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Direct detection of *Chlamydia pneumoniae* and cytomegalovirus in atherosclerotic tissue by immunohistochemistry and evaluation of serological response to these infections

Silvija Jankauskienë-Kiverytë[1*], Zita Kuèinskienë[1], Edvardas Þurauskas[1], Irena Bogušienë[1], Aida Laurinavièienë[2], Arvydas Laurinavièius[2]

[1] *Vilnius University, Faculty of Medicine, Lithuania*

[2] *National Centre of Pathology, Vilnius, Lithuania*

*Corresponding author. Vilnius University, Faculty of Medicine, M. K. Èiurlionio 21/27, LT-03101, Vilnius, Lithuania
E-mail address: silvija.jankauskiene@santa.lt

Introduction: Atherosclerosis is recognized as an inflammatory disease on a world scale. The chronic process of inflammation may be promoted by microorganisms. Many pathogens have been investigated on their etiopathogenetical role in the atherosclerotic process, but the strongest association has been found for *Chlamydia pneumoniae* and cytomegalovirus.

Materials and methods: We investigated 57 specimens of carotid, iliac, femoral and tibial arteries taken during revascularization surgery from symptomatic Lithuanian patients with arteriosclerosis obliterans and carotid artery stenosis. Also, 10 autopsy specimens of coronary arteries with atherosclerotic plaques were collected. Atherosclerotic tissue sections were studied by immunohistochemical assays for *Chlamydia pneumoniae* and cytomegalovirus antigens. Sera from the same patients (including autopsy cases) were analyzed for serological markers of infections investigated.

Results: Immunoreactivity to *C. pneumoniae* and cytomegalovirus was detected respectively in 49.3% and 31.3% of the total of 67 specimens investigated, and it was related to the level of inflammatory infiltration at the site of atherosclerosis (p values 0.002 and 0.004, respectively). *C. pneumoniae*, but not CMV, antigens present in atheroma statistically significantly correlated with the level of atherosclerosis ($p = 0.0001$). In 28 specimens out of 67 (41.7%) no antigens of infectious agents were found. In 15 samples (22.3%) antigens of both (*C. pneumoniae* and CMV) were present. We found a statistically significant correlation (Spearman's positive correlation value 0.444, $p = 0.0001$) between the level of inflammation and the grade of atherosclerosis. No elevated IgM antibodies to *C. pneumoniae* were found and only 4 patients were positive for IgM antibody to cytomegalovirus. IgA antibodies to *C. pneumoniae* at the high titers were more often elevated than IgG antibodies. IgA antibodies to *C. pneumoniae* at the highest titers (1:64) tend to be present when in the atheromata a 2nd grade inflammation was observed.

Conclusions: On the basis of the results of the present study we can state that *C. pneumoniae* and CMV are present and can be detected in atherosclerotic lesions of vessel walls. There is a statistically significant correlation between the level of inflammation and the grade of atherosclerosis. The presence of *C. pneumoniae* and CMV antigens is related to the level of inflammation at the sites of atherosclerosis. *C. pneumoniae* but not CMV antigens are related to the grade of atherosclerosis.

Key words: atherosclerosis, *Chlamydia pneumoniae*, cytomegalovirus, inflammation, immunohistochemistry

INTRODUCTION

The incidence of cardiovascular diseases (CVD) and the rates of mortality increase in Eastern Europe [1], while the decrease in cardiovsacular mortality rates in Western Europe and the USA lasting already for two decades has reached a plateau [2]. All classical risk factors cannot fully explain such a high incidence of CVD in general population. Also, many individuals with atherosclerosis lack identifiable traditional risk factors. On the other hand, atherosclerosis is recognized as an inflammatory disease

on a world scale [3]. The atherogenetic process resembles many aspects of chronic inflammation. This chronic process of inflammation may be promoted by microorganisms. They can infect arterial wall directly, as have been shown in experimental animal studies, but also, infection can influence atherogenesis indirectly through host defense to extravascular infections – the so-called "echo" hypothesis when proinflammatory cytokines produced to extravascular infections stimulate an increased expression of cellular adhesion molecules in endotheliocytes enhancing leukocyte adhesion to the endothelium. These cytokines could elicit a second wave ("echo") from inflammatory cells at sites of atherogenesis, such as arterial wall cells or macrophages (2). Proinflammatory cytokines in elevated levels and also soluble adhesion molecules can be detected in patients with CVD (4, 42).

The role of microorganisms in any disease can be proven by using postulates of Koch. Talking about the role of microorganisms in atherosclerosis, these postulates could be formulated as follows:

1. Microorganisms should be detected in atherosclerotic plaques (which are a morphologic unit of atherosclerosis) and not in healthy vessels.

2. Microorganisms should be isolated from atherosclerotic tissue.

3. In experimental animal models, the microorganisms with which animals where infected should produce a disease similar to atherosclerosis in humans.

4. Microorganisms should be isolated from animal vessels damaged by atherosclerosis.

Many pathogens have been investigated on their etiopathogenetic role in the atherosclerotic process, but the strongest association has been found with *Chlamydia pneumoniae* and CMV. Both are widely distributed, can experimentally infect blood vessel wall cells, and exhibit persistence, latency and recurrence of infection.

The first possible association of *C. pneumoniae* and CVD came from the seroepidemiologic study performed in Finland in 1988 (5). Since then, almost 500 papers have been published on this association. Different observational studies published heterogeneous results: while many earlier studies reported a positive association (6–8), some recent large well-conducted studies did not find any association between elevated IgG antibodies to *C. pneumoniae* and the risk of coronary events (9–11). The serological diagnosis of chronic or persistent infection of *C. pneumoniae* is difficult. Anti-*Chlamydia pneumoniae* IgA antibody has been promoted as a marker of chronic infection, because the biological half-life of serum IgA is less than 7 days, versus 23 days for IgG. So, the presence of IgA in the serum for a long time implies an active or persistent infection process (12).

Seroepidemiologic studies were followed by the studies in which *C. pneumoniae* was directly detected in atherosclerotic tissue by immunocytochemical staining, PCR, electronic microscopy (13–16) and in several studies this organism has been isolated (17). The results of cytomegalovirus detection in atherosclerotic tissue are not so consistent (18), the link between this virus and atherosclerosis mainly has been based on serological, experimental and clinical studies (19–21). Isolation of *C. pneumoniae* from atherosclerotic tissue remains to be the prevailing gold standard used to demonstrate current infection by *C. pneumoniae* and to establish viability and thus infectivity. However, the use of culture for detection is problematic because of the difficulty in growing *C. pneumoniae* in cell culture, especially from a tissue sample (12). M. Maas and coworkers developed a highly sensitive culture system and isolated *C. pneumoniae* from atherosclerotic tissue and by DNA sequence analysis proved a 100% sequence identity to a *C. pneumoniae* reference respiratory tract isolate (17).

In vitro studies and animal experiments also support the hypothesis that *Chlamydia pneumoniae* and CMV can be associated with atherosclerosis (22–26). This group of studies can show the strongest link between microorganisms and atherosclerosis.

Clinical treatment studies with antibiotics showed controversial results, but in most of them the effect of antibiotic treatment on adverse cardiac outcome was not found or was only temporary (27–31). Studies on the treatment of *C. pneumoniae* in animal models of atherosclerosis showed that the best effect of treatment is achieved in acute but not chronic infections, while atherosclerosis seems to be a chronic but not an acute process. CMV has been shown to have a role in atherosclerotic changes in the donor heart after heart transplantation, and one clinical treatment study showed that the incidence of CHD in donor heart after transplantation was lower in patients who where treated with ganciclovir – an antiviral drug effective against CMV (21).

Until a better understanding of the molecular mechanisms of infection-induced atherosclerosis is reached and more direct evidence for a causal pathway is presented, use of antibiotics in the prevention or treatment of CHD is premature and must not be recommended outside well-controlled trials (32, 33).

MATERIALS AND METHODS

The present study was approved by the Bioethics Committee of the Ministry of Health of Lithuania. Collection of samples was started in May, 2001. Fifty-seven specimens from symptomatic Lithuanian patients with arteriosclerosis obliterans (fragments of iliac, femoral and tibial arteries) and carotid artery stenosis (endarterectomy specimens) were collected

during revascularization surgery at the surgery departments of two Vilnius University hospitals (Vilnius University Hospital and Vilnius University Emergency Hospital). Also, 10 autopsy specimens of coronary arteries damaged by atherosclerosis were collected at the National Center of Pathology (Lithuania). Sera from the same patients were collected for serological analysis and stored at –20 °C until tested. In the cases of autopsy, sera were taken from the laboratory where they were stored if collected 24–48 hours before death. Cases with previously diagnosed rheumatic diseases and diabetes mellitus were excluded from this study, as we wanted to investigate classical cases of atherosclerosis.

Immunohistochemistry (IHC). Each artery specimen was divided into two parts. One part was fixed in 10% of buffered formalin solution, paraffin-embedded, sectioned, and stained with hematoxylin and eosin for histological examination. The other part of the specimen was frozen at –70 °C for further investigation. We investigated 67 atherosclerotic arteries for the presence of *C. pneumoniae* and CMV antigens by immunohistochemistry. Areas for histological investigation were taken from formalin-fixed artery specimen where macroscopically atherosclerotic plaques could be seen. IHC was performed on adjacent sections. For antigen visualization we used peroxidase-based ChemMate DAKO EnVision Detection Kit with diaminobenzidine (DAB). Briefly, after deparaffinization, tissue sections were subjected to epitope retrieval, endogenous peroxidase was blocked and sections were incubated with *C. pneumoniae*-specific mouse monoclonal antibody (clone RR402, DAKO, dilution 1:25) and with CMV-specific mouse monoclonal antibody (clones CCH2 + DDG9, DAKO, dilution 1:25). Then the samples were incubated with peroxidase-conjugated polymer (dextran) backbone, which, in addition, also carried goat secondary antibody molecules against mouse immunoglobulins. The antigens were visualized with DAB + Chromogen (dilution 1:50). As a positive control for *C. pneumoniae* staining we used formalin-fixed and paraffin-embedded sections of lungs (samples with chronic infection) of mice infected with *C. pneumoniae* (kindly provided by National Public Health Institute, Oulu, Finland), and for CMV staining we used commercially available slides of human lung tissue infected with cytomegalovirus (DAKO, Denmark). For use as negative controls we obtained autopsy samples from a normal carotid artery. These positive and negative control slides were processed identically to the investigated atherosclerotic samples and examined in parallel. The slides were read at 40´ and 400´ magnifications. The specimens were graded histologically for the level of atherosclerosis: 0 - normal artery; 1 - intimal thickening, smooth muscle cell damage, macrophage and foam cell infiltration; 2 - central necrotic area with overlying fibrosis; 3 - dense fibrosis, calcification, ulceration, neovascularization or haemorrhage. Also, atherosclerotic tissue specimens were graded for the level of inflammation: 0 – no inflammatory cells present, 1 – sporadic inflammatory cells present, 2 – more than 50 inflammatory cells are present in one field of vision (at 400× magnification), 3 – more than 50 inflammatory cells present in one field of vision (at 400´ magnification). Results of IHC staining were classified as positive or negative.

Microimmunofluorescence assay (MIFA). From the same 67 patients that were tested by IHC we investigated sera for the presence of *C. pneumoniae* antibodies using *Chlamydia pneumoniae* IgG/IgM and IgA Micro-IF Test kit (AniLabsystems, Finland). Sera were diluted according to manufacturer's instructions. Before testing IgM class antibodies, IgG antibodies were blocked. The microscopic slides were incubated with serum dilutions in a moist chamber. After washing the slides, they were incubated with anti-human antibodies conjugated with FITC. Finally, the slides were washed, dried and read at 1000´ magnification with a fluorescent microscope (Olympus BX40, Japan) in a dark room. Positive and negative controls are included in the kit and were processed in each testing. Thresholds for positivity were taken according to manufacturer's instructions: sera considered positive when fluorescence at a certain dilution (IgG ≤ 1:32, IgM ≤ 1:16 and IgA ≤ 1:8) was observed.

Microparticle Enzyme Immunoassay (MEIA). From the same 67 patients that were tested by IHC we analyzed sera for the presence of CMV antibodies by MEIA (a semi-quantitative method) using an AxSYM analyzator (Abbot Laboratories, USA). Assay results of ≥ 15AU/ml were considered positive for IgG antibodies, and for IgM positive samples the 0.500 Index Value cut-off was established by the manufacturer.

Statistical analysis. Statistical analysis was performed by SPSS for Windows version 11.0. Nominal data were analyzed by the χ^2 test. Ordinal data were analyzed using Spearman's rank order correlation. P values < 0.05 were considered to be statistically significant.

RESULTS

Immunohistochemistry. The areas for histological investigation were taken from formalin-fixed artery specimen where atherosclerotic plaques could be seen macroscopically, but after microscopic examination of the stained sections one specimen out of 67 was found to be at grade 0 (without microscopic evidence of atherosclerosis); 23 arteries were found to be at grade 1, 11 – at grade 2 and 32 segments – at the highest grade of atherosclerosis (Table 1). Seven

Table 1. **Grades of inflammation and atherosclerosis**

Grade of inflammation		
	Frequency	**%**
0	7	10.4
1	19	28.4
2	30	44.8
3	11	16.4
Total	67	100.0

Grade of atherosclerosis		
	Frequency	**%**
0	1	1.5
1	23	34.3
2	11	16.4
3	32	47.8
Total	67	100.0

Table 2. **Frequency *C. pneumoniae* antigens in atheroma within the grade of inflammation**

			Chpn antigens		Total
			Not found by IHC	**Found by IHC**	
Grade of inflammation	0	Count	7	0	7
		%	100.0%	0.0%	100.0%
	1	Count	10	9	19
		%	52.6%	47.4%	100.0%
	2	Count	16	14	30
		%	53.3%	46.7%	100.0%
	3	Count	1	10	11
		%	9.1%	90.9%	100.0%
Total		Count	34	33	67
		%	50.7%	49.3%	100.0%

specimens out of 67 were found to have no inflammatory cell infiltration, 19 were at the 1st grade, 30 – at the 2nd grade and 11 at the 3rd grade of inflammation. We found a statistically significant correlation (Spearman's positive correlation value 0.444, p = 0.0001) between the level of inflammation and the grade of atherosclerosis (Fig. 1.)

Of 67 arteries, 33 were found to be positive for *C. pneumoniae* antigens, and 21 out of 67 were found to be positive for CMV antigens (49.3% and 31.3%, respectively). The positive and negative controls reacted correspondingly. According to recommendations for standardization of *C. pneumoniae* assays (34), only the intracellular and granular pattern of specific brown color staining was considered as positive (Fig. 4). Antigens were most commonly found in those areas of atherosclerotic plaque in which intensive infiltration of inflammatory cells was also present (Table 2). A statistically significant correlation between *C. pneumoniae* and CMV antigens was found in atheroma and the degree of inflammatory infiltration was determined: p = 0.002 and 0.004 respectively (Fig. 2). A correlation between the degree of atherosclerosis and the antigens detected in atheroma was statistically significant only in *C. pneumoniae* cases (p = 0.0001), but not in CMV (p = 0.249). In 28 specimens out of 67 (41.7%) no antigens of infectious agents were found. In 15 samples (22.3%) antigens of both pathogens (*C. pneumoniae* and CMV) were present.

Serological analysis (MIFA and MEIA).

1. Markers of acute (recent) infection: out of 67 sera tested, **IgM** class antibodies to *C. pneumoniae* were not found, and only four patients had IgM antibodies to cytomegalovirus, indicating a recent infection (or reinfection) with CMV.

2. Chronic infection markers: 1) high titers of **IgG** (1:512) to *C. pneumoniae* were found only in 8 (11.9%) patients, IgG 1:218 were found in 30 (44.8%) and at the titer 1:32 in 51 (76.1%) patients. Twelve patients (17.9%) were totally negative for *C. pneumoniae* antibodies. The IgG class of antibodies to CMV, indicating infection with this pathogen in the past, was found in 62 (92.5%) patients, while only 5 (7.5%) of all individuals tested were totally negative for CMV antibodies. 2) **IgA** class antibodies to *C. pneumoniae* were found at high titers (1:64) in 23 (34.3%), at 1:32 titer in 35 (52.2%) and at 1:8 titer in 44 (65.7%) patients of all 67 tested. A correlation between the titers of antibodies to *C. pneumoniae* and the grades of inflammation in the atheromata was not statistically significant: according to the results of analysis, the relationship between the IgA titer 1:64 and the 2nd grade of inflammation had the p value of 0.112, and we can state a tendency that IgA antibodies (at

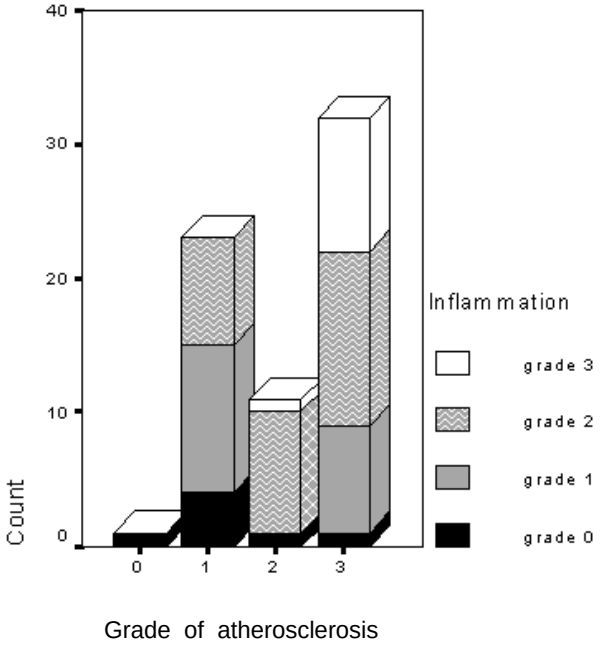

Fig. 1. Correlation between the level of inflammation and the grade of atherosclerosis in atherosclerotic tissue sections by histological examination

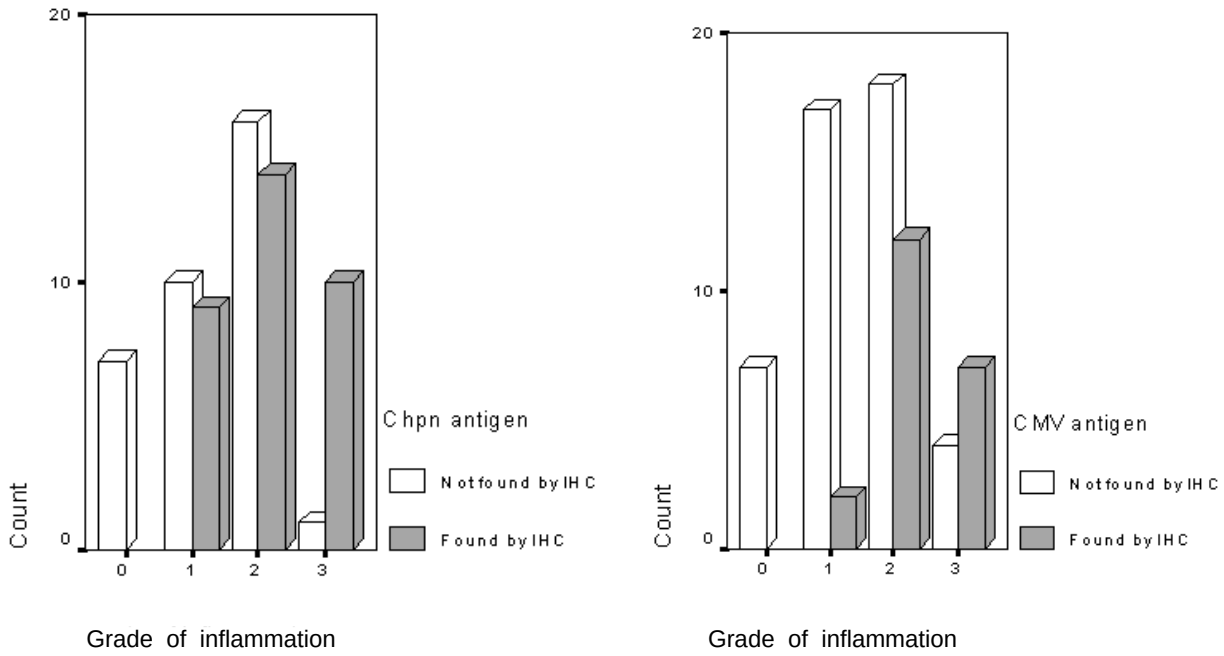

Fig. 2. Correlation between *C. pneumoniae* and CMV antigens and the level of inflammation at the site of atherosclerosis

high 1:64 titers) are more often elevated in cases with a more intensive infiltration of inflammatory cells in atherosclerotic plaques (Fig. 3).

From 12 cases that were seronegative for *C. pneumoniae*, only two specimens were positive for antigen by IHC and only one specimen was positive from five cases seronegative for CMV antibodies.

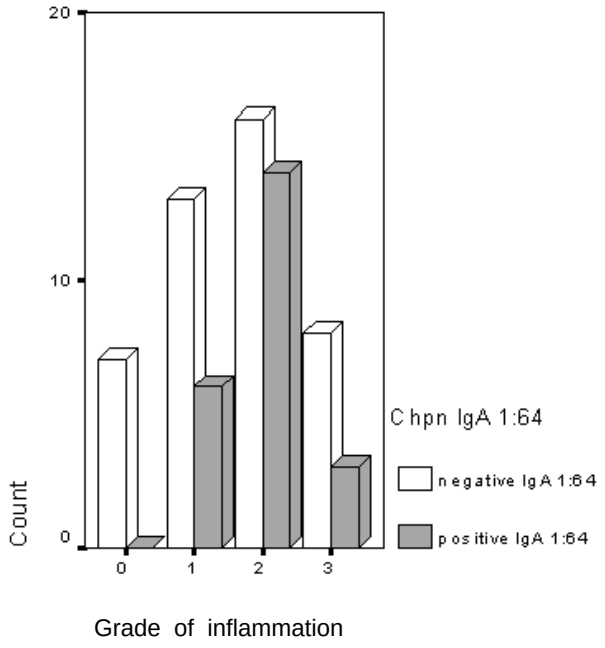

Fig. 3. Correlation between high titers of Ig A antibodies to *Chlamydia pneumoniae* and the grade of inflammation in atherosclerotic plaque (p = 0.112, not statistically significant, but showing the tendency)

DISCUSSION

This study showed that 33 out of 67 arteries were found to be positive for *C. pneumoniae* antigens, and 21 out of 67 were positive for CMV antigens (49.3% and 31.3% respectively). The detection rate is similar to those reported in studies from other countries (35–38). The immunoreactivity to *C. pneumoniae* and CMV was related to the level of inflammatory cell infiltration in atherosclerotic plaques, while a correlation with the grade of atherosclerosis was found only with *C. pneumoniae* antigen. These results received by IHC should be taken with caution as it is well known that IHC methods are prone to false positive reactions due to crossreactivity of the used antibodies with non-specific antigens (39); also, immuno-

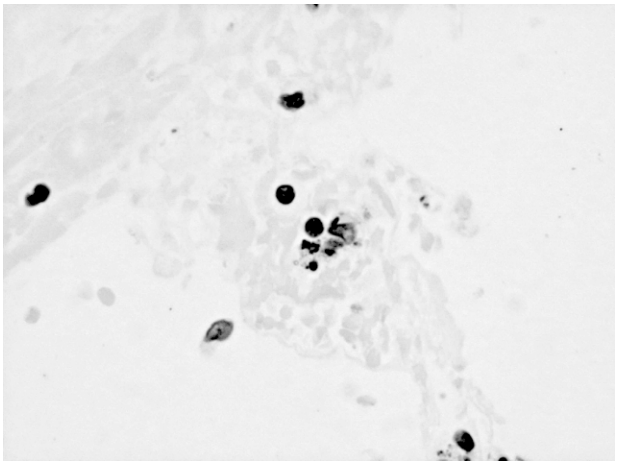

Fig. 4. Section of coronary artery: immunohistochemical staining for *C.pneumoniae* (magnification 400x, *C.pneumoniae*-specific monoclonal antibody, clone RR402, DAKO)

cytochemical techniques can give results with a decreased specificity when applied to atheromatous tissue (12, 34). Generally, the reports on the presence of *C. pneumoniae* in the vascular tissue showed variable results: detection rates varied from 0% to 83% and microbial antigens were more frequently detected than their DNA (39, 40). In most reports, all positive results obtained by any of the methods have been accepted as "true positives", despite of disagreement and lack of concordance with the other methods used (12). Ideally, positive findings should be accepted as "true positive" if they are confirmed as positive by two (or more) independent *C. pneumoniae*-specific techniques, what is difficult to achieve in practice. On the other hand, an inconsistent rate of pathogen detection and disconcordant results might be due to a focal and random distribution of the infectious agent in atheroma (14), and it is difficult to investigate the same place of atheroma by several methods.

Analysis of serological response to the investigated infections showed that IgA antibodies to *C. pneumoniae* were more often elevated than IgG antibodies. Also, the highest titers of IgA (1:64) antibodies to *C. pneumoniae* were more often detected in the cases of the 2nd grade of inflammation in the atheroma. We confirmed findings of other investigators that the presence of infectious agent's antigens in the atherosclerotic tissue is rare in individuals seronegative for *C. pneumoniae* and cytomegalovirus antibodies. From 12 cases that were seronegative for *C. pneumoniae*, only two specimens were positive for the antigen by IHC and only one specimen was positive from five cases seronegative for CMV antibodies. These findings might implicate false positive IHC or false negative serology, or may be due to a delay, or even due to a lack of immune response. On the other hand, *C. pneumonae* culture-positive infection episodes without seroconversion have been reported, especially in children (41).

Different seroepidemiologic studies furnished heterogeneous results. On the other hand, seroepidemiologic studies on the association between *C. pneumoniae* and CMV with atherosclerosis are limited by the high percentage of older adults with antibodies: 60–80% of older adults have antibodies to *C. pneumoniae* and CMV, since virtually almost everyone is infected with these pathogens during his or her lifetime. In the group of symptomatic atherosclerotic patients we have found that 62 (92.5%) out of 67 has IgG antibodies to CMV and 51 (76.1%) had IgG antibodies to *C. pneumoniae*. In addition to the limitations caused by the high seroprevalence of antibodies in the general population, discrepancies in the published results of seroepidemiologic studies might be due to the different criteria for defining CVD and inconsistent criteria used for indicators of a chronic *C. pneumoniae* infection (12). Also, different studies for antibody detection used different methods that correlate poorly and lack standardization. According to Recommendations from the Centers for Disease Control and Prevention (USA) and the Laboratory Centre for Disease Control (Canada), only the microimmunofluorescence assay (MIFA) should be used as a "gold standard" for investigation of *C. pneumoniae* antibodies (34). Despite different and inconsistent results of serological studies, it should be emphasized that serological associations neither prove the causality nor indicate the mechanisms how pathogens can contribute to atherogenesis.

CONCLUSIONS

The results of the present study allow to state that *C. pneumoniae* and cytomegalovirus are present and can be detected in atherosclerotic lesions of vessel walls. All IHC-positive samples should be confirmed by other methods to avoid false-positive results due to a crossreactivity of antibodies with non-specific antigens. There is a statistically significant correlation between the level of inflammation and the grade of atherosclerosis. The presence of *C. pneumoniae* and cytomegalovirus antigens is related to the level of inflammation at the sites of atherosclerosis. *C. pneumoniae*, but not CMV antigens are related to the grade of atherosclerosis.

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S. Jankauskienë, Z. Kuèinskienë, E. Þurauskas, I. Boguðienë, A. Laurinavièienë, A. Laurinavièius

TIESIOGINIS *CHLAMYDIA PNEUMONIAE* IR CITOMEGALOVIRUSO NUSTATYMAS ATEROSKLEROZINIAME AUDINYJE IMUNOHISTOCHEMINIU METODU IR SEROLOGINIO ATSAKO Á ÐIAS INFEKCIJAS TYRIMAS

Santrauka

Ávadas. Aterosklerozë yra lëtinis uþdegiminis procesas, kuris gali vystytis kaip atsakas á lëtiná mikrobø antigeniná dirginimà. Tyrinëjama daugelio mikroorganizmø reikðmë aterosklerozës raidai, taèiau stipriausias yra *Chlamydia pneumoniae*, citomegaloviruso (CMV) ir aterosklerozës ryðys.

Tyrimo medþiaga ir metodai. Iðtirti 57 aterosklerozës paþeistø arterijø (*a. carotis, a. femoralis*, ir *a. tibialis*) fragmentai – operacinë medþiaga, paimta endarterektomijø bei kraujotakà atstatanèiø rekonstrukciniø operacijø metu ið Lietuvos populiacinës grupës ligoniø, kuriems buvo nustatyta *arteriosclerosis obliterans* ir miego arterijos stenozë. Dar 10 ðirdies vainikiniø arterijø su makroskopiniais aterosklerozës poþymiais buvo paimta autopsijos metu. Buvo atliktos aterosklerozinio audinio pjûvio imunohistocheminës reakcijos, siekiant nustatyti *Chlamydia pneumoniae* ir citomegaloviruso antigenus. Be to, tiriamøjø asmenø kraujo serumuose nustatyti antikûnai prieð ðiuos infekcijø sukëlëjus.

Rezultatai. Ið 67-iø iðtirtø ateroskleroziniø arterijø *C. pneumoniae* ir citomegaloviruso antigenai buvo aptikti atitinkamai 49,3% ir 31,3% atvejø ir statistiðkai patikimai koreliavo su uþdegiminës làstelinës infiltracijos intensyvumu aterosklerozinëje plokðtelëje (*C. pneumoniae* atveju p = 0,002, o CMV atveju p = 0,004). Nustatyta statistiškai patikima koreliacija tarp *C. pneumoniae* antigenø ir aterosklerozës laipsnio aterosklerozinëje plokðtelëje (p = 0,0001). 28-iuose mëginiuose (41,7%) infekcijø sukëlëjø nebuvo aptikta, o 15-oje mëginiø (22,3%) aptikti abiejø sukëlëjø antigenai. Nustatyta teigiama Spearman'o koreliacija (reikðmë 0,444, p = 0,0001) tarp uþdegiminës infiltracijos intensyvumo aterokslerozinëse plokðtelëse ir aterosklerozës laipsnio jose. IgM klasës antikûnø prieð *C. pneumoniae* nebuvo aptikta ir tik 4 tiriamiesiems nustatyti IgM klasës antikûnai prieð CMV. Aukðti IgA klasës antikûnø prieð *C. pneumoniae* titrai buvo aptinkami daþniau negu IgG klasës antikûnø. Nustatyta ryðio tendencija tarp aukðtø Ig A klasës antikûnø titrø (1:64) ir II laipsnio uþdegiminës infiltracijos ateroskleroziniame audinyje.

Išvados. Atliktais tyrimais nustatyta, kad *C. pneumoniae* ir CMV antigenø yra ir juos galima rasti aterosklerozës paþeistø kraujagysliø sienelëje. Nustatyta statistiðkai patikima koreliacija tarp uþdegiminës infiltracijos aterosklerozinëje plokðtelëje intensyvumo ir aterosklerozës laipsnio joje. *C. pneumoniae* ir CMV antigenai koreliuoja su uþdegimo intensyvumu aterosklerozinіame audinyje, o *C. pneumoniae* dar ir su kraujagysliø aterokslerozës laipsniu.

Raktaþodþiai: aterosklerozë, *Chlamydia pneumoniae*, citomegalovirusas, uþdegimas, imunohistochemija