examination with routine H&E sections was sufficient for practical diagnosis of CMV and immunohistochemical staining with rabbit anti-CMV did not significantly enhance the detection of CMV infection. In situ hybridization, however, was extremely sensitive and specific and disclosed not only CMV inclusions but CMV infected cells without inclusions (Fig. 5) (Löming et al. 1986). This in situ hybridization with DMV-DNA probe was recently introduced (Löming et al. 1986; Myerson et al. 1984a, b), providing an excellent sensitivity and specificity (Keh and Gerber 1988; Masih et al. 1988; Myerson et al. 1984b) with less background staining than the immunoperoxidase technique (Walker and Lloyd 1988). In situ hybridization using commercially biotinylated CMV-specific DNA probe yielded more than twice as much CMV detection than by routine microscopic detection in H&E sections (Masih et al. 1988).

In cultured fetal fibroblasts, CMV infection can be detected unequivocally in the absence of inclusions (Fig. 5). The usage of in situ hybridization can be therefore extended to the cultured cells even before specific cytopathic changes for CMV appears in the infected cells. With immunofluorescent staining, rapid detection of CMV infection had been previously reported by shell vial cultures after 18 h incubation (DeGirolami et al. 1988) and by centrifugation culture within 16 h of incubation (Crawford et al. 1988), respectively. As seen in this study, in situ hybridization may well enhance early and specific detection of CMV in cultured cells.

The rabbit anti-CMV serum used in this study reacted strongly with nucleus and only weakly with membrane capsid, similar to the one of monoclonal antibodies to CMV reported by Tsutsui et al. (1986). Electron microscopic in situ hybridization revealed strongly positive staining in the nucleus and dense cores of intracytoplasmic viral particles, which corresponded to the positive staining of the nucleus and cytoplasm in light microscopic in situ hybridization.

In situ hybridization has dual advantages to the pathologist: early and specific diagnosis of CMV infection in tissue sections, and early and specific diagnosis of CMV infection in cultured cells especially before characteristic cytopathic changes for CMV become evident (Albrecht et al. 1980).

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