Induction of Arteriosclerosis in Normocholesterolemic Rabbits by Immunization With Heat Shock Protein 65

Qingbo Xu, Hermann Dietrich, Hans J. Steiner, Allen M. Gown, Bernd Schoel, Gregor Mikuz, Stefan H.E. Kaufmann, and Georg Wick

Previous studies have established the presence of high numbers of activated T lymphocytes and "aberrant" expression of major histocompatibility complex II antigens by endothelial and smooth muscle cells in human atherosclerotic lesions, implicating the involvement of a local cellular immune response. The identity of the antigen(s) eliciting this immune response, the extent of their effect, and the atherogenic stage at which they occur remain to be determined. In the present studies, 120 normocholesterolemic New Zealand White rabbits were immunized one or more times with various antigens, with or without adjuvants. The antigens and adjuvants included human or rabbit atherosclerotic lesion proteins, ovalbumin, Freund's complete and/or incomplete adjuvants, recombinant mycobacterial heat shock protein 65 (hsp65), and two hsp-free adjuvants, Ribi complete adjuvant and lipopeptide. In addition, some groups received a high-cholesterol diet. Sixteen weeks after the first immunization the animals were killed, and arteriosclerotic lesions in the intima of the aortic arch were found to have developed only in those animals immunized with antigenic preparations containing hsp, either in the form of whole mycobacteria or as purified recombinant hsp65, although their serum cholesterol levels were normal. No arteriosclerotic changes exceeding those of controls were found in the other groups, irrespective of the antigen used. Immunohistopathologic examination revealed that the lesions contained 20% T cells, 10-30% macrophages, and 10-40% smooth muscle cells. Analysis of the peripheral blood T-lymphocyte proliferative responses revealed that the occurrence of lesions was positively correlated with the presence of hsp65-reactive T cells, suggesting that hsp65 is involved in the induction of arteriosclerotic lesions. Furthermore, combined immunization with hsp-containing material and a cholesterol-rich diet provoked development of significantly more severe atherosclerosis and the appearance of characteristic foam cells. We conclude that an (auto)immune response to hsp may initiate the development of atherosclerosis and that a high blood cholesterol level is only one albeit a very important risk factor.


KEY WORDS • arteriosclerosis • atherosclerosis • autoimmunity • heat shock proteins • stress proteins • immunization • cholesterol

The cellular and humoral hallmarks indicative of immune system involvement in atherosclerotic pathogenesis include the occurrence of lymphocytes, notably T cells, in atherosclerotic lesions at different stages and the deposition of immune complexes in the intima.1-7 The possible (auto)antigens that may induce this reaction have not yet been identified, nor is it known whether the immunologically mediated inflammatory process is primary or secondary. It is also not clear whether a high concentration of serum cholesterol per se is necessary to initiate atherosclerosis or, as we imply, is only one major risk factor that exerts its deleterious effect when a preexisting inflammatory process exists. Several possible antigenic candidates that may initiate an immunologic process in the arterial intima have been considered, such as modified lipoproteins,8-9 virus,10-11 and denatured macromolecules derived from cellular debris.12 More recently, certain heat shock (hsp) or stress proteins, i.e., very highly conserved molecular chaperones, have been identified as autoantigens involved in the pathogenesis of various autoimmune diseases13,14 and have also been demonstrated by immunohistochemical analysis of human atherosclerotic lesions.15 However, definitive data on the involvement of hsp in atherogenesis are still lacking, and some authors propose that hsp70 plays a role in protecting lysosomal membrane integrity and arterial cell survival.16 Hsps are a group of families of approximately two dozen proteins and cognates of each family that show a high degree of sequence homology between different species from bacteria to humans.13,14,17 The

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Mycobacterium tuberculosis (Mt), we then immunized rabbits with this component alone and found that it was able to induce atherosclerosis in the same manner as FCA. Finally, a combination of immunization with heat-killed mycobacteria, was included in the study, we showed that activated T lymphocytes of the helper/inducer phenotype are among the first to infiltrate the aortic intima at the earliest stage of human atherosclerosis. To identify the possible antigens leading to this T-cell reactivity, we experimentally induced autoimmune atherosclerosis by classical immunization of healthy animals with appropriate antigens and FCA. Rabbits were chosen for their known susceptibility to diet-induced atherosclerosis and the availability of the Watanabe rabbit as a model for a spontaneous, hereditary form of the disease. We began this series of experiments by immunizing rabbits with proteins extracted from human or rabbit atherosclerotic lesions that had been emulsified with FCA. This approach was designed to identify one or more autoantigens within different types of stress, such as infections, high temperature, and chemical or mechanical stress. Mycobacteria contained in Freund’s complete adjuvant (FCA) are especially rich in hsp.

In a previous quantitative immunohistochemical study, we showed that activated T lymphocytes of the helper/inducer phenotype are among the first to infiltrate the aortic intima at the earliest stage of human atherosclerosis. To identify the possible antigens leading to this T-cell reactivity, we experimentally induced autoimmune atherosclerosis by classical immunization of healthy animals with appropriate antigens and FCA. Rabbits were chosen for their known susceptibility to diet-induced atherosclerosis and the availability of the Watanabe rabbit as a model for a spontaneous, hereditary form of the disease. We began this series of experiments by immunizing rabbits with proteins extracted from human or rabbit atherosclerotic lesions that had been emulsified with FCA. This approach was designed to identify one or more autoantigens within atherosclerotic lesions that might be responsible for inducing an autoimmune response as a first step in the pathogenesis of atherosclerosis. In contrast to our original concept, however, we observed that atherosclerosis consistently developed when FCA, i.e., an adjuvant containing heat-killed mycobacteria, was included in the immunization mixture, irrespective of the antigen itself.

Because hsp65 is the major antigenic component of Mycobacterium tuberculosis (Mt), we then immunized rabbits with this component alone and found that it was able to induce atherosclerosis in the same manner as FCA. Finally, a combination of immunization with hsp65-containing material and a cholesterol-rich diet led to the development of atherosclerosis more severe than that obtained after immunization or diet alone.

**Methods**

**Animals, Diets, and Reagents**

One hundred twenty New Zealand White male rabbits weighing between 1,800 and 2,200 g were obtained from Savo/Charles River Co. (Kisslegg in Allgäu, FRG). All animals were selected for serum cholesterol levels <100 mg/dl and were individually housed in wire-bottomed cages at 22°C with a relative humidity of 55%. All rabbits received water ad libitum and were fed a normal standard rabbit diet (containing 0.015% cholesterol; Tagger&Co., Graz, Austria) or a cholesterol-enriched diet (cholesterol content, 0.2% wt/wt). The animals were separated into 11 groups and treated as shown in Table 1.

Human atherosclerotic lesion proteins were prepared by homogenizing aortic atherosclerotic lesions obtained from vascular surgery material not later than 3 hours after operation. Rabbit atherosclerotic lesion proteins were obtained from aortic atherosclerotic lesions of rabbits that had been fed the cholesterol-rich diet for 12 weeks.

FCA (containing 0.5 mg M. butyricum/ml), Freund’s incomplete adjuvant (FIA), and killed Mt H37 Ra (lot No. 3114-33-8) were purchased from DIFCO Laboratories (Detroit, Mich.). FCA was fortified by adding 2 or 9.5 mg Mt/ml, and the mixture was sonicated on ice before emulsification with an equal volume of the appropriately diluted respective antigen solutions or phosphate-buffered saline (PBS) by means of a double-hubbed needle. Ribi complete adjuvant (ABM₃) containing monophosphoryl lipid A and trehalose dimycolate was obtained from Pan Systems (Aidenbach, FRG). Lipopeptide was a gift from W.G. Bessler (Albert-Ludwigs University, Freiburg, FRG). Recombinant M. bovis hsp65 was expressed in Escherichia coli produced and purified as described elsewhere. In short, hsp65 was purified by ammonium sulfate precipitation and anion-exchange chromatography. Ovalbumin was purchased from Sigma (Munich, FRG).

Blocks of experiments were performed over a period of 2 years, and each experiment was terminated 16 weeks after the first immunization. The data are presented here as an overall summary. Protein contents of samples were determined using the Bio-Rad protein assay (Bio-Rad GmbH, Munich, FRG).

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**Table 1. Rabbit Experimental Schedule**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Antigen (per imm/rabbit)</th>
<th>Adjuvant</th>
<th>Immunization (times)</th>
<th>Diet</th>
<th>n</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>Control</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>Normal chow</td>
<td>34</td>
</tr>
<tr>
<td>2</td>
<td>Imm</td>
<td>1 mg HLP</td>
<td>FCA + FIA</td>
<td>8</td>
<td>&quot;</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>4 mg RLP</td>
<td>FCA + FIA</td>
<td>8</td>
<td>&quot;</td>
<td>10</td>
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<tr>
<td>4</td>
<td>&quot;</td>
<td>...</td>
<td>FIA</td>
<td>8</td>
<td>&quot;</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>&quot;</td>
<td>...</td>
<td>FCA</td>
<td>3</td>
<td>&quot;</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>&quot;</td>
<td>1 mg MPL + TDM</td>
<td>ABM₂</td>
<td>3</td>
<td>&quot;</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>&quot;</td>
<td>1 mg lipopeptide</td>
<td>Lipopeptide</td>
<td>3</td>
<td>&quot;</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>&quot;</td>
<td>1 mg ovalbumin</td>
<td>FIA</td>
<td>3</td>
<td>&quot;</td>
<td>10</td>
</tr>
<tr>
<td>9</td>
<td>&quot;</td>
<td>0.1 mg hsp65</td>
<td>FIA</td>
<td>3</td>
<td>&quot;</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>Chol</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>+0.2% Chol</td>
<td>10</td>
</tr>
<tr>
<td>11</td>
<td>Imm + chol</td>
<td>...</td>
<td>FCA + FIA</td>
<td>2</td>
<td>+0.2% Chol</td>
<td>10</td>
</tr>
</tbody>
</table>

Imm, immunization; HLP and RLP, human and rabbit atherosclerotic lesion proteins; FCA, Freund’s complete adjuvant; FIA, Freund’s incomplete adjuvant; MPL, monophosphoryl lipid A; TDM, trehalose dimycolate; ABM₂, Ribi complete adjuvant; hsp, heat shock protein; chol, cholesterol. In groups 2, 3, and 11, FCA supplemented with Mycobacterium tuberculosis (Mt; 5 mg/rabbit) was only used for the first immunization. Group 5 received three immunizations with FCA (1 mg Mt/rabbit). Immunization was performed at 2-week intervals in groups 2–4 and 11 and at 5-week intervals in groups 5–9. Cholesterol diet was fed starting 2 weeks after the beginning of the experiment. All animals were killed 16 weeks after the first immunization.
Blood Cholesterol

Blood (1–2 ml) was taken from the central ear artery of rabbits that had been fasted for 16 hours. The values of serum cholesterol were measured every 4 weeks using an enzymatic procedure (Sigma). Briefly, 10 μl serum was added to 1 ml solution of cholesterol test reagent and incubated for 18 minutes at room temperature followed by photometer measurement at an excitation wavelength of 500 nm (Dynatech Laboratories Inc., Alexandria, Va.)

Immunization

Immunization schedules for the various experimental groups are shown in Table 1. As indicated, rabbits received one or more intracutaneous injections in the back region. Each milliliter of emulsion consisted of 0.5 ml protein solution or PBS and 0.5 ml FCA, FIA, or lipopeptide.

Atherosclerotic Lesion Measurement

Animals were killed by heart puncture while anesthetized with ketamine (35 mg/kg body wt) and xylazine (5–10 mg/kg body wt). Serum was stored frozen, and the aortas were removed intact from the aortic arch to the iliac bifurcation and cut longitudinally for macroscopic documentation of intimal lesions on a glass template. The templates were photographed, and the surface area covered by lesions was determined by computerized planimetry (IBM PC-AT 386 Image Analyzer, program JAVA, Gaudel Scientific) without knowledge of the source of each individual aortic sample. These data were used to calculate the area (in millimeters squared) of the intimal surface affected by atherosclerotic lesions. Further processing was performed for analysis by light, electron, and immunofluorescent microscopy. Specimens from the liver and kidney were also prepared for paraffin sectioning.

Light and Electron Microscopy

Several portions of uninvolved and lesioned aortic intimas from each group were subdivided and processed for histological and ultrastructural examinations. For conventional histology, the tissue fragments were fixed in 4% (vol/vol) buffered (pH 7.2) formaldehyde, embedded in paraffin, and sectioned for hematoxylin-eosin staining. For the transmission electron microscopy, the tissue was fixed in 2% glutaraldehyde and postfixed in 1% OsO₄ (Sigma) buffered with 0.1 M PBS. Samples were embedded in Polybed 812 (Polysciences Inc., Warrington, Pa), and ultrathin sections (80 nm) were cut with an ultramicrotome (Leica AG, Vienna, Austria) and placed on Formvar-coated 300-mesh copper grids. Sections were counterstained with lead citrate and uranyl acetate and were examined with a transmission electron microscope (Zeiss, Oberkochen, FRG).

Immunofluorescence

The sources and specificities of all antibodies used in this study are summarized in Table 2. Monoclonal antibodies against rabbit T cells and la antigens derived from hybridoma supernatants or the ascites of BALB/c mice injected intraperitoneally with the respective hybridoma cells were purified and biotin labeled in our laboratory following established procedures. Tetra- methylrhodamine isothiocyanate–labeled streptavidin, used for the visualization of biotinylated antibodies, was obtained from Jackson ImmunoResearch Laboratories (West Grove, Pa.).

The aortic specimens were stored in liquid nitrogen until use. The procedure for immunofluorescence studies has been described previously. For visualization of nuclei, sections were counterstained with the blue fluorescent DNA stain Hoechst 33258 (1 μg/ml PBS; Lambda Probes, Graz, Austria) for 3 minutes. Direct and indirect immunofluorescence tests were performed on 4-μm frozen, acetone-fixed sections at room temperature. Finally, sections were mounted in Gelvatol (Monsanto, Springfield, Mass.) and examined in an epiillumination immunofluorescence microscope equipped with appropriate filter combinations for the two-wavelength method (Leitz Ortholux II, Wetzlar, FRG).

Lymphocyte Culture

On days 9–14 after the second and third immunizations, heparinized blood (15 IU/ml preservative-free heparin; Immuno AG, Vienna, Austria) was obtained from the central ear artery and diluted 1:2 (vol/vol) in Iscove’s modified Dulbecco’s medium (GIBCO, Paisley, UK). Blood mononuclear cells were isolated by density gradient centrifugation over Lympho-Paque (Δ=1.086 g/ml; Nyegaard & Co., Oslo, Norway) as described previously. The cells (1×10⁶/well) were cultivated in triplicate in round-bottomed microtiters plates (Nunc, Roskilde, Denmark) in 0.2 ml medium supplemented with 1% (vol/vol) fresh autologous serum, 5×10⁻⁵ M 2-mercaptoethanol, streptomycin (100 μg/ml), penicillin (100 IU/ml), concanavalin A (Con A; Pharmacia, Uppsala, Sweden), and antigens including purified protein derivative of Mt (PPD) (Statens Seruminstitut, Copenhagen, Denmark) in the concentrations indicated in Table 3. The proliferative response (in counts per minute [cpm]) of the cells was determined by measuring the incorporation of [³H]thymidine (1 μCi/well; specific activity, 5 Ci/mmol or 185 GBq/mmol; Amersham, Bucks, UK) during the last 6 hours of a 48-hour culture at 37°C and 5% CO₂. The stimulation index (SI) was calculated according to the following formula:

\[ SI = \frac{cpm \text{ with antigen}}{cpm \text{ without antigen}} \]

Statistics

Statistical analyses were performed using unpaired Student’s t and linear regression tests.
normal diet for 16 weeks. Arteriosclerotic lesions can be branching points of large vessels from the immunized rabbit observed on the intimal surface of the aortic arch and the tas from control (a) and hsp65-immunized (b) animals fed a

**FIGURE 1.**

Macroscopic Assessment of Atherosclerotic Lesions

(arrow), hsp, heat shock protein.

**TABLE 3. Antigen Specificity of Proliferative Response of Rabbit Lymphocytes**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment in vivo</th>
<th>Ovalbumin, 30 µg/ml</th>
<th>PPD, 30 µg/ml</th>
<th>hsp65, 5 µg/ml</th>
<th>HLP, 100 µg/ml</th>
<th>RLP, 200 µg/ml</th>
<th>Con A, 1 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>1.05</td>
<td>2.69</td>
<td>2.18</td>
<td>1.56</td>
<td>0.82</td>
<td>50.62</td>
</tr>
<tr>
<td>2</td>
<td>HLP+FCA</td>
<td>1.85</td>
<td>47.05*</td>
<td>27.74*</td>
<td>38.46*</td>
<td>11.93*</td>
<td>85.23</td>
</tr>
<tr>
<td>3</td>
<td>RLP+FCA</td>
<td>1.07</td>
<td>33.14*</td>
<td>19.65*</td>
<td>8.85*</td>
<td>8.85*</td>
<td>68.94</td>
</tr>
<tr>
<td>4</td>
<td>FIA</td>
<td>0.85</td>
<td>2.57</td>
<td>2.98</td>
<td>ND</td>
<td>ND</td>
<td>52.14</td>
</tr>
<tr>
<td>5</td>
<td>FCA</td>
<td>0.69</td>
<td>25.51*</td>
<td>15.60*</td>
<td>4.27*</td>
<td>3.06*</td>
<td>52.35</td>
</tr>
<tr>
<td>6</td>
<td>ABM&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.82</td>
<td>3.31</td>
<td>2.96</td>
<td>ND</td>
<td>ND</td>
<td>59.71</td>
</tr>
<tr>
<td>7</td>
<td>Lipopeptide</td>
<td>1.31</td>
<td>3.12</td>
<td>3.13</td>
<td>ND</td>
<td>ND</td>
<td>50.18</td>
</tr>
<tr>
<td>8</td>
<td>Ovalbumin</td>
<td>24.99*</td>
<td>2.39</td>
<td>2.19</td>
<td>0.74</td>
<td>1.20</td>
<td>65.18</td>
</tr>
<tr>
<td>9</td>
<td>hsp65</td>
<td>1.12</td>
<td>8.90*</td>
<td>11.48*</td>
<td>3.44*</td>
<td>1.57</td>
<td>48.64</td>
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<tr>
<td>10</td>
<td>Chol</td>
<td>1.22</td>
<td>5.83</td>
<td>6.42</td>
<td>2.88</td>
<td>2.23</td>
<td>87.87</td>
</tr>
<tr>
<td>11</td>
<td>Imm+chol</td>
<td>1.40</td>
<td>16.32*</td>
<td>11.52*</td>
<td>2.26</td>
<td>1.19</td>
<td>85.13</td>
</tr>
</tbody>
</table>

PPD, purified protein derivative of Mycobacterium tuberculosis; hsp, heat shock protein; HLP, human atherosclerotic lesion proteins; RLP, rabbit atherosclerotic lesion proteins; Con A, concanavalin A; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; ABM<sub>2</sub>, Ribi complete adjuvant; chol, cholesterol. On days 9-14 after the third immunization (imm), peripheral blood mononuclear cells were isolated over Lympho-Paque. The cells (1 x 10⁶/well) were cultivated in triplicate in round-bottomed microtiter plates in 0.2 ml Iscove's medium with the indicated supplementations. The proliferative response of the cells was determined by measuring the incorporation of [³H]thymidine (1 µCi/well) that was added for the last 6 hours of a 48-hour culture. Values are means of stimulation indexes. Responses significantly exceeding control levels (*p<0.001) are marked with an asterisk. ND, not determined.

**Results**

**Macroscopic Assessment of Atherosclerotic Lesions**

Gross intimal lesions were primarily observed in the aortic arch, the descending aorta, and the branching points of large arteries. The macroscopic appearance of such lesions is shown in Figure 1. As shown in Table 4, an obviously increased incidence of aortic lesions was observed only in animals immunized with hsp65-containing material, either in the form of Mt (groups 2, 3, and 5) or recombinant hsp65 alone (group 9). Quantification of the lesion-covered areas of the aortic intima revealed significant increases in animals immunized with hsp65-containing materials, fed the cholesterol-enriched diet (group 10), as well as FCA-immunized animals simultaneously receiving a cholesterol-enriched diet (group 11).

No changes exceeding those of controls were observed with FIA (group 4), ABM<sub>2</sub> and lipopeptide adjuvants (groups 6 and 7), and ovalbumin (group 8) known to be free of hsp, while immunization with FCA alone provoked development of severe arteriosclerotic lesions. As expected, a certain fraction of untreated control rabbits (group 1) also revealed a few mild lesions exclusively localized in the aortic arch.

**TABLE 4. Atherosclerotic Lesions in Aortas**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Incidence/total No. of animals</th>
<th>Lesion area (mm²/aorta)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>5/34</td>
<td>6.8±16.2</td>
</tr>
<tr>
<td>2</td>
<td>HLP+FCA</td>
<td>10/11</td>
<td>86.9±57.6*</td>
</tr>
<tr>
<td>3</td>
<td>RLP+FCA</td>
<td>8/10</td>
<td>32.1±18.9*</td>
</tr>
<tr>
<td>4</td>
<td>FIA</td>
<td>1/10</td>
<td>5.8±13.7</td>
</tr>
<tr>
<td>5</td>
<td>FCA</td>
<td>9/10</td>
<td>90.1±34.2*</td>
</tr>
<tr>
<td>6</td>
<td>ABM&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1/5</td>
<td>15.5±17.1</td>
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<tr>
<td>7</td>
<td>Lipopeptide</td>
<td>1/5</td>
<td>11.2±3.5</td>
</tr>
<tr>
<td>8</td>
<td>Ovalbumin+FIA</td>
<td>2/10</td>
<td>17.9±34.7</td>
</tr>
<tr>
<td>9</td>
<td>hsp65</td>
<td>4/5</td>
<td>49.6±31.1*</td>
</tr>
<tr>
<td>10</td>
<td>Chol</td>
<td>9/10</td>
<td>83.5±42.4*</td>
</tr>
<tr>
<td>11</td>
<td>Imm+chol</td>
<td>10/10</td>
<td>185.1±95.9*</td>
</tr>
</tbody>
</table>

HLP, human atherosclerotic lesion proteins; FCA, Freund's complete adjuvant; RLP, rabbit atherosclerotic lesion proteins; FIA, Freund's incomplete adjuvant; ABM<sub>2</sub>, Ribi complete adjuvant; hsp, heat shock protein; chol, cholesterol; imm, immunization. All macroscopically detectable atherosclerotic lesions of the aortic intima were documented on a glass template and quantified by computerized planimetry. Values are given as means±SD per aorta in each group. Significant difference from control: *p<0.01, tp<0.001.

**FIGURE 1.** Representative macroscopic photograph of aortas from control (a) and hsp65-immunized (b) animals fed a normal diet for 16 weeks. Arteriosclerotic lesions can be observed on the intimal surface of the aortic arch and the branching points of large vessels from the immunized rabbit (arrows). hsp, heat shock protein.
The upper panels (A, C, and E) of Figure 2 represent early stages and the lower panels (B, D, and F) the advanced stages of lesions. Figure 2A depicts the histological appearance of an early intima lesion in the aortic arch of a rabbit immunized with hsp65, showing the development of several layers of intimal cells with interspersed mononuclear cells but no foam cells. Figure 2B represents a more advanced stage with intimal proliferation, mononuclear cell infiltration, and incipient participation of smooth muscle cells. In contrast, the appearance of lesions in rabbits fed a cholesterol-enriched diet alone was predominantly characterized by foam cell accumulation and lipid deposition in the intima (Figures 2C and 2D). However, a combination of immunization with hsp65-containing material and a cholesterol-enriched diet provoked severe classical atherosclerotic lesions containing mononuclear inflammatory and foam cells as well as necrotic debris (Figures 2E and 2F).

Electron microscopy revealed mononuclear cells, including activated lymphocytes, attached to the endothelial cell surface (Figure 3A) and migration into the intima underlying the endothelial cells (Figure 3B). The lesions of cholesterol-fed rabbits were characterized by intracellular and intercellular lipid accumulation extending from the endothelium to the elastica interna (Figures 3C and 3D). Figures 3E and 3F show the appearance of lesions induced by immunization with hsp65-containing material plus a cholesterol-rich diet, composed of lymphoid cells, macrophages, smooth muscle cells, and foam cells as well as deposited extracellular matrix.

**Immunofluorescence**

To identify the main cellular components in atherosclerotic lesions induced by immunization and cholesterol feeding, serial sections of the lesions were incubated with a battery of antibodies to specific cell surface markers (Table 2). As shown in Figures 2 and 3, the morphology and even the cellular composition (see below) of arteriosclerotic lesions induced by immunization with different antigens seemed to be fairly constant. Thus, all lesions induced by immunization are termed “immunization-induced lesions.”

T lymphocytes identified by monoclonal antibody L11/135 were frequently observed attached to the endothelial surface and within the lesions of immunized animals (Figure 4A) and constituted about 20% of all lesion cells revealed by nuclear counterstaining with the fluorescent DNA dye Hoechst 33258 (Figure 4B). While T cells were present, albeit in moderate numbers, in milder lesions induced by cholesterol feeding only, they...
FIGURE 3. Transmission electron photomicrographs from rabbit aortas. Leukocyte attachment to endothelium (panel A) and infiltration into the subendothelial space (panel B) are demonstrated in lesions of the descending aorta from an FCA-immunized animal fed a normal diet for 16 weeks. Typical lesions of the descending aorta from a rabbit fed a 0.2% cholesterol diet for 14 weeks show lipid droplet accumulation within the lesion from the endothelium (panel C) to the elastica interna (panel D, arrow). Atherosclerotic lesions (panels E and F) of the thoracic aortas from the animals treated with FCA immunization plus the cholesterol-rich diet for 14 weeks are characterized by a more complex composition, comprising mononuclear (including lymphocytic), smooth muscle, and foam cells; lipid-droplets; and deposited connective tissue matrix. Scale bars=1 μm. * Lumen; FCA, Freund's complete adjuvant.

occurred with high frequency in regions with advanced lesions (Figure 4C). Figure 4D shows the same visual field as in panel C but with the nuclear counterstaining. These results are similar to the observations by Hansson and coworkers in aortic lesions of rabbits fed a cholesterol-rich diet. Interestingly, about 30% of total lesion cells induced by immunization plus a high-cholesterol diet were L111/135-positive T cells (Figure 4E), of which >50% were Ia-positive (data not shown), indicating an activated state.

Cells expressing the macrophage antigen identified by the RAM11 antibody were observed in all types of atherosclerotic lesions (Figure 5), including early (Figures 5A, 5C, and 5E) and advanced (Figures 5B, 5D, and 5F) stages. In the lesions of immunized animals, macrophages appeared in early stages (Figure 5A) and more frequently in advanced lesions (Figure 5B). Depending on the stage of lesion development, 10–30% of the total lesion cells were identified as macrophages. On the other hand, almost 90% of cells in the mild lesions and, to a slightly lower degree, in advanced lesions were RAM11-positive in animals receiving the cholesterol-enriched diet alone (group 10). Most of these macrophage antigen–expressing cells appeared as foam cells (Figure 5C). Finally, atherosclerotic lesions induced by immunization plus a high-cholesterol diet revealed a macrophage distribution similar to that in human atherosclerotic lesions. One third of the total lesion cells in
FIGURE 4. Photomicrographs showing detection of T lymphocytes in aortic lesions by indirect immunofluorescence. Monoclonal antibody LI1/135 and DNA dye (Hoechst 33258) were used for double staining to identify T cells (panels A, C, and E) and cell nuclei (panels B, D, and F). T lymphocytes were observed in lesions of descending aortas from rabbits immunized with human atherosclerotic lesion proteins and fed a normal diet for 16 weeks (panel A), fed the cholesterol-rich diet only for 14 weeks (panel C), and immunized with FCA and fed the cholesterol-rich diet for 14 weeks (panel E). Note that large numbers of LI1/135-positive T cells are present in the lesion from a rabbit with immunization plus cholesterol diet (panel E). Lumen of the aorta appears at the top of each photomicrograph. Arrows denote identical cells stained with the monoclonal antibody (upper panels) and the DNA dye (lower panels). FCA, Freund's complete adjuvant. X200.

the mild lesion were macrophages (Figure 5E), some of which were foam cells. In advanced lesions, macrophages were most frequent around the necrotic core, particularly the cap and shoulder regions (Figure 5F).

α-Actin-positive smooth muscle cells appeared in the very early stages of lesion development in immunized animals. Initially, only two or three layers of intimal cells could be observed in the lesion area, some of which were identified as smooth muscle cells (Figure 6A). In advanced lesions of this group, smooth muscle cells represented 10–40% of the total lesion cells (Figure 6B). In the lesions of cholesterol-fed rabbits, very few smooth muscle cells appeared in the early stages but were more frequent in advanced lesions (Figures 6C and 6D). In contrast, α-actin–positive smooth muscle cells accounted for 10–30% of the lesion cells in animals subjected to immunization plus the cholesterol-rich diet (Figures 6E and 6F). A fraction of these cells was found to be IA-positive, depending on the presence of T cells in the immediate vicinity (data not shown). Thus, the cellular composition of atherosclerotic lesions in rabbits immunized with hsp65-containing material and also receiving a cholesterol-rich diet closely resembles that previously demonstrated in human atherosclerosis.

Effect of Immunization on Blood Cholesterol Levels

In immunized animals, the blood cholesterol levels remained <100 mg/dl, the same range as in the untreated control group (Figure 7). Thus, immunization alone does not elevate blood cholesterol levels in rabbits fed a normal diet. On the other hand, the rabbits of group 11 that were fed a diet containing 0.2% cholesterol after two immunizations with FCA and FIA showed significantly higher blood cholesterol levels than rabbits receiving the cholesterol-enriched diet alone (group 10). The reason for this phenomenon is not clear, but it may be due to interference of the immunization with the capacity of the phagocyte system to remove excess cholesterol.

Peripheral Blood Lymphocyte Responses

Table 3 summarizes the results of proliferative assays of total peripheral blood mononuclear cells cultivated for 48 hours in the presence or absence of various antigens or the T-cell mitogen Con A as a positive control. It is obvious that the peripheral blood T cells from all animals were strongly stimulated by Con A. The cells of each group reacted specifically with the respective immunizing antigen. Furthermore, T lymphocytes of rabbits immunized with hsp-containing materials or pure hsp65 responded to hsp65 as well as to PPD containing the main antigenic components of Mt, i.e., the major constituent of FCA, but not to ovalbumin, confirming that the latter is devoid of hsp. Interestingly, peripheral blood lymphocytes of all nonimmunized groups also showed a higher degree of proliferation to hsp65 or PPD than that seen in medium without antigen or even to ovalbumin, which is similar to our previous
observations in healthy humans, and which possibly reflects a former contact with bacterial hsp or reactivity against autologous hsp with high antigenic homology to mycobacterial hsp. The reactivity of T lymphocytes to hsp65 closely paralleled that of PPD and was positively correlated to the number and severity of atherosclerotic lesions in aortas of immunized and control rabbits, respectively (Figure 8), pointing to a possible involvement of hsp-activated T cells in atherogenesis. Finally, the groups immunized with the two adjuvants without hsp, ABM, and lipopeptide, showed reactivity against PPD and hsp65, similar to the untreated control or the ovalbumin-immunized groups.

**Discussion**

Based on the fact that atherosclerosis emerges prematurely in familial hypercholesterolemic patients and develops quickly in cholesterol-fed animals, the imm-

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**FIGURE 5.** Photomicrographs showing detection of RAM11-positive macrophages in aortic lesions by indirect immunofluorescence. Macrophages were encountered in various numbers in early (panels A, C, and E) and advanced (panels B, D, and F) stages of lesions of ascending aortas from animals immunized with hsp65 (panels A and B) and fed a normal diet for 16 weeks, of the descending aorta of a rabbit fed a 0.2% cholesterol diet for 14 weeks (panels C and D), or of the thoracic aorta from an animal immunized with FCA and fed the cholesterol-rich diet for 14 weeks (panels E and F). Autofluorescent elastic fibers in the media are shown at the bottom of the photomicrographs. hsp, heat shock protein; FCA, Freund's complete adjuvant. x200.

**FIGURE 6.** Photomicrographs showing detection of α-actin-positive smooth muscle cells by indirect immunofluorescence. Positively stained smooth muscle cells were observed in early (panels A, C, and E) and advanced (panels B, D, and F) stages of lesion sections of descending aortas from FCA-immunized rabbits fed a normal diet for 16 weeks (panels A and B) and from animals receiving a 0.2% cholesterol diet for 14 weeks (panels C and D) and of thoracic aortas from immunized rabbits simultaneously fed the cholesterol diet for 14 weeks (panels E and F). Note the presence of several α-actin-positive smooth muscle cells in a very early stage of the lesion (panel A). Arrows point to the elastica interna in panels A, B, and E and to the media in panels C, D, and F. FCA, Freund's complete adjuvant. x200.
portance of hyperlipidemia or disorders of lipoprotein metabolism in the pathogenesis of atherosclerosis is widely accepted. However, there is continuing debate as to whether increased lipid levels are initiating events or whether they act as secondary or modifying factors in the normal population, as fatty streaks in coronary arteries emerge in children as early as 10–12 years of age; most such children have normal blood cholesterol levels. These fatty streaks are generally considered to be precursors of the later, clinically manifested atherosclerotic lesions. In experimental atherosclerosis of animals fed cholesterol-enriched diets, the earliest cellular responses are clusters of mononuclear cells that attach to endothelial cells, subsequently penetrate between endothelial cells (peripolesis), and accumulate in the subendothelium to form foam cells. In human atherosclerosis, previous studies in our laboratory demonstrated that the attachment to and penetration of macrophages and T lymphocytes between endothelial cells represent the earliest alterations of the aortic intima in young subjects. The triggering mechanisms for these altered cellular immune responses still remain to be determined. Blood cholesterol alone is not a suitable explanation, as the high frequency of arterial fatty streaks is concomitant with normal or even relatively low blood cholesterol levels in children. Thus, we consider cholesterol to be only an additional albeit the most important risk factor in atherogenesis.

Our interest in the possibility that atherosclerosis is an immunologically mediated possibly autoimmune disease was aroused by increasing reports in the literature centering around the question of whether the humoral and cellular immunologic phenomena observed in the course of the disease are primary or secondary. Our approach to this problem was predicated on our previous experience with various animal models of experimentally induced and spontaneous autoimmune diseases, as well as the role of changes in lymphocyte lipid metabolism in the altered immune responsiveness during aging. The involvement of the immune system in atherogenesis in humans and animals has recently been extensively reviewed. Thus, it seems clear that despite the evidence for the activation of humoral immunity and the complement system during atherogenesis, T cell-mediated immune reactions, primarily of the late type of hypersensitivity with T_h effector cells, are the major factors involved. It has also been clearly shown that different cytokines are secreted by atherosclerosis-associated cells, including interleukin-1 (IL-1), tumor necrosis factor-α, IL-6, IL-8, interferon gamma, and a variety of monocyte chemotactic factors. Conversely, it has been demonstrated that IL-1 activates cytokine expression by atherosclerosis-associated cells. The role of chemotactic factors in the tunica intima of arteries and their role in the influx of mononuclear and smooth muscle cells into the intima are unclear, as is the identity of possible effector adhesion molecules during interaction of such cells with endothelial cells. Emsen and Robertson have shown that in young subjects, T lymphocytes, both CD4+ and CD8+ T cells, are present in coronary and aortic atherosclerotic lesions, with a preponderance of the CD8+ phenotype. In our previous quantitative study of the cellular composition of different stages of atherosclerotic lesions that compared specimens from younger (<35 years) and older (>60 years) donors, we emphasized the evaluation of those areas that can be considered to represent the earliest changes, i.e., the transition zone between normal intima and the core of fatty streaks. The predominant intimal infiltrating cells at that site were identified as CD4+ T cells. T-cell preponderance was more pronounced in younger versus older donors. Furthermore, as in previous observations of thyroid glands of Hashimoto thyroiditis patients, there was no evidence of a primary, aberrant major histocompatibility complex class II antigen expression on the endothelial cells of normal intima adjacent to early atherosclerotic lesions: HLA-DR, -DQ and -DP were expressed only after the influx of interferon gamma-producing T cells. Therefore, it is unlikely that a putative autoimmune process leading to atherosclerosis could be initiated by target cells, i.e., endothelial cells aberrantly expressing major histocompatibility complex class II antigens and thus capable of autoantigen presentation to T cells, similar to the situation in a variety of well-proven autoimmune diseases.

In the present study, we induced experimental atherosclerosis by the classical immunization of animals with autoantigens emulsified with FCA, postulating that...
human or rabbit lesion proteins should contain the appropriate candidate antigen(s). In the 1970s, several investigators tried to identify possible antigens responsible for the induction of arteriosclerotic lesions by experimental immunizations of animals. It was found that arteriosclerotic lesions could be induced by immunization with a variety of antigens, such as liver and kidney tissue homogenates, albumin, immunoglobulin, and β-lipoprotein, that were emulsified with FCA or even FCA alone. In these studies, large amounts of antigens (100–1,000 mg/animal) were used that led to inflammatory cell infiltration, degeneration, and necrosis of the intima and arterial media, including small arteries, subsequent to serum sickness and acute allergic injury. These authors concluded that the arteriosclerotic lesion might be a chronic inflammation based on a humoral immune reaction, i.e., an injury to arterial endothelial cells caused by antigen–antibody complex deposition.31–32 In the present experiments, we found no necrotic lesions in small and middle-sized arteries, e.g., in the kidney and liver (data not shown) from animals immunized with low doses of antigens (microgram to milligram amounts per animal), nor were necrotic lesions of the aortic media encountered in our animals. Localization and cellular make-up of arteriosclerotic lesions in rabbits immunized with hsp-containing material and simultaneously fed a cholesterol-rich diet closely parallel those characteristic of human atherosclerosis, a finding which emphasizes the potential usefulness of this model as a tool for investigating the development of arteriosclerosis.

The fact that FCA alone, irrespective of the added lesion protein or control antigens, could produce arteriosclerotic lesions was surprising, as we and others had previously immunized numerous experimental animals of different species, including rabbits, to produce antibodies against many antigens or to induce experimental autoimmune diseases of various kinds without noting the development of arteriosclerosis. Interestingly, this was described for adjuvant arthritis in rats, whereas no one had studied the possible concomitant development of arteriosclerotic lesions. Such studies are ongoing in our laboratory. The atherogenic effect of immunization with FCA alone led us to assume that the antigen involved may be an hsp, notably mycobacterial hsp65, which is abundant in FCA and which also shows significant homology with human hsp65.17,21 The logical consequence of this concept was, therefore, to immunize animals with recombinant mycobacterial hsp65, an approach that proved successful. Further proof that the two additional nonatherogenic adjuvants, ABM, and lipopeptide (in contrast to FCA), are truly free of hsp65 was provided by the fact that peripheral blood lymphocytes from rabbits immunized with these two adjuvants showed no reaction against hsp65. We thus concluded that hsp may be one of the antigens inducing atherosclerosis, and we hypothesize that the target antigen in vivo is the hsp65 mammalian analogue. The presence of hsp70 has already been demonstrated in arteriosclerotic lesions.15 This could explain why arteriosclerosis in humans and our experimental system shows a predilection for the portions of the aortic and arterial intima that are subject to excessive hemodynamic mechanical stress, such as the aortic arch and the sites of vascular branching.

Finally, although the present experiments involved a large number of animals, antigens, adjuvants, and immunization procedures, some questions remain to be answered. These include 1) investigation of the possible nature of the autoimmune disease via immunization with mammalian hsp and the demonstration of autoreactivity of peripheral blood T cells or preferably, T cells derived from atherosclerotic lesions; 2) further characterization of the infiltrating cells, primarily the use of certain T-cell receptor α/β and γ/δ V genes with preferential specificity for hsp; 3) studies using different bacterial and mammalian hsp; 4) attempts to transfer the disease by T-cell lines or clones derived from atherosclerotic lesions; and 5) parallel investigations of human material, with special emphasis on a possible anti-hsp reactivity of lesion-derived T cells. All animal experiments should, however, be performed in a species wherein inbred lines and monoclonal antibodies against appropriate T-cell receptor V gene products are available, i.e., mice or rats. We are currently engaged in attempts to establish this rodent model.

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Induction of arteriosclerosis in normocholesterolemic rabbits by immunization with heat shock protein 65.

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