Studies of Hypercholesterolemia in the Nonhuman Primate

I. Changes that Lead to Fatty Streak Formation

Agostino Faggiotto, Russell Ross, and Laurence Harker

Morphologic studies resulting from events that occur during the development of the lesions of atherosclerosis were studied in chronic, diet-induced hypercholesterolemia in a series of nonhuman primates. Within 12 days of hypercholesterolemia in Macaca nemestrina, monocytes became adherent to the surface of the endothelium. These monocytes appeared to migrate subendothelially, accumulate lipid, and become lipid-laden macrophages (foam cells). Within a month, a "serofibrinous insudate" formed together with variable numbers of subendothelial lipid-laden macrophages. By the second month, foam cells increased in number, often in multilayers, to form a fatty streak. Concomitantly, the luminal surface of the arteries became increasingly irregular due to the subendothelial accumulation of foam cells. Numerous monocytes continued to attach to the endothelial surface over the fatty streaks, and many of them appeared to enter the intima and participate in the growth of the fatty streaks. Lipid-laden smooth muscle cells appeared in small numbers and formed two to four layers between the macrophages and the internal elastic lamella at 2 to 3 months. During the third month of hypercholesterolemia, endothelial cell continuity over the lipid-laden macrophages became interrupted, exposing the underlying foam cells to circulating blood. Foam cells were then readily observed in whole blood smears, suggesting that many of the lipid-laden macrophages leave the intima and enter the circulation.

After 4 months, significant endothelial denudation was found in the iliac artery and many exposed macrophages were covered by adherent platelets in the form of a mural thrombus. Thus, the early components of atherosclerosis induced by chronic hypercholesterolemia centered around the monocyte-macrophage and its interaction with endothelium in the induction of the fatty streak. Subsequent changes that lead to macrophage-smooth muscle interactions, platelet-macrophage interactions, and platelet-endothelial interactions appeared to set the stage for the development of more advanced proliferative lesions.

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Hypercholesterolemia is the most common risk factor associated with atherosclerosis in Western society. Consequently, many different animal models and experimental designs have been used to study experimental diet-induced atherosclerosis. The rabbit has been widely studied because of its size, relative rapidity of lesion formation, and convenience of use. However, the rabbit has several limitations as a model for human atherosclerosis, partly because the lesions have a different anatomic distribution. The rat, pigeon, and dog are also useful; however, several disadvantages of these animal models have been noted. The rat, dog, and pigeon have a different aortic structure than humans, and there is a different evolution of atherosclerosis in the dog. In contrast, swine and primates develop spontaneous atherosclerosis similar to humans in structure and distribution. Consequently, studies of diet-induced atherosclerosis in these animals provide information presumably more applicable to humans.
Diet-induced atherosclerosis has been well studied in the nonhuman primate.27-32 Most of these studies have emphasized observations at single time points rather than an examination of the evolution of the lesions. The duration of the hyperlipidemic diets have varied from 3 months to 2 years and, as a result, the morphologic picture is variable and the mode of lesion development remains unclear. Moreover, genetic factors alter the susceptibility of individual monkeys to the atherosclerotic process itself, as is the case in humans.6, 11, 33

In this study we examined the cellular events that occur in the arteries and blood during the onset and progression of atherosclerosis in the hypercholesterolemic pigtail monkey (Macaca nemes trina) after 12 days and at monthly intervals up to 13 months on the experimental diet.34 The changes observed were correlated with the duration and pattern of hypercholesterolemia with time. The first segment of this report focuses on the events that occur through the first 4 months of hypercholesterolemia, and asks the following questions:

- What is the nature of the evolution of the fatty streak?
- What is the role of the monocyte and the macrophage in fatty streak formation?
- How does the presence of macrophages and of platelets relate to the integrity of the endothelial monolayer?
- What are the similarities and differences in type, sequence, and anatomic site of lesion formation as compared with humans?

**Methods**

**Nonhuman Primates**

Fourteen male pigtail monkeys (Macaca nemestrina) between 3 and 5 years of age, with an average weight of 4 to 7 kg, were used in this study. Ten were randomly assigned to the atherogenic diet and four received the control diet. Procedures followed were in accordance with the Guide for Care and Use of Laboratory Animals, as issued by the U.S. Institute of Laboratory Animal Resources.

**Diet**

The diet mixture used in this study (Table 1) contained 42% fat and was supplemented with cholesterol for a total concentration of 0.5 g of cholesterol for every 100 g of diet. While the percentage of fat is comparable to that in the average American diet,35, 36 the cholesterol supplement is approximately 2.5 times greater.36

Nine months prior to the initiation of this study, 70 monkeys were fed the same high-saturated-fat/high-cholesterol diet for 1 month to enable us to select animals with comparable susceptibility to hypercholesterolemia. Monkeys were selected for the study when they developed a total cholesterol level of between 300 mg/dl and 500 mg/dl after 1 month on the diet. On regular monkey chow these monkeys have an average plasma cholesterol level of 119 ± 29 mg/dl. When fed the atherogenic diet, they rapidly developed hypercholesterolemia, so that their plasma cholesterol levels doubled by the twelfth day on the diet. The four control animals were fed a normal, unsupplemented diet (Table 1). Two of the control animals had been screened 9 months earlier and two were randomly chosen from the untested colony of the University of Washington Regional Primate Center.

Each monkey was housed in a single cage and fed twice a day ad libitum. Dietary intake was monitored and monthly determinations were made of body weight and of plasma concentrations of cholesterol and triglycerides. Total and differential blood cell counts were also determined monthly.

**Tissue Preparation**

Before sacrifice, the left carotid and femoral arteries were removed under ketamine anesthesia and placed in 35 mm dishes over a gauze soaked in Dulbecco-Vogt medium supplemented with 20 mM HEPES (pH 7.4). The dishes were kept on ice. These arteries were then rapidly frozen in isopentane in liquid nitrogen and stored at −70°C. The sections were examined for esterase content with the alpha-naphthyl-acetate esterase reaction (Sigma Chemical Company, St. Louis, Missouri).

**Blood Samples**

Approximately 100 ml of blood was drawn into 10 ml of acid-citrate dextrose solution (ACD) for lipid and lipoprotein analysis: the concentrations of VLDL, IDL, LDL, HDL, vs. HDL, were determined according to established Standard Lipid Research Clinic procedures.37-40

Peripheral blood smears were directly evaluated on glass slides, or preparations were made by using a blood film centrifuge (Microcapillary centrifuge, Model MB, International Equipment Company). Glass slides were stained using the Diff-Quik staining procedure (Dade Diagnostics, Incorporated) and examined with a Zeiss Photomicroscope III.

**Sacrifice**

Under ketamine anesthesia and following removal of the left carotid and the femoral arteries for histochemical studies, each animal was heparinized and both femoral veins were cannulated to provide simultaneous perfusion runoff. The right carotid artery was then connected to a perfusion apparatus with a catheter and an 18-gauge vessel tip. The perfusion apparatus consisted of two fluid reservoirs in parallel. These contained: sodium phosphate-albumin buffer (4.6% wt/vol glucose, 20 mM HEPES, pH 7.4, 4%
### Table 1. Atherogenic Diet Mixture and Control Diet

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Atherogenic diet</th>
<th>Protein (g)</th>
<th>Lipid (g)</th>
<th>Carbo (g)</th>
<th>Cal/100 g of diet</th>
<th>Chol (mg)</th>
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<th>Carbo (g)</th>
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Atherogenic diet was equivalent to 0.300 mg cholesterol/cal diet; protein = 18% of calories; fat = 42% of calories; carbohydrates = 40% of calories. Atherogenic diet was supplemented with 0.36 g cholesterol/100 g diet to yield a final concentration of 0.5 g/100 g diet.

Control diet was equivalent to 0.050 g cholesterol/cal diet; protein = 22% of calories; fat = 29% of calories; carbohydrates = 49% of calories.

bovine albumin) and 2.5% glutaraldehyde in sodium phosphate buffer, pH 7.2, respectively. These were in line with a pressure gauge, a pressure source, a flowmeter in the delivery line, and valves to select each of the fluids for perfusion, and a catheter inserted into the carotid artery. Since flow is directly proportional to pressure difference, the perfusion apparatus was used to determine indirectly the Intracarotid pressure in the animal during the infusion of fixative by placing a flowmeter in the delivery tubing. Generally, the blood pressure was maintained at normal arterial values throughout fixation (i.e., 90–110 mm Hg). A flow of about 400 ml/min was required to maintain the arterial pressure within these limits when bilateral femoral venous outflow was unchecked. Fixation was begun by infusing sodium phosphate-albumin buffer at room temperature for several minutes to remove blood from the larger vessels. This was followed by 2.5% glutaraldehyde sodium phosphate buffer for 30 minutes. When the vascular system was completely washed free of blood cells with fixative, the venous outflow was clamped to conserve fixative while maintaining the intrarterial pressure at 100 mm Hg. Following in vivo pressure-perfusion fixation of approximately 30 minutes, the arterial tree was dissected intact and maintained in 2.5% glutaraldehyde in sodium phosphate buffer at 4°C for 90 minutes.
Sampling for Morphologic Studies

The entire arterial tree was divided according to the diagram in Figure 1, and each segment was placed in 2.5% glutaraldehyde with sodium phosphate buffer. Each of the cross sections was subdivided into two cylindrical segments that were fixed for 24 hours. One segment was processed for light microscopy and transmission electron microscopy, and examined on at least three levels. The other segment was prepared for scanning electron microscopy and the entire surface of the segment was examined.

Light and Electron Microscopic Procedures

Following fixation, each arterial segment (1–3 mm rings) was washed three times with 0.1 M phosphate-buffered saline. The samples were postfixed in 1% OsO₄ buffered with 0.1 M phosphate for 3 hours at room temperature. After three rinses with distilled water (10 minutes each), the tissues were immersed for 15 minutes in 50% ethanol, then transferred to 70% ethanol + 3% uranyl acetate for 1 hour. Dehydration was completed using a graded series of ethanol concentrations from 90% to 100%. Each arterial ring was subsequently embedded in Epon 812.

Three separate 1 μ sections were obtained from each arterial segment and were stained for light microscopy with basic fuchsin-methylene blue and examined and photographed with a Zeiss Photomicroscope III. Thin sections were taken from regions in each block that had been selected previously by light microscopy. Each section was stained with uranyl acetate followed by lead citrate and examined with a JEOL 100B electron microscope at 60 kV.

Scanning Electron Microscopy (SEM) Procedures

Most of the adventitial tissue was carefully removed from cross-sections of the artery to be examined by SEM. The vessel segments were opened lengthwise and pinned out on Teflon plates with minute pins (College Biological Supply, Seattle, Washington). Tissues were rinsed in phosphate-buffered saline with constant mixing seven times for 3 minutes each, placed in 1% OsO₄ (Pelco, Tustin, California) in phosphate-buffered saline for 2.5 hours and rinsed again as above. Tissues were then incubated in 1% aqueous Thiocarbodhazide (Eastman Kodak Company) at room temperature for 1 hour, rinsed as described above, and incubated in 1% OsO₄ in distilled water for 2 hours. Following the same rinsing procedure, the specimens were dehydrated through graded solutions of ethanol (30%, 50%, 70%, 90%, 95%, 98%, 100%) and subjected to critical-point drying with carbon dioxide. Tissues were then coated with a 300 Å layer of gold and examined with a JEOL 35C scanning electron microscope at 15 kV.

Electron Microscopy

Image Analysis

The areas occupied by foam cells in all of the transmission electron micrographs containing lipid-laden subendothelial macrophages were digitized using a Tektronix computer plus a digitizer board. The data were grouped according to the time interval each animal had been on the diet. Only micrographs showing the greatest diameter of cell per group were considered for data analysis as being most representative of the cross-sectional dimension of each foam cell.
Results

Effect of Diet on Blood Cell Counts and Lipid Levels

Figure 2 presents the plasma cholesterol and triglyceride levels of each animal at baseline, at 12 days (for two animals), and at monthly intervals following initiation of the atherogenic diet. The hematocrit, platelet count, and white blood cell counts were within normal range and did not significantly change in any of the animals throughout the study. Lipid and lipoprotein analyses for 14 controls, eight animals after 1 month, and four animals after 3 months on the diet, are shown in Table 2. Intake of the atherogenic diet resulted in progressive increases in plasma cholesterol; triglyceride levels remained low and relatively constant (Figure 2). Following the first month of diet, the LDL cholesterol increased approximately six times the initial value, and by the fourth month was approximately eightfold that of the controls. HDL and HDLg decreased but were variable; VLDL and IDL cholesterol were variable as shown in Table 2.

The Control Animals

Gross Examination

Two of the control animals belonged to the population of monkeys that had been previously screened for 1 month to test their response to the high-fat diet, 9 months before the beginning of this investigation. These animals had been normocholesterolemic during this interval. Two additional monkeys that had never been exposed to the high-fat diet were examined as a second set of controls. Gross examination of the arterial segments of each of these four animals did not demonstrate any visible abnormalities.

Light Microscopy

Several isolated subendothelial round cells thought to be macrophages were observed in focal areas of cross sections of arteries taken from all four control animals. Their location had no apparent relationship to anatomic level or to branches or bifurcations. Each of the two control animals that had transiently received the high-fat diet 9 months previously, contained relatively rare individual, subendothelial, lipid-laden foam cells randomly distributed throughout the arterial tree. It was not clear whether the presence of these macrophages was a natural occurrence, or if it was due to the prior exposure to the high-fat diet that had initially been used for screening purposes. Similar-appearing, lipid-containing cells were comparably distributed in the two monkeys that had not been exposed to the high-fat diet.

Transmission Electron Microscopy

The occasional lipid-containing subendothelial cells found in the control monkeys appeared morphologically to be lipid-laden macrophages, as determined by the following criteria: 1) the cells lacked a basement membrane and contained well-developed ruffles or folds on their surface, often compressed by the overlying endothelial cells or by adjacent foam cells (Figure 3A); 2) they contained numerous secondary lysosomes; 3) they had a characteristic distribution of chromatin in their nuclei that is quite distinct from that in smooth muscle cells; and 4) subendothelial macrophages with and without lipid inclusions were uniformly positive to nonspecific esterase staining (not shown). Rare lipid-containing smooth muscle cells were found immediately luminal

Table 2. Effects of Hypercholesterolemia on Lipoprotein Content of Plasma

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<tr>
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<th>Control diet (n = 14)</th>
<th>1 month (n = 8)</th>
<th>3 months (n = 4)</th>
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<tr>
<td>TC</td>
<td>124.1 ± 6.2</td>
<td>433.6 ± 18.6</td>
<td>604.7 ± 15.1</td>
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<tr>
<td>VLDL</td>
<td>6.4 ± 1.2</td>
<td>15.7 ± 2.9</td>
<td>6.5 ± 2.3</td>
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<td>IDL</td>
<td>2.1 ± 0.4</td>
<td>61.7 ± 3.6</td>
<td>31.2 ± 11.1</td>
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<tr>
<td>LDL</td>
<td>56.9 ± 2.3</td>
<td>246.0 ± 13.1</td>
<td>368.7 ± 28.4</td>
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<td>HDL&lt;sub&gt;2&lt;/sub&gt;</td>
<td>41.0 ± 2.3</td>
<td>33.2 ± 1.5</td>
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<td>5.3 ± 1.1</td>
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<td>TG</td>
<td>69.1 ± 10.9</td>
<td>36.6 ± 3.8</td>
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<td>7.2 ± 1.3</td>
<td>3.4 ± 0.7</td>
<td>2.1 ± 0.6</td>
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The values presented are mean values for total cholesterol (TC) and triglyceride (TG) broken down as lipoprotein values. The figures represent mean values ± standard error, and were derived from measurements from 14 control animals and are compared with eight monkeys studied after 1 month on the hypercholesterolemic diet and four monkeys examined after 3 months on the hypercholesterolemic diet.
to the internal elastic lamina. These rare smooth muscle cells did not appear to be enlarged or deformed by their inclusions and were surrounded by a basement membrane at their periphery (Figure 3 B). Occasional extracellular deposits of material that morphologically resembled lipid debris were present in the narrow intimal space of animals previously fed the high-fat diet, whereas those not exposed to the diet lacked any of this material.

**Scanning Electron Microscopy**

The endothelial surfaces of the aortas of the control animals were covered by smooth, structurally intact endothelium with normal cell outlines (Figure 4 A). As seen in Figure 4 B, there were occasional small focal areas that protruded into the lumen (ca. 25 μm × 10 μm). After examination by SEM, the isolated segment containing these protrusions was embedded in epoxy resin, sectioned, and examined by TEM. Cross-sectional examination of these protrusions using TEM revealed that they consisted of individual lipid-laden macrophages underlying intact endothelium, as demonstrated in Figure 3 A.

**Temporal Changes In the Lipid-Fed Monkeys**

**Twelve Days**

Upon gross examination, the arteries were morphologically similar to the control animals. However, striking changes were found when the luminal surfaces of the arteries were examined by scanning electron microscopy. All segments of the aortic arch, thoracic aorta, and 83% of the segments of the abdominal aorta and iliac arteries revealed numerous leukocytes, predominantly monocytes (as determined morphologically and by nonspecific esterase staining) adherent to the endothelial surface (Figure 5 A, B, C). Many of the attached monocytes appeared to have been fixed while migrating on the surface of the endothelium, since they exhibited ruffles at their leading edge and tail-like protrusions previously described for motile leukocytes in culture (see Figure 5 B). Several monocytes were observed between endothelial cells (as shown in Figure 5 D), perhaps in the process of entering the intima and localizing in the subendothelium, as implied by the subendothelial accumulation of lipid-laden cells following 1 month of the experimental diet (see below).

**One Month**

After 1 month of hypercholesterolemia, several of the arterial segments contained focal areas of protrusions covered by intact endothelium. These protrusions were due either to localized foam cells or to the presence of an amorphous intimal insudate located principally at branches and bifurcations and similar in appearance to the "serofibrinous insudation" de-
Figure 4. Endothelial surface in control arteries. A. This scanning electron micrograph demonstrates the characteristic appearance of the endothelium of the control monkeys. Bar = 100 μ. B. Endothelial folds overlap at the borders of adjacent cells. In this particular micrograph the endothelium covers individual subendothelial macrophages which contain lipid droplets. This specimen was taken from a monkey that had been on the high-fat diet for 1 month and then was off the diet for 9 months. Bar = 10 μ.
Figure 5. Electron micrographs demonstrating leukocytes adherent to the endothelium after 12 days on an atherogenic diet. A. Adherent leukocytes (mostly monocytes) were found scattered in patches at all levels of the aortic tree. Some of the cells appear spread on the surface, whereas most are rounded in appearance. Bar = 10 μ. B. Several leukocytes appear to have been fixed while in motion on the surface of the endothelium. Classical ruffling at the leading edge and a tail-like protrusion at the rear indicates the direction of the movement of one of the cells in this micrograph. Bar = 10 μ. C. A monocyte adherent to the endothelium. Note the interposition of surface folds of the monocyte with depressions in the surface of the endothelium. Bar = 1 μ. D. The monocyte in this micrograph has come between two endothelial cells as it was apparently in the process of entering the intimal space. Bar = 1 μ.

scribed previously.43-47 These protrusions represent 63% of all the arterial segments examined. Using transmission electron microscopy, no clearly recognizable structures were resolved in the insudate (Figure 6). Individual lipid-laden macrophages exhibiting a ruffled surface and lacking a surrounding basement membrane were present within the insudate. The endothelium over these areas was intact but convoluted and irregular, and contained numerous attached monocytes. In some regions the endothelium was 0.5 μ or less in thickness (Figure 6).

Detectable morphologic differences were observed between the macrophages in the animals fed the lipid diet for 1 month compared to animals in the
control population. Many macrophages in the former were more than double in diameter and contained numerous lipid droplets. These macrophages were more numerous in the fat-fed animals, and were located in the aortic arch and at branches and bifurcations throughout the aorta.

Two Months

Following 2 months of hypercholesterolemia, focal accumulations of subendothelial foam cells were sufficiently increased that fatty streaks were macroscopically visible in 72% of the segments of the arterial tree. Examination of these fatty streaks with the transmission electron microscope demonstrated large numbers of intimal macrophages containing variable quantities of lipid droplets. Some of the fatty streaks consisted of multilayered foam cells. The cells were four- to sixfold larger in diameter than the macrophages observed in the control animals (Figure 7), and modest numbers of lipid-laden smooth muscle cells were observed beneath the macrophages.

Three Months

The number and diameter of intimal lipid-laden macrophages was increased by seven- to eight-fold by the third month of hypercholesterolemia so that the fatty streaks contained two to three layers of macrophages and were distributed throughout the arterial tree preferentially at branches and bifurcations. Examination of these fatty streaks by scanning electron microscopy revealed a striking nodular pattern in which the lesions were covered by a highly irregular, convoluted, intact endothelium. Leukocytes continued to be observed attached to the surface of the endothelium in 19% of the segments examined (Figure 8).

Some endothelial cells were decreased in density as compared with their immediate neighbors, although they remained attached to the neighboring cells by normal-appearing junctional complexes (see Figure 9). By 3 months of hypercholesterolemia, the arterial surface contained focal sites of endothelial separation over some of the fatty streaks. This endothelial disruption resulted in exposure of macro-
Figure 7. A fatty streak containing two layers of subendothelial foam cells after 2 months of hypercholesterolemia. The large lipid-filled macrophages shown in this transmission electron micrograph are distributed focally in multilayers. The cells are four- to six-fold larger than lipid-laden macrophages observed in control animals such as those in Figure 4B. There is a small amount of intercellular matrix and some lipid debris. The macrophages maintain a close relationship to the intact endothelium. The endothelium is markedly stretched so that the cells have become very thin. Bar = 1 μ.

To test the possibility that lipid-laden macrophages could emerge into the blood, we examined peripheral blood smears from the hyperlipidemic monkeys. Foam cells (Figure 11) were present in the blood smears of 3- and 4-month hypercholesterolemic animals. The cells were 25 to 45 μ in diameter and proved to be relatively fragile, even when prepared using a blood film centrifuge. Very few of these cells were capable of adhering to plastic cell culture dishes. Little to no lipid was present in the spleen or livers of the hypercholesterolemic animals examined at autopsy.

Four Months

Following 4 months of hypercholesterolemia, all of the changes described above appeared to have progressed. Of all segments examined, 50% demonstrated some form of endothelial dysjunction over the fatty streaks. Monocytes were attached to the surrounding endothelium in 22% of all segments examined, suggesting a continuing dynamic process of entry into the fatty streaks and lesion progression. The surface of the fatty streaks became irregular and
Figure 8. A surface view of a fatty streak showing the endothelium after 3 months of hypercholesterolemia. The surface has become highly irregular and forms a striking nodular pattern with relatively deep crevices between the nodules. Leukocytes (arrow) can be seen adherent to the endothelial cells that are stretched by the accumulated subendothelial macrophages. Continuing leukocyte attachment and subendothelial migration appear to participate in continued fatty streak expansion. Bar = 10 μ.

Figure 9. The endothelium over a lipid-containing smooth muscle cell after 3 months of hypercholesterolemia. Occasional clear endothelial cells were observed in the animals fed the atherogenic diet for 3 months. These cells remained attached to normal appearing endothelium on either side. Bar = 1 μ.
Figure 10. Exposed foam cells after 3 months of hypercholesterolemia. A. A lipid-laden macrophage can be seen in what is interpreted as egress from the artery wall in a monkey that had been on the diet for 3 months. Note the numerous lamellipodia at the luminal aspects of the cell that suggest that its direction of motion may be into the arterial lumen. Bar = 10 \mu m. B. A similar group of exposed lipid-laden macrophages, as seen by scanning electron microscopy after 3 months of hypercholesterolemia. An endothelial cell bridges over an exposed macrophage (arrow) and deforms the surface of one of the egressing foam cells. Bar = 10 \mu m.

Figure 11. Foam cells in the blood. Circulating foam cells were found in increasing numbers in peripheral blood smears after the third month of hypercholesterolemia, as shown in this light micrograph. Bar = 10 \mu m.
formed crevices and domes, and were covered by extraordinarily thin endothelial cells which folded upon themselves in the depth of a crevice (Figure 12). Foam cells were often attached to the underlying intimal connective tissue despite the absence of an overlying endothelium. By transmission electron microscopy they sometimes appeared to be in the process of degeneration, since they were frequently found to have discontinuous membranes and appeared necrotic.

A particularly striking change was observed in some of the fatty streaks in the iliac arteries after 4 months of hypercholesterolemia. This consisted of separation of the endothelium over fatty streaks with subsequent loss of some of the macrophages, leading to platelets adherent to exposed foam cells (Figure 13 A) and to areas of exposed subendothelial connective tissue. When the connective tissue was exposed, it was often covered with multiple layers of adherent, spread platelets (Figure 13 A and B). Such areas of endothelial denudation were first observed in 30% of the segments of the iliac arteries at this time period.

Discussion

Previously we described the arterial changes induced in hypercholesterolemic pigtail monkeys observed after 2 years of dietary hypercholesterolemia and in hypercholesterolemia combined with mechanically (catheter) induced injury. The present study was designed to establish the temporal sequence of changes that occur in each segment of the arterial tree following the institution of dietary hypercholesterolemia. The companion report describes the changes involved in fatty streak progression to fibrous plaques.

The genetic factors influencing the development of atherosclerosis are complex and poorly understood. Consequently, in different individuals the atherosclerotic process is highly variable with time. Because of this, we chose to study a relatively homogeneous population of animals with respect to their response to an atherogenic diet. This goal was attained by choosing animals that responded in the range of 300–500 mg of plasma cholesterol/dl from 70 male monkeys in the colony that had been placed on the standard atherogenic diet for 1 month. After entry into the study, some of these animals responded to the diet by reaching, in some cases, plasma cholesterol levels as high as 1000 mg/dl.

It has long been known that high levels of LDL are associated with accelerated atherosclerosis, both in experimental animals and in humans. Evidence for the atherogenic nature of LDL has also been derived from studies of patients with familial hypercholesterolemia (Type II hyperlipoproteinemia). Cholesterol levels in heterozygotes range between 250 and 500 mg/dl, whereas homozygotes have levels between 500 and 1000 mg/dl, 80% of which is present in LDL. Both groups of patients have extensive, premature atherosclerosis, and death can occur between ages 1 and 30. Thus, the degree of diet-induced hypercholesterolemia achieved in this study (Figure 2, Table 2) is comparable to that found in some human lipid disorders. Consequently, we postulated that systematic collection of data from these animals might help to explain how lesions form and progress at these markedly elevated levels of lipid, and possibly at lower levels over longer periods of time. The data presented in this and the accompanying study help to explain the relationships between monocyte-endothelial interactions, subendothelial macrophage accumulation, endothelial injury, endothelial-macrophage-platelet interaction, and fatty streak formation and progression to fibrous plaques.

Control Animals

Occasional subendothelial macrophages were found in all control animals and their presence sug-
Figure 13. Platelet adherence at sites of endothelial denudation. A. In this scanning electron micrograph the endothelium covering a fatty streak has detached from its neighbors, contracted, and exposed part of the intimal subendothelial matrix. This matrix is covered by several layers of flattened, adherent platelets. Individual leukocytes are present together with macrophages (arrows) that were presumably constituents of the fatty streak before it lost its endothelial cover. One of the macrophages is covered by a platelet thrombus (P). This type of interaction was more commonly observed at later times.34 The cracks in the tissue are drying artifacts that developed during the processing of the tissue. Bar = 10 μ. B. Breaks in endothelial junctions and contraction of endothelium has resulted in exposure and loss of intimal foam cells. Exposure of connective tissue matrices with adherence of platelets is seen more clearly in this higher magnification of the region shown in 13A. Bar = 10 μ.
gests that these cells probably pursue their normal function as scavengers in the arteries just as they do in other tissues of the body, and that this physiological role may be amplified in hypercholesterolemia.

Macrophages are prominent in early descriptions of arterial lesions, but previous studies of experimentally induced atherosclerosis have not focused on the role of macrophages. In recent years there has been renewed interest in this cell and its role in the onset and progression of atherosclerosis. Clearly identified foam cells lacking the morphological characteristics of smooth muscle cells were found in lesions and prompted the speculation that these cells might result from smooth muscle dedifferentiation, or that these lipid-laden cells might be derived from monocytes or lymphocytes.

Comparisons between the monkeys that had been hypercholesterolemic for 1 month followed by a normocholesterolemic diet for 9 months, with the animals in the experimental group that were examined immediately after 1 month of hypercholesterolemia suggest that the previously exposed animals also developed fatty streaks consisting of accumulated macrophages that reversed over time. This conclusion is based on a greater than 50% decrease in the number of arterial segments containing subendothelial macrophages in the animals that had been taken off the diet after 1 month.

**Hypercholesterolemic Animals**

**Monocyte Attachment and Entry**

The sequential examination of the hypercholesterolemic animals demonstrates that the monocyte/macrophage is critically involved in the onset and progression of fatty streaks and possibly of fibrous plaques as well. Although some fatty streaks may be derived from pre-existing intimal macrophages, they appear to be principally derived from blood monocytes as shown in Figure 6, and as has been described in the rabbit, swine, and cynomolgus monkey. We postulate that the relatively rapid appearance of subendothelial macrophages is in response to some change in the surface of the endothelial cell and/or the macrophage coupled with a chemotactic signal, possibly like that suggested in recent in vitro studies. Chemotactic factors could result from lipoproteins or other plasma constituents that had been previously taken up, modified, and deposited in the subendothelium by the endothelium. Subendothelial macrophages could then accumulate in the intima to form fatty streaks. Furthermore, our data show that monocytes continuously enter the intima during the hypercholesterolemic state.

**Fatty Streak Formation and Progression**

Focal interstitial intimal “serofibrinous insudation” was found concomitant with the early subendothelial accumulation of macrophages, and is accompanied by alterations in the surface contours of the endothelium. The insudate may be a mixture of plasma components and/or their products. Our observations in the hypercholesterolemic monkeys and those in studies of rabbits and swine lead us to suggest that the macrophages contribute to development of the lesions of atherosclerosis by establishing the fatty streaks. They do this by initiating the process through their normal function as scavengers.

The presence of the intimal insudate that accompanies monocyte entry into the intima appears to be an early and transient phenomenon, since it was seldom observed after the second month of hypercholesterolemia. In contrast, increasing numbers of tightly apposed, lipid-laden macrophages are responsible for the development of the fatty streaks. During the first 3 months the endothelium remains intact. However, as the lesions enlarge, the endothelium becomes extremely thin and the surface of the lesions become highly deformed.

Endothelial cells in culture are capable of endocytosing and presumably transcytosing many substances, including LDL. They have also been shown to be capable of modifying LDL in culture so that such LDL becomes incorporated by macrophages via the “modified LDL receptor.” If this process occurs in vivo, modified LDL might attract monocytes and effect their conversion to foam cells. Whether the insudate is due to loss of selective permeability, to active transport of plasma components, or to a combination of these is not clear. However, data have been reported which suggest that endothelial cells are capable of creating a lipoprotein gradient that is not present if the endothelium is lost.

The proteoglycan component of atherosclerotic lesions has been shown to be capable of avidly binding to lipoproteins, particularly LDL and VLDL, to form insoluble complexes. HDL fractions containing a preponderance of arginine-rich apoproteins also bind to arterial proteoglycans. Such binding substances are apparently not present in the adventitia of arteries and are absent from veins. Consequently, the presence of such binding factors in arteries may predispose them to lipid accumulation. By 3 to 4 months of hypercholesterolemia, fatty streaks are macroscopically visible and, as observed in humans, are preferentially found in proximity to branches and bifurcations. This focal, uneven accumulation of several layers of foam cells induces a profound alteration in the surface of the artery which may affect the blood flow at these sites. This, in turn, could lead to further injury to the overlying endothelium that may assist in the progression of the lesions of atherosclerosis.

**Presence of Foam Cells in the Circulation**

Between the third and fourth months breaks are observed between endothelial cells overlying some fatty streaks, exposing single macrophages or, commonly, groups of macrophages. Similar observa-
tions were made in the rabbit by Poole and Florey\textsuperscript{46} and have been re-emphasized by recent observations in swine by Gerrity et al.;\textsuperscript{12-14} they have also been confirmed recently in rabbits by Baumgartner (personal communication).

We have confirmed and extended the important observations of Gerrity et al.;\textsuperscript{12-14} and, although individual micrographs do not establish whether foam cells are in the process of entering or leaving an artery, two observations suggest that the cells are emigrating. First, the exposed macrophages appear both singly and in large clusters surrounded by endothelium that has smooth borders. If circulating foam cells had entered the artery wall, they probably would have done so individually. Furthermore, in several instances, groups of exposed lipid-laden macrophages are partially covered by cytoplasmic extensions of endothelial cells. This would be difficult to explain if macrophages were in the process of entering rather than leaving the artery.

In vitro studies mentioned previously\textsuperscript{69} suggest that modification of LDL by endothelial cell lead to uptake of such lipoproteins by macrophages that contain receptors for this form of LDL. Modified lipoproteins as well as other substances may be part of the subendothelial milieu,\textsuperscript{45,48,64} suggesting that macrophages may attempt to clear them from the intima, since one of the principal functions of this cell is to act as a scavenger.

Finally, circulating foam cells were found in abundance by 4 months, but only rarely at earlier times.\textsuperscript{34} They appear to behave like effete cells, as they are fragile and have partially lost their ability to adhere to plastic surfaces. Examination of the spleen and liver of hyperlipidemic animals did not demonstrate accumulation of either intra- or extracellular lipids.

Endothelial Denudation

By the fourth month, focal endothelial denudation and exposure of the subendothelium was common in the iliac arteries. Platelets adhered to the subendothelium and to exposed macrophages which were also attached in these regions. This observation is important since these arteries developed proliferative fibrous plaques within the subsequent 1 to 2 months.\textsuperscript{34} In contrast, lesions in the upper abdominal aorta and thoracic aorta consisted of extensive fatty streaks after 4 months of hypercholesterolemia.\textsuperscript{34}

The factors producing endothelial cell denudation are not evident. The cholesterol content of the plasma membranes of the endothelial cells may increase due to the rapid exchange that occurs upon chronic exposure to elevated levels of lipoproteins.\textsuperscript{85} It is possible that following the increase in plasma concentrations of LDL and VLDL, endothelial cells might increase in their rate of transport, modification, and subendothelial deposition of lipoproteins and their products. Accumulation of these substances might induce subendothelial macrophages to become foam cells and might help to recruit monocytes by chemotaxis, increasing the number of intimal macrophages and foam cells to the point where fatty streaks become visible. The fatty streaks markedly distort the characteristic smooth lining of the artery. The increased tension on endothelial junctions and marked thinning of the endothelial cells induced by enlarging lipid-laden macrophages, or the increased susceptibility of endothelial cells to shear forces and eddy currents at particular anatomic sites could lead to separation of endothelial cells, exposure of foam cells, and their eventual release into the circulation.

Another possibility that needs to be explored may be that the foam cells in the fatty streaks may directly damage the overlying endothelium by the formation of products that are toxic or lytic to the cells. Oxidation products such as superoxide anion, hydroxyl radicals, or other free radicals together with both neutral and acid proteolytic enzymes and other macrophage constituents could be damaging to the endothelium.\textsuperscript{86}

Of particular interest are the recent observations by Hessler et al.\textsuperscript{87} that oxidation of LDL and VLDL renders them highly toxic to fibroblasts, and presumably endothelium and smooth muscle, in culture. These authors demonstrated that the toxic agent was a lipid-extractable moiety, and since lipid peroxides and oxidized sterols can occur in the lesions of atherosclerosis, their potential role in endothelial injury needs to be further explored.

As we discuss in the accompanying paper, these changes in some fatty streaks may show how a lesion, presumably initially a part of a protective inflammatory response, may be altered and in some cases develop into a connective tissue-rich, proliferative, smooth muscle lesion, the fibrous plaque.

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Studies of hypercholesterolemia in the nonhuman primate. I. Changes that lead to fatty streak formation.

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