Unstable atherosclerotic plaques contain T-cells that respond to
Chlamydia pneumoniae

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Abstract

Objective: Atherosclerotic lesions are characterized by an immune mediated chronic inflammation. Seroepidemiological studies support a relationship between atherosclerotic disease and infection with C. pneumoniae; an association further endorsed by immunocytochemical and DNA directed studies. However, the question arises whether C. pneumoniae acts as a causal antigen, or is merely a bystander. For this reason we have analyzed the T lymphocyte population of carotid atherosclerotic plaques of symptomatic patients for their response against C. pneumoniae.

Methods: T cell lines were generated from carotid endarterectomy tissues obtained from eight patients with symptomatic disease. The response of these T cell lines against C. pneumoniae elementary bodies was analyzed by H-thymidine incorporation. T cell clones were generated by limiting dilution from the cell lines of three patients and tested for antigen specificity in the same manner. Furthermore, cytokine profiles (Th1/Th0/Th2) were established by measuring the production of IFN-γ and IL-4.

Results: Of the eight T-cell lines five responded to C. pneumoniae. Eighteen of 69 CD4-positive clones, generated from three patients with a positive T cell lines response, responded to C. pneumoniae also. The majority (17/18, 96%) of these clones showed a Th1 cytokine profile. Conclusion: These results show that in a subpopulation of symptomatic patients C. pneumoniae can activate T cells within atherosclerotic plaques suggesting that a C. pneumoniae enhanced proinflammatory Th1 response contributes to plaque destabilization in these patients.

Keywords: Atherosclerosis; Infection/inflammation; Immunology

1. Introduction

An accumulating body of data obtained through studies in seroepidemiology, immunocytochemistry, molecular biology and tissue culture shows an association between C. pneumoniae and atherosclerotic vascular disease [1–5]. Experimental studies in a rabbit model have shown that infection with C. pneumoniae accelerates atherosclerosis, whereas this process was prevented by treatment with azithromycin [6]. In patients, however, preliminary antibiotic trials have shown contradictory results [7–10], albeit that the discrepancies may relate to the fact that different patient cohorts had been included [11]. Despite the fact that C. pneumoniae can be detected in human plaques, it is presently not known whether the microorganism is causally involved in atherosclerosis, or merely acts as a bystander [12,13].

The potential role of C. pneumoniae infections in atherosclerosis is relevant since atherosclerotic plaques contain large numbers of macrophages and activated T lymphocytes, accepted to reflect a cellular immune response, and because plaque inflammation is responsible for the production of cytokines and tissue degrading enzymes which may enhance the risk of plaque rupture [4,15]. However, as yet little is known concerning the candidate antigens responsible for local T cell activation. The observations alluded to above raise the possibility that C. pneumoniae could be one of the antigens involved. Identi-
fication of *C. pneumoniae* specific T cells in plaques of patients is one approach that provides direct evidence for a link between the presence of *C. pneumoniae* in plaques and plaque inflammation. Such an association could support a direct specific antibiotic approach of atherosclerotic patients. We have therefore analyzed the T cell population present in human atherosclerotic plaques for its antigenic specificity against *C. pneumoniae* antigens. Moreover, we tested the cytokine secretion profile of *C. pneumoniae* responsive T cell clones derived from plaques, since particular cytokines, such as interferon gamma (IFN-γ), are believed to play a role in the process of plaque destabilization that precedes plaque rupture.

2. Methods

The study was approved by the local ethical committee; all patients gave informed consent.

2.1. Tissue samples

Human carotid endarterectomy specimens were collected at surgery from eight consecutive patients (mean age: 67.4 ± 6.7 yrs), all of whom were treated for symptomatic carotid artery disease, and on that basis considered to have vulnerable (unstable) plaques. From the same patients heparinized blood was collected as a source for autologous antigen presenting cells. All patients had anti-chlamydial antibodies in their serum (IgG and/or IgA) (data not shown). A representative part of the tissue was frozen immediately in liquid nitrogen for immunohistochemical evaluation. The remaining tissue was used for the isolation of T cells.

2.2. Immunohistochemistry

The presence of T cells, T cell subsets and *C. pneumoniae* in the tissue specimens was analyzed by immunohistochemistry. Cryostat sections (5 μm) were stained with antibodies against CD3 (Leu-4, Becton & Dickinson San Jose, CA, USA), CD4 (Leu-3, Becton & Dickinson), CD8 (Dako T8, Dako A/S, Glostrup, Denmark), CD68 (Dako) and *C. pneumoniae* (clone RR402, Washington Research Foundation, Seattle, USA). For all immunostains a streptavidin biotin complex technique was used, as previously described [16]. For evaluation of the anti-*C. pneumoniae* immunostaining, cultured mononuclear cells infected with *C. pneumoniae* were used as positive controls.

2.3. Chlamydia pneumoniae elementary bodies

Propagation of *C. pneumoniae* (strain TW-183, Washington Research Foundation) was carried out essentially as described previously [17]. Briefly, *C. pneumoniae* was propagated in 6-well microtiter plates. One-day-old mono-layers of HEp2 cells (American Type Culture Collection (ATCC), Rockville MD, CCL 23) were inoculated by centrifugation at 4800 ×g for 1 h at 25°C using a MOI between 0.75 and 1.0 and incubated for 72 h in Optimem medium (Life Technologies) supplemented with 10% fetal calf serum, antibiotics, and 1 μg/ml cycloheximide [18]. Chlamydia elementary bodies were purified by centrifugation through a layer of 35% sodium diatrizoate, washed in PBS, and stored aliquoted at −80°C. Because these *C. pneumoniae* preparations also contain protein fractions derived from the host cells (HEp-2), these proteins were separately isolated from HEp2 cells by the same procedure, but without inoculation of the HEp-2 cells with *C. pneumoniae*, and used in the proliferation assays as control antigens.

2.4. Antigen presenting cells

Peripheral blood mononuclear cells were isolated from the blood of each patient by gradient centrifugation using Ficoll-paque (Pharmacia Biotech, Uppsala, Sweden). Cells were frozen and stored in liquid nitrogen until further use.

2.5. Generation of T cell lines

Primary T cell cultures (from here on designated as T cell lines) were generated as described previously, with only minor modifications [19]. Briefly, endarterectomy specimens were extensively washed in PBS to remove adhered blood cells. Visible thrombi were carefully removed. The remaining atherosclerotic tissue fragments were minced, washed and put in 24 well plates (Costar, Cambridge, MA). The fragments were cultured in T cell medium, consisting of Iscove’s Modification of Dulbecco’s medium (IMDM, Life Technologies, Breda, the Netherlands) supplemented with 10% heat inactivated pooled human serum, antibiotics (penicillin/streptomycin, Life Technologies) and 10 U/ml r-IL-2 (Eurocetus, Amsterdam, The Netherlands) for 7–10 days, resulting in spontaneous migration of T cells from the plaque tissue into the culture medium. The T cells obtained were further expanded by stimulation with 10 μg/ml phytohaemagglutinin (PHA) and irradiated feeder cells (a mixture of peripheral blood mononuclear cells from two buffycoats (CLB, Amsterdam, the Netherlands), and 10% JY cells) in T cell medium. Cultures were refreshed every 2–3 days with T-cell medium.

2.6. Generation of C. pneumoniae specific T cell clones

T cell clones were generated from the T cell lines of three patients (numbers 2, 3 and 5). Ten days after stimulation with feeder cells and PHA, T cells (from the primary cultures) were incubated with *C. pneumoniae* elementary bodies and autologous, irradiated PBMCs in T cell medium. After 5 days r-IL-2 was added, leading to a
selective outgrowth of T cells responsive with *C. pneumoniae*. The resulting T cell lines were cloned by limiting dilution (0.3 cells/well) in T cell medium, supplemented with PHA, IL-2 and feeder cells. Clones obtained were further investigated for phenotype, antigen specificity and cytokine production.

2.7. Antigen specificity

Ten days after the last stimulation with PHA, IL-2 and feeder cells, the specificity of the T cell lines or clones was analyzed by incubating $1 \times 10^5$ T-cells and $1.10^5$ autologous irradiated peripheral blood mononuclear cells for 4 days in T cell medium in either the presence or absence of *C. pneumoniae* elementary bodies or control antigen in 96 well roundbottom culture plates (Costar). The MHC dependency of positive responses was always tested using antibodies against HLA-DR (B8.11.2 [20]). In case a positive response was not HLA-DR restricted, further inhibition studies with anti-HLA-DP (B21/7), HLA-DQ (SPV L3) and HLA-ABC (W6/32, ATCC) were performed. All combinations were analyzed as triplicates. Positive controls (10 µg/ml PHA) and negative controls (irradiated antigen presenting cells only) were always included. During the last 16 h of the culture 0.3 µCi $^3$H-thymidine was present per well. Cultures were harvested and incorporated radioactivity was measured by liquid scintillation counting. The results are expressed as mean counts per minute (cpm). The stimulation index (SI) was calculated as the mean cpm of cultures in the presence of antigen divided by the mean cpm of parallel cultures without antigen. Differences between experimental conditions of the T cells were analyzed by Anova with Bonferroni correction. $P \leq 0.05$ was considered statistically significant.

*T cell lines*: A positive and specific response was defined as a significantly increased proliferation in the presence of *C. pneumoniae* elementary bodies, whilst antibodies against MHC (class I or II) antigens inhibited this proliferation and no response was observed with control antigens. Cell lines were only regarded non responding when the T cell lines showed not only a lack of response to *C. pneumoniae* elementary bodies but in addition a proliferative response with mitogen PHA, a positive control for the viability of the cell line.

*T cell clones*: A positive and specific response was defined as a proliferative (significant increased) response with a SI of 3 or more with *C. pneumoniae* elementary bodies, a lack of response with control antigens, and in which proliferation could be inhibited with anti-MHC class I or II antibodies.

2.8. Phenotype analysis

The phenotype of the T cell lines and clones was determined by FACS analysis, using a indirect staining protocol. T cells were incubated with appropriate dilutions of anti-CD4 (Leu-3, Becton & Dickinson), anti-CD8 (Dakopatts, Glostrup, Denmark), or TCR-δ1 (T cell Sciences, Cambridge, MA, USA), followed by phyco-erythrin conjugated rabbit anti-mouse immunoglobulins. Fluorescence was analyzed on a FACS Calibur (Becton & Dickinson).

2.9. Cytokine profile analysis

Cytokine production of all CD4-positive T cell clones was analyzed after stimulation of $1.10^5$ T cells with immobilized anti-CD3 monoclonal antibody (OKT-3 (ATCC), 1 µg/ml) and soluble PMA (1 ng/ml, Sigma Chemical Co, St Louis, USA) in a total volume of 200 µl/well in a 96 well flat-bottom plate (Costar). After 24 h, supernatants were harvested and stored at −80°C until tested by ELISA for IL-4, and IFN-γ. ELISA kits (PeliKin Compact) were obtained from the CLB. T cell clones that produced IFN-γ but no IL-4 (<10 pg/ml) or T cell clones with an IFN-γ/IL-4 ratio >20 were considered Th1 type, T cell clones with a IFN-γ ratio between 20.0 and 0.4 were defined as Th0 type, and T cell clones that produced IL-4 but no IFN-γ (<10 pg/ml) or with an IFN-γ/IL-4 ratio <0.4 were defined as Th2-like [21].

3. Results

Immunohistochemical analysis showed that all lesions contained an inflammatory infiltrate of T cells (CD4 and CD8-positive cells) and macrophages (CD68-positive). All intimal plaques contained mononuclear cells with positive staining for *C. pneumoniae*, albeit that marked differences in the number of immunopositive cells occurred between different specimens. T cell lines were obtained from each of the eight patients. All cell lines were mixtures of CD4 and CD8-positive lymphocytes. The percentage of the CD4-positive cells in these lines was in the range of 60–90%. TCR-γδ-positive cells were not observed.

3.1. T cell responsiveness with *C. pneumoniae*

The results of the proliferation assays of the obtained cell lines are summarized in Fig. 1. Five of eight cell lines (patients 2, 3, 5, 6 and 7) showed a significantly increased proliferation after incubation with *C. pneumoniae*. With the use of anti-HLA-DR antibodies the proliferation could be inhibited in patient 2, 3, 5 and 7, showing that the response in these lines was HLA-DR restricted. One T cell line (pt. 6) showed increased proliferation which could be inhibited with anti-HLA-ABC, but not with HLA-DR, DP or DQ antibodies. None of the T cell lines responded to the control antigens.

The *C. pneumoniae* induced T cell proliferation was dose dependent. In Fig. 2 representative examples of the
Table 1

<table>
<thead>
<tr>
<th>Patient</th>
<th>Nr. of clones</th>
<th>SI ≥ 3</th>
<th>SI &lt; 3</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Th0</td>
<td>Th1</td>
<td>Th2</td>
</tr>
<tr>
<td>Patient 2</td>
<td>22</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Patient 3</td>
<td>36</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>Patient 5</td>
<td>11</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>69</td>
<td>18</td>
<td>1</td>
</tr>
</tbody>
</table>

* All C. pneumoniae responsive T cell clones were CD4-positive.

A representative example of the results obtained with one T cell clone is illustrated in Fig. 4, where the dose–response kinetics and MHC restriction are shown. A significantly increased proliferation already could be observed with C. pneumoniae elementary bodies at a dilution of 1/6250 (SI=28), whereas a maximal response was reached using a dilution of approximately 1/250 (SI=488). This response was HLA-DR restricted, since antibodies against HLA-DR almost completely inhibited C. pneumoniae induced proliferation.

3.2. Cytokine profile analysis

The cytokine secretion profiles of all CD4-positive clones were determined, irrespective of whether they were responsive or non responsive to C. pneumoniae (Table 1).
Authors were able to generate those from Mosarin et al. [22] are basically the same. All responsiveness of intraplaque T cells. Hence, these findings in expanding the cell lines, our observations and only two patients, one of whom also exhibited T cell lines by stimulation with \( C. \) pneumoniae specific T cells within these lines. We did not contain increased numbers of bodies, which resulted in selective expansion of \( C. \) pneumoniae specific T plaque composition by inhibiting the proliferation of smooth muscle cells as well as their connective tissue (collagen) synthesis; features contributing to the development of a vulnerable atherosclerotic plaque [14].

The vast majority (94%) of \( C. \) pneumoniae responsive T cell clones (SI=3) showed a Th1 cytokine profile, except for one clone which represented a Th0 type. Among the non-responsive clones (SI<3), the majority (73%) also showed a Th1 cytokine secretion pattern, 12 (23%) were classified as Th0 and 2 (4%) as Th2.

4. Discussion

This study shows that \( C. \) pneumoniae responsive T lymphocytes can be detected within the inflammatory infiltrate of atherosclerotic plaques. Five of eight endarterectomy specimens from the carotid artery of patients treated for symptomatic disease showed a specific response upon incubation with \( C. \) pneumoniae elementary bodies. Four cell lines were HLA-DR restricted, one was HLA-ABC restricted. Our results obtained with T-cell lines generated from isolated plaque T-cells are in accordance with those recently published by Mosarin et al. [22]. These authors were able to generate \( C. \) pneumoniae specific T cell lines from carotid atherosclerotic plaques from 7 of 17 patients. However, there are important differences in the culture methods used. Mosarin et al. expanded their T cell lines by stimulation with \( C. \) pneumoniae elementary bodies, which resulted in selective expansion of \( C. \) pneumoniae specific T cells within these lines. We did not perform such a positive selection step before testing our T cell lines for \( C. \) pneumoniae responsiveness. Despite this difference in expanding the cell lines, our observations and those from Mosarin et al. [22] are basically the same. All in all, these findings strongly suggest that \( C. \) pneumoniae contributes to the process of symptomatic atherosclerotic disease; at the same time, the fact that not all T cell lines show a \( C. \) pneumoniae specific response, indicate that additional candidate antigens have to be taken into account.

Our analysis of cloned T lymphocytes generated from the T cell lines of three patients who presented a positive response, showed a marked variation regarding frequency and specificity. The frequency of \( C. \) pneumoniae responsive clones was 30% in two patients, whereas from one patient only one responsive clone was obtained, although with a much higher SI. At the present stage it remains unknown which antigenic structures of the microorganisms are recognized, since \( C. \) pneumoniae elementary bodies were used to stimulate the lines and clones. For instance, it could be that some of these clones recognize heat shock proteins (HSP), in particular HSP60 which is produced by \( C. \) pneumoniae in large quantities, but known also to show a high degree of sequence homology with human HSP60 as it occurs in atherosclerotic plaques [23,24]. Moreover, one may anticipate also that other antigens within the plaque, other than \( C. \) pneumoniae, can be involved. For example, Stemme et al. [25] generated T cell lines from carotid endarterectomy specimens which responded to oxidized low density lipoproteins.

Once subpopulations of T lymphocytes have shown to be capable of recognizing \( C. \) pneumoniae antigens, it is of major importance to get informed about the patterns of cytokine secretion of these cells. It is presently well appreciated that T lymphocytes have their own programmed cytokine secretion profile [26]. On this basis T lymphocytes are categorized as Th1 cells (securing high levels of IFN-\( \gamma \) and IL-2, but no IL-4), Th2 cells (producing high levels of IL-4, but no IFN-\( \gamma \)) and Th0 cells (not showing such a polarized cytokine secretion pattern and capable of producing both IL-4 and IFN-\( \gamma \)). Th1 cells are the principal effector cells of cell mediated immune responses against intracellular microorganisms. We found that almost all (17/18, 94%) \( C. \) pneumoniae specific T cell clones showed a Th1 cytokine profile. This observation is of particular interest, since clinically stable plaques were shown to contain only 20% Th1-positive T cell clones [19]. The clinical relevance of the present findings can be exemplified by pointing out that IFN-\( \gamma \) may modulate plaque composition by inhibiting the proliferation of smooth muscle cells as well as their connective tissue (collagen) synthesis; features contributing to the development of a vulnerable atherosclerotic plaque [14].

Recent clinical studies have shown that the peripheral blood of patients with symptomatic coronary artery disease contain increased numbers of \( C. \) pneumoniae responsive T cells [27]. In our series of eight patients \( C. \) pneumoniae T cell responsiveness in the peripheral blood was positive in only two patients, one of whom also exhibited T cell responsiveness of intraplaque T cells. Hence, these find-
ings contrast markedly with the results obtained from the analysis of the intraplaque T cells. It appears that the lack of responsiveness in peripheral blood does not rule out a C. pneumoniae reactive T cell response in the plaque and that the reverse is not true either: patient 8 showed a strong response in the peripheral blood lymphocytes, but no significant increase could be observed with plaque-derived T cells. This indicates that testing the peripheral T cell response against C. pneumoniae cannot be used as a reliable method to evaluate the existence of a C. pneumoniae induced intraplaque immune response.

Furthermore it appeared that the mere presence of C. pneumoniae antigens in plaques, as detected with immunohistochemical stains, is not necessarily associated with the presence of antigen specific T cells, since the immunohistochemical presence of C. pneumoniae was observed in all eight atherectomy specimens, whereas C. pneumoniae responsive T cell lines were only obtained from five patients.

In conclusion, the combined findings of C. pneumoniae responsive plaque T lymphocytes, immunohistochemically detectable C. pneumoniae antigens in plaques and serologic evidence for a past infection with C. pneumoniae in a subpopulation of patients, strongly suggest that C. pneumoniae could act as a trigger for inflammation in plaques in these individuals. Moreover, the fact that virtually all C. pneumoniae responsive T cells are of the Th1 subtype, programmed to secrete large amounts of IFN-γ upon stimulation, a cytokine with strong tissue remodeling capacities, could relate to the onset of plaque complications in these patients. However, the fact that not all patients showed intraplaque T cell responsiveness for C. pneumoniae once more illustrates that additional factors have to be taken into consideration, and raises questions as to the effectiveness of the administration of antibiotics in a general population of atherosclerotic disease patients.

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