Studies on Atherogenesis: The Reaction of the Corneal Model to Repeated Injections of Lipoprotein-Rich and Lipoprotein-Poor Homologous Serum*†

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How the artery wall accumulates lipids remains an important problem in the pathogenesis of atherosclerosis. The problem is emphasized by the almost invariable occurrence of fatty plaques in the arteries of adult Americans with no known excess or abnormality of their blood lipids. The observations reported here, utilizing the corneal connective tissue system as an experimental model for the artery wall, bear on this problem.

As background for these studies it had been found previously in this laboratory that the artery wall retains soluble lipoproteins selectively at sites of injury, a mural lipophagic reaction ensuing which reproduces many of the morphologic features of the early atherosclerotic plaque in man(1). In addition, it was shown that the living animal cornea, following injection with small quantities of serum from normal humans or from animals with blood cholesterols in the range of normal humans, also retains serum lipoproteins(2,3). A stromal lipophagic reaction occurs in the cornea mimicking the foam-cell stage of atherosclerosis. It may be pointed out that the corneal stroma is similar to the intimal—inner medial segment of elastic arteries in several important respects: Firstly, it is inherently avascular, yet maintains an optimal detergescence by an active fluid exchange. Secondly, it has a capillary blood supply at its periphery and thirdly, collagenous fibers are in microscopic lamellar arrangement with a rich admixture of mucopolysaccharides. An obvious difference between the serum-injected corneal model and the inner artery wall is that the latter may be subject to repeated

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episodes of increased permeability brought about by minor endothelial injury, so that repeated infiltrations of lipid-rich macromolecules from the ambient plasma may take place. The object of the present experiments was to make a thorough study of the corneal lesion following one injection of lipoprotein-rich serum and then to test, for comparative purposes, the effects of repeatedly injecting the cornea with either lipoprotein-rich or lipoprotein-poor homologous serum. Preliminary control procedures were also carried out.

MATERIALS AND METHODS

New Zealand white rabbits of either sex, weighing 3–5 kg, were utilized for the experiments. They were anesthetized with intravenous sodium pentobarbital (Nembutal, 25 mg/kg) for corneal injections. Injection of 0.1 or 0.3 ml of normal or of hyperlipoproteinemic homologous rabbit serum or of other pertinent materials was made tangentially into the cornea near the limbus through a sterile 30-gauge hypodermic needle. The resultant edematous opaque zone was 8–15 mm in diameter with its edge in contact with the limbus.

Serum was obtained by aseptic technique from normal rabbits, and an aliquot of each sample was analyzed for lipids and protein. Serum lipid fractions were determined by the following methods: cholesterol and cholesterol esters—modified Schoenheimer-Sperry(4); lipid phosphorus—a modified Youngburg procedure(5), and fatty acids—a modified Stoddard and Drury technique(6). Average serum levels were total cholesterol 35.0 mg%, free cholesterol 7.5 mg%, fatty acids 11.0 mEq/liter, phospholipids 4.0 mg%, and total protein 7.0 g%. Hyperlipoproteinemic sera were obtained from cholesterol-fed rabbits. The serum lipids ranged as follows: total cholesterol 800–1000 mg%, free cholesterol 350–500 mg%, fatty acids 60–80 mEq/liter, phospholipids 13–16 mg%, and total protein 5.0–7.0 gm%. The serum was examined microscopically to rule out the presence of chylomicra. If these were present they were removed by centrifugation at 20,000g. for 1 hr. Comparable serum from cholesterol-fed rabbits was shown by Gofman et al.(7) to have high levels of $S_f$ 10–30 class lipoproteins. This group of lipoproteins in the rabbit corresponds to the $S_f$ 10–20 lipoproteins often found to be significantly elevated in patients with known premature coronary atherosclerosis. By cellulose acetate electrophoresis the lipoprotein-rich sera utilized in the present experiments exhibited a strong band in the $\beta$ globulin zone staining for lipid by osmic acid.

Animals were fed a standard rabbit chow (Purina Mills, Inc., fat content 2.0%). For histological studies animals were killed by iv sodium pentobarbital anesthesia. In some cases the eyes were then perfused with 1% India Ink in 0.9% saline at 100 mm Hg pressure in order to demonstrate fully the capillaries in the cornea. The corneas were removed and cut in such a way as to divide the lesion into two approximately equal parts. One half of each cornea was fixed in 10% formalin, the other half in mercuric chloride fixative (two parts of saturated aqueous solution of HgCl$_2$ plus one part of absolute alcohol for 48 hr followed
by a change to 80% alcohol. The formalin-fixed tissues were cut by frozen section and were stained for lipids with Sudan IV. The adjacent tissues, fixed in mercuric chloride–alcohol, were imbedded in paraffin, cut, and stained with hematoxylin and eosin. Selected sections were also stained by toluidine blue, Masson, or von Kossa’s stain for calcium.

RESULTS

The Corneal Lesion that follows a Single Injection of Lipoprotein-rich Homologous Serum

Preliminary control procedures included microscopic examination of normal rabbit corneas and a study of the effects of the injection of physiological saline. The corneal stroma is a dense, uniform, collagenous, avascular structure with interspersed elongate stromal cells (Fig. 1). No sudanophilic material is present. Intracorneal injection of 0.1 or 0.3 ml of sterile physiologic saline solution led to the immediate production of an opaque, edematous, disc-like zone which grossly cleared completely within 24 hr. Microscopic examination of the plaque immediately after injection revealed an edematous region of separated collagen fibers. By 24 hr the edema had largely subsided, and by the end of two weeks the cornea appeared normal. There was neither sudanophilic material in the stroma nor vascularization (Fig. 2).

Thirty corneas were now injected with 0.1 ml of chylomicron-free, hyperlipoproteinemic, homologous serum. Gross examination revealed the immediate production of edematous, opaque, yellow–white plaques. By 7–10 days many fine vessels could be seen growing into the plaques from the limbus. Approximately two weeks after injection there was a narrow band of clearing of the cornea paralleling the limbus where contact had previously been made between it and the plaque. By this time a narrow zone of clearing had appeared around some of the vessels penetrating the plaque. Vascularization progressed for the next 2–3 months, by which time the plaque was usually separated from the limbus by a clear zone 2–3 mm wide. The plaque was less dense than originally, often having a mottled appearance associated with the clearing around the vessels. After this stage, the vessels gradually became smaller and fewer grossly. At the end of one year the plaque was greatly reduced in size, opacity, and vasculature but, nevertheless, persisted.

Microscopic examination of these plaques, produced by a single injection of 0.1 ml of chylomicron-free, hyperlipoproteinemic, homologous serum, revealed the following sequence of events: The retained lipid impregnated and led to swelling of collagen fibers almost immediately, and a lipophagocytic response was elicited in 4 days. The early lipophagic cells were initially distributed uniformly throughout the plaque, suggesting that these early-appearing foam cells were modified stromal cells arising in situ. Within six days after the introduction of the lipid-rich serum into the corneal stroma, many dilated capillaries were already invading the swollen, edematous, previously avascular connective tissue.
Microhemorrhages were not uncommon. By the end of a month almost all of the lipid originally present in the serum in molecular solution and shortly thereafter seen to be impregnating collagen fibers as fine extracellular granules, was contained within large foam cells (Figs. 3 and 4). At this time, much of the lipid, especially in the zone nearest the greatest number of blood vessels, i.e., the paralimbal region of the cornea, had been removed. In addition, the acute inflammatory cellular exudate seen shortly after injection had become wholly mononuclear. By the end of the second month both the foam-cellular response and vascularization reached their peaks, and the foam cells were most numerous along the ingrowing capillaries. At the end of one full year, the lipid-filled foam cells, vascularization, and damaged collagen, although quantitatively decreased from their level at two months, were still unquestionably present.

![Image 1](image1.jpg)

**Fig. 1.** Control section of cornea of normal rabbit. There is slight shrinkage of the stromal fibers due to dehydration. Hematoxylin-eosin X 450.

**Fig. 2.** Control section of rabbit corneal stroma, 14 days after injection of 0.1 ml physiological saline. The nuclei of a few stromal cells are present. There are no lipophages. Hematoxylin-eosin X 450.
The Effects on the Cornea of Injection of an Increased Amount of Lipoprotein-Rich Serum

In an attempt to accelerate and augment the changes noted above, larger plaques were produced by the injection of larger amounts (0.3 ml) of chylomicron-free, hyperlipoproteinemic rabbit serum. This procedure was carried out in eight corneas. It resulted not only in a larger plaque and more rapid and intense vascularization, but also in a more marked foamcellular response and more prominent degenerative changes in the collagen fibers. The production of so many large foam cells provided an opportunity to study the pleomorphism of these cells. Spindle-shaped cells and all the intermediate forms up to and including engorged, round, lipid-filled foam cells were seen (as is the case in human

Fig. 3. Rabbit cornea 1 month after injection of 0.1 ml lipoprotein-rich rabbit serum. Foamy lipophages in stroma (arrows). Hematoxylin-eosin X 450.

Fig. 4. Rabbit corneal stroma one month after injection of 0.1 ml lipoprotein-rich rabbit serum. Abundant sudanophilic lipophages in stroma. Sudan IV without counterstain X 100.
atherosclerosis), giving more support to the hypothesis that some of these cells may arise, or at least mature in situ.

The Effects of Repeated Injections of Lipoprotein-Rich Serum

To test the effects of repeated deposits of soluble serum lipids in the corneal stroma and to stimulate the process still further, repeated injections of 0.3 ml of hyperlipoproteinemic rabbit serum were made into the same cornea. Up to five injections of 0.3 ml—each were given at weekly intervals in 16 corneas. The majority of the animals were killed 2–3 months after the last injection, thus allowing the response to the acute injury of injection to subside. These corneas were grossly swollen and contained large, yellow, well-vascularized plaques. Microscopically, massive foam-cellular (Fig. 5), extensively vascularized lesions were

Fig. 5. Rabbit corneal stroma injected 3 weeks and 2 weeks before killing with 0.3 ml lipoprotein-rich rabbit serum. Large foamy lipophages in edematous collagen (arrows). Hematoxylin-eosin X 450.

Fig. 6. Rabbit corneal stroma. Cornea was injected 5 times at weekly intervals with 0.3 ml lipoprotein-rich rabbit serum; animal was killed 2 months after the last injection. Acicular cholesterol ester crystals in phagocytes (arrow). Hematoxylin-eosin X 450.
evident. The acicular clefts of cholesterol-ester crystals, a common feature of lesions of late human atherosclerosis, were frequent (Figs. 6 and 7), and the tissues gave positive Schultz tests (based on the Liebermann–Burchard reaction) for steroids. The collagen in all plaques was pale and swollen and in some cases also frayed and fragmented. The number of cell nuclei in these regions was decreased. Large numbers of lipophages were frequently observed in the immediate vicinity of fragmented collagen, suggesting that these cells were ingesting the swollen, degenerating, lipid-impregnated collagen fibers. Again, microhemorrhages were present near the advancing capillary tips.

The Effect of a Single Injection of Normal Lipoprotein-Poor Rabbit Serum

Fourteen rabbit corneas were studied after a single injection of 0.3 ml of homologous normal rabbit serum (serum lipid and protein content: total cholesterol 35 mg%, free cholesterol 7.5 mg%, fatty acids 11.0 mEq/liter, phospholipids 4.0 mg%, and total protein 7.0 mg%). Injection of this material produced an opaque, edematous zone immediately. Over the course of the following five days the edematous, opaque area cleared completely and no blood vessels were seen.

Microscopically, none of the edematous, normal rabbit serum-injected corneas showed sudanophilia for the first few days, but four days after injection, when the edema had decreased, there was a trace of sudanophilic material at the surfaces of the collagen fibers. This became slightly more prominent during the following week as the edema further subsided, suggesting that much of the serum water was being removed while the lipid remained and was being concentrated in the cornea in association with collagen fibers. Three of the eight corneas in this series were shown to contain sudanophilic material. Neither a foam-cellular response nor vascularization could be demonstrated.

The Effects of Repeated Injections of Normal Lipoprotein-Poor Rabbit Serum

The above observations led to an investigation of the effects of repeated injections of normal lipid-poor rabbit serum into the same cornea. First a group of control corneas was prepared and examined. The six control eyes received repeated intracorneal injections of 0.3 ml of sterile physiologic saline. Within 24 hr of each injection the resultant edematous zone had cleared completely. Four such injections at 4-day intervals, followed by two months in which no further injections were given, led to the growth of a few tiny blood vessels into the region of the transient plaques. There was no sudanophilia or lipophagocytic response histologically. Four rabbit corneas were then given four injections of 0.3 ml of homologous normal rabbit serum (total cholesterol 35.0 mg%) at 4-day intervals followed by two months in which they received no injections. Each injection produced a white edematous plaque, but within a few hours the plaque became much less opaque and swollen. In these corneas the plaques never completely cleared; they persisted as faint, hazy regions. Several small vessels could be seen growing into the plaques from the limbus. Microscopic examination of these plaques revealed, in three of the four corneas, a lesion consisting of definite
lipid-filled macrophages (Fig. 8), a sparse mononuclear infiltrate, swollen collagen fibers and a thin network of dilated, congested capillaries.

**DISCUSSION**

The major histologic features of the vascularizing serum-lipid injected rabbit cornea have been presented. This animal model and the inner segment of human elastic arteries have important structural similarities: Both the normal human arterial intima and the normal rabbit corneal stroma are avascular connective tissues, rich in mucopolysaccharides and collagenous connective tissue in lamellar

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**Fig. 7.** Corneal stroma from same animal as Fig. 4. Multiple intracorneal injections of lipoprotein-rich serum. Crystalline cholesterol ester deposits (arrows). Hematoxylin–eosin X 100.

**Fig. 8.** Rabbit corneal stroma injected 4 times at 4-day intervals with lipoprotein-poor (normal) rabbit serum. Killed 2 months after last injection. Foamy lipophages present (arrows). Hematoxylin–eosin X 1000.
arrangement; and both are near vascular beds capable of proliferation and expansion into the adjacent avascular tissue—the medial vasa vasorum into the intima of the artery and the limbal vessels into the cornea. An obvious difference between the artery and the cornea is that the former is continually subjected to pulsating intraarterial blood pressure. However, assuming that mild, episodic endothelial injury in man occurs throughout life, leading to periods of increased intimal permeability to serum lipoprotein molecules, the parallel between the human lesion and the serum-injected corneal model in the experimental animal can be preserved. It must be borne in mind, however, that evidence from any model is indirect.

From the corneas injected once with homologous hyperlipemic serum it could be seen that lipoprotein in its native serum, when placed in an avascular connective tissue, first impregnates collagen fibers as very fine granules and is later taken up into lipophagic cells where it assumes a globular form. It should also be noted that at no time during the full year of observation were foam cells seen to be degenerating or otherwise breaking down and releasing their lipid content. In the experimental conditions, once lipid is phagocytosed it remains intracellular for long periods. Further evidence for this is in the fact that once regions of established corneal plaques clear, opacity does not recur. This sequence of events relating to the fate of stromally deposited serum lipoprotein is directly opposed to the interpretation of Cogan and Kuwabara(8) that lipid in an avascular connective tissue (cornea) appears first as intracellular globules and later becomes extracellular fine granules as the lipid-containing cells degenerate. Observations of the corneal model also present strong alternative evidence to the theories that the lipid in an atheroma either is derived primarily from cellular metabolic derangements (fatty degeneration) as postulated by Virchow(9), McGill and Geer(10), or is synthesized in situ as suggested by Zilversmit et al.(11), and Holman et al.(12).

As noted by Ashton and Cook(13) in corneas vascularizing during maintained edematous states and by Cogan(14), Julianelle and Bishop(15), and Friedenwald et al.(16) in corneas vascularizing after physical or chemical injury, microhemorrhages are common in the vicinity of advancing capillary tips. This regular association of neovascularization and microhemorrhages is also seen in the rabbit corneas injected with hyperlipoproteinemic serum and should be considered as a possible mechanism for hemorrhage into atheromatous plaques.

One respect in which the once-injected cornea might fail to parallel human atherogenesis is that in man there is undoubtedly more than one episode of lipid infiltration into the arterial wall at a given site during a lifetime. Each episode of vascular injury with its attendant increase in intimal permeability may lead to an accompanying increased perfusion of the intimal connective tissue by lipoprotein macromolecules. Such repeated injury and lipid infiltration was simulated in the animal model by repeatedly injecting the same cornea. Using this technique of repeated injections and lipid-rich serum, florid lesions corresponding to the late stages of atherosclerosis are routinely produced as described above. Similarly, the same technique of repeated injections but instead utilizing lipid-
poor serum (cholesterol 35 mg\%\textsubscript{o}) also produces foam-cellular, lipid-rich, vascular plaques. Thus the corneal equivalent of an atherosclerotic lesion is produced by four injections of 0.3 ml each of serum with a cholesterol content of only 35 mg\%\textsubscript{o}, a total of only 0.42 mg cholesterol (as lipoprotein) in a total of 1.2 ml of serum. This morphologic sequence resulted probably because the water, electrolytes, and other small molecules in each volume of injected serum were removed from the avascular connective tissue while the large, comparatively less diffusible lipoproteins remained and were concentrated. With each injection of serum the process repeated itself. The result was a concentration of enough lipid from lipid-poor serum to elicit both a lipophagocytic response and vascularization and to produce early collagen changes. There is definite evidence that such concentration of lipid also occurred when lipid-rich serum was repeatedly injected into the cornea, for in this case with each repeat injection of serum, the Schultz test for steroids became increasingly positive on corneal slices. A faint or negative reaction was the rule in an equally swollen cornea after a single injection of hypercholesterolemic serum.

There is no reason to believe that this same mechanism of lipid concentration cannot or might not occur and progress to fully developed atherosclerotic plaques in human arteries in the presence of so-called normal or low serum lipid levels.

**SUMMARY**

The potential of the serum-injected rabbit cornea as an experimental model for pertinent reactions of the arterial intima is noted. Utilizing the corneal model, it has been possible to elucidate further certain controversial mechanisms of atherogenesis.

Thus it has been clearly demonstrated that after a single infiltration of avascular connective tissue by lipoprotein-rich serum, lipid appears initially as fine granules closely associated with collagen fibers. In only four days lipophagocytic cells appear. There is a steady progression of the lipid from an extra- to an intra-cellular location. During a full year of observation no necrosis of foam cells or release of lipid into the intercellular stroma could be seen. Over the course of a year there is gradual decrease in the lipid content of the plaque, suggesting that the foam cells slowly metabolize the phagocytosed lipid. Microhemorrhages, as advancing capillary aneurysms burst, are a regular feature of neovascularization of corneal lipid-connective tissue plaques, and a similar phenomenon may be a source of the larger hemorrhages in developing atheromata. By the removal of water from serum-infiltrated connective tissue, serum lipid is concentrated to such an extent that even four episodes of infiltration by lipid-poor serum leads to the development of a fatty, foam-cellular lesion. This observation suggests that repeated concentration of filtered serum lipids, and not in situ synthesis, may be responsible for the massive amounts of lipids regularly present in the atherosclerotic lesions of arteries in man.
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