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ApoE$^{-/-}$ Mice Develop Atherosclerosis in the Absence of Complement Component C5

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Previous studies have suggested that the terminal complex of complement may contribute to the pathogenesis of atherosclerosis. C5b-9 complexes colocalize with the extracellular lipid in the aortic intima of hypercholesterolemic rabbits, and C6-deficient rabbits develop less atherosclerosis than controls. To test the role of complement in atherosclerosis in a different animal model, C5 deficient (C5def) mice were crossbred with atherosclerosis susceptible apoE$^{-/-}$ mice, generating mice deficient in both apoE and C5 and control apoE$^{-/-}$ mice. Progeny were typed for C5 titer and serum cholesterol levels. Both male and female mice were fed a high fat diet from weaning until 22 weeks of age. At that time there were no significant differences in plasma cholesterol or triglycerides between apoE$^{-/-}$ control and apoE$^{-/-}$/C5def groups. Morphometric analysis of the aortic root lesions gave mean (±SEM) lesion areas for male apoE$^{-/-}$ and apoE$^{-/-}$/C5def mice of 468,176 ± 21,982 and 375,182 ± 53,089 μm^2, respectively ($n = 10$ each, P value = 0.123). In female apoE$^{-/-}$ mice ($n = 5$), the mean lesion area was 591,981 ± 53,242 μm^2, compared to 618,578 ± 83,457 μm^2 for female apoE$^{-/-}$/C5def mice ($n = 10$) (P value = 0.835). Thus neither male nor female mice showed a significant change in lesion area when C5 was not present. In contrast to the case in the hypercholesterolemic rabbit, activation of the terminal complex of complement does not play a major role in the development of atherosclerosis in apoE$^{-/-}$ mice. © 2001 Academic Press

Key Words: **atherosclerosis; C5 deficiency; apoE$^{-/-}$ mice.**

Atherosclerosis is a complex disease with a strong inflammatory component (1). The subendothelial deposition of modified lipoproteins is followed closely by the appearance of monocyte-derived macrophages and T-lymphocytes (2–6). The interaction of these cells with each other and factors in their environment initiates and perpetuates an inflammatory response. Continued inflammation leads to the release of hydrolytic enzymes, cytokines, chemokines, and growth factors (7, 8), that can induce tissue damage and cause cellular necrosis (9).

An additional contributor to many inflammatory processes is the complement system, serum proteins primarily involved in host defense. Generation of C5a, through either the classical or alternative pathways, provides a potent chemoattractant for leukocytes. In addition, C5b combines with other complement proteins to form membrane attack or terminal complex (C5b-9) that can insert into the membranes of host cells, as well as those of bacteria, causing lysis. In healthy organisms a wealth of protective proteins on the surface of cells prevents accidental deposition of terminal complexes, but in pathological states this protective mechanism can fail.

Several lines of evidence suggest that complement may contribute to the inflammatory milieu that constitutes an atherosclerotic lesion. Terminal complexes colocalize with the subendothelial, extracellular lipid in the aortic intima of hypercholesterolemic rabbits (10). In addition, terminal complexes have been immunolocalized in human atherosclerotic plaques (11). The presence of complement terminal complexes in atherosclerotic tissue suggests that complement activation occurs at those sites and may contribute to tissue injury and the perpetuation of an inflammatory response. Complement may become activated at the site of atherosclerotic lesions by modified lipoproteins present in the forming plaques. *In vitro* studies show that lipid extracted from human atherosclerotic plaques is able to activate the complement cascade and that the resulting material is avidly phagocytosed by macrophages (12). Thus, chronic activation of the al-

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ternative pathway may lead to the progression of atherosclerotic lesions by promoting foam cell formation.

Genetic deficiencies in most of the individual complement proteins have been reported both in humans (13) and animals (14). In keeping with the important role of complement in host defense, most complement deficiencies render the individual susceptible to infections. The role of complement in the development of atherosclerosis has been examined previously in studies using C6-deficient rabbits. Animals that were C6-deficient showed reduced atherosclerosis compared to control animals, when fed a high cholesterol diet (15, 16). These studies, however, used relatively few animals and a model of atherosclerosis that is dependent on diet for the induction of lesions.

To study the role of the alternative pathway in atherosclerosis, we used apoE$^{-/-}$ mice, which are genetically prone to develop atherosclerosis (17), and crossed them with a naturally occurring C5-deficient strain, B10.D2-H2 (18). Mice deficient in both apoE and C5 developed atherosclerotic lesions to the same extent as did apoE$^{-/-}$ controls, on a high fat diet. In contrast to the studies using hypercholesterolemic rabbits, this study suggests that activation of the terminal complex of complement does not play a major role in the development of atherosclerosis in a mouse model of the disease.

METHODS

Animals. ApoE$^{-/-}$ mice that had been backcrossed onto a C57BL/6J background for at least six generations and C5-deficient mice (C5def), strain B10.D2-H2 (18), which has a C57BL/10SnJ background, were bought from Jackson Laboratories (Bar Harbor, ME). All animal study protocols were approved by the Institutional Animal Care and Use Committee at Merck Research Laboratories (Rahway, NJ). All animals were cared for in accordance with the "Guide for the Care and Use of Laboratory Animals" (revised 1996, National Academy Press, Washington, DC).

ApoE$^{-/-}$ mice were crossed with C5def mice to produce F1 progeny heterozygous for apoE (apoE$^{+/-}$) and with half the normal titer of C5. The F1 progeny were interbred, and the resultant F2 progeny were tested for serum cholesterol levels and hemolytic activity. For typing, mice were bled retro-orbitally at four weeks of age, and serum was prepared in Microtainer Serum Separator tubes (Becton Dickinson, Franklin Lakes, NJ). Serum cholesterol was measured enzymatically using a standard kit (Sigma Chemical Co., St. Louis, MO). C5 titer was determined using the hemolysis assay described below. Animals that had serum cholesterol of >10.3 mmol/L (400 mgs/dL) were judged to be apoE$^{-/-}$, and animals with no C5 titer were considered C5def. Animals with both these characteristics were designated apoE$^{-/-}$/C5def. Littermates with serum cholesterol levels of >10.3 mmol/L (>400 mg/dL) and a C5 titer of 200–300 C5H50 units/ml, representing half the normal activity, were also selected and designated as heterozygotes. Heterozygotes were interbred to produce apoE$^{-/-}$ controls with full C5 titer. The mice selected as apoE$^{-/-}$/C5def from the F2 progeny were interbred to produce the experimental mice used in the study.

Hemolysis assay. The C5 titer in sera was measured in a standard hemolysis assay (Sigma, Product C1163). To avoid inactivation of complement, the sera, once made, were immediately snap frozen in liquid nitrogen and stored at −80°C until use. A non-agglutinating dose of rabbit anti-sheep IgG was used to sensitize 10^9 sheep erythrocytes (SRBC) (BioWhittaker, Walkersville, MD) at 37°C for 30 min in veronal buffer (1.82 mmol/L sodium barbital, 3.12 mmol/L barbituric acid, 144 mmol/L NaCl, 0.15 mmol/L $CaCl_2$, 0.5 mmol/L $MgCl_2$, 0.05% Human Serum, pH 7.4) plus 28 mmol/L glucose. The hemolysis assay mixture contained 5 μl of test serum, 2.5×10^7 antibody-sensitized SRBC, 20 μl C5 deficient human serum (Sigma, St. Louis, MO), and veronal buffer in a final volume of 0.5 ml. The assay mixture was incubated at 37°C for 30 min, the SRBC were pelleted at 1000*g* for 10 min, and the amount of cell lysis was determined by measuring the absorbance of the supernatant at 415 nm. 100% lysis of SRBC is A_{415} obtained from 0.5 ml containing only SRBC and distilled water. Percent lysis 'Y' was calculated by dividing the absorbance value of the test serum by that obtained for 100% lysis. Y/(100 − Y) was plotted on a log scale against the respective dilutions of serum. The complement titer was defined as the reciprocal of the serum dilution that yielded 50% lysis, that is when Y/(100 − Y) = 1 (one C5H50 unit). Sera from apoE$^{-/-}$, C5 deficient B10.D2.H2, and normal C57BL/6J mice were used as standards for C5 activity.

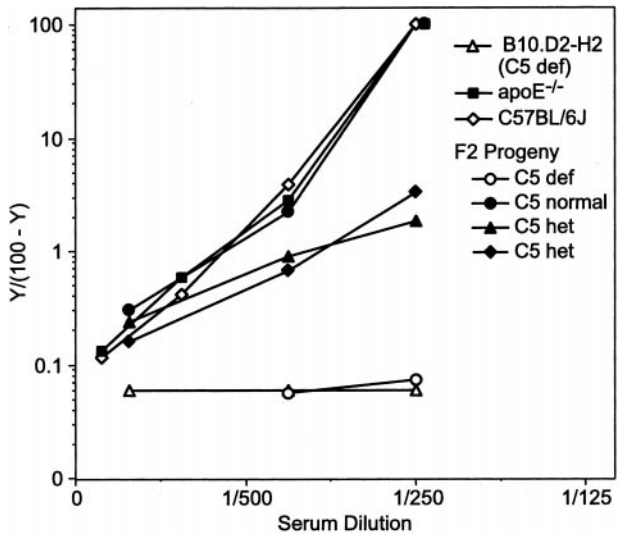

FIG. 1. Complement titers from representative F2 progeny. Interbreeding apoE$^{+/-}$/C5 heterozygous mice generated F2 progeny. Complement titers were determined using the hemolysis assay as described under Methods.

Sera from mice with elevated cholesterol ≥10.3 mmol/L (≥400 mg/dL) were tested for C5 titer at the time of weaning. Results from representative progeny are shown in Fig. 1. F2 progeny that were deficient in C5 had no C5 titer as was the case for sera from C5 deficient B10.D2-H2 mice (Fig. 1, e.g., C5def). Mice that were heterozygous for C5 had an intermediate C5 titer of 200–300 C5H50 units/ml (Fig. 1, C5het).

Study design. Mice were weaned at 4 weeks of age and placed on a high fat, Western-type diet containing 0.15% cholesterol (TD 88137, Harlan Teklad, Madison, WI). Ten male apoE$^{-/-}$/C5def and apoE$^{-/-}$ control mice received high fat diet until 22 weeks of age, at which time they were euthanized and weighed. Ten female apoE$^{-/-}$/C5def mice were fed and treated similarly. Sufficient female controls with normal complement titers were not obtained from the crosses between F2 heterozygous mice (C5 het). Therefore five female apoE$^{-/-}$ controls were obtained from a normal apoE$^{-/-}$ × apoE$^{-/-}$ mating.

Plasma cholesterol and triglycerides. At the time of euthanasia, blood was collected from the vena cava into syringes containing EDTA as an anticoagulent. Plasma was prepared by centrifugation at 850*g* for 15 min at 4°C and stored at −20°C for later evaluation of

TABLE 1

C5 Deficiency Does Not Alter Plasma Cholesterol or Triglyceride in ApoE$^{-/-}$ Mice[a]

Groups	Plasma cholesterol[b] (mmol/L ± SEM)	Plasma triglyceride[b] (mg/dL ± SEM)	Body weight (g ± SEM)
Male apoE$^{-/-}$	31 ± 11	223 ± 79	41.1 ± 14.6
Male apoE$^{-/-}$/C5def	27.6 ± 8.9	202 ± 64	38 ± 12
Female apoE$^{-/-}$	20 ± 9	126 ± 56	22.1 ± 9.9
Female apoE$^{-/-}$/C5def	25.1 ± 8	170 ± 54	34 ± 10.8*

[a] Male and female apoE$^{-/-}$/C5def and apoE$^{-/-}$ controls were fed a high fat diet until 22 weeks of age.
[b] Plasma cholesterol and triglyceride were measured as described under Methods.
* $P = 0.0005$.

plasma cholesterol and triglyceride levels. Plasma cholesterol and triglyceride measurements were made using standard enzymatic kits (Sigma).

Histology. Euthanized mice were gently perfused through the left ventricle with cold PBS. Following perfusion, the heart with about 1 mm of the proximal aorta attached was removed and prepared for cryosectioning. The portion of the heart distal to the tips of the auricles was removed by excision, and the remaining portion containing the aortic root intact was stored briefly on ice in PBS, 0.02% NaN_3 then frozen in O.C.T. (Optimal Cutting Temperature) embedding medium (Fisher Scientific, Springfield, NJ) over liquid nitrogen-isopentane.

The freshly frozen hearts were used to quantitate lesion area in the aortic root and for immunohistochemistry. Sequential 20 μm sections were cut until the aortic valve leaflets appeared. From this point, serial 6 μm sections were cut over a distance covering approximately 280 μm of the aortic root area, as described by Paigen *et al.* (19). Sections were collected on 10-well masked slides, air-dried and stored at −20°C prior to use. Ten sections at 24-μm intervals were stained with hematoxylin–phyloxine–saffron (Polyscientific, Bayshore, NY) for morphometry. Intervening sections were stained with Oil Red O (Polyscientific, Bayshore, NY) for lipids or immunolabeled for CD11b to localize macrophage-derived foam cells. For immunolabeling the slides were blocked with 1.5% normal goat serum, followed by Vector Avidin and Biotin block. Anti-CD11b antibody (anti-mouse CD11b monoclonal antibody, Endogen, Woburn, MO) was added at a 1:100 dilution for 1 h. Biotinylated anti-hamster IgG was added for 30 min, and the Vector ABC detection system was used according to the manufacturer's instructions, with diaminobenzidine as the substrate (20). Due to the unavailability of antibodies against murine C5b-9 neoantigens, terminal complexes could not be labeled immunohistologically in the aortic root.

Morphometry. Lesions in the aortic root area were quantitated by a modification of a previously published procedure (17). The entire intimal area was manually traced and quantitated using an Image Proplus (Ipwin) image analysis system in a blinded fashion. The mean lesion area was determined for each mouse by averaging the values for 8–10 sections. The individual mean lesion areas were further averaged to determine the mean lesion area for each group, with ±SEM. Statistical significance was calculated using the paired Student's *t* test, with significance achieved at $P < 0.05$.

RESULTS

C5 Deficiency Has No Effect on Plasma Cholesterol and Triglyceride Levels in ApoE$^{-/-}$ Mice

ApoE$^{-/-}$ mice were crossed with C5def mice to generate mice deficient in both apoE and C5 (apoE$^{-/-}$/C5def) and control apoE$^{-/-}$ mice with normal levels of C5. Resulting progeny were fed a high fat, Western-type diet from weaning at four weeks of age until 22 weeks of age. Plasma cholesterol and triglyceride levels for apoE$^{-/-}$ controls and apoE$^{-/-}$/C5def animals were determined at 22 weeks of age as described under Methods. Male mice with a combined deficiency in apoE and C5 showed no significant difference in plasma cholesterol ($P = 0.3405$) or triglyceride ($P = 0.5655$) compared to apoE$^{-/-}$ controls (Table 1). Similarly, there was no significant difference in plasma cholesterol ($P = 0.3090$) or triglyceride ($P = 0.1913$) between apoE$^{-/-}$ and apoE$^{-/-}$/C5def female mice (Table 1). Body weights for both groups of male mice were the same ($P = 0.0662$). However, female apoE$^{-/-}$ mice had slightly lower body weights than female apoE$^{-/-}$/C5def mice ($P = 0.0005$). Thus the absence of complement does not affect serum parameters that could influence the development of atherosclerosis.

C5 Deficiency Does Not Reduce the Development of Atherosclerotic Lesions in the Aortic Root of ApoE$^{-/-}$ Mice

Morphometry was used to quantitate the lesion area in the aortic roots of male and female apoE$^{-/-}$/C5def mice and apoE$^{-/-}$ controls (Fig. 2). The mean lesion area in male apoE$^{-/-}$/C5def mice was not significantly different from that for male apoE$^{-/-}$ controls ($P = 0.123$) (Fig. 2 and Table 2). The mean lesion area for female apoE$^{-/-}$/C5def mice was the same as that for female apoE$^{-/-}$ mice ($P = 0.835$) (Fig. 2 and Table 2). Moreover, the mean lesion area was higher for both groups of female mice compared to both groups of male mice ($P = 0.0229$ for controls; $P = 0.0241$ for apoE$^{-/-}$/C5def), as has been reported previously (17). Thus the absence of C5 did not significantly decrease the mean lesion area in either male or female apoE$^{-/-}$ mice, suggesting that the development of atherosclerotic lesions was not altered by C5 deficiency.

Atherosclerotic Lesions Have a Similar Distribution of Lipid and Foam Cells in ApoE$^{-/-}$/C5def and ApoE$^{-/-}$ Mice

In addition to the quantitative analysis of lesion area, we examined the morphology of the aortic root

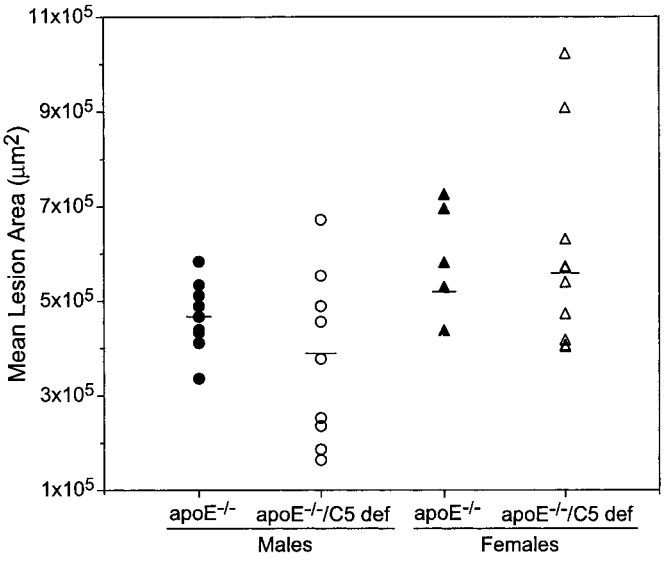

FIG. 2. The absence of C5 does not alter the mean lesion area in the aortic root of apoE$^{-/-}$ mice. ApoE$^{-/-}$/C5def and apoE$^{-/-}$ control mice were fed a high fat diet until 22 weeks of age. The lesion area in the aortic root for individual mice was measured by morphometry as described under Methods. Each symbol represents the mean lesion area for an individual animal, with the means for each group indicated by horizontal lines on the graph.

lesions in apoE$^{-/-}$/C5def and apoE$^{-/-}$ control animals. Oil Red O was used to localize lipid accumulation within the lesions, and adjacent sections were also immunolabeled with antibody against CD11b, a marker for macrophage-derived foam cells. The extent of Oil Red O positive lipid accumulation was very similar in both apoE$^{-/-}$ and apoE$^{-/-}$/C5def mice (Fig. 3). CD11b immunostaining coincided with Oil Red O staining in both groups of mice (Fig. 3), indicating the presence of foam cell rich areas. Thus, the morphology of aortic root lesions was very similar for both the apoE$^{-/-}$ and apoE$^{-/-}$/C5def mice.

DISCUSSION

Complement component C5 contributes to at least two different processes that promote host defense and could be involved in the inflammatory component of atherosclerosis. One product of C5, C5a, acts as a potent chemoattractant for leukocytes, inducing transient, integrin-mediated adhesion that is essential for leukocyte extravasation and trafficking to sites of infection. Another portion of C5, C5b, forms part of the terminal complex of complement, that inserts pore-forming complexes (C5b-9) into the membranes of bacteria, resulting in their lysis (21). Thus C5 contributes in multiple ways to the innate immune response to infection. While organisms are well-equipped with proteins that prevent the accidental activation of complement and deposition of terminal complexes, pathological conditions may lead to failures in this defense mechanism, with subsequent inappropriate activation of leukocytes and lysis of host cells. The combination of leukocytic influx and formation of terminal complexes on host tissues contributes to the inflammatory process.

The importance of C5 in the defense against bacterial infection is underscored by the phenotype of mouse strains that are deficient in C5. It has been repeatedly demonstrated that C5-deficient mice are more susceptible to infection by *Listeria* than C5-sufficient mice (22–24). The most elegant proof that C5 is directly responsible for protection against *Listeria* was the demonstration that replacement of C5 in C5-deficient mice, to produce a congenic strain with normal C5, conferred protection (23). C5 has also been implicated in defense against certain other bacteria, including *Psuedomonas aeruginosa* (25) and *Cryptococcus neoformans* (26), and against candidiasis (27). Delayed recruitment of macrophages after injury to the sciatic nerve has also been reported in C5-deficient mice. Macrophages were still present at the site of injury, suggesting that factors other than C5 have a compensatory role for recruitment of macrophages (28). Similarly, accumulation of neutrophils after experimentally induced cutaneous candidiasis has been observed in C5-deficient mice suggesting that sources other than complement-derived chemotactic factors exist (29).

Deficiencies in C5, as well as other individual complement components that contribute to the formation of terminal complexes, have been reported in humans. Like the C5-deficient mice, humans that lack C5 show susceptibilities to bacterial infection, especially infec-

TABLE 2

C5 Deficiency Does Not Reduce the Mean Lesion Area in the Aortic Roots of ApoE$^{-/-}$ Mice

	Males		Females	
Groups[a]	ApoE$^{-/-}$	ApoE$^{-/-}$/C5def	ApoE$^{-/-}$	ApoE$^{-/-}$/C5def
Mean lesion area[b] ($\mu m^2 \pm$ SEM)	468,176 ± 21,982	375,182 ± 53,089	591,981 ± 53,242	618,578 ± 83,457

[a] Mice were fed high fat diet until 22 weeks of age.
[b] Mean lesion area in the aortic root was quantitated by morphometry as described under Methods.

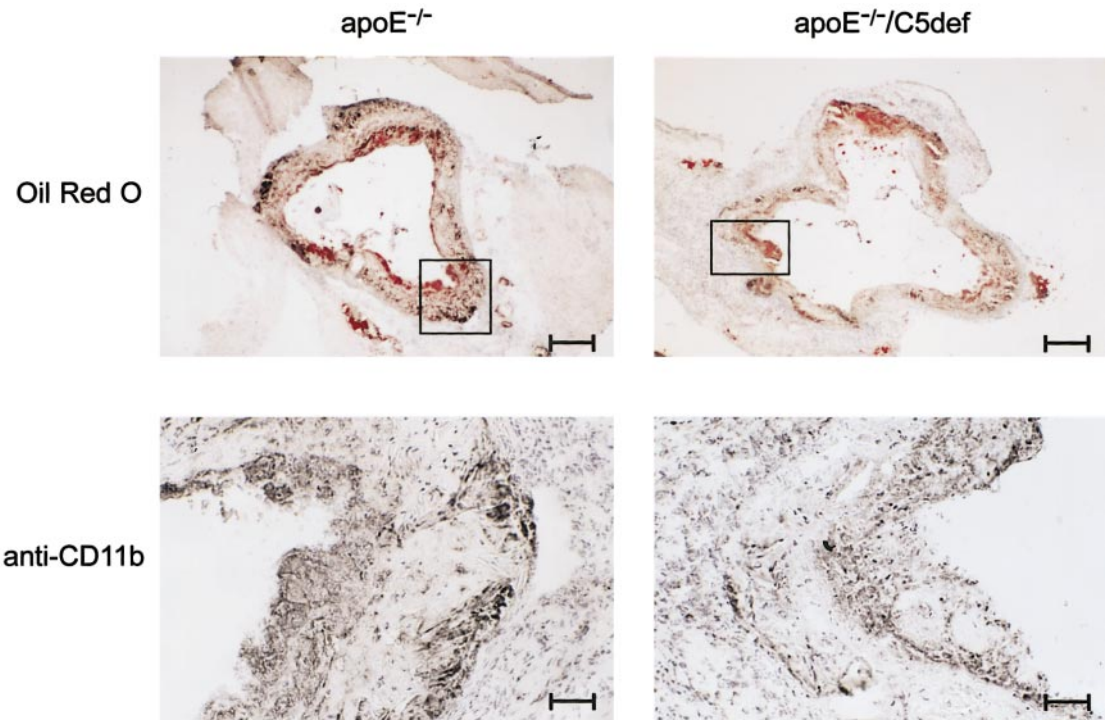

FIG. 3. Localization of foam cells by Oil Red O and CD11b immunolabeling. The top panels show representative sections of aortic roots from apoE$^{-/-}$ and apoE$^{-/-}$/C5def mice stained with Oil Red O. Bar, 300 μm. The bottom panels show immunolocalization of CD11b in sections adjacent to those in the top panels. Bar, 60 μm. The boxes in the top panels delineate areas comparable to those shown in the bottom panels.

tions caused by *Neisseria meningitidis* and *Neisseria gonorrhoea* (30). There has, however, been no systematic study of atherosclerosis or other inflammatory diseases among complement-deficient humans.

Several studies suggest that monocyte chemoattractants and complement terminal complexes may be important in the development of atherosclerosis. When mice deficient in the monocyte chemoattractant MCP-1 were crossed with LDL receptor$^{-/-}$ mice, the lack of MCP-1 resulted in a reduction in lesion area (31). Similarly, when mice deficient in the receptor for MCP-1, CCR2, were crossed with apoE$^{-/-}$ mice, there was a 50% reduction in lesion area (32). In addition, studies in C6-deficient, hypercholesterolemic rabbits suggest that complement terminal complexes may be involved in the development of atherosclerotic lesions (15, 16). Chemotaxis of monocytes is therefore an important process in atherosclerosis, and complement deposition may also play a role. The availability of mice deficient in C5 offers the opportunity to test the role of an additional monocyte chemoattractant and component of terminal complexes in this disease.

Here we demonstrate that the presence of C5 is not required for the development of atherosclerotic lesions in apoE$^{-/-}$ mice. Neither male nor female mice showed a significant difference in lesion area, whether they had normal C5 titers or were C5-deficient. Comparison of aortic root tissue from control and C5-deficient animals showed a similar distribution of macrophage-derived foam cells, suggesting that C5a was not required as a monocyte chemoattractant. It is likely that other chemoattractants, such as MCP-1, may compensate for the lack of C5, or they may be more important physiologically in this setting. While C5-deficient mice are susceptible to bacterial challenge, and infection has been recently identified as a factor that could contribute to the initiation or progression of human atherosclerosis, there is apparently no role for infection in the initiation of atherosclerosis in this particular murine model of atherosclerosis. ApoE$^{-/-}$ mice that were raised under germ-free conditions developed atherosclerosis to the same extent as did control mice raised in normal animal housing (33).

While the findings here do not support a role for the terminal complex or C5a in a murine atherosclerotic model, they do not rule out a role for the early acting components. A contribution of these proteins could be tested using mice that have been made genetically deficient in C3 or C4 (34). Similarly, while these studies rule out a role for C5 in early atherogenesis, they do not rule out a role in plaque rupture or in the response to plaque rupture, both processes of great importance to coronary heart disease in man.

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