Widespread presence of cytomegalovirus DNA in tissues of healthy trauma victims

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Abstract

***Aims*—To determine the localisation of human cytomegalovirus (CMV) DNA in abdominal aorta, spleen, and transplantable organs, such as kidney, pancreas, and liver, obtained from healthy individuals; to characterise the cell type(s) in these tissues that serve as a reservoir for latent CMV.**

***Methods*—CMV DNA was detected by dot blot DNA hybridisation and in situ DNA hybridisation with a probe for CMV major immediate early sequences (UL123) and nested PCR with primers derived from the CMV major immediate early (IE) gene exon 4 (UL123ex4). Samples of liver, abdominal aorta, spleen, kidney, and pancreas were obtained at necropsy or from donor kidneys from healthy subjects.**

***Results*—CMV DNA was detected in most tissue samples using dot blot hybridisation and nested PCR. In situ hybridisation demonstrated that, in addition to smooth muscle cells in the arterial wall, hepatocytes, tubular and glomerular kidney cells, splenic red pulp cells, and pancreatic acinar cells also harboured CMV DNA. CMV DNA was detected in seropositive and in some seronegative subjects.**

***Conclusion*—CMV DNA is widely distributed in organs of healthy subjects. CMV DNA was found in various cell types in several organs, suggesting that during latency, CMV DNA is present thoughout the body.**

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Infection by human cytomegalovirus (CMV), a herpesvirus, can result in a variety of disorders ranging from subclinical disease in normal subjects to fatal disease in immunocompromised patients.[1] CMV can establish latent infections. Virus reactivation followed by active infection has been clearly demonstrated in recipients of organ transplants and often leads to life-threatening disease.[1] Seronegative recipients of donor organs are at particular risk of developing CMV disease.[1] Molecular epidemiological studies have shown that two recipients receiving organs from the same donor excrete identical CMV strains in the urine,[2] demonstrating that CMV transmission occurs with the graft. However, the cell types or the types of graft that serve as a reservoir for latent CMV are unkonwn. Several reports have suggested that latent CMV is present in the smooth muscle cells of the arterial wall.[3–6] Using PCR, we detected CMV DNA in the arterial wall in almost 70% of CMV seropositive patients[5] and using in situ DNA hybridisation showed that arterial smooth muscle cells harboured the virus. In other reports CMV nucleic acids were found in peripheral leucocytes obtained from healthy CMV seropositive and seronegative subjects.[7 8]

In this report we describe the presence and location of CMV DNA in aorta, kidney, spleen, and pancreas of healthy organ donors.

Methods

Two groups of patients were included in this study. Group 1 comprised trauma victims (eight men, five women) available for donor nephrectomy. Samples were taken from the abdominal aorta, spleen and pancreas using strict aseptic surgical techniques. Twelve artery, 12 spleen and eight pancreatic samples were collected from these subjects. The mean age of the subjects was 35 years (range eight to 45 years). CMV DNA was detected using in situ DNA hybridisation and PCR. If there was sufficient tissue, dot blot DNA hybridisation was also done.

Group 2 comprised patients (six men, two women) with no relevant medical history who died of sudden cardiac arrest. Samples were taken at necropsy from the spleen, liver and kidney. All samples were taken within 12 hours of death using aseptic techniques. Eight spleen, eight liver, and eight kidney samples were collected. The mean age of the subjects was 65 years (range 54–75 years). In most cases CMV was detected by PCR only. Some liver and kidney samples were examined using in situ DNA hybridisation to identify the cell types harbouring CMV DNA.

All tissue samples were handled separately to avoid cross contamination. Tissues from which DNA was to be isolated were stored at 4°C in 50% ethanol. Tissues for in situ DNA hybridisation were fixed routinely in formalin and embedded in paraffin wax.

SEROLOGY

Seropositivity for CMV was determined for all patients using a latex agglutination test (CMV-scan, Becton Dickinson, Cockeysville, MD, USA), an IgG ELISA from Organon Teknika, Boxtel, The Netherlands, and an in-house ELISA based on an AD169 infected cell lysate.[9] To establish active infection, IgM anti-CMV antibodies were detected using a capture ELISA technique as described by van

Loon *et al.*[10] All serum samples were taken directly on arrival of the subjects at our hospital.

EXTRACTION OF DNA FROM TISSUE AND TISSUE SECTIONS

DNA for dot blot hybridisation and PCR analysis was extracted from the ethanol preserved tissues. Small tissue samples were collected in 1.5 ml polypropylene microcentrifuge tubes. Subsequent proteolytic digestion of all samples was carried out overnight at 50°C in a mixture of 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 100 mM NaCl, 250 µg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany), and 10 mg/ml SDS under constant agitation. The DNA was purified by phenol/chloroform extraction and ethanol precipitation, and finally dissolved in 50 µl distilled water. To exclude the possibility of cross contamination during these procedures, herring sperm DNA was included as a negative control.

OLIGONUCLEOTIDES

Oligonucleotides purchased from Eurogentec (Liege, Belgium) were derived from the CMV major immediate early gene exon 4 (UL123ex4). The nucleotide sequences of primers used for nested PCR are: first round: A1 5'-ACGAATTCGTAATGAAGCGCCGC ATTGA-3' and A2 5'-ACGAGCTGCAAT AGTGACGTGGGATCCA-3'; second round: B1 5'-ACGATTTCTCTGCATGAAGGTCT TTGC-3' and B2 5'-ACGAGCTCGTGGG ATCCATAACAGTAA-3'; internal probe: C 5'-CAGTAATTGTGGCGTAGAACAGTGA TCAGG-3'. The nested PCR product was 468 base pairs. The primer set specific for the β-globin gene has been described previously.[11]

HYBRIDISATION PROBES

A 7.0 kilobase EcoR1-Sal1 fragment from the EcoR1-J fragment of the CMV AD-169[5] strain, which contains the major immediate early gene, was used as a probe. The probe was labelled with biotin by incorporating biotin-11-dUTP (Bethesda Research Laboratories, Maryland, USA) using a random primed DNA labelling kit (Boehringer Mannheim).

DOT BLOT DNA HYBRIDISATION

Transfer of cellular DNA to nitrocellulose, hybridisation conditions, and visualisation of successful hybridisation were carried out as described previously.[5] The specificity of the biotinylated probe has been evaluated extensively.[5]

NESTED PCR

The reaction mixture contained standard PCR buffer (10× PCR buffer; Promega, USA) supplemented with $MgCl_2$ to a final concentration of 2.4 mM and 250 µM of each dNTP in a reaction volume of 50 µl. One nanogram of each primer, 1 unit DNA polymerase (Amplitaq; Perkin Elmer Cetus, USA) and 200 ng target DNA were added. After 30 cycles of one minute at 94°C, one minute at 58°C and two minutes at 72°C on a Prem III thermal cycler (LEP Scientific, UK), 1 µl of the reaction mixture was transferred to the second reaction tube containing the nested primer for the second round PCR, carried out under the same conditions. After 40 cycles, 10 µl of the second reaction mixture was subjected to electrophoresis on 2% agarose gels (Seakem GTG FMC Bioproducts, USA) and transferred to a nylon filter (Hybond N+ Amersham, Little Chalfont, UK). The internal oligonucleotide was end-labelled with ^{32}P and used as a probe for hybridisation to the nylon filters. All samples showing hybridisation were considered to contain CMV DNA.

PREVENTION OF FALSE POSITIVE PCR RESULTS

Samples and buffers were prepared in different laboratories to prevent carry over of reaction products. A panel of related DNA viruses, including herpes simplex I, herpes simplex II, Epstein-Barr virus, varicella zoster virus, human herpes virus 6, rat CMV, and a panel of negative controls, including herring sperm DNA and DNA obtained from various organs of SPF Brown norway rats, was subjected to PCR with the CMV primer set.

IN SITU DNA HYBRIDISATION

The presence of CMV nucleic acids was detected using in situ DNA hybridisation on sections 4 µm thick as described previously.[5]

IMMUNOHISTOCHEMISTRY

CMV specific antigens were detected immunohistochemically as described previously.[12] A monoclonal antibody directed against the CMV 72 kilodalton IE antigen (UL123) (Dupont Speciality Diagnostics, Wilmington, USA) and the MC 222 monoclonal antibody, directed against the 65 kilodalton tegument protein of the virus particle, pp65 (ppUL83)(PK)),[13] were used.

Table 1 Results of dot blot hybridisation, in situ hybridisation and PCR

		Subject number													
Test	*Tissue*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*Total*
Dot blot hybridisation	Aorta	+	+	NA	+	NA	NA	−	+	+	NA	NA	+	NA	6/7
	Spleen	+	+	+	+	+	NA	+	+	+	NA	NA	+	+	10/10
	Pancreas	NA	+	−	−	+	NA	−	NA	NA	NA	NA	NA	+	3/6
In situ hybridisation	Aorta	+	+	+	+	+	−	−	+	−	−	−	+	NA	6/12
	Spleen	+	+	+	+	+	NA	+	−	+	−	−	+	+	8/12
	Pancreas	NA	+	+	+	+	NA	−	NA	+	NA	+	NA	+	6/7
Nested PCR	Aorta	+	+	+	+	+	−	−	+	+	−	+	+	NA	9/12
	Spleen	+	+	+	+	+	NA	+	+	+	+	+	+	+	12/12
	Pancreas	NA	+	+	+	+	NA	−	NA	+	+	+	NA	+	6/8
Serology		−	+	−	+	+	−	+	−	+	+	+	+	+	4/13

NA = not available.

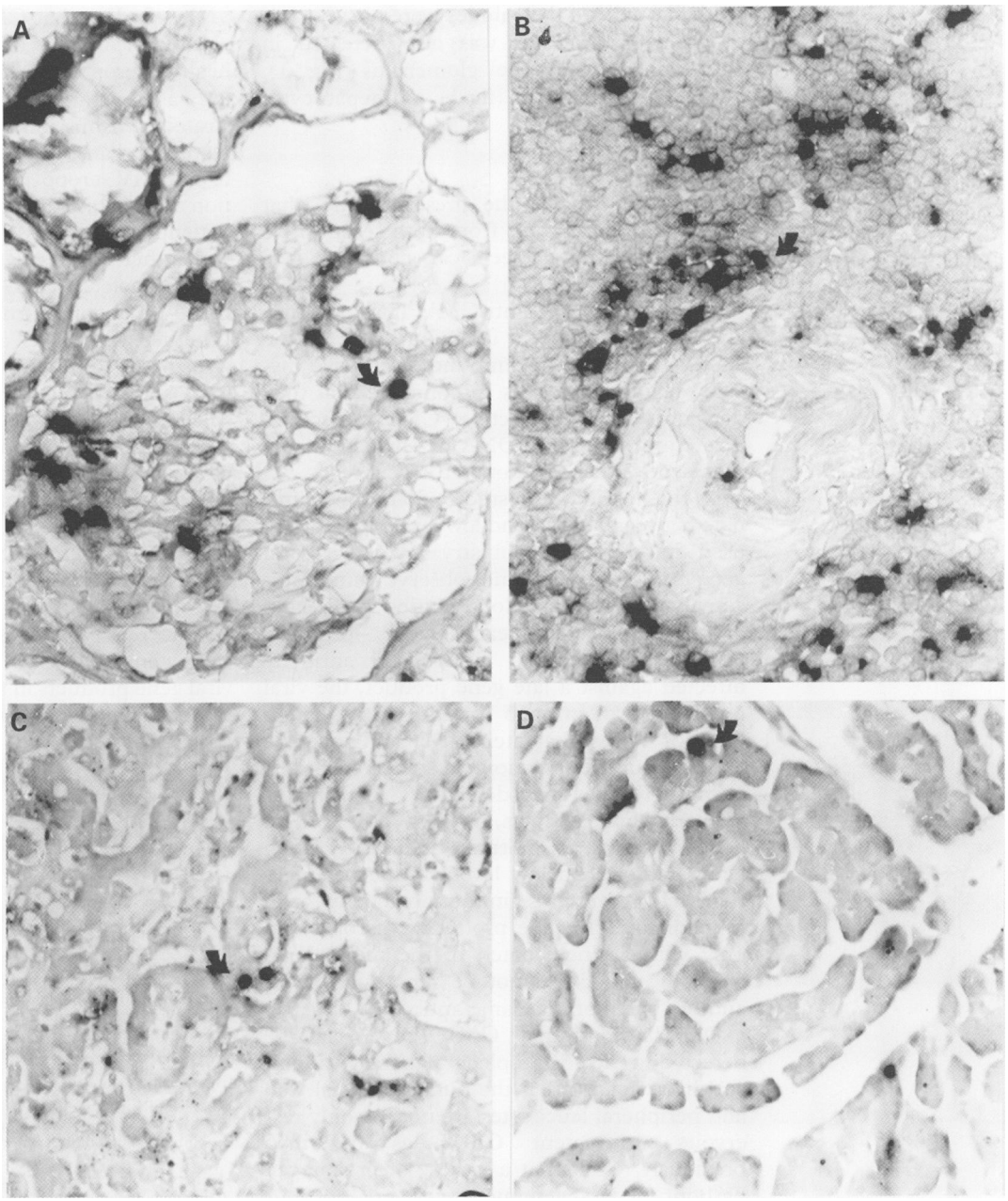

Figure 1 In situ DNA hybridisation (arrow) of samples from four organs, using CMV specific probes. All sections were counterstained with methyl green. (A) Kidney, (B) spleen, (C) liver, and (D) pancreas. (Original magnification ×400.)

Results

SEROLOGY

Serum samples from four group 1 subjects did not contain CMV antibodies. All other subjects were CMV positive. Results of all IgG tests showed 100% agreement. No CMV reactive antibodies of the IgM class were detected, indicating that none of the subjects had had active CMV disease.

IMMUNOHISTOCHMISTRY

Immunohistochemical studies were done using both monoclonal antibodies. Immunoreactivity was not observed in any of the samples tested.

DOT BLOT HYBRIDISATION

Twenty three tissue samples from group 1 subjects were subjected to dot blot hybridisation. Overall, six of seven samples of aorta, 10 of 10 samples of spleen and three of six samples of pancreas harboured CMV DNA (table 1). Tissue samples from three of four seronegative donors (two aorta, three spleen and one pancreas) were evaluated using this technique. Of these, samples of aorta and spleen contained viral DNA, while that of the pancreas did not.

PCR

In group 1 (table 1), nine of 13 abdominal aorta, 12 of 12 spleen, and six of eight pancreatic samples contained viral DNA. CMV DNA was also found in three of four seronegative subjects. In group 2, eight of eight spleen, eight of eight liver and eight of eight kidney samples harboured CMV DNA. The few samples which did not react with the CMV primers reacted well with the β-globin primers.

None of the numerous negative controls included nor any of the samples containing DNA from the other known herpesviruses were positive.

IN SITU HYBRIDISATION

The red pulp of the spleen often contained CMV positive cells (fig 1). In the kidney the

epithelial cells of the tubules were positive frequently. CMV DNA was also detected in epithelial cells of the glomerulus (fig 1). Pancreatic acinar cells (fig 1) and hepatocytes were often positive.

Discussion

We have shown, in the present study, that potential donor organs from both seropositive and seronegative subjects can contain CMV DNA.

All subjects were anti-CMV IgM negative, excluding active CMV infection. None of the subjects had had an immunosuppressive disease, again allowing us to conclude that they did not have an active CMV infection. Therefore, the CMV DNA detected probably represents latent and not active virus.

As the hybridisation studies do not discriminate between latency and low level infection we used immunohistochemistry to detect viral gene products. We did not observe immunoreactivity with a monoclonal antibody directed against immediate early gene products (UL123) nor with a monoclonal antibody directed against a late gene product, the viral tegument protein pp65 (ppUL83(pk)). These findings suggest that latent CMV and not a low level productive infection was detected. Interestingly, Toorkey *et al*[14] detected CMV immediate early gene products, but not early nor late gene products, in normal tissue suggesting latent rather than low level productive infection. However, this immunoreactivity with immediate early gene products was observed only with a single batch of the monoclonal antibody and could not be reproduced with another batch of that same antibody.

Our findings that CMV DNA can be detected in the aorta, spleen, pancreas, kidney, and liver suggest that the human arterial wall and peripheral leucocytes are not the only reservoirs for latent CMV, as described previously,[3-8] but that a variety of organs can harbour the viral genome.

PCR is very sensitive to cross contamination between specimens[15] and to contamination by previously amplified DNA.[12] However, the overall agreement of results obtained by dot blot hybridisation, in situ hybridisation and PCR, and the absence of viral DNA in the numerous negative controls included in the PCR experiments in this study preclude the possibility of cross contamination.

PCR studies of murine cytomegalovirus (MCMV) have shown that lung, spleen, brain, heart, kidney, liver, and salivary glands harbour latent MCMV DNA.[16-19] The exact nature of the cell(s) carrying this MCMV DNA remain obscure. As shown in the present study by in situ DNA hybridisation, many different cell types, such as hepatocytes, tubular and glomerular cells, and splenic red pulp cells, contained viral DNA. During active infection the virus is distributed widely and replicates in virtually all cell types as shown by the appearence of specific viral cytopathological changes, the presence of viral antigens and the presence of viral DNA in various cells in almost all organs.[20 21]

Our finding that CMV DNA is present in a variety of organs obtained from previously healthy seropositive subjects was not surprising, as several studies have clearly demonstrated transmission of CMV present in transplanted organs and peripheral blood obtained from CMV seropositive donors.[22] The observation that in this study three of four seronegative patients carried CMV DNA is in accordance with other reports describing the presence of CMV nucleic acids in leucocytes[7 8 23 24] and tissue[25 26] obtained from seronegative patients. Whether this CMV DNA represents latent virus or defective virus remains unknown.

In summary, a variety of organs such as spleen, kidney, liver, and pancreas contain CMV DNA as demonstrated by PCR and dot blot hybridisation. The presence of CMV DNA was demonstrated in seropositive and also in a number of seronegative subjects by dot blot hybridisation, in situ hybridisation and nested PCR. Arterial smooth muscle cells, hepatocytes, tubular and glomerular kidney cells, splenic red pulp cells and pancreatic acinar cells, contained CMV DNA. The absence of viral gene products in these cells suggests latent rather than low level active infection.

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