Atherosclerosis is a chronic inflammatory disease of the arterial wall characterized by progressive accumulation of lipids, cells (macrophages, T lymphocytes, and smooth muscle cells), and extracellular matrix.1 The inflammatory process is involved throughout the different stages of atherosclerosis.1 Inflammation also plays a major role in atherosclerotic plaque disruption and thrombosis2–5 and therefore greatly influences the occurrence of acute coronary syndromes and their related mortality.6

During the inflammatory reaction, anti-inflammatory cytokines are also produced and tend to modulate the inflammatory process. Whereas a large body of evidence exists to support a role for proinflammatory cytokines in atherosclerosis,1 little information is available regarding the potential role of anti-inflammatory cytokines in this setting. Interleukin (IL)-10, secreted by lymphocytes of the Th2 subtype, and also in large amounts by macrophages, is an anti-inflammatory cytokine with potent deactivating properties on both macrophages and T cells.7,8 IL-10 is expressed in early inflammatory cytokine with potent deactivating properties in macrophages and T cells and modulates many cellular processes that may interfere with the development and stability of the atherosclerotic plaque. IL-10 is expressed in human atherosclerosis and is associated with decreased signs of inflammation. In the present study, we show that IL-10–deficient C57BL/6J mice fed an atherogenic diet and raised under specific pathogen-free conditions exhibit a significant 3-fold increase in lipid accumulation compared with wild-type mice. Interestingly, the susceptibility of IL-10–deficient mice to atherosclerosis was exceedingly high (30-fold increase) when the mice were housed under conventional conditions. Atherosclerotic lesions of IL-10–deficient mice showed increased T-cell infiltration, abundant interferon-γ expression, and decreased collagen content. In vivo, transfer of murine IL-10 achieved 60% reduction in lesion size. These results underscore the critical roles of IL-10 in both atherosclerotic lesion formation and stability. Moreover, IL-10 appears to be crucial as a protective factor against the effect of environmental pathogens on atherosclerosis. The full text of this article is available at http://www.circresaha.org. (Circ Res. 1999;85:e17-e24.)

Key Words: interleukin-10 ■ atherosclerosis ■ mice ■ macrophage ■ lymphocyte ■ collagen

Materials and Methods

Mice

Female C57BL/6J IL-10+/− mice and IL-10−/− mice were obtained from Jackson Laboratory (Bar Harbor, Maine) at 7 to 8 weeks of age.
and placed on an atherogenic diet for 16 weeks. The atherogenic diet was obtained by the addition of 15% cacao butter, 1.25% cholesterol, and 0.5% sodium cholate to the standard chow diet, which contained 3% fat (UAR). Mice were kept in accordance with standard animal care requirements, housed 4 to 5 per cage, and maintained on a 12-hour light-dark cycle. Water and food were given ad libitum. The mice analyzed were housed under either SPF or CONV conditions. Morphometric Analysis

After 8 or 16 weeks on the atherogenic diet, mice were killed by ether overdose, and the basal half of the ventricles and the ascending aorta were removed, embedded in OCT compound (Tissue-Tek), frozen in isopentane, and stored at −70°C until processing for analysis of lipid accumulation in the aortic sinus. Serial 10-μm sections of the aortic sinus with valves (60 to 80 per mouse) were cut on a cryostat. Of every three sections, one was kept for immunohistological analysis and collagen detection. The others were stained with Sudan IV to detect lipid deposition. The sampling method for calculation of the mean lesion area per section per animal has been previously described in detail.19

Collagen fibers were stained with Sirius red. Morphometric analysis was performed with an automated image processor (NS 15000, Microvision) as previously described.20 The lesion collagen content was determined by measuring the relative area/density in 12 contiguous fields in each Sirius red-stained section. Protein and Lipoprotein Analysis

Cholesterol was measured with a commercially available kit (Boehringer-Mannheim). Cholesterol in plasma lipoproteins was assayed after analytical gel filtration chromatography, with a Superose 6 HR
 IL-10 expression plasmid, pCor-IL-10 and six with the control empty plasmid. Murine IL-10 cDNA was cloned into pCor backbone\textsuperscript{21} under the control of the cytomegalovirus promoter (nucleotides –522/+72) and upstream of the simian virus 40 late polYA signal to generate pXL3458. Control plasmid was a similar construct devoid of therapeutic cDNA. The IL-10 or control expression plasmid (15 μg) was injected into both tibial cranial muscles of the anesthetized mouse as previously described.\textsuperscript{22} Briefly, transcutanous electric pulses (8 square-wave electric pulses of 200 V/cm, 20 ms each, at 2 Hz) were delivered by a PS-15 electropulsator (Jouan) using two stainless steel plate electrodes placed 4.2 to 5.3 mm apart, at each side of the leg. Mice were placed on the atherogenic diet for 8 weeks and were housed under CONV conditions. In a pilot study in C57BL/6 mice, we determined the time course of IL-10 plasma levels after intramuscular administration of the IL-10 plasmid, using an immunoassay kit for the quantitative detection of murine IL-10 (Cytoscreen, BioSource International). Given that circulating levels of IL-10 were found detectable up to day 21, in vivo transfections in IL-10/– mice were repeated at day 30 to ensure long-term IL-10 production.

Statistical Analysis
The effects of genotype and environmental conditions on lesion area and lipoprotein and apolipoprotein data were determined by 2-way ANOVA. Multiple comparisons were performed using Bonferroni’s method. Simple regression analysis was performed to analyze the relation between lesion area and HDL cholesterol in IL-10/– mice. Morphometric data were compared using a t test. Data are expressed as mean±SEM. A value of P<0.05 was considered to be statistically significant.

Results
After 16 weeks on the atherogenic diet, animal weights were not different between the various groups (Table 1). After this period, total plasma cholesterol did not differ between IL-10/– and IL-10+/+ mice whether they were housed under SPF or CONV conditions (Table 1). However, IL-10+/+ mice raised under SPF conditions showed higher total cholesterol levels than those raised under CONV conditions (P<0.01; Table 1). HDL cholesterol levels were significantly lower in IL-10/– than in IL-10+/+ mice, regardless of the environmental condition (P<0.0001), but were not significantly different in IL-10/– mice housed under SPF or CONV conditions (Table 1).

Atheromatous Lesions in IL-10/– Mice
After 16 weeks on the atherogenic diet, IL-10/– SPF mice showed a significant 3-fold increase in atherosclerotic lesion

<p>| TABLE 1. Weights, Plasma Cholesterol, and ApoA-I and ApoA-II Levels in IL-10+/+ and IL-10/– Mice Raised Under SPF and CONV Conditions |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>IL-10+/+</th>
<th>SPF (n=9)</th>
<th>CONV (n=8)</th>
<th>IL-10/–</th>
<th>SPF (n=8)</th>
<th>CONV (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td>22.1±0.9</td>
<td>18.2±0.5</td>
<td>22.8±0.6</td>
<td>18.3±1.3</td>
<td></td>
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</tr>
<tr>
<td>Total cholesterol</td>
<td>429.9±15.8</td>
<td>267.9±9.5*</td>
<td>418.2±91.4</td>
<td>299.5±58.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>58.9±4.4</td>
<td>56.1±4.4</td>
<td>27.2±2.3†</td>
<td>22.7±1.9†</td>
<td></td>
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</tr>
<tr>
<td>ApoA-I</td>
<td>63.5±2.6</td>
<td>78.3±6.2</td>
<td>36.7±2.6</td>
<td>35.4±2.7</td>
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<td></td>
</tr>
<tr>
<td>ApoA-II</td>
<td>18.2±1.7</td>
<td>22.0±1.9</td>
<td>11.7±1.1</td>
<td>8.2±1.1</td>
<td></td>
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</tbody>
</table>

Female IL-10+/+ and IL-10/– mice were fed a high-fat diet for 16 weeks. Weight values are g; other values are mg/dL (mean±SEM).

* Different from SPF, P<0.001.
† Different from +/+, P<0.001.
Figure 3. Representative photomicrographs showing immunohistochemical staining for IFN-γ in atherosclerotic lesions of IL-10+/+ (A) and IL-10−/− (B and D) mice fed an atherogenic diet for 16 weeks. No IFN-γ expression was found in the lesions of wild-type mice (A). However, substantial levels of IFN-γ expression were detected in early (B) (brown staining) and advanced lesions (D) of IL-10−/− mice housed under SPF (B) or CONV (D) conditions. Panels C and E represent control staining (using irrelevant IgGs) of lesions shown in panels B and D, respectively. Original magnification ×360 (A), ×400 (B through E).
area in the aortic sinus compared with IL-10+/+ SPF mice (P<0.001; Table 2, Figures 1A and 1B). This finding underscores the natural protective role of IL-10 against diet-induced atherosclerosis.

Interestingly, the susceptibility of IL-10−/− mice to atherosclerosis was exceedingly high in the mice raised under CONV conditions (30-fold increase in lesion size compared with IL-10 (P<0.0001; Table 2, Figures 1C and 1D). Lesion development in IL-10−/− mice did not increase in the mice housed under CONV conditions and was even lower than that observed under SPF conditions, which was likely due to lower levels of plasma cholesterol (Tables 1 and 2). However, lesion area was 4.5-fold higher in IL-10−/− CONV than in IL-10−/− SPF mice (P<0.0001; Table 2, Figures 1B and 1D).

Two-way ANOVA confirmed that IL-10 and environmental conditions (SPF or CONV) significantly affected the size of the arterial lesions (P<0.0001 for IL-10 and P<0.0001 for environment), with a significant interaction between the two factors (P<0.0001). Although HDL cholesterol levels were lower in IL-10−/− than in IL-10+/+ mice (Table 1), there was no correlation between HDL levels and the size of the lesions in IL-10−/− mice. Moreover, IL-10−/− mice housed under CONV conditions had much larger lesions than those raised under SPF conditions with comparable HDL levels.

**Plaque Composition in IL-10−/− Mice**

Arterial lesions of IL-10−/− mice evolved into advanced atheromatous lesions and frequently contained a central acellular lipid core. Immunohistochemical analysis showed that the surface area occupied by macrophages (MOMA-2 staining) in the aortic sinus was proportional to the size of the atherosclerotic lesion and was significantly higher in IL-10−/− than in IL-10+/+ mice (190364±14466 versus 6038±707 μm², respectively, P<0.0001). However, the percentage of lesion cross-sectional area occupied by macrophages was not different between the two groups (58.6±4.5% versus 56.3±6.6% of lesion cross-sectional area in IL-10−/− and IL-10+/+ mice, respectively; Figure 2). Interestingly, the number of CD3+positive lymphocytes per lesion cross-sectional area was 2.5-fold higher in IL-10−/− than in IL-10+/+ mice (313.2±50.8 versus 126.3±41.2 T cells/mm², respectively, P<0.01; Figure 2). High levels of IFN-γ expression were found in lesions of IL-10−/− mice (17.2±3.2% of lesion area), whereas IFN-γ expression was barely detectable in IL-10+/+ mice (0.19±0.06% of lesion area, P=0.0004; Figure 3). However, these mice expressed detectable levels of IL-10 (9.7±2.6% of lesion area) compared with IL-10+/− mice in which IL-10 was undetectable (P=0.0045; Figure 4). Enhanced expression of iNOS was also detected in lesions from IL-10−/− mice (data not shown). These findings indicate that atheromatous lesions of IL-10−/− mice are characterized by an increased infiltration of activated T cells with a Th1 cytokine profile accompanied by an exaggerated proinflammatory response.

IL-10 and IFN-γ may regulate differently various enzymes and proteins implicated in extracellular matrix remodeling,13,15,23,24 which may have an important impact on plaque

### Table 2. Aortic Lesion Area in IL-10+/+ and IL-10−/− Mice Raised Under SPF and CONV Conditions

<table>
<thead>
<tr>
<th></th>
<th>SPF</th>
<th>CONV</th>
</tr>
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<tbody>
<tr>
<td>IL-10+/+</td>
<td>24 504±4852</td>
<td>10 720±3373†</td>
</tr>
<tr>
<td>IL-10−/−</td>
<td>72 163±10 172*</td>
<td>325 003±37 627§</td>
</tr>
</tbody>
</table>

Female IL-10+/+ and IL-10−/− mice were fed a high-fat diet for 16 weeks. Values are μm²/section (mean±SEM).

*Different from +/+, P<0.001.
†Different from +/+, P<0.0001.
§Different from SPF, P<0.05.
§Different from SPF, P<0.0001.

[Figure 4. Representative photomicrographs showing immunohistochemical staining for IL-10 in atherosclerotic lesions of IL-10+/+ (A) and IL-10−/− (C) mice fed an atherogenic diet for 16 weeks. IL-10 expression was detectable in some lesions from wild-type mice (A) (brown staining). IL-10−/− mice showed no IL-10 expression (C). Panel B represents control staining (using irrelevant IgGs) of the lesion shown in panel A. m, l, and L denote media, intima, and lumen, respectively. Original magnification ×600 (A and B), ×500 (C).]

collagen content and stability. Therefore, we determined the collagen content in atheromatous lesions of IL-10<sup>+/+</sup> and IL-10<sup>−/−</sup> mice. Because small early lesions of IL-10<sup>+/+</sup> mice consist of pure macrophage accumulation, only relatively large lesions were examined for the presence of collagen by staining with Sirius red. Despite the very large size of atheromatous lesions in IL-10<sup>−/−</sup> mice, there was a very low collagen accumulation in the lesions (Figure 5). In contrast, substantial collagen accumulation could be detected in relatively large lesions from IL-10<sup>+/+</sup> mice (Figure 5). This was confirmed by a quantitative analysis of collagen content showing a marked decrease in collagen content in IL-10<sup>−/−</sup> lesions (4.37±1.06% versus 19.79±5.02% in IL-10<sup>−/−</sup> mice, n=8, and IL-10<sup>+/+</sup> mice, n=4, respectively, P<0.002; Figure 5). Absence of IL-10 appears to favor the development of large atheromatous plaques characterized by an exaggerated proinflammatory response and a reduced collagen content, which may greatly increase the plaque susceptibility to rupture.

**In Vivo Injection of Murine IL-10 Expression Plasmid DNA in IL-10<sup>−/−</sup> Mice**

IL-10–encoding (or control) plasmid was transferred to muscle cells using a highly efficient electrottransfer procedure recently developed. In a pilot study in C57BL/6 mice (n=4), we found that peripheral circulating levels of IL-10 peaked at day 4 (1101±247 pg/mL) and remained detectable up to day 21 (68±24 pg/mL), and were 458±116 pg/mL and 102±30 pg/mL at day 7 and day 14, respectively. Therefore, to ensure long-term IL-10 production, the IL-10 or control expression plasmid (15 μg) was electrotferred into both tibial cranial muscles of mice at day 0 and day 30 on the atherogenic diet. This led to high plasma values of circulating IL-10 (1146.94±131.38 pg/mL) 4 days after the first electotransfer, similar to those found in the pilot study in C57BL/6 mice. After 8 weeks on the atherogenic diet, substantial fatty lesions were observed in the aortic sinus of IL-10<sup>−/−</sup> mice injected with the control expression plasmid (pCor) and housed under CONV conditions (47 963±10 899 mm<sup>2</sup>). In vivo intramuscular injection with the pCor-IL-10 expression plasmid resulted in a marked reduction in fatty lesion development (18 069±1565 mm<sup>2</sup>, P<0.01).

**Discussion**

Signs of chronic inflammation are present throughout the different stages of atherosclerosis, and various pathophysiological mechanisms have been identified that may influence the development and progression of the lesions. However, although a large body of evidence is accumulating on the destructive role of proinflammatory cytokines in atherosclerosis, little is known regarding the role of the anti-inflammatory component of the reaction. This information

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**Figure 5.** A and B, Representative photomicrographs showing Sirius red staining (to detect collagen fibers) in atherosclerotic lesions of IL-10<sup>+/+</sup> (A) and IL-10<sup>−/−</sup> (B) mice fed an atherogenic diet for 16 weeks. Collagen content (red staining) in the lesions was very low in IL-10<sup>−/−</sup> mice (B) (faint red staining within the lesion near the lumen) compared with that in IL-10<sup>+/+</sup> mice (A) (red staining at the base of the lesion). m, i, and L denote media, intima, and lumen, respectively. Arrowheads in panel A delimit the internal elastic lamina. Original magnification ×400. The decrease in collagen content in IL-10<sup>−/−</sup> lesions was confirmed by quantitative analysis of collagen density (C) in lesions from 4 IL-10<sup>+/+</sup> and 8 IL-10<sup>−/−</sup> mice (**P<0.002).
may be helpful because it may improve our understanding of the pathophysiology of atherosclerosis and may open the way for the identification of novel therapeutic strategies to combat this common and fatal disease.

Among the anti-inflammatory cytokines, we considered IL-10 as a cytokine with potentially potent antiatherosclerotic effects. IL-10 is produced by various inflammatory cells, especially macrophages, and could therefore be produced locally within the atherosclerotic lesion. Because IL-10 has deactivating effects on macrophages and T cells, it could play a significant role in the modulation of the local inflammatory reaction. Also, previous studies have shown that IL-10 modulates several cellular pathways that may play an important role in the development and progression of atherosclerosis, including NF-kB activation, tissue factor, and cyclooxygenase expression, metalloproteinase production, and cell death. Moreover, IL-10 is produced within advanced human atherosclerotic plaques, and we have shown recently that its expression in the plaque is associated with lower expression of iNOS and decreased cell death. Taken together, these data prompted us to examine the in vivo role of IL-10 in atherosclerosis by using IL-10–deficient mice generated on the genetic background of the inbred strain C57BL/6, a strain susceptible to the development of atherosclerosis when maintained on an atherogenic diet. Our present study demonstrates a protective role of IL-10 against atherosclerosis because the absence of this anti-inflammatory cytokine in mice raised under SPF conditions led to a marked increase in the susceptibility to diet-induced atherosclerosis.

In conditions of real life, however, animals and humans are exposed to a variety of environmental factors, including common pathogens and pathogen-derived products. Such conditions may affect the individual inflammatory responses. Previous studies have shown that the intestinal inflammation that characterizes IL-10−/− mice is exaggerated in the mice housed under CONV conditions, although no active intestinal infection can be detected. Interestingly, recent studies in humans have suggested a link between prior exposure to pathogens and subsequent development of atherosclerosis. We therefore investigated atherosclerosis development in IL-10−/− mice fed an atherogenic diet and raised under CONV conditions. Diet-induced atherosclerosis was markedly increased (4.5-fold increase) in these mice compared with that observed in IL-10+/− mice raised under SPF conditions. This marked increase in atherosclerosis occurred in the absence of active infection with known infective pathogens (data not shown) and could not be accounted for by changes in lipoprotein profiles, because total cholesterol and HDL cholesterol levels were not different in IL-10−/− mice raised under SPF or CONV conditions. HDL cholesterol levels were lower in IL-10−/− than in IL-10+/− mice, which points out to the potential role of the balance between proinflammatory and anti-inflammatory cytokines in HDL metabolism. This may have contributed in part to the higher susceptibility to atherosclerosis in IL-10−/− mice raised under SPF conditions. Previous studies have reported an association between inflammation and decreased HDL cholesterol levels, but the mechanisms are not well understood, and further studies are required to elucidate this issue.

Interestingly, fatty lesion development did not increase in IL-10−/− mice housed under CONV conditions. It was even higher under SPF conditions. This unexpected result is probably due to the higher cholesterol levels achieved, for unknown reasons, under SPF conditions. Our results indicate that the interaction between various environmental factors and the arterial wall becomes critical in the atherosclerotic process only when IL-10 is absent. We therefore propose that individual variations in the quality and extent of the inflammatory response to environmental factors (particularly variations related to IL-10 production) may greatly influence the atherosclerotic response of the arterial wall to an atherogenic diet. This hypothesis may be relevant to the situation in humans in whom individual variations in IL-10 production have been documented and may in part be under genetic control.

Clinical studies in humans have shown that the clinical prognosis of a patient with atherosclerosis depends only in part on the size of the lesions, and it is now widely accepted that the quality (plaque composition), rather than the size, of the lesion could be a better indicator of the development of ischemic events. Indeed, severe clinical manifestations of atherosclerosis (infarctions of the heart and the brain) are mainly due to vessel lumen occlusion by a thrombus formed on contact with a disrupted atherosclerotic plaque. Pathological studies have shown that vulnerable or unstable plaques (i.e., plaques prone to rupture or having ruptured) greatly differ in cell and matrix composition compared with stable plaques, not prone to rupture. The vulnerable plaques are rich in inflammatory cells (as is the case of the IL-10−/− mice lesions shown in the present study), contain a thrombogenic lipid core, and are characterized by a thin fibrous cap with a substantial loss in extracellular matrix. Apoptotic cell death contributes to the formation of the atherosclerotic lesion is considered to be a good indicator of its stability. In the present study, we examined the matrix and cell composition of the arterial lesions in IL-10−/− and IL-10+/− mice. Lesions of IL-10−/− mice showed increased infiltration of inflammatory cells, increased production of IFN-γ, and interestingly, a very low percentage of collagen in comparison with lesions of IL-10+/− mice. These findings indicate that the absence of IL-10 favors the development of atherosclerotic lesions with major signs of increased vulnerability.

Finally, we assessed the effects of in vivo transfer of murine IL-10 cDNA on fatty lesion development in IL-10−/− mice. Two in vivo intramuscular electrotransfers of pCorIL-10 plasmid DNA at a 4-week interval markedly increased peripheral circulating levels of IL-10. We were sufficient to induce a substantial 60% reduction in fatty lesions in the mice housed under CONV conditions and fed an atherogenic diet for 8 weeks. These findings strongly support the hypothesis of a protective role of IL-10 in atherosclerosis. It is likely that increasing the peripheral circulating levels of IL-10 permitted...
an interaction between IL-10 and the endothelium, which may have decreased its state of activation. Moreover, the high circulating levels of IL-10 may have permitted IL-10 to enter the arterial wall, which may have compensated for the lack of local production of IL-10.

In conclusion, our results demonstrate that the lack of the anti-inflammatory cytokine IL-10 has a profound impact on both the development and the composition of the atherosclerotic lesion and point to a major role for this cytokine in the interaction between common environmental factors and atherosclerosis. Our findings were obtained in a mouse model of atherosclerosis in which C57BL/6 mice were fed cacao butter, cholesterol, and cholate. Wild-type mice fed this diet typically develop fatty streaks but do not develop fibrous plaques. In contrast, apoE null mice and LDL receptor null mice develop not only fatty streaks but also fibrous plaques that are more typical of human atherosclerosis. Although our IL-10 null mice do not develop fatty streaks but do not develop fibrous plaques, our data still suggest that IL-10 may play a role in atherogenesis in humans. Because exogenous IL-10 reduced the atherosclerosis in our model, therapeutic strategies increasing IL-10 production may reduce the extent or severity of atherosclerosis.

Acknowledgments

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