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ORIGIN OF LIPID AND CHOLESTEROL IN EXPERIMENTAL THROMBOATHEROSCLEROSIS *

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Recently we have described (1) the sequence of phenomena that followed the experimental placement of a thrombus in the aorta of a hypercholesterolemic rabbit. It was found that the thrombus invariably elicited a hyperplastic intimal response leading eventually to a plaque (see Color Figure 1) which in its lipid and tissue characteristics bore a striking resemblance to the human atherosclerotic plaque. Moreover, necrosis, liquefaction, and even calcification of the central portions of the plaque were commonly observed.

The sudanophilia and also the excess accumulation of cholesterol occurring in these plaques first became detectable about 14 days after the induction of the thrombus itself. Although hundreds of sections of these plaques were examined before, during, and shortly after this 14-day period, the first accumulation of the lipid never was observed in the superficially located intimal tissue which was in closest contact with the luminal blood (i.e., the intimal tissue encircling the periphery of the thrombus). Invariably such lipid made its first appearance in the deeper areas of the new intimal tissue immediately adjacent to the underlying media. Indeed, not infrequently, the first appearance of lipid was detected in the inner portion of the media underlying the newly formed plaque. These observations clearly suggested to us that the excess lipid (and presumably its cholesterol congener) was not derived from luminal blood. Its accumulation, therefore, appeared to be due to its transport by blood vessels newly derived from the adventitia or to its excess production *in situ* in the newly forming intimal tissue. Which of these two possible phenomena was responsible for the accumulation was not determined in our first study.

However, because of the theoretical as well as practical importance of discovering the manner in which lipid and cholesterol accumulated in continually increasing excess in this new intimal tissue, a second study was initiated to determine this point. The results obtained by a variety of techniques left little doubt that the excess accumulation of lipid and cholesterol was due primarily to an excess escape of these substances from the newly formed intimal blood vessels because of the latter's increased permeability and frequent rupture. Subsequent retention of the lipid and cholesterol moieties, which had infiltrated, appeared to be due to cellular sequestration by hyperplastic intimal tissue.

METHODS

A. General histological study of the early developing plaque

In order to verify the earlier finding of initial sudanophilia, beginning in the basal areas of the newly developing intimal tissue, and also to determine if excess mucopolysaccharide and hemorrhages were present, thrombi were induced in the aortas of 18 rabbits by the introduction of zinc chloride-treated magnesium alloy spirals (2). Half of these rabbits were given Wayne rabbit chow diet plus added cholesterol (2 per cent) and cottonseed oil (2 per cent), and the remaining half, only the chow diet. Five of the rabbits on each of the two diets were sacrificed at the end of 18 days, and the remaining rabbits, at the end of 28 days. Blood samples obtained at the time of insertion of the coil and again at time of sacrifice were analyzed for cholesterol content (3).

Three sections were obtained from each developing plaque and the portion of artery wall subjacent and immediately adjacent to it. These were stained with Sudan IV, hematoxylin and eosin, and the Rinehart stain for mucopolysaccharide (4), respectively.

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A plaque developing in a normocholesterolemic rabbit, hence exhibiting no sudanophilia (1), will be described herein as a *thrombosclerotic* plaque. A similar plaque developing in a hypercholesterolemic rabbit, hence containing excess lipid and cholesterol (1), will be referred to as a *thromboatherosclerotic* plaque.

B. Study of hyperplastic intimal tissue under avascular conditions

In order to investigate the relevance of the newly formed blood vessels to the sudanophilia or lipid accumulation observed in the basal portions of the early thromboatherosclerotic plaque, this tissue was studied in a milieu devoid of blood vessels.

Cylinders were fabricated by us of Millipore[1] (filter sheet having a pore size of 3.0 μ) by approximating the two ends of a flat section of the material (2.5 × 2.0 cm) and then sealing this juncture and one of the two open ends with an "Epoxy" glue.[2] At this time also, a short length of silk thread was incorporated in the "Epoxy" seal. Such capsules were capable of holding approximately 0.2 to 0.3 ml of fluid.

Four normocholesterolemic rabbits, into whose abdominal aorta a thrombus-inducing spiral had been introduced 21 days previously, were killed. Under aseptic conditions, the thrombosclerotic plaques were removed, pooled, and then finely minced with a razor blade. After samples of this material had been taken for chemical and histological analysis, part of the remainder was inserted into each of 12 Millipore capsules. The open ends of the capsules were closed with the Epoxy and the capsules were partially immersed in warm Tyrode's solution for several hours as the Epoxy seal hardened (these are designated as "intact" in Table II). For control purposes, the Millipore portions of 5 of the capsules were gently pierced in 8 to 10 separate areas with a 25 gauge hypodermic needle (these are designated as "open" in Table II).

One normocholesterolemic and two hypercholesterolemic rabbits, after initial blood samples had been obtained, were operated upon, and 12 intact and open capsules containing the minced intimal tissue were inserted and secured by suture to the posterior wall of the peritoneal cavity. The number and type of capsules inserted into each rabbit are given in Table II. In addition, 1 intact Millipore capsule, containing only Tyrode's solution, was placed in the abdominal cavity of each hypercholesterolemic rabbit.

At the end of 8 days, these animals were bled again and then were reoperated. A sample of peritoneal fluid was obtained from the two hypercholesterolemic rabbits and then the capsules were removed from all of the rabbits. Tissue for histological study was obtained from all 12 capsules and there was sufficient material available in 9 of the capsules for cholesterol analysis (5). Also, fluid was aspirated from each of the 2 capsules initially containing only Tyrode's solution and this fluid was analyzed for cholesterol content. Finally, the Millipore walls of two of the closed (unpunctured or intact) capsules also were analyzed for cholesterol content.

C. Studies of the synthesis and deposition of cholesterol in the early-developing plaque

In order to determine the mechanism responsible for the excess accumulation of cholesterol previously observed (1) in the developing thromboatherosclerotic plaque of the hypercholesterolemic rabbit, studies employing acetate-1-C^{14} and cholesterol-4-C^{14} were done.

1. Acetate-1-C^{14} studies. Aortic thrombi were induced by insertion of the spiral in two normal rabbits. These rabbits ingested only Wayne rabbit chow diet. The animals were anesthetized with pentobarbital (Nembutal) 21 and 28 days, respectively, after insertion of the spiral, and each abdominal aorta was excised and placed immediately in a small volume of ice cold 0.1 M potassium phosphate buffer, pH 7.4. Potassium rather than sodium was used for the buffer to offset the tendency of excised aortic tissue to lose excessive potassium (because of its low endogenous metabolism), hence a lag in its synthesis of cholesterol (6). The tissues were kept cold from that time until put into Warburg flasks.

The adventitia was rapidly removed before cutting open the aorta longitudinally to expose the plaque consisting of residual thrombus and hyperplastic intimal tissue. This plaque together with the minimal proximal area of uninvolved aorta was used for study. The samples were approximately 9 mm in length and 3 mm in width. The thrombointimal portion of the thromboaortic samples was approximately twice the thickness of the media.

Small sections were taken for histological study, then the samples were sliced "free-hand" to a thickness less than 0.5 mm, and incubated at 37° C in Warburg flasks (in duplicate) for 130 to 150 minutes during the period of steady oxygen uptake. All flasks contained in addition to tissue: nicotinamide 0.3 M in 0.011 M sodium EDTA, 0.3 ml; 0.16 M magnesium sulfate, 0.1 ml; 0.02 M sodium acetate-1-C^{14}, 0.2 ml (2 μc); 0.1 M potassium phosphate buffer, pH 7.1, 2.2 ml; and 0.2 ml 20 per cent potassium hydroxide in the center well. Respiration was terminated by the addition of 2.8 ml 50 per cent KOH for digestion of the tissue. Fifty per cent KOH was added to three flasks prior to incubation to serve as a control on possible tracer contamination of the isolated cholesterol digitonide. The activity of all three samples was identical with background.

Aliquots for nitrogen analysis were taken from each digested sample. Cholesterol was extracted from the digest by partitioning with ethanol: ether (1:4), then precipitated with digitonin, washed, and finally the cholesterol digitonide dissolved in glacial acetic acid. An aliquot was removed for micro-scale cholesterol analysis (7) and the remainder dried in planchets to yield cholesterol digitonides of infinite thinness. Counting was done using a Tracerlab Mylar end-window TG C^{14} tube.

[1] WSWP Filters, Nylon netting, white; Millipore Filter Corp., Bedford, Mass.

[2] Devcon "2-Ton" Epoxy; Devcon Corp., Danvers, Mass.

TABLE I

Accumulation of lipid and mucopolysaccharide, and occurrence of hemorrhage in developing thromboatherosclerotic and thrombosclerotic plaques (18 and 28 days)

No. of rabbits with plaque	Av. serum cholest.*	Age of plaques	No. with lipid (sudanophilia) in: Intimal tissue Basal	Intimal tissue Superficial	Media	Adventitia	No. with excess mucopolysaccharide in: Intimal tissue Basal	Intimal tissue Superficial	Media	Adventitia	No. with hemorrhage in: Intimal tissue Basal	Intimal tissue Superficial	Media	Adventitia
	mg/100 ml	*days*												
			A. Hypercholesterolemic rabbits											
5	391	18	5	0	3	2	5	2	2	0	4	0	2	2
4	985	28	4	0	2	1	4	2	2	0	2	0	1	1
			B. Normocholesterolemic rabbits											
5	38	18	0	0	0	0	5	2	2	0	0	0	0	0
4	28	28	0	0	0	0	4	2	2	0	1	0	0	0

* Average serum cholesterol calculated as the mean of serum cholesterol values at beginning and end of experiment.

Counts per minute were corrected for a background value of 11 cpm (11.0 to 11.2 cpm). All samples were counted to a minimum 95 per cent confidence level ($p < 0.05$) or 400 counts.

2. Cholesterol-4-C^{14} studies. Aortic thrombi were induced in four rabbits, then 28 days later, each was given 5 μc of cholesterol-4-C^{14} (dissolved in 2 ml of olive oil) by gastric intubation. The rabbits subsequently were given a diet containing 2 per cent cottonseed oil for 72 hours to facilitate the absorption of the administered radioactive cholesterol. They then were fed the ordinary laboratory rabbit chow.

These four rabbits came to autopsy at 8, 22, 38, and 66 days after the intubation. The plaque area of each rabbit was stripped from the subjacent aorta and a sample of nearby, but uninvolved, aorta also was obtained. The thrombointimal portion of the thromboaortic samples was approximately the same width as that of the media. Digestion of the samples, nitrogen analysis, and cholesterol extraction and counting procedures were done as described above.

D. Studies of the permeability of newly formed blood vessels in hyperplastic intimal tissue

The observed early accumulation of lipid in the basal portions of the intimal tissue growing in response to thrombus induction suggested the possibility that the newly formed capillaries attending this intimal growth were unduly permeable to the lipoprotein aggregates present in plasma. In order to determine the existence of excessive permeability, the following studies were done.

Thrombi were induced in 87 normal rabbits subsequently maintained on ordinary rabbit chow. Then, at 14, 21, 42, and 84 days after such induction, some of the animals were injected intravenously either with solutions of Evans blue, Trypan blue, or with suspensions of Thorotrast or fine iron particles derived from decomposition of iron carbonyl.[3]

[3] Carbonyl iron powder (average diameter, 3 μ); A. D. Mackay, Inc., New York, N. Y.

Thirty-seven such rabbits received injections of Evans blue (10 mg per day for 3 days) either at 14, 21, 42, or 84 days after induction of the thrombus. Five of the rabbits were injected with a single dose of Trypan blue (15 mg) 21 days after thrombus induction. Two of the rabbits received a single injection of Thorotrast (2.5 ml) 21 days after thrombus formation. The remaining 43 rabbits were injected with a suspension containing 0.3 g of carbonyl iron particles (approximate diameter, 3 μ) twice daily for 3 days beginning either at 14, 21, 42, or 84 days after thrombus induction.

All rabbits were sacrificed 24 hours after the last injection and the aorta and the thrombosclerotic plaque inspected grossly for differences in dye or particle accumulation. In addition, the plaques and samples of uninvolved aorta of the rabbits receiving iron or Thorotrast were sectioned and stained with Berlin blue and Sudan, respectively.

RESULTS

A. Observations concerning some of the phenomena involved in the early-developing plaque. As was noted before (1), the early plaques of all nine hypercholesterolemic rabbits, whether they were examined 18 or 28 days after thrombus induction, exhibited sudanophilia in the basal layer of the newly developing hyperplastic intimal tissue. The stained lipid appeared to be deposited both intra- and extracellularly. In addition (Table I and Color Figure 2), five of these rabbits exhibited sudanophilic infiltration of the inner third of the media underlying the plaque. Indeed, the internal elastic membrane as early as 18 days after thrombus induction invariably exhibited splitting (Figure 1) or fragmentation. The adventitial area also was not spared, in that three of the nine hypercholesterolemic rabbits

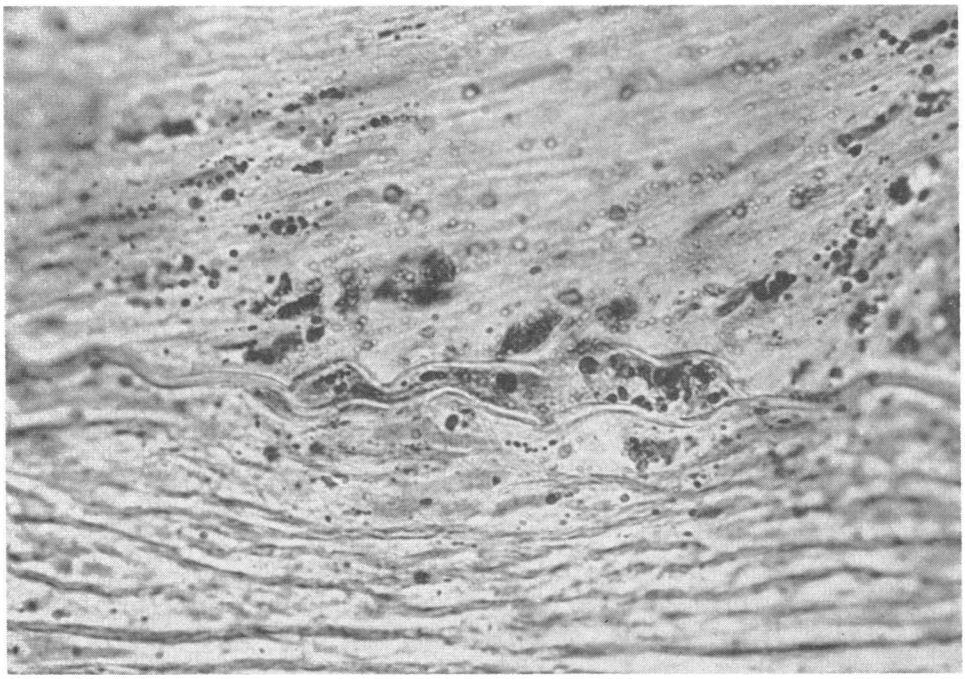


FIG. 1. RABBIT 0–3. Developing thromboatherosclerotic plaque of a rabbit fed cholesterol-oil, 28 days after intraaortic coil insertion (Sudan IV stain, ×400). A portion of the basal intimal tissue and media is shown. The splitting of a part of the internal elastic membrane can easily be discerned. The dark particles contained within this reduplication are masses of lipid taking the Sudan stain. Note the diffusely scattered extravasated red blood cells in the intimal tissue, indicating a previous hemorrhage.

exhibited a similar but somewhat less sudanophilic infiltration of the adventitia opposite the plaque. None of these nine plaques, however, exhibited any sudanophilia in the superficial intimal areas adjacent to luminal blood.

On the other hand, no significant sudanophilia was observed in any portion of the plaque or aortic wall of the nine normocholesterolemic rabbits, either in those sacrificed at 18 or 28 days after thrombus induction. However, similar splitting and fragmentation of the internal elastic membrane were observed.

The plaques of all rabbits, both normo- and hypercholesterolemic, exhibited excess mucopolysaccharide in the basal layer of the new intimal tissue (Table I and Color Figure 3). The inner media, but not the adventitia, of most of these rabbits likewise showed some excess of mucopolysaccharide. It should be pointed out that such excess material usually was found in the same areas of the plaque which exhibited sudanophilia.

Usually, too, it was in this same basal area of new intimal tissue that new blood vessels could be observed most frequently. Most of these vessels (Figure 2) appeared to be larger in size than the ordinary capillary, but they lacked the various cellular layers of the arteriole. Frequently, also, many vessels of this type were conjoined to form a labyrinth of sinusoidal channels. Such capillaries always were found to enter the base of the plaque via an area of disorganized medial tissue. In no section, however, was there evidence of the luminal origin of any capillary.

Hemorrhage (as evidenced by large accumulations of erythrocytes lying free in the tissue) occurred (Table I) in the plaques of six of the nine hypercholesterolemic rabbits, but in only one of the nine normocholesterolemic rabbits. Such hemorrhages were observed early and once again usually were found chiefly in the same basal area of the new intimal tissue (Color Figure 4) or in the inner, disorganized third of the media where sudanophilia and dense mucopolysaccharide staining also were observed. However, hemorrhage (as evidenced by pools of erythrocytes) in the adventitia immediately beneath the media (Figure

COLOR FIG. 1. RABBIT RR–19. Fully developed thromboatherosclerotic plaque, 84 days after insertion of thrombus-inducing coil in a cholesterol-fed rabbit (Sudan IV stain, ×25). The sudanophilia involving the basal and lateral areas of the plaque is clearly shown. Note too the slight sudanophilic infiltration of the adventitia underlying the plaque. Medial atrophy below the plaque is quite striking. The similarity of this plaque to that found in the diseased human coronary artery already has been pointed out (1).

COLOR FIG. 2. RABBIT 0–2. Developing thromboatherosclerotic plaque of a rabbit fed cholesterol-oil, 28 days after intraaortic coil insertion (Sudan IV stain, ×100). The sudanophilia involving the basal intimal tissue, portions of the inner third of media, and parts of the subjacent adventitia is clearly depicted. Metallic salt fragments can still be seen. Between and over such areas, newly growing intimal tissue almost devoid of sudanophilia can be observed. The transmedial source of the Sudan-stained lipid can be adduced from this photograph.

COLOR FIG. 3. RABBIT 0–5. Developing thromboatherosclerotic plaque in a rabbit fed cholesterol-oil, 28 days after insertion of coil [Rinehart and Abul-Haj (4) stain, ×25]. The excess mucopolysaccharide (staining blue) can be seen to occupy chiefly the same basal area of new intimal tissue that the Sudan-staining lipid occupies in Figure 2. The superficial intimal tissue covering the original thrombus (it has been torn from the base on the left) also exhibits some excess mucopolysaccharide. The adjacent uninvolved aorta is free of this blue stain.

COLOR FIG. 4. RABBIT 0–3. Portion of a developing 28 day old thromboatherosclerotic plaque (Sudan IV stain, ×400). The area illustrated is a basal portion of the newly developed intimal tissue lying upon a partially disintegrated inner media. The hemorrhage indicated by the extravasated red blood cells (pale cells with heavy, circular outlines) is precisely in the area where Sudan-staining material abounds. Note the presence of both red blood cells and sudanophilia in the media also.

COLOR FIG. 5. RABBITS W2–W5. Sample of the pooled tissues obtained from 21 day old thrombosclerotic plaques of four normocholesterolemic rabbits that later was placed in Millipore capsules (Sudan IV stain, ×160). The spindle-shaped cells with elongated nuclei and the complete absence of sudanophilia can easily be seen.

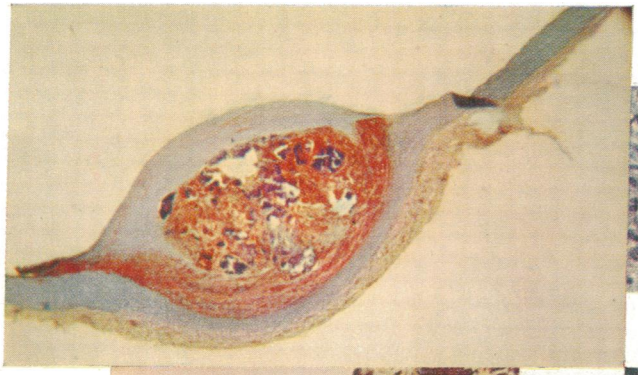
COLOR FIG. 6. RABBIT 24. Portion of the pooled tissues shown in Color Figure 5 after 8 days in an "intact" Millipore capsule (Sudan IV stain, ×160). The tissue resembles quite closely that shown in Color Figure 5. No capillaries or sudanophilia can be detected.

COLOR FIG. 7. RABBIT 24. Portion of the pooled tissues shown in Color Figure 5 after 8 days in an "open" Millipore capsule (Sudan IV stain, ×160). Note the intense, intracellularly situated sudanophila and the presence of capillaries. These findings contrast strongly with those observed in the same tissue kept in the "intact" capsule (Color Figure 6).

COLOR FIG. 8. RABBIT 42. Photograph of the opened abdominal aorta and a 14 day old thrombosclerotic plaque of a normocholesterolemic rabbit previously injected with Evans blue dye. The thrombus exhibiting a large residue of metallic fragments has been detached from the aorta revealing, at this latter site, the presence of several areas of extravasated Evans blue dye (blue).

COLOR FIG. 9. RABBIT 16. Developing 21 day old thrombosclerotic plaque of a normocholesterolemic rabbit previously injected with colloidal iron (Berlin blue stain, ×65). The blue-staining iron deposits can be observed in the basal and lateral areas of the intimal tissue abutting upon the media.

COLOR FIG. 10. RABBIT 36. Mature 84 day old thrombosclerotic plaque of a normocholesterolemic rabbit previously injected with colloidal iron (Berlin blue stain, ×65). Unlike the deposit observed in the 21 day old plaque (Color Figure 9), no iron can be detected anywhere in this older plaque.



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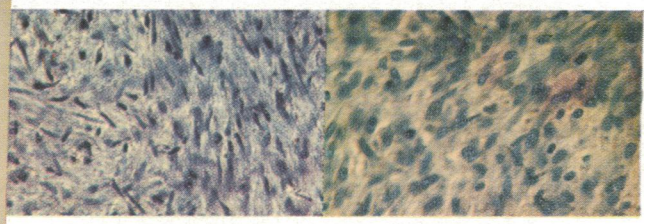

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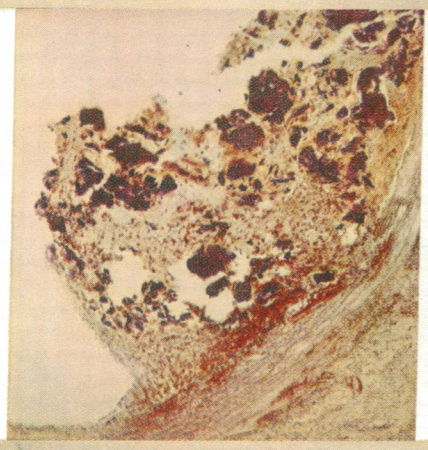

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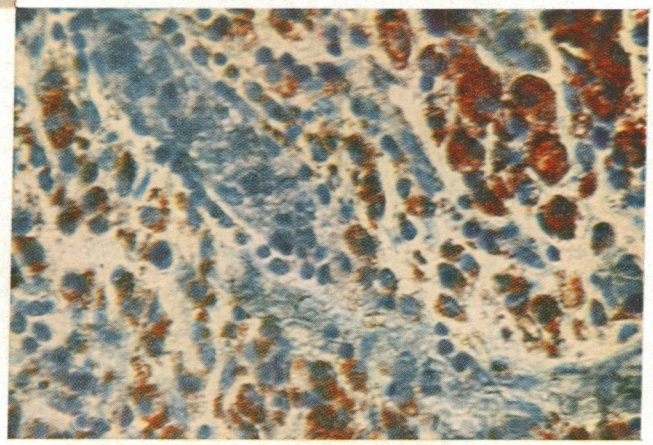

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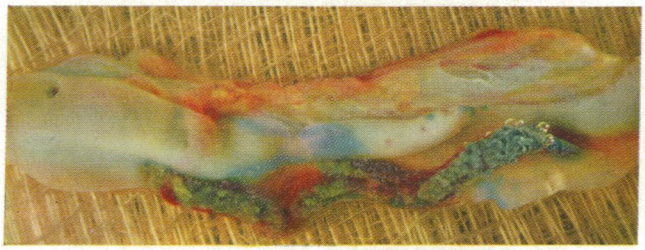

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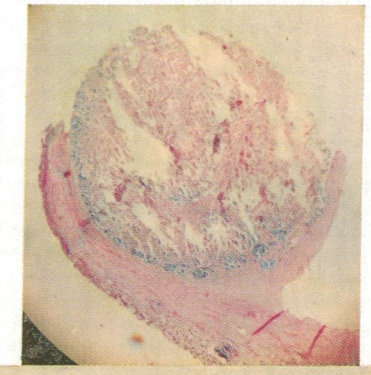

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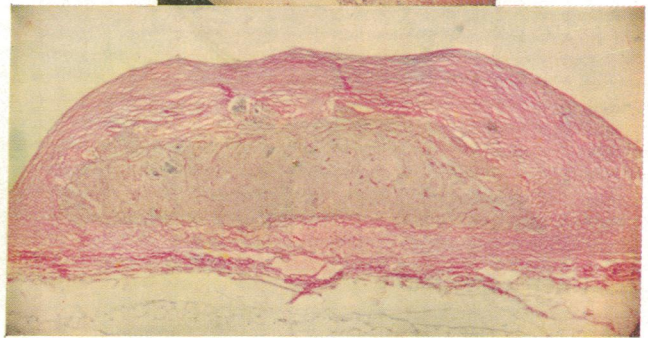

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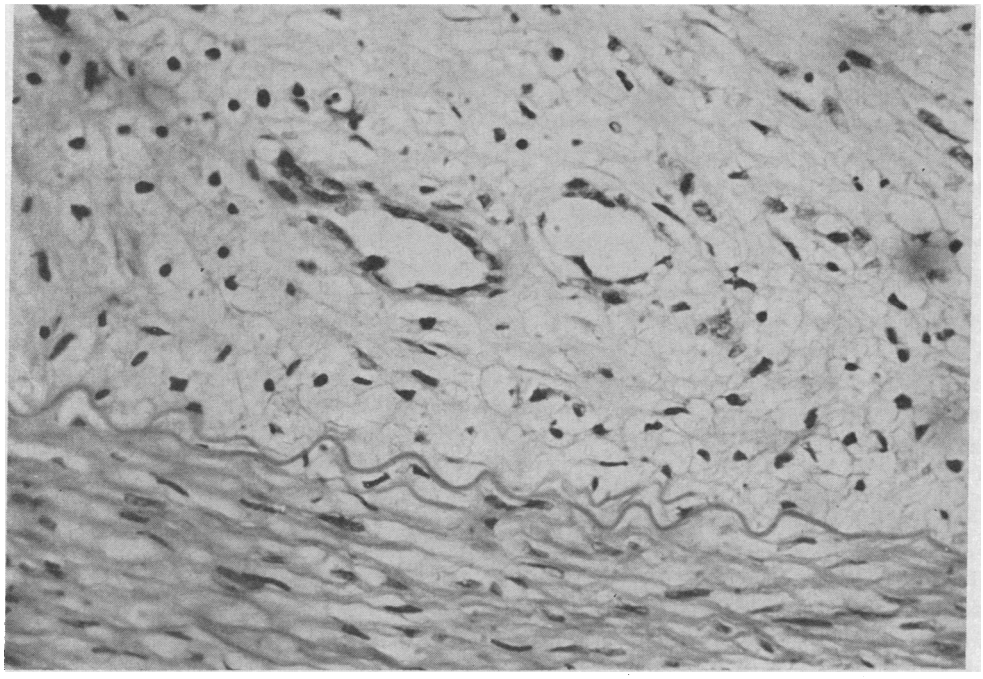

FIG. 2. RABBIT 0–4. Another plaque similar in type and age to that shown in Figure 1 (H & E stain, ×400). Two vessels can be seen in the basal intimal tissue exhibiting a wide lumen yet possessing capillary-like walls. Note the internal elastic membrane and its beginning fragmentation.

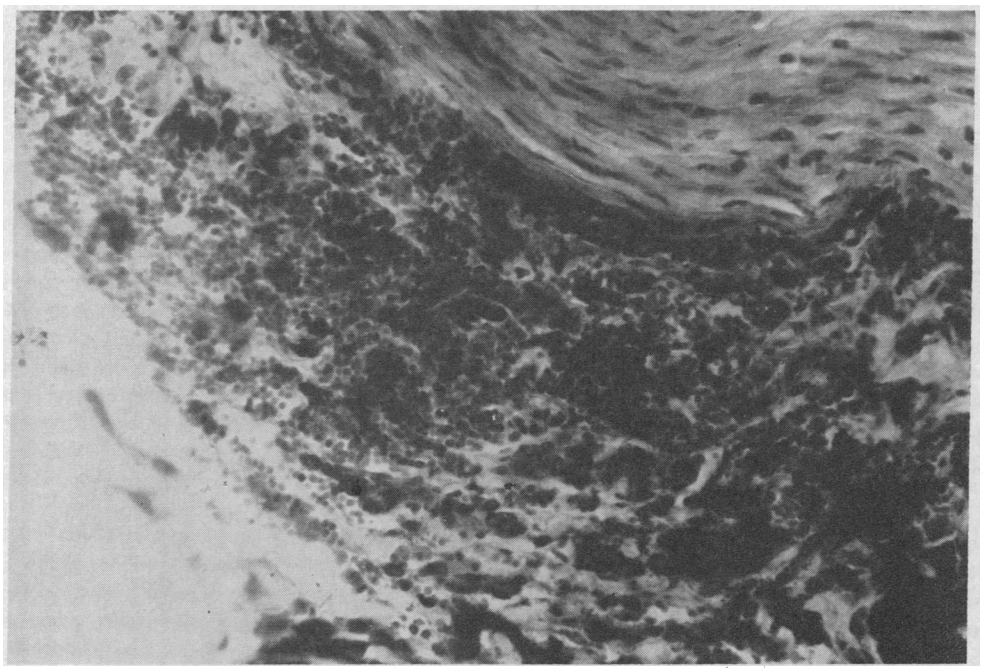

FIG. 3. RABBIT 0–4. Portion of adventitia and outer part of media subjacent to the plaque illustrated in Figure 2 (Sudan IV stain). Here a large hemorrhage (indicated by the masses of extravasated red blood cells) has occurred in the adventitia (below) immediately external to the media (above).

3) occasionally was observed near the plaques borne by the hypercholesterolemic rabbit.

B. Observations concerning the lipid and cholesterol accumulation occurring in avascular hyperplastic intimal tissue. The hyperplastic intimal tissue obtained from plaques borne by normocholesterolemic rabbits and transferred to "intact" Millipore capsules (which then were inserted in the peritoneal cavity of each of two hyper- and one normocholesterolemic rabbits) survived during the 8-day period in every instance but one. The same tissue transferred to the "open" capsules and inserted also into the peritoneal cavity of the same three rabbits survived in every instance.

Observation at autopsy revealed that all of the capsules were covered with highly vascular connective tissue. Moreover, this latter tissue was observed to have penetrated each of the "open" capsules through the openings that had been made. The "intact" capsules, however, had not been penetrated from the outside. When the capsules were opened, the tissues contained in "open" capsules exhibited a rich vascularity as observed by means of the dissecting microscope. The tissues in the "intact" capsules, however, appeared to be totally devoid of vascularity.

A second marked difference was observed between the tissue contained in the "intact" capsules and that in the "open" capsules. The tissues obtained from the four "intact" capsules inserted into the hypercholesterolemic rabbits histologically resembled without exception (see and compare Color Figure 6 with Color Figure 5), the initial proliferating intimal tissue. Both types of tissue consisted predominantly of spindle-shaped cells and both appeared totally devoid of sudanophilia. It was of interest, however, that the outer half of the Millipore material which had been in contact with hypercholesterolemic peritoneal fluid, itself stained red with the Sudan stain. No capillaries could be detected on microscopic examination in the samples of intimal tissue that had been in the "intact" Millipore capsules for the 8-day period. The samples of intimal tissue contained in the "intact" capsules placed in the normocholesterolemic rabbit appeared identical with those described in the "intact" capsules of the hypercholesterolemic animals.

TABLE II

Characteristics of newly developing thrombosclerotic tissue after survival in Millipore capsules placed in the peritoneal cavities of hyper- and normocholesterolemic rabbits

Rabbit	Av. serum cholest.*	Peritoneal fluid cholesterol†	No. of Millipore capsules inserted: With tissue Intact	With tissue Open	With Tyrode's	No. of capsular tissues surviving (8 days) Intact	Open	Av. cholest. in capsular tissues‡ Intact	Open	Av. cholest. in capsular fluid	No. of capsular tissues with sudanophilia Intact	Open	No. of capsular tissues with capillaries Intact	Open	Av. cholest. of Millipore material§
	mg/100 ml							*mg/100 g*		*mg/100 ml*					*mg/100 g*
22	1,850	394	3	1	1	2	1		8,420	393	0	1	0	1	1,795
24	2,335	1,015	2	2	1	2	2	2,905	7,505	412	0	2	0	2	894
26	55		2	2	0	2	2	2,310	2,578		0	2	0	2	

* Average serum cholesterol calculated as the mean of serum cholesterol values at beginning and end of experiment.
† Sample obtained at sacrifice of animal.
‡ Thrombosclerotic tissue at time of insertion = 2,140 mg/100 g cholesterol.
§ Millipore Epoxy assayed 0 cholesterol at time of insertion.

TABLE III

Synthesis and deposition of cholesterol in thrombosclerotic plaques

Rabbit	Age of plaque at sacrifice	Days after cholest.-4-C^{14} intubation*	Dry wt of samples		Total cholest.		Cholesterol		Tissue nitrogen		Ratio: Cholest./Nitrogen						Qo_2 (N)†	
			Plaque	Aorta	Plaque	Aorta	Plaque	Aorta	Plaque	Aorta	Plaque	Aorta	Plaque	Aorta	Plaque	Aorta	Plaque	Aorta
	days		*mg*	*mg*	*mg/100 g*		*mg plated*		*mg*	*mg*	*mg/g*		*cpm*		*cpm/mg cholest.*		*cpm/mg N*	
Acetate-1-C^{14} studies																		
RA-1	21						0.417	0.055	4.14	1.64	101	34	45	18	107	322	11	11	6.5	4.6
RA-3	28						0.301	0.040	2.50	1.52	120	26	11	8	36	192	4	5	3.1	2.2
Cholesterol-4-C^{14} studies																		
757	36	8	23.7	51.2	2,470	69	0.572	0.034	2.73	6.78	209	5	18	1	31	29	6	<1		
572	50	22	16.4	68.6	3,170	372	0.507	0.249	1.64	9.55	309	26	10	4	20	16	6	<1		
755	60	38	19.3	52.5	3,870	381	0.730	0.195	1.36	6.55	534	30	26	7	36	36	19	1		
758	94	66	13.8	55.9	2,140	281	0.281	0.149	1.86	6.65	151	22	14	3	50	20	8	<1		
Mean			18.3	57.1	2,912	276	0.522	0.157	1.90	7.41	301	21	17	4	34	25	10	1		

* All of the plaques of rabbits (752–758) were 28 days old at time of administration of cholesterol-4-C^{14}.
† μl O_2/hr/mg tissue nitrogen.

On the other hand, the three tissue samples obtained from the "open" capsules inserted in the same two hypercholesterolemic rabbits uniformly showed on microscopic examination (see Color Figure 7) a rich supply of capillaries but no hemorrhages and an intense sudanophilia. The stained lipid appeared to be exclusively intracellular. The tissues obtained from the "open" capsules inserted into the normocholesterolemic rabbit also exhibited the same rich vascularity, but no hemorrhages and no sudanophilia.

Analyses of the fluid remaining in the two "intact" capsules initially filled with Tyrode's solution (Table II), however, indicated that considerable cholesterol, ostensibly from the peritoneal fluid or from the blood supply of the new connective tissue covering the outside of the capsule, had been able to enter the capsules. Moreover, the Millipore material itself was found to have adsorbed considerable cholesterol (Table II).

Cholesterol analyses of some of these capsular tissues agreed in general with the histological results. Whereas the average cholesterol content of two tissue samples placed in "intact" capsules carried by a hypercholesterolemic rabbit was 2,905 mg per 100 g (Table II), the average cholesterol content of three similar tissues in "open" capsules carried by hypercholesterolemic rabbits was 7,810 mg per 100 g. It was of interest that the tissues contained in the "intact" capsules carried by the hypercholesterolemic rabbit did not exhibit a much higher cholesterol than that exhibited by tissues contained in the "open" capsules placed in the normocholesterolemic rabbit. Nor did such isolated tissues in the hypercholesterolemic rabbits exhibit a dramatic increase in cholesterol as compared to that present (2,140 mg per 100 g) in the intimal tissue as initially placed in the capsule.

These results appeared to us to indicate that the sudanophilia and the intense cholesterol accumulation noted in the early-developing plaque were primarily dependent upon the presence of the new vascular supply of this same plaque; for in the absence of such a new vascular supply, both phenomena failed to occur.

C. Cholesterol synthesis and deposition in the developing plaque. Table III documents the

TABLE IV

Extravasation of dyes in developing thrombosclerotic plaques

No. of rabbits with plaque	Plaque age	No. of plaques exhibiting gross dye extravasation	
		Basal area	Superficial area
	days		
A. After injection of Evans blue			
6	14	6	2
14	21	14	2
12	42	2	0
5	84	0	0
B. After injection of Trypan blue			
5	21	5	0

fact that the living tissue (i.e., the hyperplastic intimal tissue) of the early-developing plaque was capable of incorporating acetate-1-C^{14} into cholesterol. Moreover, it also is evident from the data in this table that this hyperplastic tissue appeared to incorporate as much radioactive acetate into cholesterol as the control, uninvolved section of aorta, although the rate of incorporation was quite slow in both types of tissue. At first glance, the much higher specific activity of the cholesterol isolated from the normal aorta suggests that this tissue is capable of incorporating more acetate-1-C^{14} into cholesterol than is the plaque tissue. However, the plethora of cholesterol already present in the plaques (1) prior to the study would tend to dilute the newly formed radioactive cholesterol as compared with that formed in the normal aorta. A comparison, therefore, of the respective specific activities of the cholesterol finally isolated would not reveal the respective degrees of acetate incorporation. However, when the counts per minute (cpm) of the total cholesterol content are correlated with the nitrogen content of both tissues, it can be seen (Table III) that the ratios are essentially the same. In short, these results strongly suggested that the newly formed intimal tissue was synthesizing neither more nor less cholesterol than the normal aorta. The excess cholesterol observed accumulating in these plaques, therefore, did not appear to be due to an increased cholesterol synthesis *in situ*.

The results obtained from the administration of cholesterol-4-C^{14} to plaque-bearing animals, however, strongly suggested (Table III) that the tissue making up these plaques differed markedly from normal aortic tissue in its ability to extract or retain an excess of cholesterol from the blood, or both. Thus, at whatever age the plaque-bearing animals were sacrificed, although the cholesterol moiety of the normal aorta exhibited very minimal radioactivity per milligram of tissue nitrogen, that of the plaques exhibited significant activity (at least ten times as much as that of aortic cholesterol). It also was of interest that the specific activity of the cholesterol contained in the plaques was the same as or slightly higher than that of the normal aorta (Table III), despite the diluting effect of the relatively enormous amount of nonradioactive cholesterol that had accumulated before, and continued to accumulate after, the administration of the radioactive cholesterol. These results, therefore, when considered with those obtained from the acetate-1-C^{14} experiments, strongly suggested that the excess cholesterol found in the thromboatherosclerotic plaque was being obtained primarily and chiefly from the blood supplying this tissue.

D. Permeability of the newly formed blood vessels in hyperplastic intimal tissue. The studies described above left little doubt that the excess lipid and cholesterol accumulating in the thromboatherosclerotic plaque were not formed *in situ,* but were being brought to the area by its new supply of blood vessels, arriving by way of the adventitia. It seemed probable to us that these same vessels, at least in the earlier stages of plaque development, were abnormally permeable to large size molecules such as lipoproteins. If such increased permeability were present, it should be possible to demonstrate it by other techniques.

The results obtained with the dyes, Evans blue and Trypan blue, and with the iron and Thorotrast suspensions made it clear indeed that these vessels were excessively permeable. After the injection of Evans blue and sacrifice of plaque-bearing rabbits, it was observed grossly (Table IV, A) that those plaques which were 14 and 21 days old invariably exhibited a considerable extravasation and condensation of the dye at their base; i.e., at the point where they were attached to the aorta (Color Figure 8). This area, of course, is precisely that region where lipid and

cholesterol were observed to be initially and later, maximally deposited. It is also the area wherein the majority of blood vessels was first observed. On the other hand, the peripheral intimal tissue covering the initial thrombus infrequently exhibited extravasated dye (Table IV, A). Also, no extravasation of the dye was ever detected in the intima of the nearby normal uninvolved aorta. The 21 day old plaques of the five rabbits injected with Trypan blue similarly revealed (Table IV, B) extravasation and condensation of the dye at the base of the thrombosclerotic plaque. Similar extravasation of dyes in the adjacent uninvolved aorta was never seen.

However, 42 day old thrombosclerotic plaques infrequently exhibited, and 84 day old plaques never exhibited (see Table IV, A) the basal extravasation of Evans blue dye after its antemortem injection. Apparently the increased permeability of these intimal vessels was a phenomenon limited to the first few weeks or months of their development.

Results obtained (Table V) after the injection of suspensions of carbonyl iron particles were similar in part to those obtained after the injection of the dyes. Thus, three of the four 14 day old plaques grossly exhibited iron deposition at the base of the plaque precisely as was observed in the plaques of the dye-injected animals. Nineteen of the 39 plaques (21 days old) also grossly exhibited this basal deposition of iron. However, in addition, deposits of iron also were observed (Figures 4 and 5) in the superficial intimal tissue covering many of the 14 and 21 day old plaques (Table V). The luminal blood obviously was the provenance of these deposits of iron. No deposit of such iron was ever observed in the intima of the normal uninvolved aorta. Plaques that were 42 and 84 days old uniformly failed to exhibit gross deposits of iron. Here again, as was found in the dye studies, the intimal vessels apparently became relatively impermeable with the passage of time.

Microscopic studies of these plaques confirmed, in general, the gross findings. The basal site of

TABLE V

Deposit of iron in newly developing and mature thrombosclerotic plaques

No. of rabbits with plaque	Plaque age	No. of plaques grossly exhibiting iron deposits		No. of plaques exhibiting microscopic iron deposits		
		Basal area	Superficial area	Basal area	Superficial area	Adventitia
	days					
4	14	3	3	3	4	0
39	21	19	13	36	19	20
5	42	0	0	0	0	0
5	84	0	0	0	0	0

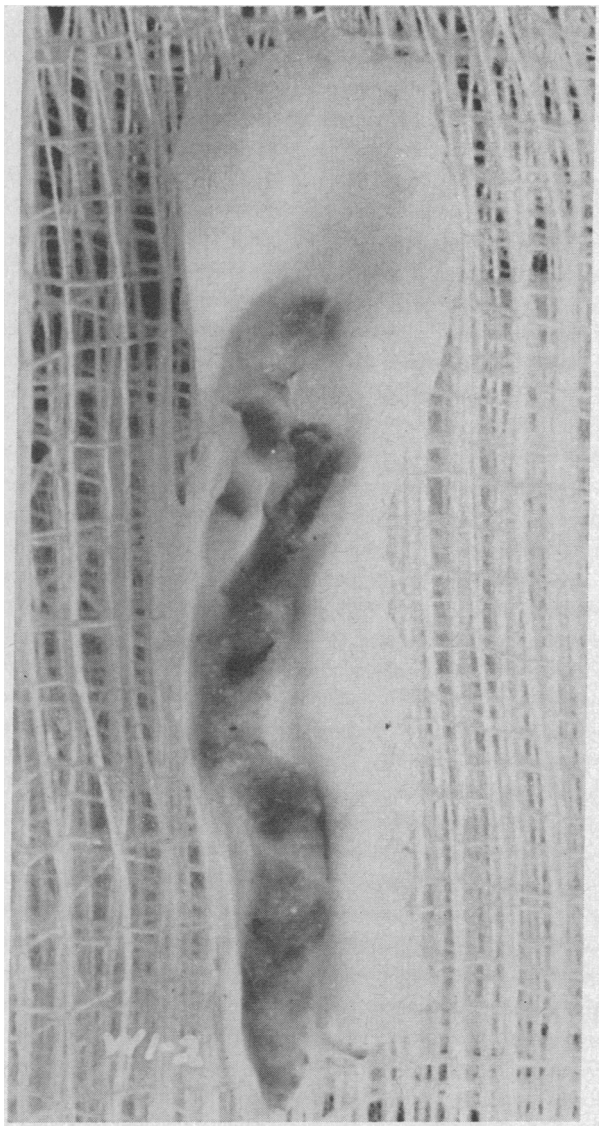

FIG. 4. RABBIT W1–2. Abdominal aorta with its attached thrombosclerotic plaque (28 days old) of a normocholesterolemic rabbit previously injected with iron particles. The superficial deposits of iron (black) in the plaque can be discerned. As Figure 5 also illustrates in cross section, the iron particles are mostly just within the enveloping intimal tissue. Note the iron-free appearance of the uninvolved normal adjacent aortic areas.

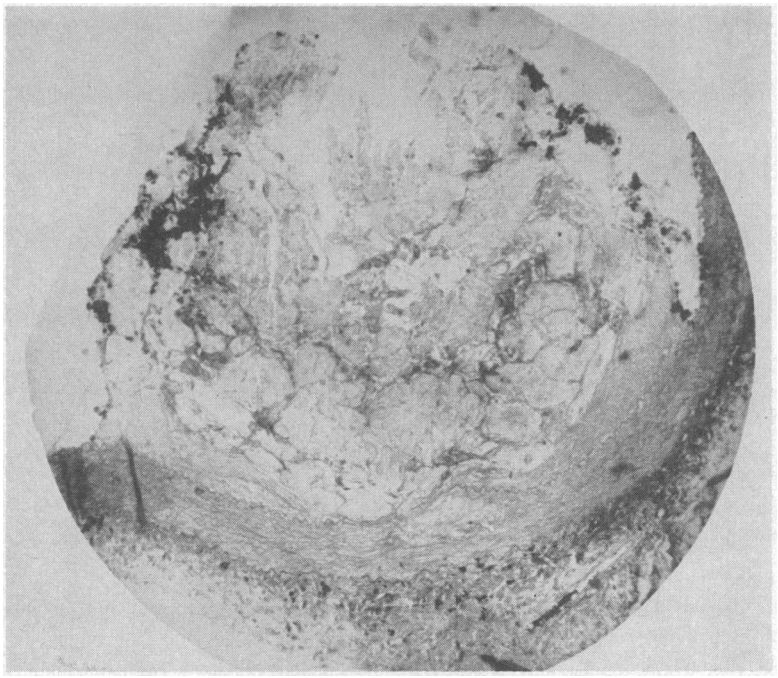

FIG. 5. RABBIT W1–2. Microscopic view of thrombosclerotic plaque also illustrated in Figure 4 (Berlin blue stain, ×65). The superficial deposits of iron (black) in the enveloping intimal cells are clearly shown. Note also the dispersed iron particles in the adventitial area subjacent to the total plaque process. The deposits of iron usually observed in the basal intimal area did not occur in this plaque.

iron deposit in the early developing plaque was clearly observed (Color Figure 9) and its absence as clearly noted in the fully developed or 84 day old plaque (Color Figure 10). Again in a number of the 14 and 21 day old plaques, deposits of iron in the more peripheral areas of intimal tissue encircling the thrombus could be observed (Figure 5). Also, in more than half of the 21 day old plaques, iron deposits were observed in that portion of the adventitia underlying the plaque (Figure 5). It was our impression that these latter iron particles were being retained by the endothelial cells of smaller, possibly newly formed adventitial vessels. In several instances, we were certain that we could detect actual phagocytosis of iron particles by the lining cells of adventitial capillaries.

The two rabbits injected with Thorotrast 21 days after thrombus induction exhibited, on sacrifice, plaques whose sections, when stained with Sudan, exhibited the characteristic minute particles of thorium (dioxide) again and only in the extravascular areas of the basal areas of the hyperplastic intima.

DISCUSSION

The preceding studies appear to us to answer the question posed in our first study of experimental thromboatherosclerosis (1) concerning the origin of the excess lipid and cholesterol detected very early in the basal layers of hyperplastic intimal tissue in response to an induced thrombus.

The present study confirmed the findings of the initial study in respect to the initial site of the lipid and cholesterol accumulation and the improbability of their derivation from the luminal blood flowing past the intimal process. Thus, the greatest mass of new blood vessels (chiefly capillaries) occurred in the same area that was observed to accumulate lipid and cholesterol. The frequent rupture of these vessels (especially in the hypercholesterolemic animal) was observed, and again in the same area in which lipid and cholesterol first accumulated in excess. Also, inti-

mal tissue, transplanted in such a manner as to ensure the absence of these blood vessels, failed to exhibit either excess lipid or cholesterol. The radioactive studies, moreover, indicated that the hyperplastic intimal tissue, while not capable of synthesizing more cholesterol than is normal aortic tissue, nevertheless appeared to extract or retain overwhelmingly more cholesterol from plasma than did normal aortic tissue. Finally, the experimental results obtained from the dye and colloidal suspensions suggested that "leakage" from the newly formed adventitia-derived capillaries was sufficiently gross to allow the escape of particles having a diameter of at least 3 μ (e.g., the carbonyl iron particles). Such "leakiness" or increased permeability of these vessels was, however, a temporary phenomenon observed only during the first few weeks of plaque development. It was of interest, too, that the superficial hyperplastic intimal tissue itself in its earliest phases of growth appeared capable of phagocytosis, as revealed by the iron injection studies.

Considered as a whole, these results suggest that the thromboatherosclerotic plaque developing in the hypercholesterolemic rabbit accumulates lipid coming from capillaries almost certainly of adventitial origin. The early or initial structure of these capillaries appears to be such that their frequent gross fracture and excessive permeability allow this accumulation. However, important as hemorrhage from these vessels probably is in this process of lipid and cholesterol deposition, it is not the chief provender. Excessive lipid and cholesterol deposit also occurred in the intimal tissue allowed to survive in the "open" Millipore capsules, although in no instance was hemorrhage ever observed in these transplanted tissues. In short, hemorrhage undoubtedly increases the lipid and cholesterol accumulation, but such accumulation will still take place even in the absence of hemorrhage.

Additional evidence that increased capillary permeability was the chief cause of the excess deposit of lipid and cholesterol in the forming thromboatherosclerotic plaque was provided by the following observation. Capillaries of a thrombosclerotic plaque (i.e., an aortic plaque placed in a normocholesterolemic rabbit) failed to exhibit this abnormal permeability to dyes and colloidal suspensions after 6 or more weeks (1). Such mature plaques also were observed (1) to exhibit extreme resistance to the accumulation of excess lipid and cholesterol if the rabbits bearing such plaques later were made hypercholesterolemic only *after* the relative maturation of the plaque.

There was little doubt when these studies had been completed that the lipid and cholesterol accumulating in the plaque of the hypercholesterolemic rabbit had escaped from abnormally permeable and frequently ruptured capillaries, or possibly from capillaries whose walls were not as yet fully formed. Nevertheless, the hyperplastic intimal tissue in its turn probably was capable of preferentially retaining lipid and cholesterol. The marked phagocytic or retentive capacity of this tissue observed in respect to escaped iron particles probably is the cause also of the frequently observed intracellular sequestration, hence probable retention of the escaped lipoproteins. Frequently, extracellular masses of Sudan-stained material also were observed even in young plaques, but invariably there was associated hemorrhage or necrosis of intimal cells. Certainly the lipid was exclusively intracellular in the intimal tissue within the "open" Millipore capsules. Such transplanted intimal tissue, moreover, never exhibited hemorrhage.

Besides the phagocytic property of new intimal tissue, a further cause of retention of the escaped lipoproteins could be the observed excess of mucopolysaccharide accumulating in the basal areas of the new intimal growth. Such mucopolysaccharide has been reported (8) to be capable of binding β-lipoprotein moieties. Finally, the possible absence of lympatic vessels in this newly growing tissue might also favor the accumulation of escaped lipoproteins therein.

The demonstration of the role of the intimal capillary in the pathogenesis of the excess lipid and cholesterol found to be accumulating in the thromboatherosclerotic plaque of the hypercholesterolemic rabbit in no manner controverts the findings of those investigators (9–14) who have found aortic tissue and even plaques capable of synthesis of certain lipids and cholesterol. In the present study we also found the aorta and plaque capable of cholesterol synthesis. Nevertheless, we believe our present results amply illustrate that, in this form of thromboatherosclerosis, the excess triglyceride and cholesterol are not derived pri-

marily from local processes of synthesis. It is of interest that the plaques in animals made atherosclerotic by feeding of cholesterol also have been found to extract relatively large quantities of either labeled cholesterol (14–16) or triglyceride (17) when either substance is administered. The atherosclerotic aorta of man also has been observed (18) to extract and retain administered labeled cholesterol.

In our first study of experimental thromboatherosclerosis, we stressed our belief that the essential sameness of all experimental atherosclerotic plaques may be due, not to the sameness of initiating agent, but to the sameness of reactive intimal hyperplasia and its characteristic properties which are elicited by various agents or actions; e.g., thrombus formation (19–21), hemorrhage (22), direct mechanical or chemical injury of the artery (23–28), spontaneous fragmentation of the internal elastic membrane (29, 30), implantation of synthetic grafts (31, 32), experimental hypercholesterolemia, and so forth. Two of these characteristic properties are now found to be: *1*) increased permeability of the capillaries accompanying the intimal hyperplasia, and *2*) phagocytosis by intimal cells. Both of these properties acting conjointly serve as an adequate explanation for the observed avidity with which this intimal tissue obtains and retains excess lipid and cholesterol in experimental thromboatherosclerosis. It is our opinion that these processes are also active in the pathogenesis of human atherosclerotic plaques, again regardless of the possible variety of agents that may initiate intimal hyperplasia in the human species.

The present study leaves unanswered the question of why the newly formed intimal capillaries exhibit increased permeability or "leakage" in the earlier stages of the development of a thrombosclerotic plaque and a loss of this property in the latter's more mature stage. As stated before (1), we are uncertain at this time whether such increased permeability is induced by some atypical inflammatory process directly injuring the capillaries, such as seemingly occurred in the arterial lesions which Waters (27, 28) produced by injections of pressor chemicals. Nor are we certain that this capillary defect also will disappear, perhaps more slowly, in a thromboatherosclerotic plaque because the latter type of plaque contains an excess of lipid and cholesterol. These two substances (or possibly only cholesterol) have been found (1) to lead to necrosis and liquefaction of the central area of plaques and are found in this present study to be associated with increased fragmentation of newly formed plaque capillaries. Thus, these substances may themselves act as chronic stimulants for compensatory intimal hyperplasia with a persistence or even intensification of the two properties characteristic of early intimal hyperplasia which were found in this study to play such a major role in the accumulation of lipid and cholesterol.

SUMMARY

The pathogenesis of the excess accumulation of lipid and cholesterol occurring in experimental thromboatherosclerotic plaques was studied by means of various techniques.

It was observed that the principal causes of the accumulation of excess lipid and cholesterol were: *1*) an increased permeability of the newly formed intimal capillaries, associated with *2*) a phagocytic capacity of the new intimal tissue, leading to sequestration and retention of the escaped excess lipoprotein molecules. The tissue of the early developing plaque did not appear to synthesize cholesterol any more rapidly than adjacent normal aortic tissue, but it was found capable of extracting much more cholesterol from the circulating plasma than was uninvolved aortic tissue. This last capacity was considered to be due primarily to the increased permeability of the newly formed intimal capillaries.

The possible role of hyperplastic intimal tissue in the pathogenesis of human atherosclerosis is discussed.

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