THE POSSIBLE RELATIONSHIP BETWEEN THE ANTIHEPARIN ACTIVITY OF SERUM AND THROMBOSIS

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Stored serum reduces the anticoagulant effect of heparin on the clotting times of normal plasma. This is also well marked with sera from patients with Christmas (factor IX) deficiency, with factor VII-deficient sera, and in sera derived from patients treated with phenindione with a gross defect in thromboplastin generation. The possible relationship between antiheparin activity of serum and heparin resistance in recent thrombosis is discussed. The antiheparin agent resembles factor VII and Christmas factor in being present in excess in serum, adsorbed and subsequently eluted from alumina. Unlike these, however, it does not appear to be appreciably reduced by phenindione treatment. It appears to have some properties in common with those described for the thrombotic agent of serum described by Wessler and his colleagues. It may play a part in the increased coagulability associated with thrombosis from the release of serum products into the circulation, although its relationship to the production of thrombosis in man remains to be established.

The observation that heparin "resistance" is associated with active thrombosis has attracted a good deal of attention in recent years. The anticoagulant effect of heparin was seen to be less marked when it was given to patients with recent thrombosis than when it was given to other types of patient (Crafoord, 1937; de Takats, 1943). A heparin tolerance test in vitro was devised by Waugh and Ruddick (1944), and this was used by Ogura, Fetter, Blankenhorn, and Glueck (1946) to demonstrate that clotting took place more rapidly in cases of thrombosis than in controls. Silverman (1948) improved the test by the use of plasma to facilitate the reading of the end-point. I have used a modification of this test, the heparin plasma clotting test, which is performed under standardized conditions. With this technique it has been shown that in cases of vascular occlusion there is a significantly shortened clotting time (Poller, 1954, 1955, and 1956). A further study of this problem showed that plasma factor VII activity was increased as was thromboplastin generation associated with shortened heparin plasma clotting times (Poller, 1957) in a group of patients with recent thrombosis. It was subsequently observed that normal stored serum and serum preparations rich in factor VII activity reduced the effect of heparin on plasma clotting times (Poller, 1959).

The aim of the present investigation was to determine whether the antiheparin effect of serum was due to factor VII or to some other agent in serum. Serum was used in preference to products of alumina adsorption, as it was found that the activity of serum in neutralizing the anticoagulant action of heparin was quite marked, and it seemed preferable to use the more natural product. Serum may be a product of little physiological importance, but it may be released into the circulation following thrombus formation, and may be responsible for heparin "resistance."

To demonstrate the agent responsible for the antiheparin activity, observations were made on the effect of sera from patients with various coagulation defects on plasma clotting in the presence of heparin. Two sets of controls were used: (1) Normal saline to control the dilution effect, and (2) serum obtained from normal healthy adults. Sera with coagulation factor deficiencies were obtained from patients with Christmas disease (factor IX deficiency), from patients with pure factor VII deficiency, and from patients being treated with phenindione who showed a gross defect in thromboplastin generation.

Method

Reagents.—The following were used:

- Calcium Chloride.—0.18%.
- Normal Substrate Plasma.—Fresh oxalated plasma was obtained from 5 ml. venous blood by adding it to a mixture of potassium and ammonium oxalates.
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(Stock solution (0.21 ml.) containing 1.6% potassium oxalate and 2.4% ammonium oxalate is put into a bijou bottle and left to dry overnight.) The blood and the oxalate anticoagulant were then thoroughly mixed after collection with the minimum of trauma and haemostasis, and the specimen spun at 1,500 r.p.m. in an M.S.E. angle centrifuge to obtain a platelet-rich plasma.

Heparin (Pularin (Evans) 10 units/ml.).—This solution was found to be stable for at least six months if kept at 4° C. when not in use, and a sterile technique employed to remove samples from the stock bottle.

Diluted Heparin.—The stock solution of heparin was diluted to a strength of 2 units/ml. immediately before use.

Test Sera.—Sera were obtained from healthy normal subjects and from patients with serum coagulation factor deficiencies from venous blood which was allowed to clot in glass test-tubes. The sera were separated by centrifuging, and the resulting serum removed and stored for 24 hours at 4° C.

Technique.—Four rows of three tubes (3 in. × ½ in.) each were placed in a rack in a 37° C. water-bath, and allowed to warm. Normal plasma (0.2 ml.) and 0.1 ml. diluted heparin were added to each tube. To the first row of tubes increasing volumes of normal saline were added, 0.1 ml. to the first tube, 0.2 ml. to the second tube, and 0.3 ml. to the third tube. To the second row of tubes normal serum was added instead of saline, in the same increasing volumes. In the third and fourth rows of tubes two different test sera from different types of coagulation defect were compared by adding them in the same increasing volumes, to their own row of tubes. The plasma clotting time was measured by adding 0.2 ml. of calcium chloride to each tube, mixing thoroughly, and tilting at 15-second intervals until a fibrin clot formed. The tubes of all four rows used in each experiment were recalculated at five-second intervals to eliminate the effects of storage in vitro on activating the coagulation mechanism. It was necessary to include a saline and normal serum control for each separate investigation, as the clotting times of normal plasma showed a good deal of variation in the presence of added heparin.

Results

The results are given in the Table. Those in the first row of tubes containing normal saline show that simple dilution with saline has no appreciable effect on the clotting time. The results in the second row of tubes containing normal serum show there is a significant shortening of the clotting time in the presence of heparin anticoagulant. All normal sera tested showed appreciable antiheparin activity.

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<th>TABLE RESULTS</th>
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<td>Control 1. Normal saline (total 15)</td>
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<td>Control 2. Normal serum (total 15)</td>
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<tr>
<td>Christmas serum (total 6)</td>
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<tr>
<td>Factor-VII-deficient serum (total 9)</td>
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<td>Serum from phenindione-treated cases with deficient thromboplastin generation (total 7)</td>
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The effect of sera obtained from cases with severe Christmas disease in reducing the anti-coagulant effect of heparin was well marked in all six cases, and, although rather less than in the group of parallel control normal sera, the difference was not significant. Thus Christmas factor does not appear to be the important antiheparin agent of serum.

Specimens were obtained from nine patients suffering from factor VII deficiency. Seven were patients treated with phenindione whose thromboplastin generation was 100% although prothrombin activity measured by Quick’s method and the "P and P" method was depressed. Two patients were subjects with congenital factor VII deficiency. In this group of nine patients serum also significantly shortened the plasma clotting times in the presence of heparin.

A further group tested was a series of seven patients on phenindione whose thromboplastin generation was less than 1%. The thromboplastin defects during phenindione treatment result from the depression of Christmas factor and Prower-Stuart factor. There was no significant difference between this group and parallel normal control sera. Unlike factor VII and Christmas factor, Prower-Stuart factor is not activated during clotting (Johnson, Ferguson, O’Hanlon, and Black, 1959), and therefore was not likely on theoretical grounds to be the active antiheparin principle of serum.

As van Creveld and Paulssen (1951) have demonstrated that an antiheparin factor is present in platelets, it seemed important to eliminate the possibility that the effect may be due to the action of platelet extracts in serum. Blood was therefore collected into a siliconed syringe, transferred
to a siliconed tube, and the platelets spun out by centrifuging at 5,000 r.p.m. The platelet-deficient plasma was then removed and allowed to clot in glass, the resulting serum being tested the following day in the usual manner. The results of six sera obtained in this manner showed no significant difference from sera obtained from the same subjects collected in the usual manner. The antiheparin effect, therefore, does not seem to depend on factors released from the platelets during clotting.

The effect of adsorption by alumina was also observed. It has previously been shown that the antiheparin agent in serum could be adsorbed and subsequently eluted from alumina. Serum was obtained from subjects without coagulation defect. It was observed for its antiheparin effect before adsorption, and then 0.1 ml. of alumina was added to 1 ml. serum and the tube left in a water-bath for four minutes. The serum was then removed from the alumina by spinning in a centrifuge, and the adsorbed serum tested for antiheparin activity. In none of the adsorbed sera tested in this manner was there any evidence of antiheparin effect, and indeed in three the clotting time was prolonged, perhaps due to antithrombins remaining in adsorbed serum.

Discussion

The demonstration of the presence of a powerful heparin antagonist in serum appears of importance in view of the established association between heparin "resistance" and recent thrombosis. Heparin "resistance" may play a part in predisposing to thrombosis, but on the other hand it may in part at least be a secondary effect resulting from the release of serum products from the thrombus into the circulation. In support of this view is the observation that the shortest heparin clotting times appeared in cases in which large blood vessels were occluded. Patients with thrombophlebitis of large veins were found to have significantly shorter heparin plasma clotting times than those with myocardial infarction (Poller, 1959).

The antiheparin activity of serum may play some part in the production of intravascular clotting, even if this is only as an aggravation of a pre-existent clotting tendency. In the last few years several investigators have claimed that measurable amounts of heparin exist in the circulating blood (Freeman, Engelberg, and Dudley, 1954; Engelberg, 1958; Nilsson and Wenckert, 1954; and others). Mast cells, the source of natural heparin, have been demonstrated in the walls of veins and arteries by Sundberg (1955) and Pomerance (1958). Both these authors reported an increase in the number of these cells in the vessels at the site of thrombus formation. The possibility that this may be a physiological response to the release of antiheparin serum products from the thrombus has been suggested (Poller, 1958).

The fact that antiheparin activity is not reduced by phenindione is somewhat surprising. When the heparin plasma clotting test was used as a method of control of treatment by dicoumarol and phenindione in a previous investigation, accelerated clotting, i.e., a clotting time shorter than the minimum normal, with further thrombotic episodes was found on many occasions at "therapeutic levels" of prothrombin activity (prothrombin times greater than twice the controls), and was regarded as an indication for additional heparin treatment. It is possible that such heparin resistance may have been due to the liberation of serum products into the circulation.

Wessler (1955) and Wessler, Reiner, Freiman, Reimer, and Lertzman (1959) have demonstrated the presence of a "thrombotic" agent in serum using a different technique. They have used experimental animals and produced thrombosis by the infusion of serum products. They have found that the serum product responsible could be adsorbed from serum, and obtained by subsequent elution as in the case of the antiheparin agent. This factor has also been found in factor-VII-rich solutions, and in later work they found that it was also present in the serum of patients on dicoumarol treatment. This led Wessler and his colleagues to the view that heparin may be preferable to dicoumarol as an anticoagulant drug (Wessler, Ballon, and Gilbert, 1956).

It appears from indirect evidence that the coagulant factor of serum demonstrated experimentally by Wessler may be related to the antiheparin agent of serum demonstrated by the author. The exact importance of such a serum product in the pathogenesis of thrombosis in man remains to be established, but there is some evidence to suggest that it may play a part in the production of the increased coagulability associated with recent thrombus formation.

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