The Anticoagulant Effect of Bacterial Polysaccharides in Normal and Thrombocytopenic Plasma of Leukemia

By Gustave Freeman, M.D.

JOPES, in his monograph on heparin, reviewed various polysaccharides in the light of their anticoagulant activities. These polymers are of both biologic and synthetic origin. None of the naturally occurring anticoagulant polysaccharides, other than heparin, are known to exist in the blood of man. However, the possible effect of polysaccharides arising from bacteria during infection, on coagulation has not been determined. Chargaff, Bancroft and Stanley-Brown, in 1936, mentioned a lack of anticoagulant activity by polysaccharides from cultures of pneumococcus, type III, and of the bacillus Calmette-Guérin, as compared to heparin. From another point of view, Shear and his co-workers have been pursuing a fruitful field of investigation related to the characterization and effect of bacterial polysaccharide on neoplastic tissue. Generally, considerable interest has been developing around the biologic role of polysaccharides and carbohydrate moieties in physiology and disease.

Bacterial autolysates have been studied in relation to their effects on blood vessels and on coagulation since 1907 when Heyrovsky described the appearance of purpura in mice injected with such materials. This is of interest partly because such autolysates contain polysaccharides, in addition to other derivatives, and because the specific residence of the purpura-producing and anticoagulant properties has not been clearly determined. Among others, Julianelle and Reimann worked with purpura-producing pneumococcal autolysates. They found that removal of fractions precipitated by acetic acid and then coagulated by heat left the active material in solution. Purpura produced in this way was reported to occur in association with a marked decrease in circulating platelets and erythrocytes in the mouse. Goodner described the anticoagulant effect of materials from pneumococcal autolysates, and by the injection of thromboplastin was able to inhibit the spreading property of such autolysates injected into experimental pneumococcal infection in the skin of rabbits.

The frequency with which clinical bleeding occurs in leukemia in the presence of massive infection has stimulated consideration of the possible role of free bacterial polysaccharide, besides other bacterial products, in this disease. This report deals with the degree of anticoagulant effect of polysaccharides derived from certain bacteria and with their relationship to thrombocytopenic plasma of leukemic patients.
ANTICOAGULANT EFFECT OF BACTERIAL POLYSACCHARIDES

METHODS AND MATERIALS

The anticoagulant effect of polysaccharides extracted from several bacterial species were tested by the recalcification method in oxalated plasma. Although such materials were reasonably pure, they could not be considered completely free of amino acid-containing molecules. Specimens of blood were obtained from a series of individuals having various concentrations of circulating platelets. Young adults with normal platelet counts were used as donors of normal plasma. Thrombocytopenic plasma was obtained from children with leukemia, some of whom were bleeding and some of whom were not. They represented various stages of disease.

Blood and plasma were oxalated and kept cold as described previously. Needles and glassware were coated with silicone. Platelet counts were made on the plasma prior to use and on finger-prick blood at the same time, both by the direct method, using 3.8 per cent sodium citrate as diluent. Tests for anticoagulant activity were carried out within three hours after the blood was drawn.

The following polysaccharide materials were used: (1) Extract from cultures of Friedländer's bacillus, type A, prepared according to the method of Heidelberger and his associates. (2) Extract from culture of Bacillus prodigiosus (Serratia marcescens) also called P-25 or "Shear polysaccharide." (3) Extract from cultures of pneumococcus, type II, prepared after the method of Heidelberger and his associates. (4) Heparin sodium prepared commercially by Abbott Laboratories, Chicago, in neutral, physiologically isotonic sodium chloride solution, so that 1 ml. contained 1 mg. of heparin.

All bacterial polysaccharides were made up in 0.85 per cent sodium chloride solution and then were adjusted to pH 7.3 to 7.5 with concentrated aqueous sodium hydroxide. Pneumococcal polysaccharide solution was prepared as stock in a concentration of 40 mg. per ml. and the other two bacterial polysaccharides in concentrations of 20 mg. per ml. of solution. At these concentrations, the bacterial polysaccharides were grossly viscous. Solutions were prepared in series, according to desired concentrations, from the stock.

Anticoagulant activity of these substances was measured in plasma in terms of recalcification time and compared with that of control plasma diluted with physiologic saline solution. (The dilution with saline did not affect the recalcification time.) All solutions coming in contact with plasma were maintained at a pH of 7.3 to 7.5 in order to simulate physiologic conditions during coagulation.

Tests were carried out as follows: into each tube were pipeted 0.1 ml. of cold plasma and 0.2 ml. of the appropriate concentration of cold polysaccharide solution. The tube was flicked sharply with the index finger five times to mix the plasma and anticoagulant adequately. The rack of tubes was then immersed in a water bath kept at 37.5 C. and was allowed to reach temperature equilibrium during a period of five minutes. Next, 0.2 ml. of 0.025 molar calcium chloride solution at 37.5 C. was pipeted into each tube of the series, and a stop watch was started simultaneously for each tube. Tests were controlled with tubes of plasma-saline mixture at each end of the series. Each series was carried out in duplicate, one being timed in descending order of concentration of polysaccharide and the other in reverse order. The results in duplicate tubes were averaged. Generally, between five and eight individual concentrations of polysaccharide were tested in a series, in addition to the controls. Concentrations were varied, depending on which were of particular interest. In many instances, concentrations were high enough to prevent clotting for indeterminate periods. Tests were carried out on normal plasma and on specimens having initially prolonged recalcification times associated with thrombocytopenia. Comparable coagulation studies were carried out using heparin in place of bacterial polysaccharide, usually in a proportion

* Dri-Film 9987 (General Electric).
** This material was supplied through the generosity of Dr. John F. Enders, Harvard Medical School and The Children's Medical Center.
† Provided by Dr. M. J. Shear of The National Cancer Institute, and described as a potent antitumor agent.
¶ Kindly supplied by Miss L. H. Wetterlow, Division of Biological Laboratories, Massachusetts Public Health Department.
of 1:1,000, by weight. Concentrations of plasma, calcium chloride, and sodium chloride were kept constant in a total volume of 0.5 ml. Results were recorded in milligrams of polysaccharide and gamma of heparin per 0.1 ml. of plasma. The nature of clot retraction was followed qualitatively by gross observation.

Prothrombin activity of plasma specimens was measured by the Rosenfield-Tuft modification of the Quick prothrombin time method.

Results

An initial qualitative observation, that an arbitrary amount of polysaccharide (Friedländer's bacillus, type A) inhibited retraction of the fibrin clot, was followed by further observations on the effect of such bacterial substances on the clotting time. Pneumococcal polysaccharide was tested for anticoagulant activity in normal and thromboctopenic plasma, and the results are illustrated in figure 1.

Platelet counts on the venous plasma and capillary blood of the same subjects are listed in table 1. Normal plasma specimens A and B required more than 2 mg. per 0.1 ml. of plasma (2 Gm. per 100 ml.) to prolong the recalcification time beyond that of the normal control. Moreover, clotting was not completely prevented unless more than 8 mg. had been used. Thromboctopenic specimens A and B required lesser concentrations of polysaccharide than normal plasma.
to prolong coagulation as well as to inhibit it completely. The effect was more pronounced in specimen A, that had a higher initial recalcification time, than in specimen B. In this instance, less than 1 mg. per 0.1 ml. was an effective concentration.

Polysaccharide from cultures of both Friedländer's bacillus and S. marcescens (P-25) proved to have greater anticoagulant effect than that from cultures of the pneumococcus. Consequently they were used exclusively in the studies shown in figures 2 and 3. Heparin (in quantities of 1:1,000 of bacterial polysaccharide by weight) was included for comparative purposes and is expressed in gamma instead of milligrams on the scale for bacterial polysaccharides.

In figure 2 there are two groups of three curves each. The lower group is the result of plotting milligrams of bacterial polysaccharides and gamma of heparin per 0.1 ml. of plasma against the clotting time in normal plasma. By contrast, the upper group of curves illustrates the exaggerated anticoagulant effect in thrombocytopenic plasma that was suggested in figure 1 by the relatively inactive pneumococcal polysaccharide. The prolonged recalcification time of the control specimen in this upper group is compatible with its concentration of 48,000 platelets per cu. mm. of plasma. The prothrombin activity was adequate.
Effective concentrations of polysaccharide appear to be as low as 0.05 mg. per 0.1 ml. (50 mg. per 100 ml.) in normal plasma. The appearance of the curves in leukemic plasma suggest that the effective concentration may be lower in thrombocytopenia.

![Graph showing curves of recalcification time indicating increases in rate of coagulation at a concentration of 0.025 mg. of polysaccharide per 0.1 ml. of normal plasma.](image)

**Figure 3.**—Curves of recalcification time indicating increases in rate of coagulation at a concentration of 0.025 mg. of polysaccharide per 0.1 ml. of normal plasma.

**Table 1.**—Platelet Concentrations in Capillary Blood and Venous Plasma, and Prothrombin Activity of Specimens Shown in Figure 1

<table>
<thead>
<tr>
<th>Specimens</th>
<th>Initial recalcification time*</th>
<th>Capillary blood platelet count</th>
<th>Venous plasma platelet count</th>
<th>Prothrombin activity, per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal A</td>
<td>120</td>
<td>224,000</td>
<td>408,000</td>
<td>50-100</td>
</tr>
<tr>
<td>Normal B</td>
<td>105</td>
<td>260,000</td>
<td>386,000</td>
<td>50-100</td>
</tr>
<tr>
<td>Thrombocytopenic A</td>
<td>525</td>
<td>6,000</td>
<td>10,000</td>
<td>40-50</td>
</tr>
<tr>
<td>Thrombocytopenic B</td>
<td>210</td>
<td>18,000</td>
<td>32,000</td>
<td>50-100</td>
</tr>
</tbody>
</table>

* Recalcification time (average of duplicates to closest 15 seconds).
† Platelets per cu. mm.

In figure 3, experiments are illustrated in which polysaccharide concentrations as low as 0.025 mg. per 0.1 ml. of normal plasma were used. It can be seen that such a concentration of polysaccharide (from Friedländer's bacillus) can prolong clotting of normal plasma containing 440,000 platelets per cu. mm. This is a concentration equivalent to 25 mg. per 100 ml. of plasma.

Although it would appear from the results that the relative strengths of heparin and the two bacterial extracts used was precisely 1,000:1, this propor-
tion did not hold at all times. The same lot of heparin was used for all tests, but for unknown reasons, the relative strengths varied from 400:1 to 1,000:1, the higher proportions being decidedly more common.

In general, gross observations of clot retraction revealed retardation approximately in proportion to prolongation of plasma clotting time. Quantitative measurements were not made.

**DISCUSSION**

These data indicate that polysaccharide extracts of some bacteria have an anticoagulant effect on normal plasma in vitro. This effect appears to be exaggerated in thrombocytopenic plasma of leukemia. In this respect, the action of these bacterial polysaccharides is qualitatively similar to that of heparin, the only known naturally occurring anticoagulant. Such protraction of coagulation time by an anticoagulant would be additive to any other deceleration, whether the basis were thrombocytopenia or any other defect likely to deter coagulation.

At present, the evidence that bacterial polysaccharide circulates freely is serologic rather than chemical, but that it exists in combination with antibody is clear. It may be pertinent at this time to review the circumstances associated with increases in protein bound serum polysaccharide measurable by several methods in general use. It appears that non-glucosamine polysaccharide of serum (measured against a standard of mannose and galactose) may increase as much as threefold (to approximately 300 mg. per cent) in disease. Neither the source of the carbohydrate nor its significance appears to be known. Certain characteristics of the protein in which excess carbohydrate is reportedly bound coincide with the particular glycoprotein described previously as “acute phase protein” of infection, or C-reactive protein, by Tillett and Francis. This protein is present only during the acute stage of infection and is characterized further by Perlman, Bullowa and Goodkind as being distinct from antibody globulin. Concentrations of bacterial polysaccharides (as low as 25 mg. per cent) found to be anticoagulant in vitro in some degree, are of a magnitude compatible with the rises reported in serum in the presence of disease. Inferences as to possible relationships are entirely speculative.

Defective coagulation in relation to the hemorrhagic diathesis is always of interest. Bleeding per se, however, would be difficult to explain without consideration of prior or concomitant changes in the vascular walls. A study of the anticoagulant activity of polysaccharides extracted from bacteria in relation to bleeding was stimulated by the frequent association of infection with bleeding in thrombocytopenic leukemia. The association among protein-bound serum polysaccharide, bleeding and fever in leukemia is being reported.

From the point of view that infection may be a mechanism through which elements influential in bleeding are introduced, the experiences of Goodner with anticoagulant filtrates from pneumococcal culture and those of Heyrovsky and Julianelle and Reimann with crude extracts from bacterial autolysates may be pertinent. Although some fractionation of the autolysates was carried out, the nature of the purpura-producing factor was not clarified.

It is quite possible that the biologic effects in question may not relate to poly-
saccharide, per se, but to unrecognized, closely associated elements derived from bacterial cultures together with the polysaccharide. Work in this direction is in progress.

**Summary**

Bacterial polysaccharides from cultures of Friedländer's bacillus and *Serratia marcescens* (Shear's P-25) tend to prolong the coagulation time of diluted plasma in vitro. This activity appears to be parallel to, but limited with respect to, that of heparin. The anticoagulant effect of bacterial polysaccharide as well as that of heparin is likely to be enhanced in thromboocyteopenic plasma of children with acute leukemia.

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