Immunopathogenesis of Atherosclerosis
Endotoxin Accelerates Atherosclerosis in Rabbits on Hypercholesterolemic Diet

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Background—On the basis of our concept that atherosclerosis has an immunopathological background, we tested whether activation of the innate immune system influences its progression.

Methods and Results—Hypercholesterolemic (0.5% wt/wt diet) rabbits received either repeated intravenous injections of endotoxin (Escherichia coli lipopolysaccharide 1.25 to 2.5 μg, once per week) or a self-limiting cutaneous Staphylococcus aureus infection with or without a quinolone antibiotic. Measured laboratory parameters, including LDL and HDL cholesterols, were similar in the different groups of hypercholesterolemic animals. All endotoxin-treated animals developed transient episodes of fever after endotoxin administration. The extent of atherosclerosis was evaluated by computer-assisted morphometry in the aortas en face (Sudan IV) and by histology at 8 weeks after start of the experiments. Endotoxin-treated animals exhibited significantly accelerated atherosclerosis compared with control animals (141±38 versus 45±16 mm³ total lesion volume, n = 7 to 9 rabbits each, P < 0.001).

Conclusions—Nonspecific stimulation of the innate immune system accelerates cholesterol-induced atherosclerosis. These data support the concept that atherosclerosis has an immunopathological component and render it improbable that a single infectious agent should assume particular importance in its initiation or progression. (Circulation. 2001;104:914-920.)

Key Words: imaging • immunology • atherosclerosis • endotoxin

Atherogenesis shares many features with inflammatory reactions (reviewed in Reference 1). Fatty streaks, the earliest lesions of atherosclerosis, are composed largely of lipid-laden macrophages derived from circulating blood monocytes that adhere to and emigrate across the endothelium of middle-sized and large arteries.1,2 One of the authors (S.B.) hypothesized that enzymatic degradation of LDL is centrally important in atherogenesis.3 Proteolytic degradation of apolipoprotein B in conjunction with cleavage of cholesteryl esters generates lipoprotein droplets that are similar to lipoprotein derivatives that were earlier isolated from atherosclerotic lesions.3-5 Enzymatically modified LDL (E-LDL) induces foam cell formation,4 binds C-reactive protein (CRP) to activate complement,6 and induces upregulation of adhesion molecules on endothelial cells to promote selective transmigration of monocytes and lymphocytes across cell monolayers.7 In contrast, oxidized LDL does not activate complement.8 Immunohistochemical evidence has been obtained that E-LDL is extensively distributed in early atherosclerotic lesions and in colocalization with CRP and complement C5b-9 complexes.9 Enzymatic transformation of LDL into a complement-activating molecule is thought to be physiologically meaningful because it initiates removal of stranded cholesterol from the vessel wall.3 Atherosclerosis is proposed to ensue when the physiological transport system suffers overload, because detrimental effects are evoked by the unhailed activation of complement and macrophages. This concept implies that atherosclerosis has an immunopathological component with central involvement of innate immune mechanisms. Inhibition of complement and/or macrophages might therefore counteract atherogenesis, whereas excessive coactivation of these components could accelerate disease progression. Evidence supporting the first expectation is available: complement C6 deficiency protects against diet-induced atherosclerosis in rabbits,10 and functional impairment of macrophages through abolishment or blockade of macrophage colony–stimulating factor is protective in mice.11 In
stained with Sudan red IV. Adventitial fat was removed, and aortas were performed in a blinded fashion. Aortas were dissected and formaldehyde. Macroscopic and microscopic analyses of the samples kidneys, and 1 lobe of the liver were removed and fixed in nary Inc). The entire aorta was removed 1 to 2 cm into the iliac experiments with T61, an anesthetic composed of embutramide, fibrinogen). Animals were euthanized on day 57 after the start of the creatinine; triglycerides; total, HDL, and LDL cholesterol; and 3 received standard maintenance diets (K-H4 pellets, Ssniff); groups 5 rabbits per group). *P vs group 4 (Wilcoxon) (n = 5 rabbits per group).

Methods

Animal Model

Forty-nine female New Zealand White rabbits (12 weeks old at the beginning of the experiments) were maintained under standardized conditions (21°C, 41% to 62% humidity) with regular day/night (10/14 hours) cycle and free access to water and laboratory diets. The animals were randomly assigned to 1 of 8 groups. Groups 1 through 3 received standard maintenance diets (K-H4 pellets, Ssniff); groups 4 through 8 received the same diet but supplemented with 0.5% (wt/wt) cholesterol (Table). In animals of groups 2, 5, and 6, subcutaneous infections with Staphylococcus aureus (strain Berlin) were established with a 2-cm cotton thread sutured into the shaved skin at the right hind leg once every week. The thread was previously soaked in 0.1 mL of saline containing 10^8 colony-forming units of S aureus/mL in normal saline. Rabbits of groups 6 and 7 received 5 mg/kg, (0.1 mL/kg) of the quinolone antibiotic enrofloxacin (Baytril, Bayer) during study days 2 through 6. Endotoxin was administered by a phenol-chloroform–petroleum ether method.

Lipid Profile and Hematological Parameters at the End of the Experiments

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>S aureus</th>
<th>Endotoxin</th>
<th>Control</th>
<th>S aureus</th>
<th>S aureus + Antibiotic</th>
<th>Antibiotic</th>
<th>Endotoxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal Diet</td>
<td></td>
<td></td>
<td>2</td>
<td>Normal Diet</td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Cholesterol, mmol/L</td>
<td>1.5 ± 0.52</td>
<td>1.46 ± 0.2</td>
<td>1.59 ± 0.34</td>
<td>19.27 ± 8.9</td>
<td>22.45 ± 8.8</td>
<td>27.81 ± 6.4</td>
<td>18.39 ± 5.9</td>
</tr>
<tr>
<td></td>
<td>LDL cholesterol, mmol/L</td>
<td>0.48 ± 0.44</td>
<td>0.51 ± 0.15</td>
<td>0.62 ± 0.53</td>
<td>18.06 ± 8.3</td>
<td>21.22 ± 8.6</td>
<td>26.52 ± 6.2</td>
<td>17.32 ± 5.4</td>
</tr>
<tr>
<td></td>
<td>HDL cholesterol, mmol/L</td>
<td>0.76 ± 0.26</td>
<td>0.65 ± 0.18</td>
<td>0.61 ± 0.33</td>
<td>0.47 ± 0.2</td>
<td>0.53 ± 0.25</td>
<td>0.67 ± 0.27</td>
<td>0.68 ± 0.32</td>
</tr>
<tr>
<td></td>
<td>Triglycerides, mmol/L</td>
<td>0.64 ± 0.21</td>
<td>0.66 ± 0.34</td>
<td>0.6 ± 0.29</td>
<td>1.65 ± 1.66</td>
<td>1.34 ± 0.74</td>
<td>1.38 ± 0.49</td>
<td>0.86 ± 0.47</td>
</tr>
<tr>
<td></td>
<td>Fibrinogen, g/L</td>
<td>3.36 ± 0.72</td>
<td>3.3 ± 0.67</td>
<td>3.24 ± 0.52</td>
<td>2.58 ± 0.29</td>
<td>2.7 ± 0.45</td>
<td>2.46 ± 0.34</td>
<td>3.25 ± 0.36</td>
</tr>
<tr>
<td></td>
<td>Leukocytes, 10^9/L</td>
<td>8.7 ± 1.76</td>
<td>8.0 ± 1.49</td>
<td>8.7 ± 1.64</td>
<td>10.3 ± 1.46</td>
<td>10.2 ± 0.15</td>
<td>12.2 ± 3.69</td>
<td>11.5 ± 1.39</td>
</tr>
<tr>
<td></td>
<td>Monocytes, %</td>
<td>5.9 ± 4.5</td>
<td>5.4 ± 1.3</td>
<td>7.5 ± 3.3</td>
<td>6.3 ± 4.2</td>
<td>5.9 ± 3.7</td>
<td>4.9 ± 2.2</td>
<td>7.1 ± 4.3</td>
</tr>
<tr>
<td></td>
<td>Neutrophils, %</td>
<td>32.5 ± 21.7</td>
<td>34.6 ± 17.4</td>
<td>40.0 ± 21.0</td>
<td>36.1 ± 15.9</td>
<td>36.6 ± 19.7</td>
<td>37.4 ± 19.8</td>
<td>45.1 ± 22.7</td>
</tr>
<tr>
<td></td>
<td>Lymphocytes, %</td>
<td>59.1 ± 21.6</td>
<td>58.4 ± 19.6</td>
<td>50.8 ± 23.2</td>
<td>55.3 ± 20.4</td>
<td>55.6 ± 23.9</td>
<td>55.9 ± 23.0</td>
<td>45.9 ± 25.8</td>
</tr>
<tr>
<td></td>
<td>Erythrocytes, 10^12/L</td>
<td>6.37 ± 0.3</td>
<td>5.98 ± 0.27</td>
<td>6.07 ± 0.23</td>
<td>3.98 ± 0.7</td>
<td>4.33 ± 0.36</td>
<td>4.45 ± 0.53</td>
<td>4.29 ± 0.86</td>
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<tr>
<td></td>
<td>Hematocrit, %</td>
<td>35.6 ± 2.0</td>
<td>34.1 ± 1.3</td>
<td>34.4 ± 0.9</td>
<td>24.1 ± 2.9</td>
<td>26.2 ± 1.1</td>
<td>26.8 ± 3.5</td>
<td>25.7 ± 3.1</td>
</tr>
</tbody>
</table>

*P < 0.05 vs group 4 (Wilcoxon) (n = 5 rabbits per group).

this study, we demonstrate that, conversely, nonspecific activation of the innate immune system markedly accelerates atherosclerosis in hypercholesterolemic rabbits.
Chromogen density and distribution in immunohistochemical stains were quantified by Photoshop-based image analysis as described. 15,16 Pixels with similar chromogen characteristics were selected with the Magic Wand tool and the Select Similar command, and gray levels/luminosities of the areas were calculated with the Histogram command in Photoshop.16 For MIF slides, which exhibited an even distribution of MIF immunoreactivity over the entire neointimal space (Figure 4, top), the immunostaining intensity was evaluated separately in the media and the neointima, and a mean luminosity value was calculated for the red channel of the color spectrum. For E-LDL slides, in which the chromogen distribution was patchy and localized in the vicinity of the lamina elastica interna (Figure 4, bottom), the chromogen distribution was assessed and calculated as percentage of neointimal space.

Statistical Analyses
Data were analyzed by the Wilcoxon test and by ANOVA analysis.

Results
All animals survived through the course of the experiment. All animals on hypercholesterolemic diet had comparable serum levels of total, LDL, and HDL cholesterol, ranging between 18.4 and 27.8 mmol/L, 17.3 and 26.5 mmol/L, and 0.47 and 0.73 mmol/L, respectively (Table). Endotoxin-treated animals developed fever on day 1 after each injection, body temperature rising from baseline temperatures between 38.4°C and 39.2°C (mean 38.6 ± 0.1°C) to values between 40.3°C and 41.1°C (mean 40.8 ± 0.02°C; *P* < 0.01 versus baseline values) at 6 hours after endotoxin injection. There was no difference between the animals of groups 3 and 8 and no difference in the development of fever over the course of the study. All animals reacted similarly, and no animal failed to develop fever after any of the endotoxin injections. Animals with the local *S aureus* infection did not develop fever. The cutaneous infections were self-limiting, resolving within 3 to 4 weeks in animals with no antibiotic treatment and within 1 week in quinolone-treated animals.

None of the animals on regular diet developed any macroscopic or histological evidence of atherosclerosis (Figure 3, groups 1 to 3). In contrast, all animals on the cholesterol-supplemented diet developed atherosclerosis, which was comparable in groups 4 through 7: similar values were observed for plaque surface area (Figure 3, top), average plaque thickness (Figure 3, middle), and overall plaque load (Figure 3, bottom). The distribution of atherosclerotic lesion area and total lesion load over the entire aorta was similar in all groups of hypercholesterolemic animals, with the majority of lesions (67% to 84%) present in the aortic arch. The infrarenal abdominal aorta contributed, 3% and the thoracic and suprarenal abdominal aortas contributed between 6% and 17%, respectively. Of particular interest was the observation that repeated local infection with *S aureus* (group 5) failed to elicit increased lesion formation (Figure 3). The fact that antibiotic treatment did not attenuate atherogenesis compared with the hypercholesterolemic control animals of group 4 underscores the specific pathogen-free environment in which the animals were kept during the experiment: previous studies...
have suggested that rabbits maintained under non–specific pathogen–free conditions can show increased atherogenesis because of infections with Pasteurella multocida and that this is reversed by antibiotic treatment.17 In contrast, weekly injections of the animals with endotoxin significantly accelerated atherosclerosis, as evidenced by increased aortic lesion area and lesion volume, but not lesion thickness (Figure 3). For lesion area and lesion volume, the difference reached statistical significance at a level of $P<0.01$ and $P<0.001$, respectively, versus the 9 animals of the hypercholesterolemic control group. Indeed, lesion volume was higher in each individual animal of the endotoxin group (group 8) than in any of the animals in the control group (group 4). This was also confirmed by ANOVA ($P<0.025$). Histological examination of hearts, kidneys, and livers showed no abnormalities other than striking fatty change in the livers of all cholesterol-fed rabbits. There was no difference between animals from the different groups, however, as assessed in a 3-tier score of fatty change severity (data not shown). Of all the hematological and clinical chemistry data assessed in a subset of 5 animals per group, only 1 parameter showed a significant difference: total monocyte counts were significantly increased in the animals of group 8 (Table). All animals showed comparable, steady weight gain over the course of the study (not shown). Also, no effect of endotoxin was observed on the level of serum triglycerides or on the level of serum and LDL cholesterol.

The image analysis of the immunohistochemical studies using E-LDL and MIF antibodies showed no significant differences between the treatment groups. For each individual tissue section, MIF immunoreactivity was stronger in the neointima than in the media (Figure 4, top), but the immunostaining intensity for neointima and media was comparable in groups 4 through 8 (data not shown). Likewise, we saw no significant differences in E-LDL–associated chromogen distribution in the neointimas of animals from groups 4 through 8 (data not shown).

**Discussion**

The present experiments were conducted to test a hypothesis that followed from a new concept about the pathogenesis of atherosclerosis.3 We propose that each LDL molecule con-
saccharide injections induced partial tolerance: the dose of endotoxin and the time interval between injections therefore had to be empirically sought to define experimental conditions under which the animals transiently developed fever without becoming critically ill. Only animals that were challenged with lipopolysaccharide developed transient fever episodes and increases in monocyte counts. Remarkably, this correlated with significant increases in the extent of atherosclerotic lesion development, in terms of both lesion area and lesion volume (Figure 3).

Our finding is in direct contrast to earlier studies, in which no proatherogenic effect of endotoxin infusion was observed in cholesterol-fed piglets and rabbits. Those studies differed fundamentally in that the efficacy of endotoxin infusion was not controlled: in the rabbit study, endotoxin doses of only 0.1 μg/kg body wt were applied, which in our pilot experiments failed to induce fever. Body temperature was also not controlled in the study on piglets, which are rather insensitive to endotoxin. Furthermore, the endotoxin doses were maintained in both studies, with induction of endotoxin tolerance not controlled for.

Atherosclerosis never developed in control animals that were kept on a normal diet. This unsurprising finding was in line with the abundant literature that underscores the essential role of hypercholesterolemia in disease induction. Antibiotic treatment alone had no effect on lesion development, which emphasizes the efficacy of our pathogen-free environment. Animals with S aureus infections did have more extensive lesions than controls, although statistical significance was not attained. The S aureus lesions resolved rapidly in quinolone-treated animals, and the extent of atherosclerosis that developed in this group was identical to that in controls.

The Photoshop software used here contains sophisticated automated tools for the selection of pixels with defined color characteristics. These selection tools are the basis for so-called map commands. We quantified Sudan-stained areas (Figure 1), mean thickness of atherosclerotic lesions (Figure 2), and chromogen densities of the immunohistochemical slides (Figure 4). 3D measures of atherosclerotic lesion load in the entire aortas were thus obtained.

Multiple effects of endotoxin may converge to accelerate atherogenesis. Endotoxin-stimulated monocytes attracted into the atherosclerotic lesions will probably be more proinflammatory and thus exacerbate lesion development. Induction of the acute-phase response with elevation and increased trapping of CRP should enhance complement activation in the lesions. Endotoxin induces procoagulant activity, elicits release of tissue factor and endothelin from endothelial cells, and damages endothelial cells. Endotoxin may foster the adhesion of circulating leukocytes to the vessel wall. Via induction of nitric oxide synthase, endotoxin could provoke excessive generation of nitric oxide and its deleterious metabolite peroxynitrite. Implantation of an endotoxin-soaked thread induces intimal infiltration of monocytes, neutrophils, and smooth muscle cells. Endotoxin can reportedly induce macrophage foam cell formation in vitro. Studies in rats suggest that endotoxemia might contribute to monocyte/macrophage recruitment into tissues by stimulating the expression of innate immune mechanisms, coactivating the innate immune system would accelerate atherosclerosis, and this is attenuated by monocytes, neutrophils, and smooth muscle cells. 29
pathogenesis of atherosclerosis should best be avoidable through reduction of the E-LDL load in tissues. The success of all clinical lipid-lowering trials as opposed to the failure of antioxidant trials is completely in line with this concept.

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References


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