High-Cholesterol Diet Facilitates *Anaplasma phagocytophilum* Infection and Up-Regulates Macrophage Inflammatory Protein–2 and CXCR2 Expression in Apolipoprotein E–Deficient Mice

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**Background.** *Anaplasma phagocytophilum* is an obligatory intracellular bacterium that infects granulocytes and causes human granulocytic anaplasmosis (HGA). This bacterium requires cholesterol for host cell infection in vitro and incorporates exogenous cholesterol into its membrane.

**Methods.** To understand the role of host cholesterol in *A. phagocytophilum* infection in vivo, we analyzed the effects of a high-cholesterol diet and reduced apolipoprotein E (apoE) activity on *A. phagocytophilum* infection in mice.

**Results.** A high-cholesterol diet significantly facilitated *A. phagocytophilum* infection in the spleen, liver, and blood of apoE-deficient (apoE/−/−) mice, compared with the level of infection in apoE+/− mice fed a normal-cholesterol diet or wild-type (WT) mice fed a high- or normal-cholesterol diet. *A. phagocytophilum* infection induced a significant elevation in the mRNA expression of macrophage inflammatory protein (MIP)–2 and an MIP-2 receptor, CXCR2, in the spleen in apoE/−/− mice fed a high-cholesterol diet, compared with the other 3 groups.

**Conclusion.** Our results suggest that high blood cholesterol levels resulting from an interaction between dietary and genetic factors facilitate *A. phagocytophilum* infection and up-regulate a proinflammatory chemokine and its receptor, which may contribute to HGA pathogenesis.
poptroteinemia), which is characterized by the accumulation of βVLDL and an increase in total serum cholesterol [7]. Therefore, defects in cholesterol metabolism may play a role in the exacerbation of HGA, and interaction between dietary cholesterol and apoE may affect the risk of *Anaplasma* infection.

The involvement of various cytokines and chemokines in *A. phagocytophilum* infection and pathogenesis has been investigated in cultured cells, patients with HGA, and experimentally infected animals [8–13]. Of the cytokines, the involvement of interleukin (IL)–8 and interferon (IFN)–γ in infection has been studied most. Patients with confirmed HGA have significantly higher concentrations of IL-8 in the serum than healthy control subjects [10]. CXCR2–/– mice, which lack CXCR2, the human IL-8 receptor homolog, have reduced amounts of *A. phagocytophilum* in the blood, compared with control mice [10]. In one study, serum IFN-γ levels were significantly higher in infected C3H mice than control mice from day 2 through day 8 of infection. IFN-γ–deficient mice had a markedly higher bacterial burden in the blood on days 5 and 8 after infection than wild-type (WT) mice, suggesting that IFN-γ may facilitate bacterial clearance during the early stage of infection [14].

In the present study, we examined whether dietary cholesterol and apoE determine the risk for *Anaplasma* infection using WT and apoE–/– mice on normal- and high-cholesterol diets. Given the importance of cytokines and chemokines in the pathogenesis of HGA, we examined cytokine and chemokine profiles in these mice to examine the possibility of cross-talk between the 2 host pathogenesis factors, cholesterol and cytokines, in facilitating *Anaplasma* infection.

**MATERIALS AND METHODS**

**Mice.** Five- to 6-week-old male apoE–/– C57BL/6 mice and congenic WT mice were purchased from Jackson Immuno-Research Laboratory. The animals were housed in a 12-h light/dark cycle and fed a normal-cholesterol diet (Formulab diet 5008; PMI) or a high-cholesterol diet (Teklad diet TD-88051; Harlan Teklad) for 4 weeks. Once each week, a blood specimen (∼100 μL) was collected from each mouse by venipuncture at the base of the mandible to track cholesterol concentrations over time using an Infinity Cholesterol Reagent Kit (Thermal Electron).

*A. phagocytophilum and mouse infection.* *A. phagocytophilum* HZ strain was cultured in HL-60 cells as described elsewhere [15]. Five mice in each group were inoculated intraperitoneally with *A. phagocytophilum*–infected HL60 cells (>90% cells infected; 1 × 10^7 cells/mouse). Ten days after inoculation, all mice in each group were killed by CO2 inhalation. Blood specimens were collected by cardiac puncture, and the peripheral blood leukocytes (PBLs) were isolated as described elsewhere [9]. Spleens and livers were harvested. These specimens were stored in RNA later (QIAGEN) at −20°C before DNA and RNA analysis. The protocol for use of animals in this study was approved by the Institutional Laboratory Animal Care and Use Committee.

*Measurement of bacterial burden in blood and tissues.* Total DNA was extracted from blood, spleen, and liver specimens with a QIAamp blood kit (QIAGEN). To determine the number of *A. phagocytophilum* organisms, the p44 competitive polymerase chain reaction (C-PCR) assay was performed to amplify p44 paralogs using primer set 1 (table 1) as described elsewhere [16]. Densitometric analysis of PCR products separated by agarose gel electrophoresis was performed as described elsewhere [9]. To normalize the input of mouse DNA across samples, PCR amplification for the mouse glyceraldehyde-3-phosphate dehydrogenase gene was performed with primer set 2 (table 1) over a linear range, as described elsewhere [9].

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**Table 1. Sequences of oligonucleotides used in competitive polymerase chain reaction (PCR) and cytokine reverse-transcriptase PCR.**

**NOTE.** G3PDH, glycerdehyde-3-phosphate dehydrogenase; HPRT, hypoxanthine-guanine phosphoribosyltransferase; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; KC, keratinocyte-derived chemokine; MIP, macrophage inflammatory protein; TNF, tumor necrosis factor.
Cytokine reverse-transcriptase (RT)–PCR. Total RNA was extracted from spleen, liver, and PBLs using an RNeasy kit (QIAGEN). The concentration and purity of the RNA were determined by measuring the A260 and the A260:A280 ratio with a GeneQuant II RNA and DNA calculator (Pharmacia Biotech). The RNA was stored at −80°C until use. Total cellular RNA (2 μg) was reverse transcribed in accordance with the manufacturer’s instructions by using SuperScript III RT (Invitrogen) and an oligo(dT)12–18 primer (Invitrogen). The cDNA (2 μL) was amplified in a 25-μL reaction mixture containing 1X PCR buffer (10 mmol/L Tris-HCl [pH 8.3], 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L deoxynucleoside triphosphates, and 0.4 μmol/L [each] 3’ and 5’ primers) (table 1). RT-PCR conditions were as follows: after 5 min of denaturation, 28 cycles of denaturation at 94°C for 45 s, annealing at 60°C (62°C for macrophage inflammatory protein [MIP]–2) for 1 min, and extension at 72°C for 1 min. The final extension was 7 min at 72°C. A mouse housekeeping gene, hypoxanthine-guanine phosphoribosyl transferase, was used to normalize the amount of input mRNA among different samples. As a positive control, splenocytes from uninfected mice were cultured in RPMI1640 growth medium with 10% fetal bovine serum at 37°C with 5% CO₂. After 24 h, RNA was extracted from the splenocytes and used as a positive control for the cytokine and chemokine RT-PCR.

Histopathologic analysis. Spleen and liver specimens from killed mice were fixed in 10% formalin, embedded in paraffin, sectioned at 4-μm thickness, and stained with hematoxylin and eosin. Slide-mounted sections were observed under a microscope using appropriate magnifications.

Statistical analyses. Statistical analysis was performed by Student’s t test or by 1-way analysis of variance (ANOVA) with the Tukey highly significant difference test. P < .05 was considered to be significant.

RESULTS

Blood cholesterol level in apoE−/− and WT mice. Four groups of 5 mice each were compared. After 1 week on a high-cholesterol diet, the plasma cholesterol concentrations in both the apoE−/− group (the AH group) and the WT group (the WH group) increased over baseline; in the AH group, plasma cholesterol concentration increased 4-fold within 1 week. In contrast, plasma cholesterol concentrations were stable during the entire period of diet feeding (4 weeks) in apoE−/− and WT mice fed a normal diet (the AN and WN groups, respectively). ANOVA of the plasma cholesterol concentration over weeks 2–4 showed that cholesterol levels were significantly higher in the AH group than in the other 3 groups (P < .05, for AH vs. AN; P < .01, for AH vs. WH or WN) (figure 1). These data are in agreement with a previous report [6] that apoE−/− mice are very sensitive to a high-cholesterol diet.

A. phagocytophilum burden in apoE−/− and WT mice fed formulated diets. A previous study reported the presence of A. phagocytophilum in various tissues of experimentally infected mice [17]. To compare the A. phagocytophilum burden among the different groups of mice, we performed C-PCR in specimens of 3 different tissues to measure the amount of bacterial DNA relative to mouse tissue DNA. Our results showed that A. phagocytophilum burdens in the blood, spleen, and liver from AH mice were ∼10-fold greater than those in the respective tissues from the other 3 groups of mice (figure 2). ANOVA revealed no significant differences among the other 3 groups of mice for each tissue type, but we did find significant differences among the tissues in the ratio of A. phagocytophilum DNA to mouse DNA: the blood was the primary site of anaplasma burden, followed by the spleen, and the burden was lowest or undetectable in the liver (figure 2).

MIP-2, MIP-2 receptor, and cytokine mRNA levels in infected apoE−/− and WT mice fed formulated diets. To explore alternative or concurrent mechanisms by which A. phagocytophilum infection is increased in apoE−/− mice fed a high-cholesterol diet, we investigated the levels of several cytokines in the mouse tissue specimens. We examined the mRNA expression levels of mouse MIP-2, a functional analog of human IL-8, and the MIP-2 receptor, CXCR2, in vivo in the mouse specimens infected with A. phagocytophilum. Our results showed that, in infected mice, MIP-2 mRNA expression level

![Figure 1](https://academic.oup.com/jid/article-abstract/195/10/1497/2191967)
Figure 2. *Anaplasma phagocytophilum* burden in mouse blood, spleen, and liver, determined by competitive polymerase chain reaction (C-PCR). *A. phagocytophilum* DNA burden in blood, spleen, and liver specimens were determined 10 days after infection in 4 groups of mice by C-PCR with primers specific for the *A. phagocytophilum* p44 gene. The *A. phagocytophilum* chromosome equivalent was calculated according to previously acquired data [16]. Each DNA sample was normalized to mouse glyceraldehyde-3-phosphate dehydrogenase DNA levels. Data are mean ± SD values of 5 mice each. "a," "b," and "c" indicate significant differences (analysis of variance) among the 4 groups in blood, spleen, and liver specimens, respectively. AH, apolipoprotein E–deficient (apoE/−/−) mice fed a high-cholesterol diet; AN, apoE+/+ mice fed a normal-cholesterol diet; WH, wild-type (WT) mice fed a high-cholesterol diet; WN, WT mice fed a normal-cholesterol diet.

was higher in the PBLs from the AH group than from the other 3 groups (P < .05), but no significant difference in CXCR2 mRNA was detected among the 4 groups (figure 3A). MIP-2 mRNA concentrations in spleen tissues were >100-fold greater in the AH group than in the WH group and were undetectable in both apoE−/− and WT mice fed a normal diet. CXCR2 mRNA was detected in the spleen in all 4 treatment groups infected with *A. phagocytophilum*, and the expression level was ∼2-fold greater in the AH mice than in the remaining 3 groups of mice (P < .01) (figure 3B). In the livers of mice infected with *A. phagocytophilum*, MIP-2 and CXCR2 mRNA were undetectable under the same PCR conditions used for blood and spleen specimens (data not shown). Chemokine keratinocyte-derived chemokine (KC) mRNA was undetectable in all mouse groups under the same conditions used for MIP-2 and CXCR2.

Previous studies reported an association of hypercholesterolemia with elevated serum concentrations of CXC chemokines: KC and MIP-2 in the human apoE*3-Leiden transgenic mouse model [18]. Given this information, we examined MIP-2 and CXCR2 expression in specimens from 4 groups of uninfected mice also fed high- and normal-cholesterol diets. MIP-2 and CXCR2 mRNA were undetectable in blood or spleen specimens from all 4 groups of uninfected mice under the same PCR conditions used for infected tissues. These results suggest that the high MIP-2 and/or CXCR2 mRNA concentrations in the blood and spleen were induced by *A. phagocytophilum* infection.

IL-1β mRNA expression was detectable after 28 PCR cycles in the spleens of AH mice infected with *A. phagocytophilum* and was ∼2-fold higher than that in the other 3 groups of mice (P < .05; data not shown), whereas IL-6, tumor necrosis factor–α, IFN-γ, and inducible nitric oxide synthase mRNAs were undetectable by RT-PCR in the spleens from all 4 mouse groups under the same conditions used for MIP-2 mRNA.  

**Histopathologic observations.** Previous studies using the murine model of HGA have shown that host-mediated immunological injury contributes to HGA pathogenesis [19, 20]. Therefore, we performed a histopathologic examination of the
spleen and liver from 4 groups of mice infected with *A. phagocytophilum* and from 4 corresponding groups of uninfected control mice.

There was marked disorganization and lymphoid depletion in the lymphoid follicles and surrounding white pulp in spleens from AH and AN mice, compared with those from WH and WN mice, before *A. phagocytophilum* infection (figure 4). *A. phagocytophilum* infection exacerbated the reduced cellularity and disorganization in lymphoid follicles and the surrounding white pulp with necrotic cells (AN, WH, and WN groups) or vacuolated cells (AH group) (figure 4). *A. phagocytophilum* infection did not cause remarkable histopathologic changes in the liver in the 4 groups of mice; differences seen among the 4 groups, including granulomatous infiltration, were associated with the high-cholesterol diet and apoE deficiency and were already present before infection (data not shown).

**DISCUSSION**

The present study examined whether high blood cholesterol levels caused by dietary factors, such as high cholesterol intake, and genetic factors, such as apoE genotype, increase the risk for HGA. We have addressed this issue by studying the *A. phagocytophilum* burden in apoE–/– and WT mice fed high- or normal-cholesterol diets. The apoE deficiency in apoE–/– mice is considered to be equivalent to the loss of function resulting from the presence of the apoE4 allele in humans [21]. The high-cholesterol diet potently and invariably enhanced *A. phagocytophilum* infection in the blood, spleens, and livers of apoE–/– mice. In view of our observations, it is tempting to speculate that humans may be more susceptible to *A. phagocytophilum* infection if they consume a high-cholesterol diet and/or have an apoE deficiency.

On infection with *A. phagocytophilum*, apoE–/– mice on a high-cholesterol diet had significantly increased levels of mRNA for spleen and blood MIP-2, the mouse homolog of human IL-8, and the MIP-2 receptor CXCR2. IL-8 is produced by neutrophils, monocytes, and other types of cells and is the most studied chemokine [22]. Neutrophils are not only producers of IL-8 but are also primary targets for IL-8, responding to this mediator by chemotaxis, release of granule enzymes, respiratory burst activity, and up-regulation of adhesion to unstimulated endothelial cells [23, 24]. Akkoyunlu et al. [10] proposed that IL-8 secretion by infected neutrophils recruits naive neutrophils to enhance *A. phagocytophilum* infection. IL-8 is also a myelosuppressive chemokine [25]. Given this information, high blood cholesterol may not be the only reason for the higher bacterial burden in the tissues of apoE–/– mice. It is possible that up-regulation of IL-8 and IL-8 receptors would participate in the enhanced infection in apoE–/– mice on a high-cholesterol diet.

Several studies have suggested that apoE protein affects innate and acquired immune responses in vitro, as evidenced by its ability to suppress lymphocyte proliferation, generate cytolytic T cells, and stimulate cultured neutrophils [26]. Recently, human apoE or fragments containing the receptor-binding domain were reported to provide innate immunity to viral in-
fection by direct disruption of viral particles and/or inhibition of viral attachment in cell culture [27]. An increasing body of evidence demonstrates that apoE−/− animals have impaired immunity after challenge with bacteria, such as Listeria monocytogenes and Klebsiella pneumonia [28, 29]. In agreement with these reports, A. phagocytophilum infection caused histopathologic observations of the spleen to show more-severe loss of follicular cells and architecture in apoE−/− mice regardless of the cholesterol content of the diet. However, because apoE−/− mice fed the normal-cholesterol diet had a much lower bacterial burden than apoE−/− mice fed the high-cholesterol diet, immunosuppression alone cannot be the reason for the much higher bacterial burden.

Bakken et al. reported that increased age is associated with the severity of HGA illness in patients [30]. A more-recent study confirmed the unusually high median age of patients with HGA (51 years), compared with patients with other tickborne diseases, such as Lyme disease (39 years) and Rocky Mountain spotted fever (38 years) [31]. The difference is not due to different species of tick vectors or reservoir hosts, because Borrelia burgdorferi, which causes Lyme disease, and A. phagocytophilum are both transmitted by the Ixodes scapularis tick, and the reservoirs are white-footed mice [32]. Generally, immune functions are weakened [33] and blood cholesterol levels are increased with advanced human age [34]. Patients who are immunocompromised by natural disease processes or medications may develop severe HGA [30]. The present study showed that, even in genetically predisposed apoE−/− mice, by providing a low-cholesterol diet, the bacterial burden was reduced to the level in WT mice. Therefore, lowering the plasma cholesterol level by dietary and pharmacological means may be beneficial in preventing and ameliorating severe HGA in elderly and immunocompromised patients in conjunction with prompt antibiotic therapy. Well-designed case-control studies in patients with HGA are desired in this regard.

In summary, we have demonstrated that the combination of apoE deficiency and high cholesterol intake results in enhanced infection with A. phagocytophilum in vivo, perhaps facilitated in part by high blood cholesterol level, up-regulation of IL-8 and IL-8 receptors, and immunosuppression. This influence of dietary and genetic factors could be relevant for the development of HGA in humans and could inform the development of an improved supportive therapy to antibiotics and preventive measures for HGA.

References