

Characterization of Heat Shock Protein-Specific T Cells in Atherosclerosis

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A role for infection and inflammation in atherogenesis is widely accepted. Arterial endothelium has been shown to express heat shock protein 60 (HSP60) and, since human (hHSP60) and bacterial (GroEL) HSP60s are highly conserved, the immune response to bacteria may result in cross-reactivity, leading to endothelial damage and thus contribute to the pathogenesis of atherosclerosis. In this study, GroEL-specific T-cell lines from peripheral blood and GroEL-, hHSP60-, and *Porphyromonas gingivalis*-specific T-cell lines from atherosclerotic plaques were established and characterized in terms of their cross-reactive proliferative responses, cytokine and chemokine profiles, and T-cell receptor (TCR) V β expression by flow cytometry. The cross-reactivity of several lines was demonstrated. The cytokine profiles of the artery T-cell lines specific for GroEL, hHSP60, and *P. gingivalis* demonstrated Th2 phenotype predominance in the CD4 subset and Tc0 phenotype predominance in the CD8 subset. A higher proportion of CD4 cells were positive for interferon-inducible protein 10 and RANTES, with low percentages of cells positive for monocyte chemoattractant protein 1 and macrophage inflammatory protein 1 α , whereas a high percentage of CD8 cells expressed all four chemokines. Finally, there was overexpression of the TCR V β 5.2 family in all lines. These cytokine, chemokine, and V β profiles are similar to those demonstrated previously for *P. gingivalis*-specific lines established from periodontal disease patients. These results support the hypothesis that in some patients cross-reactivity of the immune response to bacterial HSPs, including those of periodontal pathogens, with arterial endothelial cells expressing hHSP60 may explain the apparent association between atherosclerosis and periodontal infection.

Cardiovascular disease with an etiology of atherosclerosis is the main cause of death in Western societies. The importance of the role of infection and inflammation in the initiation and progression of atherosclerosis is now widely accepted. Controlled studies have shown that acute infections, especially bacterial respiratory infections, often precede the onset of symptoms of stroke or myocardial infarction by several weeks (18, 32, 34).

Chronic inflammatory periodontal disease is a significant oral health problem, with *Porphyromonas gingivalis* being one of the major causative organisms. Individuals with severe chronic periodontitis have been reported to have a significantly increased risk of developing cardiovascular disease, including atherosclerosis, myocardial infarction, and stroke (5, 18, 34, 37). Although this association has been challenged by some (22), tooth loss levels, used as an indicator of cumulative periodontitis, have been significantly associated with peripheral arterial disease (23) and carotid artery plaque prevalence (6). Work on animal models further supports the association between periodontal disease and cardiovascular disease. Murine models of atherosclerosis have demonstrated that repeated intravenous or oral inoculation with *P. gingivalis* resulted in atherosclerotic lesions that were more advanced and developed more rapidly than those of control animals (27, 29).

Bacterial heat shock protein 60s (HSP60s), termed GroEL, are major antigenic determinants during infection (25) and, since human and bacterial HSPs have a >55% homology, it is possible that the immune response to bacterial challenge may result in cross-reactivity by targeting host cells expressing human HSP60 (hHSP60) (40). Arterial endothelium has been shown to express hHSP60 (1) caused possibly by stress factors such as hypertension or hypercholesterolemia, and the resulting endothelial damage may contribute to the pathogenesis of atherosclerosis.

The outcome of an immune response is determined by the particular subsets of leukocytes that are recruited and subsequently activated within the lesion. A Th1 cytokine response has been shown to dominate the lesion of atherosclerosis with production of gamma interferon (IFN- γ), tumor necrosis factor alpha (TNF- α), and interleukin-2 (IL-2) (8, 36). IFN- γ appears to be a central mediator of atherosclerosis. IFN- γ -deficient mice have been shown to develop lesions of reduced size, lipid accumulation, and cellularity but with increased fibrosis and collagen content, resulting in a more stable plaque (20). The Th1-inhibitory cytokine IL-10 is expressed in human atherosclerotic lesions in association with reduced inflammation (reviewed by Greaves and Channon [19]). The Th2 cytokine IL-4, on the other hand, has been shown to be uncommon in atherosclerotic lesions (8). Monocyte chemoattractant protein 1 (MCP-1) exhibits potent chemotactic activity for monocytes and has been demonstrated in human atherosclerotic plaques (33). IFN-inducible protein 10 (IP-10) specifically chemottracts activated T cells. IFN- γ induces the expression of

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IP-10, which in turn, upregulates IFN- γ production (9), thereby contributing to the progression of the lesion. Neovascularization and healing are inhibited by IP-10, and this may increase the severity and instability of the plaque (30).

The aim of the present study was to establish and characterize T-cell lines from peripheral blood and atherosclerotic plaques obtained from subjects with atherosclerosis in terms of their cytokine and chemokine profiles and their T-cell receptor (TCR) V β expression and to investigate cross-reactivity with hHSP60, GroEL, and *P. gingivalis* outer membrane antigens.

MATERIALS AND METHODS

Patients. Peripheral blood was obtained from 20 patients undergoing carotid endarterectomy at the Royal Brisbane and Women's Hospital. Biopsy material from atherosclerotic plaques was also collected from 12 of these patients. 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 a signed consent form was obtained according to the Helsinki Declaration. Institutional ethics review committee approval to carry out the study was also obtained.

Periodontal status. Periodontal disease status was classified as either healthy (no sites with a pocket depth of ≥ 3.5 mm), moderate (≥ 1 sites with a pocket depth of ≥ 3.5 mm and < 4 sites with a pocket depth of ≥ 5 mm), or advanced (≥ 4 sites with a pocket depth of ≥ 5 mm). Five patients were edentate, and five patients did not consent to an oral examination.

Peripheral blood. T-cell lines specific for bacterial GroEL were established from the peripheral blood of 12 patients and characterized in terms of their TCR V β repertoire and cytokine and chemokine profiles as described previously (10, 14, 16). Briefly, 50 ml of blood was taken from each patient, and the mononuclear cells isolated by Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden) gradient centrifugation. A portion of the mononuclear cell suspension for use as antigen-presenting cells (APC) was placed into RPMI 1640 containing 10% dimethyl sulfoxide and glycerol and stored at -80°C . The other portion of the cell suspension was incubated at a concentration of 10^6 lymphocytes/ml with 5 ng of recombinant GroEL protein (purified from *Escherichia coli* with $>90\%$ purity; Stressgen Biotechnologies Corp., Victoria, British Columbia, Canada)/ml in RPMI 1640 containing 10% pooled human AB serum, 2 mM glutamine (ICN Biomedicals, Inc., Aurora, Ohio), 100 IU of penicillin/ml (Sigma, St. Louis, Mo.), 100 μg of streptomycin/ml (Sigma), and 1.25 μg of amphotericin B (Gibco, Paisley, Scotland, United Kingdom)/ml in 24-well plates (Nunc, Roskilde, Denmark) in a humidified atmosphere of 5% CO_2 in air at 37°C .

After 14 days, the T cells were restimulated with GroEL (5 ng/ml), together with irradiated (30 Gy) autologous peripheral blood mononuclear cells (PBMC). rHIL-2 (Boehringer Mannheim, Mannheim, Germany) was also added at this stage at a concentration of 10 U/ml so that the now specific T cells would proliferate. After a further 14 days, cells for the proliferation assay were ready for testing, whereas cells for flow cytometry were subjected to a further round of stimulation (Table 1).

Atherosclerotic lesions. Cells were extracted from the atherosclerotic lesions of 10 patients by the method of De Boer et al. (4). After Ficoll-Paque density centrifugation, the mononuclear cells were incubated with 5 μg of the T-cell mitogen phytohemagglutinin (PHA)/ml, irradiated (30 Gy) autologous PBMC (5×10^5 /ml), and IL-2 (10 U/ml). After 2 to 4 weeks, proliferation assays and flow cytometry were performed as described below. At 2 weeks, three cell lines were generated from a proportion of each PHA-expanded T-cell line by stimulation with GroEL (5 ng/ml), hHSP60 (5 ng/ml; does not contain GroEL and has low levels of endotoxin [$< 5 \times 10^{-4}$ EU/well]; Stressgen), or *P. gingivalis* outer membrane antigens (0.5 μg /ml) (14, 16), along with irradiated autologous APC. The cells were restimulated with specific antigen and APC twice more at fortnightly intervals before proliferation assays and flow cytometry were performed as for the PHA lines (Table 1).

In order to test for effects due to the presence of lipopolysaccharide (LPS) in the antigen preparations, *P. gingivalis*-specific, hHSP60-specific, and GroEL-specific lines established from one of the patients who demonstrated cross-reactivity were incubated with the appropriate antigen, along with polymyxin B at a concentration of 10 μg /ml (Sigma). Polymyxin B binds to endotoxin to inhibit its biological activity and is widely used to investigate the effect of any possible LPS contamination (35). The *P. gingivalis* outer membrane preparation has been shown to contain at least 32 antigens, 15 of which were attributed to LPS and 1 of which had a molecular mass of ~ 65 kDa, indicating that the preparation

TABLE 1. T-cell lines established, assays performed, and periodontal status of patients in this study

Patient no.	Periodontal status	Assay(s) performed ^a				
		PB line	PHA line	GroEL line	hHSP60 line	PGS line
1	Advanced	F, P				
2	Not known	F, P				
3	Advanced	F, P				
4	Not known	F				
5	Edentate	F, P				
6	Edentate	F, P				
7	Not known	F, P				
8	Not known	F, P				
9	Healthy	F, P				
10	Edentate	F, P	F, P	F, P	F, P	F, P
11	Edentate	F, P	F	F	F	F
12	Healthy		F, P	F, P	F, P	F, P
13	Advanced		F, P	F, P	F, P	F, P
14	Healthy		F, P	F, P	F, P	F, P
15	Advanced		F, P	F, P	F, P	F, P
16	Edentate		F, P	F, P	F, P	F, P
17	Healthy		F, P	F, P	F, P	F, P
18	Advanced		F, P	F, P	F, P	F, P
19	Advanced		F, P	F, P	F, P	F, P
23	Not known	F, P				

^a The assays performed on the T-cell lines were flow cytometry (F) and proliferation assay (P). PB, GroEL-specific T-cell lines were established from peripheral blood. PHA expanded, GroEL-specific, hHSP60-specific, and *P. gingivalis*-specific (PGS) T-cell lines were established from atherosclerotic artery tissue.

contained both protein, including GroEL, and LPS antigens (13) and therefore could serve as a positive control for the activity of polymyxin B. Proliferative responses and cytokine profiles were determined as described below. The cross-reactive proliferative response of the hHSP60-specific line to *P. gingivalis* outer membrane antigens after the addition of polymyxin B was also confirmed. In addition, anti-HSP60 (LK1 clone; Stressgen) and anti-GroEL (Stressgen) antibodies were added to T cells in the hHSP60- and GroEL-specific lines, respectively, to confirm specificity of these lines. Anti-GroEL antibody was also added to the *P. gingivalis*-specific line to determine the effect of any *P. gingivalis* GroEL present in the outer membrane preparation.

Lymphoproliferation assay. Lymphoproliferation assays were performed to investigate cross-reactivity of the specific T-cell lines with the remaining two antigens not used to establish the lines. Therefore, each cell line was stimulated with each of the three antigens: GroEL, hHSP60, and *P. gingivalis* outer membrane antigens. T cells from each line were cultured at a concentration of 10^5 /well in flat-bottom 96-well tissue culture plates (Nunc), along with irradiated (30 Gy) autologous APC at a concentration of 10^4 /well. The cells were stimulated with hHSP60 or GroEL at concentrations of 100.0, 10.0, 1.0, 0.1, 0.01, and 0 ng/well or *P. gingivalis* outer membrane antigens at concentrations of 10.0, 1.0, 0.1, 0.01, and 0 μg /well for 3 days. Quadruplicate wells were prepared for each antigen concentration. Cultures were pulsed with 0.5 μCi of [^3H]thymidine 24 h prior to harvesting onto glass fiber disks. [^3H]thymidine incorporation was measured by using a LS 6000SC Beckman scintillation counter.

Flow cytometry. Flow cytometry was used to examine the TCR V β expression and the cytokine and chemokine profiles of the T cells in each line. The CD4 and CD8 T cells in the cultures were stained for intracytoplasmic IL-4, IFN- γ , IL-10, IP-10, MCP-1, macrophage inflammatory protein 1 α (MIP-1 α), and RANTES as described by Gemmell et al. (10, 16). Briefly, the T cells were incubated with fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD4 or CD8 (Pharmingen, San Diego, Calif.), followed by fixation of cells with 4% paraformaldehyde, permeabilization with proteinase K, and incubation with phycoerythrin (PE)-conjugated rat anti-human IL-4, IFN- γ , IL-10, IP-10, MCP-1, MIP-1 α , or RANTES (Pharmingen). Surface membrane staining of the V β regions 3.1, 5.1, 5.2-3, 5.3, 6.7, 8.1, 8.2, 11.1, 12.1, 13.1, 13.3, 14.1, 16.1, 20.1, 21.3, and 22.1 was achieved by staining with PE-conjugated mouse anti-human CD4 or CD8 (Pharmingen), together with FITC-conjugated mouse anti-V β regions 3.1, 5.1, 5.2-3, 5.3, 6.7, 8.1, 8.2, 12.1, 13.1, and 13.3 (T-Cell Diagnostics, Cambridge, Mass.) or FITC-conjugated mouse anti-V β regions 11.1, 14.1, 16.1, 20.1, 21.3, or 22.1 (Immunotech, Marseille, France).

FITC- and PE-conjugated specific mouse or rat immunoglobulin isotypes

TABLE 2. Cross-reactive proliferative responses of T-cell lines established from peripheral blood

Patient no./line specificity	T-cell response (dpm) ^a														
	GroEL concn/well						hHSP60 concn/well					<i>P. gingivalis</i> concn/well			
	0 ng	0.01 ng	0.1 ng	1 ng	10 ng	100 ng	0.01 ng	0.1 ng	1 ng	10 ng	100 ng	0.01 µg	0.1 µg	1 µg	10 µg
1/GroEL ⁺	40,324	39,716	41,359	42,777	40,007	34,467	41,965	43,773	41,926	37,418	34,081	14,713	14,505	14,274	12,305
2/GroEL ⁺	11,893	12,182	12,716	12,449	11,910	12,055	11,386	12,940	13,022	10,291	10,009	46,080	46,618	46,103	23,690
3/GroEL ⁺	148,480	141,897	141,042	147,095	140,886	135,241	133,835	140,267	135,107	125,188	129,257	156,599	157,559	148,499	94,691
5/GroEL ⁺	98,306	98,385	98,449	100,507	98,635	88,132	95,704	97,332	100,854	94,700	90,713	100,595	105,487	101,353	95,549
6/GroEL ⁺	151,387	152,748	147,196	136,660	135,380	132,373	163,799^a	169,862^c	172,002^d	150,975	152,560	137,903	146,479	135,746	170,191^b
7/GroEL ⁺	217,363	217,765	212,453	206,950	204,095	198,364	208,914	210,655	207,186	207,228	201,256	213,197	210,723	207,554	203,342
8/GroEL ⁺	58,545	55,171	56,002	64,477	68,667	61,699	46,054	56,656	62,136	58,497	54,312	62,592	60,841	61,699	16,393
9/GroEL ⁺	127,064	127,193	127,048	130,448	126,706	127,256	125,812	127,704	137,604	130,280	124,785	123,248	129,855	151,766^b	187,047
10/GroEL ⁺	166,540	164,737	166,760	161,639	169,405	172,041	164,707	164,367	162,489	165,983	147,065	170,285	170,100	167,092	156,634
11/GroEL ⁺	62,050	57,382	62,538	61,372	61,943	65,187	58,910	59,442	58,309	58,465	60,264	69,737	62,672	63,432	58,088
23/GroEL ⁺	198,077	208,401	218,212	232,599	229,211	229,104	195,451	204,140	200,014	194,573	211,756	226,137^c	234,564^a	229,391^f	154,044

^a Values in boldface indicate mean cross-reactive proliferative responses of T cells that were significantly different from control wells (zero concentration/well). Superscript letters: a, *P* < 0.001; b, *P* < 0.002; c, *P* < 0.003; d, *P* < 0.010; e, *P* < 0.013; f, *P* < 0.017.

(Dako) were used as negative controls. Ten thousand stained cells from each sample were analyzed by using dual-color flow cytometry on a FACSCalibur (Becton Dickinson, Mountain View, Calif.), and the percent CD4 and CD8 cells in the lines positive for each antigen were determined.

Statistics. Multivariate analysis of variance with the general linear model was used to test for differences within and between the percentages of IL-4⁺, IFN-γ⁺, and IL-10⁺ CD4 and CD8 cells; MCP-1⁺, MIP-1α⁺, RANTES⁺, and IP-10⁺ CD4 and CD8 cells; and Vβ⁺ CD4 and CD8 cells. Pairs of groups were then tested for significance by using the Student *t* test. The Minitab statistical package (Minitab, Inc., State College, Pa.) was used to perform the analyses.

RESULTS

Periodontal status. Of the 15 patients who consented to an oral examination, 4 were characterized as healthy, 6 had advanced periodontal disease, and 5 were edentate (Table 1).

Lymphoproliferation assay. Variations occurred in the proliferative responses of the individual GroEL-specific T-cell lines established from the peripheral blood of atherosclerosis patients. Although some GroEL-specific T-cell lines did not respond to hHSP60 or *P. gingivalis*, 3 of the 11 lines did proliferate in response to at least one of these two antigens. One line responded to hHSP60 (*P* < 0.013) and also to *P. gingivalis* (*P* < 0.001), and two other GroEL-specific T-cell lines responded to *P. gingivalis* (*P* < 0.017) (Table 2).

Proliferation studies were carried out on artery lines from 9 of the 10 subjects. Several specific T-cell lines derived from individual patients exhibited cross-reactivity. Three cell lines established from atherosclerotic plaque tissue specific for hHSP60 showed responses to GroEL (*P* < 0.043). One GroEL-specific artery T-cell line demonstrated a significant response to hHSP60 (*P* < 0.049). In addition, the *P. gingivalis* specific line from this patient proliferated in response to GroEL (*P* < 0.004). Five GroEL-specific lines responded to *P. gingivalis* (*P* < 0.031), and three hHSP60-specific lines proliferated in response to *P. gingivalis* (*P* < 0.015) (Table 3).

Cytokines. The mean percentage of IL-4⁺ CD4 and CD8 cells in the GroEL-specific T-cell lines established from the peripheral blood of atherosclerosis patients was higher than the percentages of IFN-γ⁺ and IL-10⁺ T cells (*P* < 0.032), with the mean percentage of both IL-4⁺ and IFN-γ⁺ CD4 and CD8 T cells being higher than that of IL-10⁺ cells (*P* < 0.025) (Fig. 1).

The PHA-expanded T-cell line and the three specific T-cell lines established from each of the atherosclerotic plaques dem-

onstrated cytokine profiles similar to those of the peripheral blood lines. Again, the percentage of IL-4⁺ CD4 and CD8 cells was higher than IFN-γ⁺ and IL-10⁺ T cells (*P* < 0.046), and the percentage of IL-4⁺ and IFN-γ⁺ CD4 cells was greater than that of IL-10⁺ CD4 cells (*P* < 0.005). However, in contrast to the peripheral blood and the PHA-expanded artery T-cell lines, the artery lines specific for GroEL, hHSP60, and *P. gingivalis* demonstrated marked differences between the CD4 and CD8 populations. Although the percentages of IL-4⁺ CD4 and CD8 cells were similar, the CD8 subset demonstrated a Tc0 profile with an increase in the percentages of IFN-γ⁺ and IL-10⁺ CD8 cells (*P* < 0.014) compared to the CD4 population, which demonstrated a Th2 profile. The results clearly demonstrated a subset of T cells positive for both IL-4 and IFN-γ, and the mean percentages of CD4 and CD8 cells that expressed IL-4 only, IFN-γ only, or both IL-4 and IFN-γ are shown in (Table 4). In comparison to the peripheral blood and PHA-expanded artery T-cell lines, the percentage of IL-10⁺ CD4 cells was reduced in the GroEL- and hHSP60-specific T-cell lines (*P* < 0.021) but not in the *P. gingivalis*-specific artery T-cell line, whereas the percent IL-10⁺ CD8 cells was increased in the three specific lines (*P* < 0.015) (Fig. 1).

CD4/CD8 ratio. The mean CD4/CD8 ratio for the peripheral blood derived GroEL-specific T-cell lines was ca. 6/1, and this was significantly higher than that of the PHA-expanded and the GroEL-specific, hHSP60-specific, and *P. gingivalis*-specific lines established from atherosclerotic plaque tissue (ca. 0.6/1) (*P* < 0.001).

Chemokines. In the peripheral blood T-cell lines, the percentages of cells positive for RANTES and IP-10 were significantly greater than for MCP-1 and MIP-1α (*P* < 0.009) (Fig. 2). The PHA artery lines showed higher percentages of RANTES⁺ and IP-10⁺ cells than MIP-1α⁺ cells (*P* < 0.027), whereas the mean percentage of IP-10⁺ cells was higher than for MCP-1 (*P* ≤ 0.05). A comparison of the peripheral blood and PHA lines showed that the mean percentage of IP-10⁺ CD4 cells only in the PHA lines was significantly higher (*P* < 0.037) (Fig. 2).

As with the cytokine profiles, there were large differences in the chemokines produced by the CD4 and CD8 cells in the GroEL-, hHSP60-, and *P. gingivalis*-specific artery T-cell lines. The percentage of CD4 cells positive for IP-10 was significantly

TABLE 3. Cross-reactive proliferative responses of T-cell lines established from artery tissue

Patient no./line specificity	T-cell response (dpm) ^a														
	0 ng	GroEL concn/well					hHSP60 concn/well					<i>P. gingivalis</i> concn/well			
		0.01 ng	0.1 ng	1 ng	10 ng	100 ng	0.01 ng	0.1 ng	1 ng	10 ng	100 ng	0.01 µg	0.1 µg	1 µg	10 µg
10/GroEL ⁺	56,255	56,111	55,526	56,518	55,493	55,213	56,146	58,441	51,535	49,538	52,258	61,554	57,093	52,104	36,739
12/GroEL ⁺	112,213	102,945	100,773	107,737	113,075	109,134	107,391	97,235	106,225	98,936	100,773	114,251	117,447	95,531	58,971
13/GroEL ⁺	123,259	117,283	127,162	133,522	127,908	117,147	132,241	136,616	132,877	115,765	127,796	151,378^k	145,489	119,488	73,344
14/GroEL ⁺	114,154	102,903	106,110	115,112	120,663	112,573	102,721	96,335	105,356	100,123	98,022	114,443	104,797	84,404	55,197
15/GroEL ⁺	100,525	104,741	109,943	130,557	132,087	126,983	111,367	117,927ⁿ	89,522	86,068	91,031	108,222	110,737	92,967	66,867
16/GroEL ⁺	59,723	57,067	61,382	70,472	75,273	76,522	56,958	63,518	64,019	54,926	57,058	89,725^a	81,843^b	66,224ⁱ	48,819
17/GroEL ⁺	30,191	30,674	29,768	32,725	32,141	30,316	25,271	26,863	27,917	25,377	25,271	33,658^a	32,564^j	27,531	13,150
18/GroEL ⁺	99,787	99,236	97,817	106,899	101,896	102,921	93,846	92,831	94,783	91,383	97,086	110,437^a	111,673^b	95,291	52,427
19/GroEL ⁺	21,073	20,783	22,669	23,284	22,121	25,200	20,453	19,851	21,137	20,267	19,989	24,217^b	24,196^d	18,511	5,860
10/HSP60 ⁺	52,144	52,101	51,591	61,201	60,647	55,608	51,161	52,519	54,547	48,810	51,023	60,367	61,356	57,375	39,363
12/HSP60 ⁺	154,419	148,751	145,405	165,495^b	175,933^a	168,702	150,391	141,798	149,136	143,243	143,239	152,084	157,686	143,430	95,820
13/HSP60 ⁺	161,256	151,374	145,385	163,468	155,186	158,158	137,853	143,150	149,680	140,790	140,828	167,342	157,740	133,049	86,585
14/HSP60 ⁺	126,689	113,996	116,111	130,229	121,527	130,161	110,389	112,592	112,744	103,375	109,567	139,902	121,982	106,837	63,115
15/HSP60 ⁺	120,140	109,825	115,135	114,132	114,259	111,698	100,874	106,585	88,119	95,324	97,568	101,199	95,727	87,565	66,604
16/HSP60 ⁺	63,589	61,425	65,179	69,555	70,914	69,689^m	60,958	68,925	64,286	60,637	59,233	88,851^a	79,093^a	69,353^l	47,340
17/HSP60 ⁺	27,981	30,684^k	29,315	32,103^f	32,246^e	30,144	25,623	27,233	26,754	27,879	25,256	29,492	33,964^d	27,669	14,415
18/HSP60 ⁺	114,787	103,057	104,165	102,212	115,032	112,303	93,771	100,485	110,297	100,735	105,113	119,950	117,700	95,056	51,398
19/HSP60 ⁺	20,911	20,442	21,383	22,415	22,556	22,993	17,895	19,799	20,251	19,345	18,940	23,259	24,869^e	19,417	5,689
10/PgOM ⁺	69,378	63,054	58,996	72,562	68,740	60,627	55,622	56,546	62,051	59,298	60,543	61,107	60,732	56,489	37,161
12/PgOM ⁺	129,973	123,844	121,117	135,284	130,631	135,191	121,449	118,529	128,540	124,016	124,842	152,974	124,528	117,804	78,156
13/PgOM ⁺	171,366	167,259	165,235	166,894	169,776	184,356	153,737	162,395	164,832	151,138	163,584	194,701	162,895	148,355	99,642
14/PgOM ⁺	132,282	128,972	128,909	124,284	127,834	131,926	NA	NA	126,110	115,828	119,636	135,231	NA	NA	NA
15/PgOM ⁺	74,150	72,402	76,523	77,069	90,750^e	96,987^b	74,909	76,041	56,614	63,470	64,034	72,281	72,054	76,507	51,319
16/PgOM ⁺	84,178	83,806	85,243	85,775	82,601	80,609	68,338	80,544	80,565	75,517	77,330	90,146	81,055	73,139	48,867
17/PgOM ⁺	26,177	24,298	25,075	27,539	25,796	24,923	21,185	23,420	24,279	21,412	22,249	27,102	26,967	21,987	10,561
18/PgOM ⁺	117,828	101,951	108,620	103,925	118,959	110,349	96,179	101,419	105,731	97,194	99,004	112,778	110,039	86,605	47,112
19/PgOM ⁺	43,985	41,374	42,938	46,448	45,452	42,139	38,173	41,427	42,463	39,844	37,532	44,063	46,738	35,910	12,854

^a Values in boldface indicate cross-reactive proliferative responses of T cells that were significantly different from control wells (zero concentration/well). Superscript letters: a, *P* < 0.001; b, *P* < 0.002; c, *P* < 0.004; d, *P* < 0.005; e, *P* < 0.007; f, *P* < 0.01; g, *P* < 0.015; h, *P* < 0.021; i, *P* < 0.022; j, *P* < 0.024; k, *P* < 0.031; l, *P* < 0.036; m, *P* < 0.043; n, *P* < 0.049. NA, data not available.

greater than for MCP-1, MIP-1α, and RANTES (*P* ≤ 0.05), while there were significantly higher percentages of MCP-1⁺, MIP-1α⁺, and RANTES⁺ CD8 cells than CD4 cells (*P* < 0.027). The specific artery T-cell lines also demonstrated higher percentages of MCP-1⁺ and MIP-1α⁺ CD8 cells (*P* < 0.013) and

reduced levels of MIP-1α⁺ CD4 cells compared to the peripheral blood lines (Fig. 2) (*P* < 0.032).

TCR Vβ expression. The mean frequencies of CD4 and CD8 cells in the T-cell lines showed that all 15 TCR Vβ families examined were expressed. Approximately 38 and 36% of the

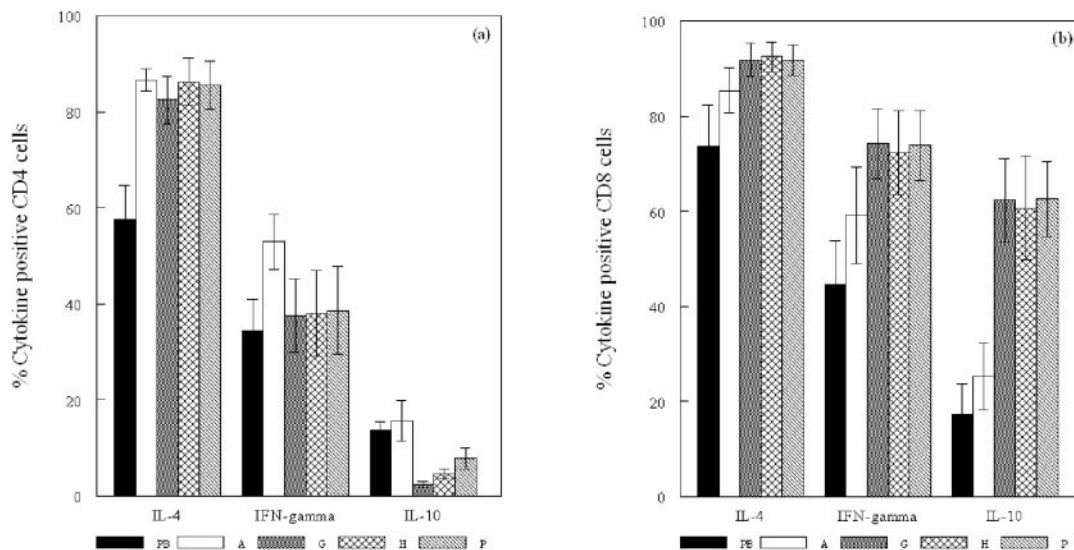


FIG. 1. Mean ± the standard error of the mean percentage of CD4 cells (a) and CD8 cells (b) positive for IL-4, IFN-γ, and IL-10 in GroEL-specific T-cell lines derived from peripheral blood (PB), PHA-expanded (A), GroEL-specific (G), hHSP60-specific (H), and *P. gingivalis* outer membrane antigen (P)-specific T-cell lines derived from atherosclerotic plaques.

TABLE 4. Th1, Th2, Th0, Tc1, Tc2, and Tc0 profiles in the T-cell lines

T-cell line source	No. of samples	Mean % ± SEM					
		CD4 cells			CD8 cells		
		IL-4 ⁻ IFN-γ ^{+a} Th1	IL-4 ⁺ IFN-γ ^{-c} Th2	IL-4 ⁺ IFN-γ ^{+b} Th0	IL-4 ⁻ IFN-γ ^{+a} Tc1	IL-4 ⁺ IFN-γ ^{-c} Tc2	IL-4 ⁺ IFN-γ ^{+b} Tc0
Peripheral blood	12	19.4 ± 2.8	42.8 ± 5.1	14.8 ± 6.7	14.8 ± 4.4	44.5 ± 9.4	29.3 ± 10.1
PHA-expanded artery	10	13.4 ± 2.2	47.1 ± 5.7	40.0 ± 6.7	13.8 ± 4.5	40.0 ± 9.8	45.4 ± 12.8
GroEL ⁺ artery	10	11.6 ± 2.8	56.5 ± 6.4	26.0 ± 9.1	8.2 ± 3.5	25.8 ± 7.3	66.1 ± 10.2
hHSP60 ⁺ artery	10	8.1 ± 3.2	56.3 ± 7.4	29.9 ± 10.0	7.1 ± 2.8	27.3 ± 8.6	65.3 ± 11.6
PgOM ⁺ artery	10	9.3 ± 2.8	56.2 ± 8.1	29.3 ± 9.6	8.3 ± 3.2	26.2 ± 7.3	65.6 ± 10.0

^a That is, % IFN-γ⁺ - (% IL-4⁺ IFN-γ⁺).

^b That is, (% IL-4⁺ + % IFN-γ⁺) - 100%.

^c That is, % IL-4⁺ - (% IL-4⁺ IFN-γ⁺).

CD4 and CD8 cells, respectively, in the GroEL-specific T-cell lines established from the peripheral blood and PHA-expanded artery lines expressed the 5.2-3 Vβ region, and this was significantly higher than that of the other 14 families, including the 5.3 region (*P* < 0.013 and *P* < 0.001, respectively), indicating that the 5.2 region was overexpressed. About 11 and 13% of the CD4 cells in the GroEL-specific peripheral blood lines and the PHA-expanded artery lines expressed the 13.1/13.3 regions, which was significantly greater than that of the other families except for 5.2-3 (*P* < 0.050 and *P* < 0.047, respectively) (Fig. 3).

The TCR Vβ profiles of the GroEL-, hHSP60-, and *P. gingivalis*-specific artery T-cell lines were all similar to the peripheral blood and PHA artery T-cell lines, with an over-representation of the 5.2-3 region. The Vβ 5.2-3 region was expressed by significantly more CD4⁺ cells than all other regions (*P* < 0.039) except for the 3.1 and 13.1/13.3 regions and the 8.1 in the GroEL-specific artery lines. The percentage of CD8⁺ cells of these lines expressing the 5.2-3 region was significantly higher than for only 6 of the other 14 families (*P* ≤ 0.05), although the mean percentage of 5.2-3 Vβ⁺ CD8 cells was reduced compared to the peripheral blood (*P* < 0.019) and PHA artery (*P* < 0.006) T-cell lines (Fig. 3).

LPS effects. The addition of polymyxin B reduced the proliferative response by ca. 30% in the case of GroEL-specific and hHSP60-specific lines at both 3 and 6 days and at day 6 in the *P. gingivalis*-specific line. Cross-reactivity of the hHSP60-specific line with *P. gingivalis* was observed as determined previously (Table 3, patient 16). When the hHSP60-specific line was stimulated with *P. gingivalis* together with polymyxin B, the proliferative response was again reduced by ca. 30% compared to *P. gingivalis* alone; however, this response was still ca. 20% higher than when the T cells were incubated with hHSP60 and polymyxin B showing that cross-reactivity occurred even in the presence of polymyxin B.

Intracytoplasmic cytokine staining of these lines demonstrated that the cytokine profiles of these specific lines remained unchanged by the addition of polymyxin B to the cultures (Table 5).

The 3-day proliferative response of the GroEL-specific line was reduced by 18% after the addition of 10 μg of anti-GroEL antibody/ml and by 13% with the addition of 2.5 μg of anti-GroEL antibody/ml. Similarly, the hHSP60-specific line showed a reduction in 3-day proliferative responses of 20% with 10 μg of anti-hHSP60 antibody/ml and 8% with 2.5 μg of anti-hHSP60 antibody/ml. The addition of anti-GroEL antibody

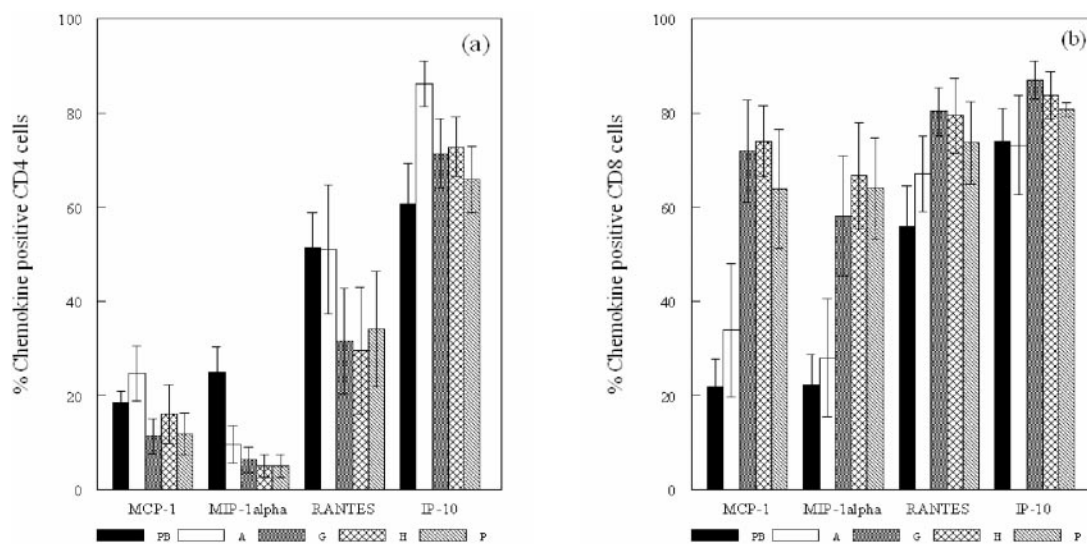


FIG. 2. Mean ± the standard error of the mean percentage of CD4 cells (a) and CD8 cells (b) positive for MCP-1, MIP-1α, RANTES, and IP-10 in GroEL-specific T-cell lines derived from peripheral blood (PB), PHA-expanded (A), GroEL-specific (G), hHSP60-specific (H), and *P. gingivalis* outer membrane antigen (P)-specific T-cell lines derived from atherosclerotic plaques.

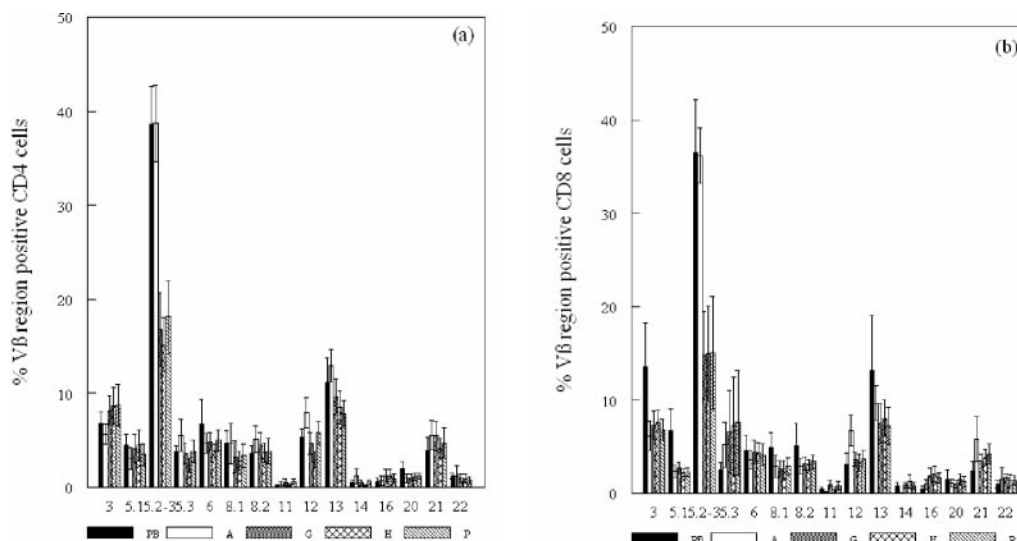


FIG. 3. Mean \pm the standard error of the mean percentage of CD4 cells (a) and CD8 cells (b) expressing 15 TCR V β families in GroEL-specific T-cell lines derived from peripheral blood (PB), PHA-expanded (A), GroEL-specific (G), hHSP60-specific (H), and *P. gingivalis* outer membrane antigen (P)-specific T-cell lines derived from atherosclerotic plaques (5.2 includes the V β regions 5.2 and 5.3).

to the GroEL-specific line and anti-hHSP60 antibody to the hHSP60-specific line therefore reduced proliferation in a dose-dependent manner. Furthermore, the addition of *P. gingivalis* at 5 μ g/ml to the *P. gingivalis*-specific line inhibited 3-day proliferative responses by 17%, whereas the addition of 10 μ g of anti-GroEL antibody/ml negated this inhibition, demonstrating that in the *P. gingivalis*-specific lines the proliferative response to *P. gingivalis* GroEL was inhibitory.

Proliferation was therefore confirmed to be due to specific antigen even though reduction in proliferative responses after the addition of polymyxin B to the T-cell lines indicated that LPS effects may have been responsible for a proportion of the response.

DISCUSSION

The results of the present study have shown cross-reactivity in a number of GroEL- and hHSP60-specific T-cell lines to hHSP60 and GroEL, respectively. Since the hHSP60 antigen used contained no GroEL, these results support the existence of molecular mimicry between GroEL and hHSP60. Other lines that did not demonstrate cross-reactivity may contain T cells which recognize non-cross-reactive epitopes. Interestingly, a GroEL-specific line and a *P. gingivalis*-specific line established from the arterial tissue of one patient cross-reacted with hHSP60 and GroEL, respectively, suggesting that the HSP component of the *P. gingivalis* preparation was an important antigenic determinant, at least in this patient. This patient also had advanced periodontitis, and it is possible that the immune response to this infection may have influenced the specificity of the T cells recruited to the atherosclerosis lesion. Indeed, Yamazaki et al. (42) have shown that in periodontitis patients but not in control subjects clonal expansion of PBMC was preferentially induced by hHSP60 and *P. gingivalis* GroEL, and this occurred in an antigen-specific manner.

The present study also demonstrated that specific T-cell lines could be established in response to each of the three

antigens from all subjects, regardless of their periodontal status. In contrast, Choi et al. (3) were able to establish *P. gingivalis*- and *P. gingivalis* GroEL-specific T-cell lines from the atherosclerotic lesions and the peripheral blood of patients with periodontitis but not from those without periodontitis. However, whereas these authors conducted dose-response assays of the *P. gingivalis* GroEL-specific lines, they were unable to demonstrate any significant responses, probably due to the magnitude of these proliferations, which were extremely low. T-cell lines maintained long term and subjected to repeated rounds of restimulation with specific antigen to ensure specificity respond differently to PBMC with regard to proliferation assays, and this has been shown previously (17). Although PBMC show a clear dose response, T-cell lines, as shown in the present study, demonstrated high proliferations even when unstimulated (i.e., zero concentration/well), probably due to the continual restimulation of these cells with specific antigen, in contrast to the PBMC that are starting from a resting or inactivated state. However, any response by the T-cell lines

TABLE 5. Effect of polymyxin B on cytokine profiles of T-cell lines

T-cell line	% Positive ^a for:					
	CD4 cells			CD8 cells		
	IL-4	IFN- γ	IL-10	IL-4	IFN- γ	IL-10
16/PgOM ⁺	84.79	74.68	3.82	89.36	86.46	62.14
16/PgOM ⁺ (polymyxin B added)	91.21	66.57	9.92	95.48	83.63	50.53
16/hHSP60 ⁺	100.00	79.13	10.40	98.24	90.55	84.67
16/hHSP60 ⁺ (polymyxin B added)	98.25	60.22	4.43	98.20	95.18	79.26
16/GroEL ⁺	68.27	11.07	4.67	81.58	78.52	80.01
16/GroEL ⁺ (polymyxin B added)	68.46	9.88	8.28	93.38	72.05	82.92

^a Values are percent positive values for each cytokine (one sample per group) of T cells treated with or without polymyxin B (10 μ g/ml).

that is significantly higher than that of the unstimulated cells must represent a true proliferative response.

The cytokine profiles of the peripheral blood T-cell lines and the PHA-expanded artery lines showed a trend toward a Th2 profile particularly in the CD8 cells with a higher percentage positive for IL-4 than for IFN- γ and only a small percentage of cells positive for IL-10. This was also the case for the specific artery T-cell lines, although the percentage of IFN- γ ⁺ and IL-10⁺ CD8 cells was greatly increased, shifting the balance of cytokines expressed to a Tc0 phenotype for the CD8 subset. This was shown by determining the percentage of cells that were possibly producing both IL-4 and IFN- γ . The proportion of these cells was increased in the artery T-cell lines compared to the peripheral blood lines, particularly in the CD8 subset. The presence of possible LPS contamination in the antigen preparations did not appear to have any effect on the cytokine profiles, as determined by the results obtained after addition of polymyxin B. T-cell clones derived from atherosclerotic plaques have previously been shown to be predominantly of a type 0 cytokine profile, although higher levels of IFN- γ compared to IL-4 were detected by using an enzyme-linked immunosorbent assay (4). In another study, IL-4 was rarely observed in atherosclerotic plaques by using immunohistochemistry and PCR analysis (8). Interestingly, a cytokine profile similar to that demonstrated in the present study has also been reported to occur in T-cell lines established from gingival tissues of periodontitis patients (39) and in the peripheral blood of patients with periodontal disease (10).

The CD4/CD8 ratios of the artery T-cell lines were much reduced compared to those of the peripheral blood lines. This ratio has been shown to decrease with periodontal disease progression, indicating a role for both CD8 cells and CD4 cells in chronic periodontitis (15). Indeed, the present study has shown that both CD4 and CD8 cells in the lines established from atherosclerosis patients express cytokines in response to stimulation with GroEL, hHSP60, and *P. gingivalis*, suggesting an involvement of both subsets in atherosclerosis. Although histologically, fewer CD8 cells have been observed than CD4 cells in the lesion of atherosclerosis (28), a higher percentage of CD8 cells was found to be positive for the cytokines IFN- γ and IL-10 in the present study. It has been suggested that CD8 T cells expand both humoral and cellular responses in vivo by the production of chemokines at the peripheral site of infection (26). In this context, the results of the present study suggest that GroEL, hHSP60, and *P. gingivalis* may preferentially activate CD8 cells in the atherosclerotic lesion and that these cells have an important regulatory function even though fewer numbers are present in vivo than CD4 cells.

The chemokine profiles of the peripheral blood lines and the PHA-expanded artery lines demonstrated higher percentages of T cells positive for IP-10 and RANTES with low percentages of MCP-1- and MIP-1 α -positive cells. Although CD4 cells in the specific artery T-cell lines had a similar profile, there was an increase in the percentages of positive CD8 cells, with no differences between the expression of the four chemokines. MCP-1 expression by monocytes has been shown to be regulated by IL-10 (38), and the clear correlation between MCP-1 and IL-10 levels demonstrated by the CD4 and CD8 cells of the specific artery T-cell lines suggests that this regulatory mechanism may also exist for T cells. Low levels of IL-10 and

therefore of MCP-1, as seen in the CD4 subset, could indicate a reduction of cell-mediated responses. The predominance of IP-10⁺ T cells in both subsets observed in the present study has been demonstrated in situ, along with expression of the IP-10 receptor CXCR3 (31), suggesting that active recruitment of Th1 cells may occur (7). IP-10, MIP-1 α , and RANTES attract Th1 CD4 cells and cytotoxic CD8 T cells (43), and therefore the expression of these chemokines especially by CD8 cells may regulate the cell-mediated component of the immune response. It has been postulated that the expression of IP-10 and RANTES, along with their respective receptors CXCR3 and CCR5, by the majority of the CD8 cells in oral lichen planus, may result in a self-recruiting mechanism involving activated effector cytotoxic T cells (24). The findings of the present study add to the evidence supporting the possibility of this mechanism also operating in atherosclerosis.

The present study has also shown that the V β 5.2 family is the predominant TCR expressed by T cells in the GroEL-specific lines established from the peripheral blood of atherosclerosis patients, the PHA-expanded line, and the GroEL-specific, hHSP60-specific, and *P. gingivalis*-specific T-cell lines established from atherosclerotic plaque tissue, although in the specific artery lines this overexpression was less pronounced. The V β 13 family appeared also to be more highly expressed. The V β 5.2 region has previously been shown to be preferentially expressed by CD4 and CD8 cells in *P. gingivalis*-specific T-cell lines established from peripheral blood of both adult periodontitis and control subjects, and this overexpression of certain V β families was shown to be due to antigen-specific rather than superantigen activation (11).

The cytokine, chemokine, and V β profiles of the GroEL-, hHSP60-, and *P. gingivalis*-specific artery T-cell lines are remarkably alike. There is a high degree of homology between GroEL and hHSP60 (40), and the proliferation data after the addition of anti-GroEL antibody to the *P. gingivalis*-specific cell line indicates that HSPs could be an important antigen of the *P. gingivalis* outer membrane preparation. This idea is supported by the results of the proliferation assays which demonstrated that cross-reactivity did occur between these antigens at least in some patients. The results also showed that this cross-reactivity was most likely to hHSP60/GroEL rather than any possible LPS contamination in the antigen preparations. A number of studies have demonstrated the presence of multiple pathogens in atherosclerotic lesions. The organisms detected include *Chlamydia pneumoniae*, cytomegalovirus, herpes simplex virus, and, more recently, the oral bacteria *P. gingivalis*, *Streptococcus sanguis*, *Tannerella forsythia*, *Actinobacillus actinomycetemcomitans*, and *Prevotella intermedia* (2, 21, 41). In these studies almost all lesions examined were positive for at least one of the pathogens studied, and multiple species were detected in the majority of cases. Due to the homologous nature of HSPs across species, it is not surprising that we were able to establish T-cell lines specific for GroEL and *P. gingivalis* outer membrane antigens for each of the patients in the present study and that cross-reactivity was demonstrated in a number of these. In contrast, Choi et al. (3) were unable to show reactivity of *P. gingivalis* GroEL-specific T-cell lines with any other periodontopathic bacteria, including *A. actinomycetemcomitans*, *Capnocytophaga sputigena*, and *Eikenella corrodens*.

In conclusion, although these in vitro techniques give valuable insight into the role of HSP-specific T cells in the immune response associated with atherosclerosis, it is important that the effects of the complex interactions that occur in the host are not overlooked. The present study has demonstrated the presence of T cells specific for GroEL in the lesion of atherosclerosis and that cross-reactivity of these specific T cells with hHSP60 occurred. The artery T-cell lines specific for GroEL, hHSP60, and *P. gingivalis* demonstrated a Th2 phenotype predominance in the CD4 subset and a Tc0 phenotype predominance in the CD8 subset with a high proportion of CD8 cells expressing the chemokines IP-10, RANTES, MCP-1, and MIP-1 α . Finally, there was an overexpression of the TCR V β 5.2 family in all lines. The cytokine, chemokine, and V β results are similar to those demonstrated previously for *P. gingivalis*-specific lines from periodontal disease patients (10–12). Taken together, these results give strong support to the hypothesis (40) that cross-reactivity of the immune response to bacterial HSPs, including those of periodontal pathogens, with arterial endothelial cells expressing hHSP60 may be a mechanism involved in the disease process of atherosclerosis at least in some patients. The relative contribution, however, of this cross-reactivity to atherogenesis remains to be determined.

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