

The effect of viable *Chlamydia pneumoniae* on serum cytokines and adhesion molecules in hemodialysis patients

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Background. *Chlamydia pneumoniae* (Cp) induces the production of cytokines and adhesion molecules in infected host eukaryotic cells. The causes for pro-inflammatory cytokine and adhesion molecule increase in hemodialysis (HD) patients have not been fully elucidated. The possibility that, in this particularly atherosclerotic population, Cp, a microorganism implicated in the infectious-based inflammatory hypothesis of atherosclerosis' is also responsible for these molecules' increase is assessed in this study.

Methods. In 130 stable HD patients, serum interleukin-1 β (IL-1), interleukin-6, tumor necrosis factor α , interleukin-10, L-selectin, E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 (VCAM-1) levels were determined. Cp presence was identified by inoculation of the patient's peripheral blood mononuclear cells (PBMCs) in Hep-2 cell lines and subsequent polymerase chain reaction (PCR) in DNA extracted from cell cultures, as well as by determination of serum IgG antibodies against Cp (IgGCp).

Results. Patients, positive or negative for IgGCp, had no statistically significant differences in all molecules measured. Patients with viable Cp in PBMCs had higher serum levels of IL-1 and soluble VCAM-1 than negative ones for IgGCp (IL-1 6.87 ± 7.35 vs. 2.34 ± 1.47 pg/mL; $P = 0.0009$ and VCAM-1 1647.16 ± 513.64 vs. 1162.14 ± 546.83 ng/mL; $P = 0.0115$, respectively). Viable Cp in PBMCs remained a significant predictor factor for IL-1 and VCAM-1 in statistical analysis, when patients' characteristics and dialysis conditions were also evaluated.

Conclusions. Our results showed that some serum cytokine and adhesion molecule increase in HD patients could be attributed to viable Cp presence in PBMCs. These findings support the Cp-based inflammatory atherogenous hypothesis and add a better understanding of these molecules' increase in HD patients.

Inflammation plays a central role in the initiation and progression of atherosclerosis [1]. A complex network

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of intercellular signaling peptides, such as cytokines, adhesion molecules, and growth factors, produced by activated cells are among the main effector molecules involved in this process [1]. *Chlamydia pneumoniae* (Cp) is the microorganism most frequently implicated in the infection-based inflammatory atherogenous hypothesis [2]. A continuously increasing amount of mainly experimental work shows that Cp is capable of infecting and activating all types of cells involved in atheroma formation and development [3].

On the other hand, hemodialysis (HD) patients are in an inflammatory state and the cytokine-adhesion molecules network is disturbed [4]. The causes for this disturbance are not fully understood. Chronic persistent infections may be a cause for these molecules' increases [4] and Cp might be associated with inflammation in these patients [5, 6].

The aim of this study was to correlate Cp presence with a series of pro-inflammatory cytokines, the anti-inflammatory interleukin 10 (IL-10), and soluble adhesion molecules determined in serum of HD patients, in an effort to explore the possibility that chronic persistent infection with Cp is a cause for these disturbances in this particularly atherosclerotic category of patients.

METHODS

One hundred forty-two stable HD patients were examined after informed consent was given. Twelve patients were excluded because of active infection the month before study initiation. Forty percent of the remaining 130 subjects were females; 61.91 ± 13.72 (mean \pm SD) (range, 18 to 87 years) years old, 23.4% were smokers, 14.6% were diabetics, 44.6% were hypertensives, 18.2% had chronic bronchitis, 32.3% were on angiotensin-converting enzyme inhibitors, 37% were on acetylsalicylic acid (80 mg/day), and 1.2% were on statins. Body mass

index was 25.72 ± 4.49 and serum albumin 4.05 ± 0.28 g/dL. The cause of end-stage renal failure was chronic glomerulonephritis for 40% of patients, hypertensive nephrosclerosis for 16.9% of patients, polycystic disease for 14.6% of patients, diabetic nephropathy for 10.8% of patients, other causes for 7.7% of patients, and the cause was unknown in 10% of patients. The time in HD was 72.77 ± 65.07 (1 to 280) months; 88.5% had an A-V fistula; 39.2% had nonfunctioning vascular accesses; 96.9% were on classic HD with bicarbonates; 3.1% on hemodiafiltration; 68.3% were dialyzed with a modified cellulose membrane, and 36.2% were dialyzed with a synthetic one. The dialyzer ultrafiltration coefficient was <10 mL/hour mm Hg in 66.2% of patients, the dialysis filter surface area 1.65 ± 0.30 m², and the water processing was common for the whole group of patients.

Blood collection

Blood samples of 15 mL were taken before dialysis from vascular access immediately following venipuncture within two consecutive days for all patients. Two 5 mL EDTA tubes were used for the cell cultures, and one 5 mL tube without anticoagulant was used for cytokines, adhesion molecules, and serum IgG antibodies against Cp (IgGCp) determination.

Serum cytokines and soluble adhesion molecule assays

Sera separated from the coagulated blood by centrifugation within 30 minutes after collection were transferred immediately to sterile tubes and stored at -20°C until use. Interleukin 1 β (IL-1) (intra-assay/interassay precision 8.5%/8.4%), interleukin-6 (IL-6) (4.3%/6.3%), tumor necrosis factor α (TNF) (5.2%/7.4%), IL-10 (5%/7.3%), L-selectin (L-sel) (6.8%/9.9%), E-selectin (E-sel) (5%/8.8%), soluble intercellular adhesion molecule-1 (ICAM-1) (4.8%/7.4%), and soluble vascular cell adhesion molecule-1 (VCAM-1) (5.9%/10.2%) were evaluated by enzyme-linked immunosorbent assay (ELISA) (R&D System Europe, Ltd., Oxon, United Kingdom). All samples and standards were assayed in duplicate.

IgGCp assay

Sera separated from blood samples were aseptically transferred to sterile tubes and stored at -20°C until use. IgGCp were determined by indirect micro-immunofluorescent assay (MIF) techniques (MRL Diagnostics, Cypress, CA, USA). The serum screening dilution was 1/16 in phosphate buffered saline (PBS). End point titers $\geq 1/16$ were considered positive, providing evidence of infection at an undetermined time.

Detection of viable Cp in PBMCs

Cp was isolated from PBMCs after inoculation of Hep-2 cell cultures with buffy coats and subsequent detection by PCR.

Preparation of buffy coats. Five mL of EDTA-whole blood from each patient was centrifuged at 3000 g for 15 minutes. The buffy coat was carefully aspirated with a sterile Pasteur pipette, transferred into 2.5 mL cryovials, and stored at -160°C of liquid nitrogen until the day of determination.

Cell cultures. Cell cultures were performed by the shell vial technique using a commercially available kit (Vircell, S.L., Granada, Spain). Cells were detached after shaking with glass beads, and the resulting homogenates were used for the PCR detection of Cp. Controls consisted of *C. pneumoniae* ATCC VR-1355 TWAR strain 2043 suspensions, which were run as the buffy coat samples.

PCR for Cp. For the detection of Cp in cell culture homogenates, a nested PCR was performed using a commercially available kit (Clonit srl, Milano, Italy). The amplification product was a 193 base pair (bp) fragment of the gene encoding the RNA polymerase beta of Cp. Amplified products were detected by conventional agarose-gel electrophoresis. Extraction procedures of DNA from cell culture homogenates were included in the kit.

The above PCR protocol succeeded in detecting about 20 to 30 *C. pneumoniae* elementary bodies in 300 mL of cell culture medium, as confirmed after staining of air-dried suspensions with anti-Cp fluorescent monoclonal antibody. This was a very good sensitivity, as compared with previous reports [7]. In the specificity control, PCR failed to detect DNA from a mixed suspension of ATCC reference strains, including *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Enterococcus faecalis*, *Candida albicans*, and *Campylobacter jejuni*.

Statistics

All molecules determined and used in our analysis with parametric techniques were transformed to their natural logarithms due to their highly skewed distribution. Results are expressed as mean \pm SD of the initial values. Spearman's correlation coefficient was calculated for the univariate analysis between initial values of the parameters. Multivariate one way analysis of variance (MANOVA) was initially used to compare the vectors of means of eight molecules (IL-1, IL-6, TNF, IL-10, E-sel, L-sel, ICAM-1, and VCAM-1), determined between the patients with or without viable Cp in PBMCs, as well as between patients with IgGCp titer $\geq 1/32$ and $< 1/32$. The results (data not shown) strongly suggested that one or more of the means were different between the two former groups, but not between the latter two. After the multivariate analysis, the univariate *t* test was applied in order to determine which one of the means differed between various groups of patients. Multiple linear regression analysis was applied as described in the **Results** section. A significance level of 0.05 was used for all statistical tests.

Table 1. Serum cytokine and soluble adhesion molecules in patients with or without *Chlamydia pneumoniae* in Peripheral Blood Mononuclear Cells and serum IgG Cp antibodies $\geq 1/32$ or $< 1/32$

	Cp in PBMCs ^a			IgGCp ^b		
	Positive (9 patients)	Negative (121 patients)	P value	$\geq 1/32$ (37 patients)	$< 1/32$ (93 patients)	P value
IL-1 ^c pg/mL	6.87 ± 7.35	2.34 ± 1.47	0.0009 ^k	2.62 ± 1.57	2.67 ± 2.91	0.8529
IL-6 ^d pg/mL	7.80 ± 5.11	8.98 ± 12.49	0.7434	7.75 ± 7.46	9.35 ± 13.52	0.8231
TNF ^e pg/mL	11.48 ± 3.70	14.11 ± 16.22	0.9991	14.08 ± 20.16	13.87 ± 23.63	0.3098
IL-10 ^f pg/mL	6.45 ± 8.21	4.61 ± 7.21	0.7241	4.13 ± 4.16	4.98 ± 8.19	0.6125
L-Sel ^g ng/mL	1185.22 ± 227.15	1199 ± 410.12	0.6382	1162.28 ± 450.63	1212.35 ± 572.30	0.3766
E-Sel ^h ng/mL	63.33 ± 22.76	76.72 ± 32.35	0.2725	74.68 ± 27.09	76.23 ± 33.74	0.9279
ICAM1 ⁱ ng/mL	572.27 ± 180.01	612.19 ± 161.61	0.9373	601.11 ± 134.68	612.74 ± 172.90	0.8979
VCAM1 ^j ng/mL	1647.16 ± 513.64	1162.14 ± 546.83	0.0115 ^k	1093.12 ± 443.29	1120.50 ± 514.73	0.2999

^a *Chlamydia pneumoniae* in PBMCs^b IgG serum antibodies against *Chlamydia pneumoniae*^c Interleukin 1 β ^d Interleukin 6^e Tumor necrosis factor α ^f Interleukin 10^g L-selectin^h E-selectinⁱ Intercellular adhesion molecule-1^j Vascular-cell adhesion molecule-1^k $P < 0.05$; Student's *t*-test was performed using natural logarithms for all molecules determined

RESULTS

Serum cytokines and soluble adhesion molecule levels for the 130 HD patients were: IL-1, 2.66 ± 2.59 pg/mL; IL-6, 8.90 ± 12.11 pg/mL; TNF, 13.93 ± 15.68 pg/mL; IL-10, 4.74 ± 7.26 pg/mL; L-sel 1198.22 ± 399.60 ng/mL; E-sel, 75.79 ± 31.89 ng/mL; ICAM-1, 609.43 ± 162.51 ng/mL; and VCAM-1, 1195 ± 556.59 ng/mL. Statistically significant correlations were found between L-sel and E-sel ($r = 0.2799$, $P = 0.013$), L-sel and ICAM-1 ($r = 0.2289$, $P = 0.0089$), E-sel and ICAM-1 ($r = 0.2682$, $P = 0.001$), ICAM-1 and VCAM-1 ($r = 0.2410$, $P = 0.0057$), and a positive correlation was found between IL-6 and TNF ($r = 0.1613$, $P = 0.0689$). IL-10 was negatively, but not significantly, correlated with IL-1 and L-sel.

Only 9 (6.9%) out of 130 patients were positive for viable Cp in PBMCs, while 64 patients (49.2%) were seropositive (titer $\geq 1/16$), and 37 patients (28.3%) had a serum IgGCp titer $\geq 1/32$.

Serum cytokines and soluble adhesion molecule levels in these patients are shown in Table 1. IL-1 and VCAM-1 were significantly higher in patients positive for viable Cp in PBMCs than in those who were negative, but not in patients with IgGCp (titer $\geq 1/32$) in comparison to those without serum IgGCp.

In stepwise multiple linear regression analysis, using the natural logarithm of IL-1 as the dependent variable and the positivity or negativity for viable Cp in PBMCs as independents, serum IgGCp $< 1/32$ or $\geq 1/32$, the rest of cytokines and adhesion molecules, as well as all patient characteristics and dialysis conditions, with entry factors at $P < 0.05$, and removing those factors no longer contributing at $P > 0.10$, only the viable Cp presence in PBMCs ($\beta = 0.9063$, $t = 3.809$, $P < 10^{-4}$) and the use of synthetic membrane ($\beta = 0.3651$, $t = 2.840$, $P < 0.005$)

were significant predictors for the IL-1 serum level. With the same statistical method and the natural logarithm of VCAM-1 as dependent factor, the predictors for this soluble adhesion molecule serum level were Cp in PBMCs ($\beta = 0.4419$, $t = 2.979$, $P < 0.003$) and ICAM-1 ($\beta = 0.006$, $t = 2.597$, $P < 0.011$).

DISCUSSION

In the present study we found that the few HD patients with Cp in PBMCs had higher levels of serum IL-1 and VCAM-1 than those without the presence of viable microorganism. Patients positive or negative for serum IgG antibodies against Cp did not have significantly different serum cytokine or adhesion molecule levels.

Cp is capable of inducing the production of all molecules examined in this study from different cell types [2]. Viable Cp presence in PBMCs, as was demonstrated by cell culture and PCR for the first time in this study, increases the probability of cellular activation and production of cytokines and adhesion molecules determined.

Why is it that only IL-1 and VCAM-1 were higher in patients positive for Cp compared to negative ones, while the rest of molecules examined were not? The possible explanations are: (1) other cytokines or adhesion molecules genes are induced (IL-1 among them) in the infected host cells by this pathogen and others are not [8]; (2) the quantities of cytokines released by Cp infected cells are not the same [9]; (3) the infection of PBMCs may have taken place at different times for each patient, noting that a time-dependent secretion of cytokines after Cp infection has been described [10]; and (4) host cell polymorphisms of genes encoding cytokines and adhesion molecules [11] may influence the final production of these molecules.

VCAM-1 was significantly increased in patients positive for viable Cp than in those without Cp in PBMCs. This finding may be of importance to the hypothesis linking Cp to the atherosclerotic process because VCAM-1 is not expressed under baseline conditions, but is rapidly induced by proatherosclerotic factors [12], as well as by components of Gram-negative bacteria (via activation of nuclear factor κ B) after toll-like receptor ligation (receptors that are also necessary for cellular activation induced by Cp) [13]. Finally, the role of VCAM-1 in early atherosclerosis seems to be important [14].

Viable Cp presence in PBMCs was the main predictor for IL-1 and VCAM-1 serum levels when, in statistical analysis, all patient characteristics or specific dialysis conditions were entered. These results are noteworthy because the number of positive patients was small, and patients' characteristics and dialysis factors examined in this study, capable of influencing these molecule's levels, sufficient. An interesting finding was that another determining factor for IL-1 levels was the use of synthetic membranes in these patients. Although this correlation appears at first view paradoxical because synthetic membranes are considered more biocompatible (and, hence, poor cytokine stimulators) than cellulosic ones, two groups of investigators [15, 16] showed that the use of synthetic membranes (versus that of nonmodified cellulosic ones) restores the down-regulated capacity of PBMCs in HD patients to produce a normal cytokine response.

The absence of correlation between serum IgGCp and molecules determined is in contrast to the studies that correlated seropositivity with inflammation in HD patients [5, 6], but seems to confirm the results from other studies [17] that serum antibodies against Cp are not a reliable indicator of the microorganism's presence.

Though the present study is confined by the same limitations that apply to all studies which try to bridge the gap between basic research and clinical medicine, basically demonstrating an association between viable Cp presence and serum levels of molecules measured and not a cause-and-effect relationship, we do consider that our results support data from experimental and clinical studies linking Cp and the inflammatory atherogenous process. This consideration is based mainly on the facts that IL-1 and VCAM-1 serum levels were significantly higher only in patients with viable Cp in PBMCs, applying a method of identification that proves viability (cell cultures) and determines with sensitivity and specificity (PCR) this microorganism presence, and circulating monocytes have a central role in Cp systemic dissemination as well as in the atherosclerotic process [1, 2].

Lastly, the possibility that the Cp microorganism con-

tributes to the cytokine and adhesion molecule disturbances in HD patients opens the discussion of possible new causes needing further investigation, such as chronic persistent infections, responsible for cellular activation and release of these molecules in this category of patients.

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