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In situ hybridization for the detection of cytomegalovirus (CMV) infection*

Application of biotinylated CMV-DNA probes on paraffin-embedded specimens

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Summary. Five autopsy cases of cytomegalovirus (CMV) infections were studied. Conventional light microscopy disclosed characteristic cytopathic effects in lungs, kidneys, and brain. In one case, electron microscopy was carried out and revealed typical herpesvirus particles.

In situ hybridization was done with biotin-labeled CMV-DNA probes and an avidin-alkaline phosphatase detection system. 4/5 cases were observed to contain hybridizing cells in different organs. Intensity of hybridization was related to the severity of CMV infection, roughly estimated by counting cytomegalic cells. In addition to cytomegalic cells, a high number of normal-looking epithelial and mesenchymal cell types were positive. These latter cells showed nuclear hybridizations in contrast to cytomegalic cells which hybridized both within the nuclei and the cell bodies.

This modified in situ hybridization procedure is a rapid and valuable tool for the detection and final demonstration of virus infection, and will be of particular help for the examination of paraffin-embedded specimens.

Key words: Cytomegalovirus – in situ hybridization – Paraffin sections – Biotinylated probes

Introduction

Among the human herpesviruses cytomegaloviruses contain the largest genome measuring approximately 240 kb (Fleckenstein et al. 1982). Whereas the members of the herpesvirus family differ strikingly in their DNA compo-

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sition, different viral strains of CMV are genetically closely related showing only 10% genetic variability as deduced from DNA-DNA renaturation kinetics and restriction endonuclease maps (Davis et al. 1980). The frequency of CMV infections is high, documented by the demonstration of serum antibodies in 20% of infants and 50%–60% of adults in middle Europe (Lennartz and Piesbergen 1983). However, disease is rare and most commonly reported in newborns following intrauterine or perinatal infections (Seifert and Oehme 1957; Huang et al. 1980; Spector 1983) and in adults as a consequence of virus reactivation in pregnancy, following blood transfusion or in transplant recipients or immunocompromised tumour patients etc. (Pass 1985; Krämer et al. 1985; Winkelmann et al. 1985; Kotler et al. 1985). In childhood, the most serious complication of CMV infection is mental retardation, while in adults the clinical spectrum ranges from mild febrile episodes to severe CMV pneumonia, myocarditis, hepatitis and gastroenteritis. The lungs, kidneys, liver, pancreas, salivary glands are most often reported to show typical cytopathic effects (CPE, Seifert and Oehme 1957). These CPE comprise nuclear and cytoplasmatic inclusions, perinuclear vacuolization, cellular enlargement, and loss of intercellular adhesion (Seifert and Oehme 1957; Seifert 1973). The CPE are the consequence of the intranuclear replication and the intracytoplasmic packaging of the virus DNA. Early and late antigens are parts of the nucleocapsid and the lipoprotein envelope, respectively.

Clinical monitoring of CMV infections is most frequently done by measuring IgM and IgG response (Cappel et al. 1978; Schmitz et al. 1980; Yolken and Leister 1981; Yolken and Stopa 1980; Pozzetto and Gaudin 1981; Grint et al. 1985).

The major disadvantages of this method are:

1. The primary humoral immune reaction (IgM response) against the nucleocapsids takes place 3–4 days after the onset of clinical symptoms. Most often, however, CMV infections are encountered several weeks after the IgM reaction has already been switched and replaced by IgG antibodies. In this situation, even a close follow-up of serum titer changes may not be helpful for the diagnosis of CMV disease.

2. Antigenic heterogeneity of different CMV strains can cause false negative results (Faix 1985; Volpi and Britt 1985).

3. Serological methods often fail to detect virus antibodies (low titres) in immunocompromised patients, such as transplant recipients or patients suffering from AIDS.

Apart from these problems, the requirements for an accurate morphological diagnosis emerge from two interrelated demands of clinical care: Firstly if no antibody response is measurable, cytological and/or histological diagnosis is asked for, for the sake of the patient and for the prevention of a nosocomial outbreak. Precise diagnosis is even more demanded since specific antiviral chemotherapy is now being evaluated increasingly (Ringden et al. 1985). Secondly, if a virus infection without any clinical and microbiological diagnosis is suspected, post mortem examination is asked for in order to get information about nosocomial infections and of course for

increasing the clinical experience in diagnosis and management of infections of unknown aetiology.

To date, the accuracy of viral diagnosis was often hampered by the failure of pathologists to define the virus in paraffin-embedded tissues. Cytopathic effects are often not reliable enough to make a clear-cut diagnosis, and monoclonal antibodies especially against viral envelopes frequently do not work on paraffin sections. Modern molecular biological methods are expected to become an additional diagnostic tool in virus infections, since they allow us to substantiate not only the mere presence of the viral genome, but also the degree of amplification at the DNA level and the degree of transcriptional activity at the RNA level (Chou et al. 1983; McCarrey et al. 1984). Thus, in this study we examined the accuracy of nucleic acid hybridization on paraffin sections. We decided to begin with a small series of autopsy cases of CMV infections, since in CMV infected organs cytopathic effects are easy to detect and to relate to the aetiological agent.

Our aims were:

1. to improve the in situ hybridization method in order to obtain a rapid and easy detection system readily to handle in every laboratory.
2. to compare in situ hybridization with simple evaluation of cytopathic effects of CMV infections of different organs.

Material and methods

Material

Paraffin-embedded specimens of different organs from four cases assumed to have CMV infection were collected in the last two years (Table 1). Since in a further autopsy case, investigated in 1965, paraffin blocks of brain tissue were also available we included these specimens in our study.

All samples were sectioned and routinely stained with Haematoxylin-Eosin in addition to the in situ hybridization with nucleic acid probes.

Methods

Electron microscopy. In case No. 4, glutaraldehyde-fixed specimens were postfixed in 2 per cent osmium tetroxide, dehydrated and embedded in epoxy resin. Thin and ultrathin sections were cut with either glass or diamond knives on a Reichert OmU2 (Reichert, Vienna, Austria). The ultrathin sections were contrasted with alcoholic uranyl acetate and lead citrate and examined with a Zeiss 9 S-2 electron microscope.

In situ hybridization. Paraffin sections were adhered on poly-D-lysin-coated glass slides and hybridizations were performed according to Brigati et al. (1983) with some modifications: Sections were dewaxed in Xylene, rehydrated, and treated sequentially with 0.02 N HCl; 0.01% Triton X-100 in PBS; 1 mg/ml Pronase E (Calbiochem, Frankfurt, FRG) in 0.05 M Tris-HCl, pH 7.6, 5 mM EDTA; 100 µg/ml ribonuclease B from pancreas (Sigma, St. Louis, MO, USA) and 100 units/ml ribonuclease T1 (Sigma) in PBS; 100 µg/ml avidin (Sigma) in PBS; and 4% paraformaldehyd in PBS. After washing in PBS, the sections were dehydrated and air-dried.

For hybridization, each section was covered with 20 µl of the following hybridization solution: 2 × SSC, 45% (v/v) formamide, 10% (w/v) dextran sulfate, 0.1 mg/ml herring sperm DNA, and 1.5 µg/ml biotinylated CMV-DNA (Cytomegalovirus Bio-Probe™ labeled probe, Enzo, New York, USA). This commercially available CMV probe consists of a mixture of two CMV-DNA sequences of 25.2 kb and 17.2 kb cloned into the Bam H1 restriction cleavage

Table 1. Synopsis of clinical and morphological data

Case	Age	Sex	Clinical diagnosis	Pathology	CMV infection, affected organs and hybridizing cell types
1	19 d	m	Prematurity, resp. distress syndrome, perforation of the intestine, renal insufficiency	Hyaline membrane syndrome, arterial thrombosis of the truncus coeliacus and the A. mesenterica superior, intestinal, renal and splenic infarctions; renal and discrete pulmonary CMV infection	Kidney: glomerular, interstitial and endothelial cells proximal tubule epithelium Lung: no reactivity
2	22 y	m	Accident, cerebral contusion sepsis of unknown aetiology	Contusio cerebri, candida sepsis with metastatic abscesses in lungs, heart, liver, spleen, kidneys and lymph nodes; pulmonary CMV infection	Lung: pneumocytes, interstitial cells, endothelial cells intravascular leucocytes
3	53 y	m	Phenacetin nephritis, renal insufficiency, renal transplantation, cerebral haemorrhage	Chronic interst. nephritis, chronic rejection of the renal transplant, uraemia, pulmonary CMV infection	Lung: as in case 2
4	39 y	m	AIDS, pneumocystis carinii pneumonia	Lymphadenopathy syndrome, pneumonia with pneumocystis carinii, discrete pulmonary CMV infection	Lung: no reactivity
5	2 d	m	Prematurity, microcephaly	Paraventricular encephalitis	Brain: astroglia and endothelial cells

site of the plasmid pBR 322 and biotinylated by nick translation with a biotinylated nucleotide, Bio-11-dUTP. Sections and DNA probes were denatured together in a 90° C water bath for 10 min, then hybridized for 1 h at 37° C. After hybridization the sections were washed 2×10 min in $1 \times$ SSC, 45% formamide, 42° C (washing under stringent conditions), then 3×5 min in $2 \times$ SSC at room temperature.

For the detection of the biotinylated DNA, the sections were incubated overnight at 4° C with rabbit anti-biotin-antibody (Enzo) diluted 1:100 in $2 \times$ SSC + 1% bovine serum albumin (BSA). After extensive washing in $2 \times$ SSC, they were incubated with a 1:200 dilution of biotinylated anti-rabbit-IgG (Vector) for 1 h at 37° C, followed by washing and a 1:500 dilution of avidin-alkaline phosphatase (Bresa, Adelaide, Australia) in 100 mM Tris-HCl, pH 7.5, 1 M NaCl, 2 mM $MgCl_2$, 0.05% Triton X-100 for 1 h at 37° C. After extensive washing in 100 mM Tris-HCl, pH 9.5, 1 M NaCl, 5 mM $MgCl_2$ the sections were incubated with 0.3 mg/ml

nitro blue tetrazolium and 0.2 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$ for 15 min in the dark. In order to inhibit endogeneous alkaline phosphatase activity 1 µl/ml levamisole (Sigma) was added to the substrate solution in some experiments. The colour reaction was blocked with 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and the sections were photographed without and with counterstaining.

Results

Report of autopsies

Four autopsy cases suggested to have CMV infection were observed in the last two years. As seen in Table 1, each case was unique with respect to the clinical background and the underlining organ failures.

Case 1. A 19 day old premature male infant (weight: 1950 g, length: 41 cm), born at the 27th week of gestation. He developed a respiratory distress syndrome soon after birth. Autopsy revealed thrombosis of the truncus coeliacus and the A. mesenterica superior which probably occurred as a consequence of arterial catheterization. The arterial thrombosis caused haemorrhagic infarction of the small intestine and anaemic infarctions in kidneys and spleen.

Microscopical investigation revealed discrete cytological signs of CMV infection in the lung in addition to hyaline membrane disease. The CMV infection was much more pronounced in the kidneys. The kidneys as well as liver and spleen showed severe ischaemic reaction. The liver and spleen and the other organs however, did not contain additional cytomegalic cells.

Case 2 was a 22 year old man who – following a car accident – suffered from a severe head injury. His condition was aggravated by a pulmonary infection leading to septic episodes. He died $2^1/_2$ weeks later.

Post mortem findings comprised cerebral contusion, confluent pneumonia in both lungs, pleural and pericardial empyema, and septic abscesses in lungs and within the myocardial tissue. Microscopical examination revealed mycotic abscesses in lungs, heart, liver, spleen, kidneys, and lymph nodes. Yeasts were found to be candida albicans, and candida sepsis leading to metastatic abscesses was our major diagnosis. Surprisingly, additional CMV infection was detected which, however, was only observed in pulmonary necrotizing foci.

Case 3 was a 53 year old man with chronic interstitial nephritis (analgetica nephritis) and renal insufficiency. The patient received a kidney graft few months before death. Chronic rejection of the kidney transplant was followed by uraemia, gastrointestinal and cerebral bleeding. Microscopical examination disclosed the end stage of analgesic nephritis including papillary sclerosis as remnants of papillary necrosis, bilateral atrophy and scarring of the organs. The renal transplant showed severe arterial thickening and marked interstitial fibrosis. CMV infection was found in the lungs only, in addition to hyaline membrane disease. Interestingly, CMV infection was obvious neither in the nephritic kidneys nor in the kidney graft.

Case 4 was a 39 year old homosexual man with AIDS. HTLV III infection was probably acquired six years ago. At the time of admission, the patient showed multiple Kaposi sarcomas at cutaneous and mucous membrane sites. Death was attributed to severe pulmonary infection caused by pneumocystis carinii. Confluent pneumonia was the major autopsy finding.

Microscopical investigation confirmed pneumocystis carinii as the causative agent of pneumonia. Additional CMV infection was observed in the lungs. Cytomegalic cells were scanty however. With the electron microscope, we succeeded in finding virus particles (Fig. 1), which consisted of a viral core and envelope and had a diameter of about 150 nm. Thus, these viruses belonged to the herpesvirus family and could very well be CMV virus particles.

The lymph nodes showed the end stage of HTLV III infection. The lymphoid tissue was almost completely eradicated. Kaposi sarcomas were observed in the skin and the penile and oral mucosa.

In addition to these very recent cases of CMV infection we obtained paraffin blocks (brain) from an autopsy case of an infant examined in 1965. With this case we had the opportunity to see whether or not nucleic acid hybridization can be carried out even on the very old cases in our paraffin files.

In situ hybridization

For technical reasons, this study was focussed on a comparison of conventional light microscopy and in situ hybridization in those organs and in those tissue sections (lungs, kidneys, brain), in which there was already some evidence of CMV infection. This included cellular enlargement, nuclear inclusions (basophilic to amphophilic), an intranuclear halo, and cytoplasmic inclusions (acidophilic to amphophilic). In this way, the presence of cytomegalic cells, likely to contain viral DNA, served as an intrinsic control of efficacy of our in situ hybridization procedure.

Lungs

We found cytomegalic cells in the lung specimens of all cases tested. The number of cytomegalic cells, however, differed largely within these cases and within different tissue sections. Thus, we found less than 5 cytomegalic cells in cases No. 1 and 4, in contrast to more than 10 cytomegalic cells in cases No. 2 and 3. Taking into account this distribution, it was interesting that in situ hybridization only yielded positive results in cases No. 2 and 3. In these cases, however, a large number of cells were found to be hybridized including not only cytomegalic cells, but also normal-looking pneumocytes, interstitial cells, endothelial cells, and even intravascularly located leucocytes (Figs. 1, 2a, b). Whereas cytomegalic cells showed both hybridizing nuclei and cytoplasmic inclusions, all the other CMV infected cells reacted only within their nuclei.

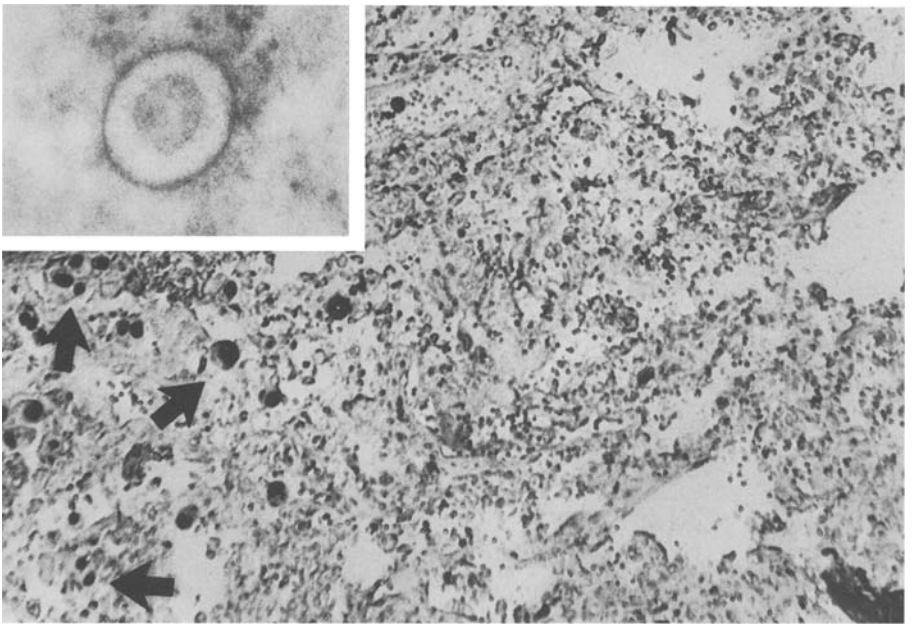

Fig. 1. In situ hybridization (case No. 3). Small foci of hybridizing cells (arrows) within the lung tissue. At his low magnification, accuracy of the technique and preservation of tissue structure is documented. Mag. × 80. *Inset:* Electron micrograph of an enveloped virus particle of the herpes virus family (supposed to be cytomegalovirus, case No. 4). The particle is composed of a central core, a surrounding pale zone and a thin membranous shell. Mag. × 87000

Kidneys

Renal CMV infection was only observed in case No. 1. We found cytomegalic cells in the renal cortex within the proximal parts of the tubule. Again, the number of cytomegalic cells varied markedly within different tissue blocks and tissue sections ranging between 10 and 20 owl eye cells/section. As already mentioned, we found discrete signs of pulmonary CMV infection (less than 5 cytomegalic cells) in the same case. In contrast to the renal infection, however, we failed to substantiate CMV infection in the lungs by in situ hybridization.

In situ hybridization disclosed positive glomerular, interstitial and endothelial cells in addition to hybridizing cytomegalic cells (Figs. 3, 4a). As in the pulmonary infections, hybridization was restricted to nuclei except for the cytoplasmic inclusions in cytomegalic cells within the proximal tubular system. Also in the kidney, leucocytes were seen to be labeled within and around the vessels.

Brain

CMV infection of the central nervous system was only observed in one case of paraventricular encephalitis, in which post mortem examination was performed in 1965. This case was particularly interesting, since cytomegalic

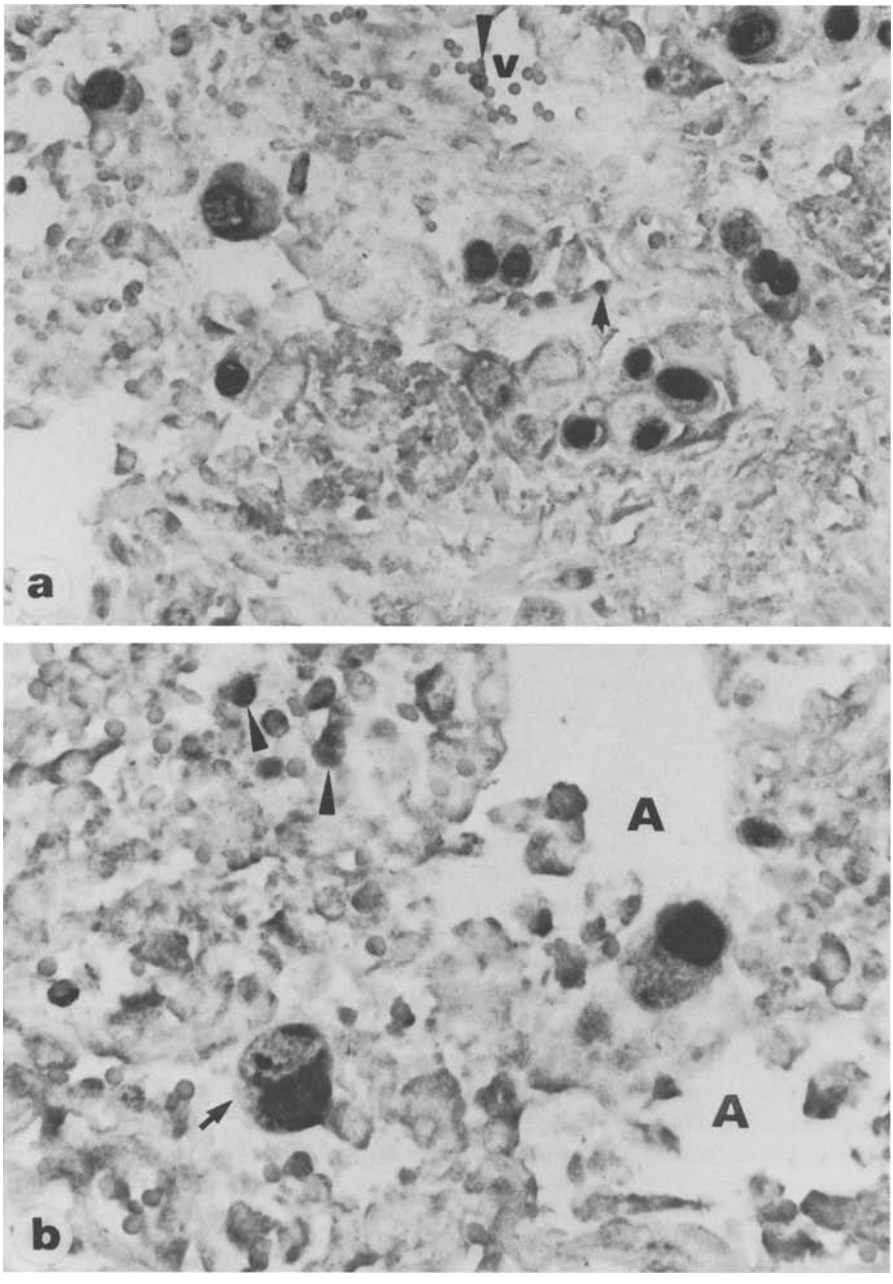

Fig. 2a. Pulmonary CMV infection (case No. 3). In situ hybridization. In addition to labeled cytomegalic cells, positive endothelial cells (arrow) and leucocytes (arrowhead) within vessels (V) are seen. Mag. × 320. **b** Higher magnification of another CMV infected zone. Cytomegalic cells show nuclear and granular cytoplasmic (*arrow*) hybridization. Cytomegalic cells are desquamated and are located within the alveolar space (*A*). Some positive interstitial cells (*arrowheads*) are seen, too. Mag. × 500

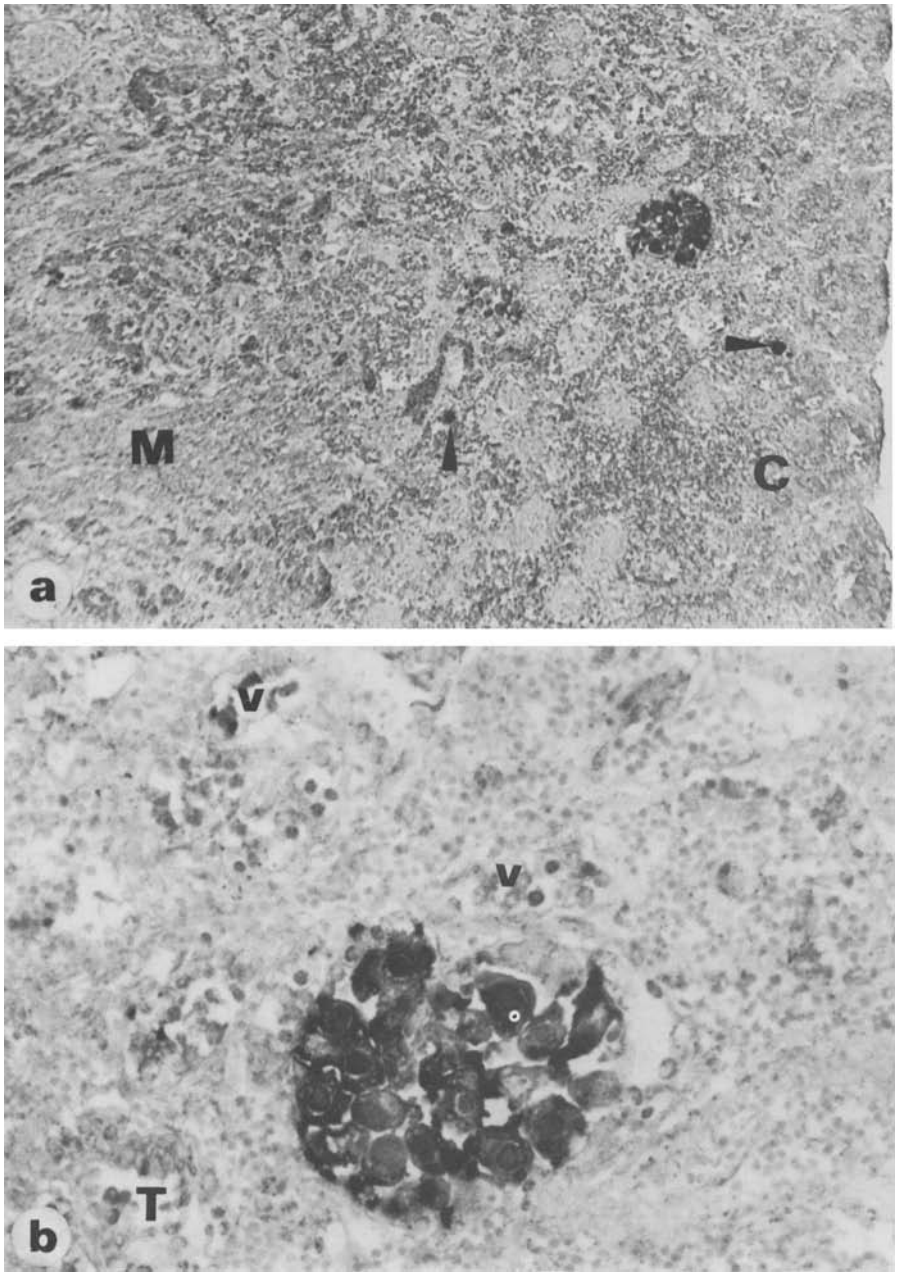

Fig. 3a. Renal CMV infection (case No. 1). Low magnification documents structural preservation of renal cortex (*C*) and medulla (*M*). In addition to some scattered hybridizing cells (*arrowheads*) two larger zones of CMV infection are clearly disclosed by in situ hybridization. Mag. × 80. **b** Higher magnification. Cytomegalic cells in the centre of the figure show nuclear and cytoplasmic hybridizations. In addition, further labeled cells are recognized in the tubular system (*T*) and within small vessels (*V*). Mag. × 320

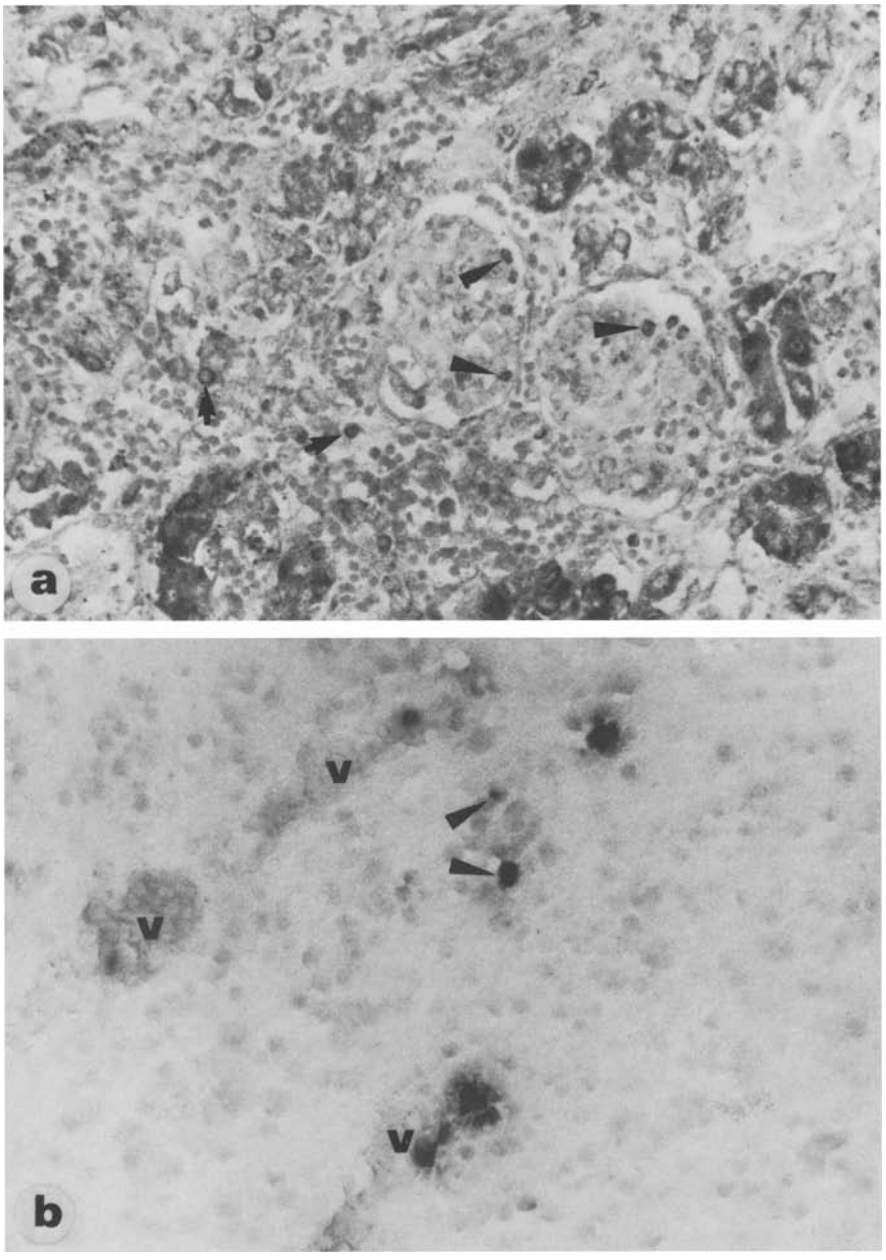

Fig. 4a. Renal CMV infection. Demonstration of hybridizing cells within glomeruli (*arrowheads*) and the interstitial spaces (*arrows*) in addition to intense hybridization of tubular epithelial cells. Mag. × 320 **b** CMV infection of the brain (case No. 5). In situ hybridization discloses positive endothelial cells (*V* vessels) and few labeled glial cells (*arrowheads*)

cells were hard to recognize (less than 5 suspicious cells), and the diagnosis of toxoplasmosis was made initially. In situ hybridization clearly determined CMV infection, showing hybridizing nuclei of some astroglial and endothelial cells (Fig. 4b). Neurones were not seen to hybridize in these sections.

Specificity

The specificity of the biotinylated CMV probe and of the applied alkaline phosphatase detection system was scrutinized by testing the CMV probe on tissue sections infected by other viruses (herpes simplex-, hepatitis B-, papilloma-), examining other virus probes (herpes simplex-, papilloma-) on CMV infected tissues, testing the biotinylated vector pBR 322 without viral inserts, and running the procedure without any biotinylated probe. In addition, the possibility of hybridizing cellular nucleic acids homologous to CMV DNA was examined in non-infected tissues (lung, kidney, bladder, lymph node). No staining occurred in these experiments.

Discussion

Our results demonstrate that in situ hybridization can be carried out on paraffin embedded material with an excellent degree of specificity and with a surprisingly well conserved tissue structure. Biotinylated nucleic acid probes shorten the method, in that results are available within two days. In addition our modified alkaline phosphatase detection system is sensitive (detecting 5 pg of biotinylated DNA on blots), easily applied and barely interferes with endogenous enzyme activities.

Major technical problems were overcome by coating the slides with poly-D-lysine to avoid section floating and blocking endogenous avidin-binding activity by incubation of the sections with avidin before hybridization. The simple two-step biotin-avidin detection method was changed by introducing anti-biotin antibodies and creating a three-step detection system in order to produce amplification. Finally endogenous alkaline phosphatase was blocked by levamisole. Blocking is particularly needed in those organs (liver, kidney) with high enzyme activities.

In our early experiments, we tested peroxidase and β-galactosidase detection systems for detection of the biotinylated virus DNA. In our hands, the latter immunoenzyme methods gave inconsistent results because of low sensitivity and substrate precipitations (β-galactosidase) and nonspecific staining and disturbing background dots (peroxidase), respectively. Myerson et al. (1984a; 1984b) used in situ hybridization with cosmid-cloned CMV DNA representing 56 and 100% of the entire genome, respectively. Their probes contained inverted repeat sequences subdividing the DNA into a large and a small segment (Fleckenstein 1982), which were shown to hybridize to mammalian cellular DNA (Peden et al. 1982). The commercial probe used in our study did not contain these inverted repeats, but two 25.2 and 17.2 kb sequences from the large and the small segment of the CMV genome. Thus, it did not cross-hybridize to cellular DNA or to the DNA

of other herpesviruses. In situ hybridization is superior to electron microscopy and tissue culture techniques which need more time and larger numbers of intact virus particles to get positive results (Lee et al. 1978; Gregory and Menegus 1982). Immunohistochemistry with monoclonal CMV-antibodies is not successful with paraffin sections. A comparison of immunohistochemistry with a commercial polyclonal antibody (Polysciences) and in situ hybridization is in progress. Our results confirm those of Myerson et al. (1984b) who found CMV DNA not only in cytomegalic owl eye cells, but also in entirely normal-looking cells in many organs. In addition to their results, we found CMV-positive cells in the brain of one patient. This latter case is a good example of in situ hybridization applied to the very old tissue samples which we have often carefully collected over the last decades.

From our results, and at least for the organs examined in this study, it has become evident that CMV infection is either not as widespread as discussed in recent papers (Myerson et al. 1984a, b) or that the sensitivity of the biotin-avidin technique is much lower than in situ hybridization carried out with radioactive probes (Gissmann, personal communication). In our hands, hybridization was always confined to small foci in the respective organs and, in a roughly quantitative way, positive reactions could be expected when more than five cytomegalic cells were seen. Thus, we missed hybridizing cells in pulmonary CMV infection of cases No. 1 and 4, in which the morphological signs of CMV infection were discrete. In case No. 4, we found particles of the herpes virus type by chance with the electron microscope, and this finding was obtained for one cell in a large series of ultrathin sections examined. Of course, in this case, our diagnosis of CMV infection is a doubtful one, and primarily based on the scarcity of cytomegalic cells (less than 5) in some lung specimens. Microbiological and serological attempts, however, were even more disappointing in this case, thus being an example of the situation which clinicians frequently encounter in immunocompromised patients.

Opprotunistic CMV infections play an increasingly important role in transplant recipients, tumour patients, and patients suffering from AIDS. Additionally, CMV was found associated with certain tumours, especially Kaposi sarcomas and colorectal carcinomas (Rüger and Fleckenstein 1985; Grail and Norval 1985), but the significance of these results is doubted by other authors (zur Hausen et al. 1974). In situ hybridization should be used as an additional rapid and sensitive method for the diagnosis of CMV infections.

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