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Inflammation, heat shock proteins and periodontal pathogens in atherosclerosis: an immunohistologic study

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Background: Inflammation is a significant component of atherosclerosis lesions. Bacteria, including periodontopathogens, have been demonstrated in atherosclerotic plaques and cross-reactivity of the immune response to bacterial GroEL with human heat shock protein 60 has been suggested as a link between infections and atherosclerosis.
Methods: In this study, the nature of the inflammatory infiltrate and the presence of human heat shock protein 60 and GroEL were examined in 31 carotid endarterectomy specimens. Additionally, monoclonal antibodies were used to detect the presence of six bacteria, including those implicated in periodontal disease.
Results: The inflammatory cell infiltrate of the lesions was dominated by $CD14^+$ macrophages and $CD4^+$ T cells. Most cells of the infiltrate as well as the endothelium were HLA-DR^+, indicating activation; however, there was an absence of CD25 expression, demonstrating that the activated T cells were not proliferating. Few $CD1a^+$ and $CD83^+$ cells were noted. Human heat shock protein 60 expression was evident on endothelial cells and cells with the appearance of smooth muscle cells and lymphocytes. GroEL and bacteria were detected within intimal cells. *Chlamydia pneumoniae, Porphyromonas gingivalis, Fusobacterium nucleatum, Tannerella forsythia, Prevotella intermedia*, and *Actinobacillus actinomycetemcomitans* were found in 21%, 52%, 34%, 34%, 41%, and 17% of arteries, respectively.
Conclusion: These results give evidence for a specific immune response associated with atherosclerosis. Whether bacteria initiate the observed inflammation in atherosclerotic lesions is not clear; however, the present study shows that maintenance of inflammation may be enhanced by the presence of periodontopathic bacteria.

Key words: atherosclerosis, heat shock proteins, immunohistology, inflammation, periodontopathogens

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The importance of the role of infection and inflammation in atherosclerosis is now widely accepted. In atherosclerosis, low density lipoprotein (LDL) accumulates and becomes oxidized in the intima. Monocytes and T cells migrate into the arterial wall after adhesion to endothelial cells. The recruited monocytes and smooth muscle cells further accumulate oxidized low density lipoprotein and eventually die, contributing to the central necrotic core. The macrophages and T cells produce proteins which induce changes in smooth muscle cells. These cells then respond to growth factors from cells in the lesion, leading to a generalized immune reaction (2) with the formation of the established plaque.

Infection may initiate and facilitate the progression of atherosclerosis as a result of the immune response to bacterial heat shock proteins (HSPs). All cells, both prokaryotic and eukaryotic, express HSPs on exposure to various forms of stress

including temperature, oxidative injury and infection (23, 27). Factors such as bacterial lipopolysaccharide, cytokines, and mechanical stress can induce the expression of host protective (h) HSP60 on endothelial cells. Due to the homologous nature of HSPs among species (9), cross-reactivity of an immune response to bacterial HSP (GroEL) with hHSP60 on endothelial cells may result in endothelial dysfunction and the subsequent development of atherosclerosis (32). The presence of risk factors such as high blood cholesterol would enhance the expression of hHSP60 and adhesion molecules by endothelial cells and result in progression from early fatty streak lesions to severe and irreversible atherosclerotic alterations.

Periodontal disease is one of the most common chronic infections in humans, with *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Actinobacillus actinomycetemcomitans* being important bacteria associated with periodontal disease progression. Individuals with severe periodontitis have been reported to have an increased risk of developing cardiovascular diseases including atherosclerosis (7, 31) and this association been strengthened further by animal models which demonstrated acceleration of atherosclerosis following repeated inoculation with *P. gingivalis* (21, 22).

Studies controlling for other cardiovascular disease risk factors have shown that acute infections, especially bacterial respiratory infections, often precede the onset of symptoms of stroke or myocardial infarction by several weeks (14, 26, 30). A strong association of cardiovascular disease with the common respiratory pathogen, *Chlamydia pneumoniae* has been demonstrated and this organism has been detected in atherosclerotic plaques (4, 5, 34). It has been suggested that the bacteria are transported in the blood from the respiratory organs to the arterial wall, where they enter the plaque through the disrupted endothelium or small vessels in the arterial wall (34). The presence of multiple pathogens, including periodontal pathogens, in atherosclerotic plaques has been reported (4, 17). We have recently quantified bacterial numbers of *P. gingivalis*, *Fusobacterium nucleatum*, *T. forsythia*, *C. pneumoniae*, *Helicobacter pylori* and *Haemophilus influenzae* in atherosclerotic plaques using real time polymerase chain reaction (10); up to five of these species were detected in the same specimen.

The aim of this study was to investigate the relationship of periodontopathic bacteria and the expression of hHSP60 and bacterial GroEL to the inflammatory infiltrate and to examine the nature of this infiltrate in atherosclerotic lesions obtained from patients undergoing carotid endarterectomy for the treatment of atherosclerotic disease.

Material and methods

Patients

Endarterectomy specimens were obtained from 31 patients undergoing surgical treatment of atherosclerosis at the Royal Brisbane and Women's Hospital. Informed consent to use tissue that would otherwise have been discarded was obtained from each patient at the time of surgery. A written explanation of the purpose of the study was provided, and signed consent according to the Helsinki Declaration was obtained. Institutional ethics review committee approval to carry out the study was also obtained.

Preparation of tissue

After surgery, each specimen was embedded in Tissue Tek® OCT (Sakura Finetek U.S.A., Inc, Torrance, CA), quenched and stored in liquid nitrogen. Serial cryostat sections of 5 μm thickness were cut from each specimen, air-dried and fixed in equal parts acetone and chloroform for 5 min and stored at − 20°C.

Identification of infiltrating cells

$CD4^+$ and $CD8^+$ T cell subsets, $CD14^+$ cells, $CD19^+$ B cells, $CD25^+$ cells, HLA-DR^+ cells, $CD1a^+$ cells (immature dendritic cells), and $CD83^+$ cells (mature dendritic cells) were labeled using an avidin-biotin immunoperoxidase method (12). Briefly, the sections were depleted of endogenous peroxidase using 0.3% hydrogen peroxide in phosphate-buffered saline and then placed in 1% bovine serum albumin in phosphate-buffered saline to block nonspecific sites. The sections were then incubated with the following primary monoclonal IgG antibodies: mouse antihuman CD4, CD8, CD14, CD19, HLA-DR, CD1a (DAKO, Glostrup, Denmark), CD25 and CD83 (Pharmingen, San Diego, CA) (1 : 20). This was followed by biotinylated rabbit antimouse immunoglobulins (DAKO) and, lastly, streptavidin peroxidase (DAKO). The peroxidase was developed using a liquid DAB substrate-chromagen system (DAKO). Nuclei were counterstained with Mayer's hematoxylin. Tonsil sections prepared in the same way as the artery specimens were used as positive controls. Monoclonal mouse antihuman epithelial antigen (IgG1; DAKO) used as the primary antibody acted as negative controls.

Identification of hHSP60 and GroEL

hHSP60 and bacterial GroEL present in sections of the atherosclerotic lesions were labeled by the immunoperoxidase method described above using mouse antimammalian HSP60 (LK-1 clone, Stressgen Biotechnologies Corp., Victoria, British Columbia, Canada) (1 : 20) and mouse anti-GroEL (Stressgen) (1 : 400).

Identification of bacteria

The immunoperoxidase technique described above was also used to identify a panel of six bacteria in the sections using the following primary monoclonal antibodies: *C. pneumoniae* (DAKO), *P. gingivalis* lipopolysaccharide (CB5.C5), *T. forsythia*, *F. nucleatum*, *Prevotella intermedia,* and *A. actinomycetemcomitans* (1 : 160) (1). As an additional method to detect bacteria, Gram staining of the sections was carried out using the Hucker Conn modification. Due to a lack of tissue for two of the specimens, 29 of the 31 samples were used to identify the presence of bacteria.

Cell analysis

The morphology of each specimen and the extent of the inflammatory cell infiltrate were determined using sections stained with hematoxylin and eosin. For each specimen, three representative fields involving the inflammatory cell infiltrate (× 400) were viewed. The total number of cells within each field and the total number with positive membrane staining were counted and the mean percent positive cells determined as a percentage of the total number of cells in the three fields counted. The percentages of $CD4^+$, $CD8^+$, $CD14^+$, $CD19^+$, $CD25^+$, $CD1a^+$, and $CD83^+$ cells were determined. Expression of HSPs and HLA-DR and the presence of the specific bacteria were noted. Lesions were graded according to the magnitude of colonization by the specific bacteria and lesions with no bacteria detected were given a colonization score of 0. Lesions with 1–5 cells containing positively staining bacteria per representative field (× 400) were scored 1 (low bacterial load); 6–20 cells per field containing bacteria scored 2 (moderate bacterial load); and

>20 cells per field containing bacteria scored 3 (high bacterial load).

Statistical analysis

Multivariate analysis of variance using the general linear model was used to test for differences in the inflammatory cell infiltrate for lesions positive for bacteria by immunohistology and those in which none of the bacteria examined was detected. These groups were then tested for significance using Student's *t*-test. The Minitab statistical package (Minitab Inc., State College, PA) was used to perform the analyses.

Results

Nature and location of the inflammatory cell infiltrate

The atherosclerotic lesions generally consisted of a fibrous cap beneath which the main cell type observed was the lipid-laden macrophage or foam cell. There were also areas of extracellular lipid (cholesterol clefts) and areas of intimal hyperplasia (myofibroblasts). Lymphocytes were distributed throughout the lesion, but particularly at the periphery or shoulder of the central lipid rich core of the lesion. Infiltration was also observed in parts of the arterial wall that were not associated with the primary lesion.

Generally, the atherosclerotic lesion consisted mainly of $CD14^+$ macrophages (29.6% ± 3.8%), mostly as foam cells. The remainder of the inflammatory cells in the lesion were almost entirely T cells, with a 2.4 : 1 ratio of $CD4^+$ T cells (22.2% ± 3.0%) to $CD8^+$ T cells (8.6% ± 1.4%). Very few $CD19^+$ B cells were present (2.0% ± 0.7%). As well as inflammatory cells, there were abundant smooth muscle cells and a proportion of these cells were $CD14^+$. Since the smooth muscle cells appeared to function as part of this infiltrate, they were included in the total cell counts (Fig. 1A–C). Approximately one third of cells with the appearance of infiltrating lymphocytes were positive for HLA-DR (Fig. 2A,B). Few CD25, CD1a or CD83 positive cells were detected.

HSP expression

hHSP60 was expressed by endothelial cells and some cells with the appearance of smooth muscle cells as well as inflammatory cells (Fig. 3A). GroEL was observed primarily within cells and occasionally extracellularly in areas of lipid accumulation (Fig. 3B).

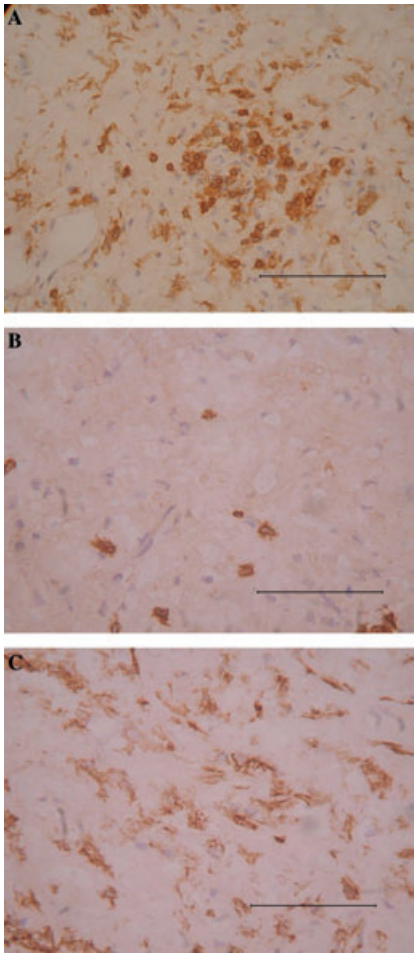

Fig. 1. $CD4^+$ T cells (A), $CD8^+$ T cells (B), and $CD14^+$ macrophages, many of which are foam cells, as well as cells with the morphology of smooth muscle cells (C) of the inflammatory cell infiltrate in serial sections of a human atherosclerotic plaque (original magnification × 400).

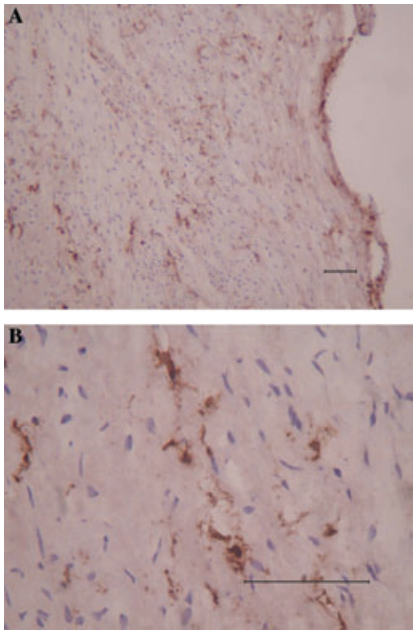

Fig. 2. A) Endothelial cells, lymphocytes and macrophages/foam cells expressing HLA-DR in a human atherosclerotic lesion (A – original magnification × 100, B – original magnification × 400).

Detection and localization of bacteria

Gram staining of the lesions demonstrated the presence of bacteria in 62% of the 29 arteries examined. The organisms were found almost exclusively within intimal cells. They were typically gram-negative cocci or small rods in large pale staining cells, with irregular cell outlines. Using immunohistology, *C. pneumoniae, P. gingivalis, F. nucleatum, T. forsythia, P. intermedia*, and *A. actinomycetemcomitans* were detected in 6 (21%), 15 (52%), 10 (34%), 10 (34%), 12 (41%), and 5 (17%), respectively, of the specimens examined (Table 1, Fig. 3C, D). Seventy-six percent of the arteries contained one or more of these bacterial species, with a single species from the panel present in 10%, two species in 35%, and three or more species in 30% of the specimens. Positive staining for these specific bacteria in the arteries generally correlated with positive Gram staining. The bacteria appeared in the same regions as demonstrated with the Gram stain, namely within cells in the central parts of the atherosclerotic lesions and occasionally in the extracellular lipid spaces.

The nature of the infiltrate in lesions positive for bacteria by immunohistology was compared with lesions in which none of these bacteria was detected. Lesions which stained positively for at least one of the bacteria examined demonstrated an infiltrate which contained a significantly greater percentage of $CD8^+$ T cells compared with lesions in which none of the test bacteria was detected ($P < 0.046$). The CD4/CD8 ratio of these lesions (2.5) was reduced compared with lesions negative for the test bacteria (3.6). A trend towards increased percentages of $CD4^+$, $CD14^+$, and $CD19^+$ cells in positive lesions was also shown (Table 2).

Discussion

The results of the present study showed that the atherosclerotic lesion consisted of a central lipid rich region surrounded by a zone with more normal arterial wall

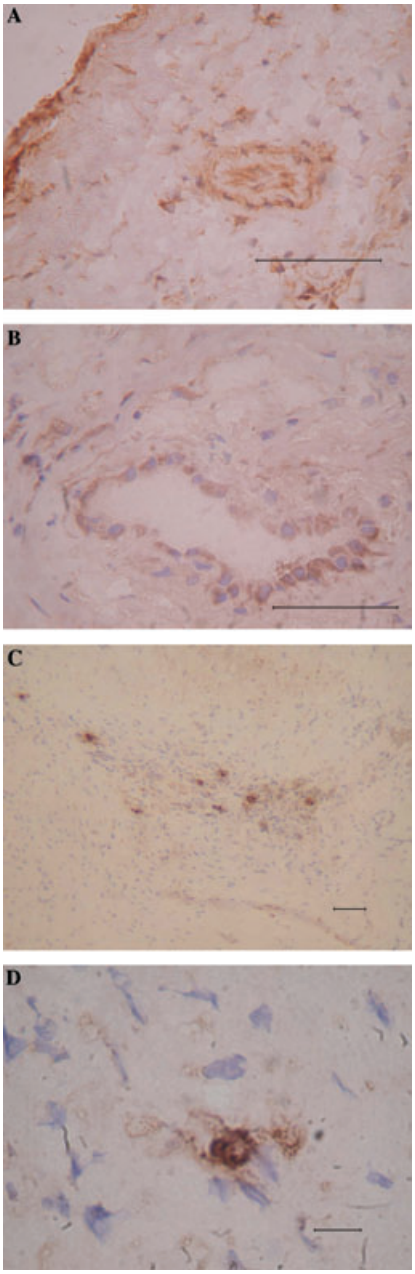

Fig. 3. hHSP60^{+} (A) and GroEL^{+} (B) endothelial cells of small vessels in the artery wall (original magnification × 400) and cells containing *P. intermedia* (C – original magnification × 100, D – original magnification × 1000) in serial sections of a human atherosclerotic artery. Scale shown represents 100 μm except in Fig. 3D, in which it represents 10 μm.

architecture. The central lesion was characterized by accumulations of lipid, both extracellularly as cholesterol clefts, and intracellularly as foam cells. An inflammatory cell infiltrate was present and was dominated by macrophages. In some areas, many of these macrophages were engorged with lipid and appeared as large cells, the foamy, pale staining cytoplasm of which caused the nucleus to appear flattened against the cell wall. T cells, with higher numbers of CD4^{+} than CD8^{+} cells, and very low numbers of B cells were also present. The periphery of the lesion typically contained fewer deposits of lipid and therefore cell density was higher in this region.

The role of T cells in atherosclerosis lesions is still not completely understood. We have previously demonstrated the presence of T cells specific for GroEL, hHSP60, and *P. gingivalis* in human atherosclerotic lesions (11). The present study showed high levels of expression of the major histocompatibility complex Class II antigen, HLA-DR by endothelial cells, lymphocytes, macrophages and smooth muscle cells, indicating an activated state; this is in agreement with previous reports (16, 29). The absence of CD25 expression, however, suggested that the T cells, although activated, were not proliferating, so antigen presentation was unlikely to have been occurring at the site of the lesion. Others have shown CD25 expression by T cells in atherosclerotic lesions (16); however, this was at quite low levels, confirming that significant T cell proliferation was not occurring in the lesion.

A lack of CD25 expression could also suggest that CD4^{+} CD25^{+} regulatory T cells were not present in the lesion. Since regulatory T cells appear to be involved in the induction and maintenance of tolerance to self antigens such as hHSP60, it is possible that their absence could lead to the persistence of antihHSP60 T-cell activity (15, 25). This does not discount the presence of other regulatory T cell subsets which could act to suppress self-reactive T cells, offering another explanation for the low levels of T-cell proliferation observed in the lesion. Very few dendritic cells were detected in the lesions, in contrast to a previous study which correlated the presence of dendritic cells with rupture-prone regions (35). As CD83 has also been shown to be expressed by cells other than dendritic cells (12), it is possible that the authors (35) included CD83^{+} cells that were not dendritic cells. In the present study, only those CD83^{+} cells with the morphological characteristics of dendritic cells were included. Additionally, as previously stated, the presence of dendritic cells appears to be related to plaque type, and therefore the reduced CD83^{+} cell counts in the present study may also be due to differences in the specimens obtained for analysis.

Although it is conceivable that macrophages and smooth muscle cells could present antigens from the lesion to the T cells, a lack of interleukin-2 receptor expression in the present study suggests that this was not occurring, as proliferation would be expected to occur following antigen presentation. This finding is in contrast to previous assumptions that macrophages present antigen to T cells in the lesion. It may be that although a specific immune response is involved, antigen presentation is occurring at remote sites of infection, such as gingival tissues in the case of periodontopathic bacteria, after which the dendritic cells circulate to the lymph nodes where antigen would be presented to specific T cells that would proliferate before homing back to the arterial and gingival tissues containing the specific bacterial antigens. In this respect, it has been shown that specific T cells migrated preferentially and were retained in gingival tissues infected with *A. actinomycetemcomitans* (19). As well, studies have shown that increased numbers of activated T cells and elevated interleukin-2 levels in the peripheral blood are associated with coronary artery disease (18), further supporting the involvement of a systemic immune response in atherosclerosis.

In the present study, GroEL was observed intracellularly, with staining occurring in areas similar to those positive for specific bacteria, therefore at least a proportion of the GroEL could be attributed to the specific bacteria detected. hHSP60 expression was noted on endothelial and some inflammatory cells and has been reported previously (20, 33). This study has therefore demonstrated the presence of hHSP60 and GroEL in atherosclerotic lesions and that these antigens appeared closely associated with an inflammatory infiltrate of activated T cells and macrophages.

The presence of multiple species of bacteria in atherosclerotic lesions has previously been demonstrated (4, 10, 17). It has been suggested that the presence of multiple intracellular pathogens in endothelial cells and the resulting endothelial dysfunction may be a mechanism contributing to the initiation of atherosclerosis and increased progression of established disease (28). These and other studies have provided serologic evidence (8) that the total pathogen burden of an individual is an important risk factor for atherosclerosis. *C. pneumoniae* exists within macrophages (3) and it has recently been shown that *P. gingi-*

Table 1. Colonization scores of microorganisms detected using immunohistologic analysis of endarterectomy specimens from 29 patients (0 = no bacteria detected, 1 = low bacterial load, 2 = moderate bacterial load)

Patient No.	Gram stain	*P. gingivalis*	*F. nucleatum*	*T. forsythia*	*P. intermedia*	*A. actinomycetemcomitans*	*C. pneumoniae*	No. of species detected
1	0	0	1	1	2	0	0	3
2	1	2	0	0	0	0	0	1
3	1	0	0	0	0	0	0	0
4	2	0	1	1	1	0	1	4
5	2	1	1	1	1	1	1	6
6	2	0	1	0	0	1	0	2
7	1	1	2	1	1	0	1	5
8	1	1	0	0	0	1	0	2
9	1	0	1	1	1	0	0	3
10	1	1	0	1	1	0	1	4
11	1	1	1	0	0	0	1	3
12	1	0	1	0	1	0	0	2
13	2	1	0	0	0	0	1	2
14	0	0	0	0	0	0	0	0
15	0	1	1	1	1	0	0	4
16	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0
19	1	1	0	0	1	1	0	3
20	0	1	0	1	0	0	0	2
21	0	0	0	1	1	0	0	2
22	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0
25	0	1	0	1	0	0	0	2
26	1	1	0	0	1	0	0	2
27	0	0	1	0	1	0	0	2
28	1	1	0	0	0	0	0	1
29	2	1	0	0	0	1	0	2
30	1	0	0	0	0	0	0	0
31	1	1	0	0	0	0	0	1
% positive arteries	62	52	34	34	41	17	21	

Table 2. Percentage of cells positive for inflammatory cell surface markers in 29 atherosclerotic lesions according to the presence of bacteria determined by immunohistology

Cell surface marker	Bacteria detected in lesion	No bacteria detected in lesion
CD4	25.0 ± 3.3	11.9 ± 5.1
CD8	10.0 ± 1.6*	3.3 ± 1.1*
CD14	33.1 ± 4.6	20.1 ± 5.9
CD19	2.6 ± 0.9	0.1 ± 0.1

* $P < 0.046$.

valis is able to adhere to and invade these cells (13) as well as endothelial cells (6). These organisms are able to persist and multiply intracellularly (24), allowing the development of a chronic low grade infection, which is consistent with our findings of low numbers of bacteria located within cells of the inflammatory infiltrate in the present study.

Previous studies of periodontal pathogens in atherosclerotic lesions have primarily used polymerase chain reaction to detect the presence of the organisms. Our recent study quantified a panel of microorganisms using real time polymerase chain reaction, which showed that the periodontopathic bacteria *P. gingivalis*, *F. nucleatum*, and *T. forsythia* were found more frequently than the other commonly associated pathogens, *C. pneumoniae*, *H. pylori*, and *H. influenzae* (10). The detection rates in that study were significantly higher than those reported in the present study. As well as the increased sensitivity of the real time polymerase chain reaction technique compared with immunohistologic detection, three separate sites for each lesion were analyzed in the former study, and as the presence of bacteria throughout the lesion is not homogeneous, this further increased the detection rates for that study. Immunohistologic detection of specific bacteria in the lesions, however, provides important information regarding the localization of the bacteria in relation to various cell types and their state of activation.

Interestingly, when the inflammatory infiltrate was examined according to the presence of any of the six bacteria tested, an increase in the percentage of $CD8^+$ T cells and a reduction in the CD4/CD8 ratio were shown in positive lesions. Although fewer CD8 cells were observed than CD4 cells in the atherosclerosis lesion in the present study, we have previously found a higher percentage of CD8 cells than CD4 cells to be positive for the cytokines interferon-γ and interleukin-10 and for the chemokines MCP-1, MIP-1α, and RANTES in T-cell lines derived from atherosclerosis lesions (11). $CD8^+$ T cells would therefore appear to have an important role in mediating the immune response occurring in atherosclerosis even though present in fewer numbers than CD4 cells, and in the present study this role appeared to be up-regulated in the presence of any of the bacteria examined.

In conclusion, the inflammatory cell infiltrate of the lesion of atherosclerosis has been described. Macrophages and $CD4^+$ T cells were found to dominate the lesions. Although many of these cells, including the endothelium, were activated, as seen by expression of HLA-DR, significant proliferation of T cells may not be occurring as CD25 expression was not evident. hHSP60 expression occurred on endothelial cells and other cells of the infiltrate and this was noted in close association with activated lymphocytes. Bacteria were observed in 83% of the 29

arteries examined using Gram staining and immunohistology, with at least one of the oral bacteria detected in 75% of the specimens. Bacteria and GroEL were detected within cells of the infiltrate. These results strongly support the concept of a specific immune response associated with atherosclerosis lesions. The finding of GroEL, some of which is likely to be from periodontal organisms, within the lesion in close association with activated inflammatory cells suggests that this antigen is likely to be involved in evoking this response, with cells expressing hHSP60 as possible targets. This adds further to the evidence of the involvement of infections, including periodontitis, with atherosclerosis. Immunohistologic detection of specific bacteria in atherosclerosis lesions in this study has provided important information regarding the localization of the bacteria in relation to lesional cells and their state of activation. The expression of hHSP60 and GroEL and the presence of *P. gingivalis* in the atherosclerotic plaques, in association with activated inflammatory cells, support the hypothesis of an immune response to HSPs and periodontopathic bacteria as a mechanism involved in atherosclerosis.

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