Virchows Arch (2000) 436:539–552

ORIGINAL ARTICLE

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Cytomegalovirus genome and the immediate-early antigen in cells of different layers of human aorta

Received: 10 June 1999 / Accepted: 16 November 1999

Abstract A number of data suggest that reactivation of cytomegalovirus (CMV) latent in arterial wall cells may contribute to atherogenesis; however, there is no direct evidence available. To address this issue, we have examined, using in situ hybridization or immunohistochemical staining, the frequency of occurrence of cells containing viral genome and of those expressing the IE 70 viral antigen in the endothelial layer and in deeper layers of human aortas with or without visible atherosclerotic lesions. Using endothelial cell cultures or tissue endothelial preparations, we found CMV-hybridizing endothelial cells in 6 of 8 grossly normal aortas and in 16 of 18 lesioned aortas. Antigen-positive endothelial cells were detected in 1 of 5 grossly normal vessels and in 6 of 7 lesioned vessels. Infected endothelial cells were abundant in areas adjacent to orifices of intercostal arteries of grossly normal aortas and in fatty spots of lesioned aortas, but no infected endothelial cells were observed in most plaques examined. In paraffin sections of grossly normal vessels, we detected CMV genome in cells adjacent to lumen and in cells randomly scattered through subendothelial intima and the media; however, no immunoreactive viral protein was found in the same tissue samples. In sections of lesioned vessels, clusters of CMV-hybridizing cells were found in the media in addition to infected cells randomly scattered through the intima and the media. In these samples of lesioned vessels, viral antigen was detected in cells adjacent to lumen and in cells clustered at the intima/media border. We found antigen-positive cells in grossly normal areas of lesioned aortas and in fatty lesions, but not in plaques of the same vessels. The data suggest that accumulation of the immediate-early CMV antigen in cells of endothelial layer and development of antigen-positive cell clusters in deeper layers of vascular wall accompany early atherogenic events in human aorta.

Key words Human · Atherosclerosis · Cytomegalovirus

This research was undertaken within the framework of the US–Russia Agreement for Cooperation on Biomedical Research and Public Health, Program Area 1: Pathogenesis of Arteriosclerosis

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Introduction

Cytomegalovirus (CMV) is an important human pathogen with an ability to cause disease in immunocompromised subjects. In humans without clinical symptoms of infection, the virus is present in the arterial wall [34]. The demonstration of herpes virus-induced atherosclerosis in an animal model [8] provides a basis for the assumption that reactivation of latent CMV infection may contribute to atherogenesis in humans [19]. In seroepidemiologic studies, high levels of CMV antibodies were found to be associated with clinically manifested atherosclerosis [1]. It was further demonstrated that heart-transplant recipients, who are actively infected with CMV, are particularly prone to develop accelerated atherosclerosis in the transplanted organ [10]. Again, a high amount of tumor suppressor protein p53 was found in smooth muscle cells in coronary restenosis of patients after angioplasty, and the association of the p53 with the IE 84 antigen of CMV was demonstrated [26].

In view of these findings, many efforts have been made to look for a causal relationship between the presence of the CMV genome in the artery wall and atherosclerotic lesion formation; however, the question has not yet been resolved. Strong evidence has been collected

that CMV DNA sequences are present both in grossly normal vessels and within arterial lesions [11, 18]. Since attempts to isolate infectious virus from arterial tissue have failed, a possibility for activation of a complete cycle of viral replication in lesioned vessels remains questionable. Similarly, there are no data available that could support or disprove the assumption that CMV reactivation restricted by early viral functions accompanies atherogenesis. In fact, the CMV antigens were detected in vessels exhibiting atherosclerotic injury, and some antigen-positive cells were found to correspond to endothelial cells or smooth muscle cells on the basis of morphology and tissue location [34]. However, this finding cannot be interpreted in terms of virus reactivation, since pattern of CMV genome expression in arterial wall cells before the appearance of atherosclerotic lesions is not yet known. Thus, to understand the relevance of vascular CMV infection with regard to atherosclerosis, it is necessary to determine what cells serve as a reservoir of CMV infection in grossly normal vessels and whether any alterations of CMV genome expression in these cells accompany atherosclerotic lesion formation.

It is reasonable to certify whether the CMV infection is active or latent by evaluation of the immediate-early CMV antigens which, being the first CMV gene products expressed after the virus infects the host cell, are critical requirements for sequential viral gene expression [20]. With this in mind, we have examined, using polymerase chain reaction (PCR), in situ hybridization, or immunohistochemical staining, the frequency of occurrence of cells containing the CMV genome and of those expressing the IE 70 viral antigen in the endothelial layer and in deeper layers of the human aorta with or without visible atherosclerotic lesions. The data suggest that accumulation of the immediate-early CMV antigen in cells of the endothelial layer and development of antigen-positive cell clusters in deeper layers of vascular wall accompany early atherogenic events in the human aorta.

Materials and methods

The thoracic segment of aorta taken at autopsy within 4 h postmortem from trauma victims aged 16–73 years was used in this study.

Cell cultures

Human aortic endothelial cells (HAEC) were harvested from the vessel as described by Antonov et al. [3]. Cells were cultured in Medium 199 (Gibco) supplemented with 10% human donor serum and antibiotics. For immunodetection of the CMV IE protein, cells isolated from the vessel were seeded in 2-cm^2 wells (~10^5 cells/well) on gelatin-coated coverslips and grown at 37°C in humidified air with 5% CO_2 for 2 days before fixation. For detection of the CMV genome, cells isolated from the vessel were stored in a 25-cm^2 flask for 1 week to eliminate blood cell contaminants, then either frozen for PCR or seeded in 2-cm^2 wells (4×10^4 cells/well) on polylysine-coated coverslips and fixed on the next day for in situ hybridization. Mitotic cells were not observed in human aortic endothelial cell (HAEC) cultures throughout the first week after isolation. Endothelial origin and purity of HAEC cultures were confirmed by immunostaining with polyclonal antibodies to human von Willebrand factor (vWF) (Sigma).

As an additional control, human umbilical vein endothelial cells (HUVEC) were tested for the presence of the CMV genome. Endothelial cells were harvested from umbilical vein according to standard protocol [2] and propagated in Medium 199 supplemented with 10% human donor serum, 100 µg/ml endothelial cell growth factor (bovine), 12 U/ml sodium heparin, and antibiotics. Cells were passed weekly at a ratio of 1:2. HUVEC culture of the third passage was used for PCR and in situ hybridization.

Human embryo lung fibroblasts (HELF) non-infected or infected with CMV (AD 169) served as negative or positive controls for PCR, in situ hybridization, and immunohistochemistry. HELF were propagated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics. HELF cultures of the 10th passage were used for experiments. For acute infection, a confluent monolayer of HELF was inoculated with CMV at a multiplicity of 1 PFU/cell. Cells were fixed between day 7 and day 10 post-infection.

Polymerase chain reaction

To detect the CMV DNA in cultured endothelial cells, two-round PCR with nested primer oligonucleotides was carried out. Cells (5×10^5) were lysed in 200 µl 0.5% Tween 20 in 10 mM Tris-HCl (pH 8.0) and digested with 100 µg/ml proteinase K at 56°C for 1 h followed by heating to 95°C for 10 min. The tubes were centrifuged for 3 min at 6000 rpm. A 3-µl aliquot of the supernatant was used for the PCR.

Four oligonucleotide primers relevant to the human CMV major immediate-early exon 3–exon 4 region were synthesized using DNA sequence data. The outer primers, upstream C1 (5'-GCAACGAGAACCCCGAGAAAG-3') and downstream C4 (5'-AAGCCATAATCTCATCAGGG-3'), produced a 698-bp amplimer. The inner primer set consisted of C0 (5'-GCGGCATAGAATCAAGGAGCACATG-3') and C2 (5'-CAAGCCATCCACATCTCCCGC-3') flanking a 222-bp amplimer. PCR was carried out in a 30-µl volume containing 3 µl of the tissue extract, 0.3 µM of each primer, 2.5 mM $MgCl_2$, 16 mM ammonium sulfate, 67 mM Tris-HCl (pH 8.8), 0.01% Tween 20, 0.2 mM of each dNTP, and 1 U *Taq* polymerase (Promega). A total of 30 cycles were performed, each involving denaturing at 95°C for 1 min, annealing at 50°C for 1 min, and extending at 72°C for 1 min. Extending during the final cycle was continued for 7 min at 72°C. In the second round of amplification with primers C0 and C2, the annealing was performed at 55°C for 1 min. To confirm the CMV-specific nature of the final DNA product, the 222-bp amplimer was digested with *Taq* I restriction endonuclease, producing 132-bp and 90-bp fragments. Amplified DNA fragments and digested DNA were subjected to electrophoresis in 1.5% agarose gels, stained with ethidium bromide and visualized using ultraviolet (UV) fluorescence. As a positive control, PCR was performed with a set of primers amplifying the human steroid sulfatase gene.

Prehybridization processing of cell and tissue samples

Prehybridization processing was carried out according to Schrier et al. [25]. Cells grown on coverslips were fixed with periodate–lysine–paraformaldehyde fixative (PLP) for 20 min, then with 95% ethanol for 15 min. After treatment with 0.2 M HCl for 10 min, the samples were permeabilized with 1% Triton X-100 for 5 min at room temperature, postfixed for 4 min with PLP, dehydrated through alcohol gradient, and air dried.

For detection of the CMV genome in the intact endothelial layer of human aorta, the preparations of intact aortic endothelium (IAE) were used. The thoracic aorta was incised lengthwise, washed in PBS, and fixed with PLP for 20 min. Thereafter, the aortic segment was immersed in PBS, and fragments of about 10 mm^2 were excised from grossly normal areas of vessel and areas containing fatty lesions or plaques. The thin layer of intima adjacent to the lumen was separated using fine forceps, post-fixed

with 95% ethanol for 15 min, and air dried following the completion of prehybridization processing (see above). Just before the hybridization procedure, IAE preparations were stained with antibodies against vWF to visualize endothelial layer as described later in detail.

In some IAE preparations, the endothelial layer was visualized by means of silver impregnation of cell borders using a modification of the method by Poole et al. [23]. The thoracic aorta was fixed with PLP for 5 min, treated for 2 min with 5% silver nitrate diluted in 5% glucose, washed in water, rinsed with methol-hydrohynon developer, and fixed with PLP for a further 15 min. The aortic segment was immersed in PBS, and approximately 10-mm^2 fragments were excised from grossly normal areas of vessel and areas containing fatty lesions or plaques. The thin layer of intima adjacent to the lumen was separated, post-fixed with 95% ethanol for 15 min, and air dried following the completion of prehybridization processing. Prior to hybridization, silver-impregnated IAE preparations were treated for 15 min with 3 mg/ml pepsin in 0.2 M HCl. It should be noted that a similar pattern of intranuclear staining was detected by in situ hybridization in CMV-infected HELF non-treated or treated with silver nitrate by the same manner as the preparations of intact aortic endothelium.

In addition to IAE preparations, paraffin-embedded tissue samples were prepared from the same vessels. Tissue pieces were excised from grossly normal areas and areas containing fatty lesions or plaques immediately after the initial fixation of thoracic aorta with PLP. The samples were fixed with PLP for a further 1 h and embedded in paraffin by routine procedure. Paraffin sections were dewaxed, treated for 15 min with 3 mg/ml pepsin in 0.2 M HCl and then with 1% Triton X-100 for 5 min at room temperature, dehydrated through alcohol gradient, and air dried.

Combination of in situ hybridization with immunohistochemistry

To identify infected endothelial cells in cell cultures or in IAE preparations, immunostaining for vWF was performed just before the hybridization procedure as described previously [24]. Cell or tissue samples were sequentially incubated with polyclonal rabbit antibodies to vWF (Sigma) and alkaline phosphatase-conjugated goat anti-rabbit antibodies (Vector). Bound antibodies were visualized with 5-bromo-4-chloro-3-indolylphosphate/nitrobluetetrazolium substrate kit (Vector). After inactivation of endogenous peroxidase, the hybridization procedure was carried out. It should be noted that the specimens subjected to the prehybridization processing up to heating at 95°C demonstrated the same pattern of staining for vWF as those tested immediately after the initial fixation with PLP, namely, blue-stained Weibel-Palade bodies were clearly visible. However, homogeneous blue staining of cytoplasm was detected following the completion of in situ hybridization in accordance with the results obtained by Roberts et al. [24]. In IAE preparations, the endothelium was detected as a thin layer of blue color visible at the one side of each specimen.

In situ hybridization

In situ hybridization with biotin-labeled CMV DNA probe (Enzo Diagnostics) was carried out in cultured cells, in IAE tissue preparations, and sections of paraffin-embedded tissue samples. The hybridization procedure employed was a modification of a protocol described by Brigati et al. [4]. Prior to hybridization, all the samples were treated for 20 min with 0.3% H_2O_2 in methanol to inactivate endogenous peroxidase. The hybridization mixture consisted of 50% deionized formamide, 10% dextran sulfate, 2×sodium saline citrate (SSC; 0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0), 5× Denhardt's solution, 400 µg/ml of herring sperm DNA, and 1 µg/ml human CMV DNA probe. About 20 µl of the hybridization solution was added to each sample and heated at 95°C for 10 min in a hermetically closed container to denature the probe and cell DNA simultaneously. Following overnight incubation at 37°C, the samples were washed twice with 50% formamide in 2×SSC at 37°C, twice in 2×SSC at 37°C, once in 1×SSC at 37°C, and once in 1×SSC at room temperature. For detection of a hybridized probe, the samples were incubated with avidin-biotinilated horseradish peroxidase complex (Vector), goat biotin-labeled anti-avidin (Vector) and again with avidin-biotinilated horseradish peroxidase complex. The last two steps were replicated. Bound conjugate was visualized using a substrate kit containing 3-amino-9-ethylcarbazole (Vector). The samples were mounted in Aqua-Poly/mount medium (Polysciences).

The specificity of hybridization signals was evaluated by testing of non-infected or CMV-infected HELF cultures and paraffin-embedded samples of retina obtained from trauma victims or from subjects with acute CMV infection. To confirm the specificity of hybridization signals in IAE preparations, a part of samples within each tissue group examined (grossly normal pieces, lipid spots, plaques) were processed with the omission of the CMV probe.

Immunodetection of the immediate-early CMV protein

A monoclonal antibody against the 70-kDa immediate-early viral polypeptide (Virostat) that is present in the nucleus of productively infected fibroblasts for all times of infection was used for detection of infected cells in HAEC cultures, IAE tissue preparations, and paraffin sections of the aortic wall. To detect the CMV antigen in cultured endothelial cells, indirect double immunofluorescent staining for p70 and vWF was carried out. Cells grown on coverslips were fixed for 10 min with 3.7% formaldehyde in PBS, permeabilized with 1% Triton X-100 for 10 min at room temperature, blocked with casein and sequentially incubated with monoclonal antibody to p70, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma), polyclonal antibodies to vWF (Sigma), TRITC-conjugated goat anti-rabbit IgG (Sigma), and DNA dye 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) at a concentration of 1 µg/ml. The coverslips were mounted in Moviol containing 0.1% phenylenediamine and viewed with an Opton epifluorescence photomicroscope III.

To detect the CMV antigen in IAE tissue preparations, indirect immunohistochemical staining for p70 was combined with silver impregnation of endothelial cell borders. Thoracic aorta was incised lengthwise, washed in PBS, fixed with PLP for 5 min, washed in 20 mM Hepes, pH 7.4, and treated for 2 min with 5% silver nitrate diluted in 5% glucose solution prepared in 20 mM Hepes. Following a rinse in Hepes buffer, the aortic segment was immersed in PBS, and fragments of 5–10 mm^2 were excised from grossly normal areas of the vessel and fatty lesions. The thin layer of intima adjacent to the lumen was separated using fine forceps, post-fixed with PLP for 5 min, permeabilized with 1% Triton X-100 for 15 min at 37°C, and treated with 0.3% H_2O_2 in methanol for 20 min to inactivate endogenous peroxidase. Following overnight block with 0.4% casein in PBS, preparations were sequentially incubated with antibody to p70, biotin-labeled goat anti-mouse IgG (Amersham), and avidin-biotinilated horseradish peroxidase complex (Vector) each for 30 min at 37°C using a shaker. It should be noted that similar results were obtained if the samples were incubated in each reagent for 2 h at room temperature without a shaker. The bound conjugate was visualized using a substrate kit containing 3-amino-9-ethylcarbazole (Vector). Thereafter, preparations were treated with methol-hydrohynon developer for 1 min to visualize endothelial cell borders. The samples were mounted in Aqua-Poly/mount medium (Polysciences). Non-infected or CMV-infected HELFs treated in the same manner as IAE preparations were used as negative or positive controls, respectively. To monitor monotony of immunostaining along the endothelial layer, some IAE preparations were stained with monoclonal antibody to α-tubulin or to vimentin (Sigma).

To detect the CMV antigen in sections of paraffin-embedded aortic samples, indirect immunohistochemical staining for p70 was performed. Sections were dewaxed, treated with 3 mg/ml pepsin in 0.2 M HCl for 10 min, then with 1% Triton X-100 for 5 min at room temperature. Following inactivation of endogenous peroxidase with 0.3% H_2O_2 in methanol and blocking in casein, sections were sequentially incubated with antibody to p70, biotin-labeled goat anti-mouse IgG (Amersham), and avidin-biotinilated horse-

radish peroxidase complex (Vector). Bound conjugate was visualized using a substrate kit containing 3-amino-9-ethylcarbazole (Vector). The samples were mounted in Aqua-Poly/mount medium (Polysciences).

As controls for nonspecific binding, cultured cells, IAE preparations, and paraffin sections were reacted with irrelevant murine IgG (Sigma).

Results

CMV genome in the aortic endothelium

To identify CMV-infected endothelial cells in grossly normal or lesioned human aorta, we tested primary cultures of aortic endothelium for CMV DNA using PCR or in situ DNA hybridization. PCR was performed on HAEC cultures obtained from three vessels, namely, a grossly normal thoracic aorta (female aged 35 years) and two aortas exhibiting severe atherosclerotic injury (males aged 43 years and 53 years). As an additional control, HUVEC culture of the third passage was analyzed. The CMV DNA sequences were detected using PCR in all examined HAEC cultures but not in HUVEC culture (Fig. 1). To verify the endothelial identity of cells containing the CMV genome, in situ hybridization combined with immunostaining for vWF was carried out on HAEC cultures obtained from the aortas of 11 subjects aged 16–73 years (cases 1–11 in Table 1). HUVEC culture served as a negative control. In accordance with the PCR data, CMV-hybridizing cells were detected in most of the HAEC cultures but not in HUVEC culture. vWF-positive cells that display hybridization signals were found in both grossly normal and lesioned vessels (Fig. 2A–C). Interestingly, in endothelial cells isolated from grossly normal vessels, signals of the CMV probe were visible in the nucleus (Fig. 2B), whereas, in cultures isolated from vessels with atherosclerotic lesions, some vWF-positive cells exhibited hybridization signals both in nuclei and in cytoplasm (Fig. 2C). The number of endothelial cells containing viral genome varied from 2% to 50% (Table 1). It should be noted that the total number of cells isolated from different vessels also varied over a wide range.

To evaluate the distribution of CMV-infected endothelial cells over the endothelial layer of human aorta with or without visible atherosclerotic lesions, in situ hybridization was carried out in the IAE preparations of thoracic aortas of 15 subjects aged 21–70 years (Table 2). The thoracic segment of each vessel studied was previously grossly examined to estimate the distribution of atherosclerotic lesions. In 3 of 15 vessels (cases 1–3 in Table 2), there was no visible abnormality detected. In 6 vessels (cases 4–9 in Table 2), only fatty lesions were found that looked like yellow-colored, elevated regions in the dorsal part of the aortic segment. Fatty spots were located in close proximity to the orifices of intercostal arteries; some arterial orifices were uninvolved. In some cases, fatty streaks oriented along the blood stream were also seen. In six other vessels (cases 10–15 in Table 2), atherosclerotic plaques were visible as white-colored, firm, elevated regions. Plaques were exclusively located around the orifices of intercostal arteries in five of six vessels. In one vessel, some plaques were also found between the artery orifices. Fatty lesions in vessels with plaques were located either closely to plaque-free orifices or between plaques. The results of our gross examination are in agreement with previous observations establishing arterial branches as atherosclerosis-prone regions [6, 13, 15]. In view of these data, special attention was given to the frequency of occurrence of infected endothelial cells in aortic regions adjacent to orifices of intercostal arteries.

To detect CMV-infected cells in the intact endothelial layer, in situ hybridization was combined either with immunostaining for vWF or with silver impregnation. A

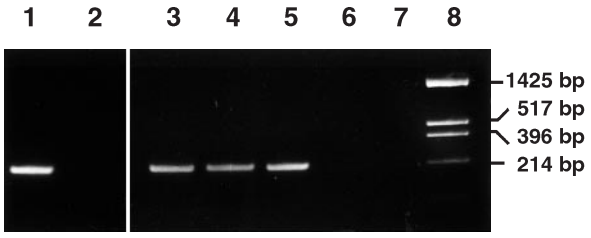

Fig. 1 Polymerase chain reaction (PCR)-amplified cytomegalovirus (CMV) IE gene DNA fragment (222 bp) separated in ethidium bromide-stained agarose gels. *Lane 1*, positive control cell culture (human embryo lung fibroblasts infected with CMV strain AD 169); *lane 2*, negative control cell culture (non-infected human embryo lung fibroblasts); *lanes 3–5*, endothelial cells isolated from grossly normal (*3*) or lesioned (*4, 5*) human aortas; *lanes 6 and 7*, endothelial cells isolated from human umbilical vein; *lane 8*, molecular weight standard (pUC-19 DNA digested with *Hin*dI)

Table 1 Number of cytomegalovirus (CMV)-infected endothelial cells in human aortic endothelial cell (HAEC) cultures prepared from thoracic aortas of 14 subjects aged 16–73 years, as determined by in situ DNA hybridization (1–11 cases) or by immunostaining for the IE antigen (12–14 cases). Atherosclerotic injury of thoracic aorta was determined macroscopically before enzymatic treatment: *I* vessels without visible abnormalities; *II* vessels containing fatty lesions; *III* vessels with fatty lesions and plaques

Case	Age	Gender	Vessel injury type	No. of infected cells (%)
1	16	Female	I	5
2	42	Male	I	10
3	44	Female	I	0
4	50	Male	I	0
5	62	Male	I	8
6	35	Female	II	30
7	42	Male	II	50
8	45	Female	II	5
9	33	Female	III	2
10	41	Male	III	10
11	73	Male	III	5
12	22	Female	I	0
13	25	Male	II	10
14	45	Female	II	2

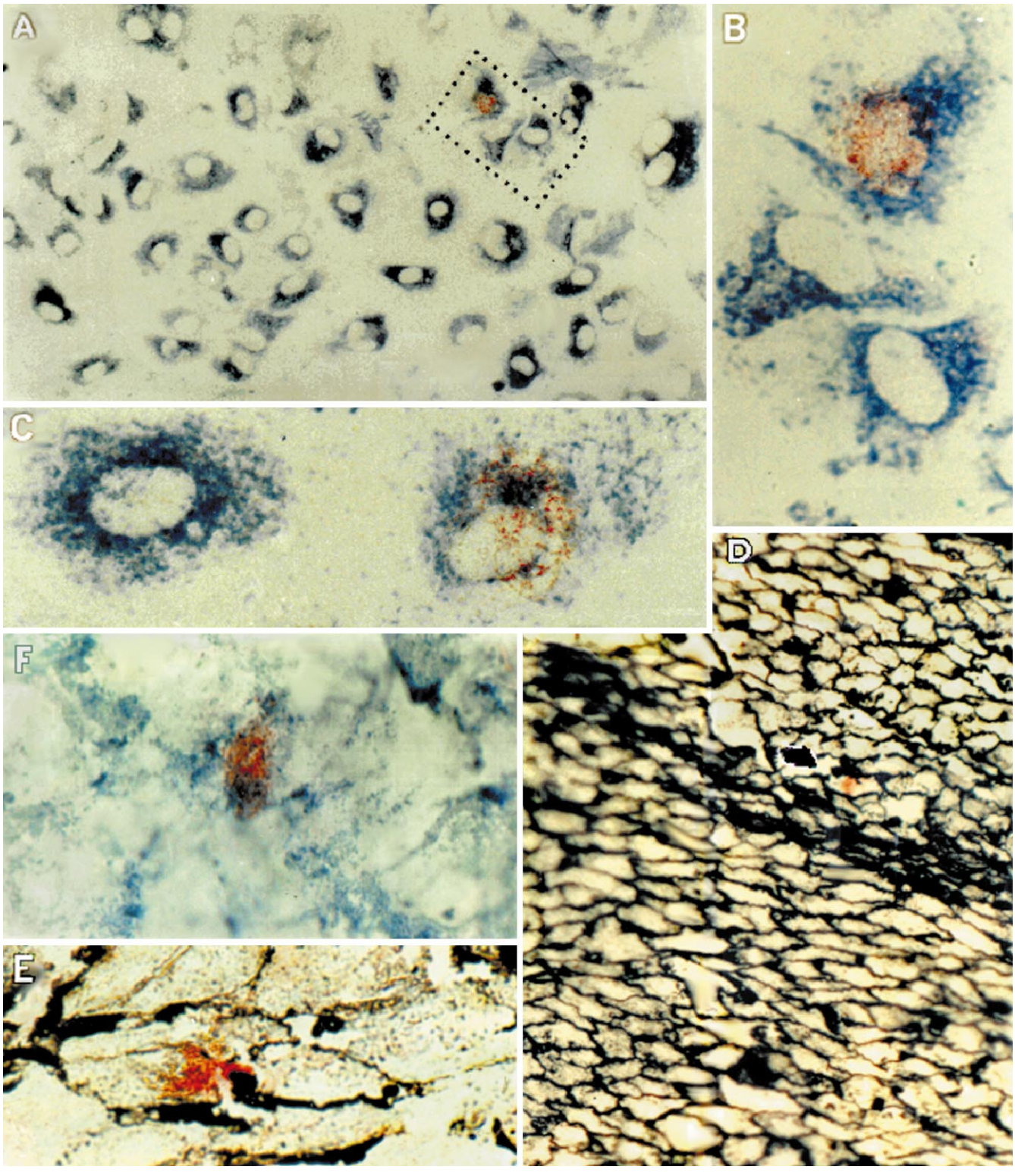

Fig. 2 In situ hybridization for the cytomegalovirus (CMV) genome in primary cultures of human aortic endothelial cells (A–C) or in intact aortic endothelial layer (D–F). Endothelial cells are visualized by immunostaining for von Willebrand factor (blue color; A–C, F) or by silver impregnation (D, E) as described in Materials and methods. Positive hybridization signals appear as *red grains*. **A**, **B** Endothelial culture from grossly normal human aorta (Table 1, case 1). The area limited in **A** by a *broken line* is presented in **B** at a higher magnification. Hybridization signals are visible in the nucleus of endothelial cell (B, *upper*). **C** Endothelial culture from lesioned aorta (Table 1, case 10). Hybridization signals are visible in the nucleus and in cytoplasm of endothelial cell (*right*). **D** The preparation of intact aortic endothelium (IAE) from grossly normal human aorta (Table 2, case 2). *Arrow* indicates endothelial cell displaying hybridization signals. **E** Endothelial cell exhibiting hybridization signals in the IAE preparation of fatty lesion (Table 2, case 5). **F** Hybridization signals within endothelial layer in the IAE preparation from grossly normal area of lesioned aorta (Table 2, case 11). Magnifications **A**, **D** ×750; **B**, **C**, **E**, **F** ×3000

Table 2 Detection of the cytomegalovirus (CMV) genome in endothelial cells (ECs) covering grossly normal areas, fatty lesions, and plaques in thoracic aortas of 15 subjects aged 21–70 years (in situ hybridization). All infected ECs were counted in fragments of about 10 mm² in each area: – no infected cells; + several infected cells; ++ several tens of infected cells; *I* vessels without visible abnormalities; *II* vessels containing fatty lesions; *III* vessels with fatty lesions and plaques; *N* grossly normal fragments; *F* fatty lesions; *P* atherosclerotic plaques. Underlines indicate fragments adjacent to orifices of intercostal arteries

Case	Age	Gender	Vessel injury type	Fragment injury type	CMV-infected ECs
1	21	Female	I	N	++
1	21	Female	I	N	+
1	21	Female	I	N	+
1	21	Female	I	N	–
1	21	Female	I	N	–
1	21	Female	I	N	–
1	21	Female	I	N	–
2	35	Female	I	N	++
2	35	Female	I	N	++
2	35	Female	I	N	–
2	35	Female	I	N	–
3	50	Male	I	N	++
3	50	Male	I	N	++
3	50	Male	I	N	–
3	50	Male	I	N	+
3	50	Male	I	N	–
4	29	Male	II	N	+
4	29	Male	II	N	+
5	33	Male	II	N	+
6	40	Male	II	N	–
7	43	Male	II	N	–
9	70	Female	II	N	+
9	70	Female	II	N	–
9	70	Female	II	N	–
10	45	Female	III	N	+
10	45	Female	III	N	–
11	55	Male	III	N	+
11	55	Male	III	N	–
11	55	Male	III	N	–
12	57	Male	III	N	–
12	57	Male	III	N	–
13	58	Male	III	N	+
13	58	Male	III	N	+
13	58	Male	III	N	–
4	29	Male	II	F	+
4	29	Male	II	F	–
5	33	Male	II	F	++
6	40	Male	II	F	++
7	43	Male	II	F	–
8	47	Male	II	F	–
9	70	Female	II	F	+
9	70	Female	II	F	++
10	45	Female	III	F	–
10	45	Female	III	F	–
11	55	Male	III	F	+
11	55	Male	III	F	–
12	57	Male	III	F	+
12	57	Male	III	F	++
13	58	Male	III	F	+
13	58	Male	III	F	+
14	65	Male	III	F	+
10	45	Female	III	P	–
10	45	Female	III	P	–
10	45	Female	III	P	+
11	55	Male	III	P	–
11	55	Male	III	P	–
11	55	Male	III	P	–
12	57	Male	III	P	–
12	57	Male	III	P	–
13	58	Male	III	P	–
13	58	Male	III	P	+
14	65	Male	III	P	–
14	65	Male	III	P	–
14	65	Male	III	P	–
15	50	Male	III	P	–
15	50	Male	III	P	–
15	50	Male	III	P	–
15	50	Male	III	P	+

similar pattern of endothelial staining with the CMV probe was found in IAE preparations in which endothelial layer was visualized using different methods (Fig. 2D–F). Either several units or several tens of CMV-hybridizing cells randomly scattered through endothelial layer were observed in different IAE preparations, each about 10 mm^2 in area. As shown in Table 2, infected cells were found in 13 of 15 vessels studied. In vessels without visible abnormalities (cases 1–3), infected cells were observed in areas adjacent to orifices of intercostal arteries. In vessels containing atherosclerotic lesions (cases 4–15), infected endothelial cells were observed in grossly normal regions and in fatty lesions regardless of their position with respect to arterial orifices. Interestingly, at the majority of plaques examined, endothelial cells containing the CMV genome were not detected (Table 2).

CMV antigen in the aortic endothelium

To find out whether naturally infected endothelial cells could express the IE 70 viral protein, double immunofluorescent staining for p70 and vWF was performed in HAEC obtained from grossly normal or lesioned vessels (cases 12–14 in Table 1). p70-Positive cells containing vWF were found in HAEC cultures from lesioned vessels (Table 1). Unexpectedly, the p70 immunostaining was visible like a cluster of granules or vesicles in cytoplasm of endothelial cells, while nuclei were unstained (Fig. 3A–C). No positivity for irrelevant murine IgG was observed in vWF-positive cells on day 2 after isolation from aorta.

To determine whether CMV-infected endothelial cells express the IE 70 viral protein in thoracic aorta in situ, immunostaining for p70 combined with silver impregnation of endothelial cell borders was carried out in IAE tissue preparations. It should be noted that using this method, we found a typical distribution of the cytoskeleton components in aortic endothelial cells, whereas IAE preparations processed with irrelevant murine IgG were unstained (Fig. 4A–C). Endothelial expression of the CMV antigen was examined in the aortas of nine subjects aged 31–70 years (Table 3). Antigen-positive endothelial cells were found in one of four grossly normal vessels and in four of five lesioned vessels (Table 3). Again, the p70 immunostaining was visible like a cluster of small vesicles in cytoplasm of endothelial cells (Fig. 4D–G). To detect nuclei, all the IAE preparations in which endothelial staining were observed were freed from mounting media in water and counterstained with Bemer hematoxylin (Fig. 4F, G). Up to five p70-positive cells randomly scattered through the endothelial layer were found in different IAE preparations, each about 5 mm^2 in area.

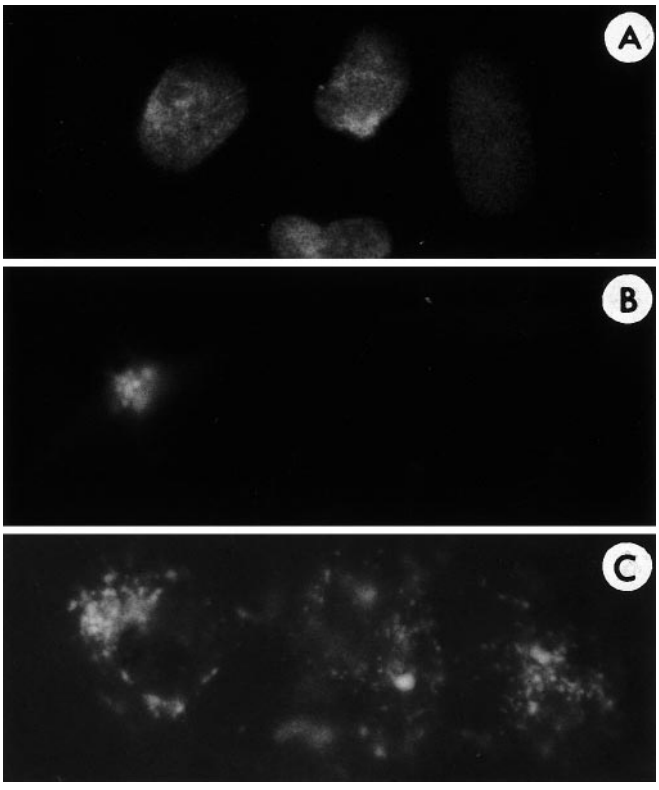

Fig. 3 Fluorescent staining of endothelial cells isolated from lesioned human aorta (Table 1, case 13) with antibody to p70 (B) followed by antibodies to von Willebrand factor (C) and DNA dye DAPI (A) as described in Materials and methods. **A**–**C** The same microscope field. Weibel-Palade bodies (C) are visible in three cells, one of which shows p70-positive vesicles (B, *left*) clustered near the nucleus (A). Magnifications **A**–**C** ×2500

CMV genome and antigen in the intima and the media of aorta

To evaluate how many of the infected cells express the viral antigen in grossly normal or lesioned aortas, we analyzed the frequency of occurrence of CMV genome-positive cells and antigen-positive cells in the same paraffin-embedded aortic sample. The samples were obtained from the vessels that were previously studied with tissue endothelial preparations, namely, from the aortas of nine subjects aged 21–70 years (Table 2). Randomly selected sections of each tissue sample tested were distributed into two groups – one group was subjected to in situ hybridization and the other to immunostaining procedures.

CMV-hybridizing cells were found in sections of all vessels studied (Table 4). In both grossly normal and lesioned vessels, the CMV genome was detected in cells adjacent to the lumen (Fig. 5A) and in cells randomly scattered through subendothelial intima and the media. In subendothelial intima, we regularly found about one infected cell per ten sections, and, in the media, about one infected cell per two or three sections. Interestingly, loose clusters of CMV-hybridizing cells were occasion-

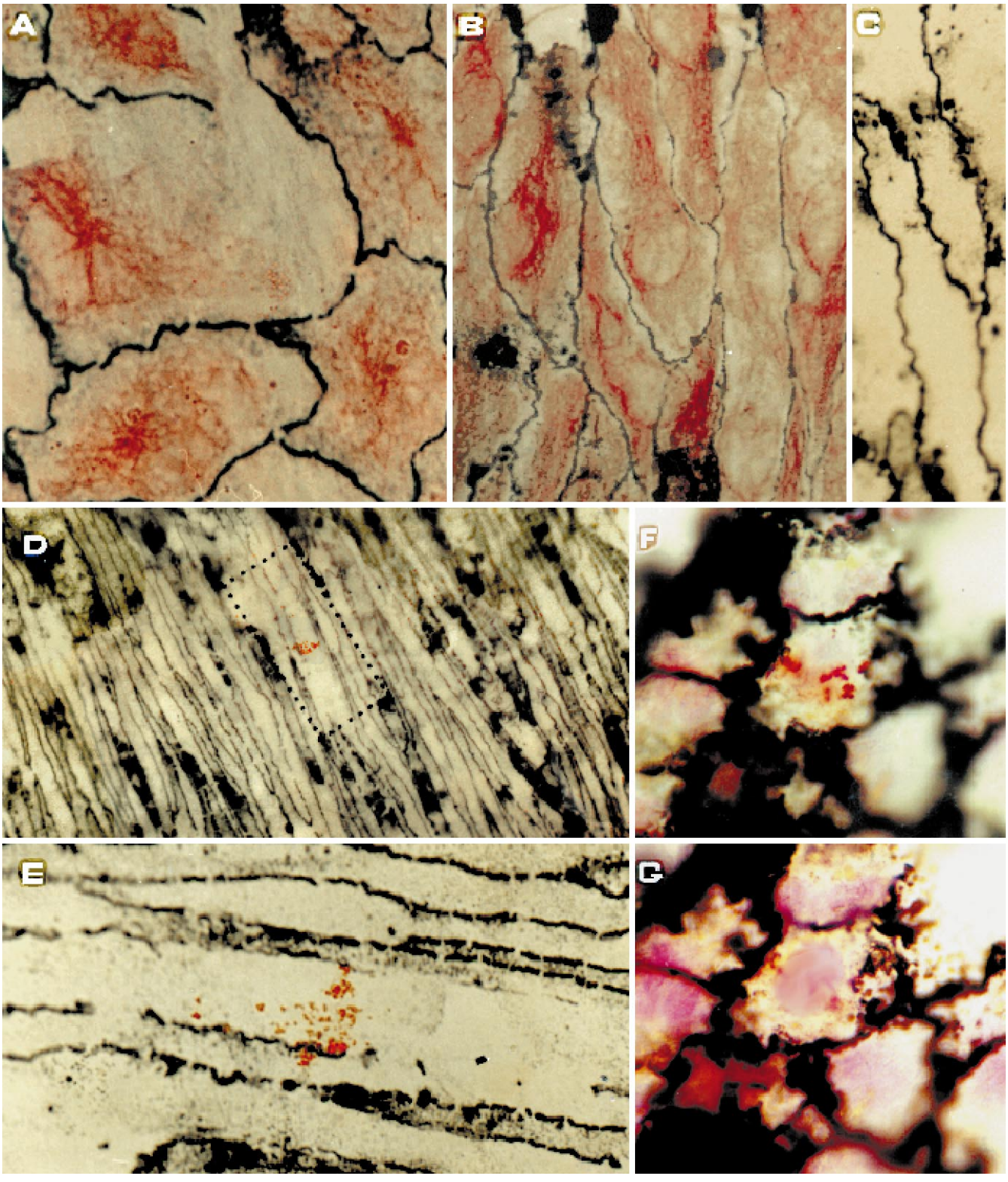

Fig. 4 The immunostaining of intact endothelial layer for cytoskeletal cell components (*red color*, A–C) or for the IE viral antigen (*red color*, D–G). Endothelial cells are visualized by silver impregnation as described in Materials and methods. **A**–**C** Microtubule-organizing center is visible in each cell within endothelial layer stained with antibody to α-tubulin (A), while perinuclear filament bundles are visible in cells stained with antibody to human vimentin (B). No positivity for irrelevant IgG was observed within the intact aortic endothelial layer (C). The IAE preparations were obtained from the grossly normal area (A, B) and the fatty lesion (C) of human aorta. **D**, **E** p70-Positive endothelial cell in the IAE preparation from grossly normal region of lesioned aorta (Table 3, case 7). The area limited in **D** by a *broken line* is presented in **E** at a higher magnification. The p70-immunostaining looks as a cluster of granules. **F**, **G** Two microphotographs focused on different planes of the same endothelial cell in the IAE preparation of fatty lesion (Table 3, case 8). The preparation was counterstained with hematoxylin. The p70-immunostaining is visible as a cluster of granules (F) located near the nucleus (G) of the endothelial cell. Magnifications **A**–**C**, **E**–**G** ×3000; **D** ×750

Table 3 Detection of the cytomegalovirus (CMV) IE antigen in endothelial cells (ECs) covering grossly normal areas and fatty lesions in thoracic aortas of nine subjects aged 31–70 years (immunocytochemical staining). *I* vessels without visible abnormalities; *II* vessels containing fatty lesions; *III* vessels with fatty lesions and plaques; *N* grossly normal aortic fragments; *F* fatty lesions. Underlines indicate fragments adjacent to orifices of intercostal arteries

Case	Age	Gender	Vessel injury type	Fragment injury type	p70-positive ECs
1	39	Male	I	N	–
1	39	Male	I	N	–
1	39	Male	I	N	–
1	39	Male	I	N	–
1	39	Male	I	N	–
1	39	Male	I	N	–
1	39	Male	I	N	–
1	39	Male	I	N	–
2	42	Male	I	N	–
2	42	Male	I	N	–
2	42	Male	I	N	–
2	42	Male	I	N	–
2	42	Male	I	N	–
2	42	Male	I	N	–
2	42	Male	I	N	–
3	43	Male	I	N	–
3	43	Male	I	N	–
3	43	Male	I	N	–
3	43	Male	I	N	–
3	43	Male	I	N	–
3	43	Male	I	N	–
3	43	Male	I	N	–
3	43	Male	I	N	–
4	47	Male	I	N	–
4	47	Male	I	N	–
4	47	Male	I	N	–
4	47	Male	I	N	–
4	47	Male	I	N	+
4	47	Male	I	N	+
4	47	Male	I	N	+
5	31	Male	II	N	–
5	31	Male	II	N	–
5	31	Male	II	N	–
5	31	Male	II	N	–
5	31	Male	II	N	–
5	31	Male	II	F	–
5	31	Male	II	F	–
5	31	Male	II	F	–
5	31	Male	II	F	–
6	40	Male	II	N	–
6	40	Male	II	N	–
6	40	Male	II	N	–
6	40	Male	II	N	–
6	40	Male	II	F	–
6	40	Male	II	F	–
6	40	Male	II	F	+
6	40	Male	II	F	–
6	40	Male	II	F	+
6	40	Male	II	F	+
7	51	Male	II	N	–
7	51	Male	II	N	–
7	51	Male	II	F	–
7	51	Male	II	F	+
7	51	Male	II	F	+
7	51	Male	II	F	–
8	57	Male	III	N	–
8	57	Male	III	N	–
8	57	Male	III	N	–
8	57	Male	III	N	+
8	57	Male	III	N	+
8	57	Male	III	F	+
8	57	Male	III	F	+
8	57	Male	III	F	+
8	57	Male	III	F	+
8	57	Male	III	F	+
9	70	Male	III	N	–
9	70	Male	III	N	–
9	70	Male	III	N	–
9	70	Male	III	N	–
9	70	Male	III	F	–
9	70	Male	III	F	+
9	70	Male	III	F	–
9	70	Male	III	F	+
9	70	Male	III	F	+

Table 4 Detection of cytomegalovirus (CMV) in the intima and in the media of thoracic aortas of nine subjects aged 21–70 years by in situ hybridization or by immunostaining for the IE antigen. *E* cells located on luminal surface; *SubE* deep layers of the intima; *nt* not tested. Case numbers correspond to those shown in Table 2. The designations of vessel or fragment injury are the same as in Table 2. No less than ten randomly selected sections were analyzed either by in situ hybridization or by immunocytochemical staining in each tissue sample tested. Brackets indicate clusters of antigen-positive cells located at the intima/media border

Case	Age	Gender	Vessel injury type	Fragment injury type	CMV DNA		CMV antigen	
					Intima E SubE	Media	Intima E SubE	Media
1	21	Female	I	N	+ +	–	– –	–
1	21	Female	I	N	nt	nt	– –	–
1	21	Female	I	N	– –	+	– –	–
2	35	Female	I	N	+ –	+	– –	–
2	35	Female	I	N	nt	nt	– –	–
2	35	Female	I	N	nt	nt	– –	–
2	35	Female	I	N	– –	+	– –	–
3	50	Male	I	N	– +	+	– –	–
3	50	Male	I	N	– –	–	– –	–
3	50	Male	I	N	– –	+	– –	–
5	33	Male	II	N	nt	nt	+ –	–
16	41	Male	II	N	nt	nt	– –	–
16	41	Male	II	N	– +	+	– –	–
9	70	Female	II	N	– –	–	– –	–
5	33	Male	II	F	nt	nt	– –	–
16	41	Male	II	F	+ +	+	– –	–
9	70	Female	II	F	– –	+	– –	–
9	70	Female	II	F	nt	nt	– –	–
10	45	Female	III	N	– –	–	– (+	+)
10	45	Female	III	N	nt	nt	– –	–
10	45	Female	III	N	nt	nt	– –	–
11	55	Male	III	N	– +	+	– +	+
11	55	Male	III	N	nt	nt	– –	–
12	57	Male	III	N	– +	+	– (+	+)
10	45	Female	III	F	+ –	+	+ (+	+)
10	45	Female	III	F	nt	nt	– –	–
10	45	Female	III	F	nt	nt	– –	–
11	55	Male	III	F	nt	nt	– –	–
12	57	Male	III	F	– +	+	– (+	+)
12	57	Male	III	F	nt	nt	– –	–
12	57	Male	III	F	nt	nt	– –	–
10	45	Female	III	P	– –	–	– –	–
10	45	Female	III	P	nt	nt	– –	–
10	45	Female	III	P	nt	nt	– –	–
10	45	Female	III	P	nt	nt	– –	–
11	55	Male	III	P	– –	–	– –	–
11	55	Male	III	P	– +	+	– –	–
11	55	Male	III	P	nt	nt	– –	–
12	57	Male	III	P	– –	+	– –	–
12	57	Male	III	P	– –	–	– –	–
12	57	Male	III	P	nt	nt	– –	–

ally seen in the media of lesioned vessels in addition to randomly scattered infected cells (Fig. 5E).

Although CMV genome-positive cells were observed in the majority of tissue samples from both grossly normal and lesioned vessels, the viral antigen was detected only in lesioned aortas (Table 4). Antigen-positive cells were found to be localized in the luminal surface of the aorta or at the intima/media border. It should be noted that both viral genome and viral antigen were rarely detected in endothelial cells that were present in randomly selected sections. So, antigen-positive cells adjacent to lumen were found in 2 of 31 samples of lesioned vessels (Table 4). It is significant that in such rare cells found, the p70 immunostaining was visible like granules (Fig. 5B–D). A granular pattern of the p70 immunostaining was never observed in any of the cells located in the subendothelial intima or in the media. In deep layers of aortic wall, we found loose clusters of antigen-positive cells that display homogeneous staining of nuclei (Fig. 5F–I). The bulk of cells constituting a cluster was located in the media, and rare cells were seen in the intima. In each cluster, spindle-shaped cells and cells resembling mononuclear infiltrates were seen (Fig. 5G–I). Occasionally, several nuclei in the middle of a cell cluster exhibited more size than the other antigen-positive nuclei in the same section (Fig. 5G, H). Interestingly, clusters of antigen-positive cells were detected in grossly normal regions and fatty lesions of lesioned aortas but not within plaques of the same vessels (Table 4). It should be noted that although antigen-positive clusters were found in aortic areas that were identified macroscopically as grossly normal regions, all of the samples containing p70-positive cell clusters are characterized by highly thickened intima that is approximately equal to the media in magnitude.

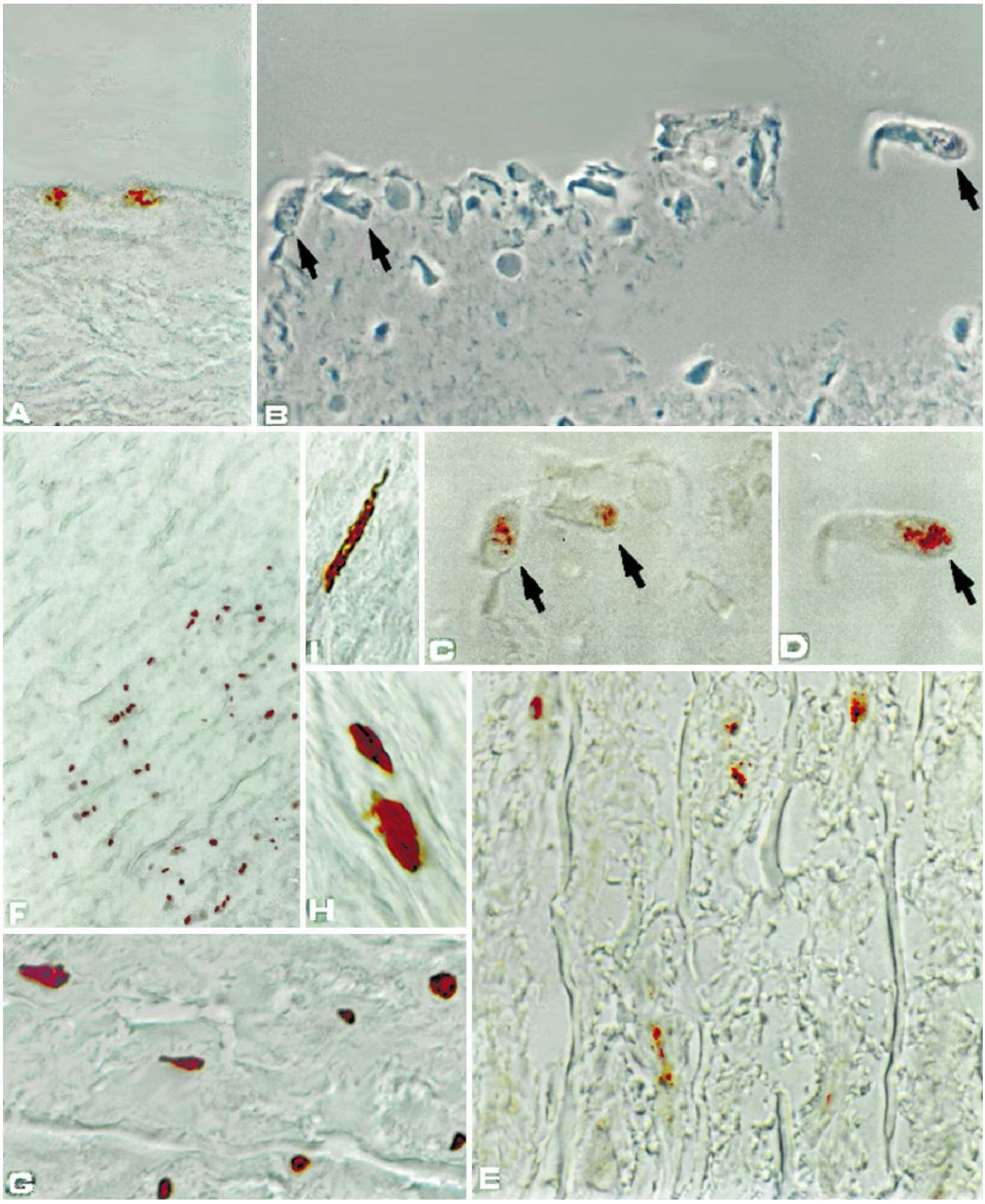

Fig. 5 Detection of cytomegalovirus (CMV)-infected cells in paraffin sections of aortic wall by in situ hybridization (*red color*, A, E) or by immunostaining for the IE antigen (*red color*, B–D, F–I) as described in Materials and methods. **A** Hybridization signals in cells located on luminal surface of grossly normal aorta (Table 4, case 1). **B**–**D** Phase-contrast (B) and standard light microscopy (C, D) of the same section of fatty lesion (Table 4, case 10). Cells located on luminal surface of aorta (B, *arrows*) display a granular pattern of the p70-immunostaining (C, D, *arrows*). **E** Several CMV-hybridizing cells from a large cluster found in the media of the lesioned vessel (Table 4, case 11). **F** Cluster of antigen-positive cells in deep layers of fatty lesion (Table 4, case 12). **G**–**I** Homogeneous nuclear staining is visible in antigen-positive cells clustered at the intima/media border in fatty lesion (G, H) or in grossly normal region (I) of lesioned aorta (Table 4, case 12). Magnifications **A**, **B**, **E**, **G**–**I** ×3000; **C**, **D** ×3750; **F** ×750

Discussion

A number of clinical and experimental studies suggest that reactivation of latent CMV in arterial tissue may contribute to atherogenesis; however, there is no direct evidence available. To this end, we have examined the frequency of occurrence of CMV-infected cells in different layers of the human aorta and attempted to evaluate how many cells containing viral genome express the immediate-early viral antigen p70 in aortas with or without visible atherosclerotic lesions.

In this study, we found that CMV genome-positive endothelial cells are usually present in both grossly normal and lesioned human aortas. Indeed, CMV-hybridizing endothelial cells were observed in 6 of 8 grossly normal vessels and in 16 of 18 lesioned vessels (Table 1 and Table 2). Our data confirm previous reports on the presence of CMV-infected cells on the luminal surface of lesioned aorta [34] and suggest that CMV infection of aortic endothelium is widespread in the adult population. The presence of CMV genome-positive endothelial cells in grossly normal vessels of young trauma victims (case 1 in Table 1 and Table 2) raises a possibility that infection of aortic endothelia with CMV occurs long before atherosclerotic lesions begin to appear rather early after primary infection. This means that latently infected endothelial cells, if they undergo mitotic division, could be accumulated over the years in those aortic areas that are characterized by enhanced proliferative activity of endothelial cells, for instance, in regions adjacent to orifices of intercostal arteries [33]. Interestingly, in both grossly normal and lesioned vessels that contain CMV-hybridizing endothelial cells, we found infected cells in the media (compare Table 2 with Table 4). Our data taken together with those showing a high probability of occurrence of CMV-infected smooth muscle cells in aortas of immunocompetent individuals [12, 18] provide a basis for the assumption that both types of vascular wall cells, endothelial cells, and smooth muscle cells of the media, appear to be obligatory constituents of the reservoir of latent CMV in the human aorta.

Our data imply that the IE 70 antigen expression in aortic endothelia is of rather a high incidence among immunocompetent individuals. Indeed, we found antigen-positive endothelial cells in 5 of 9 vessels tested (Table 3). Given that the CMV genome was detected in endothelia of 22 of 26 studied vessels (Table 1 and Table 2), one can conclude that only in part of CMV-infected aortas does the number of endothelial cells expressing viral antigen approximate to the number of endothelial cells containing viral genome. Interestingly, diminished probability of occurrence of antigen-positive endothelial cells characterizes grossly normal vessels, while in the endothelial layer of lesioned vessels the CMV genome and antigen were detected with about equal frequency. Indeed, the CMV genome was detected in endothelia of 16 of 18 lesioned vessels (Table 1 and Table 2), and antigen-positive endothelial cells were found in 6 of 7 vessels (Table 1 and Table 3). With regard to grossly normal aortas, we detected the CMV genome in endothelia of 6 of 8 vessels (Table 1 and Table 2), whereas only one grossly normal aorta out of five aortas tested exhibited endothelial expression of the CMV antigen (Table 1 and Table 3). The data suggest that, unlike in lesioned vessels, in most of the grossly normal aortas exhibiting endothelial CMV infection, infected cells do not contain the immediate-early viral protein at least at the level sufficient for immunodetection. This suggestion is supported by the results of comparative study of IAE endothelial preparations and paraffin-embedded samples prepared from the same vessel. In IAE preparations of grossly normal aortas, numerous CMV-infected endothelial cells were found in aortic fragments adjacent to orifices of intercostal arteries (Table 2); namely, infected cells were observed in 7 of 9 aortic fragments adjacent to artery orifices and, in 5 such fragments, we found more than 20 infected endothelial cells. In paraffin sections of the same vessels, cells containing viral genome were detected on the luminal surface of two of four tissue samples taken from aortic areas adjacent to artery orifices, but no antigen-positive cells were observed in these tissue samples and in three others with the same vascular location (Table 4). Unlike in grossly normal aortas, in lesioned vessels only several infected cells were detected in most of the IAE preparations studied (Table 2). However, in paraffin sections of the same aortas (Table 4), we found both cells containing the CMV genome (in 2 of 14 samples) and antigen-positive cells (in 2 of 31 samples) on the luminal surface of the aortic wall. Taken together, our data allow the assumption that accumulation of the IE 70 antigen in infected endothelial cells accompanies development of atherosclerotic lesions in the human aorta.

Although the CMV genome was detected by in situ hybridization in endothelial layer and in deeper layers of both grossly normal and lesioned vessels, cells exhibiting immunoreactivity for the IE 70 viral protein were found almost exclusively in lesioned vessels. In fact, we detected antigen-positive cells in 10 of 13 lesioned vessels, but only in 1 of 8 grossly normal vessels (Table 1, Table 3 and Table 4). Interestingly, there was a remarkable difference found between the endothelial layer and deeper layers of lesioned vessels with regard to the relative frequency of occurrence of CMV-hybridizing cells and antigen-positive cells. In both cell cultures or IAE preparations and paraffin sections of aortic wall, we found antigen-positive endothelial cells with about the same frequency as CMV-hybridizing endothelial cells; again, a spatial distribution of antigen-positive endothelial cells in aorta in situ corresponded to that of CMV-hybridizing endothelial cells. Unlike in the endothelial layer, in deeper layers of lesioned vessels, the number of antigen-positive cells never approximates to the number of CMV-hybridizing cells. Indeed, CMV-hybridizing cells randomly scattered through subendothelial intima and the media were frequent in both grossly normal and lesioned aortas, but no antigen-positive cells with the same tissue location were observed (Table 4). Presum-

ably, only a small part of infected cells located in the deep layers of aortic wall express the immediate-early antigen p70, namely, those clustered at the intima/media border. Our data thus imply that infected cells randomly scattered through subendothelial intima and the media of human aorta, unlike those in endothelial layer, do not contain detectable amount of the IE viral antigen, whether or not visible atherosclerotic lesions are present. The data are suggestive of an intriguing difference between the host mechanisms controlling latent state of the CMV infection in two types of vascular wall cells, such as endothelial cells and smooth muscle cells. It cannot be ruled out that the IE gene expression of CMV latent in endothelial cells is under the control of circulating anti-CMV antibodies as it has been observed in cultures of persistently infected fibroblasts [21]; however, too little is known about the qualitative and quantitative changes in the humoral immune response to CMV that accompany serum CMV antibody elevation found in patients with atherosclerosis.

Unexpectedly, we found that antigen-positive cells scattered within the endothelial layer differ from those clustered at the intima/media border in staining pattern for p70. A cluster of granules or vesicles was visible in the cytoplasm of endothelial cells, whereas a typical homogeneous staining of nuclei was observed in the cells of deeper layers of the aortic wall. The data imply that p70 is not transported into the nucleus of endothelial cells, but accumulated in a membrane compartment of cytoplasm. It has been reported that some products of the CMV IE 1 gene can be associated with cytoplasm membranes in infected fibroblasts [22]; however, there are no data demonstrating nuclear exclusion for IE gene products, DNA-binding proteins that are thought to regulate subsequent viral and cellular gene expression [27]. Given that CMV infection leads to remarkable and predominant cytoplasmic sequestration of tumor suppressor protein p53 in productively infected endothelial cells, but not in fibroblasts [9, 16], and that the IE gene products can interact with p53 [26], one cannot eliminate the possibility that cytoplasmic accumulation of p70 could be a particular feature of endothelial CMV infection. Interestingly, our data suggest that one of the IE 1 gene products which, as known, have an important role in the immune response to CMV [7, 17], appears to be accumulated in cytoplasm of endothelial cells of immunocompetent individuals. Considering that antigen-presenting capacity of endothelial cells has been reported [5], and that CMV-infected endothelial cells are capable of directly activating $CD4^+$ T cells from CMV-seropositive individuals [30, 31], one can conclude that antigen-positive endothelial cells could provoke extensive emigration of mononuclear blood cells in underlying tissue. Notably, in deep layers of aortic wall, we found clusters of antigen-positive cells, among which smooth muscle-like cells and cells resembling mononuclear infiltrates were observed. Whether focal clustering of antigen-positive cells appears to be due to infection of neighboring cells by virus released in this aortic site or proliferating of infected cells or both, remains to be determined. If local spreading of secondary infection takes place in the aortic wall, emigrated blood cells appear to be the most probable source of infectious virus. Indeed, CMV-infected T lymphocytes and monocytes are usually present in peripheral blood of seropositive individuals [25, 28], and at least the last of them, being activated, become more permissive to viral gene expression [14, 29]. Our data thus imply that, in the course of atherogenesis, CMV-infected cells found in different layers of the human aorta could trigger different immune mechanisms in a cascade manner. In future research, it will be important to determine the histogenesis of antigen-positive cells in deep layers of the aortic wall and the extent of CMV genome expression in these cells.

Our data suggest that formation of macroscopically noticeable plaque in the human aorta is associated with a decline in local density of infected endothelial cells. Indeed, endothelial cells containing viral genome were regularly found in atherosclerosis-prone regions of grossly normal aortas and in fatty lesions, but occasional infected cells were detected in rare plaques (Table 2). Given that antigen-positive endothelial cells were found in grossly normal areas and fatty lesions of injured vessels (Table 3), and that antigen-positive cell clusters were found in grossly normal areas and fatty lesions but not in plaques (Table 4), one can propose that the elimination of antigen-positive endothelial cells and a simultaneous inactivation of antigen-positive clusters take place within bounds of area in which plaque is forming. Our findings agree with the fact that a much higher percentage of primary cultures of smooth muscle cells from uninvolved aortic tissues of patients with advanced atherosclerosis exhibited CMV antigens than did cultures from plaque tissue [18]. Interestingly, a similar observation has been made in an animal model of virus-induced atherosclerosis. In chickens infected with Marek's disease herpes virus, viral antigens were detected in cells of the media in vascular regions exhibiting early lesions, but never in atherosclerotic plaques [8]. In this context, it seems reasonable to consider plaque formation as a response to injury caused by activated CMV infection. It has become necessary to understand whether CMV-infected cells can provoke a chronic inflammatory/immune reaction that accompanies atherogenesis [32].

Taken together our data fit in with the idea that reactivation of latent CMV in the artery wall may contribute to atherogenesis. Results allow us to suggest that accumulation of the immediate-early viral antigen p70 in endothelial cells may be the earliest change in vascular tissue. Our findings further suggest that endothelial accumulation of p70 and development of antigen-positive cell clusters in deeper layers of aorta accompany early atherogenic events, but these factors become inactivated during plaque growth. Further information about regularities of CMV genome expression in arterial wall cells could allow us to gain more insight into the relevance of immune factors to the origin and progression of atherosclerosis.

Acknowledgements The authors thank Dr. Hamida B. Qavi and Dr. Ervin Adam, Baylor College of Medicine, for expert assistance in performing of in situ DNA hybridization, helpful discussions, and generous use of their laboratories for part of the work.

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