Leukocytosis in Rabbits With Diet-Induced Atherosclerosis

David L. Feldman, Therese C. Mogelesky, Barbara F. Liptak, and Ross G. Gerrity

In cholesterol-fed rabbits the extent of monocyte involvement in atherogenesis may be influenced by the level of circulating leukocytes during hypercholesterolemia. We characterized the leukocytosis in rabbits fed either a 0.25% or a 0.1% cholesterol-enriched diet (0.25% or 0.1% rabbits, respectively). Circulating leukocytes were elevated by 1 week of feeding, and the elevation was sustained for at least 30 weeks. Differential counts were unchanged. Immature leukocytes were not seen, indicating that the leukocytosis was not due to premature release of bone marrow cells. Animals were free of bacterial or parasitic disease; selected rabbits with leukocytosis had normal body temperatures. Spleen weights averaged at least 100% higher in 0.25% rabbits but did not show histological evidence for hematopoiesis that could account for the leukocytosis. At approximately 22 weeks there was a second rise in leukocytosis in bilirubinemic 0.25% rabbits, suggesting that in the late stages of hypercholesterolemia, leukocytosis is related to liver failure. Cholesterol-fed rabbits also showed thrombocytosis. Existing leukocytosis and hypercholesterolemia were reversed to pretreatment levels by switching the rabbits to chow diets. In bone marrow from 0.25% rabbits, the mean number of cells per gram was greater (p<0.05) than that from normocholesterolemic rabbits. In 0.25% rabbits, the fraction of blood mononuclear cells showing phagocytosis of immunoglobulin G–coated red blood cells did not differ from that of controls, suggesting an unchanged population of these cells with regard to Fc and phagocytic function during hypercholesterolemia. These data suggest an effect (direct or indirect) of hypercholesterolemia on the production of leukocytes in the bone marrow and/or on the circulation kinetics of leukocytes in the blood. (Arteriosclerosis and Thrombosis 1991;11:985–994)

Leukocytes, specifically monocytes, play an important role in atherogenesis. Cells thought to be of monocytic origin are present in developing foam cell lesions1–11 where they engulf lipid and form most of the volume of fatty streaks. Recent evidence suggests that such cells migrate from the lesion back into the blood,1,2,12 possibly serving as a lipid clearance system for the artery,3 at least during the early stages of hypercholesterolemia. An understanding of the involvement of monocytes in atherogenesis, therefore, is of great importance, especially because monocyte-derived foam cell lesions (fatty streaks) may be the precursors to the occlusive “fibromuscular” atherosclerotic lesions.13,14 Chemotaxis undoubtedly plays an important role in recruitment of monocytes to potential lesion sites.9,15–18 Although macrophage proliferation in experimental lesions has recently been reported,19 it is unlikely that this terminally differentiated cell type undergoes large numbers of divisions. Therefore, the number of monocytes available for foam cell formation is expected to be dependent largely on the number of circulating leukocytes (monocytes) that are available for chemotactic recruitment. Thus, implicit to an understanding of the formation of foam cell lesions is a knowledge of the status of the circulating pool from which foam cells ultimately arise. The present study was undertaken as a first step in a broader attempt to understand the relation between the number of circulating leukocytes and the formation of foam cell lesions. In these experiments we report the effect of prolonged hypercholesterolemia on the number of circulating leukocytes in experimental atherosclerosis and the characteristics of the hematologic changes during this time.

Methods

**Animals and Standard Procedures**

Male New Zealand White (NZW) rabbits weighing 2.5–3.0 kg were used. All procedures conformed to the guidelines of the Animal Care Committee at Hoffmann-La Roche. On arrival all animals were quarantined for 2 weeks, during which time exami-
weeks of cholesterol feeding in experiment 1 and were measured weekly. Body weights and food consumption differential counts were made on Wright's-stained preparations performed with a Coulter S Plus IV counter, and terms 2, 3, and 4. Complete blood counts were monitored periodically except for experiment 4).

In all experiments leukocyte levels and serum chemistry parameters were monitored periodically from blood collected from the marginal ear vein. Blood was drawn between 9 and 11 AM. Measurements of serum cholesterol, total bilirubin, albumin, and total protein were performed on a Cobas Bio centrifugal analyzer. Blood for complete blood counts and differentials was taken beginning at 7 centafugal analyzer. Blood for complete blood counts and total protein were performed on a Cobas Bio centrifugal analyzer. Blood for complete blood counts from the cholesterol-fed rabbits. Additional studies were conducted that sought information on the number and state of activation of mononuclear phagocytes from hypercholesterolemic rabbits. First, we sought histochemical confirmation of the estimates of the number of circulating monocytes that had been obtained from cytological examination of blood smears from the cholesterol-fed rabbits. Second, we examined the effect of hypercholesterolemia on the ability of mononuclear cells to ingest immunoglobulin G (IgG)-coated sheep erythrocytes, an index of activation. 

For histochemical studies of mononuclear cells, experimental animals were fed a 0.25% cholesterol/2% peanut oil diet and were killed after 8, 16, or 32 days. Peanut oil was added as part of another study. Previous work (T. C. Mogerlesky, B. F. Liptak; unpublished data) showed that leukocyte levels were unaffected by the addition of 2% peanut oil to the 0.25% diet when fed for 32

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*Sacrificed due to gastric hairball. †Not eating; sacrificed 3 weeks early.

### Experimental Design

Several studies were conducted to determine the changes in circulating leukocyte levels during early and late stages of moderate and severe hypercholesterolemia. Experiment 1 tested the effect of long-term cholesterol feeding on the number of circulating leukocytes in rabbits with severe hypercholesterolemia (Table 1). Experimental animals were fed Purina Rabbit Chow ad libitum supplemented with 0.25% cholesterol (in this article, termed "0.25% diet") and killed after 4, 7, or 10 months.

In experiment 2 we studied the effect of moderate hypercholesterolemia on circulating leukocyte levels. In this study rabbits were fed Purina Rabbit Chow supplemented with 0.1% cholesterol ("0.1% diet") and killed at the same time intervals as in experiment 1. A balanced design with two replicates was used (Table 1). In this study the development of leukocytosis was monitored from the initiation of cholesterol feeding.

In experiment 3 we tested whether diet-induced leukocytosis was reversible. Fifteen rabbits were fed the 0.25% diet for 13 weeks (Table 1). At this time leukocytosis had been established, and half of the group was then switched to an unsupplemented chow diet while the remaining animals continued to receive the atherogenic diet. All rabbits were killed 31 weeks after the beginning of the experiment.

A separate experiment was conducted to determine whether bone marrow from hypercholesterolemic rabbits contained more cells compared with that from normcholesterolemic rabbits. Age-matched male NZW rabbits were fed Purina Rabbit Chow concurrently with experimental animals for each experiment (except for experiment 4).
days. Control rabbits were fed unsupplemented rabbit chow for the same times. Mononuclear cells from control and experimental animals were separated in parallel from sodium citrate–treated whole blood by the use of Ficoll-Paque (Pharmacia, Piscataway, N.J.), cytocentrifuged onto glass slides, and stained for acid phosphatase (Sigma Chemical Co., St. Louis, Mo., kit No. 386). The specificity of staining was confirmed by incubating the cells in the absence of substrate and by inhibition with tartrate. The percentage of cells showing acid phosphatase–positive granules (monocytes) was determined by counting 200 cells per slide. Cyttoplasmic staining for acid phosphatase–positive granules was classified as negative (very few granules present), intermediate (sparse number of granules), or extensive staining (cells filled with granules). Counts were made by an observer who was unaware of the treatment group.

For studies of the ability of mononuclear cells from rabbits to ingest IgG-coated erythrocytes, rabbits were fed the 0.25% cholesterol diet or unsupplemented chow for varying periods. Monocyte-enriched populations of mononuclear cells were isolated from whole blood by density gradient centrifugation. Glass-adherent cells from these preparations were greater than 98% acid-phosphatase positive (monocytes). Such cells were incubated for 1 or 2 hours at 37°C with IgG-coated erythrocytes in eight-well chamber slides (Lab-Tek, Miles Scientific, Naperville, Ill.). Parallel control monocytes from normocholesterolemic and hypercholesterolemic rabbits were treated with IgM-coated erythrocytes or phosphate-buffered saline-treated erythrocytes. Adherent (noningested) erythrocytes were removed by brief hypotonic lysis, and the slides were stained with May-Grünwald Giemsa stain. The number of monocytes that contained one or more erythrocytes was counted and expressed as the percentage of the 500 cells that were counted per well. The observer was unaware of the treatment group.

Additional Measurements

To address the possibility that leukocytosis in cholesterol-fed rabbits was part of an inflammatory reaction, rectal body temperatures were measured in seven leukocytotic rabbits from experiment 1. Measurements were taken during a 2½-hour period with a multichannel temperature recorder equipped with automatic readout. The body temperature at the end of this period was recorded.

Sacrifice

Animals were killed by an overdose of Nembutal, immediately followed by perfusion fixation with 2% paraformaldehyde in 0.13 M phosphate buffer (pH 7.3). Aortic samples were taken for histological analysis; the results of these evaluations will be reported elsewhere.

Livers and spleens were removed, weighed, and processed for histology. Hematoxylin- and eosin-stained paraffin sections of liver were evaluated for general pathology and especially for the presence of Coccidia. Similarly stained sections of spleen were assessed for general pathology and extramedullary hematopoiesis.

Statistics

The Shapiro-Wilk test was used to test the data for normal distribution. Statistical significance (p<0.05) in parameters from serum chemistries and complete blood counts was determined by the Mann-Whitney U test. For circulating leukocytes, probability values obtained by the Mann-Whitney U test were subjected to Bonferroni corrections to account for "multiple looks." Cell counts from acid phosphatase staining and Fc-mediated phagocytosis were analyzed by Student’s t test after arcsin transformation of the percentages.

Results

General Health/Mortality

Apart from the symptoms related to liver failure (mentioned below), the rabbits did not show outward signs of poor health. Most experimental animals survived the dietary treatments and gained weight during the respective studies. Three rabbits that had stopped eating their respective diets were killed for humane reasons before their scheduled sacrifice; they were eliminated from further analysis after this time. None of the experimental animals showed signs of malnutrition, based on body weights and serum protein levels.

Experiments 1 and 2: Effect of Severe and Moderate Hypercholesterolemia on Leukocyte Levels

Serum cholesterol. Feeding of the 0.25% diet resulted in a prompt steady increase in serum cholesterol levels. The mean serum cholesterol level over the 43 weeks of feeding was 886.9 mg/dl. Hypercholesterolemia peaked at 14 weeks of feeding and declined thereafter (Figure 1). This decline was not accompanied by a reduction in food consumption. In contrast, serum cholesterol levels in rabbits fed the 0.1% diet rose more slowly (Figure 1), and the mean serum cholesterol level was 245.5 mg/dl. The combined mean serum cholesterol levels of control rabbits in both experiments remained unchanged (28.9 mg/dl).

Hematology. Mean values for total circulating leukocytes were elevated with respect to those of controls by 54% (p<0.05, Figure 2) after 7 weeks of feeding the 0.25% diet. After 18 weeks of feeding, a secondary more pronounced leukocytosis was seen only in bilirubinemic animals. The onset of this leukocytosis was temporally correlated with the onset of bilirubinemia.

In rabbits fed the 0.1% cholesterol diet, mean leukocyte counts were elevated after 1 week of feeding (Figure 3), reaching a value that was 24% greater than that of controls at 7 weeks. As with the 0.25% diet group, bilirubinemic animals contributed to wide variations in leukocyte levels. However, such variations were not seen until 34 weeks and were much less frequent than with the 0.25% diet.
The temporal relations between the onset of hypercholesterolemia and leukocytosis were studied in rabbits fed the 0.25% diet in Experiment 3 (Figure 4). Leukocyte levels were elevated slightly by 1 week and rose in parallel with serum cholesterol levels. A qualitatively similar relation was seen in rabbits fed the 0.1% diet in Experiment 2 (data not shown). On a statistical basis however, leukocytosis was poorly correlated with serum cholesterol levels at all intervals in each study, regardless of whether the absolute

**Figure 1.** Line plot of serum cholesterol levels (mg/dl) in rabbits that were fed cholesterol-enriched diets (●, ●) and their respective controls (○, ○). Severe hypercholesterolemia was induced with the 0.25% cholesterol-enriched diet, while a more moderate hypercholesterolemia was seen after feeding the 0.1% cholesterol-enriched diet. Bars indicate SEM. Different n's over time (weeks) reflect animal sacrifice.

**Figure 2.** Line plot of circulating leukocyte levels (10⁶/mm³) in rabbits that were fed the 0.25% cholesterol-enriched diet (●) or chow diet (○). Leukocytosis is evident by 7 weeks of feeding and is sustained through 43 weeks. Asterisks indicate p<0.05 from the Mann-Whitney U test. When data were subjected to Bonferroni correction, points at weeks 7–14 still achieved statistical significance at the p<0.0045 required by this correction. Bars indicate SEM. Different n's over time (weeks) reflect animal sacrifice.

**Figure 3.** Line plot of circulating leukocyte levels (10⁶/mm³) in rabbits fed the 0.1% cholesterol-enriched diet (●) or chow diet (○). Sustained leukocytosis is shown throughout the experiment although it is less severe than that in rabbits in Figure 2. Asterisks indicate p<0.05 from the Mann-Whitney U test. When data were subjected to Bonferroni correction, no points achieved statistical significance at the p<0.0038 required by this correction. Bars indicate SEM. Different n's over time (weeks) reflect animal sacrifice.
Leukocyte counts or their percentages above the control leukocyte levels were used in the computation. Thus, the highest correlation between leukocytes and cholesterol levels was \( r = 0.776 \). Despite this high correlation between leukocytosis and serum cholesterol, in rabbits with established leukocytosis withdrawal from the 0.25% cholesterol diet resulted in a sharp reduction of serum cholesterol (Figure 5) and a slower but progressive diminution in circulating leukocytes to pretreatment levels (Figure 6).

Because cholesterol feeding was associated with anemia, a compensatory increase in hematopoiesis was considered as a possible mechanism for leukocytosis. Therefore, the temporal relation between leukocytosis and anemia was examined in rabbits from experiment 3. While leukocytosis began within 1 week of cholesterol feeding, anemia was not seen until 6–8 weeks (data not shown), indicating that the leukocytosis was not due to a compensatory mechanism.

Regardless of the atherogenic diet used, differential counts did not show a consistent statistically significant increase in any cell type in experimental rabbits. Differential counts for monocytes were especially variable. In the 0.25% diet group, neutrophil levels tended to be elevated at 22 weeks of feeding and thereafter. Immature leukocytes were seen in differentials only at 22 weeks in one chow-fed and one 0.25% cholesterol–fed animal.

The lack of an effect of cholesterol feeding on the number of circulating monocytes (as a percentage of the total leukocytes), as assessed by cytological criteria, was confirmed histochemically in mononuclear preparations. Significant differences were not revealed in the percentage of cytocentrifuged mononuclear cells that stained positive for acid phosphatase in chow-fed versus cholesterol–fed leukocytotic animals, regardless of the duration of hypercholesterolemia. Furthermore, there was no significant difference in pretreatment versus treatment values for acid phosphatase staining in hypercholesterolemic animals.

Thrombocytosis was evident at 18 weeks and thereafter in the 0.25% cholesterol group (Figure 7). In rabbits fed the 0.1% atherogenic diet, platelets were elevated \((p<0.05)\) above those of controls between 3–18 weeks (Figure 8), although pretreatment levels were elevated over those of controls to a similar degree in these experimental rabbits.

**Figure 4.** Line plot showing the temporal relation between the onset of hypercholesterolemia and leukocytosis in rabbits that were fed the 0.25% cholesterol–enriched diet. Leukocyte levels \((\bullet, 10^3/mm^3)\) rose in parallel with serum cholesterol levels \((\circ, \text{mg/dl})\) after initiation of the 0.25% cholesterol–enriched diet. Each point represents the mean value for 15 animals.

**Figure 5.** Line plot showing cholesterol levels in 15 rabbits that were fed the 0.25% cholesterol–enriched diet \((\bullet)\) for 13 weeks; at that time eight rabbits were switched to the chow diet \((\circ)\) and seven rabbits remained on the cholesterol–enriched diet. Removal of the cholesterol diet resulted in a prompt reduction of serum cholesterol levels \((\text{mg/dl})\) while continued consumption of the cholesterol–enriched diet resulted in further increases in serum cholesterol levels. Bars indicate SEM.
The mean cellularity of bone marrow samples from leukocytotic hypercholesterolemic rabbits (4.52×10⁸ cells/g bone marrow±7.36×10⁷[SEM]) was statistically greater (p<0.05) than that in normocholesterolemic rabbits (2.45×10⁸ cells/g bone marrow±3.73×10⁷) when group means were compared. However, in individual animals the presence or extent of leukocytosis did not correlate with cellularity in the bone marrow.

Fc-mediated phagocytosis. The percentage of monocytes that had phagocytosed IgG-coated eryth-

![Figure 6](image)

**Figure 6.** Line plot showing leukocyte levels in rabbits identical to those in Figure 5 that were fed the 0.25% cholesterol-enriched diet (●) for 13 weeks; at that time eight rabbits were switched to the chow diet (○) and seven rabbits remained on the cholesterol-enriched diet. Removal of the cholesterol diet resulted in a gradual decline in circulating leukocyte levels (10⁶/mm³) while continued consumption of the cholesterol-enriched diet resulted in a continued leukocytosis. Bars indicate SEM.

![Figure 7](image)

**Figure 7.** Line plot of circulating platelet levels in rabbits that were fed the 0.25% cholesterol-enriched diet (●) or chow diet (○). In the early phase of the dietary regimen, rabbits fed the 0.25% cholesterol-enriched diet showed levels of circulating platelets (10⁶/mm³) comparable with those of chow-fed rabbits. However, elevations in circulating platelets are seen at 18 weeks and thereafter. Bars indicate SEM. Different n's over time reflect animal sacrifice.

![Figure 8](image)

**Figure 8.** Line plot of circulating platelet levels in rabbits that were fed the 0.1% cholesterol-enriched diet (●) or chow diet (○). Elevated platelet levels (10⁶/mm³) existed between 3–18 weeks in rabbits fed the 0.1% cholesterol-enriched diet, but in these animals pretreatment platelet levels were higher than those of controls. Bars indicate SEM. Different n's over time reflect animal sacrifice.
rocytes was not consistently different when normo-cholesterolemic and hypercholesterolemic groups were compared (Figure 9). Furthermore, no relation was apparent between the level or duration of hypercholesterolemia and erythrophagocytosis by monocytes. Attempts to count the number of erythrocytes per monocyte were unsuccessful due to the compression and consequent obscuring of erythrocytes in the monocytes. Monocytes that were exposed to phosphate-buffered saline-treated or IgM-coated erythrocytes did not show uptake of erythrocytes.

**Body temperature.** Rectal body temperatures were measured in seven rabbits that were confirmed to be leukocytotic 3 days earlier and in three age-matched control rabbits. Body temperatures did not vary significantly between control and experimental groups.

**Autopsy and histology.** Pathogenic bacteria or parasites were not found in nasal and rectal swabs or in fecal samples from rabbits during quarantine before the animals were allocated to the experiments. Furthermore, randomly selected leukocytic rabbits that were similarly evaluated during the studies were free of pathogenic microorganisms. Gross autopsies did not reveal evidence for infection in any animal. Histological sections of livers were negative for parasites in all animals.

Microscopic examination of spleens from experimental animals fed the 0.25% and 0.1% diets showed large numbers of foam cells, destroyed leukocytes, and hemosiderin in macrophages. The germinal centers appeared normal. Occasional islands of hematoPoiesis (mostly erythroid) were seen, especially in spleens showing less deposition of foam cells.

The aortas from every experimental animal in experiment 1 showed grossly visible raised atherosclerotic lesions in the aortic arch and thoracic aorta. The abdominal aorta usually showed extensive lesions in rabbits that consumed either atherogenic diet for 7 or 10 months. Histologically, the lesions were similar to those described elsewhere and ranged from well-developed foam cell lesions to advanced fibromuscular lesions with collagen and necrotic foci.

In rabbits fed the 0.1% atherogenic diet, grossly visible aortic lesions were seen in three of six, six of six, and five of seven rabbits after 4, 7, and 10 months of cholesterol feeding. Microscopic comparisons between lesions from rabbits fed the two atherogenic diets will be reported elsewhere.

**Discussion**

In this study we have demonstrated sustained panleukocytosis associated with cholesterol feeding in rabbits. Two levels of dietary hypercholesterolemia were induced to ensure that leukocytosis was not due solely to the supraphysiological hypercholesterolemia resulting from the 0.25% diet. Leukocytosis has been described in cholesterol-fed rabbits and in rats, but not in animals subjected to the same duration or level of hypercholesterolemia as in the present studies. Evidence for leukocytosis in hypercholesterolemic humans has also been described.

The mechanisms for cholesterol-induced leukocytosis are unknown. The number of circulating leukocytes (and therefore monocytes available for plaque formation) appears to be dependent on factors that influence their production, release, life span, and transit time in the blood. Therefore, direct stimulation of hematopoiesis in the bone marrow seems to be the most plausible mechanism for leukocytosis in cholesterol-fed rabbits. The finding that, as a group,
the bone marrow of hypercholesterolemic leukocytic rabbits contained almost twice the number of cells per unit weight than did that of normocholesterolemic rabbits supports this concept. If such a mechanism is operating, the stimulus is likely to be acting at the level of a totipotential stem cell in the marrow because the numbers of granulocytes and platelets are also elevated during cholesterol feeding. Recent studies of hypercholesterolemic swine in one of our laboratories (R.G.G.) have also shown an increase in the number of bone marrow leukocyte progenitor cells, ranging from 50% at 4 months to eight- to ninefold after 6–9 months of hypercholesterolemia. Unlike the current results in rabbits, however, hypercholesterolemic swine demonstrated a marked monocytosis rather than a leukocytosis and a preferential production of monocyte colonies by bone marrow progenitor cells when they were placed in culture. Furthermore, hypercholesterolemic swine serum induced preferential formation of mononuclear colonies by normal bone marrow cultures from both normal and hypercholesterolemic swine. Further studies have shown that hypercholesterolemia swine serum acts directly on progenitor cells to produce this effect, indicating the presence of a monocyte colony-stimulating factor in hypercholesterolemic serum in this model. On the basis of the present results in rabbits, it is possible that colony-stimulating factors are also being produced under hypercholesterolemic conditions. Whether the production of a generalized leukocytosis in rabbits, as opposed to the monocytosis seen in swine, is a species difference (with respect to response of the bone marrow to putative colony-stimulating factors) or is a result of greater levels of hypercholesterolemia in the rabbit model, is unknown.

Premature release of leukocytes from the bone marrow is an unlikely explanation of the leukocytosis seen in this study because immature leukocytes were seen extremely infrequently in blood smears. Extramedullary hematopoiesis was not seen in spleen sections to an extent or with a consistency that could account for the observed leukocytosis. It is also unlikely that anemia induced a compensatory increase in leukocyte production in the marrow because leukocytosis developed several weeks before anemia did. The possibility that altered turnover of circulating leukocytes is responsible for leukocytosis cannot be ruled out.

In the present study it is unlikely that the basis of leukocytosis is an inflammatory reaction elicited or potentiated by hypercholesterolemia; no evidence for infection or general inflammation was obtained based on body temperatures, bacteriological and parasitological data, and gross or microscopic autopsy observations.

The close association between leukocytosis and cholesterol feeding was shown by two findings. First, leukocytosis began within 1 week of the onset of diet-induced hypercholesterolemia and increased in parallel with hypercholesterolemia. Second, leukocytosis is reversible after withdrawal from cholesterol feeding. The lack of correlation between leukocyte levels and serum cholesterol levels in individual animals indicates that it is the presence of hypercholesterolemia and not its severity that is the determining factor for inducing and maintaining leukocytosis. Thus, although hypercholesterolemia and the onset of leukocytosis are temporally correlated, on the basis of data from individual animals neither serum cholesterol levels nor circulating leukocytes were predictive of the other parameter. In apparent contradiction to the above poor correlation, leukocyte levels in rabbits fed the 0.25% diet were higher (absolute levels and percentage above control) than in rabbits fed the 0.1% diet, suggesting that levels of dietary cholesterol rather than serum cholesterol may determine the degree of leukocytosis.

The reversibility of leukocytosis as described herein may have a bearing on studies showing regression of experimental atherosclerotic lesions in rabbits. In such experiments the termination of cholesterol feeding results in the reduction of monocyte-derived foam cells in presumably preexisting lesions. This type of lesion regression may be mediated in part by a reduction in the number of circulating monocytes available to enter the lesion. This in turn may alter the ratio of monocytes and monocyte-derived foam cells migrating into and out of the lesion, favoring reduction in lesion size.

The current study was not designed to relate the degree of leukocytosis with the extent or severity of atherosclerotic lesions. Such studies would be more profitably conducted during the early stages of lesion formation when monocyte adherence could be monitored. In well-developed lesions it is unlikely that correlations between lesion size and degree of leukocytosis would yield meaningful information. The dimensions of such lesions are not only related to the ability of monocytes to engulf lipid, in addition to the number of monocytes present in the lesion, but also to the involvement of smooth muscle cells and enhanced amounts of connective tissue.

The secondary leukocytosis seen in the later stages of the 0.25% atherogenic regimen seems to be associated more with liver failure than with hypercholesterolemia per se because this leukocytosis was temporally correlated with the appearance of bilirubinemia. Although the importance of this secondary leukocytosis in early atherogenesis is doubtful because atherosclerotic lesions are well developed by this time (about 20 weeks), its effect on the progression of established lesions may be important to consider.

Although the numbers of circulating monocytes were not preferentially increased in hypercholesterolemic rabbits, it was of interest to assess the state of activation of this leukocyte population because of its known role in atherogenesis. Furthermore, during hypercholesterolemia alterations have been reported in the phagocytic function of monocytes and peritoneal macrophages and in the adhesive
properties of peritoneal macrophages. Activated macrophages display enhanced phagocytic capabilities and increased Fc receptors. Thus, Fc-mediated phagocytosis of IgG-coated erythrocytes can be used as an indication of monocyte activation. In the current experiments there was no change in the percentage of phagocytic cells as a result of hypercholesterolemia. These data suggest that in the adherent monocytes studied, there is no shift in the population toward more numbers of activated cells, as assessed by this technique. However, we cannot exclude the possibility that a population of more activated monocytes adheres to and penetrates sites of developing lesions, making these monocytes inaccessible for study. These results do not preclude possible increases in phagocytic activity on a per cell basis; such evaluations were not possible with this assay. Neither do these results rule out activation, as manifested by other physiological parameters.

The thrombocytosis seen in hypercholesterolemic rabbits in these experiments is not likely to be important in the initiation of atherosclerotic lesions because the onset of thrombocytosis occurs later than the onset of lesions. However, thrombocytosis conceivably could influence the cellular composition of atherosclerotic lesions in long-term hypercholesterolemia by making more platelets available for interaction with the vessel wall. In primates, Faggiotto and Ross described platelet deposits at sites of endothelial retraction. These sites subsequently developed lesions rich in smooth muscle cells, suggesting a role for the release of platelet-derived growth factor by adherent platelets in transforming foam cell lesions into fibromuscular lesions. Recently, Rosenfeld et al. reported platelet deposition in areas of endothelial cell retraction in Watanabe heritable hyperlipidemic and cholesterol-fed rabbits. In both studies the authors proposed a potential role for platelet-derived growth factor in the recruitment of monocytes and proliferation of smooth muscle cells.

The relevance of leukocytosis to the formation of foam cell lesions is unknown. The onset of leukocytosis (1 week after initiation of cholesterol feeding) occurred before the time at which lesions would be expected to develop. Therefore, it is possible that the number of circulating monocytes in hypercholesterolemic animals influences the rate or extent of the formation of foam cell lesions. Studies in chronically leukocyte-depleted animals would help clarify this point. However, such studies are difficult to complete due to the high probability of recurrent infections. In one such study, cyclophosphamide inhibited diet-induced atherosclerosis in rabbits. However, the authors did not quantify hematological changes, and they interpreted the results from the standpoint of reduced humoral immune response rather than from an orientation toward leukocytes. Further work on the relation between hypercholesterolemia, the number of circulating leukocytes, and lesion formation may provide new insights into the formation of atherosclerotic lesions.

Acknowledgments

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References

15. Gerrity RG, Goss JA, Soby L: Control of monocyte recruitment by chemotactic factor(s) in lesion-prone areas of swine aorta. Arteriosclerosis 1985;5:55-66


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