Great Amount of *C.pneumoniae* in Ruptured Plaque Vessel Segments at autopsy. A Comparative Study with Stable Plaques

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A possible relationship between C.pneumoniae *(CP) infection, atherosclerosis and acute myocardial infarction is a debated matter. Now we performed the search of CP in histological segments of fatal ruptured plaques and of stable plaques by histochemistry (Macchiavello stain), immunohistochemistry and* in situ *hybridization techniques. Electron microscopy and confocal laser microscopy techniques were used in two additional cases. The semi-quantitification of CP + cells (0-4+) and quantification of lymphocytes demonstrated greater amount of CP + cells and more inflammation in the adventitia of vulnerable plaque vessel segments than of stable ones, larger amount of CP + cells in adventitia than in the plaque and high frequency of CP + cells in all groups studied. This preliminary study strongly suggests a direct pathogenetic involvement of adventitial CP in the rupture of the atheromatous plaque, development of acute myocardial infarction and also in the development of atherosclerosis.*

The relationship between *C.pneumoniae* (CP), atherosclerosis and acute myocardial infarction was first suggested at the time of an epidemiological study in Finland [1]. Many works have demonstrated by different techniques, the presence of C.*pneumoniae* in atherosclerotic plaques. However, the reported incidence varies from 0% to 100% [2-5]. A clear morphological demonstration of *C.pneumoniae* was made only in aorta with aneurysm [4]. Such difficulty in demonstrating the *C.pneumoniae* in coronary specimens led authors to question the relationship between the bacteria, atherosclerosis and plaque instability. Most of the current data indicating such association have come from clinical trials. A pilot trial (ROXIS) using antibiotic therapy suggested the clinical benefits in preventing death and re-infarction for at least 6 months after the initial treatment [6].

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Received on 13/2/00
Accepted on 29/2/00

We have previously demonstrated that unstable atheromas are larger than stable ones, and more frequently have positive remodeling [7]. Adventitial inflammation, disappearance of collagen fibrosis and neovascularization are associated with plaque instability and positive remodeling of the vessel [8]. The inflammatory infiltrate in the adventitia is more intense than the inflammation inside the plaque, and this could support the hypothesis that the adventitia may be the main entrance of some infectious agents. We concluded that the unstable plaque is associated with pan-arteritis, which frequently evolve to aneurysmatic vessel enlargement. Such positive vessel remodeling may favour the development of larger fat plaques and plaque instability.

In the present work we looked for *C.pneumoniae* in the adventitia and in the plaque, using different techniques for detection of *C.pneumoniae in situ* in unstable and stable plaques in order to clarify whether *C.pneumoniae* is involved in the etiopathogenesis of such adventitial inflammation in unstable plaques.

Methods

Three groups of necropsy atheromatous coronary lesions were retrospectively studied: Group A - 11 ruptured thrombosed plaques responsible for fatal acute myocardial infarction from 11 patients; Group B - 11 stable plaques from the same patients of group A, presenting similar grade of obstruction but in another coronary branch; Group C - 11 stable plaque from 11 distinct patients who were submitted to elective by-pass surgery due to stable angina and did not die due to acute myocardial infarction.

Serial paraffin embedded 5 µm- thick sections were performed in order to detect *C.pneumoniae*, using 3 techniques– Macchiavellos's method (modified) [9]; Immunohistochemistry (monoclonal antibody, DAKO Co. USA); *In situ* hybridization (Oligonucleotide probe end-labeled with biotin, synthesized by GIBCO- BRL, USA). The amount of *C.pneumoniae* positive (CP+) cells was graded in 0 (absence); 1+ (scarce CP+ cells), 2+ (moderate number of CP+ cells) and 3+ (foci with many CP+ cells) and 4+ (many foci with a lot of CP+ cells), in slides stained by immunohistochemistry technique and Macchiavello's

method. Both methods demonstrated the same amount of CP+ cells.

Two additional recent cases were also studied by electron microscopy and confocal laser microscopy in order to certify that the positivity for *C.pneumonia* in macrophages, fibroblasts and smooth muscle cells detected by immunohistochemistry, histochemistry and *in situ* hibridization were reliable and specific. These two additional cases were from patients who died due to acute myocardial infarction and whose coronary arteries were perfused with formalin. The culprit lesion was detected macroscopically and selected for study through the 5 above described techniques of *C.pneumoniae* diagnosis. These two cases were not included in the comparative study of the 3 groups previously described.

The quantification of lymphocytes have already been performed in a study with the same cases [8]. These data were used to test the correlation between number of inflammatory cells and score of CP+ cells.

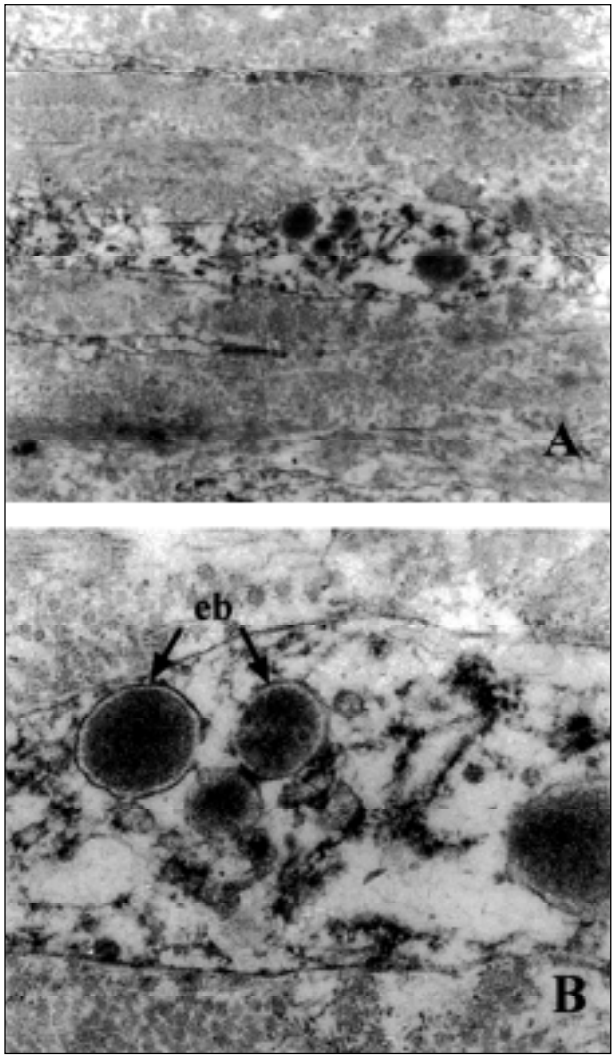

Fig. 1 - Electron micrography of an unstable plaque arterial segment showing one adventitial fibroblast containing many intracellular *C.pneumoniae* elementary bodies, the infective form of the bacteria (A- original magnification X 4200). A closer view is shown in B; original magnification X10000.

Results

All of the techniques showed many fibroblasts and macrophages positive for *C.pneumoniae* in adventitia (figures 1,2,3,4 and 5), in the adventitia of ruptured thrombosed plaque segments. There were also many positive macrophages at the base of the plaque. The number of positive cells was greater in the adventitia than in the plaque. The frequency of *C.pneumoniae* detection was very high in groups A, B and C: 100%; 100% and 82% and the mean scores of CP+ cells were 2.73; 1.55; 1.09 respectively. There was a significantly higher amount of CP+ cells (t test) in Group A than Group B ($p<0.005$) and C ($p<0.001$), but no significant difference between Groups B and C. There was no linear correlation between the amount of CP+ cells and number of inflammatory cells. However, there was a significant positive association between high score (2 or 3) of CP+ cells and moderate or severe (>15 lymphocytes/mm^2) adventitial inflammation ($p<0,05$- Chi-2 test).

Discussion

Most of clinical and epidemiological trials have pointed to an influence of *C.pneumoniae* in acute myocardial

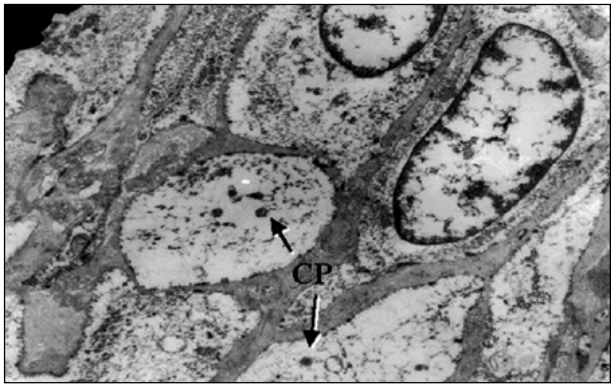

Fig. 2 - Electron micrography of adventitial cells presenting forms of *C.pneumoniae* (CP- arrows); original magnification X 2600.

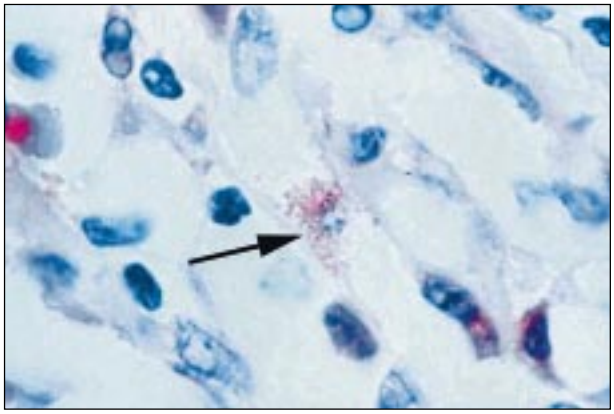

Fig. 3 - Adventitial inflammatory infiltrate from an unstable plaque arterial segment exhibiting a macrophage labeled for *C.pneumoniae* (arrow) by immunohistochemical reaction (red granules); original magnification X1000.

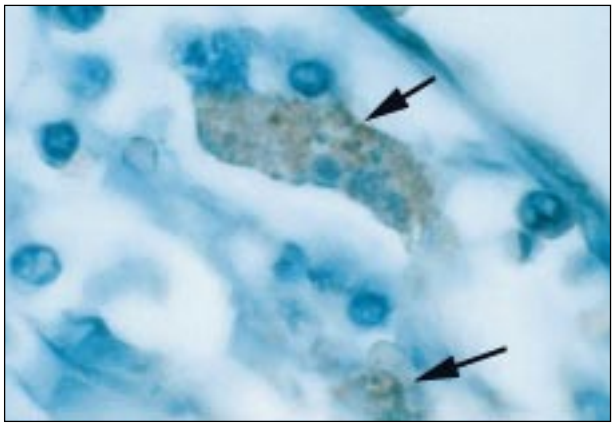

Fig. 4 - Inflammatory infiltrate at the base of the plaque showing macrophages positive for *C.pneumoniae* (arrows) by *in situ* hybridization reaction (brownish granules); original magnification X1000.

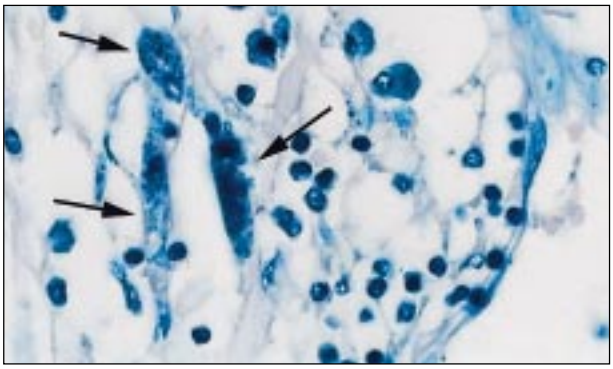

Fig. 5 - Adventitial inflammatory infiltrate from an unstable plaque arterial segment stained by Macchiavello's method, showing macrophages with cytoplasmic granules (arrows); original magnification X 630.

infarction or unstable angina. However, the lack of morphological data demonstrating presence of *C.pneumoniae in situ* in the plaque has led many authors to attribute the inflammation in the plaque to autoimmune process, questioning the direct role of the bacteria [10] or pointing to an indirect action of it by heat shock protein pathway [11] in the development of plaque instability.

Other authors have recently demonstrated CP membrane protein positivity by immunohistochemistry not only in the atheromatous plaque but also in the adventitia from old patients [12]. In the present work, we reported for the first time a significantly larger amount of *C.pneumoniae* + cells in ruptured fatal thrombosed plaques than unstable ones. Moreover, we could demonstrate by electron microscopy that the intact bacteria (not only their fragments)are present mainly in the adventitial layer. Moderate to severe inflammation is associated with high numbers of parasitized cells. These results strongly favour the concept that *C.pneumoniae* is directly involved in the development of adventitial and plaque inflammation (pan-arteritis), leading to plaque rupture. A high frequency of *C.pneumoniae* in all groups suggests its involvement in the pathogenesis of atherosclerosis.

Conclusion - Fatal vulnerable atheromatous plaques are associated with larger amount of *C.pneumoniae* + cells and severe inflammation in the plaque and adventitia, strongly suggesting a direct pathogenetic involvement of *C.pneumoniae* in the rupture of the atheromatous plaque and development of acute myocardial infarction.

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