Brief Definitive Report

Infectious Agents Are Not Necessary for Murine Atherogenesis

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Abstract

Recent work has revealed correlations between bacterial or viral infections and atherosclerotic disease. One particular bacterium, *Chlamydia pneumoniae*, has been observed at high frequency in human atherosclerotic lesions, prompting the hypothesis that infectious agents may be necessary for the initiation or progression of atherosclerosis. To determine if responses to gram-negative bacteria are necessary for atherogenesis, we first bred atherosclerosis-prone apolipoprotein (apo) $E^{-/-}$ (deficient) mice with animals incapable of responding to bacterial lipopolysaccharide. Atherogenesis was unaffected in doubly deficient animals. We further tested the role of infectious agents by creating a colony of germ-free apo $E^{-/-}$ mice. These animals are free of all microbial agents (bacterial, viral, and fungal). Atherosclerosis in germ-free animals was not measurably different from that in animals raised with ambient levels of microbial challenge. These studies show that infection is not necessary for murine atherosclerosis and that, unlike peptic ulcer, Koch's postulates cannot be fulfilled for any infectious agent in atherosclerosis.

Key words: atherosclerosis • apolipoprotein E knockout • germ-free animal • Toll • cholesterol

Introduction

Several types of studies have suggested that infectious agents may play a role in atherosclerosis and coronary heart disease (CHD). Atherosclerosis in chickens is exacerbated by infection with Marek's disease virus, a member of the herpes virus family (1, 2), and numerous studies have suggested an association of viral pathogens such as CMV and bacterial pathogens such as *Helicobacter* and *Chlamydia* with human atherosclerosis (3–5). Recent data has drawn special attention to the association between *Chlamydia pneumonia* and CHD. Most (6–8) though not all (9, 10) studies have found that patients with CHD are more likely to carry anti-*Chlamydia* antibodies than healthy subjects, and several studies have demonstrated that *Chlamydia* can be detected immunohistologically in >70% of atherosclerotic lesions, whereas it is virtually undetectable in undiseased arteries (5–8). *C. pneuomonia* is a gram-negative bacteria that may produce persistent infection through intracellular growth in macrophages (11), a cell type that plays a dominant and necessary role in atherosclerotic lesions (12, 13). These observations have led to the hypothesis that *Chlamydia* may play a causal role in atherosclerosis and CHD (14–18). This hypothesis is made plausible by studies on the association of *Helicobacter pylori* and duodenal ulcer (19, 20). This condition was originally thought to be caused by excess gastric acid and was treated by reducing or neutralizing acid. It is now clear that ulcers are most commonly caused by infection with *H. pylori*, and the most effective treatment involves antibiotics. In the same way, atherosclerosis is currently thought to be caused by excess plasma cholesterol and is treated by agents that reduce cholesterol levels. If *Chlamydia* or any other pathogen causes atherosclerosis, then a more effective treatment may be an antibiotic or vaccine.

We have sought evidence for a causal role of infectious agents in atherosclerosis using the murine apolipoprotein (apo) $E^{-/-}$ (deficient) model. Because of the deficiency of apo E, these animals have high levels of plasma cholesterol and develop atherosclerosis with a reproducible time course (21–23). In a first experiment, we bred the apo $E^{-/-}$ with a strain of mouse that is unable to respond to bacterial LPS,

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but found no alteration in the rate or extent of atherogenesis. In a second study, we developed a colony of germ-free apo $E^{-/-}$ animals. We observed no effect of gnotobiosis on the progress of atherosclerosis. We conclude that neither the response to infectious agents nor the presence of infectious agents themselves is absolutely necessary for atherogenesis.

Materials and Methods

Mice with the *lps*^{d} gene crossed onto a C57BL background (C57BL/10ScN) were the gift of Dr. Steven K. Chapes (Kansas State University, Manhattan, KS). These mice exhibited minimal increases in serum levels of TNF-α and IL-6 in response to intraperitoneal injection of 50 μg of LPS, whereas C57BL and apo $E^{-/-}$ animals exhibited >100-fold elevations in both (not shown). C57BL/10ScN mice were crossed with apo $E^{-/-}$ mice on a C57Bl/6 background (Jackson Laboratories). Apo $E^{-/-}$/*lps*^{d} animals were identified in the F2 progeny by the presence of plasma cholesterol levels >500 mg/dl and the absence of plasma elevations of TNF-α 90 min after injection of 50 μg LPS.

Germ-free and control apo $E^{-/-}$ mice were generated and maintained at Taconic Farms. Sterility was verified by established procedures (standard operating procedure [SOP] no. G-LB-300.04).

For all mice in this study, offspring were weaned at 4 wk of age onto a high-fat Western-type diet containing 21.22% (g/100 g) fat, 17.01% protein, 48.48% carbohydrate, and 0.15% cholesterol (TD88137; Harlan Teklad) and maintained on the diet for the remainder of the study. Autoclaved food and water were provided ad libitum. The Institutional Animal Care and Use Committee of Merck Research Laboratories approved the animal use for experimentation, and all animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (1996, National Research Council).

To measure aortic cholesterol and cholesteryl ester, mice were killed and gently perfused through the left ventricle with cold PBS with 5 mM EDTA. All branches and any adipose tissue connected to the aorta were removed, and each aorta was carefully excised from the aortic root to the right renal artery. The aortas were blotted dry, minced, and extracted with chloroform/methanol (2:1) according to the method used by Folch et al. (24). Total and free cholesterol in the aortic extracts were determined using an enzymatic fluorometric assay modified from previously described methods (25, 26), and data are expressed on a per aorta basis. Plasma cholesterol and triglyceride levels were determined using standard enzymatic kits (Sigma Chemical Co.).

For histology, mouse hearts were perfused in situ with PBS and removed with 1 mm of proximal aorta attached. The top half of the heart was embedded in O.C.T. embedding medium (Fisher Scientific) and prepared for cryosectioning. 6-μm sections were collected of the aortic root area, defined as having aortic valve leaflets present, and mounted on 10-well masked slides. Sections were stained with hematoxylin-phyloxine-saffron for morphology. Additional sections were immunolabeled with mAb L3T4 for CD4 or M1/70 for CD11b (PharMingen) using the horseradish peroxidase method with 3-3′-diamino benzadine as substrate.

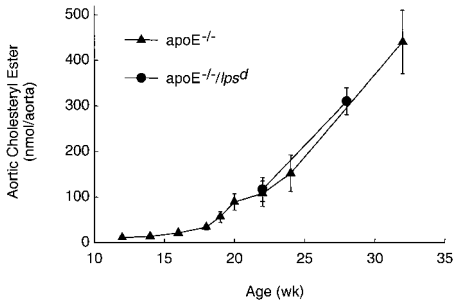

Figure 1. Accumulation of cholesterol ester in the aortas of apo $E^{-/-}$ and apo $E^{-/-}$/*lps*^{d} mice fed a high-fat diet. Animals were weaned onto a high-fat diet, and at the indicated times, aortic cholesterol measurements were performed as described in Materials and Methods. Each data point represents the average value from 7–10 animals, with error bars indicating the SE.

Results

The time course of atherosclerogenesis in apo $E^{-/-}$ mice was followed by measuring aortic cholesteryl ester (Fig. 1), a parameter that tracks with histological progression of atherosclerosis (23; data not shown). To determine a role for gram-negative bacteria such as *Chlamydia*, we first used *lps*^{d} mice (27), animals with a congenital deficiency in responses

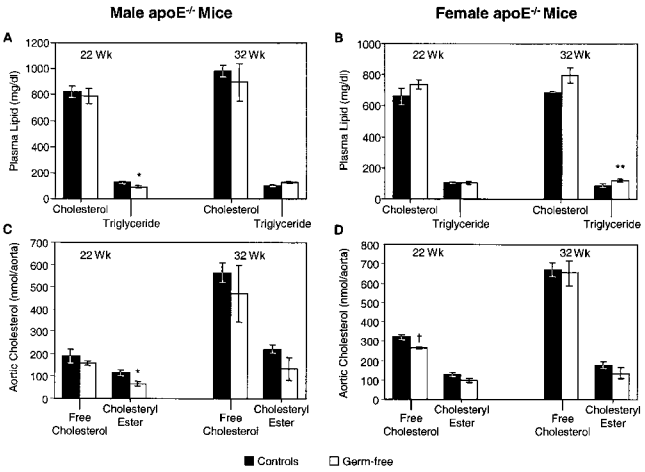

Figure 2. Plasma lipid profiles and aortic cholesterol levels of germ-free (white bars) and control (black bars) apo $E^{-/-}$ mice. Apo $E^{-/-}$ mice were reared germ free or in the presence of ambient pathogens. At 22 or 32 wk of age, animals were killed, blood was collected for analysis of plasma cholesterol and triglyceride, and aortas were harvested and extracted for determination of cholesterol content. (A and B) Comparisons of plasma cholesterol and triglyceride levels in control and germ free yield nearly identical values in male (A) and female (B) apo $E^{-/-}$ mice at both 22 and 32 wk. None of the small differences reached statistical significance, with the exception of decreased triglyceride levels for the male germ-free animals at 22 wk ($^*P < 0.005$) and increased triglyceride levels for the female germ-free animals at 32 wk ($^{**}P = 0.03$). (C and D) Comparisons of aortic free cholesterol and cholesteryl ester levels in male (C) and female (D) apo $E^{-/-}$ mice at 22 and 32 wk show a trend toward lower levels in the germ-free animals at both time points, although statistical significance was achieved only for the cholesteryl ester in germ-free males at 22 wk ($^*P < 0.005$) and for the germ-free cholesterol in germ-free females at 22 wk ($^{\dagger}P = 0.05$). However, data shown are expressed on a per aorta basis, and after correction for the decreased body weight of the germ-free mice, none of the differences attained statistical significance. For each time point and sex, group size was ≥10 animals, with the exception of the 32-wk male germ-free mice, which were reduced to 3 by mortality caused by aggressive behavior.

to the principal inflammatory component of these bacteria, LPS (endotoxin). LPS is known to stimulate expression of cytokines (TNF, monocyte chemotactic protein 1 [MCP-1], and others), adhesion molecules (vascular cell adhesion molecule 1 [VCAM-1], intercellular adhesion molecule 1 [ICAM-1], β2 integrins), and enzymes (inducible nitric oxide synthetase [iNOS], matrix metalloproteinase 9 [MMP9]) observed in atherosclerotic lesions (28) and is a prime candidate to mediate possible proatherogenic effects of *Chlamydia*. *lps*^{d} mice fail to respond to LPS because of a deficiency in Toll-like receptor 4 (TLR-4 [29, 30]), a transmembrane protein with homology to the IL-1 receptor. *lps*^{d} animals were crossed with apo E$^{-/-}$ animals to yield doubly deficient animals. The inability of these animals to respond to LPS was verified by measurements of plasma TNF in response to LPS challenge, and their deficiency in apo E was verified by measurements of plasma cholesterol (described in Materials and Methods). We observed that development of atherosclerotic lesions in mice fed a high-fat Western-type diet was not altered by the deletion of TLR-4 (Fig. 1). Further studies showed that plasma cholesterol was also not affected by the absence of TLR-4 (data not shown). This result suggests that responses to gram-negative bacterial products are not critical for murine atherogenesis.

To more thoroughly ascertain the role of infectious agents in atherogenesis, we generated a germ-free colony of apo E$^{-/-}$ mice. Founder germ-free apo E$^{-/-}$ pups were delivered by Caesarian section and reared by germ-free foster mothers. The colony resulting from these founders was maintained germ free: weekly tests showed that no bacteria could be grown from feces, bedding, or swabs of the isolator. In addition, animals chosen at random showed no antibodies against any of several viral pathogens. The absence of intestinal bacteria is known to cause enlargement of the caecum (31), and dramatically enlarged caeca were observed in all of the animals from the colony.

The progress of hyperlipidemia and atherogenesis in germ-free animals was compared with that of a second set of animals taken from the germ-free colony and reared with ambient pathogens. As expected, control apo E$^{-/-}$ animals exhibited extremely high levels of plasma cholesterol and moderately high triglyceride at both 22 and 32 wk of age (Fig. 2, A and B). Germ-free animals showed an essentially identical plasma lipid profile, and lipid changes are thus unlikely to obscure effects of gnotobiosis on atherogenesis.

In both male and female animals, we observed that the exclusion of infectious agents caused no consistent differences in the rate or extent of free cholesterol or cholesteryl ester accumulation in the aorta (Fig. 2, C and D). We did observe slightly reduced aortic cholesterol ester in the germ-free animals that attained statistical significance at 22 wk of age. However, these small differences disappeared upon correction for reduced body weight observed in the germ-free animals at the 22-wk time point (28 ± 0.6 g for germ-free males vs. 34 ± 0.8 g for control males, and 22 ± .02 g for germ-free females vs. 24 ± 0.6 g for controls; P = 0.001). The values for aortic cholesteryl ester in germ-free mice were also similar to those seen in a control colony that had never been made germ free (values in Fig. 1).

The similarity of atherogenesis in control and germ-free animals was confirmed by histologic examination of the aortic root in animals taken at the 22-wk time point. Well-

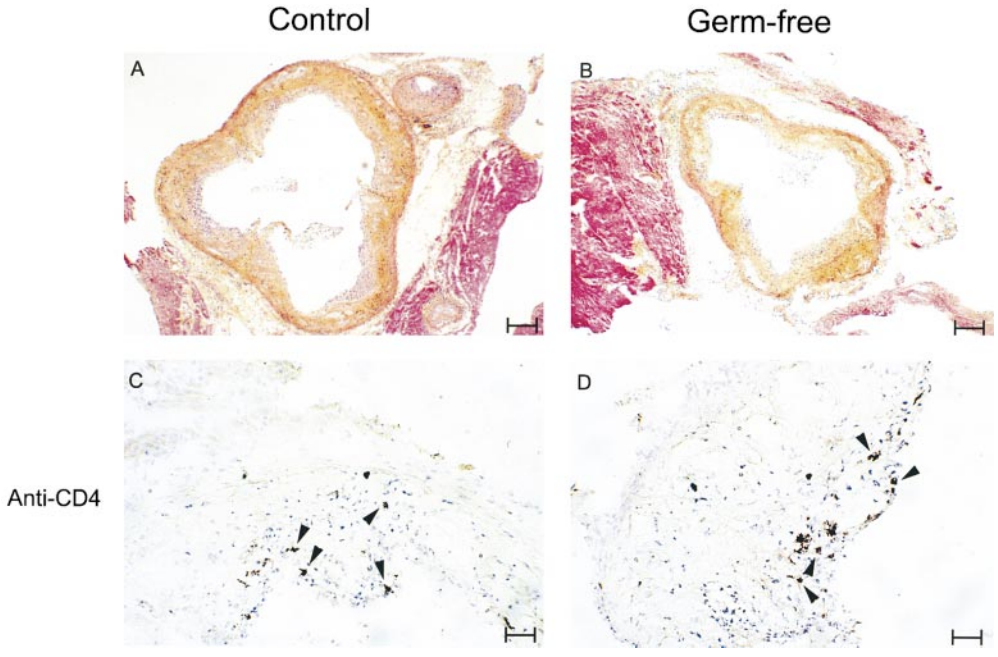

Figure 3. Histology of aortic root lesions in germ-free and control apo E$^{-/-}$ mice. (A and B) Cryosections of the aortic root area in mice at 22 wk of age were stained with hematoxylin-phyloxine-saffron. Lesions in both types of animal have fibrous components (indicated by yellow staining) and large areas that are rich in foam cells (indicated by light purple staining). White rice grain–shaped spaces indicative of the extracellular deposition of cholesterol are also visible in lesions from control and germ-free mice. The tissue staining red is surrounding muscle. Bar = 156 μm. (C and D) These sections are stained brown for the T lymphocyte marker CD4. Arrowheads indicate the location of T lymphocytes, present in both control and germ-free animals. Bar = 31 μm. Hearts from five mice of each type for both males and females were examined histologically, and the sections shown are representative of the results from all of the mice. The same morphology and cellular composition was observed in both female and male mice. Histology of hearts from two mice of each type at 32 wk of age similarly revealed no differences between germ-free apo E$^{-/-}$ and control apo E$^{-/-}$ mice (not shown). Additionally, staining for the macrophage marker CD11b revealed comparably stained areas in control and germ-free animals (not shown).

developed lesions with necrotic cores, large foam cells, and fibrous caps were observed in both control and germ-free specimens (Fig. 3, A and B), and neither quantitative nor qualitative differences could be detected in observations of either male or female lesions. A difference in aortic size reflecting the smaller body size of the germ-free animals (31) was readily detected histologically. T cells are found in human and murine atherosclerotic lesions (12, 23), suggesting activity of the adaptive immune system. We identified T cells by immunohistochemistry in both control and germ-free animals (Fig. 3, C and D), indicating that pathogens are not required to drive the influx of T cells into atherosclerotic lesions.

Discussion

The observations presented here indicate that infectious agents, whether bacterial, viral, or fungal, are not necessary for murine atherogenesis. Infectious agents do not appear necessary either for initiation or progression of atherosclerosis, nor does the presence of infectious agents alter the morphology or cellular composition of the atherosclerotic lesions. Finally, the presence of infectious agents does not appear to alter the pattern of distribution of lesions along the aorta (not shown). Rather, it appears that the high-circulating cholesterol levels in the apo $E^{-/-}$ animals are sufficient to initiate and drive atherogenesis. We conclude that Koch's postulates cannot be fulfilled for any infectious agent in murine atherosclerosis. Atherosclerosis in apo $E^{-/-}$ mice closely resembles that in humans with respect to histology, progression, and dependence on circulating cholesterol (23), and we may thus speculate that infectious agents may not be necessary for the development of human atherosclerosis.

We wish to emphasize that the observations reported here relate to atherogenesis, not to plaque rupture, thrombus formation, or myocardial infarction. It is possible that infectious agents play an important role in one or more of these acute processes. Several current studies are seeking to define a role for bacterial infection in human CHD by studying endpoints such as myocardial infarction in patients randomized to placebo or antibiotic therapy of varying duration. The largest of these studies to report results thus far has failed to demonstrate a decline in cardiovascular events in the 6 mo after treatment with antibiotic (32). However, definitive results must await completion of larger, more extended trials.

Although our results suggest that pathogens do not serve as etiologic agents for atherosclerosis, they may still have a role in exacerbating the disease. Recent reports suggest that inoculation of atherosclerosis-prone mice with high doses of *Chlamydia* may cause an approximately twofold increase in the size of atherosclerotic lesions (33, 34). It is important to note that stresses such as infection provoke a set of physiological responses that are uniformly proatherogenic (35–37). These include insulin resistance and hyperglycemia, elevation of plasma triglycerides, elevated white blood cell counts, elevated acute phase reactants (e.g., C-reactive protein and fibrinogen), and depression of high density lipoprotein (HDL) cholesterol levels. It is thus possible that infection may exacerbate atherosclerogenesis, and antibiotics may ameliorate atherogenesis not through the action of an etiologic agent, but through indirect effects on metabolism and known risk factors.

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