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The Presence of Cytomegalovirus Nucleic Acids in Arterial Walls of Atherosclerotic and Nonatherosclerotic Patients

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The presence of cytomegalovirus (CMV) nucleic acids was demonstrated in abdominal aortas and femoral arteries of patients with and without atherosclerosis by dot blot and* in situ *DNA hybridization using a DNA probe derived from immediate early genomic regions. Viral antigens could not be detected by immunohistochemistry and infectious virus could not be recovered from the arterial wall by virus isolation techniques. The high percentage (55%) of vascular wall specimens containing CMV nucleic acids, in atherosclerotic as well as in control material and the location of CMV-containing cells in arteries without gross changes indicative of atherosclerosis suggest that the human arterial wall may be a site of latency for this virus. (Am J Pathol 1989, 134:1151–1157)

Infections with cytomegalovirus (CMV), a member of the herpes virus group, are common. A characteristic feature of these viruses is that they persist in the host after primary infection and frequently reactivate from this latent state. This is clearly demonstrated in renal transplant recipients, who almost invariably show evidence of active CMV infection.[1]

Recently, several reports have suggested a role for CMV in the etiology of atherosclerosis. Studies in an animal model (chicken) have shown that infection with Marek's disease virus (MDV), a herpes virus, results in an extensive atherosclerosislike disease in normocholesterolemic chickens. Using *in situ* DNA hybridization techniques it was possible to show that MDV nucleic acid sequences are present in the arterial walls of the infected chickens.[2]

In humans the presence of herpes viruses in the arterial wall also was demonstrated by DNA *in situ* hybridization and immunohistochemical techniques. Benditt and coworkers[3] detected herpes simplex virus (HSV) by *in situ* DNA hybridization of aortic wall specimens obtained from patients undergoing coronary bypass surgery. Melnick et al[4] described the presence of CMV antigens in smooth muscle cells cultured from tissues obtained from patients undergoing endarterectomy or coronary bypass surgery. Four years later these findings were confirmed by the same group using *in situ* DNA hybridization techniques.[5] Recently the presence of HSV and CMV in the coronary arteries and thoracic aortas of young trauma victims without overt heart disease could be demonstrated by *in situ* DNA hybridization and immunohistochemical techniques.[6] Most of these reports focus on the presence of CMV in arterial tissues obtained from patients with symptomatic atherosclerotic disease of the coronary and/or carotid arteries. It remains possible that the presence of CMV nucleic acids in atherosclerotic arterial walls reflects a site of latency, as proposed by others,[4–6] and that the virus will also be present in healthy arterial vessels.

In this report we describe the presence of CMV in arterial wall tissues in patients with overt atherosclerosis as well as trauma victims and patients without visible atherosclerosis at autopsy. In this study, virus isolation, immunohistochemistry, and DNA hybridization techniques with a probe derived from Immediate Early genomic regions were used.

Materials and Methods

Patients and Controls

To study the presence of CMV in the arterial wall, tissue samples were obtained from the femoral artery or abdominal aorta of CMV-seropositive patients undergoing reconstructive vascular surgery for atherosclerotic disease. The patients received femoropopliteal bypass grafts or synthetic bifurcation grafts. Controls consisted of CMV-seropositive patients on whom autopsy was performed. Only

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patients dying of nonatherosclerotic disease and with maximally grade I atherosclerosis of the aorta as shown at autopsy were included. In addition, a small group of controls consisting of young trauma victims available for donor nephrectomy were included in this study. In the control groups, tissue samples were obtained from the abdominal aorta.

Virus Isolation

A 10% (wt/vol) homogenate of arterial wall specimens was inoculated onto monolayers of diploid human embryonic fibroblasts (HEF, Flow 2002). Inoculation was performed under a centrifugal force of 1000*g* for 1 hour at 37 C. Subsequently, cultures were placed on maintenance medium consisting of Eagle's minimal essential medium (MEM, GIBCO, Grand Island, NY) containing 10% fetal calf serum (FCS, GIBCO) and antibiotics. The cultures were incubated at 37 C and examined microscopically twice weekly during a period of 5 weeks for the development of a cytopathologic effect (CPE). After 1 and 5 weeks the presence of viral antigens in the monolayer was analyzed using the indirect immunofluorescence technique. For the detection of viral antigens monoclonal antibodies (Dupont, Wilmington, DE) directed against CMV immediate early antigens (IEA) were used.

Fixation of Tissues

Arterial tissues for *in situ* hybridization were fixed in a modified Carnoy's solution containing 73,5% absolute ethanol, 24,5% glacial acetic acid, and 2% formaldehyde (37%). After overnight incubation at 4 C, the tissues were rehydrated by successive 30-minute washes in graded alcohols (100, 95, 85, 80, and 70% ethanol, respectively). Final storage of specimens was in 70% ethanol at 4 C. Arterial specimens for dot blot hybridization were finally minced, placed in RPMI-1640 (GIBCO) medium and an equal volume of 95 to 100% ethanol was added slowly under gentle agitation. Fixed tissues were stored at 4 C in 50% ethanol.

Monoclonal Antibodies

Monoclonal antibody H88 directed against immediate early antigens of CMV, prepared following standard procedures, was used in this study. The method employed for producing hybridomas secreting CMV antibodies has been described for rat CMV (RCMV) in detail previously.[7] Briefly, Balb/c mice were immunized with sonicated suspension of CMV strain AD169 infected HEF. After 4 weeks their spleen cells were fused with mouse myoloma cells of the SP 2/0 Ag-14. Hybrid cells were selected in hypoxanthine-aminopterin-thymidine medium and CMV antibody-producing clones were identified by enzyme-linked immunosorbent assay and indirect immunofluorescence staining on CMV-infected HEF monolayers as described previously for RCMV.[7] Selection of the clones producing antibodies to the immediate early (IE) antigens of CMV was done using infected HEF blocked with cycloheximide and actinomycin D to only induce immediate early antigens.[12]

Monoclonal antibody H88, which selectively precipitates a 68 kd protein from infected fibroblasts, was used in this study. This monoclonal antibody gives a uniform nuclear fluorescence in CMV infected fibroblasts within as little as 12 hours after infection. No cross-reactivity with other herpes viruses could be detected. High-titer mouse ascitic fluid was produced by infecting the antibodyproducing clone intraperitoneally into pristane-treated mice.

Immunohistochemistry

Thin tissue sections of approximately 4 μ mounted on glass slides were deparaffinized and washed in 90% ethanol, followed by blocking of endogenous peroxidase activity by incubation in 100% methanol with 0.3% H_2O_2 for 30 minutes.

The sections were then washed in phospate-buffered saline (PBS), preincubated with PBS containing 2% bovine serum albumin (BSA) for 30 minutes, followed by incubation for 60 minutes with one of the mouse MAbs, washed again in PBS for 5 minutes, followed by incubation with biotinylated affinity purified sheep anti-mouse Ig (Amersham) 1:200 in PBS containing 2% BSA for 60 minutes, and washed again for 5 minutes in PBS followed by incubation with biotin-streptavidin-HRP complex (Amersham) 1:400 in PBS containing 2% BSA for 45 minutes.

After washing again in PBS the sections were incubated for 10 minutes in diaminobenzidine-4HCl-substrate 0.05% in TRIS-HCl buffer pH 7.4, containing 0.002% H_2O_2. The color reaction was stopped by washing in PBS, followed by counterstaining for 1 minute in hematoxylin, dehydration, and mounting in Entellan (Merck, Darmstadt, FRG).

DNA Probes

Plasmid pBR 328 containing the 7,0 Kb ES fragment, an EcoRI-Sal1 subdigestion of the EcoRI J. Fragment of CMV strain AD 169 (Figure 1) was provided by Dr. J. Geelen, University of Amsterdam, The Netherlands. Plasmid was transfected into an *Escherichia coli* mutant J.M. 109 and

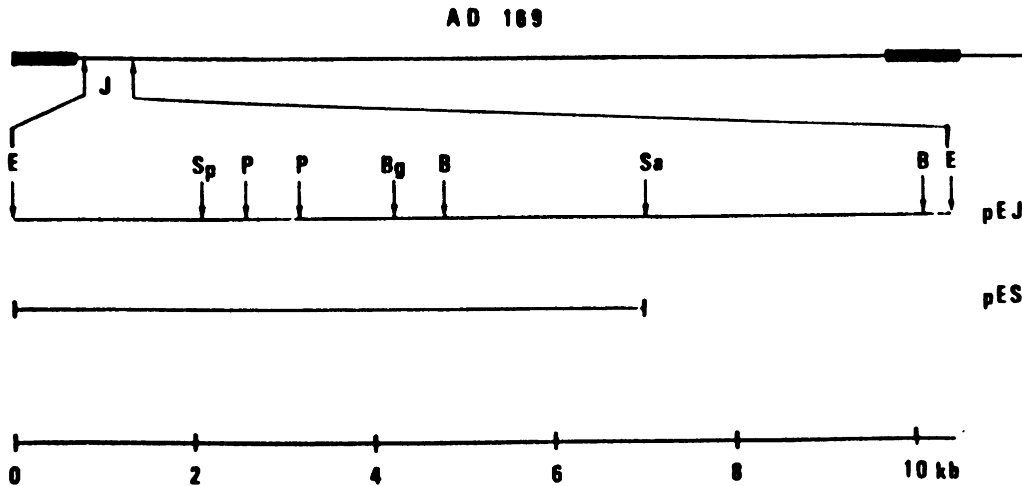

Figure 1. *Restriction endonuclease cleavage map of the cloned EcoRI-J fragment and the subcloned 7.0 kb pES fragment. The top line represents the 236 kb genome of HCMV strain AD169 and shows the location of the EcoRI-J fragment in the prototype orientation. pES indicates the cloned EcoRI-J-Sal1 fragment. P(PvUII); E(EcoRI); B(BamHI); Bg(BglII); Sa(Sal1); Sp(SphI) represent the restriction sites present in the pES fragment.*

pure ES fragment DNA was recovered using standard procedures.[8] Controls consisted of total CMV strain AD 169 DNA, total rat cytomegalovirus (RCMV) DNA,[9] and plasmid pBR 328 DNA without inserts. In all DNA biotin was inserted by incorporation of biotin-11-dUTP (Bethesda Research Laboratories, Bethesda, MD) using a random primed DNA labeling kit (Boehringer). The specificity of the biotinylated DNA probes was evaluated in uninfected HEF, HEF infected with CMV laboratory strain AD 169, and in arterial samples using both dot blotting and *in situ* hybridization. The *in situ* hybridization procedures also were performed without DNA probes to evaluate nonspecific reactions.

Dot Blot Hybridization

Extraction of cellular DNA was performed essentially as described previously.[10] After extensive washings the ethanol fixed tissues were digested overnight at 50 C in a mixture containing 10 mM TRIS-HCl, pH 7,5, 5 mM EDTA, 100 mM NaCL, 0,2 mg/ml proteinase K (Boehringer), and 10 mg/ml SDS. Proteins were removed using successive phenol-chloroform and chloroform extractions and DNA was recovered by ethanol precipitation. RNA was digested by incubating the precipitate for 30 minutes at 37 C in a mixture of 10 mM TRIS-HCl pH 7,5, 1 mM EDTA and 75 μg RNAse A (Sigma Chemical Co., St. Louis, MO). DNA was again ethanol precipitated, air dried, and redissolved in distilled water. DNA was quantified by spectrophotometry at 260 and 280 nm. For hybridization 5 to 10 μg DNA (NaOH denatured) was spotted onto nitrocellulose paper (BA 85, Schleicher and Schull). After incubation at 80 C for 2 hours, filters were prehybridised for 4 hours at 42 C in a mixture containing 50% deionised formamide 5× SSC, 5× Denhardt's solution, 25 mM sodium phosphate buffer, pH 6.5, and 0.5 mg/ml heat denatured herring sperm DNA. After this period the prehybridization solution was replaced with 150 ng/ml heat denatured dUTP probe and 5% (wt/vol) dextran sulphate in the same solution. Hybridization was carried out at 42 C for 12 hours in a roller drum at 1 to 2 rpm. Filters were washed in two changes of 2× SSC, 0.1% SDS at room temperature for 5 minutes each, twice in 0.2 SSC, 0.1% SDS at room temperature for 5 minutes each, twice in 0.16 SSC, 0.1% SDS at 55 C for 15 minutes each, and finally once in 2× SSC at room temperature for 1 minute. Visualisation of successful hybridization was performed with the BLU gene kit (Bethesda Research Laboratories). The specificity of the biotinylated probes was evaluated with DNA extracted from CMV strain AD 169 infected HEF and uninfected HEF spotted onto nitrocellulose paper. Only ES probe and total CMV probe showed hybridization with DNA extracted from infected HEF but not with DNA extracted from uninfected HEF. RCMV and plasmid probes did not show hybridization.

In Situ *Hybridization*

Arterial specimens fixed in a modified Carnoy's solution were embedded in paraffin blocks using routine histopathologic procedures. Sections 3 to 4 μ thick were cut from these blocks and placed on chromium-alum-gelatin coated and glutaraldehyde activated slides.[11] After overnight incubation at 52 to 54 C, sections were dewaxed in xylene, washed once in 100% ethanol, and air dried.

Table 1. *Characteristics of Patients Series Under Investigation*

Patients	Biopsies	Mean age (years ± SD)	Range (years)	Men	Women
Grade III atherosclerosis (surgical patients)	Femoral artery	70 ± 9.5	55–88	10 (45%)	12 (55%)
	Abdominal aorta	62 ± 10.0	44–80	20 (90%)	2 (10%)
No atherosclerosis (autopsy)	Abdominal aorta	69 ± 13.6	45–91	10 (43%)	13 (57%)
Trauma victims	Abdominal aorta	32 ± 18	9–60	6 (75%)	2 (25%)

Aldehyde groups were inactivated by incubating the slides with 1% ammoniumchloride in PBS. Proteolytic digestion with proteinase K (Boehringer) (2.5 to 20 μg/ml) in 20 mM TRIS-HCl, pH 7.4 containing 2 mM $CaCl_2$ was carried out for 30 minutes at 37 C, and the reaction was subsequently stopped by washing the slides with PBS containing 2 mg/ml glycine. Slides were then dehydrated by successive washings in graded alcohols (30, 60, 80, 95 and, 100% ethanol, respectively) and air dried. When needed, RNA digestion was carried out by incubating the slides with 100 μg/ml RNAse A (Sigma) in PBS at 37 C, 1 hour, and the reaction was stopped by 2 successive washings in PBS. Slides were again dehydrated by successive washings in graded alcohols. Immediately after these pretreatment steps, samples were overlaid with 48% deionised formamide, 10% dextran sulphate, 5 × SSC, 5 × Denhard's solution, 50 mM sodium phosphate, pH 6.4, 0.1 μg/ml herring sperm DNA, and 200 μg/ml probe DNA covered with a silicon-coated coverslip and sealed with rubber cement. Cellular and probe DNA were denatured by heating the slides to 83 C and immediately immersing them in ice-cold water. Hybridization was carried out overnight at 42 C. Washing procedures and visualization of formed hybrids was performed as described for dot blot hybridization.

The specificity of the biotinylated probes was evaluated on CMV strain AD 169 infected HEF monolayers and on uninfected HEF monolayers. Only ES probe and total CMV probe showed hybridization with infected HEF monolayers, but not with uninfected HEF monolayers. RCMV and plasmid probes never showed hybridization.

Statistical Analysis

Statistical analysis was carried out using Fisher's exact tests. Results were considered significant when $P < 0.01$.

Results

Patients and Controls

Tissue samples were obtained from 44 patients undergoing vascular surgery (Table 1). Twenty-two samples were taken from the abdominal aorta (20 men, 2 women) and 22 samples were taken from the femoral artery (10 men, 12 women). The mean age of these patients was 66 years. Controls consisting of 8 trauma victims (6 men, 2 women) with a mean age of 32 years were used. Arterial samples were taken from the abdominal aorta during donor nefrectomy.

The autopsy-control series contained 23 patients (10 men, 13 women) with a mean age of 69 years. The abdominal aorta samples were taken within 12 hours after death. Mean ages of the atherosclerotic and autopsy control group were in the same range, as was the distribution among the sexes. Statistical analysis of these data showed no significant differences, so the groups were suitable for comparison. The mean age of the trauma victim group, however, was too low for comparison.

Virus Isolation

Virus isolation experiments were performed on fresh arterial samples only. In none of the tested samples was a cytopathologic effect (CPE) observed in the HEF monolayers during a 5-week period. Using the indirect immunofluorescence technique with monoclonal antibodies directed against CMV, immediate early antigens (IEA) viral proteins were not detected in the HEF monolayers, indicating the absence of infectious virus.

Immunohistochemistry

Immunohistochemical studies were performed on all arterial samples. Immunoreactivity was not observed in any of the tested samples using MAbs against IEA.

Dot Blot Hybridization

Using the dot blot hybridization technique (Table 2) CMV nucleic acid sequences were detected in 4 of 22 (18%) samples of the femoral artery and in 7 of 22 (32%) samples of the abdominal aorta of patients with severe atherosclerosis. Statistical analysis revealed this difference be-

Table 2. *Results of Dot Blot and* In Situ *DNA Hybridization of Arterial Tissues*

Patients	Biopsies	CMV hybridization: Dot blot number positive/ total number (%)	CMV hybridization: *In situ* number positive/ total number (%)
Grade III atherosclerosis (surgical patients)	Femoral artery	4/22 (18%)	9/22 (41%)
	Abdominal aorta	7/22 (32%)	10/22 (45%)
No atherosclerosis (autopsy)	Abdominal aorta	5/23 (22%)	17/22 (77%)
Trauma victims	Abdominal aorta	4/8 (50%)	5/8 (63%)

tween the two groups to be not significant ($P < 0.447$). By evaluating the extracted DNA in the samples obtained from the abdominal aorta of patients without signs of atherosclerosis, CMV nucleic acid sequences were detected in 5 of 23 (22%) cases, which is not statistically different from the atherosclerosis samples ($P < 0.479$). Of samples from trauma victims, 4 of 8 (50%) were positive for CMV DNA but this group was too small for statistical analysis. Thus, we could conclude that 30% of the samples tested contained CMV nucleic acids using this technique.

In Situ *Hybridization*

In situ DNA hybridization (Figure 2) of sections of arteries obtained from patients with severe atherosclerosis showed that 9 of 22 (41%) biopsies of femoral arteries and 10 of 22 (45%) biopsies of abdominal aortas contained CMV nucleic acid sequences; statistical analysis showed no significant differences between these groups ($P < 0.220$). Evaluation of the samples of the abdominal aorta of patients without atherosclerosis at autopsy revealed that 17 of 22 (77%) contained CMV DNA, which is not statistically different ($P < 0.240$) from the group with atherosclerosis. Five of 8 (63%) samples obtained from the abdominal aorta of trauma patients were positive for CMV nucleic acids; however, the number of trauma victims was too small for statistical analysis. Thus, we could conclude that 55% of the samples tested contained CMV nucleic acids using this technique. Tissue samples that showed extensive *in situ* hybridization reactivity were analyzed further by RNA digestion before *in situ* DNA hybridization. After RNAse treatment the tissue sections showed a less intense hybridization reaction, and in some cases the reactivity disappeared altogether.

Histologically, the CMV nucleic acid sequences detected with the ES probe were located mainly in apparently normal arterial tissue without an inflammatory reaction or atheromatous changes. Sequential sections, stained with hematoxilin and eosin, showed that the DNA hybridization reactivity was located mainly in smooth muscle cells in the arterial media.

Discussion

This study have demonstrated the presence of CMV nucleic acid sequences in biopsies of femoral arteries and abdominal aortas of atherosclerosis patients as well as controls. CMV nucleic acids were distributed equally among patients with and without this disease. No differences were observed in CMV distribution between biopsies taken from femoral arteries or from abdominal aortas.

The percentage of samples containing CMV nucleic acid (30%) by dot blot hybridization techniques equals the percentage found by others using *in situ* DNA hybridization techniques[5,6] or immunohistochemistry.[4] Using *in situ* hybridization, however, we found that a considerably higher percentage (55%) of the samples investigated contained CMV nucleic acids. There are two possible explanations for this phenomenon.

First, our *in situ* DNA hybridization technique did not include RNAse treatment of the tissue sections. It is therefore likely that the probe DNA formed not only DNA-DNA hybrids but also RNA-DNA hybrids.[11] The finding that tissue samples that showed extensive *in situ* hybridization reactivity showed a less intense hybridization reaction and in some cases became negative after RNAse treatment seems to corroborate this possibility. It is therefore likely that *in situ* mRNA-DNA hybrids contribute significantly to the obtained reactivity. In contrast, extraction of DNA for dot blot hybridization always included RNAse treatment and consequently only the samples sufficiently rich in viral DNA showed hybridization reactivity using this technique.

Second, in our view the most likely explanation of the observed difference is the choice of the CMV DNA probe, which was prepared from the EcoRI-J fragment of CMV strain AD169, containing sequences coding for immediate early transcripts (Figure 1).[12] Recently, latency associated transcripts (LAT) were described in neurons during latent herpes simplex virus (HSV) infection,[14,15] originating from genomic regions coding for immediate early transcripts of HSV.[16-18] These HSV mRNA transcripts could be detected by DNA probes prepared from the immediate early genomic regions.[14] In CMV the immediate early tran-

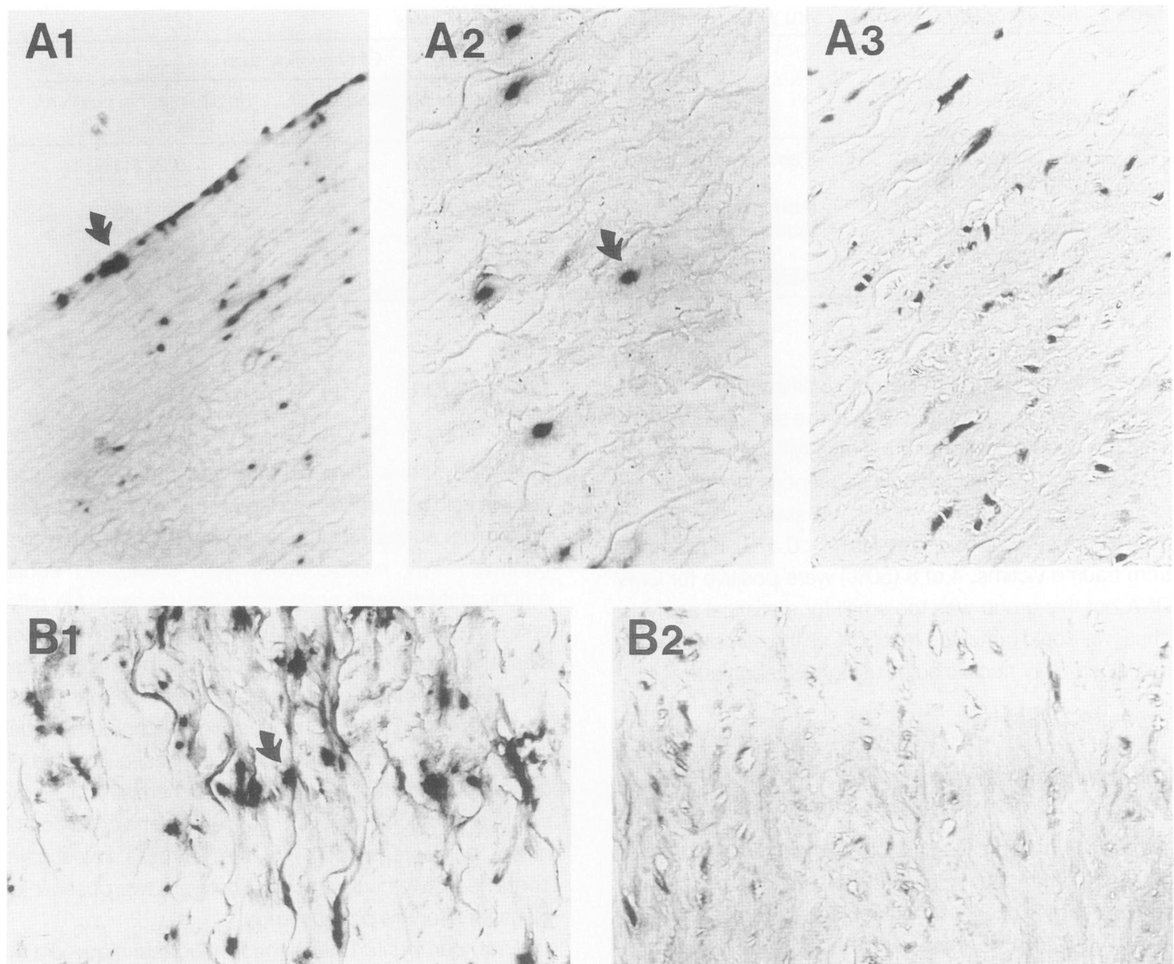

Figure 2. *Photomicrographs of* in situ *hybridization of sections of arterial walls using CMV-specific probes and hemotoxylin and eosin (H&E) staining of sequential sections of arterial walls of two patients (A and B).* **A1**: *An* in situ *hybridization of the intima and media of an arterial wall. The dark spots (arrow) represent the hybridization (×100).* **A2**: *Magnification of A1 showing discrete nuclear hybridization (×250).* **A3**: *Sequential section of A2, stained with H&E, showing a normal arterial architecture.* **B1**: *Another example of an in situ* hybridization. **B2**: *Sequential section stained with (H&E, ×250).*

scripts might therefore not only be transcribed during reproductive viral cycles,[13] but also during latent infections. The high percentage (55%) of arterial samples containing CMV nucleic acids in this study can therefore be explained by the use of the ES probe, because in other studies[5,6] probes coding for nonimmediate early regions were used. Together with the negative results from the virus isolation and the negative results from the immunohistochemical studies, we conclude that the arterial wall may be a site of latency for CMV, as already proposed by others.[4–6]

Although the present results do not suggest a direct role for CMV in the pathogenesis of atherosclerosis, the presence of (latent) virus in the vessel wall might contribute to the process of atherogenesis. The ability to reactivate from the latent status leading to a local lytic infection in the vessel wall might result in repeated local inflammatory reactions, eventually leading to vascular injury. This injury, in cooperation with other established risk factors, could play an as yet unknown role in atherogenesis.

Further studies are needed to elucidate the factors leading to reactivation of the CMV and the development of atherosclerosis as a consequence of a local productive infection.

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