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In situ hybridization analysis of cytomegalovirus lytic infection in Kaposi's sarcoma associated with AIDS

A study of 14 autopsy cases*

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Summary. Cytomegalovirus (CMV) was assayed by in situ hybridization with commercially available biotin-labeled CMV-DNA probes in 45 formalin-fixed paraffin-embedded autopsy specimens with Kaposi's sarcoma from 14 cases of the acquired immune deficiency syndrome (AIDS). In seven of the 14 cases, a few scattered hybridizing cells were detected in Kaposi's sarcoma, but not all specimens from the same case showed such cells. Most of the positive cells were peculiarly swollen and not typical of Kaposi's sarcoma cells. All positive cases had at least some CMV-infected organs with typical cytomegalic cells containing nuclear inclusions while five of the 7 negative cases revealed no CMV-infected tissue by conventional light microscopy. Our results suggest that this in situ hybridization procedure using biotin-labeled DNA probes only reveals generalized CMV infection that is a consequence of impairment of immune mechanisms in AIDS patients.

Key words: Kaposi's sarcoma – Cytomegalovirus – In situ hybridization – Autopsy

Introduction

From epidemiological findings a recent outbreak of Kaposi's sarcoma has been associated with the acquired immune deficiency syndrome (AIDS) (Friedman-Kien and Ostreicher 1984). Of malignancies appearing in AIDS patients Kaposi's sarcoma is the most common, followed by malignant non-Hodgkin B-cell lymphoma (Schmidts et al. 1986). However, among AIDS patients an extremely high prevalence of human cytomegalovirus (CMV) infection has been documented (Drew et al. 1981). Since Giraldo et al. (1972a) initially succeeded in isolation of human CMV from tissue cell lines derived from the classic Kaposi's sarcoma in Africa, significant serological correlation between CMV infection and patients with Kaposi's sarcoma was reported (Giraldo et al. 1972; Giraldo et al. 1975; Giraldo et al. 1978) and human CMV-DNA homologous sequences, CMV-specific RNA-DNA, CMV-specific antigen and herpes-type virus particle were verified in biopsy specimens of Kaposi's sarcoma using DNA-DNA reassociation (Boldogh et al. 1981; Giraldo et al. 1980), RNA-DNA in situ hybridization (Boldogh et al. 1981; Fenoglio et al. 1982), immunofluorescence (Boldogh et al. 1981; Giraldo et al. 1977; Giraldo et al. 1980) and electron microscopy (Walter et al. 1984), respectively. However, the question remains unresolved whether CMV infection is only a secondary phenomenon in Kaposi's sarcoma resulting from immune deficiency, or whether human CMV is the causal agent of Kaposi's sarcoma (Huang 1984).

To reassess CMV infection in Kaposi's sarcoma, we employed in situ hybridization technique in autopsy specimens from various organs, using a commercially prepared biotin-labeled CMV-DNA probe (Löning et al. 1986; Unger et al. 1986). Applying similar modifications of this method conflicting results have been obtained by several investigators (Fenoglio et al. 1982; Löning et al. 1986; Unger et al. 1986). For this reason we give

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Table 1. 14 autopsy cases of AIDS with Kaposi's sarcoma

Case No.	Age	Sex	CMV-infected organs detected by conventional light microscopy
1	35	M	lung, gastrointestinal tract, adrenal gland, salivary gland, cerebellum
2	49	M	lung, gastrointestinal tract, pancreas, salivary gland, spleen, testis, cerebrum
3	37	M	not detected
4	49	M	lung, adrenal gland, cerebrum
5	46	M	not detected
6	37	M	lung, gastrointestinal tract, adrenal gland
7	42	M	lung, gastrointestinal tract, tongue
8	44	M	not detected
9	31	F	not detected
10	34	M	lung, gastrointestinal tract, tongue, pancreas, adrenal gland, thyroid gland
11	35	M	lung, adrenal gland, thyroid gland
12	54	M	lung, oesophagus, gastrointestinal tract, tongue, pancreas, gallbladder, adrenal gland, thyroid gland, kidney, spleen, lymph node
13	26	M	not detected
14	31	M	lung, thyroid gland

a detailed description of the method used in our study.

Materials and methods

Routinely processed, formalin-fixed paraffin-embedded autopsy tissues from 14 AIDS patients with Kaposi's sarcoma, examined during the period from May 1984 to April 1986, were examined (Table 1). All patients were LAV/HTLV-III/HIV antibody-positive. After reviewing all the microscopic slides, we selected 45 representative specimens with Kaposi's sarcoma from various organs for in situ hybridization with nucleic acid probes.

In situ hybridization was performed according to Brigati et al. (1983) with some modifications (Haase et al. 1984; McDougall et al. 1986; Myerson et al. 1984; Rijntjes et al. 1985; Unger et al. 1986). Paraffin sections were attached to chrome-gelatine-coated glass slides, allowed to dry at room temperature, and baked overnight at 60° C. The sections were dewaxed by three 10-min incubations in xylene, placed twice in absolute ethanol for 5 min, and then hydrated by sequential two 3- min incubations in ethanol-H_2O mixture containing 96%, 70%, 30% and 0% ethanol, respectively. The hydrated sections were soaked briefly in PBS and then placed for 20 min in 0.2 N HCl. After washing twice for 3 min in PBS, the sections were incubated for 1.5 min in PBS containing 0.01% Triton X-100, and again washed twice for 3 min in PBS. The sections were then treated with proteinase K (Merck, Darmstadt, FRG; 0.005 mg/ml) dissolved in 20 mM Tris-HCl, pH 7.5 containing 2 mM $CaCl_2$ for 15 min at 37° C. After washing twice for 3 min in PBS containing 2 mg/ml glycine, the sections were soaked in PBS containing 4% paraformaldehyde for 30 min, and washed twice for 3 min in PBS containing 2 mg/ml glycine. The sections were then dehydrated through a graded ethanol-H_2O series containing 30%, 70%, 96% and 100% ethanol (twice each for 3 min) and air-dried.

The hybridization cocktail consisting of 50% (v/v) deionized formamide, 10% (w/v) dextran sulfate (Sigma, St. Louis, MO, USA), 4 × SSC buffer (0.6 M NaCl, 0.06 M sodium citrate sulfate) pH 7.0, 400 μg/ml sonicated herring sperm DNA and 2.3 μg/ml biotin-labeled CMV-DNA (Cytomegalovirus BIO-PROBE™ Labeled Probe, ENZO, New York, USA) (Löning et al. 1986; Unger et al. 1986) was previously denatured at 100° C for 2 min and quickly cooled on ice. The prepared sections were overlaid with 10–40 μl of the hybridization cocktail and covered with a coverglass onto a frame of modelling clay. The sections with the cocktail were then denatured in a 90° C water bath for 6 min and incubated overnight at 37° C. After hybridization and removal of the coverglasses, the sections were washed three times for 3 min in 50% deionized formamide in 2 × SSC buffer (0.3 M NaCl, 0.03 M sodium citrate) pH 7.0 at 34° C, twice for 3 min in 2 × SSC at 34° C, and finally for another 3 min in 2 × SSC at room temperature.

For the detection of biotin-labeled hybridized probes, the following two different methods were used:

(1) Avidin-biotinylated alkaline phosphatase complex (ABAP) method (Crum et al. 1986; Löning et al. 1986; Unger et al. 1986). The washed sections were immersed for 5 min in blocking buffer, 3% BSA in Tris-saline-triton 7.5 (0.1 M Tris HCl pH 7.5, 0.1 M NaCl, 5 mM $MgCl_2$, 0.01% Triton X-100), and incubated in a 1:50 dilution of freshly prepared avidin-biotinylated alkaline phosphatase complex (if the complex is refrozen after the first thawing following delivery, a highly increased background staining and false-positive staining of many cells especially lymphocytes due to the resulting instability of the complex will occur) (DETEK I-alk, ENZO, New York) in dilution buffer, 1% BSA in Tris-saline-triton 7.5 for 1 h at 37° C. After washing three times for 5 min in Tris-saline-triton 7.5 and soaking for 5 min in propandiol buffer pH 8.7, the alkaline phosphatase label was revealed with 15-min incubation in Naphthol AS-BI Phosphate (Sigma, St. Louis, MO, USA, N-2250) plus Fast Red TR Salt (Sigma, St. Louis, MO, USA, F-1500) in propandiol buffer 8.7 containing levamisole (Sigma, St. Louis, MO, USA). The sections were rinsed in distilled water and lightly counterstained with haematoxylin.

(2) Avidin-biotinylated peroxidase complex (ABC) method (Hsu et al. 1981). The washed sections were placed for 30 min in methanol containing 1% H_2O_2 for inactivation of endogenous peroxidase activity, and washed twice for 5 min in 2 × SSC. The sections were then incubated for 10 min in 10% normal rabbit serum (Dako, Denmark) diluted in 2 × SSC. The probe was then detected with sequential treatments with a 1:100 dilution of avidin DN (Vector Laboratories, Burlingame) in 2 × SSC for 30 min at 37° C, two 5-min washes in 2 × SSC, a 1:250 dilution of goat anti-avidin serum (Vector) in 2 × SSC for 1 h at 37° C, two 5-min washes in 2 × SSC, a 1:200 dilution of biotinylated rabbit anti-goat IgG (Vector) in 2 × SSC for 30 min at 37° C, two 5-min washes in 2 × SSC, Vectastain ABC complex diluted in 2 × SSC for 1 h at 37° C, and two 5-min washes in 2 × SSC. The site of location of the peroxidase label was identified by formation of brown reaction products utilizing 0.5 mg/ml diaminobenzidine (DAB) (Rudolf Walter K.G., Kiel, FRG) dissolved in Tris HCl containing a final concentration of 0.01% H_2O_2 for 5 min. The sections were rinsed in distilled water and lightly counterstained with haematoxylin.

Almost no background staining occurred using both signal systems as described above. Although sensitivity of the two methods for the detection of biotin-labeled hybridized probes was essentially equivalent, the reaction was finally evaluated with avidin-biotinylated alkaline phosphatase complex (ABAP), method because the red reaction products were more easily identified in tissue with many pigments such as haemosi-

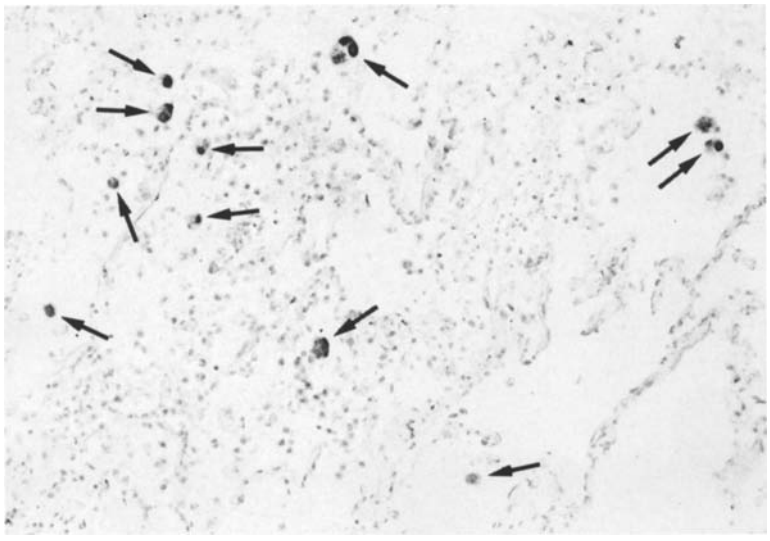

Fig. 1. Case 2. Infected lung tissue adjacent to Kaposi's sarcoma containing scattered CMV-DNA hybridizing alveolar lining cells and macrophages (arrows). ABAP method counterstained with haematoxylin, ×116

Table 2. In situ hybridization in tissues with Kaposi's sarcoma

Case No.	Skin	Lymph node	Lung	Gastrointestinal tract		Mouth	Liver	Adrenal gland	Ureter	Thoracic duct
1	− (−)	− (−)								
2	+ (−)	+ (−)	+ (++)	jejunum	+ (++)					
3	− (−)	− (−)	− (−)	duodenum	− (−)	− (−)				
4	+ (−)	+ (−)								
5		− (−)								
6		− (−)	− (+)	colon	+ (++)				− (−)	
7		− (−)	− (+++)	ileum	+ (++)	+ (+)				
8	− (−)	− (−)		ileum	− (−)					
9	− (−)	− (−)	− (+)	stomach	− (−)		− (−)			
10			− (+++)	colon	+ (+)	+ (+)		+ (++)		+ (−)
11	− (−)	− (−)		stomach	− (−)					
12	− (−)					+ (−)				
13		− (−)								
14	− (−)	− (−)	+ (+)				+ (−)			

−, negative in Kaposi's sarcoma: +, a few positive cells in Kaposi's sarcoma: (−), negative in the adjacent tissue: (+), (++), (+++); a few, a moderate number of, many positive cells in the adjacent tissue, respectively

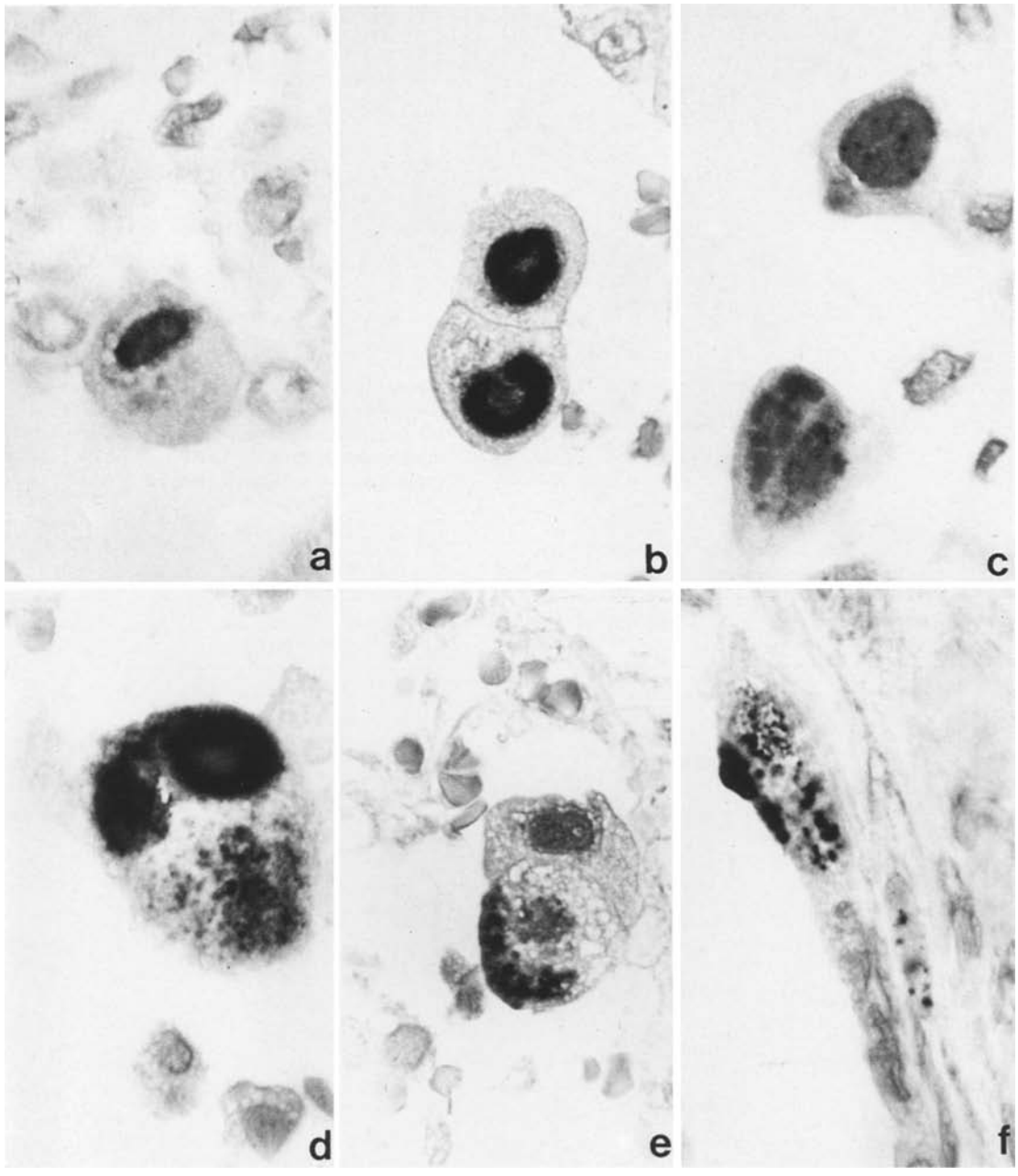

Fig. 2. High magnification of CMV-DNA hybridizing alveolar lining cells and macrophages showing various patterns of the reaction. ABAP method counterstained with haematoxylin, ×1200. A nuclear reaction exhibits a central core corresponding to a nuclear inclusion (**a**), circular condensation (**b, d**) and a reticular or granular pattern (**c, e**). Granular reaction products in the cytoplasm are diffuse (**d**) or peripheral (**e**). Figure 2f shows a relatively flattened hybridizing alveolar lining cell

derin pigments and dust particles phagocytized by macrophages, than were the brown reaction products by avidin-biotinylated peroxidase complex (ABC) method.

Possible non-specific binding of DNA was examined by applying biotin-labeled virus probes of herpes simplex type 1 and 2, adenovirus 2 and the plasmid pBR 322 (Cytomegalovirus BIO-PROBE™ is cloned into the BAM H 1 restriction cleavage site of the plasmid pBR 322) (ENZO, New York, USA) to sections of CMV-infected lung in cases 7 and 10. The possibility of hybridizing cellular nucleic acids homologous to CMV-DNA was tested in non infected tissues. In addition the specifity of both the alkaline phosphatase and peroxidase detection system was tested by running the procedure without any biotin-labeled probe. No staining was present in any of these control specimens.

Results

In nine (69%) of the 14 cases with Kaposi's sarcoma, cells strongly suggestive of cytomegalovirus

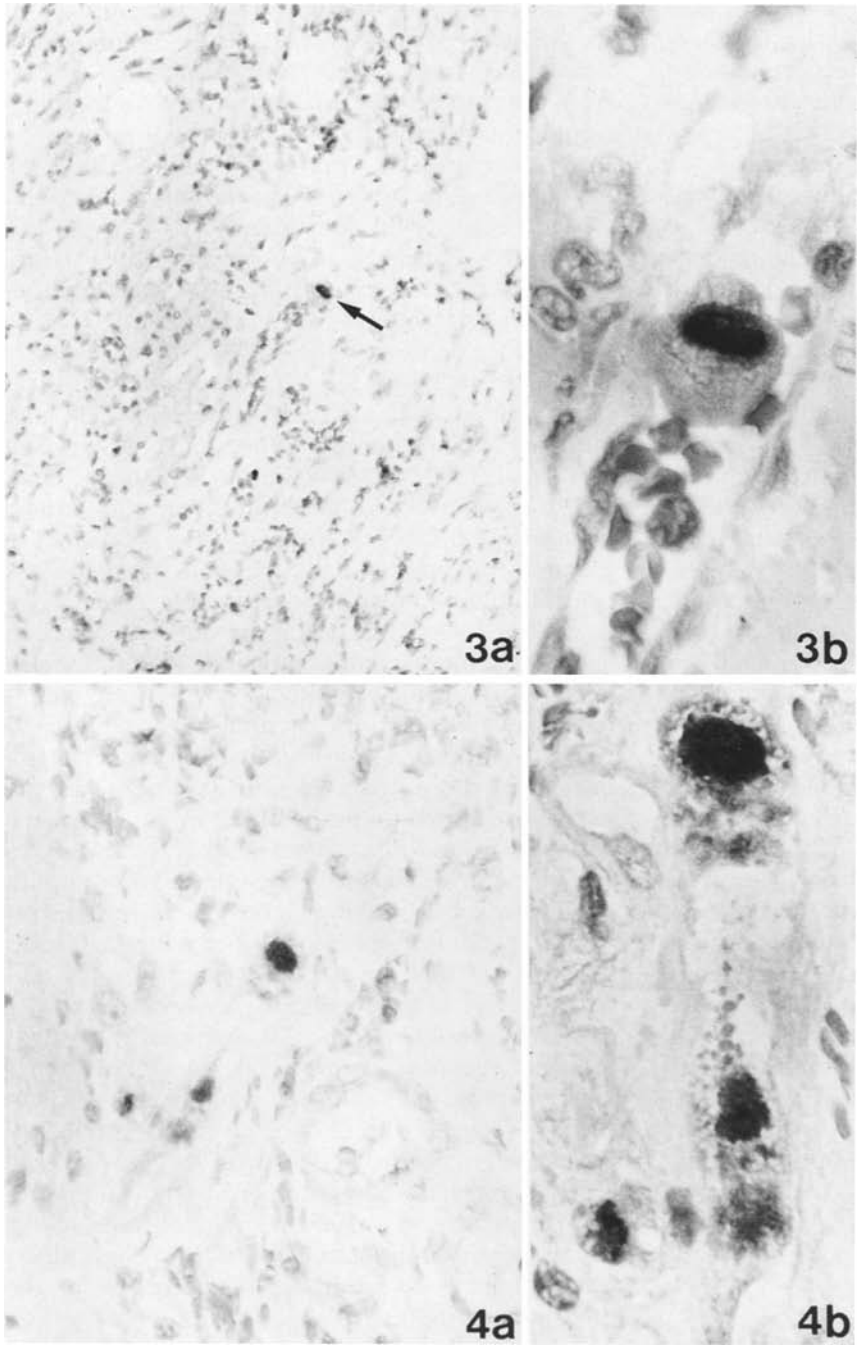

Fig. 3. Case 10. (**a**) A single positive cell (*arrow*) in Kaposi's sarcoma in the capsule of the adrenal gland. ABAP method counterstained with haematoxylin, ×230. (**b**) Higher magnification of the hybridizing cell appearing to be a diagnostic cell of CMV infection with a nuclear inclusion. The cell may be an endothelial cell of the non-neoplastic blood vessel. ×1200

Fig. 4. Case 7. (**a**) A few hybridizing cells, which are not diagnostic of CMV infection by conventional light microscopy, in the submucosa of the ileum. ABAP method counterstained with haematoxylin, ×460. (**b**) Higher magnification of the positive cells showing peculiarly swollen and granular cytoplasms. ×1200

(CMV) infection were detected at least in some of organs available for examination by conventional light microscopy (Table 1). These cells were enlarged, and often contained an intranuclear basophilic or amphophilic inclusion surrounded by a halo and intracytoplasmic amphophilic or basophilic granular inclusions.

In the preexisting tissue adjacent to Kaposi's sarcoma, in situ hybridization for CMV-DNA showed infected cells in six of 14 cases (6 of 7 specimens from the lung, 4 of 8 specimens from the gastrointestinal tract, 2 of 4 specimens from the mouth and one specimen from the adrenal gland). The positive reaction was recognized in alveolar lining cells or alveolar macrophages of the lung (Figs. 1 and 2), epithelia and endothelial cells of the gastrointestinal tract, salivary ducts and endothelial cells of the mouth and endothelial cells and parenchymal cells of the adrenal gland. The nuclear reaction products showed not only an in-

tense solid core corresponding to the nuclear inclusion but also circular condensation in the periphery of the nuclear inclusion or a diffuse reticular or granular pattern (Fig. 2). Granular reaction products in the cytoplasm probably representing cytoplasmic inclusions were not always present in the positive cells (Fig. 2). Reaction products were not seen in any normal-appearing cells. In one (case 9) of the six positive cases no cells with diagnostic inclusions had been detected by conventional light microscopy, although subsequent careful search of the corresponding area of the lung in the haematoxylin-eosin stained section revealed a single infected swollen alveolar lining cell with basophilic granular cytoplasm.

A few scattered hybridizing cells were detected in the Kaposi's sarcoma in seven (50%) of the 14 patients examined, but in not all specimens from the same patient (Table 2). In all the positive cases, there were at least some CMV-infected organs with diagnostic cells containing a nuclear inclusion confirmed by conventional light microcopy, while no diagnostic CMV-infected cells were found anywhere in five of the remaining seven cases without hybridizing cells in Kaposi's sarcoma. Most of the positive cells were large oval or large plump and spindle shaped and had red reaction products restricted to nuclei, or within both nuclei and the cytoplasm (Figs. 3 and 4). Careful correlation of areas containing these positive cells with corresponding areas in routinely stained sequential serial sections allowed identification of peculiarly swollen but not always diagnostic cells of CMV infection. In no case, did other spindle cells of Kaposi's sarcoma, which have been described in literature as the typical tumour cells of Kaposi's sarcoma, reveal a positive reaction. It was sometimes very difficult to assess whether the positive cells were tumour cells, stromal endothelial cells or macrophages.

Discussion

Kaposi's sarcoma is known to be the most common of malignancies appearing in patients with the acquired immune deficiency syndrome (AIDS) (Friedman-Kien and Ostreicher 1984; Schmidts et al. 1986), and among these AIDS patients extremely high frequency of human cytomegalovirus (CMV) infection has been reported (Drew et al. 1981). However, the question whether human CMV is a causative agent for Kaposi's sarcoma or whether CMV infection is the consequence of immune deficiency is still unresolved. Since the initial search by Giraldo and his colleagues (1972a) for viral agents, resulting in the demonstration of the herpes-type virus particles and isolation of human CMV from tissue culture cell lines derived from the classic type of African Kaposi's sarcoma, significant seroepidemiologic association between CMV infection and Kaposi's sarcoma has been reported (Giraldo et al. 1972b; Giraldo et al. 1975; Giraldo et al. 1978), and human CMV DNA homologous sequences, CMV-specific RNA and CMV-specific antigen existing in tumour biopsies of Kaposi's sarcoma were demonstrated using DNA-DNA reassociation (Boldogh et al. 1981; Giraldo et al. 1980), RNA-DNA in situ hybridization (Boldogh et al. 1981; Fenoglio et al. 1982) and anticomplement immunofluorescence tests (Boldogh et al. 1981; Giraldo et al. 1977; Giraldo et al. 1980), respectively. In addition, Drew et al. (1982) demonstrated CMV RNA and CMV antigen(s) in Kaposi's sarcoma in AIDS patients by in situ hybridization and immunofluorescence, respectively.

In half of our cases, the in situ hybridization using a commercially prepared biotin-labeled CMV-DNA probe exhibited a few hybridizing infected cells in Kaposi's sarcoma in formalin-fixed paraffin-embedded autopsy specimens. The frequency of the positive cells in Kaposi's sarcoma varied from one organ to another even in the same patients. In addition, in all of the positive cases there were at least some CMV-infected organs with diagnostic cells containing a nuclear inclusion confirmed by conventional light microscopy while no diagnostic CMV-infected cells were found anywhere in five of the remaining seven cases without hybridizing cells in Kaposi's sarcoma. In contrast to some previous reports (Fenoglio et al. 1982; Löning et al. 1986) and in accordance with the results of Unger et al. (1986), no positive cells appeared to be "normal" or typical Kaposi's spindle cells by conventional light microscopy. After careful reevaluation of the corresponding area in the haematoxylin-eosin stained section at least slight morphological deviations in the structure of the nucleus and the cytoplasm were found. The method used in this study may be able to detect degenerating infected cells only in secondary generalized CMV infection occurring as a consequence of the impairment of immune mechanisms produced by LAV/HTLV-III/HIV virus infection in AIDS patients. Even if there are CMV-infected proliferating Kaposi's sarcoma cells as suggested by Giraldo et al. (1972a, 1972b, 1977, 1980), Boldogh et al. (1981), Fenoglio et al. (1982) and Huang (1984), it seems to be impossible, within the limitation of sensitivity of this system, to detect a small fraction of the viral genome of CMV retained in the trans-

forming cells. Crum et al. (1986) estimated in a report of in situ hybridization analysis of human papillomavirus 16 DNA sequences in early cervical neoplasia, that in situ hybridization analysis with biotin-labeled probes will be negative if cells containing greater than 800 genome equivalents per cell are not represented in the sections. A similar sensitivity was observed by Brigati et al. (1983) in an earlier report. For this reason it seems to be doubtful whether in situ hybridization in combination with a biotin-labeled CMV DNA probe is apt to detect low genome equivalents possibly responsable for the malignant transformation of endothelial cells resulting in Kaposi's sarcoma. An additional decrease of sensitivity may arise from the commercial available reagents used in our study.

Although the in situ hybridization technique examined in this study is believed not to be sensitive enough to identify small portions of viral DNA fragments, the diagnostic sensitivity of the in situ hybridization technique performed on paraffin or cryostat sections appears to be equivalent to immunofluorescent demonstration of CMV antigens in frozen sections (Müller et al. 1986; Myerson et al. 1984). Thus this method seems to be very useful for additional rapid detection of CMV infection among cells with changes in morphological features suggestive of active viral replication. The reaction reveals very little background and nonspecific staining; immunohistochemical demonstration of CMV has not been successful in formalin-fixed specimens, and viral culture needs more time than our in situ hybridization technique. Especially, rapid diagnosis for opportunistic CMV infection in transplant recipients of organs such as kidney, liver and heart is very important in therapy. Further improvement of the sensitivity of the in situ hybridization technique may be achieved by using plasmid-free fractions of viral DNA for instance. The detection of a small fraction of a viral genome in latent viral infection and oncogene analysis in chromosomal DNA in standard histochemistry laboratories may thus be facilitated.

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