Effect of Various Surface Active Agents on Heparin Binding and Clot Formation on Graphite Surfaces.* (29235)

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Heparin adsorbed onto graphite and graphite coated plastic provides a surface for intravascular prostheses which is superior to silicone in preventing thrombus formation(1). The critical importance of a surface active agent in bringing about adsorption or binding of heparin to graphite surfaces was observed in our earlier studies(2). The present study was undertaken to delineate more clearly the role of the surface-active agent. A comparison was made of the ability of cationic, anionic, and non-anionic surface-active agents to augment heparin adsorption to graphite and thus produce a surface resistant to clot formation.

Methods. Both *in vitro* and *in vivo* studies were performed. *In vitro* studies consisted of determination of whole blood clotting times in graphite-coated plastic test tubes. After cleansing with ethanol, cellulose nitrate test tubes (8 mm diameter) were coated with Dag 154 colloidal graphite,† diluted 3 to 1 with ethanol. The tubes were then placed in a 130°F forced draft oven for 48 hours to remove diluting solvents. On the day of the test, one group of test tubes was filled with a surface active agent (.005 N) for 15 minutes and then given a single saline rinse. In a second group of test tubes, the surface active agent and saline rinse were followed by a 15 minute exposure to heparin (10 mg per cc) and 10 saline rinses. Each rinse involved filling the test tube to the top with saline and then inverting the tube to discard the rinse. Blood was drawn from the right atrium of an anesthetized dog (pentobarbital sodium, 26.4 mg/kg) with a silicone-coated syringe and 1 cc was placed in each of the test tubes. Blood clotting times were observed. Six anionic, 3 non-ionic, and 5 cationic surface active agents were evaluated (Table I). Five *in vitro* clotting studies were performed for each surface.

Control clotting studies were performed in plain graphite-coated test tubes, and graphite-coated tubes that had a fifteen minute exposure to heparin (10 mg per cc) and ten saline rinses. Similar control studies were performed with plain glass and silicone coated glass test tubes.

In vivo studies involved the use of graphite-coated plastic rings. The polycarbonate (Lexan) rings were 9 mm long with an internal diameter of 7 mm and a wall thickness of 0.5 mm. The rings were coated by dipping in colloidal graphite solution and were then placed in a 130° oven for 48 hours. One group of rings was soaked in a surface-active agent (.005 N) for 24 hours. A second group of rings was soaked in a surface-active agent for 24 hours, given a saline rinse and then placed in dilute heparin saline solution (.625 mg per cc) for one hour. A ring was then placed in either the thoracic inferior vena cava or superior vena cava of a dog through an atriotomy, using a short period of inflow occlusion as described before(2). This is an extremely severe *in vivo* clotting test, since previous studies have shown that both plastic and silicone-coated plastic rings will be completely occluded or contain large amounts of thrombus after only 2 hours(1,3). In the present study the ring was removed after 2 hours and the amount of thrombus was noted. No vessel was used twice. Glass tube clotting times (37°C) were used to screen all animals for clotting abnormalities and all were normal.

Results. Clotting time of blood in graphite-coated test tubes treated with any surface active agent was slightly longer than that in plain glass but less than that in plain graphite (Table I). The graphite-coated tubes prepared with anionic and non-ionic surface ac-

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† Dag 154 Colloidal Graphite—Acheson Colloids Co., Port Huron, Mich.

TABLE I. Test Tube Clotting Studies of Various Surfaces Showing Average Clotting Times in Minutes with S.E.M.

Surface and treatment			Without heparin	With heparin and rinse
Controls		Glass	6.6 ± .4	8.2 ± .8
		Silicone coated glass	17.4 ± 1.6	19.0 ± 1.7
		Graphite coated plastic	21.2 ± .8	52.6 ± 3.0
Graphite coated	Anionic	Sodium caprylate	12.6 ± 1.0	40.2 ± 5.6
		" laurate	13.6 ± 1.9	51.6 ±12.9
		" stearate	12.8 ± 2.0	58.2 ±17.9
		" oleate	11.2 ± 1.5	45.0 ±13.2
		" lauryl sulfate	11.8 ± 1.2	34.4 ± 3.7
		Triton X-200[a]	13.2 ± 1.5	35.6 ± 6.6
	Non-ionic	Victawet 12[e]	12.8 ± 1.0	66.4 ±11.9
		Triton X-100[b]	14.2 ± 1.3	52.6 ± 9.6
		Lauroyl diethanolamide	12.8 ± 1.0	51.8 ± 7.0
	Cationic	Hyamine 1622[c]	13.4 ± 1.1	>600
		Hyamine 10-X[d]	13.0 ± .4	>600
		Cationic amine 220[f]	13.6 ± .9	>600
		Cetyl pyridinium chloride	14.4 ± 1.0	>600
		Benzalkonium chloride	14.2 ± .5	>600

[a] Sodium alkyl aryl polyether sulfonate; [b] iso-octyl phenoxy polyethoxy ethanol; [c] di-isobutyl phenoxy ethoxy ethyl dimethyl benzyl ammonium chloride monohydrate; [d] di-isobutyl cresoxy ethoxy ethyl dimethyl benzyl ammonium chloride monohydrate (Rohm & Hass Co., Philadelphia, Pa.); [e] 1-hydroxy-ethyl-2-hepadecenyl glyoxalidine (Union Carbide and Carbon Corp., New York); [f] di-polyoxyethylene-n-alkyl phosphonate (Victor Chemical Works, Chicago, Ill.).

tive agents plus heparin showed little difference from graphite-coated tubes treated with heparin alone. The remarkable finding was that none of the graphite-coated tubes treated with cationic agents plus heparin followed by 10 saline rinses, showed any gross evidence of clotting in 10 hours.

In vivo studies (Fig. 1) showed that heavy thrombus formation occurred in all the graphite-coated rings treated with any of the surface active agents alone, and in the graphite-coated rings treated with either anionic or non-ionic agents plus heparin. The only rings free of thrombus were those in which a cationic surface-active agent was applied to the graphite surface before heparin exposure (Fig. 2).

Discussion. Surface-active agents are substances that alter the energy relationships at gas, liquid, and solid interfaces, causing a lowering of the surface or interfacial tension. Each agent contains one or several oil soluble (and water repelling) structural groups and one or more water soluble (and oil repelling) structural groups. The surface-active agents are divided by their electrical charge into 4 categories: (1) Cationic or positively charged agents, (2) Anionic or negatively charged agents, (3) Non-ionic or agents without an electrical charge, and (4) Amphoteric, or agents in which charge is dependent upon the pH of the medium.

Because of their wetting and emulsifying ability, the surface-active agents are widely used in laundering as detergents. In general, the agents are protein denaturants, or form complexes with proteins. The cationic agents have had wide usage in bacteriology and medicine because of their bactericidal prop-

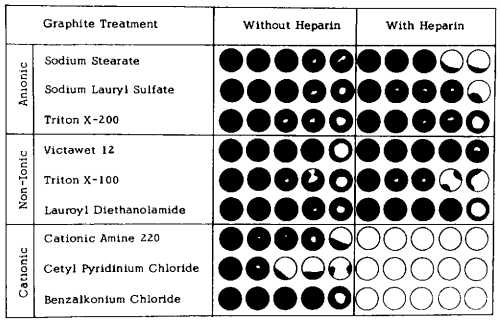

FIG. 1. Thrombus formation in graphite coated plastic rings treated with various surface active agents and with or without a dilute heparin solution. All the rings had been in a canine thoracic vena cava for 2 hr and degree of thrombus in each ring is shown diagrammatically.

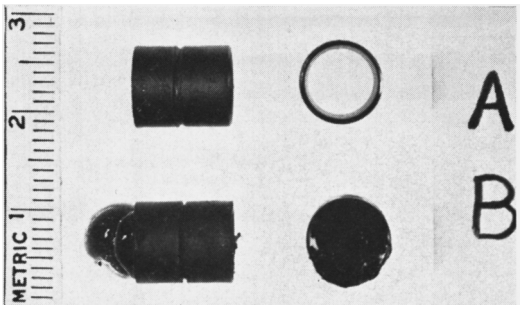

FIG. 2. A. Graphite coated polycarbonate rings treated with a cationic agent (cetylpyridium chloride) and heparin showing no evidence of thrombus formation after a 2-hr period in the vena cava. B. Similar graphite coated rings treated with a non-ionic agent (lauryl diethanolamide) and heparin showing complete occlusion by thrombus after 2 hr in the vena cava.

erties(4). They are effective against a wide range of both gram positive and gram negative bacteria. Some of the anionic agents show weak effectiveness against gram positive bacteria only(4).

Various investigators have felt that the formation of thrombi on intravascular prostheses placed in the dog is in some cases initiated by bacteria in the blood stream(5, 6). Bacteriastasis cannot account for the results of this study since cationic detergents have no bactericidal action in the presence of plasma(7) or phospholipids(8) and furthermore are ineffective in preventing thrombosis without heparin.

The present study has shown that only cationic surface agents aid adsorption or binding of heparin to graphite surfaces. The anionic and non-ionic agents were ineffective. This suggests that while the ability of cationic agents to lower surface tension may be involved in augmenting heparin adsorption by graphite, their most important property is their positive electrical charge. Heparin, a multisulfonated mucopolysaccharide, is the strongest negatively charged organic substance in the body. It is then not surprising to find that when a solution of heparin is added to a solution of cationic (positively charged) surface active agent, such as benzalkonium chloride, a heavy white precipitate forms. A precipitate is not formed when anionic or non-ionic surface active agents are mixed with heparin. The present study suggests that the oliophilic portion of the cationic detergent is adsorbed onto the graphite, allowing the strong positive charge of the quaternary nitrogen of the hydrophilic portion to bind the strongly negative sulfonic and sulfate groups of the heparin molecule.

The stability of this graphite-cationic agent-heparin complex is unknown. There is evidence that while 100 full test-tube washes of saline will not remove the heparin bonded on the graphite surface of the test-tubes, some heparin does eventually pass into blood placed into the tubes(1). The clot-preventing property of the surface, however, appears to remain. The ability of the surface to resist thrombus formation has in some instances been observed to persist *in vivo* as long as one year and may possibly persist indefinitely(1,2). It is felt that at least initially the prevention of thrombus formation is due to the graphite-cationic agent-heparin complex. The heparin-loaded graphite surface when initially introduced onto the blood stream may be similar to normal vascular endothelium since numerous investigators feel that the cement substance and tissue mast cells of the endothelium are rich in heparin-like anticoagulant mucopolysaccharides(9,10, 11,12). It is possible that as exogenous heparin is removed, other substances that might decrease thrombus development such as heparin-like mucopolysaccharides or negatively charged proteins or phospholipids are in time adsorbed onto the graphite from the blood stream, preventing thrombus formation in the long term observations.

Preliminary studies in this laboratory of the graphite-benzalkonium-heparin surface using C^{14} benzalkonium chloride have shown the presence of this cation on the graphite even after a 2-month exposure to the blood stream.

Summary. Various surface-active agents were evaluated for their ability to bind heparin to graphite-coated plastic and thus produce a prosthetic surface resistant to thrombus formation. Both *in vitro* and *in vivo* studies indicated that only cationic agents bound heparin to graphite. After the positively charged cationic agent is adsorbed to the graphite surface, it can in turn bind the

negatively charged heparin and thus present to the blood stream, at least initially, a surface rich in heparin.

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Production of a Polysaccharide by *Staphylococcus aureus*. III. Action of Penicillins and Polysaccharides on Enzymic Lysis. (29236)

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It was previously reported(1) that a polysaccharide component of *Staphylococcus aureus* accumulates within cells that are grown in the presence of subinhibitory concentrations of penicillin G or nafcillin. Further study(2) suggested that subinhibitory levels of the semisynthetic penicillins nafcillin, oxacillin and methicillin produce a disturbance or disorganization of the cell wall fabric of *S. aureus* resulting in an intracellular accumulation of the polysaccharide. In addition, treatment with nafcillin renders the normally lysozyme-resistant cells of *S. aureus* susceptible to partial lysis by lysozyme, and the concomitant addition of trypsin results in further dissolution of the cell bodies.

In this present work, by a comparative study of the penicillins nafcillin, oxacillin and cloxacillin, the following problems have been investigated: (a) effect of penicillin level on synthesis of the polysaccharide; (b) basis for superiority of one penicillin over another penicillin in production of the polysaccharide; (c) enzymic lysis of cells previously treated with various levels of the penicillin; and (d) effect of the polysaccharide and related polysaccharides on the enzymic lysis of nafcillin-treated cells.

Materials and methods. A penicillin resistant strain CHP of *S. aureus* was used. Conditions for growth of the organism and production and isolation of the polysaccharide have been described(1). Briefly, the organism was grown at 37°C for 18 hours on the surface of a casein hydrolysate-agar medium. Cells were harvested from the medium by centrifugation and washed twice with distilled water. They were suspended in saline that was standardized to contain 8.0×10^{10} cells/ml, were autoclaved at 120°C for 10 minutes, and were cooled and centrifuged at 3,000 rpm for one hour at 5°C. The supernatant fluid containing the crude polysaccharide was collected and titrated by a turbidimetric procedure(3).

Crystalline egg white lysozyme (Armour Laboratories), lysozyme substrate in the form of powdered, ultraviolet-treated cells of *Micrococcus lysodeikticus* (Difco Laboratories), crystalline trypsin, and soybean trypsin inhibitor (Worthington Laboratories) were used. Potassium hyaluronate prepared from a Group A *Streptococcus pyogenes* according to a method recently described(4) and chondroitin sulfate (Mann Laboratories) were also tested.