FURTHER STUDIES ON THE REVERSIBILITY OF SERUM SICKNESS CHOLESTEROL-INDUCED ATHEROSCLEROSIS

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Previous reports from this laboratory (1-3) and others (4-6) have shown that the combination of serum sickness and hypercholesterolemia causes a higher incidence of atheromatous lesions in the aortas and coronary arteries of rabbits than either stress factor alone. In some of these studies (1-3), data were presented which implied that lesions induced as a result of the combined stressing were more slowly reversible than those caused by either serum sickness or high blood cholesterol treatment alone. The following studies were done to further investigate this reversibility. Serum sickness was induced in groups of rabbits by giving single intravenous injections of bovine serum albumin (BSA),1 according to the procedure used by Germuth (7), and high levels of blood cholesterol were obtained by feeding the animals a cholesterol-supplemented diet of rabbit pellets for 2 wk. Following the period of lesion-induction by either cholesterol diet alone or both treatments in combination, animals were returned to normal diet for varying periods of time. The incidence of atheromatous lesions in the “rested” animals was then determined, along with that of earlier sacrificed controls, in order to compare lesion reversibility.

Materials and Methods

31 male, New Zealand white rabbits (2.0-2.5 kg, Adams Caviary, San Gabriel, Calif.) were divided into groups of five to seven animals each, and treated as shown in Table I. On day 0, each rabbit in groups I-III received a single intravenous injection of BSA (2x crystallized, Nutritional Biochemicals Corp., Cleveland, Ohio) dissolved in physiological saline (250 mg/kg body weight). Rabbits of groups IV-V received no BSA. Also beginning on day 0, all animals (groups I-V) were placed on 100 g per day of Purina rabbit pellets impregnated with sufficient cholesterol (Merck and Co., Inc., Rahway, N. J.) to effect a 1% (by weight) concentration. Water was given ad lib. All animals were maintained on the cholesterol-supplemented diet for 2 wk, at which time rabbits from groups I and IV were sacrificed with intravenously administered sodium thiopental.

Other rabbits (groups II, III, and V) were returned to regular Purina Rabbit Pellets (ad lib) after 2 wk of cholesterol diet. The animals of group II were retained on regular diet for 5 wk,

1 Abbreviations used in this paper: BSA, bovine serum albumin; H & E, hematoxylin and eosin.
and sacrificed, while group III and V rabbits were sacrificed after being maintained on normal feed for a total of 8 wk.

At sacrifice, all hearts were removed, trimmed, and fixed in 10% buffered formalin. Hearts were serially sectioned at 8-12 μ on a freezing microtome through the ascending aortas from the base of the semilunar aortic valves to a level just above the coronary ostia. Alternate sections were prepared with hematoxylin and eosin (H & E), Weigert’s resorcin-fuchsin, and oil red O stains, and examined with the light microscope for evidence of intimal lesions.

Individual sera were collected weekly, from day 0 to the time of sacrifice, and were tested for total cholesterol content using an AutoAnalyzer, (Technicon Instruments Corp., Ardsley, N. Y.).

### TABLE I

| Group | No.* | BSA‡ | Cholesterol diet§ | Regular diet | Total time of experiment |
|-------|------|------|-------------------|--------------|-------------------------|
| I     | 7    | Yes  | 2                 | —            | 2                       |
| II    | 5    | Yes  | 2                 | 5            | 7                       |
| III   | 6    | Yes  | 2                 | 8            | 10                      |
| IV    | 7    | No   | 2                 | —            | 2                       |
| V     | 6    | No   | 2                 | 8            | 10                      |

* Number of rabbits.
‡ Intravenously administered, 250 mg/kg body weight.
§ 1% cholesterol by weight.

### RESULTS

**Cholesterol Blood Levels.**—Fig. 1 illustrates the weekly course of serum cholesterol levels in animals of experimental group V, each of which received cholesterol-supplemented diet for 2 wk, and were then returned to regular diet for 8 wk (up to the time of sacrifice). Within 1 wk after beginning the supplemented diet, mean cholesterol values had risen to seven times the baseline levels. As shown by the standard errors noted in Fig. 1, there were large variations in individual cholesterol values within this group. Mean values did not change significantly during the second week of cholesterol feeding, but by the end of the first week after returning to regular diet (week three of the experiment), a definite downward trend in cholesterol levels was noted. After 1 additional week of regular diet, values had returned to baseline levels, where they stayed for the remainder of the experiment. The pattern seen in Fig. 1 is representative of the course of cholesterol levels seen in the other experimental groups.

**Microscopic Studies of Heart Sections.**—Table II shows that all seven rabbits of group I (BSA + 2 wk of cholesterol diet) developed atheromatous intimal lesions. These lesions were generally distributed along the walls of the ascending aortas, but seemed especially prevalent at or near the bases of the semilunar
SEROUS SICKNESS CHOLESTEROL-INDUCED ATHEROSCLEROSIS

aortic valves. In six rabbits, lesioned areas occurred on the aortic walls at the origins of the coronary arteries, both right and left, and in three of these animals lesions were present within the coronaries as well. Elastic tissue was visible within the lesions (using the Weigert elastic-tissue stain) in one of the seven positive animals of this group. Lipid stains (oil red O) showed that all lesions contained intra- and extracellular lipid deposits.

Four of five animals of group II (Table II) which had received 5 wk of rest...
subsequent to the 2 wk combined treatment, showed lesions of the type described above. Lipid was seen in lesions of three of the four positive animals, while one animal was questionable in this regard. Elastic tissue was detected in lesions from three rabbits of this group, as shown in Fig. 2. Two of the lesioned aortas showed involvement at the coronary ostia, and one of these animals was lesioned within the coronary artery itself (Fig. 2). As noted in Table II, in group III five of six animals were seen to have sustained lesions over an 8-wk rest period. No difference could be detected in distribution of aortic lesions in groups I, II, and III, but none of the group III animals had lesions within their coronary arteries. In addition, less lipid was apparent in the lesions of group III than had been seen in the preceding groups; one of the five positives showed lipid-free lesions, and another two evidenced only slight presence of fat. One rabbit showed a particularly large aortic lesion (Figs. 3 and 4), which contained lipid and was heavily infiltrated with elastic tissue. This lesion was grossly visible in stained section. Lesions in another positive animal of this group also contained elastic fibers.

Table II shows that five of seven animals of group IV (sacrificed after 2 wk of cholesterol diet) showed microscopic atheromatous lesions in the aortas. All lesions were heavily infiltrated with lipid, and they appeared on aorta walls in

![Fig. 2. Intimal lesion in the left anterior descending coronary artery of a rabbit from group II, which received a BSA injection plus 2 wk feeding of cholesterol-supplemented diet, and was returned to regular diet for 5 wk prior to sacrifice. Note the presence of elastic tissue within the lesion (arrows). Weigert's resorcin-fuchsin stain. X 400.](image)
Fig. 3. Lesion (arrows) in the ascending aorta of a rabbit treated as in Fig. 2, but rested 8 wk prior to sacrifice (group III). Weigert's stain. X 100.

Fig. 4. Magnified view of a section of the lesion shown in Fig. 3. Note the pronounced deposition of elastic fibers within the lesion (arrow 1), as well as the disruption of the internal elastic membrane (arrow 2). Weigert's stain. X 400.
Fig. 5. Atheromatous, intimal lesion in the ascending aorta of a rabbit from group IV, which was fed cholesterol-supplemented diet for 2 wk and sacrificed. This stain shows intense deposition of lipid within the intimal lesion (arrow 1), as well as within the medial layer of the aorta (arrow 2). The large opening at the base of the lesion (arrow 3) is artefact, and should be disregarded. Oil red O. X 400.

Fig. 6. Intimal lesion found in the aorta of a rabbit treated as in Fig. 5, but rested 8 wk prior to sacrifice. This photomicrograph reveals the presence of lipid within the lesion (arrow 1), with no lipid deposition in the medial layer (arrow 2). Oil red O. X 400.
general as well as near areas of the coronary artery origins. No lesions were seen within the coronaries of this group, and none of those detected showed the presence of elastic tissue. Fig. 5 shows a representative lesion from this group.

The final group of animals (group V) was rested for 8 wk after having received 2 wk of cholesterol diet. In contrast to the high percentage of animals which showed lesions in group IV (five of seven), only two of six animals of group V were lesioned. Lesions seen in the two positive animals of group V (represented by Fig. 6) were geographically arranged as described for those of group IV, contained lipid, and showed no trace of elastic tissue. The drop in incidence between groups IV and V (71 to 33 %) is in contrast to the relative stability seen between groups I, II, and III (Table II).

DISCUSSION

In earlier reports (1-3), we showed that rabbits receiving a combined treatment of BSA injections in conjunction with the feeding of cholesterol-supplemented diet developed atheromatous lesions more frequently than those treated for similar periods with cholesterol diet alone. In addition, we noted (2, 3) that no reduction in lesion incidence occurred during 1- or 2-wk rest periods subsequent to the 2-wk combined treatment period. These data raised the question as to whether the persistence of the lipid-containing lesions implied early occurrence of pathologic changes in the vessel of a degree or type sufficient to cause irreversible damage. The reversibility of such lesions was examined in the present study by comparing lesion incidence during prolonged rest periods in animals receiving either combined BSA-cholesterol diet or cholesterol diet alone.

The data presented here show that a marked drop in the incidence of vascular lesions occurred 8 wk after a return to regular diet in animals receiving cholesterol diet alone; in contrast, rabbits which received combined BSA-cholesterol treatment showed little or no reduction in incidence during 5- or 8-wk rest periods. These results imply that atheromata caused by the combined treatment were less reversible than those induced by cholesterol diet alone. Since it has been shown (1-3, 7) that uncomplicated serum-sickness lesions regress within 3-4 wk after antigenic priming, it is probable that BSA-cholesterol diet-induced lesions are of potentially more pathological significance than vessel damage caused by either treatment alone.

One question raised by this study concerns the characteristic(s) of combined treatment lesions which allows them to persist in high incidence for prolonged periods of time. We previously raised the possibility (3) that the presence of lipid might account for this relative irreversibility. However, the disappearance of pathology noted between animals of groups IV and V implies that early lipid deposition alone could not be responsible for the lesion persistence seen in groups II and III, since lipid was prevalent in the damaged areas of group IV animals. In fact lipid deposits grew increasingly more difficult to demonstrate
histologically in lesions of 5- or 8-wk rested animals, whether they had received cholesterol diet only, or BSA as well. Lipid seemed to be leaving the lesions after animals were returned to regular diet and hypercholesterolemia subsided. This finding is in agreement with the recent data of Bortz (8), which showed a lowering of aortic cholesterol content in rabbits returned to regular diet after receiving cholesterol diet for 2–3 wk.

On the other hand, a consistent difference was seen between BSA–cholesterol- and cholesterol-only-induced lesions with respect to the presence of elastic tissue therein. Elastic tissue could only be demonstrated histologically (Weigert's resorcin-fuchsin stain) in lesions of animals treated with combined BSA and cholesterol diet. Elastic fibers were seen in lesions of several animals of groups I, II, and III (combined treatment), while no similar deposition was detected in the lesioned animals of groups IV and V. The infiltration of elastic tissue probably represents a more profound reaction to vessel injury than the simple accumulation of foam cells seen after 2 wk of cholesterol feeding alone, and implies the presence of a more damaging reaction than seen in uncomplicated lesions. In addition, if smooth muscle cells are more susceptible to damage by lipid uptake than “foam cells,” as postulated by Wissler and Vesselinovitch (9), then the mixed lesions seen in some of our combined-treatment animals would probably be more susceptible to additional damage from prolonged hypercholesterolemia than the foam-cell lesions caused by diet alone. Data presented by Minick et al. (6) showing increased fatty change in the thoracic aortas of rabbits receiving the combined type of treatment are in agreement with the above hypothesis.

While this hypothesis is speculative and based on a small number of animals, the very presence of elastic tissue fibers in atheromatous rabbits is perhaps worthy of note. Rabbits have been used for years as disease models for human atherosclerosis, but a continuing problem has been to produce the fibro-muscular type of lesion seen in the human disease (9–11). Elasticity of atheromatous lesions in rabbits has previously been induced by causing direct local damage to vessels with enzymes, hormones, and mechanical implements, or by intermittently forcing cholesterol levels to extremely high levels by feeding cholesterol for long periods of time (11). In our work, however, we have apparently stimulated the development of elasticity within atheromata by combining two relatively mild stressing regimens, i.e. BSA and a short period of cholesterol feeding, neither of which is capable of producing prolonged or elasticized lesions independently.

In spite of the apparent persistence of the combined-treatment lesions, semi-quantitative examination of our data indicated that these lesions were decreasing in size during the prolonged rest periods. The seven animals of group I showed lesions in an average of 41 tissue slices from the ascending aorta, while the positive animals of both groups II and III had an average of only 20 aortic
sections which showed pathology. Thus it seems that while the vascular damage persisted in most animals for 5 or 8 wk after return to regular diet, the degree of aortic involvement appeared to be less extensive in rested than in nonrested animals. Similarly, the aortic damage of cholesterol-only animals seemed to decrease with time, since an average of 32 positive tissue sections were noted per animal in group IV, while the animals rested for 8 wk (group V) had an average of only 16 positive sections.

A few words might be said about the geographical distribution of lesions in the various groups. While the walls of the ascending aortas were always involved in lesioned animals, some variation was noted between groups. These differences involved the occurrence of pathology around the coronary ostia and within the coronary arteries themselves. Six of the lesioned animals of group I showed aortic damage at one or both coronary ostia, and three of these animals had lesions within left, right, or both coronary arteries. Of the four lesioned animals of group II, one showed aortic involvement around both coronary ostia, and within the left anterior coronary as well. Two of five positives in group III had lesions at the coronary ostia, but neither of these showed arterial involvement. Lesions within the coronaries were never detected within the cholesterol-only animals of groups IV and V, although the incidence of aortic involvement at the coronary ostia was about the same as in the combined-treatment animals. In none of the animals treated with both BSA and cholesterol diet was coronary arterial damage seen, unless it was also accompanied by aortic lesions at the matching coronary ostium; however, the reverse situation was frequently noted. It would appear then that the combination of BSA plus cholesterol diet somehow made the coronaries more susceptible to development of atheromatous lesions than did diet alone. These data are in agreement with reports of other workers (4–7), and could be important evidence for using this type of procedure as a model for human atherosclerotic disease, since coronary lesions which may progress to total occlusion or thrombotic blockage in man are of prime interest (10, 12, 13). It is interesting that the frequency of coronary artery involvement decreased with time after the return to regular diet, although these vessels showed pathology in a high percentage of lesioned animals early in the study. Evidence for regression of coronary lesions is in agreement with a report by Rodbard, et al. (14), in which arterial, but not aortic, atheromata were shown to regress in the chicken.

**SUMMARY**

Rabbits were induced to form atheromatous cardiovascular lesions by subjecting them to treatments of a single BSA injection plus a 2-wk period of cholesterol diet, or to the diet alone. Microscopic examination of the hearts at the end of the 2-wk induction period, or after having been returned to regular diet for 5 or 8 wk, showed that lesion incidence in the cholesterol-only animal
decreased markedly during the 8-wk rest period, while little change in incidence occurred in animals with lesions from the combined treatment. This finding was taken to mean that the latter type of lesion was less reversible, and therefore perhaps more pathologically significant, than the former type. It was felt that lipid deposition was not solely responsible for this prolongation, since it was present in diet-only lesions which disappeared with time, and also seemed to be disappearing from the prolonged lesions in all treatment groups. On the other hand, elastic tissue was demonstrated only in lesions arising from the combined treatment, and it is hypothesized that this feature is implicated in the prolongation of these lesions.

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