

DETECTION AND RESTRICTION ANALYSIS OF CYTOMEGALOVIRUS DNA PERSISTING IN HUMAN ATHEROSCLEROTIC PLAQUES USING POLYMERASE CHAIN REACTION

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ABSTRACT

The polymerase chain reaction (PCR) as applied to detection of a foreign DNA in clinical specimens could provide a sensitive instrument for rapid detection of viral DNA persisting in tissues of patients suspected of latent infection. Human cytomegalovirus (HCMV) DNA was found in arterial plaques of patients with atherosclerotic lesions using a PCR assay with nested primer oligonucleotides derived from the major immediate early (MIE) exon 4 region of the genome. In order to approach a possible part of HCMV DNA in the mechanism of atherogenesis, the nature of found MIE exon 4 sequence was intimated using restriction endonuclease mapping of the amplified DNA. Comparison of the restriction fragments length polymorphism (RFLP) produced by endonuclease treatment of viral DNA amplified from urine, blood, culture and arterial plaques displayed a distinct difference in the DNA alignment for arterial specimens compared to that of other sources. This approved the specific origin of the MIE DNA found in plaques and suggested involvement in endothelial cell metabolism changes. It could thus be established that PCR has exhibited the promise for investigation of the role of latent viral infection in the process of atherosclerosis.

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INTRODUCTION

It is agreed that atherosclerosis is one of the most important causes of complications and death of patients with organic disease. Several theories explain the mechanism of the pathogenesis and give recommendations for prevention of the disease. The recent discovery of a herpesviral and chlamidial DNA in specimens from atherosclerotic patients suggested a trigger role of systemic latent infection in atherogenesis.^{4,6,19} It had been proposed that penetration of virus particles into endothelial cells could stimulate the changes in cell metabolism and lead to uncontrolled proliferation and local fibrin deposition with forming atherosclerotic plaques.^{7,8} Finding out the key elements of latent infection, especially the type of pathogen involved could help in search-

ing for an efficient way of treatment of patients or even of prevention of the disease by vaccination.

Constant efforts were mounted to discriminate between viruses probably taking part in the triggering of atherogenesis.^{9,19} The methods used for identification of virus suspected of infection included immunocytochemistry, serological diagnosis, and DNA hybridization. However, there is no common conclusion about the type of herpesvirus which is involved in the process. The DNA of HSV-I, cytomegalovirus and even Epstein-Barr virus has been found in plaque specimens.^{10,12}

In this study, the detection of cytomegalovirus (HCMV) DNA in clinical samples was done using a nested PCR (nPCR) assay with genome-specific outer primers and identification of amplified DNA by second-round PCR with inner oligonucleotides. Restriction en-

donuclease mapping of the PCR products disclosed the specific nature of viral DNA persisting in the sample examined.

MATERIAL AND METHODS

Clinical specimens tested

Blood and arterial specimens were obtained from patients referred to Shariati Hospital in Tehran (affiliated to Tehran University of Medical Sciences) and from the Forensic Medical Center of Tehran. Patients with vascular occlusion, angina pectoris undergoing reconstructive vascular surgery or coronary angioplasty, and patients with ischemic disease on whom autopsy was performed were included in this experiment. Patients with no vascular disease were entered in a control group. All of them were without any evidence of HCMV-related disease. Biopsy and autopsy samples were collected from the abdominal and thoracic aorta.

Specimen processing for PCR

Blood specimens were treated as recommended.^{13,20,21} Briefly, 2 mL of blood was used for isolation of leukocytes by density gradient centrifugation in Ficoll-Hypaque. Approximately 10^5 purified leukocytes were incubated in 0.3 mL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) with 0.05% sodium sarcosylate at 37°C for 15 min and centrifuged for 10 min at 10,000 rpm. One mL of supernatant fluid was used directly in the PCR assay. For the biopsy sample, 2-3 pieces of sliced specimens were washed and incubated in TE buffer containing 0.05% sodium sarcosylate and 200 mg/mL of proteinase K at 55°C for 1h. The content was dispersed by vigorous homogenizing and further incubated for 1h at 55°C. They were frozen at -20°C and thawed and cell debris was sedimented briefly. This procedure (mixing, heating and freeze-thawing) was repeated three times and the final supernatant was centrifuged at 10,000 rpm for 15 min. Protease was inactivated by incubation at 95°C for 10 min and the supernatant fluid was stored until use for PCR.

Primer oligonucleotide and amplification conditions

Oligonucleotide primers and amplification conditions for PCR screening of the specimens have been elaborated previously.^{13,20} Briefly, four primers relevant to DNA encoding HCMV MIE DNA were selected using DNA sequence data (GenBank, accession number M21295) and "Oligo" software.¹⁴ The primers were synthesized with a DNA synthesizer (Applied Biosystems, Inc.) and purified according to the manufacturer's recommendations. The outer primers, forward C1 5'-GCAACGAGAACCCCGAGAAAG-3' and reverse C4 5'-AAGCCATAATTCATCAGGG-3', produced an

amplimer 698 bp long. The inner set consisted of forward C2 5'-CAAGCCATCCACATCTCCCGC-3' and reverse C0 5'-GCGGCATAGAATCAAGGAGCACATG-3' flanking a 222 bp amplimer (Fig. 1).

The PCR mixture (50 to 100 mL) contained 67 mM Tris-HCl, pH 8.8, 16 mM $(\text{NH}_4)_2\text{SO}_4$, 3.0 mM MgCl_2 , 0.001% gelatin, 0.2 mM of each dNTP, 0.4-0.5-Pc of each outer or inner primer, and 0.03 units/mL of recombinant Taq DNA polymerase (BioTaq; Biomaster, Russia). The reaction mixture was covered with 50-100 mL of mineral oil and the thermocycling was performed in a DNA thermal cycler (Pharmacia). Negative controls were run which included purified human DNA as a template or no template at all. The temperature cycling numbered 30 cycles of denaturation at 94°C for 0.5 min, annealing at 55°C for 1.2 min, and extension at 72°C for 1.2 min followed by final extension at 72°C for 3.0 min. The PCR products were separated in a 1.5% agarose gel prepared in TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.0) and inspected after staining with ethidium bromide (1 mg/mL). For two-round PCR, after the first round with primers C1-C4, 2-5 mL of the tenfold diluted reaction mixture was injected in a tube for the second round of amplification (with primers C2 and C0) at the same thermal and time protocol with the only exception for annealing step at 51°C for 1.2 min instead of corresponding parameter of the first round. During all the procedures we followed the necessary recommendations to prevent false-positives. Sensitivity of the procedure was performed by ten-fold serially diluted in TE buffer. Up to 10 ag DNA that was equivalent to one-two complete viral genomes was amplified to a visible specific band in the nested PCR with both pairs of primers. All the samples were analyzed in three independent experiments. The samples with negative result were checked for DNA integrity and probable contamination by substances inhibiting Taq DNA polymerase with a primer ser reactive with the human exon 3 of the steroid sulfatase (STS) gene.

To prepare a PCR product suitable to restriction fragment length polymorphism (RFLP) analysis, another set of primers flanking HCMV MIE exon 4 was used for amplification. It included outer primers RI 5'-GGTACCGAATTCTCATGTGTTTAGGCCCG-3' and HI 5'-GTGACGTGGGATCCATAACAG-3', and an inner pair consisting of the above primer C1 and primer D2 5'-CAGCACCATCCTCCTCTTCTCTGG (Fig 1). The latter was identical to the published primer for MIE DNA.¹⁵ Thermocycling conditions for the amplification were specific for Vent thermostable DNA polymerase (Biolab Co., New England) used instead of Taq DNA polymerase including an appropriate buffer (10 mM KCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, Tris-HCl, pH 8.8, 2 mM Mg_2SO_4 , 0.1% Triton X-100). The PCR products were incubated

with one of the restriction endonucleases AluI, HaeIII, HinfI, and RsaI and analyzed in a polyacrylamide gel in 0.5x TBE (45 mM Tris-Borate, 45 mM boric acid, 1 mM EDTA, pH 8.0) containing 0.5 mg/mL ethidium bromide.

RESULTS

In order to prevent any confusion due to the active HCMV-related disease, blood specimens of all 29 patients were tested using nPCR assay. Only one of 21 specimens from the patients who had comprised the experimental group was MIE-positive. The result was also positive in one of eight patients of the control group. Then, specimens from 27 patients who were negative by this testing were taken through the DNA extraction procedure and nPCR assay with primers C1-C4 and C0-C2 (Fig. 1). Of the 20 samples that were obtained from patients with evidence of atherosclerosis, 16 (80.0%) were positive for HCMV MIE DNA by nested PCR (Fig. 2). Of the seven specimens from the control patients, HCMV DNA was only detected in two of them (28.6%). All negative samples were positive on the STS gene testing, proving that conditions were sufficient for PCR analysis.

To gain a better insight into the nature of persisting viral DNA, the restriction maps of the amplified DNA from an arterial specimen and other virus-positive

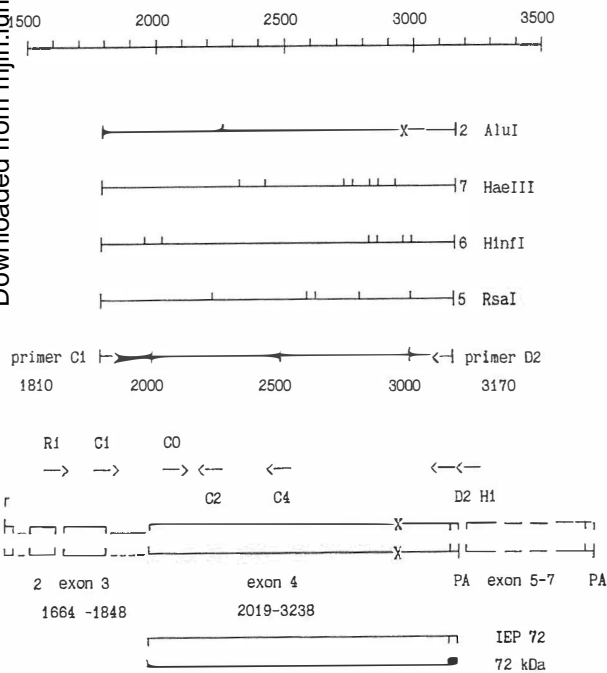


Fig. 1. Schematic exon-intron map of HCMV MIE region with position of primer oligonucleotides and restriction sites on the amplified part of MIE exon 4 region. Vertical bars (^) represent the position of cutting sites; location of Alu I plaque-specific sites is marked (X).

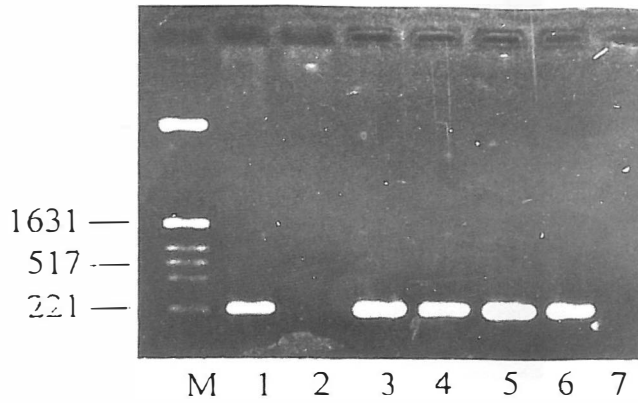


Fig. 2. The nested PCR products of arterial specimens obtained from patients with atherosclerosis. For each lane, 1 mL (2-6) of treated arterial specimens was subjected to nested PCR and run on a 1.5% agarose gel: 1-positive control (10⁻⁴ fold dilution of HCMV culture supernatant in TE buffer); 7-negative control (human DNA); M-DNA molecular weight markers (pBR 322 digested with HinfI).

samples were compared.¹³ For this purpose an nPCR with another set of primers capable of amplification of 1361 bp of MIE (complete exon 4) has been chosen (Fig. 1). HCMV positive DNA samples extracted from urine, leukocytes, plaques and culture *in vitro* were used for comparison. Two sets of primers, outer RI, HI, and inner C1, D2, provided PCR products of 1361 bp for all samples in an amount sufficient for RFLP analysis.

On the basis of simulated restriction digests calculated from strain AD169 sequence information available on the respective target (MIE exon 4), several endonucleases with different cutting patterns were chosen. AluI (2 sites), HaeIII (7 sites), HinfI (6 sites), RsaI (5 sites) (Fig. 1). The PCR products were treated with the selected enzymes and analyzed by electrophoresis in a polyacrylamide gel (Fig. 3). Digestion of the DNA amplified from clinical specimens (arterial plaques, leukocytes, urine) and from standard strain AD169 with HaeIII or HinfI or RsaI endonucleases showed the corresponding patterns identical for all specimens. However, a reproducible difference in the length of short DNA fragments after AluI digestion of plaque sample has been found when compared with fragments of the samples from other sources (Fig. 3). Amplified samples from blood, urine and control viral strain produced a 223-bp fragment along with two others after digestion with this enzyme. However, the digestion of the amplified DNA from plaque specimen resulted in clear-cut decrease in the length of this fragment shifting forward its position in the gel. That indicated in a favor of changing position of one of the AluI cutting sites in DNA isolated from the plaque specimen suggesting the distinct difference in the nucleotide sequence in the corresponding region.

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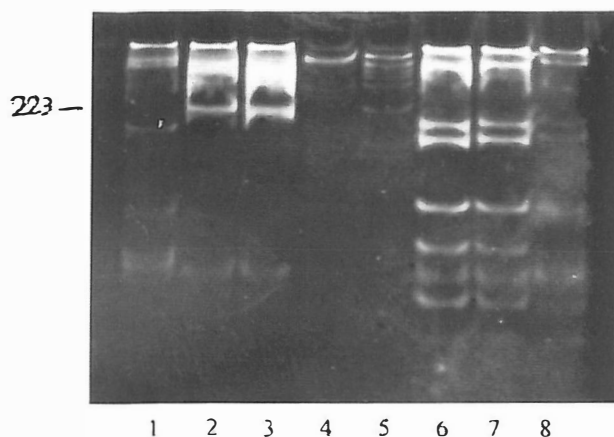


Fig. 3. Restriction endonuclease analysis of amplification products from clinical samples in polyacrylamide gel. Samples from arterial plaques (1.8), urine (2.7) and HCMV strain AD169 culture (3.6) were subjected to nested PCR and the PCR products and pBR322 (4.5) were digested with Alu I (1-4) and Hinfl (5-8).

DISCUSSION

The direct identification and localization of the latent virus in the normal host using standard culture or Ag-specific immunoassay is difficult. When *in vitro* PCR amplification followed by probe hybridization was recently applied to detect persisting HCMV DNA extracted from tissues, it allowed the detection of viral DNA in patients with latent form of infection not detected by standard Southern hybridization.^{10,16} However, this method is not capable to prepare target DNA in the amount suitable for enzyme digestion or other analysis. Therefore, the question connected with invention and probation of nPCR assay with high reliability and capability to accumulate target DNA in an amount significant for analysis was under study.

A simplified nPCR procedure for detection of HCMV MIE DNA was described recently which involved two-round thermocycling per specimen.^{13,21} The advantage of the method over previously used analogues was its high sensitivity and possibility to detect PCR product without steps of hybridization with labeled DNA probe. In our study, this modification of PCR based method was applied for detection and preparation of HCMV MIE DNA persisting in plaque samples obtained from patients with atherosclerosis. We demonstrated the prevalence of PCR samples containing viral MIE genes among specimens obtained from patients with atherosclerosis. The substantial difference in relative number of MIE-positive specimens obtained from the suspected and control patients could be interpreted as evidence of HCMV DNA persisting in the plaques dependent on the latent form of infection. Although these results do not indicate a direct

part for the virus in the mechanism of pathogenesis, they suggest a role for HCMV in the pathogenesis of the disease. The ability to reactivate from the latent status leading to a local lytic infection in a 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.

Several possible explanations exist for the PCR-negative results for some specimens from patients with atherosclerosis. First, the possibility that target sequences complementary for primers were changed can not be excluded. Second, the samples might accumulate regions of viral DNA other than MIE, that expelled them from the targets for primers used. The nested PCR assay is clearly effective as a mean of rapid detection and isolation of viral DNA persisting in plaques. However, use of PCR alone is likely to lead to confusion in some cases. This could occur because of the possibility of false-positive results due to contamination of amplified products. Thus, it is important to confirm the specific nature of persisting viral sequences using restriction mapping of amplified DNA. The persisting viral genes could be different in their structure of viral counterparts.^{17,21} The results of AluI digestion of MIE exon 4 demonstrated evident difference in arrangement of endonuclease susceptible sites for DNA amplified from plaque specimens in comparison with control viral DNAs obtained from specimens of urine, blood and culture. However, these DNAs were shown to be indiscernible using other restriction endonucleases (HaeIII, Hinfl, RsaI).

A question of practical importance is whether the obtained data provided by PCR-RFLP analysis have any relevance to mechanism of induction of atherogenesis in patients. Recent studies suggest that this is likely to be the case.^{9,17} One of them were conducted on quails susceptible to cholesterol-induced atherosclerosis. Viral genes complementary to Marek's disease herpes virus (MDV) DNA, another member of the herpesvirus family, were detected in atherosclerotic aortas by DNA hybridization.¹⁷ Southern blot analysis demonstrated that restriction mapping of aortic DNA was specific and different from that of wild MDV strain. Functional MDV was not displayed by a number of methods. Authors suggested that this quail possesses a modified portion of the MDV genome in the germline, and the viral genes are taking part in induction of atherosclerosis. We have found the modification of persisting viral DNA sequences in the 3'-region of mRNA coding for IEP 72, the early viral protein switching cell metabolism upon infection. Recently, a role of another early MIE protein, IEP 84, has been found crucial for restenosis, which shares many pathophysiological features with atherogenesis.¹⁸ Further studies are needed to elucidate the factors leading to the

development of atherosclerosis in humans.

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