THE ROLE OF *CHLAMYDIA PNEUMONIAE* AND CYTOMEGALOVIRUS IN CARDIOVASCULAR DISEASES

Ph.D. Thesis

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1. Introduction

Atherosclerosis (AT) is a multifactorial, chronic inflammatory disease and mediators of innate and acquired immunity have important role in various stages of AT. Earlier the aetiology of AT has focused on traditional risk profiles, including hyperlipidemia, hypercholesterinemia, hypertension, diabetes, tobacco abuses, age, sex and familial history of premature vascular disease. These risk factors, however, account for approximately half of the patients with clinically apparent AT. Thus, the search continues for other potential causes of AT. Since early AT lesions have been detected in the arteries or aorta in the majority of autopsy specimen from children aged 10-14, it is accepted that development of AT begins during childhood. Infection with certain pathogens in the early childhood might cause the initial damage in the arterial wall that develop into full AT in adults by the chronic presence of the initiating agents in the arteries, and by the contribution of well known additional risk factors. First it was shown that chickens fed cholesterol-rich diets remained free of arterial lesions, whereas those infected with the Marek's disease herpes virus and fed regular diets had lymphomatosis and developed AT in the aorta. The AT closely resembled the human disease, and could be prevented by a vaccine, prepared from the herpes virus of turkeys. Recently, the most strongly implicated infectious agents that might be involved in the development of AT are the human cytomegalovirus (HCMV) and *Chlamydia pneumoniae* (*C. pneumoniae)* bacteria. Lines of evidence suggesting that pathogens are associated with AT consist of detection of pathogens in AT tissue, seroepidemiological studies, animal models and *in vitro* studies.

HCMV, an ubiquiter pathogen**,** is a member of the herpes virus family. The prevalence of antibodies to HCMV in the general population is common. HCMV causes a wide spectrum of disorders in humans, ranging from severe birth defects to a mononucleosis-like syndrome in immunocompetent children and adults, to a disseminated disease process in immunocompromised patients. Whereas other herpes viruses are known to develop chronic and latent infections in specific sites, it is unclear where HCMV latency occurs. Endothelial cells and macrophages have been implicated as sites of CMV persistence and latency.

The presence of HCMV in AT plaques has been verified by electron microscopy, immunocytochemistry and PCR, but HCMV has also been found in normal areas of the vasculature. Animal models could provide more direct evidence of the role of infectious agents in AT process. However, AT is a multifactorial disease, developing slowly, thus it might be difficult for any infectious agent to fulfil Koch's postulates for causality. With mouse model it has been shown that MCMV accelerates AT in ApoE deficient mice. Moreover Balb/c mice with normal genetic background inoculated with MCMV expressed viral antigens in the endothelial and smooth muscle cells of the aortic wall. There is a strong evidence that HCMV infections play a role in the process of transplant-associated AT in humans, and infection with rat CMV enhances smooth muscle cell proliferation and intimal thickening in rat aortic allografts.

HCMV infection induces *in vitro* expression of molecules involved in the firm adhesion of leukocytes to endothelial cells, such as ICAM-1 in human arterial endothelial cells. It was shown that HCMV-encoded chemokine receptor US28 induces arterial smooth muscle cell migration. One of the molecular mechanisms by which HCMV may lead to an increased cellular proliferation is the binding of the IE2 product of the virus to the tumour suppressor protein p53, thereby inhibiting its function. It was demonstrated that infection of human smooth muscle cells with HCMV increases the uptake of oxidised LDL, an effect mediated, at least in part, by the class A scavenger receptors In addition, HCMV infection inhibits programmed cell death of HeLa cells in response to tumour necrosis alfa. If such an effect also occurs in infected smooth muscle cells, inhibition of apoptosis (which is a normal component of the healing response to injury) could lead to the excessive accumulation of

HCMV-infected smooth muscle cells, thereby contributing to an increase in the mass of restenosis lesions. HCMV has been demonstrated to induce procoagulant activity of endothelial cell, and these effects include inhibition of plasminogen activator, reduction of endothelial thrombomodulin concentrations and reductions of heparin sulphate proteoglycan.

Chlamydia pneumoniae is an obligate intracellular pathogen of humans. It has been adapted to humans so successfully that it can persist in the host for a long period of time after an acute infection. This microorganism can achieve a state of "latency" in which it is viable but dormant and does not multiply. Chlamydial immune evasion may also contribute to the persistence.

C. pneumoniae DNA has been detected by PCR in the AT lesions of the aorta, coronary arteries, carotid artery and arteries of the lower extremities and in the middle cerebral arteries. *C. pneumoniae* was cultured from the coronary arteries of a patient with coronary AT and from carotid plaques, indicating the presence of live bacteria in the AT tissue. In 1988 Saikku et al investigated a possible association between *C. pneumoniae* and coronary heart disease. Sixty-eight percent of patients with myocardial infarction and 50% of those with chronic coronary disease showed elevated IgG or IgA antibody levels to the *C. pneumoniae* antigen, both significantly greater than in the control group. In mice with low-density lipoprotein (LDL) receptor deficiency, in the presence of high-cholesterol diet, infection with *C. pneumoniae* significantly exacerbated the hypercholesterolemia-induced AT. As a control for the *C. pneumoniae* specificity, *C. trachomatis* MoPn strain failed to induce enhanced AT lesion, suggesting that the *C. pneumoniae* species may possess a unique atherogenic property. In ApoE-/- mice fed a normal chow diet, repeated *C. pneumoniae* infection accelerated the development of the AT lesions.

There are several pathogenic mechanisms by which *C. pneumoniae* infection could directly or indirectly induce atherogenesis, thrombosis, and plaque rupture. *C. pneumoniae* can gain access to the vasculature from the respiratory tract. Infected CD3+ T cell population and an adherent cell population consisting of monocytes/macrophages serve as targets to disseminate an infection from the lung to other susceptible tissues including arteries. It is noteworthy that *C. pneumoniae* can survive an anti-chlamydial therapy within monocytes *in vitro* and *in vivo*. The *in vivo* presence of viable pathogen not eliminated by azithromycin was shown by cultural recovery of *C. pneumoniae* from the circulating monocytes of patients with coronary artery disease. Infection of human vascular endothelial cells resulted in increased levels of MCP-1, PDGF-B activity and expression of adhesion molecules that support leucocyte adhesion. In addition, *C. pneumoniae* infection of human endothelial cells *in vitro* stimulated transendothelial migration of inflammatory cells, and triggered secretion of inflammatory mediators. AT lesions are infiltrated by macrophages and T lymphocytes, potentially reactive to pathogens. C. *pneumoniae*-specific T cells were detected in the plaques of *C. pneumoniae* seropositive patients and the plaque-derived Th1 cells expressed cytotoxicity and proapoptotic activity. More direct evidence of the possible participation of *C. pneumoniae* in atherogenesis comes from the fact that macrophages infected with *C. pneumoniae* begin to uptake LDL cholesterol and transform into foam cells, the key cells in AT, and the molecule responsible for this phenomenon is chlamydial LPS. Heat shock proteins (Hsp), which are evolutionarily very conservative and are present in both pathogen and host cells, seem to be important in the development of autoimmunity. *C. pneumoniae*-reactive T lymphocytes, specific to chlamydial Hsp60, were detected in AT plaques of carotid artery. Chlamydial Hsp is seen co-localised with human Hsp60 in AT plaques and induces the expression of adhesion molecules and oxidation of LDL molecules. Chlamydial Hsp60 also activates macrophage expression of matrix metalloproteinases that may weaken AT plaques and make them susceptible to rupture. *C. pneumoniae* may contribute to AT, and particularly

to the onset of acute AT syndromes, by increasing procoagulant properties of endothelial cells and smooth muscle cells.

Our research group started work on the project of the interactions of infectious agents and AT in 1997. At that time, very few data were available as concerns seropositivity and antibody titres to *C. pneumoniae* and HCMV in patients with CAD in Hungary. Nor was it clear whether there was an association between hHsp60 antibodies and *C. pneumoniae*/HCMV seropositivity in AT. Moreover, no data were available as to how genetic disorders such as mannose binding lectin (MBL) variant alleles influence AT in association with *C. pneumoniae* seropositivity. It was not clear then, and it remains controversial, how a dual infection with *C. pneumoniae* and CMV would affect the development of AT lesions. The possible role of histamine in AT was described earlier, but it has not been investigated whether *C. pneumoniae* infection has any effect on HDC production.

2. Aims

The present study was designated to address the following aims:

Aim 1. To determine the association of *C. pneumoniae/HCMV* and hHsp60 antibody levels with CAD, and to investigate the presence of MBL variant alleles in the *C. pneumoniae* seropositive individuals.

Aim 2. To investigate the effect of MCMV and *C. pneumoniae* infection in the aortas of mice with normal genetic background.

Aim 3. To determine whether *C. pneumoniae* infection induces HDC expression in the mouse lung, and to determine the cytokine pattern after infection.

3. Materials and Methods

Formázott: Felsorolás és számozás

3.1. Study population for the association of C. pneumoniae*, HCMV and hHsp60 antibodies with CAD*

The study population of 405 subjects (aged 35 to 77 years) was divided into 4 groups. Group 1 consisted of 248 patients with severe CAD who underwent coronary angiography in 1995 to 1996 at the National Institute of Cardiology, Budapest, Hungary, with significant stenosis (>50% occlusion), clinical signs of stable or unstable angina pectoris, and typical ECG abnormalities. Group 2 comprised 28 patients with mild CAD who underwent angiography at the same institute, with nonsignificant stenosis (<50% occlusion), clinical signs of stable or unstable angina pectoris, and typical ECG abnormalities. Group 3 comprised 53 control patients with clinical symptoms of stable angina pectoris and no coronary alterations detected on angiography. Finally, group 4 was composed of 76 blood donors who were apparently healthy on physical examination and were without history of CAD. All patients in group 1 received aortocoronary bypass graft by open heart surgery. Patients in groups 1, 2, and 3 received either antihypertensive therapy (when systolic/diastolic blood pressure measured in sitting position was >140/90 mm Hg) or lipid-lowering therapy (when serum total cholesterol or triglycerides exceeded 5.6 mmol/L or 2.2 mmol/L, respectively). In some analyses, data from group 1 (severe CAD) and group 2 (mild CAD) were combined (combined group A), and data from group 3 (patient controls) and group 4 (blood donors) were combined (group B). Smoking habit of patients in groups 1, 2, and 3 was recorded.

3.2. Study population for the association of C. pneumoniae *antibodies with MBL variant alleles*

The study was performed in 210 Hungarian patients with CAD (50 women and 160 men, aged 58.8±8.2 years) with signs of severe stenosis and clinical signs of unstable angina pectoris with typical ECG changes. Diagnosis was confirmed by coronary angiography. All patients underwent bypass surgery. Serum and DNA samples were collected from the patients

between 1995 and 1996. A questionnaire on the occurrence of different events (death of the patient, new myocardial infarction, necessity of bypass reoperation, coronary angioplasty, stroke, carotid endarterectomy, etc) was mailed to each patient in December 2000. In 4 months, 150 patients sent back filled questionnaires that could be properly evaluated. By contacting the family doctors, information on an additional 27 patients was obtained. Thus, in 177 of 210 patients (84.3%) the number and types of events that occurred during the 65±5.8-month follow-up period were known. Healthy controls included 93 women and 164 men, all Hungarian (blood donors and volunteers) and aged 46.5±11.9 years. All control subjects were examined and asked about any diseases, including CAD, in their medical history. Only healthy subjects without suspicion of CAD were enrolled in this study. A history of past and current cigarette smoking was obtained for each patient and control. Those who had stopped smoking >20 years ago were considered not to have smoking as a risk factor. Patients were considered to have hypertension if they had received a diagnosis with an arterial pressure >140/90 mmHg.

3.3. C. pneumoniae *antibodies*

Sera were tested at a 1:128 dilution for *Chlamydia*-specific IgG antibodies with a microimmunofluorescence assay (ServiMif *Chlamydia*, SERVIBIO), according to the manufacturer's instructions. Sera were designated as positive (titre ≥1:128) or negative (titre <1:128) based on typical fluorescence associated with evenly distributed Chlamydia organisms. Sera positive for *Chlamydia trachomatis* or *Chlamydia psittaci* antigens were excluded from the study.

3.4. HCMV-IE1 antibodies

Serum IgG antibodies to HCMV-IE1 antigen were determined by in-house–developed ELISA. For antigen production, chicken embryo fibroblasts were infected with a recombinant canarypox virus expressing HCMV-IE exon4 protein. Parental canarypox virus–infected cell lysate served as control antigen. Serum samples were tested at a dilution of 1:100 in a standard ELISA. Optical density (OD) values measured on parental canarypox antigen were subtracted from OD values on canarypox HCMV-IE exon4 antigen. Antibody levels were considered "low" at a calculated OD <1.00 and "high" at OD≥1.00.

3.5. hHsp60 antibodies

The level of hHsp60 antibodies was determined in the laboratory of Prof. George Fust, 3rd Department of Internal Medicine, Semmelweis University, Budapest.

3.6. Determinations of MBL variant alleles

The variant alleles were determined in the laboratory of Prof. Peter Garred, Department of Clinical Immunology, Rigshospitalet, Copenhagen, Denmark.

3.7. Statistical analyses

All statistical analyses were performed with SPSS for Windows program version 9.0. Differences between groups in continuous variables were estimated with independent-sample *t* test, nonparametric Mann-Whitney *U* test, or Kruskal-Wallis test. For dichotomous variables, χ^2 test or Fisher's exact test was used. ORs and 95% CIs (confidence intervals) were calculated. All tests were 2-tailed. Logistic regression was used to evaluate potential confounding by covariables and to calculate adjusted ORs. To assess the effect of high hHsp60 antibody levels on CAD development, dichotomous variables were created for hHsp60 antibody levels, ie, high (highest quartile) versus low (lower 3 quartiles of the distribution). This approach was selected because of skewed distributions of hHsp60 antibodies and because the risk associated with hHsp60 antibodies did not differ in the lower quartiles of distribution. Sets of 4 binary indicators were created for each interaction investigated. In the joint-effect analyses, subjects with low levels of both antibodies of interest

were used as reference to estimate the relative risk of the other 3 combinations. Differences were considered significant at $P<0.05$.

3.8. C. pneumoniae *preparation*

C. pneumoniae strain TWAR (purchased from the American Type Culture Collection, ATCC), was propagated in McCoy cells (ATCC) in 6-well plates, in modified Eagle's medium supplemented with 10% fetal calf serum, 0.3 mg/ml L-glutamine, 0.5% glucose, 4 mM HEPES, 25 μg/ml gentamycin and 1 μg/ml cycloheximide at 37°C in a 5% CO_2-saturated humidified incubator. Infected cells were harvested on day 4 and disrupted by two freeze/thaw cycles and sonication. Different harvests were pooled. After centrifugation at 1000 rpm for 5 minutes to remove cell debris, bacteria were concentrated by high-speed centrifugation at 25,000 g for 25 minutes. Pellets were resuspended in PBS, mixed with an equal volume of sucrose-phosphate-glutamic acid buffer (0.22 M sucrose, 10 mM NaH_2PO_4, 3.8 mM KH_2PO_4, 5 mM glutamic acid), aliquoted and frozen at -70°C until use. *C. pneumoniae* stocks were titered by indirect immunofluorescence (IF) assay. Briefly, McCoy cells were infected with serial dilutions of *C. pneumoniae*, incubated for 48 hours, fixed with acetone, and stained with a mouse monoclonal antibody to the major outer membrane protein (MOMP) (Dako, England), followed by FITC-labelled goat anti-mouse IgG $F(ab')_2$ (Sigma-Aldrich, Hungary). After counting inclusions under a fluorescence microscope, bacterial titres were expressed as inclusion forming units (IFU)/ml.

Bacterial titre in lungs was determined by infectivity assays. Homogenised suspensions of whole lung were freeze-thawed twice, centrifuged at 500 g for 5 minutes at 4°C to remove tissue debris, and frozen at -70°C until tested. Infectious titre expressed as IFU/lung was determined as described for bacterial stocks. The detection limit of infectious bacteria in our assay was 5×10^2 IFU/lung.

3.9. MCMV propagation

MCMV (Smith strain, purchased from ATCC) was passaged first in Balb/c mice by intraperitonally (i.p.) inoculation to increase the virulence of the strain. On day 14 after infection, mice were sacrificed and salivary glands were removed and mechanically homogenised. MCMV obtained from the salivary glands was propagated once in mouse embryonic fibroblast cells in RPMI 1640 medium supplemented with 10% foetal calf serum, 0.3 mg/ml glutamine, 25 μg/ml gentamycin. Five days before harvesting the virus, the medium was removed and replaced with 1% mouse serum supplemented RPMI 1640. Upon total cytopathic effect, the supernatant was harvested and mixed with an equal volume of sucrose-phosphate-glutamic acid buffer for storage at -70°C. MCMV was quantitated by plaque titration on mouse embryonic fibroblast cells.

3.10. Inoculation of mice

BALB/c, mice (Charles River), 6- to 8-week-old were mildly sedated by i.p. injection of 180-200 μl sodium pentobarbital (7,5 mg/ml) and inoculated intranasally (i.n.) with a dose of $2\text{-}4 \times 10^4$ or 1×10^6 *C. pneumoniae* in 30 μl of PBS. Control mice were inoculated with 30 μl McCoy cell lysate. MCMV was injected i.p. (4×10^5 PFU, 150 μl) into mice, which were γ-irradiated at a sublethal dose (450 rad, Terragamm Cobalt 60) at the time of inoculation, control mice were inoculated with tissue culture-adapted or with heat-inactivated salivary gland-adapted MCMV.

In some experiments, as indicated in the text, HDC knockout and control mice (heterozygous and homozygous wild-type mice with a CD1 background) were γ-irradiated at a sublethal dose 1 h before bacterial infection. HDC knockout mice and control mice were fed a histamine-free diet. All experiments complied with the Szeged University Guidelines for the Use of Laboratory Animals.

Törölt: intranasally

3.11. In vitro coinfection with C. pneumoniae *and MCMV*

McCoy cells were seeded into 24-well plates. After 24 hours the confluent cultures were infected with a multiplicity of infection (MOI) of 0.1 PFU MCMV or with 0.1 IFU *C. pneumoniae* or with both microorganisms simultaneously. The infected cells were centrifuged at 2000 rpm for 60 min (Heraeus, Megafuge1.0). Samples were taken daily from day 2 to 6. The titre of MCMV was determined by plaque titration on embryonic mouse fibroblast cells. The titre of *C. pneumoniae* was determined with indirect IF.

3.12. Histology

Mice were sacrificed at different times after infection, and lungs and hearts were obtained. Hearts were cut below the tips of the atria, and the upper portion was mounted on a cryostat with OCT (SAKURA, Netherlands) or fixed in periodate-lysine-paraformaldehyde (PLP) fixative. After 48 hours, PLP was changed to 70% ethyl alcohol and hearts were kept in the ethyl alcohol until paraffin embedding. Sections (6 μm thick, 30 μm apart) were obtained from the ascending aorta as and stained with hematoxylin and eosin (H&E) or Verhoeff's elastin stain. Lungs were fixed in PLP fixative, sectioned and stained with H&E. Histopathology of the aorta was evaluated in a blind fashion. For comparing sizes of inflammatory foci, images of each aortic section were captured with a high-resolution video camera (Hamamatsu, model C4742-95-10NR) attached to a microscope and stored in digital format in a computer using an imaging program (Qued Imaging Inc., Pittsburgh, PA). Inflammatory areas were measured by an image analysis software (Image J version 1.07 National Institute of Health, downloaded from the RSB home page (http://rsb.info. nih.gov) and expressed in μm^2. Student's two-tailed *t*-test was used to compare the sizes of inflammatory foci. P values were determined using Microsoft Excel 98 (Microsoft, Redmond, WA).

3.13. Immunofluorescence staining

Sections of paraffin embedded organs were processed to remove the paraffin. Slides were washed 3 times for 5 minutes each in xylol and twice for 2 minutes each in 96% alcohol. Sections were saturated in 4% milk powder PBS supplemented with 5% goat serum for 20 minutes. Pooled sera from *C. pneumoniae* - or MCMV-immunized mice at a dilution of 1:25 or 1:100, respectively, were used as first antibody, followed by FITC-labelled anti-mouse antibody (Sigma) at a 1:100 dilution.

3.14. In situ hybridisation

In situ hybridisation was carried out in the laboratory of Prof. Eva Gönczöl, Wistar Institute, Philadelphia, USA.

3.15. Cytokine ELISA assay

Törölt: *4*

3.15.1. IL-3, IL-6, IFN-γ ELISA

Homogenised lungs were centrifuged at 10 000 rpm in a microcentrifuge for 10 min, and the supernatants were collected and stored frozen at –70 °C until they were assayed for cytokine content. Murine IL-3, IL-6 and IFN-γ were detected with a commercial ELISA OptEIA kit (Becton Dickinson) according to the manufacturer's instructions. The IL-4 level in the supernatants was detected with a house-made sandwich ELISA.

3.15.2. Propagation of hybridomas and purification of antibodies

11B11 and BVD6 hybridomas producing IL-4-specific antibodies were kindly provided by Dr Giorgo Trinchieri, The Wistar Institute, Philadelphia, USA. Hybridoma cells were cultured in RPMI-1640 medium supplemented with 10% FCS, 4 mM HEPES, 25 μg/ml gentamycin until optimal cell density and sufficient volume. Supernatants were harvested by centrifuging 20 min at 250g and were filtered by 0.45 μm pore size filter. Protein G column

(Mab Trap^{TM} GII, Pharmacia, Biotech) was loaded with 200 ml supernatant. During elution step fractions were collected and monitored for protein content. The 11B11 monoclonal antibody was used as the coating antibody in ELISA test. The BVD6 monoclonal antibody fractions with highest protein content, usually the second and third fractions, were subjected to biotinylation.

3.15.3. Biotinylation of monoclonal antibody

The purified BVD6 monoclonal antibody was dialysed by ultrafiltration in colloid tube against 0,1 M $NaHCO_3$, pH=8,4 for 3 hours at 4 °C. The antibody mixed with Biotin-xx-N-succinimide ester (Clontech Laboratories) dissolved in dimethylformamide (SIGMA) was placed into15 ml tube, and incubated at room temperature for 2 hours with gentle rocking. The antibody sample was diluted approximately 2x with Na-azide containing PBS and transferred back into colloid tube, and dialysed against 2 changes of cold PBS (100 times sample volume) over night at 4 °C. The antibody sample was then transferred into storage tube and protein content was adjusted to approximately 0,5-1 mg/ml with PBS and BSA was added to give a final concentration of 1%. Biotinylated BVD6 monoclonal antibody was used as detecting antibody in IL-4 ELISA test.

3.16. The determination of the HDC expression

The determination of the HDC expression in the mouse lung was carried out in the laboratory of Prof. András Falus, Department of Genetics, Cell- and Immunobiology, Semmelweis University, Budapest.

4. Results and Discussion

Formázott: Felsorolás és számozás

4.1. Aim 1. To determine the association of C. pneumoniae*/HCMV and hHsp60 antibody levels with CAD, and to investigate the presence of MBL variant alleles in* C. pneumoniae *seropositive individuals.*

4.1.1. HCMV is not but C. pneumoniae *seropositivity is a risk in patients with severe/mild CAD versus control patients/blood donors.*

The demographic data and the HCMV-IE1, hHsp60, and *C. pneumoniae* antibody levels in the sera of patients with severe and mild CAD (combined group A) versus patient controls and blood donors (combined group B) were compared. Because of a slight dissimilarity in age distribution, a difference of borderline significance in the mean age of the two groups was seen. The percentage of males was significantly different. There was no significant difference between CMV-IE1 antibody levels (high or low) in the 2 groups. The most striking differences were found in (1) hHsp60 antibody levels, calculated as either median and range of units (continuous variables) or as percentage of individuals with high levels (highest quartile) of anti-hHsp60, and (2) *C. pneumoniae* seropositivity (titre ≥1:128). Logistic regression analysis of hHsp60 antibody levels (high and low) adjusted for sex and age confirmed a significantly higher percentage of subjects with high hHsp60 antibody levels in combined group A than in combined group B (P=0.0004; OR 5.4; 95% CI 2.1 to 14.0).

Sera of subjects in the clinically more homogeneous groups 1 and 3 were also compared for total serum cholesterol, HDL, LDL, triglyceride, and Lp(a) levels, and smoking habit was recorded. Age, sex, and total and LDL cholesterol levels, as well as CMV-IE1 antibody levels, were similar in both groups. HDL cholesterol was lower and triglyceride and Lp(a) levels were higher in group 1 than in group 3. There was a borderline significant difference in the percentage of smokers. Antibody levels to hHsp60 and *C. pneumoniae* remained significantly higher in group 1 than in group 3. When data were adjusted for age, sex, smoking habits, and HDL cholesterol, triglyceride, and Lp(a) levels by logistic regression analysis, anti-hHsp60 levels (high or low, P=0.0037, OR 9.8, 95% CI 2.1 to 45.9) and the

percentage of *C. pneumoniae* seropositives (*P*=0.047, OR 2.4, 95% CI 1.01 to 5.72) were significantly higher in group 1 than group 3. The same parameters were also compared in 35 patients (group 1) and 35 controls (group 3) selected on the basis of same age ±4.5 years, same sex, and similar cholesterol values; HCMV-IE1 antibody levels were not significantly different, whereas statistically significant differences were seen for *C. pneumoniae* seropositivity (*P*=0.009, OR 4.6, 95% CI 1.5 to 14.7) and for hHsp60 antibodies (high or low: *P*=0.012, OR 3.4, 95% CI 1.0 to 12.5). Furthermore, in group 1, there were 40, 28, and 189 patients undergoing treatment with statin, fibrate, and aspirin, respectively, at the time of blood sampling. No differences in the percentage of *C. pneumoniae* seropositives or serum concentrations of hHsp60 antibodies were found between patients with or without treatment.

4.1.2. Lack of correlation of C. pneumoniae *seropositivity with hHsp60 antibody levels; correlation with smoking habit.*

Comparison of hHsp60 antibody levels (continuous variable) in *C. pneumoniae*-negative and *C. pneumoniae*-positive individuals revealed no statistical difference between the 2 groups in the total study population, in either CAD group, or in either control group. Moreover, in either CAD group, no statistical difference was seen between *C. pneumoniae* seropositives and seronegatives in the percentage of subjects with high-level (highest quartile) hHsp60 antibodies (*P*=0.260 to 0.869) (not shown). By logistic regression analysis of data for *C. pneumoniae* seropositivity adjusted for sex, age, and hHsp60 antibodies (continuous variable), the probability value remained significant in all combinations of case and control groups, and ORs varied between 2.2 and 2.6. No correlation between hHsp60 and HCMV-IE1 antibodies was observed. In groups 1, 2, and 3, *C. pneumoniae* seropositivity was significantly associated with smoking; 81.1% of smokers but only 68.4% of non-smokers tested positive for *C. pneumoniae* antibodies (*P*=0.018).

4.1.3. Joint effects of microbial and hHsp60 antibodies in the development of AT

The simultaneous occurrence of high hHsp60 and high microbial antibodies was analysed in combined group A versus combined group B with respect to the relative risk of CAD. In subjects with *C. pneumoniae* antibody titres ≥1:128 and with high hHsp60 antibody levels (highest quartile), the risk of CAD was dramatically increased relative to subjects with no or low levels of *C. pneumoniae* antibodies and low levels of hHsp60 antibodies (lower 3 quartiles) (nonadjusted OR 83.3; adjusted for age and sex, OR 82.0). ORs for subjects with high hHsp60 antibodies and *C. pneumoniae* seropositivity (titre ≥1:128) in group 1 were also high compared with group 3 (adjusted for age and sex, *P*=0.0007, OR 38.3, 95% CI 4.7 to 312.5). However, the simultaneous presence of high CMV-IE1 and high hHsp60 antibody levels was not associated with increased risk. The simultaneous presence of high levels of CMV-IE1 and *C. pneumoniae* antibodies did not change the ORs.

4.1.4. Frequencies of MBL variant alleles in patients with CAD and healthy controls in relation to C. pneumoniae *status*

Antibodies to *C. pneumoniae* and the frequency of various MBL alleles were assessed in the 210 patients with CAD and in the 257 control subjects. No significant difference between *C. pneumoniae*–seropositive and -seronegative individuals in the frequency of MBL variant alleles was revealed [*P*=0.75, odds ratio 0.94 (95% CI 0.63 to 1.39)]. However, in *C. pneumoniae*–seropositive subjects the carriers of variant MBL alleles (*A/O* and *O/O*) occurred more frequently among patients than controls [*P*=0.091, odds ratio: 1.48 (0.94 to 2.31)], whereas no difference between patients with CAD and controls in MBL allele distribution was observed in the group of *C. pneumoniae*–seronegative.

4.1.5. Dependence of the association of **C. pneumoniae** *seropositivity with CAD on the MBL polymorphisms*

Patients were stratified according to MBL alleles, and homozygous carriers of normal allele (*A/A*) were compared with heterozygous and homozygous carriers of the variant MBL alleles (*A/O* and *O/O*). Odds ratios adjusted for variables that were found to be significantly different at univariate analysis. For the total number of patients and controls, after adjustment, *C. pneumoniae* positivity was associated with CAD only with a marginal significance. When only subjects carrying MBL variant *O* alleles were considered, however, a high [2.62 (1.07 to 6.44)] and significant (P=0.035) adjusted odds ratio of the patients with *C. pneumoniae* positivity was found (Fig. 2). Similar results were obtained when the results were adjusted for age, sex, serum triglyceride concentration, body mass index (BMI) as well as for the percentage of smokers and subjects with hypertension. In contrast, no association between *C. pneumoniae* positivity and CAD was seen in subjects homozygous for the normal *A* allele.

4.1.6. Predictive value of polymorphisms of the MBL of the major outcomes with severe CAD in the **C. pneumoniae***–positive and* **C. pneumoniae***–negative patients*

One hundred and seventy seven of 210 patients (84.3%) could be monitored up to 65±5.8 months. The predictive value of the MBL polymorphisms on the development of major outcomes of CAD (new myocardial infarction and/or new bypass operation and/or cardiovascular death) was calculated. Altogether, 12 patients developed myocardial infarction, and bypass operation was performed on an additional patient who did not develop myocardial infarction in the meantime. An additional patient died of heart attack without previous myocardial infarction or bypass operation. Therefore, major outcomes occurred in 14 patients during the follow-up period. These patients were divided according to MBL polymorphisms and *C. pneumoniae* serostatus measured in the baseline serum samples and predictive values of these variables were determined with the use of multiple logistic regression analysis. We also adjusted the analysis to other variables that may affect the outcomes. Major outcomes occurred more frequently in the carriers of the variant MBL alleles (adjusted odds ratio: 2.40 (95% CI 0.96 to 5.96), P=0.060). This association was, however, restricted to the patients positive with *C. pneumoniae*. In this group, 9 events occurred in 11 carriers of the variant alleles. The MBL variant allele carriers had a 3.27 (CI: 1.1 to 9.71, P=0.033) higher adjusted odds ratio to develop major outcomes than noncarriers. By contrast, MBL polymorphisms did not predict the development of events in patients negative for *C. pneumoniae*.

Aim 2. To investigate the effect of MCMV and **C. pneumoniae** *infection on the aortas of mice with normal genetic background.*

4.2.1. MCMV can induce advanced inflammatory lesions in the mouse aorta late after virus inoculation

Previous study revealed inflammatory foci resembling early AT plaques in humans in the aortic wall of MCMV-infected mice on weeks 4-5 after infection, and colocalization of MCMV antigens at the site of these foci. To examine the duration of the inflammatory foci and the expression of MCMV-IE transcripts in the mouse aorta, BALB/c mice in groups of 6-17 were infected with MCMV or not infected, sacrificed on weeks 6,11,18, and hearts were processed for histopathology and *in situ* hybridisation. No aortic changes were observed in uninfected mice or in mice infected with tissue culture-adapted MCMV or with heat-inactivated salivary gland-adapted MCMV and sacrificed at any time after infection. By week 6, inflammatory foci were seen in 8 of 17 salivary gland-adapted MCMV-infected mice, and *in situ* hybridisation revealed MCMV-IE-1 mRNA expression in the aortic walls of the same animals. Pathological aortic changes were detected in only 1 of 11 and 1 of 6 mice by 11 and

18 weeks after infection, respectively. These results indicate that the presence of early lesions coincides with MCMV-IE-1 mRNA expression and that most early lesions resolve over time. Some aortic sections obtained from mice sacrificed 11 and 18 weeks after virus inoculation revealed subendothelial lesions that were similar in appearance to advanced AT foci seen in humans. These were characterised by significant expansion of the subendothelial space due to collections of foamy, amorphous material, mononuclear inflammatory cells, and apparent myocytes. Elastic fibre staining revealed expanded subendothelial spaces with no elastic fibres. In a separate experiment, 30 mice were infected with MCMV and sacrificed on weeks 28-30 after infection; aortas from 4 of the mice had foci resembling advanced inflammatory foci. Thus, lesions resembling advanced AT seen in humans developed in some mice at late times after MCMV inoculation. It should be mentioned that because of the focal nature of the disease, it is possible that some foci were overlooked and that the number of mice with such subendothelial lesions was underestimated.

4.2.2. Infection of mice with a single dose of C. pneumoniae *does not induce aortic lesions, but repeated inoculations induce periaortic inflammation*

To determine whether *C. pneumoniae* alone induces inflammatory changes in the aorta of BALB/c mice with a normal genetic background, groups of 5-10 mice were inoculated with a McCoy cell preparation as controls, or 2-4x10^4 IFU *C. pneumoniae* one to three times and aortas and lungs were examined. On days 3-8 after a single inoculation with *C. pneumoniae* mice showed weakness and ruffled fur but none died. Bacteria were cultured from lung homogenates on day 3, 7 and 11 after infection. On day 19, *C. pneumoniae* antigens were detected by IF in a few cells of the lung of all tested infected mice, but no infectious bacteria could be cultured from the lung, consistent with other studies. *In situ* hybridisation disclosed *C. pneumoniae ompA* DNA on day 7 and 14 after infection in a few cells of heart muscle cells from all mice, and in aortic endothelial cells of one of three mice at each time point. Except for a very mild lymphocyte infiltration, no inflammatory foci were seen in the aortic wall of any infected mice sacrificed on days 25-50 after infection. No inflammatory foci were seen in the aortic walls in mice inoculated with uninfected McCoy cell preparation or *C. pneumoniae* once or twice (4-week interval) and sacrificed 14 to 50 days after second inoculation. Lungs of mice inoculated three times with *C. pneumoniae*, and sacrificed on day 14 after the last inoculation showed severe interstitial pneumonitis with extensive infiltration of lymphocytes and polymorphonuclear leukocytes, and exudate in alveolar spaces. In addition, massive periaortic inflammation consisting of lymphocytes and polymorphonuclear leukocytes concentrated around the cusps of the aorta was seen in four of seven mice inoculated three times with *C. pneumoniae*, and sacrificed 14 days after the last inoculation, suggesting an immunopathological mechanism for the development of lesions. *C. pneumoniae* antigens were detected also by IF in the aortic wall.

4.2.3. Superinfection of mice with C. pneumoniae *aggravates inflammatory foci induced by MCMV in the aorta*

The effect of superinfection with a single *C. pneumoniae* dose on inflammatory changes in the aortas in mice previously infected with MCMV was tested together with mice left uninfected or inoculated with MCMV but not superinfected 4 weeks later with *C. pneumoniae*. Mice were sacrificed 7 weeks after MCMV infection and processed for quantitative histological evaluation by computerised measurement of the size of inflammatory areas, as described in the Materials and Methods, in 4 sections (30 μm apart) of the ascending aorta from each mouse. A greater number of mice were positive for aorta histopathology in groups infected with MCMV first and with *C. pneumoniae* later (7 of 8 mice) than in the groups infected with MCMV only (4 of 9 mice). The mean area of inflammation in the

ascending aorta of doubly infected mice was increased 2.92 x 10^5 ± 1.6 x 10^5 μm^2, as compared with 1.04 x 10^5 ± 9 x 10^4 μm^2 in mice inoculated with MCMV only ($p < 0.01$). Uninfected mice and mice infected only with *C. pneumoniae* were negative for histopathology. Separately, mice were infected with MCMV and then with *C. pneumoniae* 4 weeks later or were left uninfected. Hearts were obtained later than in earlier experiments (11 weeks after initial infection) and processed for histopathological analysis. Similar to the previous results for the prevalence of aortic lesions, 9 of 17 doubly infected mice exhibited inflammatory foci as compared with only 1of 11 MCMV-infected mice in the same period.

4.3. Aim 3. To determine whether C. pneumoniae *induce HDC expression in the mouse lung, and to determine the cytokine pattern after infection.*

4.3.1. Detection of C. pneumoniae *and HDC in mice infected with* C. pneumoniae

To investigate the role of HDC in *C. pneumoniae* infection, 50 Balb/c mice were inoculated intranasally with 30 µl of *C. pneumoniae* (1 x 10^6 IFU). Ten mice were sacrificed on days 1, 3, 7, 16 and 31 after infection. The whole lungs from 4 mice were homogenised and the *C. pneumoniae* titres were determined by serial dilutions and IF test, the HDC contents by Western blotting and the cytokine contents by ELISA in the suspensions of the individual lungs. Lungs from 3 mice at each time point were frozen-sectioned for the *in situ* determination of *C. pneumoniae* antigens by the IF test, and lungs from 3 mice were paraffin-embedded for the *in situ* immunohistological determination of HDC expression. The infectious bacterial titres were below the level of detectability at 24 h after infection, but had increased to 1.75x10^6 IFU/lung by day 3. The peak titre of *C. pneumoniae* was on day 7, at 3.2x10^6 IFU/lung, and on day 16 after infection the titre had decreased below the detectability level. The correlation between the *C. pneumoniae* titre and the expression of HDC was examined by using the individual lung lysates from 4 mice at each time point on days 1, 3, 7 and 16 in a Western blot assay. The strongest bands of HDC were seen in each lysate obtained on day 1, when viable *C. pneumoniae* could not be cultured from the lung; weaker bands were observed in lysates obtained at later times after infection. The intensity of the bands detected in the lung lysates of uninfected mice was similar to that of detected in the lung lysates obtained on days 7 and 16 after infection, indicating a strongly increased HDC expression on day 1 and a slightly increased expression on day 3. The localisation of *C. pneumoniae* MOMP antigen and HDC-positive cells were tested on day 3 after infection. The MOMP antigen was determined in the interstitial cells of the lung by IF. The HDC-positive cells were observed especially among the epithelial cells of the bronchi, and only a few positive cells were found in the interstitium of the lung by immunohistochemistry. No HDC immunostaining was seen in the uninfected control mice. Cells stained with control IgY showed minimal immunoreactivity.

4.3.2. Production of cytokines in the infected lungs

The quantities of cytokines were determined by ELISA in the centrifuged supernatants of the homogenised lungs. *C. pneumoniae* infection induced IL-3 production in the lungs of Balb/c mice. The IL-3 production was at peak level on day 1 after infection (1.9 ng/ml) and had decreased to the level of the control mice on days 3, 7, 16 and 31. The IL-3 production correlated with the kinetics of HDC expression seen in the Western blot assay. *C. pneumoniae* produced in the lung cells did not increase the quantity of IL-3.

The IL-6 level was increased on day 1 after infection, but the peak level was on day 3 at 2.78 ng/ml. The quantity of IL-6 was decreased on day 7, and on days 16 and 31 it was similar to the level in the control mice.

The quantity of IL-4 was increased on day 3, a maximum level (32.9 U/ml) was observed on day 7, and the level was still 3 times higher than in the control lungs on day 16 after infection. The essential role of IFN-γ against *C. pneumoniae* has been described, and by showing an increased IFN-γ production with a peak on day 7 our results are consistent with these earlier observations. Our findings are supported by the detection of an increased level of IFN-γ in sera obtained from 7 of the 10 mice on day 7 (mean level of 1.71 ±0.46 ng/ml versus 0.55 ng/ml in the control sera); the IFN-γ level in the sera from 3 mice was not higher than that in the control mice.

4.3.3. C. pneumoniae *infection in HDC knockout mice*

In order to investigate the role of histamine synthesised by HDC in early *C. pneumoniae* infection, HDC knockout mice and control mice with the same genetic background were infected with *C. pneumoniae* and monitored for mortality. Before intranasal infection with 30 µl of *C. pneumoniae* preparation (1 x 10^6 IFU/mouse), all of the 20 mice (10 mice in each group) were sublethally gamma-irradiated (450 rad) to decrease the immune clearance of the bacterium.

From the group of 10 HDC knockout mice, 1 mouse died on each days of 1, 6, 7 and 8, with 6 survivors, while from the group of 10 control mice 4 mice died on day 6, 1 on day 7, 2 on day 8 and 2 on day 11, with 1 survivor. Thus, in the HDC knockout group 40% of the mice died within 8 days after infection, while the remaining mice recovered. Two months after the infection, these mice were sacrificed and the lungs were sectioned for histopathology. In a few segments, chronic infection with mononuclear infiltration was seen. In the group of control mice, the death rate was 90%.

The following of our results are considered novel

- *C. pneumoniae* and hHsp60 antibodies are independent risk factors for CAD.
- The coincidence of *C. pneumoniae* infection and high level of autoantibodies to hHsp60 is a strong risk factor for CAD development.
- *C. pneumoniae* seropositive patients carrying MBL variant alleles have increased risk for CAD.
- MCMV can induce advanced inflammatory lesions in the mouse aorta late after infection.
- Infection of mice with a single dose of *C. pneumoniae* does not induce, but repeated inoculations induce periaortic inflammation.
- Superinfection of mice with a single dose of *C. pneumoniae* aggravates inflammatory foci and increases the number of inflammatory foci induced by MCMV.
- *C. pneumoniae* infection induces HDC expression in the mouse lung.
- *C. pneumoniae* infection induces IL-3, IL-4 and IL-6 production in the mouse lung.

Summary

Atherosclerosis (AT) is an inflammatory, multifactorial disease. The development of AT in arterial walls, with complications such as chronic coronary heart disease, acute myocardial infarction and ischemic stroke, has remained one of the leading causes of death and morbidity in western industrialised countries. The traditional risk factors for these disease entities do not explain all of their clinical and epidemiological features. Thus, the search continues for additional putative risk factors involved in the development of the disease. An increasing body of evidence suggests that infections also play a role, an idea proposed by Sir William Osler at the beginning of the 20th century. The most strongly implicated infectious agents are *Chlamydia pneumoniae (C. pneumoniae)*, an obligate intracellular bacterium that causes acute and chronic respiratory disease, and human cytomegalovirus (HCMV), a

ubiquitous herpesvirus, that often infects humans very early in life and establishes latency in the infected host. Autoimmune mechanisms, including the increased expression of human heat shock protein 60 (hHsp60) and the corresponding antibodies, have also been suggested to contribute to the development of the AT disease.

In our study, elevated levels of *C. pneumoniae* and hHsp60 antibodies, but not HCMV-IE1 antibodies, proved to be significantly associated with a population of angiographically confirmed severe and moderate coronary artery disease patients versus patient controls and blood donors, and with a population of severe coronary artery disease patients versus patient controls. Multiple logistic regression analysis and subanalyses of selected subjects showed that these associations were independent of age, sex, the smoking habit and serum lipid levels. Antibodies to hHsp60 and *C. pneumoniae* were present independently in the study population, but a potent joint effect of high levels of hHsp60 and *C. pneumoniae* antibodies was observed, indicating that the coincidence of *C. pneumoniae* infection and a high level of autoantibodies to hHsp60 is a strong risk factor for coronary artery disease development. On the other hand, a joint effect of the simultaneous presence of high hHsp60 and high HCMV-IE1 antibodies was not revealed, suggesting differences between microbial infections in the interactions with hHsp60 antibodies. In another group of coronary artery disease patients, the association between *C. pneumoniae* seropositivity and the frequency of the mannose-binding lectin (MBL) variant allele was examined. After logistic regression analysis adjusted to age, sex and serum lipid levels, the frequency of MBL variant alleles proved significantly higher in the *C. pneumoniae*-seropositive coronary heart disease patients. In contrast, a significant difference was not seen in those homozygous for the normal MBL allele.

Mice with a normal genetic background were infected with murine cytomegalovirus (MCMV). By week 6, inflammatory foci were seen in the aortic walls, and the *in situ* hybridisation revealed MCMV-IE mRNA. Pathological aortic changes were seen on weeks 28-30, the lesions being similar in appearance to advanced AT foci seen in humans. No inflammatory changes were observed in the aortic walls of mice inoculated once or twice with *C. pneumoniae*. However, mice inoculated three times with *C. pneumoniae* exhibited massive periaortic inflammation, indicating an immunopathological mechanism in the development of the lesions. Inoculation of mice first with MCMV, and then with *C. pneumoniae*, a sequence suggested for infections with these common pathogens in humans, induced more inflammatory foci in the aorta, for a longer period of time, and in a higher number of mice, than after a single inoculation with MCMV. The enhanced aorta pathology in mice infected first with MCMV, and then with *C. pneumoniae,* might reflect a higher susceptibility to *C. pneumoniae* infection in MCMV-induced aortic lesions than in the normal aortic wall.

We have investigated the expression and role of histidine decarboxylase (HDC) during acute *C. pneumoniae* infection in mice, and have demonstrated that *C. pneumoniae* infection induced the expression of HDC in the lungs. The level of the most important maturation factor of histamine, IL-3, was also increased, and correlated with the expression of HDC. We have further established that *C. pneumoniae* infection induces IL-4 and IL-6 production in mice.

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