Objective—Growing evidence suggests that immune reactions to heat shock protein 60 (HSP60) are involved in atherogenesis. Because of the high phylogenetic conservation between microbial and human HSP60, bacterial infections might be responsible for breaking the tolerance to self-HSP60, which is expressed on the surface of stressed arterial endothelial cells.

Methods and Results—We purified serum antibodies to Escherichia coli HSP60 (GroEL), the 60-kD chlamydial HSP, and HSP65 of Mycobacterium tuberculosis by affinity chromatography from clinically healthy subjects with sonographically proven carotid atherosclerosis. Reactivity of the purified antibodies with overlapping human HSP60 peptides was measured, and 8 shared common epitopes, recognized by all anti-bacterial HSP60/65 antibodies, were identified. Antisera specific for these cross-reactive epitopes were produced by immunizing rabbits with peptides derived from human HSP60. By immunohistochemistry, the epitopes were found to be present in the arterial wall of young subjects during the earliest stages of the disease.

Conclusions—Antibodies to microbial HSP60/65 recognize specific epitopes on human HSP60. These cross-reactive epitopes were shown to serve as autoimmune targets in incipient atherosclerosis and might provide further insights into the mechanisms of early atherogenesis. (Arterioscler Thromb Vasc Biol. 2003;23:1060-1065.)

Key Words: epitopes ■ autoimmune ■ atherosclerosis ■ heat shock protein 60 ■ aging
We have previously reported on 3 dominant epitopes of mHSP65 that are recognized by serum antibodies from subjects with atherosclerosis. Interestingly, 2 of those seemed to associate into a single conformational structure, as determined by computer-assisted localization on an hHSP60 structural model. The aim of the present study was to identify linear cross-reactive B-cell epitopes shared among HSP60s from *Escherichia coli*, *Chlamydia trachomatis*, *Mycobacterium tuberculosis*, and humans.

**Methods**

**Purification of Serum Anti-HSP60 Antibodies**

Blood samples were taken from participants in the Bruneck study, a large, population-based study of atherosclerosis. Antibodies against mHSP65 were determined by ELISA by following an established protocol. For purification of anti-HSP antibodies, sera from 5 subjects with titers ≥1:1280 and sonographically proven atherosclerotic lesions in the carotid artery were pooled. Affinity chromatography of serum was performed by using a previously described method. In brief, pooled sera was heat-inactivated, and immunoglobulins were precipitated by using a standard ammonium sulfate procedure. The precipitate was suspended in PBS (pH 7.2) and allowed to run through a chromatography column loaded with 2 mL agarose gel beads (Affi-Gel 15, Bio-Rad) coupled with 3 mg recombinant mHSP65. 

**Epitope Mapping With Spot Membranes**

One hundred thirteen synthetic 13-mer peptides, comprising the whole sequence of hHSP with 8–amino acid overlap, were used for epitope mapping. Peptides were immobilized on a nitrocellulose spot membrane with a (f-Ala), space (Sigma-Genosys). After being blocked with 2.5% BSA (Sigma) in PBS overnight, the membrane was probed with affinity-purified human anti-HSP60 antibodies diluted from 1 in 20 to 1 in 20 480, were incubated for 1 hour at room temperature. The reaction was considered positive when, after incubation with secondary anti-rabbit immunoglobulins (P217, DAKO) and detection with ABTS (Sigma), the optical density at 410 nm exceeded 0.2.

**Dot Blots**

Recombinant proteins (1 µg for bacterial HSP60s, 500 ng for hHSP60, 1 µg ovalbumin) were air-dried on a nitrocellulose membrane (Protran, Schleicher & Schuell) at 37 °C for 1 hour. After being blocked with 5% dry skim milk (Merck) in PBS for 30 minutes, the membranes were probed with purified anti-epitope antibodies (10 µg/mL in 5% dry skim milk) for 1 hour. Reactions were visualized by an enhanced chemiluminescence kit (ECL, Amersham Pharma-cia) after incubation with peroxidase-conjugated swine anti-rabbit immunoglobulins (P217, DAKO).

**Immunohistochemistry**

Four-micron-thick frozen carotid arterial sections of 7 young (8, 9, 16, 19, 25, 26, and 27 years old) clinically healthy donors who had died of accident or suicide were used for immunohistochemistry. After air-drying the slides for 30 to 60 minutes at room temperature, they were fixed in acetone for 10 minutes. Sections were then incubated for 15 minutes with 10% normal human serum (heat-inactivated at 60°C for 30 minutes) in Tris-buffered saline (TBS, pH 7.4). Excess serum was blotted off, and the primary antibody-purified, epitope-specific rabbit antibody, diluted 1:10 in TBS, a pooled mixture of all epitope-specific rabbit antibodies at a final dilution for each antibody of 1 in 100 in TBS, or, as a positive control, polyclonal rabbit anti-HSP60, 1 in 100 in TBS (SPA-805, Stressgen), was applied directly without any further washing procedures and incubated for 30 minutes. Slides were rinsed 3 times in TBS, and the secondary antibody, conjugated to alkaline phosphatase (swine anti-rabbit immunoglobulin, DAKO), was incubated for another 30 minutes followed by rinsing in TBS. Visualization was done by adding fast red–naphthol (Sigma), and for better histologic orientation, sections were counterstained with hematoxylin (Merck).

**Results**

**Epitope Mapping**

The aim of this study was to identify sequence motifs that are shared between human and bacterial HSP60s and that are recognized by purified anti-bacterial HSP60 antibodies from human sera. Titters of the recovered affinity-purified antibodies were high and similar to the titer of the serum pool, whereas no reactivity to ovalbumin was measurable (data not shown). As demonstrated in a previous study by western blot analysis, the purified antibodies not only reacted with the HSP for which they were purified but also recognized their bacterial and mammalian homologues. Anti-mHSP65, anti-chHSP60, and anti-GroEL antibodies were probed on 3 spot membranes, and their reaction with each peptide was densi—
tometrically quantified. Interestingly, antibodies from both serum pools showed virtually no difference in their epitope pattern. All experiments were performed twice, and only peptides that reacted in all experiments were considered positive. As summarized in Figure 1, the anti-mHSP65 antibodies recognized 25 peptides from hHSP60 (22%), forming 17 linear epitopes. Anti-chHSP60 antibodies showed a reaction with 35 human peptides (31%), which were clustered into 18 epitopes. Forty-five peptides, forming 18 epitopes, were recognized by anti-GroEL antibodies. Eight continuous epitopes on hHSP60 were found to be reactive with all anti-bacterial HSP60 antibodies tested. Four of them consisted of a single 13-mer peptide, and 4 were assembled by 2 adjacent peptides.

Sequence Alignments and Comparative Modeling
The amino acid sequences of hHSP60, mHSP65, GroEL, and chHSP60 were aligned by using CLUSTALW software for multiple sequence alignment and the amino acid scoring function for isomorphic replacement by Tudos et al., which is suitable for epitope analysis. Epitopes 2, 4, and 8 are located in highly conserved regions, with amino acid identity and homology >50% and 80%, respectively (Figure 2). The regions in which the other epitopes are located show average identity and homology. Because there are no experimental structural data available yet for hHSP60, we used a model derived by comparative modeling (SwissModel), based on Protein Data Bank (http://www.rcsb.org/pdb/) entries of 4 bacterial HSP60s (1GRL, 1OEL, 1AON, and 1JON). Owing to sequence homology, the model should be very high (root mean square deviation for backbone atoms). In the 3-dimensional structure of hHSP60, the epitopes are found to be distributed over the whole protein, and a single immunodominant domain cannot be identified (see Figure 3). Epitopes 1, 2, 3, 7, and 8 are located in the large equatorial domain of HSP60; epitope 4 lies in the intermediate domain; and epitopes 5 and 6 are located in the apical domain. All epitopes are at least partly surface-exposed and accessible to the antibodies.

Peptide Synthesis and Production of Epitope-Specific Antibodies
The 8 identified linear cross-reactive hHSP60 epitopes, recognized by all anti-bacterial HSP antibodies tested, were synthesized by using the concept of MAPs. The MAPs obtained had molecular weights between 12 and 16 kDa and were directly immunogenic without coupling to a carrier protein. For each peptide, a rabbit was immunized 4 times (0, 2, 4, and 14 weeks) with 1 mg MAP in Freund's incomplete adjuvant. After 16 weeks, antiserum was collected. All antisera showed high and specific antibody titers against the peptide used as the immunogen, whereas cross-reactivity with the other peptides was not observed (data not shown). Epitope-specific antibodies were purified by affinity chromatography with the respective peptide. The purified anti-
epitope antibodies, which were raised against peptides derived from hHSP60, reacted not only with hHSP60 but also showed cross-reactivity to nearly all bacterial HSPs by dot blot analysis (Figure 4). The exceptions were anti-epitope 3 antibody, which did not recognize mHSP65, and anti-epitope 7, which did not react with GroEL and chHSP60.

Immunohistochemistry
To determine whether the identified cross-reactive epitopes are present in early atherosclerotic lesions, purified epitope-specific antibodies were tested on arterial sections of 8 young, clinically healthy donors. Identifiable atherosclerotic alterations were already present in the arteries of 4 of these subjects, whereas 2 even showed severe atherosclerotic lesions (Table 1). The antibody against epitope 8 was found to be reactive with 7 of 8 tested arterial sections. In specimens from apparently healthy arteries, this antibody stained cells on the surface of the vessel (Figure 5B). Double-staining experiments with von Willebrand factor identified these cells as endothelial cells (Figure 5C). Antibodies specific for epitopes 1, 2, 3, and 7 were found to be reactive with endothelial cells, subendothelial cells, and foam cells in sections with already detectable atherosclerotic alterations (Figure 5D). Antibodies specific for epitopes 4, 5, and 6 showed significant staining in only 1 arterial section with severe atherosclerotic lesions (for summary, see Table 1). A pooled mixture of all epitope-specific antibodies was applied to the late lesions. Compared with a commercially available rabbit anti-HSP60 antibody, which was produced by immunization with whole protein, a similar staining pattern was observed (Figure 5E and 5F).

Discussion
Autoimmunity to hHSP60, triggered by cross-reactivity of the protective immune response directed against microbial HSP60s, is believed to be involved in many autoimmune diseases, including atherosclerosis.3 In addition to their proven diagnostic potential, a putative causal role for circulating anti-HSP60 antibodies in atherogenesis might involve an autoimmune reaction to endothelial cells that express HSPs as a consequence of different forms of stress, such as local infections, hemodynamic stress, toxins, or biochemically modified LDL.31–34 In this study, we provide the first evidence that anti-HSP60 antibodies purified from subjects with carotid atherosclerosis recognize distinct, continuous epitopes shared between HSP60 from humans, Mycobacterium, Chlamydia, and E.coli. In addition, we demonstrate that some of these epitopes are expressed to different degrees in normal vessels and atherosclerotic lesions.
Other studies have mapped epitopes on HSP60 homologues from different bacterial species, best recognized by serum antibodies in infectious diseases. However, because highly autoreactive B cells and helper T cells would have been eliminated during maturation of the immune system, it is unlikely that the strongest-binding antibodies recognize motifs that are shared between self and non-self molecules of pathogens. The previously reported bacterial HSP60 epitopes seem to be distributed throughout the linear sequence of the molecule, and a general immunodominant region cannot be found. We confirmed these findings on a 3-dimensional structure of the human homologue, where our identified cross-reactive epitopes are also spread over the entire molecule. However, owing to spacial proximity in the modeled tertiary structure of hHSP60, the linear epitopes 2, 3, and 7 and 5 and 6 seem to be assembled into 2 conformational regions (Figure 3).

The nature of the epitopes identified in this study is intrinsically cross-reactive, because we have demonstrated that antibodies raised in rabbits against human peptides representing our epitopes also recognize bacterial HSP60. Recent publications emphasize the possibility that autoantibodies to hHSP60 might be inborn and at least partly inherited, rather than resulting from stimulation with cross-reacting microbial or biochemically altered autologous epitopes. Herein we provide evidence for atherosclerosis-associated epitopes shared between hHSP60 and bacterial HSP60. Because we have been unable to identify humans without anti-bacterial HSP60 antibodies, the question whether bona fide non–cross-reactive anti-hHSP60 autoantibodies contribute to atherogenesis can only be delineated after identification of additional hHSP60-specific epitopes.

Thus, evidence suggests that HSP60 might localize to the surface of eukaryotic cells, although less is known about its function and structure on the cell surface. Recent investigations provide evidence for the presence of an HSP60 receptor, indicating that the HSP found on the cell surface might come not only from the cell itself but also originate from external sources. We have previously reported that the anti-hHSP60 monoclonal antibody II-13 is cytotoxic for stressed endothelial cells, whereas another monoclonal antibody, ML-30, which recognizes a different epitope, is not. Thus, only distinct epitopes are accessible for antibodies, suggesting that the surface orientation of HSP60 is important. It also cannot be excluded that just single domains of hHSP60 are present on the outer surface of the cells.

It is known that in Western countries >50% of children aged 10 to 12 years have already ongoing atherosclerotic alterations in their coronary arteries. Because most of them lack any known classic risk factor, we have studied the arteries of children or young adults by immunohistochemistry. In manifest atherosclerotic lesions, all 8 epitopes were recognized, and intimal cells and foam cells stained particularly strongly (Figure 5A–5D). Interestingly, in very early lesions, a different situation was found: Here, only antibodies directed against epitope 8 were especially very reactive in apparently healthy arterial specimens of children, particularly at branching arterial regions, which are subjected to turbulent blood flow stress. Thus, antibodies reactive to epitope 8 might be involved in the development of early inflammatory disorders in prestressed regions of the arterial wall.

However, a pooled mixture of the epitope-specific antibodies showed immunohistochemical recognition patterns similar to a commercially available anti-HSP60 antibody (Figure 5E and 5F). In the case of late atherosclerotic lesions, inflammatory processes, including infiltration of mononuclear cells and the release of locally produced cytokines, might be strong HSP60 inducers, which make most of the epitopes accessible to antibodies. From these findings, we conclude that our cross-reactive HSP60 epitopes identified in sera from subjects with incipient atherosclerosis might provide further insights into the mechanisms of early atherogenesis and serve as promising targets for future diagnostic and therapeutic approaches.
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Cross-Reactive B-Cell Epitopes of Microbial and Human Heat Shock Protein 60/65 in Atherosclerosis

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