American Journal of Pathology, Vol. 151, No. 4, October 1997

Respiratory Infection in Lipid-Fed Rabbits Enhances Sudanophilia and the Expression of VCAM-1

Mary Richardson, Marnie De Reske, Kathleen Delaney, Andrew Fletch, Lindsey H. Wilcox, and Raelene L. Kinlough-Rathbone

From the Department of Pathology, McMaster University, Hamilton, Ontario, Canada

The pathogenesis of atherosclerosis has been related to infection of the arterial wall, but it is not clear whether this occurs before or after the development of lipid-containing lesions. Respiratory bacterial infection increases the expression of vascular cell adhesion molecule-1 (VCAM-1). We therefore examined whether a similar infection would enhance atherosclerosis in New Zealand White rabbits fed chow supplemented by 15% (w/w) egg yolk for 50 days. Rabbits with naturally acquired respiratory infection by *Pasteurella multocida*, pathogen-free (SPF) animals infected by *P. multocida* in the laboratory, and age-matched SPF rabbits maintained in a disease-free environment were used. Endothelial cells expressing VCAM-1 in the aorta between intercostal arteries 3 and 5 were identified using anti-VCAM-1 (Rb1/9) and an alkaline-phosphatase-linked secondary antibody and quantified in Häutchen preparations. The remainder of the aorta was stained with Sudan IV to show lipid deposition. The expression of VCAM-1 (mean ± SEM per 10,000 cells) was 22 ± 8 (n = 5) in the lipid-fed SPF rabbits, significantly different from that in the lipid-fed rabbits with naturally occurring infection (190 ± 51 (n = 5)) or from rabbits infected in the laboratory (106 ± 25 (n = 5)). The extent of Sudanophilia was significantly greater in the naturally infected rabbits (8.3 ± 1.2%) or infected SPF rabbits (10.3 ± 1.8%) than in the SPF rabbits (2.7 ± 0.8%; $P < 0.05$). Antibiotic treatment in naturally infected rabbits reduced the number of cells expressing VCAM-1 and the extent of the Sudanophilia to baseline levels. Thus, Sudanophilia is enhanced by bacterial infection in rabbits fed egg yolk and is associated with a significant increase in VCAM-1. *(Am J Pathol 1997, 151:1009–1017)*

There are various pathophysiological conditions that lead to the development of atherosclerosis. Recently, attention has focused on the possible role of an infectious process within the vessel wall as the initiating lesion.[1] Organisms such as *Chlamydia pneumoniae*[2,3] and members of the Herpesviridae[4] including cytomegalovirus[5–7] have been identified within lesions in human arteries. In an experimental setting, atherosclerotic lesions have been described in a rabbit model of *C. pneumoniae* pneumonia[8] and herpes simplex virus infection of smooth muscle cells *in vitro* will induce lipid accumulation.[9] However, the question remains of whether the presence of organisms within a lesion is indicative of a causal role or whether an already established lesion becomes infected, thereby enhancing its development.

It is generally accepted that stimulation or injury to the arterial endothelium is crucial in the pathogenesis of atherosclerosis.[10] Macrophages and adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) have been described in human atherosclerotic lesions.[11,12] In experimental diet-induced hyperlipemia, or rabbits with familial hypercholesterolemia, the accumulation of mononuclear white blood cells on the luminal surface and within the arterial intima[10] and the expression of leukocyte adhesion molecules, such as VCAM-1,[13] are early features of the development of lipid-containing lesions. The expression of adhesion molecules has been proposed as an initial endothelial alteration in atherogenesis[10].

Increased VCAM-1 expression is associated with other pathologies identified as risk factors for the development of atherosclerosis, including experimental[14] and human[15] cardiac allograft vasculopathy and in endothelial cells that have regenerated after balloon catheter removal.[16] In alloxan-diabetic rabbits the number of VCAM-1-positive aortic endothelial cells was increased compared with that in nondiabetic animals and was further increased in lipid-fed diabetic rabbits.[17] The increased expression of VCAM-1 seen in hyperglycemia was proposed as part of the explanation of the enhanced atherosclerosis associated with diabetes. In an earlier study we reported that, compared with age-matched control animals, the number of aortic endothelial cells that express VCAM-1 was increased in normolipemic rabbits during a respiratory infection caused by *Pasteurella multocida*.[18]

Supported by a grant-in-aid from the Heart and Stroke Foundation of Ontario (T2963) and the Medical Research Council of Canada (MT1309).

Accepted for publication July 9, 1997.

Address reprint requests to Dr. Mary Richardson, Department of Pathology, Rm2N35, McMaster University HSC, 1200 Main Street W, Hamilton, Ontario, Canada L8N 3Z5.

P. multocida is a common pathogen in laboratory rabbits, and this increase in VCAM-1-positive endothelial cells was seen in conventionally raised rabbits with a naturally occurring infection and in specific-pathogen-free (SPF) animals infected in the laboratory. The present study examines whether the increase in numbers of endothelial cells that express VCAM-1 observed during respiratory infection in rabbits will influence the arterial response to a cholesterol-enriched diet.

Materials and Methods

Animals

SPF rabbits (Charles River, St. Constance, Quebec, Canada) and conventionally raised non-SPF laboratory male New Zealand White rabbits (Maple Lane Farms, Clifford, Ontario, Canada) were used. All rabbits were 2.0 to 2.3 kg body weight at the start of the experiment. The health status of the SPF rabbits was monitored by the supplier by monthly checks including serology, parasitology, and bacteriology. They were documented to be free of infection by *Pasteurella sp.* and *Bordetella sp.* on arrival. All rabbits were initially fed rabbit chow (Ralston Purina, Woodstock, Ontario, Canada) with water *ad libitum*.

The uninfected control SPF rabbits received this diet for the duration of the experiment. The lipid-supplemented diet (EYD) consisted of 15% w/w egg yolk (Dominion Egg Co., Mississauga, Ontario, Canada) and standard rabbit chow.

Induction of P. multocida *Infection*

A suspension of *P. multocida* containing 1×10^6 colony-forming units per ml was prepared as described by Ringler et al[19] using an organism initially isolated from a nasal culture from an animal showing nasal discharge on arrival.

After a 7-day period of acclimatization, SPF rabbits were anesthetized with isoflurane (AErrane, Ohmeda, Liberty Corner, NJ) and oxygen. A 22-gauge, 1-inch angiocatheter (Becton Dickinson Vascular Access, Sandy, UT) was introduced into each nasal passage in turn and 0.5 ml of the *P. multocida* suspension was introduced via a syringe.

Experimental Groups

Six experimental groups were examined: group 1, SPF rabbits uninfected and fed a normal diet (n = 4 animals); group 2, SPF rabbits uninfected and fed EYD for 50 days (n = 7 animals); group 3, SPF rabbits, uninfected, fed EYD, and treated with a broad-spectrum antibiotic, enfloxacin, a quinilone carboxylic acid derivative (Baytril, Haver, Bayvet Division, Chemagro, Etobicoke, Ontario, Canada), 200 mg/L in drinking water for the duration of the lipid feeding (n = 4 animals); group 4, SPF rabbits infected with *P. multocida* by nasal inoculation with a suspension of live organisms 14 days before EYD and euthanized after 50 days on EYD (n = 5 animals); group 5, conventionally raised rabbits with nasal discharge that on culture was positive for *P. multocida* (these animals were fed EYD as soon as the positive culture was received and the diet was continued for 50 days before euthanasia; n = 7 animals); group 6, conventionally raised rabbits with clinical signs of respiratory infection and positive nasal cultures for *P. multocida*, fed EYD for 50 days and treated with Baytril as above (n = 4 animals).

Maintenance

The uninfected SPF rabbits (groups 1, 2, and 3) were maintained in barrier rooms with positive pressure to an anteroom. Drinking water was autoclaved and chow was supplied from a dedicated supply stored within the barrier facility. Personnel entering the barrier rooms donned sterile gowns, gloves, bonnets, booties, and face masks and had no contact with conventionally raised rabbits in the previous 48 hours. The infected SPF rabbits (group 4) were housed in a separate barrier room and maintained in the same way as the noninfected SPF rabbits of groups 1, 2, and 3. The infected conventionally raised animals (groups 5 and 6) were housed in rooms separate from other rabbits.

Microbiological Evaluation

Samples for microbiological examination were submitted to the Department of Laboratory Medicine, Chedoke McMaster Hospitals, Hamilton, Ontario, Canada. Nasal swabs were collected from all animals on arrival and at euthanasia and from the animals in group 4 at 14 and 28 days after infection. One section of lung was collected at euthanasia and submitted for microbiological examination. All samples were transported to the microbiology laboratory in charcoal transport medium (Kulture, NCS Diagnostics, Etobicoke, Ontario, Canada) and cultured on plates containing 5% horse blood agar, chocolate agar, and MacConkey agar. Blood-containing media were incubated at 35°C in 5% CO_2 in air and the MacConkey agar in ambient air. Plates were examined after 24 and 48 hours for growth. Organisms were identified using established microscopic and biochemical tests.[20]

Hematology and Blood Chemistry

Blood samples were collected from an ear artery. Samples anticoagulated with EDTA were sent for complete blood counts, and serum was submitted for total cholesterol levels. The SPF rabbits were handled within the barrier room in which they were housed. Samples were collected from all rabbits on arrival and at 2-week intervals thereafter. Analysis of blood samples was carried out by the Department of Laboratory Medicine, Chedoke-McMaster Hospitals.

Clinical Evaluation

All rabbits were examined on arrival and daily throughout the experiment. Symptoms of infection, ie, nasal dis-

charge, coughing, sneezing, dyspnea, and skin abscesses were recorded. Rabbits were weighed periodically during the experiment.

Tissue Preparation for Morphological Examination

Aorta: VCAM-1

Four or five animals in each group were prepared for quantification of VCAM-1 expression by the aortic endothelial cells as previously described.[17,18] Under deep general anesthesia (35 mg/kg intravenous Somnitol, MTC, Cambridge, Canada), the right carotid artery was cannulated (PE190, Intramedic, Clay Adams Division of Becton Dickinson, Parsipanny, NJ) and the animals exsanguinated. The aorta was removed and immersed in ice-cold Tris (hydroxymethyl amino-methane)-buffered saline (TBS). Samples of the thoracic aorta at the level of the third and fifth intercostal arteries were processed for immunohistochemical identification of VCAM-1. The immunostaining was carried out at room temperature and was initiated as quickly as possible after removal of the tissue from the animal. The 1-cm, unopened aortic segments were placed into 10-ml scintillation vials containing 0.5 ml of protein blocking agent (AS/AP immunostaining kit from Bio/Can Scientific, Mississauga, Canada) for 10 minutes and then transferred to fresh scintillation vials containing the primary antibody to VCAM-1 (monoclonal antibody Rb1/9 supplied by Dr. M. I. Cybulsky, Brigham and Womens' Hospital, Boston, MA) for 2 hours. The tissue was washed three times for 5 minutes each in TBS to ensure that all of the unbound primary antibody was removed. The secondary antibody (alkaline-phosphatase-conjugated horse antibody to murine IgG; Vector Laboratories, Burlingame, CA), was then added to the tissue for 1 to 2 hours. To evaluate whether there was any nonspecific reaction to the IgG used above, immunohistochemical staining was also carried out using nonimmune mouse serum or an irrelevant, isotype-matched IgG monoclonal antibody (anti-cytomegalovirus; DAKO-CMV, CCH2, Dimension Laboratories, Mississauga, Ontario, Canada) at the same concentrations.

The endothelial cells were removed as a Häutchen preparation using cellulose acetate paper. The secondary antibody was visualized by using AS/AP substrate chromogen (Dimension Laboratories) and the nuclei was counterstained with hematoxylin. The preparation was mounted in a 30% glycerol/distilled water mixture and examined by light microscopy. The number of endothelial cells stained for VCAM-1 was counted and the total number of endothelial cells was counted by recording the number of consecutive fields defined by a calibrated graticule, in which the total number of endothelial cells was known.[17,18] Approximately 70,000 endothelial cells were examined from each aorta.

Aorta: Sudan IV

Sudan IV staining was carried out according to the method described by Mitchell and Schwartz[21] on the aortic surface from the aortic arch to the iliac bifurcation with the exception of the two segments of thoracic aorta removed for VCAM-1 evaluations. The extent of staining was evaluated by measuring the total area and the stained area using Sigma Scan (Jandel Scientific, San Rafael, CA) software and an IBM PC.

Aorta: Ultrastructure

The aortas of two uninfected lipid-fed SPF rabbits (group 2) and two conventionally raised, lipid-fed infected animals (group 5) were prepared for ultrastructural examination of the aortic endothelium by perfusion-fixation of the vascular system *in situ*.[17] One-micron toluidine-blue-stained sections from samples taken at the level of the third, fourth, and fifth intercostal arteries and the right renal artery were examined for the presence of foam cell lesions, and lesion areas were selected for ultrathin sections, which were examined in a Philips 301 transmission electron microscope (Eindhoven, The Netherlands).

Respiratory Tract

Sections of lung were removed, fixed in 10% buffered formalin, and embedded in paraffin wax. Hematoxylin and eosin (H&E)-stained 5-μm sections were examined by light microscopy by two pathologists with no knowledge of the treatment of the rabbits.

Statistical Analysis

Comparisons were carried out using a one-way analysis of variance, significance being assumed when $P < 0.05$. Data are presented as mean ± SEM.

Results

The numbers of endothelial cells per 10,000 cells expressing VCAM-1, the extent of Sudanophilia, the total cholesterol, and the total leukocyte count at euthanasia are presented in Table 1. The Sudan IV staining is illustrated in Figure 1, and an example of the immunohistochemical stain for VCAM-1 is illustrated in Figure 2. Representative micrographs illustrating the morphology of the aortic lesions are presented in Figures 3 and 4. The clinical response to infection in each group is presented in detail below, and the pulmonary changes are illustrated in Figure 5.

Effect of Lipid Feeding on VCAM-1 Expression

The number of aortic endothelial cells expressing VCAM-1 in uninfected SPF rabbits was 12 ± 3 per 10,000 cells (n = 4). In uninfected, lipid-fed SPF animals it was significantly increased ($P < 0.05$) to 22 ± 8 per 10,000 (n = 5). In lipid-fed SPF rabbits infected by *P. multocida* it was 106 ± 25 per 10,000 endothelial cells (n = 5), which is a highly significant increase over both uninfected normal-fed and lipid-fed rabbits. In conventionally

Table 1. Response by Infected and Uninfected SPF and Conventionally Raised Rabbits to EYD

	Total cholesterol (mmol/L)	VCAM-1 (per 10^4 cells)	Sudan IV (% aortic surface)	Total WBC ($\times 10^9$/L)	Weight gain (g/day)
Group 1, uninfected SPF (n = 4)	>1.3	12 ± 3	0	5.3 ± 0.3	19.2 ± 1.7
Group 2, uninfected SPF fed EYD (n = 5)	13.1 ± 1.2	22 ± 8	2.7 ± 0.8	8.2 ± 0.3	17.3 ± 2.5
Group 3, uninfected SPF, fed EYD, antibiotic treated (n = 4)	14.9 ± 4	18 ± 5	3.9 ± 1.5	6.3 ± 1.1	17.6 ± 2.8
Group 4, infected SPF, fed EYD (n = 5)	12.5 ± 1.0	106 ± 25	10.3 ± 1.8	13.4 ± 1.3	5.3 ± 1
Group 5, natural infection, fed EYD (n = 5)	13.5 ± 1.2	190 ± 51	8.3 ± 1.2	11.4 ± 0.5	16.1 ± 1.8
Group 6, natural infection, fed EYD, antibiotic treated (n = 4)	8.9 ± 0.6	96 ± 25	4.8 ± 1.4	10.4 ± 0.7	23.3 ± 1.1

Data are presented as mean ± SEM. significance was determined by $P < 0.05$ using one-way analysis of variance. VCAM-1, $P < 0.01$ for group 1 *versus* groups 2, 4, and 5, $P < 0.05$ for group 5 *versus* group 6, and no significant difference for group 3 *versus* group 6; Sudan IV, $P < 0.05$ for group 2 *versus* groups 4 and 5 and for group 6 *versus* group 5; white blood cell (WBC) count, $P < 0.01$ for group 1 *versus* group 2 and for group 5 *versus* group 6, $P < 0.05$ for group 2 *versus* groups 4 and 5, and no significant difference for group 3 *versus* group 6; weight gain, $P < 0.01$ for group 4 *versus* all other groups. There was no significant difference for group 2 *versus* group 3.

raised rabbits with naturally occurring infections, including *P. multocida*, there was a similar significant increase in the number of endothelial cells expressing VCAM-1 (190 ± 51 per 10,000 cells; n = 10), although the numbers were more variable. Treatment of infected conventionally raised rabbits with a broad-spectrum antibiotic, enfloxacin, significantly reduced the number of VCAM-1-positive endothelial cells (96 ± 25; n = 5) compared with the infected counterparts (190 ± 51) but did not affect the response in lipid-fed, uninfected SPF rabbits (18 ± 5 per 10,000; n = 4), which is different neither from the numbers seen in uninfected, untreated, lipid-fed SPF rabbits nor from the treated, infected rabbits.

Sudanophilia and Total Cholesterol Levels in Infected Rabbits

Feeding an egg yolk-supplemented diet significantly increased the total cholesterol to similar levels in all animals. The extent of Sudanophilia in uninfected SPF rabbits was 2.7 ± 0.8% and was significantly increased in infected rabbits, either those with naturally occurring infection (8.3 ± 1.2%) or those infected in the laboratory (10.3 ± 1.8%). It was significantly decreased in the animals with naturally occurring infection treated with antibiotic (4.8 ± 1.4%) and was no longer different from control levels. Examples of Sudan-IV-stained aortas are illustrated in Figure 1.

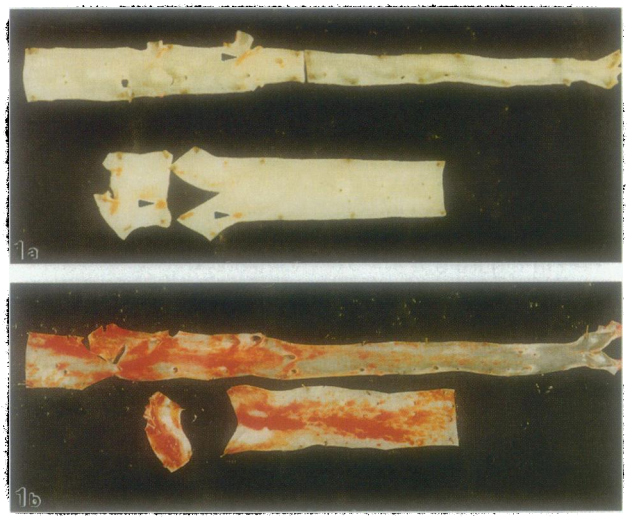

Figure 1. Aortas stained with Sudan IV. **a:** From an uninfected, SPF rabbit fed egg yolk diet for 50 days. The total lipid staining (**arrowheads**) involves 0.3% of the surface. **b:** From a naturally infected, lipid-fed rabbit. The Sudanophilic area occupies 20% of the surface.

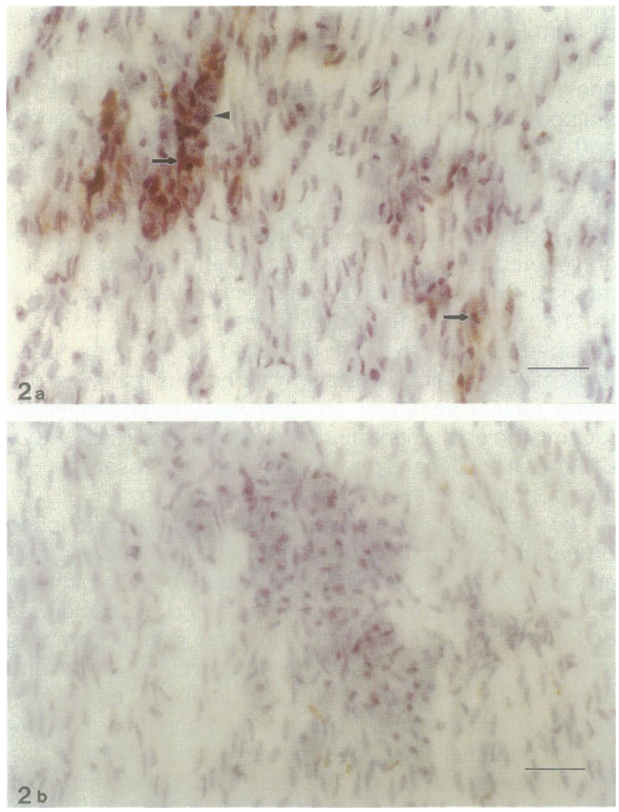

Figure 2. a: A portion of a Häutchen preparation form an egg-yolk-fed, infected SPF rabbit including a lesion area. The VCAM-1-positive endothelial cells are stained brown by the peroxidase end product and are present at the site of the disorganized cells over the lesion area (**arrow**) as well as remote from the immediate vicinity of the lesion. Foam cells can be seen within the lesion (**arrowhead**). The nuclei are counterstained blue by hematoxylin. **b:** A portion of a Häutchen preparation from an adjacent segment of the same aorta exposed to irrelevant isotype-matched antibody in place of the specific anti-VCAM-1. There is no reaction product in cells adjacent to a similar lesion. Bar, 100 μm.

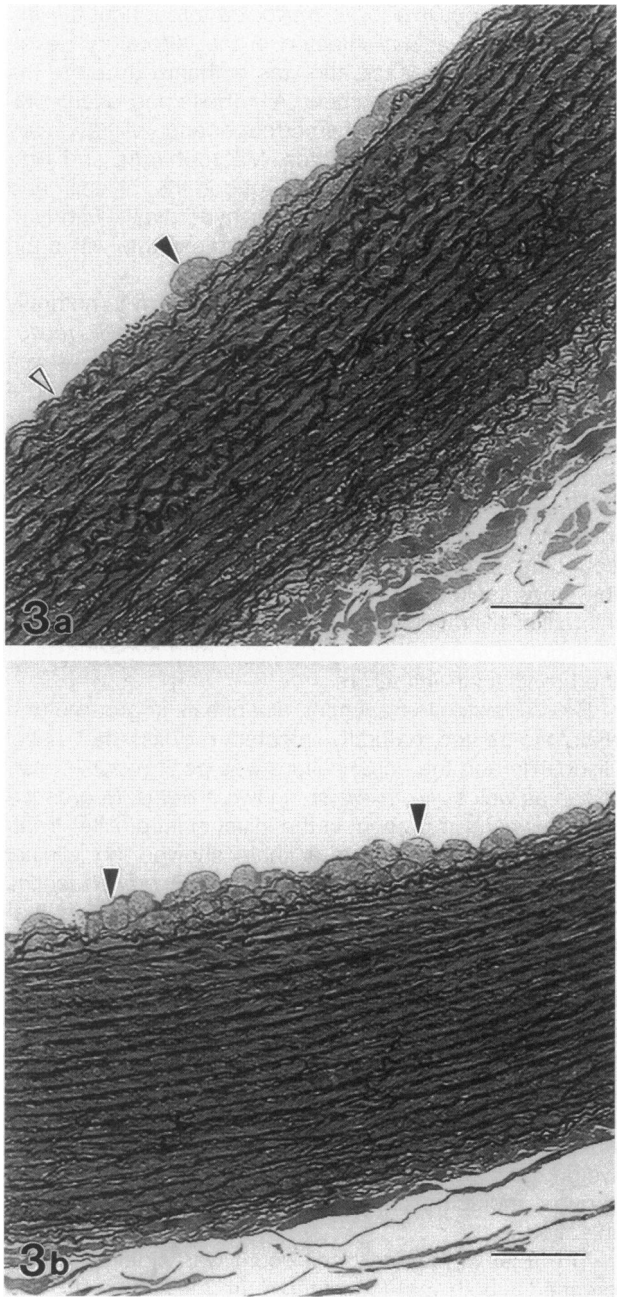

Figure 3. Light micrographs representative of lesions identified in the thoracic aorta of an uninfected, lipid-fed SPF rabbit (**a** and a naturally infected lipid-fed rabbit (**b**. Both contain intimal foam cells (**arrowhead**), but the lesion in the infected rabbit is deeper and extends over more of the vessel surface than does the lesion in the uninfected rabbit where uninvolved endothelium is evident (**open arrow**). Bar, 50 μm.

Morphology of Lesion Areas

Light microscopy of sections of the aortic arch and thoracic aorta from lipid-fed, conventionally raised, infected rabbits and uninfected SPF rabbits showed some intimal thickening in both regions. Intimal foam cell lesions were present in the aortic arch and thoracic aortas of rabbits from both groups, but in the infected animals the lipid-containing lesions were thicker and involved a larger portion of the vessel wall (Figure 3). Transmission elec-

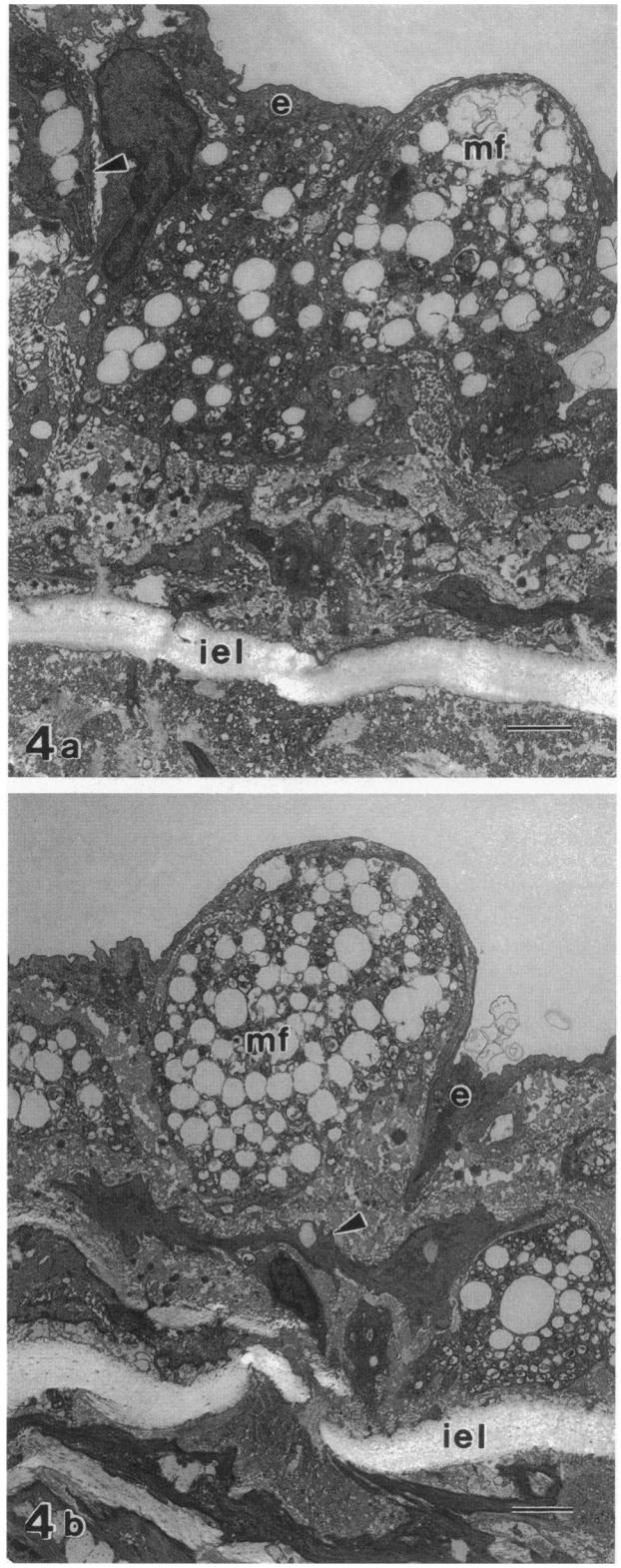

Figure 4. Transmission electron micrographs of foam cell lesions from an uninfected, lipid-fed SPF rabbit (**a** and a naturally infected lipid-fed rabbit (**b**. Both lesions contain macrophage-derived foam cells (mf) and lipid-containing smooth muscle cells (**arrowhead**). e, endothelial cell; iel, internal elastic lamina. Bar, 2 μm.

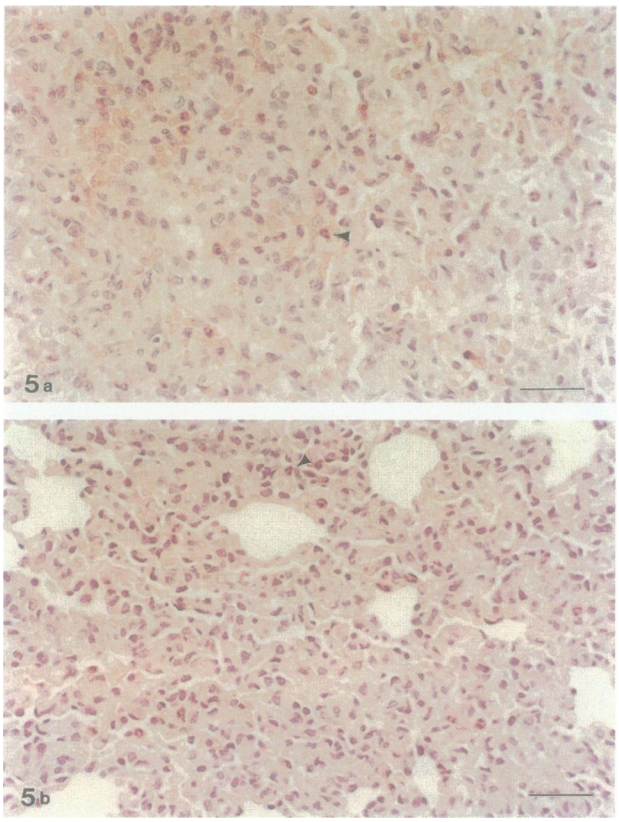

Figure 5. Light micrographs of portions of the lung. **a:** From a naturally infected rabbit. The tissue was positive for *P. multocida* on culture. There is evidence of polymorphonuclear leukocyte infiltration (**arrowhead**) and pneumonitis, with interstitial hyperplasia. **b:** From an antibiotic-treated naturally infected rabbit. There are fewer polymorphonuclear leukocytes and evidence of resolving interstitial inflammation. Bar, 100 μm.

tron microscopy revealed no major differences in the composition of the lesions that contained macrophage-derived foam cells, with some extracellular lipid and some lipid-containing smooth muscle cells (Figure 4). There was no evidence of microorganisms or inclusion bodies.

Clinical Symptoms and Microbiology

The uninfected animals (groups 1, 2, and 3) remained symptom-free and gained weight at a similar rate, irrespective of the diet or treatment with antibiotics. No abnormalities were detected on histological examination of the lungs. Both groups of lipid-fed rabbits (groups 2 and 3) showed a significant increase in total white blood cell count, 8.2 ± 0.3 × 10^9/L in untreated (group 2) and 6.3 ± 1.1 × 10^9/L in antibiotic-treated (group 3) animals, compared with the normal-fed uninfected rabbits (5.3 ± 0.3 × 10^9/L). Antibiotic treatment had no effect on any of the parameters measured in the lipid-fed SPF rabbits.

The nasal cultures from the uninfected SPF animals (groups 1, 2, and 3) at all times during the experiment grew *Branhamella catarrhalis* and *Moraxella sp*. At euthanasia, there was no bacterial growth on lung cultures.

After infection of the SPF rabbits in the laboratory (group 4), all animals had nasal discharge, and nasal swabs were positive for *P. multocida* throughout the experiment. One animal infected in the laboratory developed a severe abscess and was euthanized before the termination of the experiment. All rabbits had pneumonitis with focal areas of hemorrhage and infiltration by polymorphonuclear leukocytes. Macrophages and lymphocytes were scattered throughout the alveoli, and there was marked peribronchiolar hyperplasia with lymphocyte-containing lesions and some exudate within the airways.

Initial nasal swabs from all of the rabbits with naturally occurring infection (group 5) were positive for *P. multocida;* four were also positive for *Bordetella bronchiseptica* as well as *Branhamella catarrhalis* and *Moraxella sp*. The microbiological profile remained essentially the same for each rabbit throughout the experiment, and these organisms were cultured from lung samples taken at sacrifice. All animals displayed clinical symptoms of respiratory infection including nasal discharge and sometimes coughing. On histological examination the alterations in the lungs included pneumonitis characterized by focal interstitial infiltrate of alveolar macrophages and polymorphonuclear leukocytes. There was a mild hyperplasia of the bronchiolar epithelium.

Before antibiotic treatment, all animals in group 6 (the antibiotic-treated naturally infected rabbits) had nasal discharge and the nasal culture was positive for *P. multocida* as well as *Moraxella sp*. In two animals, *B. bronchiseptica* was also present in the initial culture. After treatment with antibiotic, the animals showed no clinical symptoms and neither *P. multocida* nor *B. bronchiseptica* was grown from nasal swabs or from the lungs at euthanasia.

The lung histology showed a slight reduction in the numbers of interstitial polymorphonuclear leukocytes and reduced interstitial thickening, consistent with a resolving pneumonitis. There was extensive accumulation of peribronchiolar lymphatic tissue (Figure 5).

The weight gain in all groups was similar except for the animals infected in the laboratory. In these rabbits, the weight gain was significantly less than any of the other groups, including the animals with naturally occurring infection.

The total white blood cell count was significantly increased in both lipid-fed infected groups (13.4 ± 1.3 and 11.4 ± 0.5 per 10^9/L), compared with uninfected, lipid-fed control animals (8.2 ± 0.3 per 10^9/L). Antibiotic treatment significantly ($P < 0.01$) reduced the total white blood cell count in naturally infected, lipid-fed rabbits (10.4 ± 0.7 per 10^9/L) when compared with untreated naturally infected animals (11.4 ± 0.5 per 10^9/L). This value was not different from that in antibiotic-treated, lipid-fed SPF rabbits (group 3; 6.3 ± 1.1 per 10^9/L).

Discussion

These observations demonstrate that a pulmonary bacterial infection is associated with enhanced deposition of arterial lipid in response to a cholesterol-enriched diet in SPF rabbits infected in the laboratory or in rabbits in-

fected by a naturally occurring exposure. The number of aortic endothelial cells expressing the leukocyte adhesion molecule VCAM-1 was increased approximately twofold by the infection[18] or by the hyperlipemia, but in combination, the increase was almost 10 times that seen in aortas of uninfected, normal-fed rabbits. It is therefore likely that the enhanced response to lipid feeding in rabbits with *P. multocida* pneumonia is in part due to the increased expression of leukocyte adhesion molecules such as VCAM-1. The response to lipid feeding is enhanced in other settings that will stimulate or injure endothelial cells, including immunological injury[22,23] and endothelial removal by a balloon catheter.[24] In these studies, intimal hyperplasia[23] and an accumulation of connective tissue, especially proteoglycan, was observed,[24] and it has been proposed that the potentiation of lipid deposition is related to the formation of a lipid-proteoglycan complex within the hyperplastic intima.[24,25] In the present study, in addition to an increased number of endothelial cells that express VCAM-1, there was morphological evidence of endothelial injury in the aortas of normal-fed rabbits with pneumonia.[18] This included increased numbers of leukocytes associated with the luminal surface of the aortic endothelial cells, which also showed blebs and craters, disorganized cell margins, and occasional fibrin-like deposits. There was extensive vacuolation of the endothelial cells and an increased expression of dilated rough endoplasmic reticulum. Evidence of increased intimal thickening in the aortas of infected rabbits compared with the control animals was minimal, but the egg-yolk-enriched diet was introduced after only 2 weeks of infection, and in the animals with naturally occurring infection, although the duration of the infection was unknown, it was not likely to have been prolonged. It is possible that a long-lasting infection may result in other intimal alterations including an accumulation of proteoglycan, and this would also be likely to potentiate further any accumulation of lipid.

The present observations have important implications in the development of atherosclerotic lesions associated with other types of infections such as *C. pneumoniae*, in that an initiating step, ie, the increased expression of leukocyte adhesion molecules, can occur in response to an inflammatory condition remote from the vessel wall, and this alone is sufficient to enhance the development of lipid-containing macrophage-rich lesions. Once established, these lesions would then be vulnerable to invasion by microorganisms by either direct entry, as leukocyte adhesion molecules are also adhesion sites for a number of virus particles,[26] or by transport within infected macrophages.

The induction of endothelial cells to express adhesion molecules such as VCAM-1 may be a consequence of a number of stimuli. VCAM-1 is induced by hyperlipemia *in vivo*[13] and by lysophosphatidylcholine, a component of atherogenic lipoproteins *in vitro*.[27] The expression of VCAM-1 by the aortic endothelium of rabbits has been observed *in vivo* in endotoxin-treated rabbits.[13] *In vitro* studies have demonstrated that cytokines, including tumor necrosis factor-α,[28] interleukin-1, and granulocyte/macrophage colony-stimulating factor will induce the expression of VCAM-1 and other adhesion molecules.[29–31] Thus, it is possible that the response to an infection such as *P. multocida* pneumonitis will be associated with the production of bacterial endotoxins and/or cytokines, which may result in the induction of endothelial-leukocyte adhesion molecules at sites remote from the infection. Furthermore, infection of endothelial cells *in vitro* with cytomegalovirus will induce VCAM-1[32] and ICAM-1.[33] ICAM-1 is also induced by herpes simplex 1 virus.[34]

There is an apparent synergistic effect of hyperlipemia superimposed on the pulmonary infection on the numbers of endothelial cells that express VCAM-1, and this can be anticipated from previous observations. Oxidized low-density lipoprotein will augment cytokine-induced VCAM-1 expression by endothelial cells *in vitro*.[35] In addition, the increase in numbers of cells that express VCAM-1 may be related to the accumulation of macrophages that have already been activated by the infection within the intima. VCAM-1 is generally seen in endothelium that overlies an intimal macrophage.[17] Macrophages accumulate within the intima in response to lipid feeding, and endothelial injury *in vitro* induced by alveolar macrophages is enhanced in the presence of *P. hemolytica*-derived lipopolysaccharide due to the increased release of macrophage-derived tumor necrosis factor-α and interleukin-1.[36] Thus, the enhanced expression of VCAM-1 in the presence of infection and hyperlipemia may be related to increased secretion of intima macrophage-derived cytokines consequent on activation by an infectious process, and the effect of cytokine stimulation may be further potentiated by oxidized low-density lipoprotein.

The dietary regime used in this study, although resulting in high levels of plasma cholesterol, was of short duration and was designed to induce some lipid deposition in all rabbits and thus to examine the initial phase of the development of atherosclerosis in infected and noninfected animals. The response to an egg-yolk-supplemented diet is similar to that seen after other cholesterol-enriched diets that induce a rise in plasma levels of total cholesterol. In addition to the formation of foam-cell-rich lipid-containing lesions, hyperlipemia has been associated with leukocytosis,[37] as in the present study. This increase was not altered by antibiotic treatment. The duration of the feeding in the present study resulted in intimal lesions containing macrophage-derived foam cells with some lipid-containing smooth muscle cells; thus, Sudan IV uptake, which allows for the measurement of vessel surface involvement, provided a valid evaluation of the extent of atherosclerosis. The weight gain in lipid-fed animals was not different from normal-fed animals or animals with naturally occurring infection but was significantly greater than in the animals infected in the laboratory fed the same diet. The enhanced atherosclerosis was seen in both groups of infected animals but was not dependent on differences in total cholesterol or weight gain.

The response by the animals with naturally occurring infection as shown by both Sudanophilia and cells expressing VCAM-1 was very variable. The variability may be related to the duration of the infection, which is not

known, and/or to the range of organisms involved and to the site of the infection. Although these animals were selected because they were infected by *P. multocida*, other potentially important pathogens were seen on nasal culture. It is also possible that the enhanced atherosclerosis seen in some animals was due to infection within the lesion, but no evidence of any microorganism was observed on transmission electron microscopic examination of the lesions.

Treatment of the animals with naturally occurring infection with a broad-spectrum antibiotic resulted in *P. multocida*-free nasal swabs, a reduction in the number of endothelial cells expressing VCAM-1, and no enhancement of the atherosclerosis. The total leukocyte count was significantly reduced compared with egg-yolk-fed naturally infected animals and was not statistically different from that in antibiotic-treated, uninfected, egg-yolk-fed SPF rabbits. Alterations in pulmonary histology showed less evidence of inflammation, with interstitial thickening, consistent with limited recovery from an infection. Thus, at least in the early stages of the response to infection, the exacerbation of the response to lipid-feeding appears to be related to the inflammatory response induced by the infection and responds to antibiotic therapy. Additional support for an inflammatory process remote from the vessel wall being implicated in vascular disease is provided by a recent prospective study of apparently healthy men in which treatment by aspirin reduced the circulating levels of the acute-phase reactant, C-reactive protein, and lowered the risk for myocardial infarction.[38] This beneficial effect was considered to be related to the anti-inflammatory properties of aspirin rather than its anti-platelet effects.

From our previous study of the response by normal-fed rabbits to *P. multocida* pneumonia,[17] we suggested that such a respiratory infection by an organism that is a common pathogen in laboratory rabbits has the potential to adversely affect observations such as alterations in vascular pathology. The present results illustrate this possibility. It must be noted, however, that the animals in this study showed marked clinical symptoms of rhinitis and coughing, which would be unlikely to be ignored in any study to examine the response to an atherogenic diet.

These observations demonstrate that an antibiotic-sensitive bacterial pneumonia will enhance the development of dietary atherosclerosis in rabbits and that this enhanced response is related to endothelial perturbation and the expression of leukocyte adhesion molecules.

Acknowledgments

We thank Dr. M. A. Packham for helpful discussion and advice.

References

1. Hajjar DP: Viral pathogenesis of atherosclerosis: impact of molecular mimicry and viral genes. Am J Pathol 1991, 139:1195–1211
2. Wissler RW: Significance of Chlamydia pneumoniae (TWAR) in atherosclerotic lesions. Circulation 1995, 92:3376
3. Kuo C, Shor A, Campbell LA, Fukushi H, Patton DL, Grayston JTJ: Demonstration of Chlamydia pneumoniae in atherosclerotic lesions of coronary arteries. J Infect Dis 1993, 167:841–849
4. Gyorkey F, Melnick L, Guinn GA, DeBakey ME: Herpesviridae in the endothelial and smooth muscle cells of the proximal aorta in atherosclerotic patients. Exp Mol Pathol 1984, 40:328–339
5. Bruggeman CA, Van Dam-Mieras MCE: The possible role of cytomegalovirus in atherogenesis. Prog Med Virol 1991, 38:1–26
6. Melnick JL, Hu C, Burek J, Adam E, DeBakey ME: Cytomegalovirus DNA in arterial walls of patients with atherosclerosis. J Med Virol 1994, 42:170–174
7. Datta SK, Tumilowicz JJ, Trentin JJ: Lysis of human smooth muscle cells infected with Herpesviridae by peripheral blood mononuclear cells: implications for atherosclerosis. Viral Immunol 1993, 6:153–160
8. Fong IW, Chiu B, Viira E, Fong MW, Jang D, Mahony J: Rabbit model for Chlamydia pneumoniae infection. J Clin Microbiol 1997, 35:48–52
9. Hajjar DP, Pomerantz KB, Falcone DJ, Weskler BB, Grant AJ: Herpes simplex virus infection in human arterial cells: implications in atherosclerosis. J Clin Invest 1987, 80:1317–1321
10. Ross R: The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 1993, 362:801–809
11. O'Brien KD, Allen MD, McDonald TO, Chait A, Harlan JM, Fishbein D, McCarty J, Ferguson M, Hudkins K, Benjamin CD: Vascular cell adhesion molecule-1 is expressed in human coronary atherosclerotic plaques: implications for the mode of progression of advanced coronary atherosclerosis. J Clin Invest 1993, 92:945–951
12. Wood KM, Cadogan MD, Ramshaw AL, Parums DV: The distribution of adhesion molecules in human atherosclerosis. Histopathology 1993, 22:437–444
13. Li H, Cybulsky MI, Gimbrone MA Jr, Libby P: An atherogenic diet rapidly induces VCAM-1, a cytokine-regulatable mononuclear leukocyte adhesion molecule, in rabbit aortic endothelium. Arterioscler Thromb 1993, 13:197–204
14. Ardehali A, Laks H, Drinkwater DC, Ziv E, Drake TA: Vascular cell adhesion molecule-1 is induced on vascular endothelia and medial smooth muscle cells in experimental cardiac allograft vasculopathy. Circulation 1995, 92:450–456
15. Briscoe DM, Yeung AC, Schoen EL, Allred EN, Stavrakis G, Ganz P, Cotran RS, Pober JS: Predictive value of inducible endothelial cell adhesion molecule expression for acute rejection of human cardiac allografts. Transplantation 1995, 59:204–211
16. Tanaka H, Sukhova GK, Swanson SJ, Clinton SK, Ganz P, Cybulsky MI, Libby P: Sustained activation of vascular cells and leukocytes in the rabbit aorta after balloon injury. Circulation 1993, 88:1788–1803
17. Richardson M, Hadcock SJ, DeReske M, Cybulsky MI: Increased expression in vivo of VCAM-1 and E-selectin by the aortic endothelium of normolipemic and hyperlipemic diabetic rabbits. Arterioscler Thromb 1994, 14:760–769
18. Richardson M, Fletch A, Delaney K, DeReske M, Wilcox LH, Kinlough-Rathbone RL: Increased expression of vascular cell adhesion molecule-1 by aortic endothelial cells of rabbits with *Pasteurella multocida* pneumonia. Lab Animal Sci 1997 47:52–60
19. Ringler DH, Peter GK, Chrisp CE, Keren DE: Protection of rabbits against experimental pasteurellosis by vaccination with a potassium thiocyanate extract of *Pasteurella multocida*. Infect Immun 1985, 49: 498–504
20. Balows A, Hausler WJ, Herrman KL, Isenberg HD, Shadomy HJ (editors): Manual of Clinical Microbiology, ed 5. Washington, DC, American Society for Microbiology, 1991, p 410
21. Mitchell JRA, Schwartz CJ, Zinger A: Relationship between aortic plaques and age, sex, and blood pressure. Br Med J 1964, 1:205–209
22. Lamberson HV Jr, Fritz KE: Immunological enhancement of atherogenesis in rabbits: persistent susceptibility to atherogenic diet following experimentally induced serum sickness. Arch Pathol 1974, 98: 9–16
23. Hardin NJ, Minick CR, Murphy GE: Experimental induction of atherarteriosclerosis by the synergy of allergic injury to arteries and lipid rich diet. Am J Pathol 1973, 73:301–327
24. Alavi MZ, Galis Z, Li Z, Moore S: Dietary alterations of plasma lipoproteins influence their interactions with proteoglycan enriched extracts from neointima of normal and injured aorta of rabbit. Clin Invest Med 1991, 14:419–431

25. Alavi MZ, Wasty F, Li Z, Galis ZS, Ismail N, Moore S: Enhanced incorporation of [14C]glucosamine into glycosaminoglycans of aortic neointima of balloon-injured and cholesterol-fed rabbits in vitro. Atherosclerosis 1992, 95:59–67
26. Haywood AM: Virus receptors: binding, adhesion strengthening, and changes in viral structure. J Virol 1994, 68:1–5
27. Kume N, Cybulsky MI, Gimbrone MA Jr: Lysophosphatidylcholine, a component of atherogenic lipoproteins, induces mononuclear leukocyte adhesion molecules in cultured human and rabbit arterial endothelial cells. J Clin Invest 1992, 90:1138–1144
28. Carlos TM, Schwartz BR, Kovach NL, Yee E, Rosso M, Osborn L, Chi-Rosso G, Newman B, Lobb R, Harlan JM: Vascular cell adhesion molecule-1 mediates lymphocyte adherence to cytokine-activated cultured human endothelial cells. Blood 1990, 76:965–970
29. Klein CL, Köhler H, Bittinger F, Wagner M, Hermanns I, Grant K, Lewis JC, Kirkpatrick CJ: Comparative studies on vascular endothelium in vitro. I. Cytokine effects on the expression of adhesion molecules by human umbilical vein, saphenous vein and femoral artery endothelial cells. Pathobiology 1994, 62:199–208
30. Pober JS, Bevilacqua MP, Mendrick DL: Two distinct monokines, interleukin 1 and tumor necrosis factor, each independently induce biosynthesis and transient expression of the same antigen on the surface of cultured human vascular endothelial cells. J Immunol 1986, 136:1680–1687
31. Vades MA, Gamble JR, Smith WB: Regulation of myeloid blood cell-endothelial interaction by cytokines. Adhesion: Its Role in Inflammatory Disease. Edited by Harlan JK, Liu DY. New York, WH Freeman, 1992, pp 65–81
32. Waldman WJ, Knight DA: Cytokine-mediated induction of endothelial adhesion molecule and histocompatibility leukocyte antigen expression by cytomegalovirus-activated T cells. Am J Pathol 1996, 148: 105–119
33. Sedmak DD, Knight DA, Vook NC, Waldman JW: Divergent patterns of ELAM-1, ICAM-1, and VCAM-1 expression on cytomegalovirus-infected endothelial cells. Transplantation 1994, 58:1379–1385
34. Brankin B, Hart,MN, Cosby SL, Fabry Z, Allen IV: Adhesion molecule expression and lymphocyte adhesion to cerebral endothelium: effects of measles virus and herpes simplex 1 virus. J Neuroimmunol 1995, 56:1–8
35. Khan BV, Parthasarathy SS, Alexander RW, Medford RM: Modified low density lipoprotein and its constituents augment cytokine-activated vascular cell adhesion molecule-1 gene expression in human vascular endothelial cells. J Clin Invest 1995, 95:1262–1270
36. Sharma SA, Olchowy TWJ, Yang Z, Breider MA: Tumor necrosis factor-α and interleukin 1α enhance lipopolysaccharide-mediated bovine endothelial cell injury. J Leukocyte Biol 1992, 51:579–585
37. Feldman DL, Mogelesky TC, Liptak BF, Gerrity RG: Leukocytosis in rabbits with diet-induced atherosclerosis. Arterioscler Thromb 1991, 11:985–994
38. Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH: Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. N Engl J Med 1997, 336:973–979