Detection of *Chlamydia pneumoniae*—Reactive T Lymphocytes in Human Atherosclerotic Plaques of Carotid Artery


**Abstract**—Linkage between *Chlamydia pneumoniae* infection and atherosclerosis has been confirmed in several studies, but the precise role of this organism in the disease process is not known. We investigated the relation and reactivity of T lymphocytes from human carotid plaques to *C pneumoniae* antigens. Tissue specimens were obtained from 17 patients who underwent carotid endarterectomy. Immunohistological staining and/or in situ hybridization revealed the presence of *C pneumoniae* in 11 (64%) of the 17 of the cases. Inflammatory infiltration seen in the vessel walls consisted primarily of CD45RO+ T-memory lymphocytes (median 80%, range 50% to 90%), whereas CD20+ B cells and monocytes were in minor proportion. In vivo activated T lymphocytes were propagated from the specimens with interleukin-2, and the antigen specificity of the established T-cell lines (TLLs) was analyzed against *C pneumoniae* elementary body antigen. TLLs were established from all 17 carotid tissues but none from the control specimens of ascending aorta. *C pneumoniae* was recognized as a specific T-cell–stimulating antigen in 7 (41%) of 17 cases. Further analyses of the *C pneumoniae*—reactive TLLs showed that chlamydial 60-kDa heat-shock protein induced specific proliferation in 5 (71%) of 7 cases and revealed 2 haplotype (DRB1*1502 and DQB1*06) binding motifs in human 60-kDa heat-shock protein. *C pneumoniae* was identified as a specific microbial antigen recognized by 41% of TLLs propagated from in vivo activated plaque T cells. Our results suggests that cell-mediated immunity to *C pneumoniae* plays a role in the atherosclerotic process and that this response may involve autoimmunity. *(Arterioscler Thromb Vasc Biol. 2000;20:1061-1067.)*

**Key Words:** atherosclerosis ■ *Chlamydia pneumoniae* ■ activated T cells

Atherosclerotic plaque is characterized as a chronic inflammatory condition involving the interaction of endothelial cells, smooth muscle cells, macrophages, and lymphocytes. Secretion of inflammatory cytokines and a broad spectrum of oxidative and proteolytic substances by macrophages contributes to the plaque size and the disease process. Macrophages and T lymphocytes are found in the lesions at an early stage of the disease, but the biological role of intraskeletal T lymphocytes is not defined. The expression of interleukin-2 receptor and various costimulatory molecules on a small number of these T cells indicates recent local contact with a specific antigen and active involvement of the inflammatory reaction in the disease process. Although target antigens for the in vivo activated T lymphocytes are not known, bacterial and viral infections have recently received considerable attention in the etiopathogenesis of atherosclerosis.

Several seroepidemiological studies associate *Chlamydia pneumoniae* with atherosclerosis and promote the organism as a major etiopathological factor of atherosclerosis. *C pneumoniae* is an obligate intracellular pathogen that infects several different cell types typical in the pathogenesis of atherogenesis, including monocytes and macrophages. Macrophages are thought to spread infection from the respiratory tract to other organ systems. Although human monocytes can restrict development of infectious progeny of *C pneumoniae*, we have recently demonstrated that the organism remains metabolically active for at least 10 days in macrocytes infected in vitro and stimulates the proliferation of T cells. *C pneumoniae* antigen induces secretion of proinflammatory cytokines (tumor necrosis factor–α, interleukin [IL]-1, and interferon-γ) from monocytes and T cells in vitro, suggesting a possible immunopathogenetic link between the organism and atherosclerosis.

To study the relation of acquired immunity to *C pneumoniae* and atherosclerosis, we established T-cell lines (TLLs) from in vivo activated lymphocytes of carotid ather-
oma by using T-cell growth factor and IL-2 and analyzed their reactivity to chlamydial antigens and to peptides from the human 60-kDa heat-shock protein (HSP60).

**Methods**

Atherosclerotic specimens of the carotid artery were obtained from 17 consecutive male patients, aged 64±8 (mean±SEM) years, undergoing carotid endarterectomy at Oulu University Hospital. The indication for surgery was transient ischemic attack, amaurosis fugax, or hemiplegia. The stenotic lesion was examined before surgery by duplex ultrasonography imaging and by carotid angiography, with the degree of carotid stenosis varying from 80% to 95%. As control specimens, cylinder-shaped pieces (5 mm in diameter) from macroscopically normal ascending aorta were obtained from 6 male patients aged 69±10 (mean±SEM) years undergoing coronary artery bypass surgery for treatment of severe coronary heart disease. A part of each tissue specimen was immediately placed in RPMI 1640 tissue culture medium (Sigma Chemical Co) supplemented with glutamine and antibiotics and transported to the laboratory. Adjacent slices were immersed in 10% phosphate-buffered formalin and liquid nitrogen for immunohistochemical studies. Peripheral blood samples were drawn for immunologic studies before the surgery. On the basis of carefully performed clinical examinations, the patients had no immune diseases, inflammatory diseases, or malignancies and were free of acute infections at the time of operation. The study protocol was approved by the Ethical Committee of the Faculty of Medicine, University of Oulu.

**Immunohistochemistry**

Immunohistochemical analyses of formalin-fixed paraffin-embedded tissues were performed by the streptavidin-biotin-peroxidase method with use of a Vectastain ABC kit (Vector Laboratories) or a Histostain SP kit (Zymed) and diaminobenzidine or aminoethylcarbazole as substrates, respectively. Hematoxylin was used as a counterstain, and normal aorta and myometrium served as negative control tissues.

*C. pneumoniae* and HSP60 antigens were detected in the tissues by using specific monoclonal antibodies, RR 402 (Washington Research Foundation) and ML-30 (kindly provided by Prof. J. Ivanyi, Medical Research Council, London, UK), for C pneumoniae and HSP60 immunostaining, respectively. ML-30 antibody was raised corresponding to the sequence, 286 MLQDMAILTG -200) per sample and is expressed as percentages high-power fields (×200) per sample and is expressed as percentages of total cell number.

*C. pneumoniae* and HSP60 antigens were detected in the tissues by using specific monoclonal antibodies, RR 402 (Washington Research Foundation) and ML-30 (kindly provided by Prof. J. Ivanyi, Medical Research Council, London, UK), for C pneumoniae and HSP60 immunostaining, respectively. ML-30 antibody was raised against Mycobacterium tuberculosis HSP60 protein, and its epitope spans over a highly conserved region of bacterial and human HSP60 corresponding to the M tuberculosis sequence, 286 MLQDMAILTG-GQV290. We have previously shown that ML-30 antibody also recognizes the sequence 2APGFGDRRKMLAIDIALTGQELG10 in chlamydial HSP60 (CHSP60). Monoclonal antibodies to human T and B cells (CD45RO and CD20, BioGenex) were used for immunophenotyping of the lymphocyte population. The population of mononuclear lymphocytes was further subtyped by immunostaining frozen tissue sections with CD4 and CD8 antibodies (DAKO) and FITC-conjugated goat anti-mouse secondary antibody (DAKO). Macrophages, plasma cells, and neutrophils were counted in routine hematoxylin and eosin stainings. The number of different cell types was counted in 10 high-power fields (×200) per sample and is expressed as percentages of total cell number.

**In Situ Hybridization**

In situ hybridization was performed in formalin-fixed tissue sections placed on silane-coated slides, as previously described by Alakärppä et al.,12 with 463-kb digoxigenin-labeled C pneumoniae 16S rRNA fragment used as a probe.

**Antigens and Peptides**

Purified and formalin-killed C pneumoniae elementary body (EB) antigen1 was used for T-cell propagation in a final concentration of 0.3 μg/mL, and for antigen specificity tests of the TLLs in 0.3 to 0.03 μg/mL. CHSP60 recombinant protein (20 to 2 μg/mL), Chlamydia trachomatis EB antigen (0.5 μg/mL), and tetanus toxoid (0.3 μg/mL, National Public Health Institute) were used as control antigens in the antigen-specificity tests of the TLLs.

Epitope scanning of HSP60-reactive T cells involved 71 overlapping peptides (length was 15 amino acids each, overlapping the neighboring peptides by 7 or 8 amino acids) representing the total human HSP60 protein. The peptides were synthesized by Fmoc chemistry with use of a cleavable peptide kit (Chiron Microtopes Ltd, Ptg), as described by Reece et al14 and Lehtinen et al.15

**T-Cell Lines**

TLLs were generated from the carotid atheroma tissue specimens according to Halme et al16 as follows: Contaminating peripheral blood was washed from the tissue by vigorous shaking with sterile water for 20 seconds, followed by 3 rinses with RPMI 1640 medium. Tissue was minced into small pieces (2×2 mm) and placed on a plastic Petri dish (Corning) in 10 mL of RPMI 1640 medium supplemented with glutamine, 20 μg/mL streptomycin, and 10% heat-inactivated human AB serum (Finnish Red Cross). In vivo activated T cells expressing IL-2 receptors were propagated by adding 10% (vol/vol) IL-2 (Biotest) into the culture medium. The cultures were incubated without antigen in a humidified 5% CO2 atmosphere at 37°C for 10 days. Half of the RPMI 1640 medium containing IL-2 was changed every third day. Thereafter, the growing lymphocytes were harvested from the Petri dish, washed once with RPMI 1640 (1600 rpm, 10 minutes), and transferred onto 24-well tissue culture plates (Sarlin Ltd) and further augmented by stimulation with C pneumoniae EB antigen (0.3 μg/mL) in the presence of irradiated autologous peripheral blood mononuclear lymphocytes (PBLs, 106 cells per well) as antigen-presenting cells and IL-2—containing culture medium in a total volume of 1 mL.16 Stimulation for the expanding TLLs was repeated as described above 2 to 3 times over periods of 10 days to achieve a cell number that was sufficient to perform antigen-specificity tests. IL-2 was added no later than 4 days before the specificity test. PBLs were isolated from heparinized blood by Ficoll-Paque (Pharmacia Biotech) density gradient centrifugation and suspended in RPMI-1640/AB medium.

The PBLs that were used as antigen-presenting cells were stored in the presence of 60% AB serum and 7.5% dimethyl sulfoxide (MERCK) at −150°C for later use.

Continuously growing TLLs were derived also from in vivo activated lymphocytes (106 cells) from peripheral blood by use of the protocol described above.

**TLL Analyses**

The antigen specificity of the TLLs was tested by culturing 20 000 cells in triplicate in 96-well round-bottomed microtiter plates (Sarlin Ltd) in the presence of 200 irradiated autologous PBLs and antigen suspended in RPMI 1640 supplemented with 10% AB serum in a total volume of 200 μL. Cultures were incubated in humidified 5% CO2 at 37°C for 72 hours as described by Halme et al.16 [Methyl-3H]Thymidine (0.2 Ci per well, Amersham Life Sciences) was added to the wells for the last 18 hours. The cells were harvested from each well on nitrocellulose filters (Wallac) with the use of an automatic cell harvester (Skatron AS), and the lymphocyte proliferation responses were measured in counts per minute of radioactivity incorporated into the proliferating cells by use of a liquid scintillation counter (Wallac).

The results are expressed as mean counts per minute or as stimulation indexes (SIs, the ratio of counts per minute in the presence of antigen to the counts per minute in its absence) calculated from triplicate cultures.

For the HLA restriction analysis, purified monoclonal antibody to the HLA-DR (L243), HLA-DQ (SPVL3), or HLA class I (W6/32) molecule was added (5 to 90 μg/mL) to the microtiter plates with autologous antigen-presenting cells. C pneumoniae EB antigen and TLL cells were added, and the cultures were incubated as described above.

Surface antigens of the TLLs were stained by use of FITC-conjugated anti-CD4 and -CD8 monoclonal antibodies (Caltag Laboratories) for immunofluorescence flow cytometric analysis by FACScan (Becton Dickinson and Co).
Results

Histology

Hematoxylin and eosiin staining of carotid artery walls showed severe atherosclerosis with foam cells, extracellular accumulation of lipids, and calcification. The numbers of inflammatory cells in the specimens varied markedly, ranging from <10 to >500 cells per 10 high-power fields. Immunophenotyping of the lymphocytes showed that the infiltrate consisted mainly of CD45RO+ T-memory cells (median 80%, range 50% to 90%), whereas CD20+ B cells were <5% (range 0% to 30%). Immunofluorescence staining showed the majority (70%) of T cells to be CD4 positive.

Twenty percent of the cells were macrophages (10% to 30%), and 10% were plasma cells (5% to 20%), as counted by hematoxylin and eosiin staining; neutrophils were found only occasionally. Only occasional lymphocytes were observed by hematoxylin and eosiin staining of the control specimens (<5 cells per 10 high-power fields). They did not involve in vivo activated T cells because no lymphocyte propagation was found when the IL-2– containing medium was used.

Eleven (65%) of the 17 specimens were positive for C pneumoniae immunostaining by using RR402 monoclonal antibody (Table 1). Of these, 3 were also found positive for C pneumoniae by in situ hybridization. Expression of HSP60 protein was detected in 14 (82%) of the 17 specimens by immunohistochemistry with the use of ML30 antibody (recognizes endogenous and chlamydial protein).

T-Cell Specificity to C pneumoniae

Using IL-2– containing medium without antigen, we were able to propagate lymphocytes from all 17 carotid specimens and PBL cultures but none from the 6 nonatherosclerotic specimens. The cells (range of cell number 3×10^4 to 5×10^5) were augmented by stimulation with C pneumoniae antigen. The antigen-specificity test was performed thereafter, and C pneumoniae was shown to induce a positive (SI >3) proliferative response in 7 (41%) of the 17 TLLs derived from the carotid tissue (Figure 1).

Table 1. Summary of Findings in Atherosclerotic Carotid Artery Walls

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Degree of Inflammation*</th>
<th>Immunohistochemistry†</th>
<th>Cpn In Situ Hybridization‡</th>
<th>Cpn-Specific TLL§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cpn</td>
<td>HSP60</td>
<td>Hybridization</td>
</tr>
<tr>
<td>1</td>
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<td>–</td>
<td>–</td>
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<td>17</td>
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<td>–</td>
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<td>–</td>
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<tr>
<td>Total positive</td>
<td></td>
<td>11/17</td>
<td>14/17</td>
<td>6/16</td>
</tr>
</tbody>
</table>

Cpn indicates C pneumoniae; ND, not done.

*Number of lymphocytes counted in 10 high-power fields in hematoxylin- and eosiin-stained sections: +/– indicates <10; +, 10–50; ++, 50–100; and ++++, >500.
†Grading of immunopositivity: – indicates no positive staining; +/–, occasional cells positive; and + to ++++, number of cells showing positive staining.
‡Grading of hybridization positivity: –, indicates no positive staining; +/-, ambiguous finding; and +, positive finding.
§Result in lymphocyte proliferation test: – indicates SI <3; +, SI >3.
and 3 of those 7 specimens were \textit{C pneumoniae} positive by immunochemistry (Table 1). \textit{C pneumoniae} antigen was recognized by the TLLs derived from tissue and peripheral blood in 4 cases, and in 2 cases, the antigen was recognized by only the TLLs derived from blood, indicating that in vivo activated cells were also circulating in the blood. The remainder of the established TLLs (10 cases) consisted of IL-2–dependent cells, showing no specificity to \textit{C pneumoniae} antigen (SI, 3).

Median response to PHA mitogen was 10 506 cpm (interquartile range 5408 to 13 774), indicating that each TLL consisted of viable lymphocytes.

Proliferative responses of the 7 \textit{C pneumoniae}–specific carotid TLLs were strong in the presence of the whole \textit{C pneumoniae} EB antigen and differed markedly from the responses of the 10 \textit{C pneumoniae} nonspecific TLLs (Table 2). \textit{C trachomatis} EB antigen was recognized by 4 of 7 \textit{C pneumoniae}–specific TLLs, but the responses were lower than responses to \textit{C pneumoniae} EB antigen. The antigen specificity of the proliferating TLLs was limited to \textit{Chlamydia}, because stimulation of the cells with tetanus toxoid did not induce a positive response in any of the TLLs (Table 2).

**Phenotype of the TLLs and Dependence of HLA Molecules**

Surface antigen analyses of the \textit{C pneumoniae}–specific TLLs revealed that >96% of the cells were CD4 positive and that <5% were CD8 positive. Antigen presentation to the specific T cells was primarily dependent on the HLA-DR molecule in 2 of 3 and on the HLA-DQ molecule in 1 of the 3 TLLs tested (Figure 2).

**T-Cell Reactivity to HSP60 Proteins**

The antigen specificity of the 7 \textit{C pneumoniae}–reactive carotid TLLs was further analyzed against the HSP60 protein of \textit{C trachomatis} (CHSP60). The CHSP60 protein was found to induce a positive response (SI >3) in 5 of the 7 \textit{C pneumoniae}–specific TLLs. The proliferative response of the TLLs was dependent on the concentration of the CHSP60 protein and was comparable to the responses stimulated by the \textit{C pneumoniae} EB antigen (Figure 3).

The hypothesis that immune responses to CHSP60 may be directed at autologous protein was evaluated by testing the reactivity of the CHSP60–specific TLLs against 72 overlapping 15-mer peptides derived from the amino acid sequence of human HSP60. One of the 5 CHSP60–reactive TLLs (HLA-DRB1*1301, -1501, and -DQB1*06) that was dependent on the HLA-DQ molecule (Figure 2) showed positive T-cell responses (SI >3) to 3 peptides, ie, amino acids 9 to 23, 170 to 184, and 305 to 319 (Figure 4). Sequences of the recognized peptides are conserved between the human HSP60 and CHSP60, but some differences disturbing the major histocompatibility complex class II binding motifs of the chlamydial peptides can be seen when these sequences are compared with the corresponding sequences on human HSP60 (Table 3).

**Discussion**

In the present study, we identified \textit{C pneumoniae} as a microbial antigen recognized by TLLs propagated from in vivo activated T lymphocytes isolated from atherosclerotic plaque. Collectively, 41% of the established TLLs, each

**Figure 2.** Role of HLA molecules on T-cell proliferation to \textit{C pneumoniae} antigen as a percentage of blocked proliferative response. Monoclonal antibodies to HLA-DR (\(\bullet\)), HLA-DQ (\(\bigtriangleup\)), or class I (\(\triangle\)) were used for blocking in a final concentration of 5 \(\mu\)g/mL (1), 60 \(\mu\)g/mL (2), or 90 \(\mu\)g/mL (3) in the T-cell cultures in the presence of antigen-presenting cells and \textit{C pneumoniae} antigen (0.3 \(\mu\)g/mL).

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**TABLE 2.** Proliferative Responses of the 7 \textit{Cpn}-Specific (SI >3) and 10 Nonspecific (SI <3) TLLs Established From Carotid Artery Walls to \textit{Cpn} (0.3 \(\mu\)g/mL) or to \textit{C trachomatis} (0.3 \(\mu\)g/mL) EB Antigen and to Tetanus Toxoid (0.3 \(\mu\)g/mL) Control Antigen

<table>
<thead>
<tr>
<th>Lymphocyte Stimulation</th>
<th>\textit{Cpn}-Specific TLLs</th>
<th>\textit{Cpn}-Nonspecific TLLs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median Response, cpm</td>
<td>IQR</td>
</tr>
<tr>
<td>No antigen</td>
<td>232</td>
<td>202–1632</td>
</tr>
<tr>
<td>\textit{Cpn}</td>
<td>14031</td>
<td>6560–22196</td>
</tr>
<tr>
<td>\textit{C trachomatis}</td>
<td>3002</td>
<td>1646–5481</td>
</tr>
<tr>
<td>Tetanus toxoid</td>
<td>516</td>
<td>189–2720</td>
</tr>
</tbody>
</table>

IQR indicates interquartile range. Spontaneous proliferation of the TLLs was measured in the absence of antigen. Statistical comparison was by Mann-Whitney \( U \) test.
representing T cells from plaques of one patient, reacted against C pneumoniae EB antigen. Our results suggest that C pneumoniae, which is commonly detected in atherosclerotic plaque of the carotid and coronary arteries,5,6 may cause T-cell activation and accumulation and thus contributes to the maintenance of the inflammatory reaction in atherogenesis. In addition, most (5 of 7) of the C pneumoniae–reactive TLLs also responded to CHSP60. Our results and those of Kol et al9 suggest a possible role for HSP60 in the pathogenesis of atherosclerosis.

The presence of C pneumoniae–reactive T cells in atherosclerotic carotid artery walls was not consistent with detection of chlamydial antigen and/or DNA in the tissue. Only 3 lesions containing C pneumoniae–reactive T cells were positive by immunohistochemistry for Chlamydia. Similar situations have been observed in atherosclerotic lesions,17–19 ie, the presence of specific serum antibodies in the absence of C pneumoniae antigen or DNA. These discrepancies might result from an active anti-chlamydial immune response, which results in decreased replication of the organisms in the atherosclerotic tissue, or from the limited amount of tissue that can be analyzed histologically. Alternatively, activation of C pneumoniae–reactive T cells in the absence of detectable C pneumoniae antigen in the plaques may be sustained by cross-reactive antigens, such as heat-shock proteins.

The apparent lack of antigen-specific T cells in the 8 C pneumoniae–positive lesions may also result from a low level of antigen, resulting in few activated T cells in the atherosclerotic plaques,5,4 which were not subsequently enriched by our methodology. The former correlates with the occasional lack of serum antibodies in the presence of C pneumoniae antigen in atheromas, which Kuo et al18 postulated to be partly due to an insufficient level of the chlamydial antigen in atheromas for stimulating antibody secretion. A deficient immune mechanism or relative immune suppression in some patients is also a possibility. The ability of C pneumoniae to infect nonprofessional phagocytic cells, such as endothelial cells or smooth muscle cells,5,6 which do not normally express HLA class II molecules and are therefore ineffective in presenting antigen to immune cells, may also explain the inability of establishing TLLs from some patients.

In spite of the probable involvement of C pneumoniae antigen in the inflammatory reaction in more than one third of human atheromas, the role for other immunogenic agents, especially in progressed disease, should not be underestimated. Accordingly, Stemme et al20 have demonstrated that atherosclerotic plaque contains T cells showing immune specificity for oxidized LDL. In addition, high levels of HSP60-specific antibodies,21 which under certain conditions

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**Figure 3.** Mean proliferative response (cpm, mean±SEM) of carotid T-cell lines to C pneumoniae (shaded bar) and CHSP60 (open bar) antigen in a final concentration of 0.3, 0.05, and 0.025 µg/mL and of 20, 10, and 2 µg/mL, respectively.

**Figure 4.** Proliferative response (SI) of carotid T cells obtained from a HLA-DRB1*1301–, HLA-1501–, and HLA-DQB1*06–positive patient to 15-mer peptides (0.5 µg/mL) derived from the amino acid sequence of human HSP60. Positive T-cell responses (SI >3) in the presence of autologous antigen-presenting cells were detected against the following peptides: amino acids 9 to 23 (*), 170 to 184 (®), and 305 to 319 (#).
TABLE 3. Sequences of Human HSP60 Peptides That Stimulated Carotid Tissue–Derived T Cells of an HLA-DRB1*1301–, HLA-1501–, and HLA-DOB1*06–Positive Patient, Aligned With Corresponding AA Sequences of Cpn and C trachomatis HSP60

<table>
<thead>
<tr>
<th>AA 9–23</th>
<th>AA 170–184</th>
<th>AA 304–319</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human 60 kDa</td>
<td>GADARAMLOGVDLL</td>
<td>LGIVTKDGKTLNDE</td>
</tr>
<tr>
<td>Cpn</td>
<td>NEEARKIKIKOKVTL</td>
<td>NGSITIVEAKGETV</td>
</tr>
<tr>
<td>C trachomatis</td>
<td>NEEARKIKIKOKVTL</td>
<td>NGSITIVEAKGETV</td>
</tr>
</tbody>
</table>

Bold indicates T-cell receptor binding motif; underlining, anchor for DQB1*0602 binding motif; double underlining, anchor for DRB1*0602 binding motif; *, no change; and :, conserved change.

Atherosclerosis is a multifactorial disease involving several risk factors and risk markers, but its etiopathogenesis is still largely unknown. It has been acknowledged in recent years that immune mechanisms and inflammation play a role in the progression of disease, and the possibility of an infectious etiology of atherosclerosis has received increasing attention. Although the causal link between C pneumoniae infection and the development of atherosclerosis continues to be debated, our results suggest that C pneumoniae is a specific microbial antigen that causes atherosclerotic T cells to proliferate. This cell-mediated immune response to Chlamydia, which may also involve autoimmune responses generated by human HSP60, may play a role in mediating the inflammatory process in atherosclerotic plaques.

Acknowledgments

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References


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