Hypercoagulability Induced by Hyperlipemia in Rat, Rabbit and Man

ROLE OF PLATELET FACTOR 3

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ABSTRACT

Feeding a butter-rich diet to rats induced a "hypercoagulable state," since multiple large thrombi were observed in response to injection of S. typhosa endotoxin but the same stimulus was ineffective in rats fed a corn oil-rich diet or laboratory chow. In vitro hypercoagulability was noted in blood from the butter-fed rats, the recalcification plasma clotting time (PCT) being reduced by 34 to 45% in comparison with the controls. When a phospholipid suspension replaced the platelets in the plasma clotting test, the reduction was only 5 to 15%, but it was 31 and 43% when the platelets of these rats were washed and resuspended in a given standard plasma. Since lipid extract of these platelets could largely reproduce their activity, the factor involved must have been factor 3.

A similar result was obtained in rabbits fed a butter or corn oil diet, or laboratory chow, indicating that, in this species also, the hypercoagulability in response to hyperlipemia was largely due to factor 3. In addition, in rats and rabbits fed butter, the platelets were more susceptible to thrombin-induced aggregation.

In 27 human subjects of either sex and various ages, the state of coagulability as determined by the PCT was also mostly related to the platelet factor 3 activity, which was negatively correlated with serum cholesterol.

ADDITIONAL KEY WORDS platelet aggregation plasma clotting time cephalin clotting time factor 3 clotting time S. typhosa endotoxin thrombosis

In Virchow's triad (stasis, endothelial lesions, hypercoagulability), considered to be essential for the production of thrombosis, hypercoagulability is probably the factor whose existence has been most seriously questioned over the years. Although it has been clearly shown that hyperlipemia is associated not only with coronary heart disease (1), but also with venous thrombosis (2-4), the relationship between hyperlipemia and hypercoagulability does not seem to have been convincingly demonstrated so far. From an extensive review of the literature, Merskey and Marcus (5) concluded that "much effort has been expended to show that dietary ingestion of fat significantly alters blood coagulation and promotes a hypercoagulable state. To date this thesis remain unproved."

In terms of in vivo thrombosis, hypercoagulable blood has been defined as blood "that forms thrombi more readily in vivo than other blood when subjected to identical stimuli" (6). Such a hypercoagulable state is found in rats fed certain high-fat diets which, unlike normal animals, consistently develop multiple large thrombi in response to a given stimulus (endotoxin, epinephrine) (7). This state could be more satisfactorily defined as a thrombotic tendency. In terms of in vitro clotting, "blood that clots more quickly than..."
other blood as a result of identical stimuli is likely to be hypercoagulable" (6). In hyperlipemic rats highly susceptible to thrombosis, we have repeatedly noted a 40 to 50% shortening in the recalcification plasma clotting time (PCT) (7). This clotting time in siliconized material can be considered a valuable index of the whole blood coagulability, because it is highly reproducible (8) and evaluates most of the clotting factors.

The purpose of the present experiments was to investigate the clotting factor involved in this hyperlipemia-induced hypercoagulable state in the rat. The first series of experiments demonstrated that most of the hypercoagulability was due to a platelet factor; the activity of this factor was then studied in detail in rats and rabbits fed various diets and in normal men and women of various ages. The results of this study are reported here.

Materials and Methods

Patients.—We studied 20 normal men and 7 normal women, ranging in age from 21 to 80 years. Most of them were hospital employees.

Animals.—In experiment 1, we used 125 male Holtzman rats, with an initial body weight of 160 to 180 g. The rats were housed three per cage in a constant-temperature environment and given tap water and food ad libitum. These animals were fed one of the three following diets for 10 weeks: group 1, laboratory chow (Purina laboratory chow, Ralston Purina Co. of Canada) exclusively; group 2, corn oil (32% + 6% water); and group 3, butter (38%). These fats were purchased from local stores and added to a basic purchased from local stores and added to a basic proportion by the supplier (Nutritional Biochemicals Corporation, Cleveland, Ohio).

In experiment 2 we used 16 male New Zealand albino rabbits, purchased from a local breeder, the rabbits initially weighed 1.0 to 1.5 kg. For 9 months before removal of blood, group 1 was fed laboratory chow (Purina rabbit chow checkers, Ralston Purina Co. of Canada) alone, group 2 was fed laboratory chow (89.9% with butter (5%) + corn oil (3%) + cholesterol (0.1%)), and group 3, chow (89.9%) + butter (10%) + cholesterol (0.1%).

Removal of Blood.—In each experiment, blood was obtained after overnight fasting by a clean venepuncture with 20- or 18-gauge needles; the first few drops of blood were used for cholesterol determination. To obtain a sufficiently large amount of blood from rats and rabbits without hemolysis, blood was taken from the jugular vein, under light ether anesthesia, according to a technique already described for the rat (11) and used in previous studies (7-10). Blood was collected in silicon-coated syringes containing the appropriate anticoagulant with which it was immediately mixed by inverting the syringe three times.

CLOTTING TESTS

For all the clotting tests, the glassware used was coated at least six times with silicone (General Electric Dri-film, SC-87, in carbon tetrachloride). However, the final clotting determinations were performed in plastic (polyethylene no. 2804, International Equipment Co.) instead of siliconized tubes, previous experience having shown that these afford more accurate results (11).

Plasma Clotting Time (PCT).—The test used has been described in detail elsewhere (11). Blood (1 ml) was collected in a 2-ml syringe containing 0.1 ml of 3.8% sodium citrate adjusted to pH 7.35 with 10% citric acid. The anticoagulated blood was immediately stored at 30°C and centrifuged (700 g, 6 minutes) at room temperature within 30 minutes.

The plasma was then removed with a silicon coated Pasteur pipet and stored again at 30°C for periods up to 2 hours. At this temperature, the results do not change with time, as previously reported (8).

Before the determination, 0.1 ml of plasma and 0.1 ml of saline (0.95%) were placed in the polycarbonate tube, gently mixed, and incubated for 10 minutes at 37°C. The test was started by blowing 0.1 ml of CaCl₂ (0.02M) into the diluted plasma.

Cephalin Clotting Time (CEP-CT).—The same general procedure as for the PCT was used, except that this time the blood was centrifuged for 20 minutes at 1000 g to remove most of the platelets. In addition, instead of being diluted in the plastic tube with saline alone, the plasma was diluted with a suspension of cephalin (Platelin, Warner-Chilcott, Morris Plains, New Jersey) in 10 ml of 0.76% NaCl (the procedure recommended by the manufacturer is to dilute Platelin with 2.5 ml of distilled water).

Factor 3 Clotting Time (F₃-CT).—For this test, a standard plasma must first be prepared for each animal species. A large amount of blood was collected from several animals or human subjects with citrate as the anticoagulant (1 volume of sodium citrate for 9 volumes of blood), omitting...
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Fuged 30 minutes at 2000 g to remove practically all the platelets, and the plasma pooled. Each such pool was prepared for each of the animal species studied (rat, rabbit, man); it was then distributed in small containers (1 to 2 ml) and frozen. Each of these pooled plasma samples gave the same clotting time when tested after various periods of time and could be referred to as standard plasma. The clotting times of such plasma, measured under the conditions of the PCT without the addition of platelets or cephalin, was 420, 480 and 520 seconds for the rat, rabbit and human plasma, respectively.

As a second step, blood was collected for each subject (animal or man), this time with EDTA as the anticoagulant. In hyperlipemic rats, 2 ml of blood was removed in a 3-ml syringe containing 0.5 ml of the anticoagulant (EDTA, di-sodium salt, 0.5 g; NaCl, 0.86 g; dextrose, 0.1 g; distilled water to 100 ml). In rats fed laboratory chow, 4 ml of blood were taken with 1 ml of the anticoagulant. In rabbits and humans, 9 ml of blood were removed in a 10-ml syringe containing 1 ml of the following anticoagulant: EDTA, 1.6 g; NaCl, 0.66 g; dextrose, 0.1 g; and distilled water to 100 ml. This blood was stored at room temperature, and was centrifuged for 8 minutes at 100 g. The platelet-rich plasma was then removed and centrifuged again at 1000 g for 20 minutes. After removal of the plasma, the platelets were washed twice in incomplete Tyrode’s solution containing no Ca and Mg (NaCl, 8.7; KCl, 0.2; NaHCO₃, 0.6; dextrose, 1.0; NaH₂PO₄-H₂O, 0.05; in grams and distilled water to 1000 ml). The pH was adjusted to 6.8 with 1 N HCl immediately before use. At the last washing, the platelets were counted and resuspended in incomplete Tyrode’s solution, this time at pH 7.4, at a concentration of 10⁸ platelets per ml. After storage for at least 30 minutes at 30°C, 0.1 ml of this suspension was then added to 0.1 ml of the previously prepared, standard frozen plasma, also stored for 30 minutes at 30°C. After incubation of this mixture for 10 minutes at 37°C, the test was performed by recalcification of the plasma with 0.02M CaCl₂ as for PCT. Figure 1 summarizes, schematically, the different steps involved in the three clotting tests described above.

Factor 3 Lipidic Extract Clotting Time (F₃EX-CPT)

FIGURE 1
Schematic summary of the technical details involved in the plasma clotting time (PCT), cephalin clotting time (CEP-CT), and factor 3 clotting time (F₃-CT).

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For this test, after storage for 30 minutes at 30°C, 1 ml of the suspension of platelets used for the F₃-CT was extracted according to the technique of Folch et al. (12), with 25 ml of a mixture of 2:1 chloroform-methanol, under strong agitation (1 minute). After filtration, 5.2 ml of 0.73% NaCl was added to the mixture, which was agitated for 20 seconds. After removal of the upper layer, the chloroform extract was evaporated to dryness at 52 to 54°C, with nitrogen; 1 ml of the same Tyrode's solution (pH = 7.4) used for platelet suspension was added to the container, and the lipids extracted were resuspended by sonication for 60 seconds. This extract was then stored at 30°C and added (0.1 ml) to the standard plasma as for the whole platelets.

PLATELET AGGREGATION

In experiment 1, the platelet aggregation test in citrated platelet-rich plasma was performed on the pooled plasma of three rats (6 determinations per group), according to the technique already reported (13). Results in cm² indicate the area under the tracing calculated by triangulation. In experiment 2, in rabbits, preliminary experiments had indicated that significant aggregation curves to thrombin could not be obtained in citrated plasma without accompanying clotting. Therefore, the platelet aggregation test was performed on each animal with washed platelets. For this purpose, blood was collected with the anticoagulant used for rats (EDTA 0.5%, of which the exact composition is described above, 1 volume/4 volumes of blood), the platelets were washed only once with Tyrode’s solution, pH = 6.6, and resuspended (350,000/mm³) in complete Tyrode’s solution with added gelatin (13). Since the aggregation curves obtained did not show a peak, the height (in cm) of the tracing at 10 cm from the origin was taken to express the extent of platelet aggregation.

Cholesterol Analysis.—Total cholesterol on serum was determined on individual samples by a manual adaptation of the Technicon Autoanalyzer automated ferric chloride technique (14).

Thrombosis in Rat.—Thrombosis was induced by an intravenous injection of S. typhosa (0901, Boivin type) lipopolysaccharide (0.3 mg/kg in saline), and evaluated as reported in previous studies (13).

Atherosclerosis in Rabbit.—These lesions were evaluated macroscopically under binocular examination after opening the aorta longitudinally. Results were verified by routine histologic examination of the aorta.

Results

Experiment 1.—Figure 2 illustrates the results obtained in 18 rats per group, group 1 being fed laboratory chow, group 2 the corn oil diet, and group 3 the butter-rich diet. Determinations of serum cholesterol, PCT, CEP-CT, F₃-CT and evaluation of the severity
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of thrombosis were performed on the same animals, while the platelet aggregation studies were carried out on three other groups of 18 rats fed the same diets as above.

As compared with laboratory chow, feeding of high fat diets, especially the butter diet induced marked changes in the serum cholesterol in rats. In addition, severe lesions of thrombosis were noted in the animals fed the butter diet solely. In this group, the platelets were also the most susceptible to thrombin-induced aggregation.

The animals fed butter showed a 45% reduction in their PCT as compared with the rats fed laboratory chow and a 34% reduction as compared with the rats fed corn oil. When the platelets were replaced by cephalin in the CEP-CT, this reduction in the clotting time was only 16 and 5%, respectively. Finally, in these three groups of rats, the clotting time of platelets (F3-CT) resuspended in a given standard plasma exhibited approximately the same differences between the groups as those noted with the PCT. The reduction in this F3-CT was 43 and 31% when compared with the rats fed laboratory-chow and those fed corn oil, respectively.

Figure 3 shows the correlation \( r = 0.97 \) between the PCT and the F3-CT in the same 54 rats seen in Figure 2.

In Figure 4, the clotting time of whole platelets (F3-CT) is compared with that of the lipidic extract (F3EX-CT) in six rats fed laboratory chow, six fed corn oil, and five fed the butter-rich diet. Although the extract was from 20 to 33% less active in the clotting test than the whole platelets, the correlation between the values of these two tests was highly significant \( r = 0.89 \).

Experiment 2.—(Figure 5) Butter or corn oil fed diets fed to rabbits for 9 months induced changes in the serum cholesterol comparable to those noted in the rats of experiment 1. Only the group fed butter had atherosclerotic lesions in the aorta. These lesions were considerably raised over the normal intima and were spread along the entire length of the aorta. Histologically, they were composed mostly of foam cells, but fibrosis was also consistently observed. Practically no lesions could be observed in the rabbits fed corn oil.

The butter-fed rabbits presented a marked shortening of the PCT as compared with the two other groups, which had values compara-
ble to each other. This same shortening of the clotting time was also noted with the F3-CT, while the CEP-CT of the butter-fed rabbits was only slightly reduced as compared with the other groups.

Experiment 3.—In 27 subjects a correlation ($r = 0.80$) was also found between the PCT and the F3-CT (Fig. 6). This platelet clotting test ($F_3$-CT) appears to be inversely related ($r = -0.51$) to the serum cholesterol (Fig. 7), while no negative correlation ($r = 0.23$) could be found between this last variable and the cephalin time (CEP-CT). In addition, in the eight subjects with a $F_3$-CT of 400 seconds and more the serum cholesterol was 167 mg% and the average age was 38 years. In the seven subjects with a $F_3$-CT of 320 seconds and less, the values were 216 mg per 100 ml and 50 years, respectively. In these latter subjects, the mean reduction of the $F_3$-CT was 26% as compared with the former subjects.

**Discussion**

It has been shown by several investigators that a change occurs in several plasma clotting factors in rats fed certain thrombogenic diets (15-17). Although the diet used in the present experiments is not identical to that of these earlier workers, similar changes could be present in our hyperlipemic rats, although
Further experiments would be necessary to elucidate this point. However, the only pertinent question is whether the changes in plasma factor may significantly contribute to the hypercoagulable state resulting from the hyperlipemic diet. The results of the present experiments suggest that in response to hyperlipemia, the plasma factors contribute very little to the hypercoagulability, the key role being attributed to a platelet factor, not only in rats but also in rabbits and in man.

To demonstrate this platelet activity, special emphasis must be placed on the technique employed. Because platelets are highly susceptible to various stimuli, extreme care has to be taken in the collection of blood, silicone-coating of all the material used, and mixing of the blood in the syringe with the anticoagulant. Under these conditions and with other minor details discussed elsewhere (7), highly reproducible results have been obtained in rats and in man, over a period of several years, with the recalcification plasma clotting time, which is a basic test of the present study. As recently underlined by other investigators (18), this test affords a critical evaluation of platelets in addition to testing other coagulation components. In contrast to this, results obtained with the cephalin clotting test utilized here, in which the platelets were replaced by a phospholipid suspension, indicated only a minor effect of the hyperlipemia on plasma clotting factors. To verify that it was mostly the platelets that were responsible for the hypercoagulability demonstrated by the plasma clotting time, a test had to be devised in which only the platelets would be different from one sample to another, while the plasma would remain the same. Such a test was performed (F3-CT), and the values obtained in relation to the lipemia were practically identical to those of the plasma clotting time. We consider the results of this experiment as evidence that the hyperlipemia-induced hypercoagulability is largely due to a platelet factor.

Although 20 to 35% of the original activity of the platelet factor was lost in the extraction procedure, the activity of the whole platelets at least in the rat, is still closely related to that of their lipidic extract. Therefore, the platelet factor connected with hypercoagulability seems to be platelet factor 3. This factor 3 has been described as a lipoprotein of which the
lipid part comprises several phospholipids (19).

Of special interest regarding the relationship between factor 3, lipemia, and hypercoagulability is the work with synthetic phospholipids demonstrating that phosphatidylcholine had the greatest clotting activity when it contained oleic acid but not saturated fatty acids (20). We have previously reported (13) that butter and long-chain saturated fatty acids, the dietary thrombogenic components in rat, markedly increase oleate but not saturated fatty acids in the platelet total lipids.

The experiment in rabbits fed only a small amount of cholesterol (0.1%) indicated an effect of the dietary fat similar to that noted in the rat. Only the butter caused hypercholesterolemia and atherosclerosis and shortened coagulation. And in these animals also, the hypercoagulability appears to be due to an increased activity of platelet factor 3.

In the normal human subjects so far examined, it is also obvious that the coagulability was largely dependent upon the activity of their platelet factor 3. Some of these subjects were in a hypercoagulable state compared with those whose values were normal. Also of interest is the absence of a negative correlation between the plasma clotting factors and the serum cholesterol; this correlation was observed with platelet factor 3. This result confirms our previous report concerning the relationship between serum cholesterol and the PCT in man (11). Many more experiments are needed to determine whether an increased activity of platelet factor 3 may be responsible for thrombotic episodes in man. However, if hyperlipemia predisposes to thrombosis, it appears logical to consider that it may act mostly through the sole lipidic clotting factor.

Another effect of hyperlipemia is to increase the susceptibility of platelets to aggregation, particularly by thrombin. We have been able to observe this in rats (21, 7), in man (13), and now in rabbits. Therefore, the hypothetic "hypercoagulable state" (thrombotic tendency) in response to hyperlipemia may be due primarily to blood platelets through two interrelated mechanisms: an increased factor 3 activity that could be mostly responsible for in vitro hypercoagulability, and an increased susceptibility of platelets to thrombin-induced aggregation.

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