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In Situ *Hybridization for Cytomegalovirus DNA in AIDS Patients**

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Infection by cytomegalovirus (CMV) is a frequent cause of morbidity and mortality in patients with acquired immune deficiency syndrome (AIDS). The authors studied the distribution of CMV in 4 patients with AIDS using a commercially available, biotin-labeled CMV DNA probe for *in situ* hybridization and immunohistochemical staining for the detection of CMV antigen in formalin-fixed paraffin-embedded tissues. The sensitivity and specificity of the hybridization procedure was demonstrated by appropriate controls. The immunohistochemical test for the detection of CMV antigen in routine histologic sections was less sensitive than the *in situ* hybridization method. CMV DNA was detected not only in cytomegalic inclusion cells, but also in nuclei and cytoplasm of histologically normal-appearing cells such as endothelial cells, pneumocytes, hepatocytes, biliary epithelium, gastrointestinal epithelium, Langerhans islet cells, acinar and duct epithelium of pancreas, adrenal cortical and medullary cells, and prostate epithelium. In addition, CMV DNA, but not CMV antigen, was found in polymorphonuclear leukocytes. These cells may serve as intermediate host or reservoir of CMV and may transmit posttransfusion CMV infection. *In situ* hybridization on routine histologic sections with a biotinylated CMV DNA probe is a rapid, sensitive, and specific method for diagnostic and experimental pathology. (Am J Pathol 1988, 131:490–496)

CYTOMEGALOVIRUS (CMV) is a common cause of infection in newborn infants[1,2] and immunocompromised patients, such as patients with organ transplants[3–5] or acquired immune deficiency syndrome (AIDS).[6–8] The diagnosis of CMV infection is made by detection of distinctive nuclear and cytoplasmic inclusions in cytomegalic cells,[9,10] rising antibody titers to CMV antigens in the serum,[11–13] isolation of the virus in tissue culture,[14,15] immunohistochemical detection of CMV antigens,[16–19] or *in situ* hybridization for CMV DNA in tissues.[20–22] Recently, a specific biotinylated CMV DNA probe has become available commercially for the detection of CMV DNA in routinely fixed paraffin-embedded tissues.[20–22] We used *in situ* hybridization to define the distribution of CMV DNA in various tissues of 4 patients with AIDS and compared this method with immunohistochemical staining by anti-CMV antibodies.

Materials and Methods

Specimens were obtained at autopsy from 4 male patients with AIDS. Pertinent clinical information and autopsy findings are summarized in Table 1.

Preparation of Tissues

The tissues were fixed in 10% buffered formaldehyde and processed for routine paraffin embedding. Five-micron-thick tissue sections were cut, placed on acid-cleaned Denhardt's medium-treated Elmer's glue-coated glass slides according to Haase's method,[23] and baked at 65 C overnight. Sections were dewaxed by three 5-minute incubations in xylene and hydrated in a graded alcohol series, and endogenous peroxidase activity was blocked by incubation in absolute methanol containing 3% H_2O_2 for 5 minutes. The sections were then treated with freshly prepared pronase (CalBiochem, San Diego, Calif) solution, 0.5

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Table 1—Summary of Clinical and Pathological Data in 4 AIDS Patients

Case	Age	Sex	AIDS risk factor	Time from initial diagnosis to death	Premortem diagnosis	Premortem CMV serology	Additional postmortem findings
1	33	M	Homosexual	7 months	PCP, salmonella sepsis	Borderline positive	CMV* pneumonia with systemic involvement
2	25	M	Homosexual	4 months	PCP	NA	CMV pneumonia
3	33	M	Homosexual	5 months	Kaposi's sarcoma of skin, lung and lymph node; toxoplasma pneumonia; non-Hodgkin's lymphoma	NA	CMV pneumonia
4	41	M	IV drug abuser	19 months	PCP	NA	No evidence of CMV infection

PCP, pneumocystis carinii pneumonia; CMV, cytomeglovirus; NA, not available.

mg/ml in 50 mM Tris buffer, pH 7.4, containing 5 mM EDTA, at 37 C for 10 minutes, then washed two times in phosphate buffered saline (PBS pH 7.4 containing 5 mg/ml glycine to inhibit residual pronase activity), dehydrated in a graded alcohol series, and air-dried.

Preparation of Hybridization Probes

The biotinylated CMV DNA probe was purchased from Enzo Biochem, Inc., New York, New York. This probe consists of a mixture of two clones of CMV DNA sequences in the Bam HI site of pBR 322. The insert sizes are 17.2 Kb and 25.2 Kb, representing approximately 18% of the CMV genome. According to the manufacturer, the CMV DNA probe does not cross-hybridize with herpes simplex virus I, herpes simplex virus II or Epstein-Barr virus DNA. Before *in situ* hybridization, 4 μl of carrier DNA (supplied with the labeled probe) was mixed with 20 μl of the CMV DNA probe (probe/carrier DNA mixture). Twenty microliters of this mixture was then added to 50 μl of formamide, which had been deionized with mixed bed resin (Bio-Rad AG 501-X8D), 20 μl of 50% dextran sulfate (Pharmacia, Piscataway, NJ), and 10 μl of 20× SSC (3.0 M sodium chloride, 0.3 M sodium citrate). This probe hybridization mixture of 100 μl is sufficient for hybridization to 5 samples each covering an area on a slide of 20 × 20 mm and can be stored at −20 C for at least 6 months.

In Situ Hybridization

The *in situ* hybridization method was modified after Brigati et al.[24] Twenty microliters of probe hybridization mixture was applied to each tissue section, gently covered with a glass coverslip, and denatured in an 85 C waterbath for 10 minutes. The slides were cooled to room temperature and allowed to hybridize at that temperature usually for 14 hours. Shorter hybridization time (2 hours) resulted in a weaker signal. After careful removal of the coverslips, the slides were immersed in 50% formamide in 0.1× PBS for 10 minutes at room temperature and rinsed in 0.05% Triton X-100 in PBS for 2 minutes for blocking nonspecific binding of detection complex, followed by PBS for 5 minutes. Then 100 μl of biotin recognizing peroxidase-Streptavidin detection complex (DETEK from Enzo), freshly prepared in 200-fold dilution, was added to each section, the sections were incubated at 37 C for 30 minutes, and the slides were washed in 2× SSC for 5 minutes and PBS for 5 minutes. The sections were developed in 0.01% 3-amino-9-ethyl carbazole (Sigma Chemical Co., St. Louis, Mo) in 0.5 M acetate buffer, pH 5.2, containing 0.03% H_2O_2 at room temperature for 2–4 minutes, resulting in a red reaction product. The slides were counterstained with Mayer's hematoxylin, washed, and mounted with Elvanol.

Controls

The following specificity controls were employed:

1. *In situ* hybridization with the CMV DNA probe (18 μg/ml) was performed on normal and adenovirus-infected tissues.
2. CMV DNA-positive sections were incubated with DNA probes for adenovirus (4 μg/ml) and hepatitis B virus (25 μg/ml) (Enzo Biochem) by the same procedure as described above. These probes stained the appropriate viruses in cultured cells and paraffin sections of tissues.
3. Selected sections were digested before the dehydration step with 1 mg/ml of ribonuclease-free DNAse (Sigma) in 20 mM Tris (pH 7.4), 10 mM $MgCl_2$ for more than 24 hours at 37 C. The high DNAse concentration and prolonged incubation time used

Table 2—Detection of Cytomegalovirus in 4 AIDS Patients: Comparison of H & E-Stained Slides With ISH

	Case 1	Case 2	Case 3	Case 4
Heart	+/+*	−/+	ND	−/−
Lung	+/+	+/+	+/+	−/−
Liver	+/+	−/+	−/−	−/−
Pancreas	−/+	−/+	−/+	−/−
Adrenal	+/+	−/+	−/+	−/−
Thyroid	−/−	−/−	−/−	−/−
Kidney	−/+	−/−	−/−	−/−
Colon	+/+	ND	ND	−/−
Spleen	−/+	−/−	ND	−/−
Lymph node	+/+	−/−	−/−	ND
Total	6/9	1/5	1/3	0/0

* Presence of CMV on H & E/ISH.
ND, not done.

resulted in reproducible digestion of DNA in formalin-fixed tissues.

4. *In situ* hybridization was carried out after omission of DNA denaturation or pronase treatment, by replacing the biotinylated CMV DNA probe by biotin or by omitting the CMV DNA probe in the hybridization mixture.

Immunohistochemical Staining

ABC staining of formalin-fixed, paraffin-embedded tissue sections with anti-CMV antibodies (polyclonal goat anti-CMV antibody from Polysciences, Inc., Warrington, Pa) was carried out with the appropriate controls as established and described previously in our laboratory.[27]

Results

In situ hybridization with a biotinylated CMV DNA probe was positive on various organs from 3 of 4 patients with AIDS (Table 2). CMV DNA was clearly demonstrated as nuclear and/or cytoplasmic staining with minimal background (Figures 1 and 2). The nuclear reaction appeared as intense, homogeneous to finely granular staining with or without halo, reminiscent of Cowdry type B inclusions. The cytoplasmic staining consisted of many coarse irregularly shaped granules. *In situ* hybridization was demonstrated to be specific based on the following results of control reactions: 1) no staining when sections of normal or adenovirus infected tissues were hybridized with biotin-labeled CMV probes; 2) no staining when CMV-infected tissues including the lung of Case 1 (containing *Pneumocystis carinii* and polymorphonuclear leukocytes) were hybridized with unrelated probes such as adenovirus DNA probe or hepatitis B virus DNA probe; 3) DNAse digestion abolished the specific staining (Figure 3); and 4) no staining of CMV-infected tissues after the control reactions described above. The *in situ* hybridization method employed was considered to be sensitive because 1) CMV DNA was detected not only in cytomegalic inclusion cells but also in nuclei and cytoplasm of cytomegalic cells without typical inclusions or cells without cytomegaly—so-called occult infection (Figure 4); and 2)

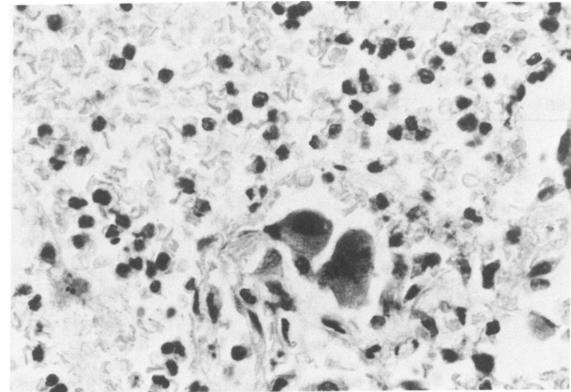

Figure 2—Lung. Parallel section of that in Figure 1 shows two cytomegalic cells in the center of an inflammatory exudate, which consists mainly of polymorphonuclear leukocytes. (H&E, ×400)

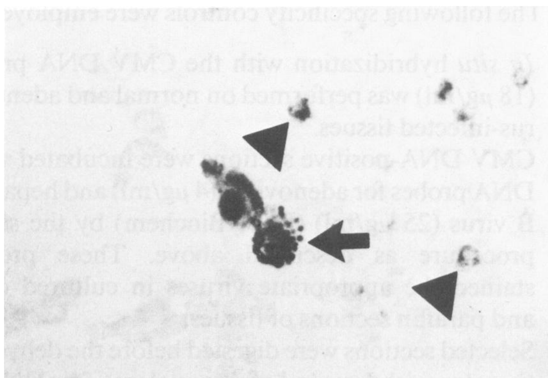

Figure 1—Lung. Two cytomegalic cells exhibit staining of nuclear and cytoplasmic inclusions (*arrow*) after hybridization to biotinylated CMV DNA probe. Several polymorphonuclear leukocytes (*arrowheads*) in the inflammatory exudate are also stained. (*In situ* hybridization, ×400)

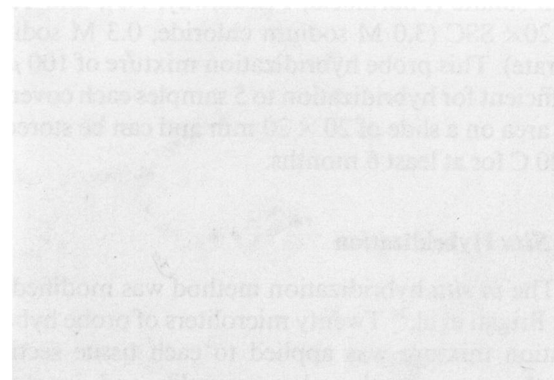

Figure 3—Lung. DNAse treatment of parallel section of that in Figure 1 abolished the reaction with the CMV DNA probe. (*In situ* hybridization, ×400)

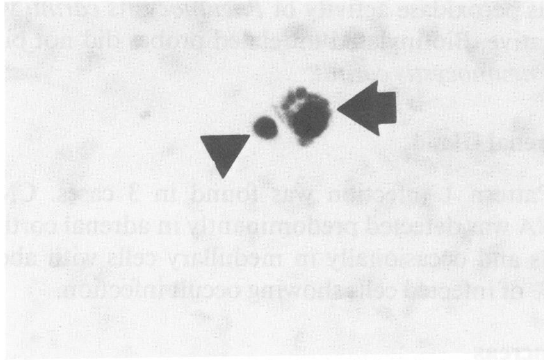

Figure 4—Lung. An intraalveolar cell of normal size (*arrowhead*) and a cytomegalic cell (*arrow*) show binding of the CMV DNA probe. (*In situ* hybridization, ×400)

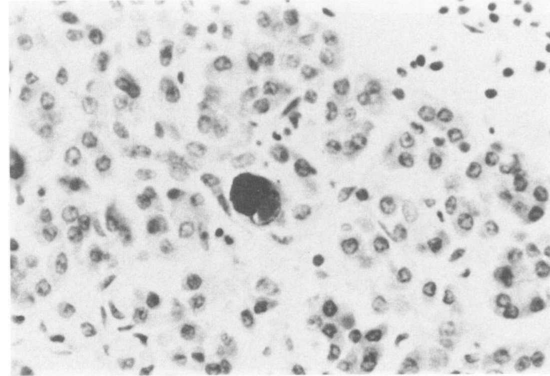

Figure 6—Pancreas. Immunocytochemical reaction with anti-CMV antibody reveals cytoplasmic and nuclear staining of a cytomegalic acinar cell. (ABC method, ×400)

CMV DNA was detected in organs in which no cytomegalic inclusion cells were found on H&E-stained slides (Table 2). The occult infected cells ranged from 25% to 55% of the total number of infected cells and were found in many organs. They were alveolar lining cells, hepatocytes, bile duct epithelial cells, pancreatic acinar and duct epithelial cells, Langerhans islet cells, adrenal cortical and medullary cells, endothelial cells (Figure 5), and fibroblasts.

Immunohistochemical reaction with anti-CMV antibodies on sections from different organs of the four patients revealed staining of some, but not all cytomegalic cells (Figures 6 and 7), even after enhancement by digestion with increased pronase concentrations. On comparison of sequential sections after *in situ* hybridization versus immunohistochemical staining, significantly more cells reacted with CMV DNA probe than with the anti-CMV antibodies (Figures 8 and 9).

Three general patterns of CMV infection were identified in patients with AIDS. They were similar to the findings in organ transplant patients reported by Myerson et al.[25] Pattern 1 was the necrotizing type. It represented a focus of CMV infection with CMV DNA-containing cells at the periphery and a necrotic center with nuclear debris, often associated with many polymorphonuclear leukocytes and few mononuclear cells. Pattern 2 was the diffuse type. It was characterized by the diffuse distribution of groups of infected cells in relatively normal parenchyma with scanty or no inflammatory cell infiltration. Pattern 3 was the small isolated type, characterized by the scattered distribution of one or more infected cells in normal parenchyma with little or no inflammation. Each organ had either one pattern or a mixture of two or three patterns. The findings in different organs by *in situ* hybridization were as follows:

Lung

The lungs showed alveolar cell hyperplasia, acute and chronic inflammation with hemorrhage, interstitial fibrosis and fibrinous alveolar exudate with *Pneumocystis carinii* in 3 cases and toxoplasma in 1 case. In three lungs, CMV DNA was detected as Patterns 2 and 3 in alveolar lining cells, bronchial epithelial cells,

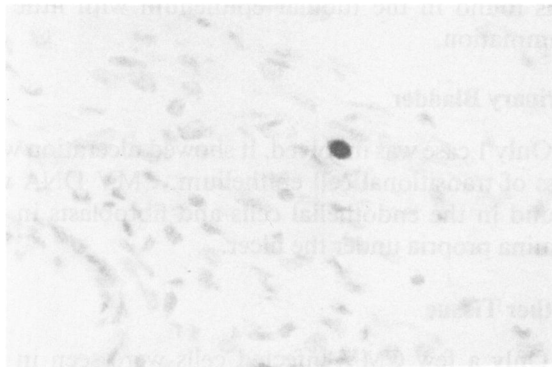

Figure 5—Kidney. Slightly enlarged endothelial cell of a vessel in renal pelvis reveals reaction with the CMV DNA probe. (*In situ* hybridization, ×400)

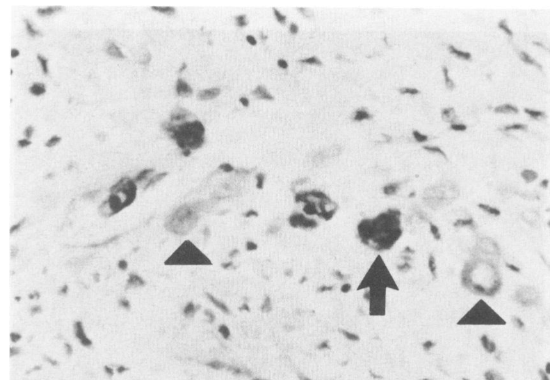

Figure 7—Colon. Immunocytochemical staining discloses CMV antigen in several cytomegalic cells (*arrow*). Other cytomegalic cells (*arrowheads*) do not react with anti-CMV antibody. (ABC method, ×400)

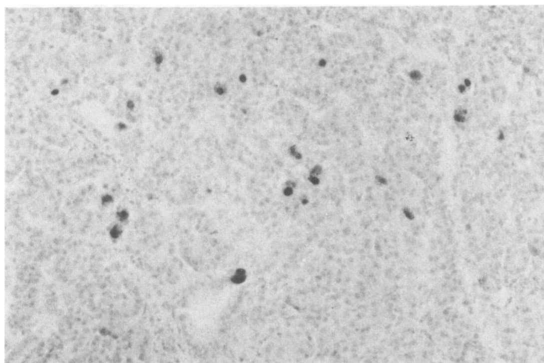

Figure 8—Pancreas. Numerous cytomegalic and normal sized cells after incubation with CMV DNA probe can be detected under scanning power. (*In situ* hybridization, ×100)

endothelial cells, and some spindle-shaped mesenchymal cells, presumably fibroblasts. About half of the positive cells appeared histologically normal. In addition, in Case 1, CMV DNA was also found in the cytoplasm of polymorphonuclear leukocytes, as recognized by their multi-lobed nuclei (Figures 1 and 2). The reaction was considered to be specific because all control reactions were carried out on this case as described above and gave the expected results, including digestion by DNAse (Figure 3) and absence of detectable endogenous peroxidase in polymorphonuclear leukocytes. In other tissues and in the other 3 cases, these inflammatory cells were present, but not reactive.

Pneumocystis carinii bound the CMV DNA probe in the three infected lungs. However, we considered this reaction to be nonspecific because the staining was not abolished by pretreatment of the sections with DNAse or by omission of tissue DNA denaturation. In addition, we studied 10 cases of pneumocystis pneumonia without CMV infection and observed binding of the CMV DNA probe to *Pneumocystis carinii* in these tissues. Omission of the CMV DNA probe eliminated the staining and the reaction for endogenous peroxidase activity of *Pneumocystis carinii* was negative. Biotinylated unrelated probes did not bind to *Pneumocystis carinii.*

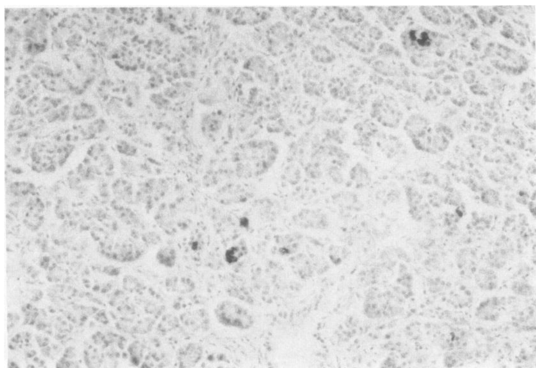

Figure 9—Pancreas. Parallel section of that in Figure 8 demonstrates only few positive cells after immunocytochemical staining with anti-CMV antibody. (ABC method, ×100)

Adrenal Gland

Pattern 1 infection was found in 3 cases. CMV DNA was detected predominantly in adrenal cortical cells and occasionally in medullary cells with about 55% of infected cells showing occult infection.

Pancreas

Pattern 3 infection was observed in 2 cases. CMV DNA was detected in pancreatic duct epithelium, acinar cells, and Langerhans islet cells. Most of the infected cells were not associated with inflammation except for several minute foci of necrosis with polymorphonuclear leukocyte infiltration (Figure 8).

Liver

Pattern 3 infection was seen in 2 cases. CMV DNA was detected predominantly in bile duct epithelial cells and less commonly in hepatocytes and sinusoidal lining cells. There was little inflammation.

Gastrointestinal Tract

Two CMV-infected foci were found in 1 case, one in an ulcer at the gastroesophageal junction and the other in the colon. Pattern 2 infection was observed in the ulcer and in the endothelial cell of vessels with vasculitis in the underlying lamina propria and submucosa. CMV DNA was detected also in mucosal cells and fibroblasts. Numerous cells with occult infection were found among the cytomegalic inclusion cells.

Kidney

CMV DNA was detected in only 1 case, mostly in endothelial cells (Figure 5). Occasionally, CMV DNA was found in the tubular epithelium with little inflammation.

Urinary Bladder

Only 1 case was involved. It showed ulceration with loss of transitional cell epithelium. CMV DNA was found in the endothelial cells and fibroblasts in the lamina propria under the ulcer.

Other Tissue

Only a few CMV infected cells were seen in the lymph nodes, spleen, heart, bone marrow, and skin in 1 case.

Discussion

In situ hybridization with a biotinylated DNA probe for detection of CMV DNA in routine sections is a rapid and sensitive histochemical method. It detects CMV DNA not only in cytomegalic inclusion cells, but also in histologically normal-appearing cells, which account for a significant portion of the infected cells (range, 25–55%) in systemic CMV infection in patients with AIDS. Other methods with similar sensitivity as *in situ* hybridization are the isolation of CMV in tissue culture[26] and the detection of CMV antigen by specific polyclonal or monoclonal antibodies in fresh or frozen cells.[15–17] However, *in situ* hybridization is superior to both because it can be completed in less than 24 hours after sectioning of the tissue,[28] whereas tissue culture tests may take several weeks. In addition, it requires only routinely fixed, paraffin-embedded tissues in which immunohistochemical demonstration of CMV antigen is less sensitive than *in situ* hybridization, as shown in this and other studies.[29] Similar observations have recently been reported for human papillomavirus infection.[30]

The *in situ* hybridization method has several other distinct advantages[31]: 1) The biotinylated probes can be prepared quickly and stored in the hybridization mixture over an extended period of time because of their chemical stability. 2) Many slides can be processed with different probes at the same time, and findings can be visualized clearly at the scanning power by microscopic examination (Figure 8). 3) Preservation of cell and tissue morphology is excellent. The procedure is so simple that any laboratory equipped to perform routine immunohistochemical experiments can handle it well.

The hybridization procedure described here is specific because reactions were consistently negative in tissues without markers of CMV infection and after hybridization of CMV-infected tissues with probes lacking CMV DNA sequences. The specificity was further confirmed by abolishment of staining by DNAse treatment. These control reactions are essential for recognition of nonspecific staining of *Pneumocystis carinii,* which commonly coexists, particularly in patients with AIDS.[32]

CMV infects primarily epithelial and endothelial cells.[6–8,15] Our retrospective study of patients with AIDS by *in situ* hybridization suggests that the virus was more widespread than previously thought. It infected many types of cells in a variety of organs and was frequently present in cells showing no cytopathic effect. We detected CMV DNA also in polymorphonuclear granulocytes by *in situ* hybridization. Blood cells such as neutrophils, lymphocytes, and monocytes have been suspected to be responsible for the posttransfusion CMV infection in transplanted patients.[33–37] Although CMV was isolated more readily from the polymorphonuclear leukocyte fraction of the blood of patients with CMV mononucleosis than from the mononuclear leukocyte fraction,[38] CMV DNA in polymorphonuclear leukocytes has not been demonstrated in tissue sections before. Our finding supports the evidence that these cells may serve as a reservoir for CMV or as carrier for CMV maintained elsewhere with constant or periodic seeding.[35,36] We did not detect CMV antigen in polymorphonuclear leukocytes by immunohistochemical staining. This may be related to the lower sensitivity of the immunomorphologic method discussed above. However, because we do not have clear evidence of viral replication or production of viral antigens in polymorphonuclear leukocytes, we cannot exclude the possibility that the CMV DNA signals detected represent phagocytized virus or viral DNA fragments which do not contribute to the spread of CMV.

Most of the CMV-infected cells in AIDS patients were associated with little inflammation. Occasionally, we found necrotizing foci in the adrenal cortex and ulcerations in the gastrointestinal tract. They were characterized by a necrotic center with nuclear debris, surrounded by polymorphonuclear leukocytes. CMV DNA staining was seen at the periphery of the foci or in the endothelial cells of nearby venules or lymphatic vessels. These findings suggest that CMV may spread along or within small vessels. It may compromise the blood supply to the surrounding tissues and may cause necrosis and inflammation. The epithelium of bile ducts and pancreatic ducts was also infected by CMV, but many infected cells were histologically normal and not associated with inflammation. These findings suggest that CMV may also spread along the ducts and that inflammation will not occur as long as the basement membranes of the ducts are intact.

In summary, *in situ* hybridization with a biotinylated DNA probe on routine histologic sections is useful for the study of the tissue distribution and pathologic manifestations of CMV infection. It is a rapid, sensitive, and specific method for the pathologist to use as a diagnostic and research tool for the detection of CMV infection.

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