The Journal of Immunology

Human 60-kDa Heat Shock Protein Is a Target Autoantigen of T Cells Derived from Atherosclerotic Plaques

Marisa Benagiano, Mario M. D'Elios, Amedeo Amedei, Annalisa Azzurri, Ruurd van der Zee, Alessandra Ciervo, Gianni Rombolà, Sergio Romagnani, Antonio Cassone and Gianfranco Del Prete

J Immunol 2005; 174:6509-6517; ;
doi: 10.4049/jimmunol.174.10.6509
http://www.jimmunol.org/content/174/10/6509

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Human 60-kDa Heat Shock Protein Is a Target Autoantigen of T Cells Derived from Atherosclerotic Plaques[1]

Marisa Benagiano,[2]* Mario M. D'Elios,[2]*† Amedeo Amedei,*† Annalisa Azzurri,* Ruurd van der Zee,‡ Alessandra Ciervo,§ Gianni Rombolà,† Sergio Romagnani,*† Antonio Cassone,§ and Gianfranco Del Prete[3]*†

Epidemiological studies suggest the potential importance of an inflammatory component in atherosclerosis and support the hypothesis that immune responses to Ags of pathogens cross-react with homologous host proteins due to molecular mimicry. Protein candidates involved may be the stress-induced proteins known as heat shock proteins (HSP). In this study, we report that atherosclerotic plaques harbor in vivo-activated CD4$^+$ T cells that recognize the human 60-kDa HSP. Such in vivo-activated 60-kDa HSP-specific T cells are not detectable in the peripheral blood. In patients with positive serology and PCR for *Chlamydia pneumoniae* DNA, but not in patients negative for both, most of plaque-derived T cells specific for human 60-kDa HSP also recognized the *C. pneumoniae* 60-kDa HSP. We characterized the submolecular specificity of such 60-kDa HSP-specific plaque-derived T cells and identified both the self- and cross-reactive epitopes of that autoantigen. On challenge with human 60-kDa HSP, most of the plaque-derived T cells expressed Th type 1 functions, including cytotoxicity and help for monocyte tissue factor production. We suggest that arterial endothelial cells, undergoing classical atherosclerosis risk factors and conditioned by Th type 1 cytokines, express self 60-kDa HSP, which becomes target for both autoreactive T cells and cross-reactive T cells to microbial 60-kDa HSP via a mechanism of molecular mimicry. This hypothesis is in agreement with the notion that immunization with HSP exacerbates atherosclerosis, whereas immunosuppression and T cell depletion prevent the formation of arteriosclerotic lesions in experimental animals. ***The Journal of Immunology,*** **2005, 174: 6509–6517.**

Atherosclerosis is a multifactorial disease for which a number of different pathogenic mechanisms have been proposed. In the last two decades, attention has been given to the inflammatory processes associated with atherogenesis. In addition to classical risk factors for atherosclerosis, such as high levels of low-density lipoprotein (LDL)[4] cholesterol or oxidized LDL, free radicals, hyperglycemia, and genetic susceptibility to endothelial damage (1, 2), a pathogenic role for infections in atherosclerosis is suggested by the detection of pathogens (e.g., *Chlamydia pneumoniae*, CMV, or herpes viruses) in the arterial vessels and the association between atherosclerosis and increased Ab levels to these pathogens (3). Observations in humans and animals suggest that atherosclerotic plaques derive from specific cellular and molecular mechanisms that can be ascribed to an inflammatory disease of the arterial wall, the lesions of which consist of macrophages and T lymphocytes (4–6). Activated macrophages and T cells would be responsible for in situ production of enzymes, cytokines, and chemokines that further expand the process. If inflammation continues unabated, it results in increased numbers of plaque-infiltrating macrophages and T cells, which contribute to remodeling of the arterial wall (7). Within the T cell population of the plaque, most of the cells are activated CD4$^+$ cells expressing HLA-DR and CD25 of the IL-2R (8). *C. pneumoniae* DNA and *C. pneumoniae*-specific T cell clones were found in the plaques of anti-*C. pneumoniae* seropositive patients. The specificity repertoire of such CD4$^+$ T cells included the *C. pneumoniae* 60-kDa heat shock protein (CpHSP60), the 10-kDa HSP, the outer membrane protein 2, or undefined Ags of the *C. pneumoniae* elementary bodies. T cell clones recovered from *C. pneumoniae* DNA-negative plaques of anti-*C. pneumoniae* seronegative patients did not react to *C. pneumoniae* Ags (9).

In this study, we focused on the analysis of the T cell infiltrates of atherosclerotic plaques of anti-*C. pneumoniae* seronegative patients, making the hypothesis that human proteins expressed under stress conditions, such as the human 70-kDa HSP (hHSP70) or human 60-kDa HSP (hHSP60), might be autoantigens recognized by plaque-infiltrating T cells.

Materials and Methods

Patients

Carotid plaques were obtained by endoarterectomy from eight patients (five males and three females; mean age, 67 years; range, 62–73 years) with atherosclerotic arteriopathy. Four patients were seronegative for anti-*C. pneumoniae* Abs (Cp-neg), whereas the other four patients were seropositive for anti-*C. pneumoniae* Abs (Cp-pos), as shown by both commercial ELISA tests (Eurospital) and standard microimmunofluorescence

*Department of Internal Medicine, University of Florence, and †Laboratory of Immunogenetics, Department of Biomedicine, Azienda Ospedaliero-Universitaria di Careggi, Florence, Italy; ‡Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; and §Department of Infectious, Parasitic and Immune-Mediated Diseases, Istituto Superiore di Sanità, Rome, Italy

Received for publication September 17, 2004. Accepted for publication February 16, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants from the Italian Ministry of University and Research, the Ministery of Health, the University of Florence, the Associazione Italiana per la Ricerca sul Cancro, and the Istituto Superiore di Sanità.

[2] M.B. and M.M.D. contributed equally to this publication.

[3] Address correspondence and reprint requests to Dr. Gianfranco Del Prete, Department of Internal Medicine, Viale Morgagni 85-50134 Florence, Italy. E-mail address: gdelprete@unifi.it

[4] Abbreviations used in this paper: LDL, low-density lipoprotein; HSP, heat shock protein; CpHSP60, *C. pneumoniae* 60-kDa HSP; hHSP70, human 70-kDa HSP; hHSP60, human 60-kDa HSP; Cp-neg, seronegative for anti-*C. pneumoniae* Ab; Cp-pos, seropositive for anti-*C. pneumoniae* Ab; MI, mitogenic index; BCG, bacillus Calmette-Guérin; MbHSP65, *Mycobacterium bovis* BCG 65-kDa HSP; EBV-B, EBV transformed B; TF, tissue factor.

0022-1767/05/$02.00

assay (cutoff value, 32). Samples of PBMC were obtained from each patient. Their MHC haplotypes were as follows: HLA-A2, B7, B51, DRB1*14, and DRB1*16 in patient 1; HLA-A1, A2, B13, B18, DRB1*03, and DRB1*07 in patient 2; HLA-A24, B15, B40, DRB1*01, and DRB1*16 in patient 3; HLA-A1, A2, B18, DRB1*04, and DRB1*11 in patient 4; HLA-A24, B44.03, B58.01, DRB1*0701, and DRB1*16 in patient 5; HLA-A2, B15, B44, and DRB1*13 in patient 6; HLA-A1, B8, B27, DRB1*03, and DRB1*07 in patient 7; and HLA-A2, A24, B7, B51, DRB1*0701, and DRB1*11 in patient 8.

Detection of C. pneumoniae *in atherosclerotic plaques*

The presence of *C. pneumoniae* was investigated by nested PCR, as reported elsewhere (9, 10). Briefly, DNA was extracted from fragments of all the endoarterectomy specimens by QIAamp DNA kit (Qiagen). Nested PCR consisted of two rounds of amplification using two sets of primers, each in a 50-μl volume. On completion of primary PCR (37 cycles), 2 μl of the PCR product were added into fresh reaction mix containing the second set of primers and amplified for 25 cycles. The amplified DNA products were analyzed by electrophoresis in 1.5% agarose gel, stained with ethidium bromide, and hybridized as reported previously (10). The nested PCR for *C. pneumoniae* included an outer primer pair (HL-1, HR-1) and an inner pair (HM-1, HR-2) that generated a product of 204 bp. The details of primers and probe are as follows: HL-1, -5′-GTTGTTCATGAAGGCCTACT-3′ end; HR-1, -5′-TGCATAACCTACGGTGTGTT-3′ end; HM-1, -5′-GTGTCATTCGCCAAGGTTAA-3′ end; HR-2, -5′-ACCTGTCCAAGGTTCATCCT-3′ end; and DNA probe, -5′-GTGTCATTCGCCAAGGTTAAAGTCTACGTT-3′ end.

Generation of T cell clones from atherosclerotic plaques and peripheral blood

Fragments of atherosclerotic plaques and samples of PBMC were cultured for 7 days in RPMI 1640 medium supplemented with IL-2 to expand in vivo-activated T cells. Single T cell blasts were then cloned under limiting dilution (9, 11–13). Briefly, single T cell blasts were seeded in microwells (0.3 cells/well) in the presence of 2×10^5 irradiated (5000 rad) allogeneic PBMC, PHA (0.5% vol/vol), and IL-2 (50 U/ml). At weekly intervals, irradiated allogeneic PBMC and IL-2 were added to each microculture to maintain the expansion of growing clones. Ag specificity of T cell clones was assessed by measuring [^{3}H]thymidine uptake after 60 h of coculture with irradiated autologous PBMC in the presence of medium, recombinant hHSP70 (Sigma-Aldrich) (10 μg/ml), recombinant hHSP60 (Sigma-Aldrich) (10 μg/ml), or recombinant CpHSP60 (10 μg/ml), prepared as endotoxin-free material (14). No significant proliferation of T cell clones was found in response to irradiated autologous PBMC alone. The mitogenic index (MI) was calculated as the ratio between mean values of cpm obtained in stimulated cultures and those obtained in the presence of medium alone. MI >5 was considered positive.

T cell clones reactive to hHSP60 were also tested for proliferation in response to the recombinant HSP65 protein of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) (MbHSP65) (Aalto Bio Reagents) and to the recombinant 60-kDa chaperonin GroEL of *Escherichia coli* (Sigma-Aldrich) (10 μg/ml).

All of the 26 plaque-derived T cell clones reactive to hHSP60 and the 18 clones reactive to both hHSP60 and CpHSP60 expressed a $CD3^+CD4^+CD8^-$ phenotype and showed a single peak of fluorescence intensity. The repertoire of the TCR Vβ chain of HSP60-specific T cell clones was analyzed with a panel of 22 mAbs specific to the following: Vβ1, Vβ2, Vβ4, Vβ7, Vβ9, Vβ11, Vβ14, Vβ16, Vβ18, Vβ20, Vβ21.3, Vβ22, and Vβ23 (Beckman Coulter Immunotech); and Vβ3.1, Vβ5.1, Vβ5.2, Vβ5.3, Vβ6.7, Vβ8, Vβ12, Vβ13, and Vβ17 (AMS Biotechnology); and isotype-matched nonspecific Ig were used as negative control. Data acquisition was performed in a FACSCalibur flow cytometer using the CellQuest software program (BD Biosciences). From each T cell clone, mRNA was extracted by mRNA direct isolation kit (Qiagen). For cDNA synthesis, the same amount of mRNA (50 ng) was used, and cDNA was synthesized by Moloney murine leukemia virus-reverse transcriptase (New England Biolabs) and oligo(dT) primers according to enzyme supplier's protocol. cDNA mix of all samples was amplified under equal conditions by a 30-cycle PCR using a Vβ TCR typing amplimer kit for Vβ10, Vβ15, and Vβ19 (BD Clontech) according to the manufacturer's instructions.

Submolecular specificity of plaque-derived T cell clones reactive to hHSP60 or to both hHSP60 and CpHSP60

To span the 573 aa sequence of hHSP60 and the 544 aa sequence of CpHSP60, 113 and 107 overlapping 15-mer peptides with a 10 (5 on each side) aa overlap, respectively, were prepared by automated, simultaneous multiple peptide synthesis, as described previously (15) Homologies between the two series of peptides were screened by using the basic local alignment search tool server of the National Center for Biotechnology Information.

Two series of 15-mer peptides corresponding to a number of sequences of the HSP65 protein of *M. bovis* BCG (MbHSP65) and of the 60-kDa chaperonin GroEL of *E. coli* (aa 21–35, 26–40, 31–45, 46–60, 51–65, 56–70, 121–136, 126–140, 131–145, 141–155, 146–160, 151–165, 161–175, 166–180, 171–185, 181–195, 186–200, 191–205, 211–225, 216–230, 221–235, 406–420, 411–425, 416–430, 421–435, and 426–440) were also prepared. Equal amounts of each component of the two series of overlapping peptides of hHSP60 and of CpHSP60 were pooled to have two series of 11 peptide pools. T cell blasts (4×10^4) from each clone were cultured in triplicate for 3 days together with irradiated autologous mononuclear cells (1.5×10^5) in the presence of medium, hHSP60 (10 μg/ml), CpHSP60 (10 μg/ml), or equal aliquots from each of the 22 pools in which each peptide component was present at a 10 μg/ml final concentration. After 60 h, [^{3}H]TdR uptake was measured. T cell blasts of each clone were then retested for proliferation to the individual peptide components of the pool that had induced a MI >5.

MHC class II restriction of hHSP60 epitope recognition by plaque-derived T cell clones

The effect of anti-HLA-DR (clone G46–6) or anti-HLA-DQ (clone TU169; BD Biosciences Pharmingen) (5 μg/ml final concentration) mAbs or their isotype control (mouse IgG2a) on T cell clone proliferation induced by hHSP60 or CpHSP60 was assessed. The MHC class II restriction of the proliferative response of T cell clones to hHSP60 or CpHSP60 peptides was assessed by using irradiated allogeneic APC. To this end, PBMC from both patients and healthy donors sharing with patients one of the *DRB1** alleles were stimulated with PHA followed by IL-2 to obtain polyclonal lines of activated T cells to be used as irradiated (3000 rad) allogeneic APC in coculture experiments with T cell clones in the presence of the HSP60 peptide to which they reacted.

Assessment of the cytokine profile of T cell clones

To assess the cytokine production of hHSP60-specific clones on Ag stimulation, 5×10^5 T cell blasts of each clone were cocultured for 48 h in 0.5 ml of medium with 5×10^5 irradiated autologous PBMC in the absence or presence of hHSP60 or CpHSP60 (10 μg/ml). At the end of culture period, duplicate samples of each supernatant were assayed for IFN-γ, TNF-α, and IL-4 (BioSource International) (9, 13). T cell clones able to produce IFN-γ, but not IL-4, were categorized as Th1; clones able to produce IL-4, but not IFN-γ, were categorized as Th2; and clones producing both IFN-γ and IL-4 were categorized as Th0.

Perforin-mediated cytotoxicity and Fas-Fas ligand-mediated proapoptotic activity

Perforin-mediated cytolytic activity of T cell clones was assessed as reported previously (16). T cell blasts of hHSP60-specific clones were incubated at ratios of 10:1, 5:1, and 2.5:1 with ^{51}Cr-labeled autologous EBV transformed B (EBV-B) cells preincubated with hHSP60 (10 μg/ml). After centrifugation, microplates were incubated for 8 h at 37°C, and 0.1 ml of supernatant was removed for measurement of ^{51}Cr release, as reported previously (17). The ability of hHSP60-specific T cell clones to induce Fas-Fas ligand-mediated apoptosis was assessed using Fas^+ Jurkat cells as target. T cell blasts from each clone were cocultured with ^{51}Cr-labeled Jurkat cells at E:T ratios of 10:1, 5:1, and 2.5:1 for 18 h in the presence of PMA (10 ng/ml) and ionomycin (1 mmol/l), as reported previously (17, 18).

Assay for T cell clone helper function for monocyte tissue factor (TF) production

T cell blasts of hHSP60-specific clones (8×10^5/ml) were cocultured for 16 h with autologous monocytes (4×10^5/ml) in the presence of medium, hHSP70 (10 μg/ml), hHSP60 (10 μg/ml), or CpHSP60 (10 μg/ml). At the end of culture period, TF protein was measured by a specific ELISA (American Diagnostica) in duplicate samples of the supernatants obtained from cell suspensions after solubilization of membrane proteins with Triton X-100 and ultracentrifugation, as reported previously (19).

Results

Autoreactive $CD4^+$ T cell clones specific for hHSP60 are present in atherosclerotic lesions

In vivo-activated T cells resident in the plaques or in the peripheral blood were expanded in vitro in IL-2-conditioned medium and

Table 1. *Atherosclerotic plaques, but not peripheral blood, harbor CD4$^+$ T cells specific for hHSP60*[a]

Patients and Source of T Cells	Total Number of CD4$^+$ T cell Clones Tested	Number (%) of Clones Reactive to	
		hHSP60	Both hHSP60 and CpHSP60
C. pneumoniae-negative patients			
1. Plaque	40	6 (15)	0
PBMC	40	0	0
2. Plaque	38	6 (16)	0
PBMC	38	0	0
3. Plaque	36	5 (14)	0
PBMC	36	0	0
4. Plaque	37	4 (11)	0
PBMC	37	0	0
C. pneumoniae-positive patients			
5. Plaque	20	7 (35)	5/7 (71)
PBMC	20	0	0
6. Plaque	25	3 (12)	3/3 (100)
PBMC	25	0	0
7. Plaque	33	6 (18)	4/6 (67)
PBMC	33	0	0
8. Plaque	37	7 (19)	6/7 (86)
PBMC	37	0	0

[a] Equal numbers of clones derived from plaques or PBMC were screened for responsiveness to hHSP60 and CpHSP60 in the presence of irradiated autologous mononuclear cells by measuring ^{3}[H]-thymidine uptake after 60 h.

then cloned by a procedure that has proved useful and accurate for studies of tissue-infiltrating T cells in various diseases (9, 11–13). A total number of 151 CD4$^+$ and 23 CD8$^+$ T cell clones were obtained from the plaques of the four Cp-neg patients, whereas 115 CD4$^+$ and 21 CD8$^+$ were the T cell clones derived from the plaques of the four Cp-pos patients. Nested PCR on endarterectomy specimens showed *C. pneumoniae* genomic material in each of the plaques obtained from the four Cp-pos patients but not in the plaques from the Cp-neg patients (data not shown).

For each patient, randomly selected CD4$^+$ and CD8$^+$ T cell clones derived from PBMC were matched to the corresponding plaque-derived T cell clones and assayed for proliferation in response to

Table II. *Epitope specificity of plaque-derived CD4 T cell clones reactive to hHSP60 but not to CpHSP60*[a]

T Cell Clone (TCR Vβ)	Proliferative Response (MI) to			Peptide Amino Acid Sequence (position)
	hHSP70	hHSP60	hHSP60 Peptide	
1.10 (Vβ11)	<2	36	49	MLRLPTVFRQMRPVS (1–15)
1.30 (Vβ1)	<2	27	53	MRPVSRVLAPHLTRA (11–25)
1.54 (Vβ17)	<2	21	23	VKDGKTLNDELEIIE (201–215)
1.26 (Vβ5.1)	<2	92	118	LEIANAHRKPLVIIA (261–275)
1.43 (Vβ18)	<2	102	97	TLNLEDVQPHDLGKV (331–345)
1.08 (Vβ7)	<2	111	78	MAGDFVNMVEKGIID (506–520)
2.14 (Vβ14)	<2	168	139	TVFRQMRPVSRVLAP (6–20)
2.08 (Vβ20)	<2	69	84	KFGADARALMLQGVD (31–45)
2.58 (Vβ21.3)	<2	46	52	KLVQDVANNTNEEAG (96–110)
2.40 (Vβ5.2)	<2	91	68	NPVEIRRGVMLAVDA (136–150)
2.53 (Vβ2)	<2	47	71	GGAVFGEEGLTLNLE (321–335)
2.11 (Vβ6.7)	<2	63	88	MAGDFVNMVEKGIID (506–520)
3.18 (Vβ8)	<2	473	439	TVFRQMRPVSRVLAP (6–20)
3.40 (Vβ4)	<2	28	42	KNIGAKLVQDVANNT (91–105)
3.53 (Vβ12)	<2	144	198	RRGVMLAVDAVIAEL (141–155)
3.43 (Vβ3.1)	<2	19	28	TLNDELEIIEGMKFD (206–220)
3.24 (Vβ16)	<2	467	488	MAGDFVNMVEKGIID (506–520)
4.13 (Vβ9)	<2	126	184	MLRLPTVFRQMRPVS (1–15)
4.07 (Vβ4)	<2	52	41	PYFINTSKGQKCEFQ (226–240)
4.31 (Vβ22)	<2	29	33	EIIKRTLKIPAMTIA (466–480)
4.25 (Vβ11)	<2	68	91	VEKIMQSSSEVGYDA (491–505)
5.02 (Vβ8)	<2	55	63	MRPVSRVLAPHLTRA (11–25)
5.14 (Vβ12)	<2	71	113	GGAVFGEEGLTLNLE (321–335)
7.31 (Vβ14)	<2	80	98	TVFRQMRPVSRVLAP (6–20)
7.12 (Vβ11)	<2	32	47	MAGDFVNMVEKGIID (506–520)
8.30 (Vβ5.2)	<2	102	178	MLRLPTVFRQMRPVS (1–15)

[a] T cell blasts from each clone were cocultured with irradiated autologous APC in the presence of medium alone, hHSP70, hHSP60, or hHSP60 peptides (10 μg/ml), and proliferative responses (MI) were measured after 3 days. Results are reported as mean values obtained in quadruplicate cultures, SD values being <14% of means. Letters *underlined* in the recognized hHSP60 peptides indicate the amino acids shared with CpHSP60 along the sequence. Patients 1–4 were anti-*C. pneumoniae* seronegative, whereas patients 5–8 were seropositive.

Table III. *Epitope specificity of CD4 T cell clones reactive to both hHSP60 and CpHSP60 obtained from the atherosclerotic plaques of anti-*C. pneumoniae *seropositive patients*[a]

T Cell Clone (TCR Vβ)	Proliferative Response (MI) to hHSP60	hHSP60	*Peptide* (sequence)	CpHSP60	CpHSP60	*Peptide* (sequence)
5.05 (Vβ17)	38	81	(DGVTVAKSIDLKDKY 76–90)	73	89	(KDGVTVAKEIELEDK 51–65)
5.16 (Vβ11)	75	113	(EEIAQVATISANGDK 166–180)	43	107	(HKEIAQVATISANND 141–155)
5.19 (Vβ13.1)	49	37	(DAYVLLSEKKISSIQ 241–255)	67	44	(EDAL I LIYDKKISGI 216–230)
5.18 (Vβ14)	20	33	(VGGTSDVEVNEKKDR 406–420)	31	39	(VGAATEIEMKEKKDR 381–395)
5.12 (Vβ9)	34	61	(PTKVVRTALLDAAGV 521–535)	22	58	(LDPTKVTRSALESAA 496–510)
6.37 (Vβ5.1)	107	76	(TVIIEQSWGSPKVTK 61–75)	88	51	(RHVVIDKSFGSPQVT 36–50)
6.08 (Vβ14)	23	57	(VIAELKKQSKPVTTP 151–165)	21	48	(VVVDELKKISKPVQH 126–140)
6.41 (Vβ8)	33	27	(LKVGLQVVAVKAPGF 291–305)	29	41	(RLRAGFRVCAVKAPG 266–280)
7.18 (Vβ9)	44	61	(LLADAVAVTMGPKGR 46–60)	21	67	(KTLAEAVKVTLGPKG 21–35)
7.16 (Vβ4)	89	113	(LEIIEGMKFDRGYIS 211–225)	147	131	(VLDVVEGMNFNRGYL 186–200)
7.03 (Vβ1)	45	37	(GCALLRCIPALDSLT 441–455)	28	42	(GTALVRCIPTLEAFL 416–430)
7.22 (Vβ13.2)	121	215	(RCIPALDSLTPANED 446–460)	120	91	(RCIPTLEAFLPMLAN 421–435)
8.22 (Vβ18)	51	78	(VAVTMGPKGRTVIIE 51–65)	39	71	(AVKVTLGPKGRHVVI 26–40)
8.26 (Vβ12)	136	104	(VATISANGDKEIGNI 171–185)	96	122	(QVATISANNDSEIGN 146–160)
8.01 (Vβ5.2)	27	40	(KKVGRKGVITVKDGK 191–205)	23	31	(MEKVGKNGSITVEEA 166–180)
8.14 (Vβ9)	76	68	(IVLGGGCALLRCIPA 436–450)	71	89	(ILPGGGTALVRCIPT 411–425)
8.07 (Vβ22)	417	398	(VNMVEKGIIDPTKVV 511–525)	548	377	(AYTDMIDAGILDPTK 486–500)
8.18 0(Vβ11)	142	201	(ASLLTTAEVVVTEIP 536–550)	170	226	(LLTTEALIADIPEEK 516–530)

[a] Culture conditions are reported in the legend to Table II. *Underlined* letters indicate the amino acids shared between hHSP60 and CpHSP60.

hHSP70, hHSP60, and CpHSP60. None of the $CD8^+$ clones derived from either plaques or PBMC showed proliferation to those Ags. Likewise, none of the 266 $CD4^+$ clones generated from the PBMC of either Cp-neg or Cp-pos patients showed significant proliferation to the Ags tested (Table I), although they proliferated in response to mitogen stimulation (data not shown). In contrast, a variable proportion between 11 and 35% of the $CD4^+$ T cell clones generated from plaque-infiltrating T cells of either Cp-neg or Cp-pos patients proliferated significantly to hHSP60 (Table I) but not to hHSP70 Ag (MI <2). Under the same conditions, none of the 21 hHSP60-specific $CD4^+$ clones from the plaques of the four Cp-neg patients proliferated significantly to CpHSP60. In contrast, in the series of the 23 hHSP60-specific $CD4^+$ clones from the plaques of the four Cp-pos patients, 18 (78%) proliferated equally well to both hHSP60 and CpHSP60 (Table I). Evidence for clonality of the $CD3^+CD4^+CD8^-$ T cell clones, which is critical for interpretation of data, was provided by the cytofluorimetric patterns of single TCR-Vβ expression and the staining by only one of the TCR-Vβ-chain-specific mAbs, with a single peak of fluorescence intensity (data not shown).

Submolecular specificity of autoreactive hHSP60-specific T cell clones

T cell blasts from each of the T cell clones reactive to hHSP60, but not to CpHSP60, were screened for proliferation in response to the 113 overlapping peptides for the hHSP60 (Table II). Each of these autoreactive T cell clones proliferated almost equally well to both the entire hHSP60 protein and to an epitope of such autoantigen. Interestingly, some hHSP60 epitopes, such as the 1–15, 6–20, and 506–520, were recognized by clones from different donors, despite their different MHC class II haplotypes or different TCR-Vβ expression by T cell clones (Table II). Also, the five T cell clones recovered from atherosclerotic plaques of Cp-pos patients that proliferated to hHSP60, but not to CpHSP60, recognized the 1–15, 6–20, 11–25, 321–335, and 506–520 epitopes of hHSP60 (Table II). The lack of responsiveness to CpHSP60 of the 26 hHSP60-specific clones was confirmed by their inability to proliferate in response to the CpHSP60 peptide corresponding to the hHSP60 epitope to which they were reactive (data not shown), although a number of the hHSP60 epitopes recognized by this series of clones shared a few (up to 7) amino acids with the corresponding CpHSP60 peptides (Table II).

Each of the 18 T cell clones that proliferated to both hHSP60 and CpHSP60 was screened for proliferation in response to the 113 overlapping peptides for the hHSP60 and the 107 peptides for the CpHSP60 (Table III). As expected, each of these cross-reactive T cell clones proliferated not only to both the entire hHSP60 protein and to an epitope of such autoantigen but also to the entire CpHSP60 and to a CpHSP60 peptide. However, the relevant cross-reactive CpHSP60 peptides showed high-sequence homology (from 7 to 12 of 15 aa) to the corresponding stimulatory hHSP60 epitopes. Interestingly, the panel of hHSP60 epitopes recognized by cross-reactive clones included 18 different peptides, with only some overlap, mainly in the 436–460 aa sequence for clones 7.03 and 7.22 of patient 7 and clone 8.14 of patient 8 (Table III).

Due to the high homology between the human and the bacterial 60-kDa HSPs, all of the hHSP60-specific clones were tested for their ability to proliferate in response to graded concentrations of the MbHSP65 of BCG or to the 60-kDa chaperonin GroEL of *E. coli*. None of the 26 T cell clones reactive to hHSP60, but not to CpHSP60, showed detectable proliferation to MbHSP65 or to GroEL, even at a dose as high as 50 μg/ml (data not shown). In contrast, 11 of the 18 T cell clones reactive to both hHSP60 and CpHSP60 also showed poor, but detectable, reactivity (range of MI, 2.8–16.1) to comparable concentrations of MbHSP65. However, at MbHSP65 concentrations <2 μg/ml, none of the 11 clones showed MI >2. Likewise, 9 of the same 18 clones showed poor reactivity to GroEL (range of MI, 2.4–17.2), but at lower Ag doses (<2 μg/ml) no proliferation was detectable. Each of the 11 clones potentially cross-reactive to MbHSP65 and each of the 9 clones potentially cross-reactive to GroEL were also tested for proliferation in response to the MbHSP65 and GroEL peptides that partially overlapped their specific hHSP60/CpHSP60 epitopes. As shown in Table IV, at a concentration of 1 μg/ml, neither MbHSP65 nor GroEL proteins or their potentially relevant peptides were able to induce T cell clone proliferation, whereas both hHSP60 and CpHSP60 and their relevant peptides were still stimulatory. It is interesting to note that a single amino acid difference (S instead of T at position 79 of hHSP60 or at position 55 of CpHSP60) accounted

Table IV. *Cross-reactivity to MbHSP65 and GroEL or to their peptides of CD4 T cell clones reactive to both hHSP60 and CpHSP60 obtained from the atherosclerotic plaques of anti-*C. pneumoniae *seropositive patients*[a]

T Cell Clone	Ag or Peptide (position and sequence)			Proliferative Response (MI)[a]		
				10 μg/ml	1 μg/ml	0.1 μg/ml
5.05	hHSP60			43	19	< 2
	hHSP60	76–90	DGV**T**VAKSIDLKDKY	98	56	23
	CpHSP60			68	29	< 2
	CpHSP60	51–65	KDGV**T**VAKEIELEDK	92	61	31
	MbHSP65			8	< 2	< 2
	MbHSP65	51–65	DGV**S**IAKEIELEDPY	21	< 2	< 2
	GroEL			13	< 2	< 2
	GroEL	51–65	KDGV**S**VAKEIELEDK	28	< 2	< 2
5.16	hHSP60			93	34	6
	hHSP60	166–180	EEIAQVATISANGDK	138	71	39
	CpHSP60			68	29	< 2
	CpHSP60	141–155	HKEIAQVATISANND	101	66	23
	MbHSP65			6	< 2	< 2
	MbHSP65	141–155	QIAATAAISAGDQSI	11	< 2	< 2
	GroEL			17	< 2	< 2
	GroEL	141–155	SEEVAQVGTISANGD	31	2	< 2
8.22	hHSP60			40	19	< 2
	hHSP60	51–65	VAVTMGPKGR**T**VIIE	69	37	25
	CpHSP60			31	14	< 2
	CpHSP60	26–40	AVKVTLGPKGR**H**VVI	62	29	11
	MbHSP65			16	< 2	< 2
	MbHSP65	26–40	KVTLGPKGR**N**VVLEK	22	< 2	< 2
	GroEL			5	< 2	< 2
	GroEL	26–40	AVKVTLGPKGR**N**VII	31	2	< 2
8.26	hHSP60			151	78	22
	hHSP60	171–185	VATISANGDKEIGNI	129	84	37
	CpHSP60			113	65	30
	CpHSP60	146–160	QVATISANNDSEIGN	165	91	36
	MbHSP65			< 2	< 2	< 2
	MbHSP65	146–160	TAA ISAGDQSIGDLI	9	< 2	< 2
	GroEL			8	< 2	< 2
	GroEL	146–160	QVATISANGDKQVGL	22	< 2	< 2

[a] Culture conditions are reported in the legend to Table II. *Underlined* letters indicate the amino acids shared between hHSP60 and the other HSP60. *Bold* letters indicate amino acid changes apparently crucial for peptide recognition by T cell clones 5.05 and 8.22.

for a strong reduction of stimulation of clone 5.05. Likewise, a single amino acid change (N instead of T at position 61 of hHSP60) hampered the recognition by clone 8.22 of the 26–40 epitope of either MbHSP65 or GroEL, whereas the substitution of T with H at position 37 of CpHSP60 did not affect cross-recognition.

The relative potency in inducing T cell clone proliferation of the self (hHSP60) and/or the corresponding cross-reactive CpHSP60 protein and peptides was assessed by comparison of dose-response curves. At 10 μg/ml, Ag proteins and peptides were almost equally potent in inducing T cell clone proliferation (Fig. 1 and Table IV). At lower doses (such as 1 or 0.1 μg/ml), the MI obtained with the appropriate self- or cross-reactive peptide was consistently higher than that obtained with the corresponding entire Ag.

Data presented in Tables II and III indicate for each clone the most stimulatory peptides of either hHSP60 or CpHSP60. However, a number of clones proliferated at lower degree also in response to one or both of the adjacent peptides, suggesting that their specific epitope was present in more than one single peptide (Fig. 2).

To know the MHC restriction elements required for Ag recognition, hHSP60-specific T cell clones were stimulated by hHSP60 or CpHSP60 in the presence of irradiated autologous APCs treated with anti-HLA-DR or anti-HLA-DQ mAbs. Anti-HLA-DR resulted consistently in virtual abrogation of the proliferative response by T cell clones to either hHSP60 or CpHSP60, whereas anti-HLA-DQ was unable to affect self- or cross-reactive HSP60-induced T cell clone proliferation (data not shown). Because anti-HLA-DR Abs may hamper T cell responses, even if the specific response under study is not MHC class II restricted, T cell clones were stimulated with the relevant hHSP60 peptide in the presence of either autologous-irradiated PHA-induced T cell blasts or allogeneic-irradiated T cell blasts from donors matched for one single *DRB1* allele. As shown in Table V, for most of plaque-derived clones, the proliferative response to the specific peptide was restricted by one of the *DRB1* alleles, whereas for a few clones, both *DRB1* alleles allowed peptide presentation.

Functional profile of autoreactive and cross-reactive hHSP60-specific T cell clones

All plaque-derived hHSP60-specific clones were assessed for their cytokine profile on Ag stimulation. In the series of hHSP60-specific clones not cross-reactive to CpHSP60, 22 (84.6%) secreted IFN-γ and TNF-α but not IL-4 (Th1 profile), whereas in 4 clones, stimulation with hHSP60 resulted in the production of IL-4 as well (Th0 profile).

Likewise, in the series of 18 hHSP60/CpHSP60 cross-reactive clones, stimulation with either hHSP60 or CpHSP60 disclosed a Th1 profile in 15 clones (83.3%) and a Th0 profile in the other 3.

The cytolytic potential of hHSP60-specific autoreactive or cross-reactive T cell clones was assessed by using Ag-pulsed ^{51}Cr-labeled autologous EBV-B cells as targets. At an E:T ratio of 10:1, 36 of the 37 (97%) Th1 and 5 of 7 (71%) Th0 clones lysed hHSP60-presenting autologous EBV-B cells (range of specific ^{51}Cr release, 18–63%), whereas autologous EBV-B cells pulsed with hHSP70 (control) Ag and cocultured with the same clones

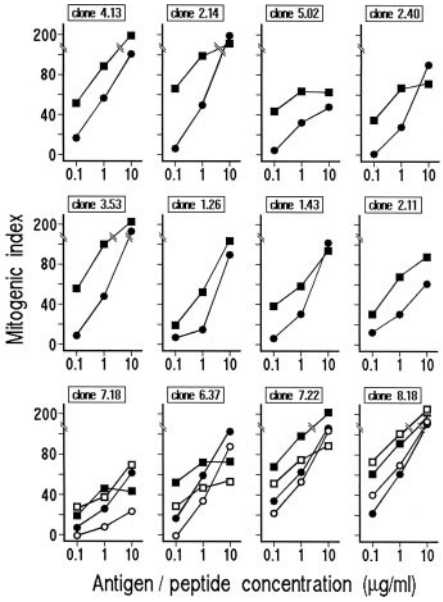

FIGURE 1. Dose-response effect of graded Ag or peptide concentrations on the proliferative response to hHSP60 and CpHSP60 of plaque-derived T cell clones. T cell blasts from each clone were cocultured with autologous-irradiated APC in the presence of graded concentrations of hHSP60 (●), CpHSP60 (○), or of the appropriate hHSP60 peptides (■), or CpHSP60 peptides (□). Results represent mean values of MIs measured in quadruplicate cultures of 8 representative of 26 clones reactive only to hHSP60 (*upper* and *middle panels*) and in 4 representative of 18 clones reactive to both hHSP60 and CpHSP60 (*lower panels*).

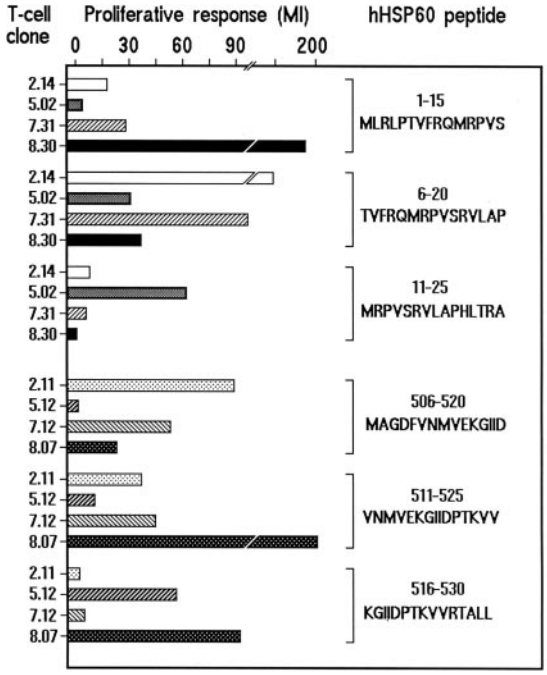

FIGURE 2. Proliferative response of hHSP60-specific T-clones to series of partially overlapping peptides of hHSP60, including the specific epitopes. T cell blasts from clones derived from the plaques of four patients sharing the *DRB1*07* allele were cocultured with autologous-irradiated APC in the presence of series of 3 partially overlapping 15-mer peptides, including the most stimulatory one, and a couple of adjacent peptides. Results represent mean values of MIs measured in quadruplicate cultures of eight representative T cell clones.

were not lysed. The relative potency of Ag-induced cytotoxic activity of HSP60-specific T cell clones against autologous EBV-B cells pulsed with hHSP60 or CpHSP60 was assessed by comparison of levels of the specific ^{51}Cr release at different E:T ratios (Fig. 3). Because activated effector T cells can also kill their targets by inducing apoptosis through Fas-Fas ligand interaction (17, 20), we evaluated the ability of activated hHSP60-specific clones to induce ^{51}Cr release by Fas$^+$ Jurkat cells undergoing apoptosis. On mitogen activation, 32 of 37 Th1 (86%) and 4 of 7 (57%) Th0 clones were able to induce apoptosis in target cells (range of specific ^{51}Cr release, 21–59%).

hHSP60-activated plaque-infiltrating T cells help monocyte TF production

Because plaque rupture and thrombosis are notable complications of atherosclerosis, we asked whether stimulation with hHSP60 or CpHSP60 might enable plaque-infiltrating autoreactive or cross-reactive T cells to express helper function for TF production by monocytes. All clones were cocultured with autologous monocytes in the absence or presence of medium alone, hHSP70, hHSP60, or CpHSP60, and TF protein was measured. In the presence of medium alone or hHSP70, none of the 44 plaque-derived clones expressed helper function for monocyte TF production, ruling out the possibility that monocytes expressed hHSP60 suitable for T cell activation (Fig. 4). In contrast, apart from 2 Th0 clones (one autoreactive and one cross-reactive), in 42 (95%) hHSP60-specific clones, stimulation with hHSP60 resulted in the expression of substantial help for TF production by monocytes. Likewise, stimulation with CpHSP60 enabled 17 of 18 (94%) cross-reactive T cell clones to induce monocyte TF production, whereas it failed to elicit any helper function for TF production in the 25 autoreactive clones that proliferated in response to hHSP60 but not to CpHSP60 (Fig. 4).

Discussion

A number of Ags are suspected to play a role in atherosclerosis-associated immune reactions. Among other candidates, the list includes modified LDL, such as oxidized low-density lipoprotein (21), viral, and bacterial components, and a family of phylogenetically highly conserved proteins known as HSPs, which are expressed in prokaryotic and eukaryotic cells under physiological conditions and in response to various forms of stress. Under both normal and stressed conditions, HSPs, as molecular chaperones, facilitate the folding of nascent proteins, resolubilize protein aggregates, assist in refolding denatured proteins (22), and help in molecular transport across the intracellular membranes (23). Cells of the arterial wall are stimulated to produce high levels of HSPs in response to a number of factors including infections, fever, mechanical or oxidant stress, or exposure to cytokines, heavy metals, alcohol, or inhibitors of energy metabolism (24). Studies in experimental models indicate that 60- to 65-kDa HSP may play a proatherogenic role. Atherosclerosis-like lesions were induced in normocholesterolaemic rabbits by immunization with mycobacteria or recombinant mycobacterial HSP65 (25), and T cells isolated from these lesions were found to respond specifically to HSP65 in vitro (26, 27).

In this study, we demonstrate that atherosclerotic patients harbored in their carotid plaques in vivo-activated CD4$^+$ T cells that reacted specifically to self HSP60. In addition, all four patients with positive serology and PCR detection of *C. pneumoniae* DNA had in their carotid plaques at least two populations of hHSP60-specific T cells: one reactive only to self hHSP60, and the other reactive to both the self and the *C. pneumoniae* analog HSP60. Blocking experiments with anti-DR and anti-DQ Abs and coculture of T cell clones with appropriate allogeneic APC showed that

Table V. *MHC class II restriction of proliferative response of plaque-derived T cell clones reactive to selected hHSP60 peptides*[a]

Code and MHC Class II Haplotype of Clones		MHC Class II Haplotype of Irradiated APC	MI in Response[a] to hHSP60 Peptide (position)
2.08	DRB1∗03–07	Autologous	108 (31–45)
		DRB1∗03	111
		DRB1∗07–11	3
2.40	DRB1∗03–07	Autologous	97 (136–150)
		DRB1∗03	88
		DRB1∗07–11	63
2.53	DRB1∗03–07	Autologous	66 (321–335)
		DRB1∗03	7
		DRB1∗07–11	52
3.40	DRB1∗01–16	Autologous	71 (91–105)
		DRB1∗01–04	4
		DRB1∗11–16	65
3.43	DRB1∗01–16	Autologous	36 (206–220)
		DRB1∗01–04	29
		DRB1∗11–16	2
4.31	DRB1∗04–11	Autologous	54 (446–480)
		DRB1∗01–04	< 2
		DRB1∗11–16	49
4.25	DRB1∗04–11	Autologous	112 (491–505)
		DRB1∗01–04	89
		DRB1∗11–16	3
7.18	DRB1∗03–07	Autologous	68 (46–60)
		DRB1∗03	79
		DRB1∗07–11	< 2
		Autologous	59 (21–35 of CpHSP60)
		DRB1∗03	41
		DRB1∗07–11	17

[a] T cell blasts (10^5/well) from each clone were cocultured with irradiated autologous or allogeneic activated T cell blasts as APC (10^5/well) in the presence of medium alone or the specific hHSP60 or CpHSP60 peptide (10 μg/ml), and proliferative responses (MI) were measured after 3 days. Results are reported as mean values obtained in quadruplicate cultures, SD values being $< 16\%$ of means.

DR represents the MHC restriction element in the T cell response to either hHSP60 or CpHSP60. It is of note that, despite good viability and IL-2-induced growth, PBMC-derived T cell clones from all patients consistently failed to proliferate in response to hHSP60, CpHSP60, or hHSP70. In the absence of obvious explanations due to technical pitfalls, the reason for the inability to detect hHSP-specific T cells in the peripheral blood remains unclear. One possibility is that hHSP60-specific T cells are present in the peripheral blood in resting state, like most of circulating T cells, and are not suitable for in vitro activation and expansion induced by IL-2. Another possibility is that very low numbers, if any, of in vivo-activated hHSP60-specific T cells are present in the peripheral blood, whereas they concentrate into the lesions of arterial walls, where they find their specific Ag(s) and participate in the pathology of atherosclerosis by expressing their effector mechanisms. That such activated T cells infiltrating the atherosclerotic

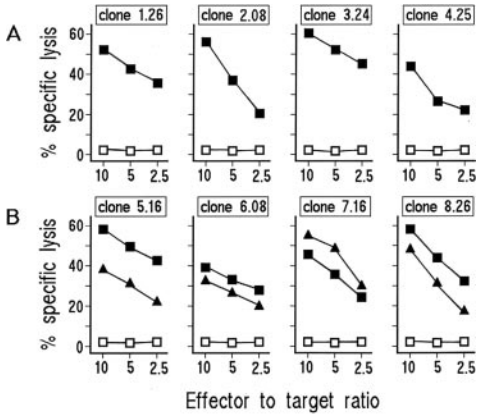

FIGURE 3. Dose-response effect of graded E:T ratios on the cytotoxic activity of plaque-derived T cell clones specific for hHSP60 against Ag-pulsed autologous APC. To assess their perforin-mediated cytotoxicity, T cell clones reactive to hHSP60 (*A*) or to both hHSP60 and CpHSP60 (*B*) were cocultured at different E:T ratios with ^{51}Cr-labeled autologous EBV-B cells pulsed with hHSP70 (10 μg/ml) (□) or hHSP60 (10 μg/ml) (■) or CpHSP60 (10 μg/ml) (▲), and ^{51}Cr release was measured as index of Ag-induced specific target cell lysis. Results represent mean values of ^{51}Cr release measured in triplicate cultures of eight representative T cell clones.

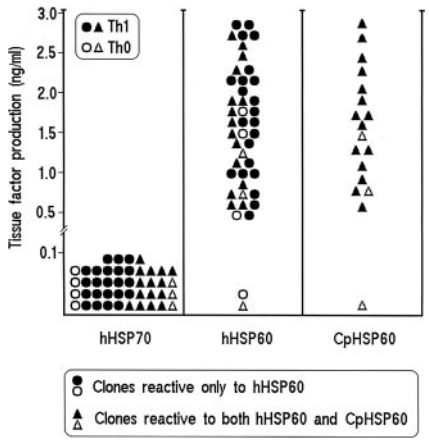

FIGURE 4. Monocyte TF production induced by plaque-derived T cell clones stimulated with hHSP60 or CpHSP60. T cell blasts from each clone were cocultured with autologous monocytes in the presence of hHSP70 (control Ag) or the specific Ag (hHSP60 or CpHSP60), and TF production was assessed by a specific ELISA.

plaques actually participate in the disease pathogenesis is supported by the observation that the formation of arteriosclerotic lesions by immunization of rabbits with HSP65-containing material could be abolished by immunosuppression and T cell depletion with an anti-CD3 Ab plus prednisolone (27).

The presence in the plaques of Cp-positive patients of T cells reactive to CpHSP60 is in agreement with the observations by other laboratories (28, 29) and with our earlier demonstration in a similar series of patients of T cells reactive to *C. pneumoniae* Ags, such as sonicated elementary bodies, the 10 kDa HSP, the outer membrane protein 2, and the CpHSP60 (9). In the present study, however, the presence of plaque-derived T cell clones specific for *C. pneumoniae* Ags different from CpHSP60 was not investigated.

T cell recognition of either hHSP60 or CpHSP60 resulted in both proliferation and expression of functional properties by T cell clones, i.e., a predominant Th1 profile. In addition, on appropriate stimulation, the great majority of plaque-derived HSP60-specific clones induced both perforin-mediated cytolysis and Fas-Fas ligand-mediated apoptosis in target cells. Based on these findings, it is tempting to hypothesize that in the inflammatory setting of the atherosclerotic plaque in which HSP60-specific autoreactive or cross-reactive Th1 cells are activated, endothelial cells may acquire APC function for HSP60 and, together with professional APCs (30), can become targets of the cytotoxic and proapoptotic activity of HSP60-specific Th1 cells. The outcome of this process would be the expansion of the plaque and the formation of the necrotic cores characteristic of complicated and unstable atherosclerotic lesions. A linkage has been suggested between the degree of macrophage apoptosis and plaque rupture, to which apoptotic death of smooth muscle cells may also contribute (31–33). Moreover, it is reasonable to suspect that HSP60-activated Th1 cells and their cytokines can play a role in driving the up-regulation of TF production by monocytes within atherosclerotic plaques, thus contributing to the thrombogenicity of lesions (34). Indeed, the Th1 polarization of T cell responses and the poor production of Th2 cytokines occurring within the plaque may represent local risk factors of thrombosis (19), which associate with platelet adhesion to dysfunctional endothelium.

Our findings support the hypothesis that a crucial component of atherosclerosis is represented by Th1 cell-mediated immune responses to self and/or foreign Ags. More than 95% sequence homology exists between HSP60s from various bacteria, and even between bacterial and hHSP60 a 50–55% sequence homology exists, and in highly conserved regions it reaches >70% (35). The analysis of the submolecular specificity of T cell clones reactive only to hHSP60 and of clones reactive to both hHSP60 and CpHSP60 showed that the former recognized their epitope in portions of relatively poor or no homology between the two proteins, whereas the latter found their specific epitope in regions of high-sequence homology. Therefore, a number of hHSP60 T cell epitopes are "private," such as the 1–15 and 6–20 N-terminal or the 506–520 C-terminal sequences recognized by different clones of different Cp-negative patients and by a few clones of Cp-positive patients, whereas other hHSP60 epitopes are similar to, and cross-reactive with, T cell epitopes of CpHSP60. T cell clones specific for private or cross-reactive epitopes of hHSP60 do, however, express similar predominant Th1 profile and may contribute equally to inflammation in the setting of atherosclerosis.

HSP60 are released in soluble form from the surface of stressed or damaged cells and can be found in the supernatant of cell cultures in vitro or in the serum in vivo (36, 37). Whether soluble hHSP60 released in the inflammatory setting of the plaque may undergo biochemical changes, becoming the target of bona fide autoimmunity (3), remains to be investigated. Likewise, it remains to be established whether autoimmunity to hHSP60 results from a breakdown of tolerance associated with chronic inflammation and aging, and what is the role of the molecular mimicry between hHSP60 and the HSP60 of pathogens, such as *C. pneumoniae*, detected in the atherosclerotic lesions. The mycobacterial homologue MbHSP65 and the *E. coli* homologue GroEL (38) might be other candidates for cross-reactive recognition by hHSP60-reactive T cell clones in atherosclerotic plaques. In this study, however, none of the 26 T cell clones reactive to hHSP60, but not to CpHSP60, also reacted to MbHSP65 or to GroEL. Only 11 and 9 of the 18 T cell clones reactive to both hHSP60 and CpHSP60 showed poor or negligible response to MbHSP65 or GroEL, respectively, on the basis of dose-response curves. Neither MbHSP65 nor GroEL peptides that partially overlapped specific hHSP60/CpHSP60 epitopes were able to induce T cell clone proliferation at intermediate or low peptide concentrations, arguing against the hypothesis that MbHSP65 or GroEL might represent major targets of plaque-infiltrating T cells in our patients.

Data obtained in this study support the hypothesis that two major mechanisms, partially overlapping and not mutually exclusive, may be responsible for the T cell-mediated immunopathology of atherosclerosis. The first one would imply that arterial endothelial cells, undergoing the effects of classical stress factors associated with atherosclerosis and conditioned by cytokines produced by plaque-infiltrating Th1 cells, express self 60-kDa HSP. Such an autoantigen would be presented by professional APC and endothelial cells, becoming a target of autoreactive T cells specific for private epitopes of hHSP60. T cell-mediated cytotoxic and apoptotic killing of stressed endothelial cells expressing self HSP60 may activate a vicious circle of self maintenance of such Th1-mediated autoimmune mechanism of endothelial damage. The second mechanism, active in patients who failed to clear *C. pneumoniae*, would be mediated by plaque-infiltrating Th1 cells specific for *C. pneumoniae* Ags, among which CpHSP60-specific T cells that cross-recognize shared epitopes of the hHSP60 via a mechanism of molecular mimicry. The availability of self HSP60 expressed by the vascular endothelium would contribute to a second branch of the vicious circle of self maintenance of the immune response that would be mediated by CpHSP60-specific Th1 cells that cross-react to self HSP60. These possibilities are consistent with the results obtained in several experimental animal models. In these models, a central role for T cells specific for HSP60s has indeed been established. Immunization with HSP65 of LDL receptor-deficient (LDL-R$^{-/-}$) mice induced specific T cell reactivity against HSP65 as well as mammalian HSP60, and transfer into nonimmunized mice of lymphocytes or purified IgG of immunized animals enhanced the size of vascular lesions (39). In contrast, nasal or oral immunization with HSP65 of hypercholesterolemic ApoE$^{-/-}$ and LDL-R$^{-/-}$ mice resulted in reduced T cell reactivity to HSP and attenuated atherosclerosis (40, 41). In contrast, arthritogenic and arthritis-preventing HSP60 epitopes have been identified in the model of adjuvant arthritis in rats (42, 43) and human rheumatoid arthritis (44). The identification in present study of a number of atherosclerosis-associated T cell epitopes of HSP60 may be of importance for designing strategies on preventive or therapeutic approaches aimed to inhibit the immune and autoimmune pathogenic mechanisms of atherosclerosis.

Disclosures

The authors have no financial conflict of interest.

References

1. Saikku, P., M. Leinonen, K. Mattila, M. R. Ekman, M. S. Nieminen, P. H. Makela, J. K. Huttunen, and V. Valtonen. 1988. Serological evidence of an

association of a novel *Chlamydia*, TWAR, with chronic coronary heart disease and acute myocardial infarction. *Lancet* 2: 983–986.
2. Shi, W., N. J. Wang, D. M. Shih, V. Z. Sun, X. Wang, and A. J. Lusis. 2000. Determinants of atherosclerosis susceptibility in the C3H and C57BL/6 mouse model: evidence for involvement of endothelial cells but not blood cells or cholesterol metabolism. *Circ. Res.* 86: 1078–1084.
3. Wick, G., M. Knoflach, and Q. Xu. 2004. Autoimmune and inflammatory mechanisms in atherosclerosis. *Annu. Rev. Immunol.* 22: 361–403.
4. Ross, R. 1999. Atherosclerosis: an inflammatory disease. *N. Engl. J. Med.* 340: 115–126.
5. Epstein, S. E., Y. F. Zhou, and J. Zhu. 1999. Infection and atherosclerosis: emerging mechanistic paradigms. *Circulation* 100: 20–28.
6. Jonasson, L., J. Holm, O. Skalli, G. Bondjers, and G. K. Hansson. 1986. Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. *Arteriosclerosis* 6: 131–138.
7. Hansson, G. K., L. Jonasson, P. S. Seifert, and S. Stemme. 1989. Immune mechanisms in atherosclerosis. *Arteriosclerosis* 9: 567–578.
8. Kleindienst, R., Q. Xu, J. Willeit, F. R. Waldenberger, S. Weimann, and G. Wick. 1993. Immunology of atherosclerosis: demonstration of heat shock protein 60 expression and T lymphocytes bearing α/β or γ/δ receptor in human atherosclerotic lesions. *Am. J. Pathol.* 142: 1927–1937.
9. Benagiano, M., A. Azzurri, A. Ciervo, A. Amedei, C. Tamburini, M. Ferrari, J. L. Telford, C. T. Baldari, S. Romagnani, A. Cassone, et al. 2003. T helper type-1 lymphocyte-driven inflammation in human atherosclerotic lesions. *Proc. Natl. Acad. Sci. USA* 100: 6658–6663.
10. Khan, M. A., and C. W. Potter. 1996. The nPCR detection of *Chlamydia pneumoniae* and *Chlamydia trachomatis* in children hospitalized for bronchiolitis. *J. Infect.* 33: 173–175.
11. Del Prete, G., M. De Carli, M. M. D'Elios, P. Maestrelli, M. Ricci, L. Fabbri, and S. Romagnani. 1993. Allergen exposure induces the activation of allergen-specific Th2 cells in the airway mucosa of patients with allergic respiratory disorders. *Eur. J. Immunol.* 23: 1445–1449.
12. D'Elios, M. M., M. Manghetti, F. Almerigogna, A. Amedei, F. Costa, D. Burroni, C. T. Baldari, S. Romagnani, J. L. Telford, and G. Del Prete. 1997. Different cytokine profile and antigen-specificity repertoire in *Helicobacter pylori*-specific T cell clones from the antrum of chronic gastritis patients with or without peptic ulcer. *Eur. J. Immunol.* 27: 1751–1755.
13. Amedei, A., M. P. Bergman, B. J. Appelmelk, A. Azzurri, M. Benagiano, C. Tamburini, R. van der Zee, J. L. Telford, C. M. Vandenbroucke-Grauls, M. M. D'Elios, and G. Del Prete. 2003. Molecular mimicry between *Helicobacter pylori* antigens and H^+,K^+-ATPase in human gastric autoimmunity. *J. Exp. Med.* 198: 1147–1156.
14. Ciervo, A., P. Visca, A. Petrucca, L. M. Biasucci, A. Maseri, and A. Cassone. 2002. Antibodies to 60-kilodalton heat shock protein and outer membrane protein 2 of *Chlamydia pneumoniae* in patients with coronary heart disease. *Clin. Diagn. Lab. Immunol.* 9: 66–74.
15. Van der Zee, R., S. M. Anderton, C. A. F. Buskens, E. Alonso de Velasco, and W. Van Eden. Heat shock protein T cell epitopes as immunogenic carriers in subunit vaccines. In *Peptides 1994, Proceedings of the Twenty-Third Peptide Symposium.* H. L. S. Maya, ed. ESCOM, Leiden, p. 841.
16. Del Prete, G., M. De Carli, M. Ricci, and S. Romagnani. 1991. Helper activity for immunoglobulin synthesis of T helper type 1 (Th1) and Th2 human T cell clones: the help of Th1 clones is limited by their cytolytic capacity. *J. Exp. Med.* 174: 809–813.
17. Vergelli, M., B. Hemmer, P. A. Muraro, L. Tranquilli, W. E. Biddison, A. Sarin, H. F. McFarland, and R. Martin. 1997. Human autoreactive $CD4^+$ T cell clones use perforin- or Fas/Fas ligand-mediated pathways for target cell lysis. *J. Immunol.* 158: 2756–2761.
18. D'Elios, M. M., M. P. Bergman, A. Azzurri, A. Amedei, M. Benagiano, J. J. De Pont, F. Cianchi, C. M. Vandenbroucke-Grauls, S. Romagnani, B. J. Appelmelk, and G. Del Prete. 2001. H^+,K^+-atpase (proton pump) is the target autoantigen of Th1-type cytotoxic T cells in autoimmune gastritis. *Gastroenterology* 120: 377–386.
19. Del Prete, G., M. De Carli, R. M. Lammel, M. M. D'Elios, K. C. Daniel, B. Giusti, R. Abbate, and S. Romagnani. 1995. Th1 and Th2 T-helper cells exert opposite regulatory effects on procoagulant activity and tissue factor production by human monocytes. *Blood* 86: 250–257.
20. Kagi, D., F. Vignaux, B. Ledermann, K. Burki, V. Depraetere, S. Nagata, H. Hengartner, and P. Golstein. 1994. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science* 265: 528–530.
21. Stemme, S., B. Faber, J. Holm, O. Wiklund, J. L. Witztum, and G. K. Hansson. 1995. T lymphocytes from human atherosclerotic plaques recognize oxidized low density lipoprotein. *Proc. Natl. Acad. Sci. USA* 92: 3893–3897.
22. Schlesinger, M. J. 1990. Heat shock proteins. *J. Biol. Chem.* 265: 12111–12114.
23. Morimoto, R. I. 1993. Cells in stress: transcriptional activation of heat shock genes. *Science* 259: 1409–1410.
24. Lamb, D. J., W. El-Sankary, and G. A. A. Ferns. 2003. Molecular mimicry in atherosclerosis: a role for heat shock proteins in immunisation. *Atherosclerosis* 167: 177–185.
25. Xu, Q., H. Dietrich, H. J. Steiner, A. M. Gown, B. Schoel, G. Mikuz, S. H. Kaufmann, and G. Wick. 1992. Induction of atherosclerosis in normocholesterolaemic rabbits by immunisation with heat shock protein 65. *Arterioscler. Thromb.* 12: 789–799.
26. Xu, Q., R. Kleindienst, W. Waitz, H. Diertrich, and G. Wick. 1993. Increased expression of heat shock protein 65 coincides with a population of infiltrating T lymphocytes in atherosclerotic lesions of rabbits specifically responding to heat shock protein 65. *J. Clin. Invest.* 91: 2693–2702.
27. Metzler, B., M. Mayr, H. Dietrich, M. Singh, E. Wiebe, Q. Xu, and G. Wick. 1999. Inhibition of arteriosclerosis by T-cell depletion in normocholesterolaemic rabbits immunised with heat shock protein 65. *Arterioscler. Thromb. Vasc. Biol.* 19: 1905–1911.
28. Curry, A. J., I. Portig, J. C. Goodall, P. J. Kirkpatrick, and J. S. Gaston. 2000. T lymphocyte lines isolated from atheromatous plaque contain cells capable of responding to *Chlamydia* antigens. *Clin. Exp. Immunol.* 121: 261–269.
29. Mosorin, M., H. M. Surcel, A. Laurila, M. Lehtinen, R. Karttunen, J. Juvonen, J. Paavonen, R. P. Morrison, P. Saikku, and T. Juvonen. 2000. Detection of *Chlamydia pneumoniae*-reactive T lymphocytes in human atherosclerotic plaques of carotid artery. *Arterioscler. Thromb. Vasc. Biol.* 20: 1061–1067.
30. Millonig, G., C. Schwentner, P. Mueller, C. Mayerl, and G. Wick. 2001. The vascular-associated lymphoid tissue: a new site of local immunity. *Curr. Opin. Lipidol.* 12: 547–553.
31. Kolodgie, F. D., J. Narula, A. P. Burke, N. Haider, A. Farb, Y. Hui-Liang, J. Smialek, and R. Virmani. 2000. Localization of apoptotic macrophages at the site of plaque rupture in sudden coronary death. *Am. J. Pathol.* 157: 1259–1268.
32. Geng, Y. J., L. E. Henderson, E. B. Levesque, M. Muszynski, and P. Libby. 1997. Fas is expressed in human atherosclerotic intima and promotes apoptosis of cytokine-primed human vascular smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol.* 17: 2200–2208.
33. Mallat, Z., and A. Tedgui. 2001. Current perspective on the role of apoptosis in atherothrombotic disease. *Circ. Res.* 88: 998–1003.
34. Toschi, V., R. Gallo, M. Lettino, J. T. Fallon, S. D. Gertz, A. Fernandez-Ortiz, J. H. Chesebro, L. Badimon, Y. Nemerson, V. Fuster, and J. J. Badimon. 1997. Tissue factor modulates the thrombogenicity of human atherosclerotic plaques. *Circulation* 95: 594–599.
35. Young, R. A., and T. J. Elliott. 1989. Stress proteins, infection, and immune surveillance. *Cell* 59: 5–8.
36. Liao, D. F., Z. G. Jin, A. S. Baas, G. Daum, S. P. Gygi, R. Aebersold, and B. C. Berk. 2000. Purification and identification of secreted oxidative stress-induced factors from vascular smooth muscle cells. *J. Biol. Chem.* 275: 189–196.
37. Xu, Q., G. Schett, H. Perschinka, M. Mayr, G. Egger, F. Oberhollenzer, J. Willeit, S. Kiechl, and G. Wick. 2000. Serum soluble heat shock protein 60 is elevated in subjects with atherosclerosis in a general population. *Circulation* 102: 14–20.
38. Hartl, F. U., and M. Hayer-Hartl. 2002. Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* 295: 1852–1858.
39. George, J., A. Afek, B. Gilburd, Y. Shoenfeld, and D. Harats. 2001. Cellular and humoral immune responses to heat shock protein 65 are both involved in promoting fatty-streak formation in LDL-receptor deficient mice. *J. Am. Coll. Cardiol.* 38: 900–905.
40. Harats, D., N. Yacov, B. Gilburd, Y. Shoenfeld, and J. George. 2002. Oral tolerance with heat shock protein 65 attenuates *Mycobacterium tuberculosis*-induced and high-fat-diet-driven atherosclerotic lesions. *J. Am. Coll. Cardiol.* 40: 1333–1338.
41. Maron, R., G. Sukhova, A. M. Faria, E. Hoffmann, F. Mach, P. Libby, and H. L. Weiner. 2002. Mucosal administration of heat shock protein-65 decreases atherosclerosis and inflammation in aortic arch of low-density lipoprotein receptor-deficient mice. *Circulation* 106: 1708–1715.
42. Barker, R. N., A. J. Easterfield, R. F. Allen, A. D. Wells, C. J. Elson, and S. J. Thompson. 1996. B- and T-cell autoantigens in pristane-induced arthritis. *Immunology* 89: 189–194.
43. Anderton, S. M., R. Van der Zee, B. Prakken, A. Noordzij, and W. van Eden. 1995. Activation of T cells recognizing self 60-kD heat shock protein can protect against experimental arthritis. *J. Exp. Med.* 181: 943–952.
44. Gaston, J. S. 1998. Role of T-cells in the development of arthritis. *Clin. Sci.* 95: 19–31.