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STAPHYLOCOAGULASE AS A HEMOSTATIC AGENT¶

Staphylocoagulase, a protein produced by *Staphylococcus aureus* reacts with a plasma protein designated the Coagulase Reacting Factor (CRF) which is either prothrombin or is presumed to represent some active component of the prothrombin molecule.[1,2] The interaction of these two substances leads to the conversion of fibrinogen to fibrin, a reaction that proceeds *in vitro* in the absence of calcium and in the presence of anticoagulants such as heparin and dicoumarol. The administration of large intravenous doses of coagulase to rabbits may result in *in vivo* defibrinogenation, sequestration of fibrin in tissues such as the lungs, and even in acute death.[3-8] The question has remained open, however, whether appropriate lower doses of coagulase may function as systemic hemostatic agents. It is well established, as in studies of coagulase antigenicity, that some levels of coagulase are very well tolerated by experimental animals, without evidence of any untoward effects. The present observations were therefore undertaken principally to determine whether systemically administered coagulase may shorten the whole blood clotting time of the experimental animal, such as the rat, and whether indeed any significant reduction in blood loss and in the duration of bleeding may be achieved.

MATERIALS AND METHODS

Staphylocoagulase was prepared by Drummond from *S. aureus* #104 according to a previously described method.[8] This preparation contained 0.31 mg. of protein per mg. of dry weight. The activity was such that when equal volumes of coagulase and non-inhibitory human plasma were incubated for 18 hours, 0.0001 mg. of coagulase in 0.5 ml. of peptone-saline produced a firm, invertable clot.

CRF titrations consisted in making a series of doubling dilutions of cell-free plasma, generally in the range of 1:10 to 1:160. To 0.2 ml. of plasma dilution, in M/15 phosphate buffer at pH 6.85, 0.2 ml. of bovine fibrinogen (Armour) at a concentra-

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tion of 250 mg./100 ml., in physiological saline, was added. Finally, 0.2 ml. of coagulase in M/15 phosphate buffer at a pH of 6.85 was introduced into each tube as the stop watch was started. The first visible floccules or wisp of fibrin was taken as the clotting time.

Rats were of the Sprague-Dawley strain, as specified.

General procedure of administering coagulase and withdrawing blood for whole blood clotting determinations

Under Nembutal® anesthesia, the aorta was cannulated through the common carotid artery, using polyethylene tubes PE 10 (Clay-Adams).[9] The coagulase was introduced directly through the cannula into the aorta, and samples of blood were withdrawn from the same cannula at the indicated time intervals. The cannulas were thoroughly washed with saline, and no anticoagulants were introduced. White virgin female rats were used, weighing an average of 217 grams., with 22 receiving coagulase and 14, subjected to identical surgery and cannulation, serving as the controls.

Clotting time determinations

Three to five drops of blood were allowed to flow freely from the cannula on a clean glass plate, taking care that the drops were of uniform size. The blood was gently teased with the tips of a pair of fine forceps, and observed for the first indication of fibrin formation. It was found that 3-5 drops of blood collected simultaneously, checked within 2-3 seconds of each other by the stop watch. All determinations of clotting were made on 3-5 drops to establish the validity of each reading.

Collection of plasma for CRF and hematocrit determinations

Blood was withdrawn from the aortic cannula, 0.9 ml. into a syringe containing 0.1 ml. of 3.8 percent sodium citrate for the CRF determinations, and 0.02 ml. for the hematocrit determinations. The cells were removed by centrifugation.

Experimental procedure for determining the action of coagulase on surgically induced bleeding

White female rats averaging 230 grams in weight were used. Following the technique of Popovic and Popovic[9] the left jugular vein was exposed under Nembutal® anesthesia through a small neck incision, and a polyethylene cannula, PE #10 (Clay-Adams) was inserted. Coagulase (0.05 mg.) in physiological saline was introduced into the cannula of the experimental animals, while the control animals were similarly treated except that saline alone was introduced instead of coagulase. The test and control animals were placed in identical positions on their right sides, and the heads were supported in a slightly raised position. The tongues were held in place by threading the base and taping the thread to the table. Thirty minutes after the inoculation of the coagulase, approximately 0.5 cm. of the tip of the tongue was excised, and the blood permitted to flow freely into a graduated centrifuge tube for direct measurement of the blood volume and of the duration of bleeding from the cannula. Eighteen minutes following the excision of the tip of the tongue, a small cut was made on the toe of the left hind foot, and the blood loss was similarly measured.

Miscellaneous observations

The rats were observed for overt evidence of distress, such as changes in behavior

or muscle tone. The heart rate, respiratory rate, and systolic blood pressure, determined by a Beckman Dynograph, Type RS, were also monitored.

RESULTS

The effect of coagulase on the whole blood coagulation time at intervals up to 24 hours

In order to determine the extent and duration of coagulase action on the whole blood clotting time, frequent measurements were made over a span of 24 hours. These results were obtained with several different doses of coagulase, and the average values based on 22 test animals and on 14 controls are summarized in Figure 1. Following a single dose of coagulase, a fall in the clotting time is evident within 10 minutes, persists for some four hours, and returns to the base line thereafter. Since the control animals were similarly handled in every respect, the differences noted must be ascribed to the coagulase preparation rather than to the operative manipulations.

Differences in the whole blood clotting times of individual rats before and after coagulase administration

Preliminary titrations indicated considerable individual variation among rats in their whole blood clotting times. For example it was found that while most rats responded with a shortening of the blood clotting time even at the highest dose of coagulase tested, 0.25 mg., one rat exhibited markedly prolonged clotting time, suggesting that in this animal *in vivo*

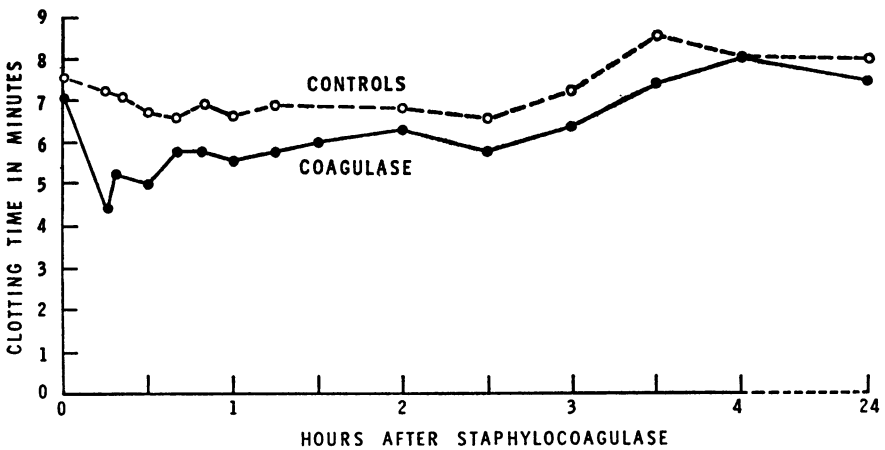

FIG. 1. The effect of coagulase on the coagulation time at intervals up to 24 hours. Each point represents the average of 22 coagulase-treated animals and 14 control animals respectively.

defibrinogenation had occurred. Accordingly, lower doses were selected, such as 0.05 mg. and 0.12 mg. for most of the tests summarized in Table 1. The clotting time recorded in 22 coagulase-treated rats and in 14 controls represents the shortest clotting times selected from serial observations made over a period of four hours. It was found that the mean of the reduction in the clotting time was 3.02 minutes for the coagulase-treated group, and 1.24 minutes for the control series. The Analysis of Variance Test gave an F value of 6.084, indicating a probability of less than 0.5 percent that this difference may be ascribed to chance.

TABLE 1. THE EFFECT OF COAGULASE ON THE CLOTTING TIME OF INDIVIDUAL RATS

	Experimental animal			*Control animal*	
	Coagulation time in minutes			*Coagulation time in minutes*	
No. of rat	*Dosage of coagulase in mg.*	*Before coagulase*	*After coagulase*	*Initial C. T.*	*Shortest C. T. during experiment*
1	0.05	6.50	5.50	6.16	5.60
2	0.05	6.83	4.50	6.16	6.00
3	0.05	5.83	5.00	6.16	5.50
4	0.05	6.50	5.50	6.25	5.50
5	0.05	5.62	4.00	6.25	5.50
6	0.05	6.00	4.50	5.66	5.10
7	0.05	10.90	5.50	10.00	6.00
8	0.05	6.16	5.00	7.25	7.00
9	0.05	9.66	6.00	12.50	9.00
10	0.05	8.50	5.30	5.25	4.50
11	0.05	6.50	3.00	8.33	7.00
12	0.05	6.16	3.50	5.41	4.50
13	0.12	13.50	4.00	5.66	5.00
14	0.12	6.50	5.50	5.12	2.50
15	0.12	7.33	5.50		
16	0.12	11.37	4.00		
17	0.12	5.66	5.00		
18	0.25	9.33	0.50		
19	0.25	7.00	3.50		
20	0.25	5.25	3.50		
21	0.25	5.83	3.50		
22	0.25	7.33	5.50		
Average values		7.46	4.44	6.86	5.62
Difference between average values		3.02 minutes		1.24 minutes	

TABLE 2. PLASMA CRF LEVELS OF NORMAL RATS AND 30 MINUTES AFTER COAGULASE (Clotting times in seconds)

Plasma dilution	*Uninoculated rats**	*0.2 mg. coag./kg.***	*0.04 mg. coag./kg.†*
1:10	134	133	124
1:20	147	151	137
1:40	170	176	168
1:80	231	264	247
1:160	378	448	399

* Average of 14 rats.
** Average of 10 rats.
† Average of 3 rats.

CRF levels of rat plasma after the administration of coagulase

Since the fall in the clotting times after coagulase inoculation indicates *in vivo* activity, it became pertinent to determine whether the level of CRF of the plasma was affected. The CRF levels of 14 normal rats were first determined, and when compared to a noninhibitory human plasma, it was found that normal rat plasmas contain approximately 25-50 percent of CRF activity present in the human. The CRF content was then determined in ten rats receiving 0.2 mg. of coagulase per kg. and in three animals given 0.04 mg. per kg. The results are summarized in Table 2. The values represent CRF levels 30 minutes after the inoculation of coagulase when a maximal drop in clotting time occurs. It is evident that the plasma level of CRF is essentially unaffected by the coagulase injection.

The effect of coagulase on surgically induced bleeding in the rat

In this study, the excision of the tip of the tongue proved far more satisfactory technically than the wound inflicted on the foot pad. While the volume and duration of blood loss was easily measurable from the tongue, the blood flow from the foot lesion was often so small that accurate measurement was not possible. The results are summarized in Table 3. It was determined that the amount and the duration of bleeding from the tongue was reduced in the test animals by approximately 50 percent. The reduction in blood loss from the toe lesion was similar, but it was not feasible to measure the duration accurately. The Analysis of Variance Test gave an F value of 8.79 for the amount of blood loss from the tongue, indicating a probability of less than 1 percent that the difference of blood loss in the treated and control groups was due to chance. A similar analysis of the differences in the duration of bleeding from the tongue gave an F value of 15.442, indicating a probability of less than 0.5 percent that the results obtained were due to chance.

TABLE 3. THE EFFECT OF COAGULASE ON SURGICAL BLEEDING IN THE RAT

	Bleeding with coagulase treatment			*Bleeding in control animals*		
Rat no.	*Bleeding from tongue (ml.)*	*Tongue bleeding time (min.)*	*Bleeding from toe (ml.)*	*Bleeding from tongue (ml.)*	*Tongue bleeding time (min.)*	*Bleeding from toe (ml.)*
1	0.20	—	0.00*	2.00	—	0.70
2	0.30	—	0.00	0.70	—	0.00
3	0.80	4	0.25	3.40	16	0.75
4	0.30	2	0.05	2.70	12	0.10
5	0.85	5	0.00	3.20	15	0.00
6	2.40	6	0.00	2.00	12	1.15
7	2.10	5	0.00	1.90	6	0.00
8	3.50	9	0.00	0.85	4	0.25
9	0.60	4	0.00	3.60	10	0.00
10	1.50	4	0.00	1.40	14	0.50
11	1.40	7	0.50	3.20	15	0.40
12	1.05	3	0.00	3.30	5	0.00
13	1.70	5	0.30	2.50	7	0.60
14	2.00	4	0.00	2.40	6	0.00
15	0.30	3	0.00	1.30	6	0.00
Avg.	1.26 ml.	4.69 min.	0.07 ml.	2.29 ml.	9.84 min.	0.3 ml.

* Volume too small to measure.

Miscellaneous observations

All test and control animals survived, and there was no evidence of distress or constitutional disturbance. No significant changes were noted in hematocrit values, heart rate, respiratory rate, or the weight of the animals.

DISCUSSION

The present findings indicate that appropriate doses of coagulase lower the glass clotting time of rats and reduce the volume of blood loss and the duration of bleeding following surgical trauma. The tongue lesions proved to be most satisfactory for monitoring the blood loss. The Analysis of Variance Test indicates that the results are statistically significant.

Many systemically introduced substances are known to induce transient hypercoagulation states.[10,11] None of these has found clinical acceptance for the amelioration of bleeding problems, barring those designed to correct a specific clotting deficiency, as in the case of antihemophilic globulin. Of unique interest is ellagic acid[11-14] which, like coagulase, reduces traumatic bleeding in the rat,[12] and which lowers the silicone and glass clotting times.[13] The duration of activity in the rat is brief, about 20 minutes, and no un-

toward effects have been observed. All available evidence implicates the activation of Factor XII as the basis of ellagic acid action.

In contrast to ellagic acid, coagulase does not require the participation of the chain of clotting factors initiated by the activation of Factor XII. Coagulase reacts directly with prothrombin and/or Coagulase Reacting Factor (CRF) of appropriate animal species to form "coagulase-thrombin," which then induces the conversion of fibrinogen to fibrin. The reported reduction in traumatic bleeding by ellagic acid in the rat[12] is more striking than achieved by coagulase. However, it should be noted that the bleeding was monitored immediately following ellagic acid inoculation, while 30 and 48 minutes elapsed after coagulase administration before the tongue and foot pad trauma respectively was inflicted. The reduction of glass clotting time following ellagic acid[18] lasted approximately 10 minutes in the rat, and up to 60 minutes in other species, while with coagulase the significant reduction in glass clotting time persisted over four hours. Thus, in addition to the difference in the presumed mode of action of the two agents, the available evidence suggests that the coagulase effect is more sustained and prolonged than the one induced by ellagic acid.

It may be of interest to speculate on the possible utility and limitations of coagulase as a systemic hemostatic agent. It is noteworthy that the *in vivo* introduction of coagulase leaves the established blood clotting factors singularly undisturbed, excepting, with appropriate doses, fibrinogen. Extensive studies in Poland[7] and in France[8] failed to show any significant impact on platelets, Factors II, V, VIII, IX, X, XI, XII. No consumption of CRF was observed, as confirmed by the present study. Coagulase action requires prothrombin and/or CRF, and fibrinogen. It seems theoretically possible that coagulase may favor a hypercoagulable state in situations of simple blood loss, as in trauma, surgery, peptic ulcers, etc. Since coagulase acts in the presence of such anticoagulants as heparin and dicoumarol, it is conceivable that it may counteract excessive bleeding induced by such anticoagulants. There is no obvious reason why coagulase may not be effective in counteracting bleeding resulting from some specific clotting factor deficiency, such as antihemophilic globulin, as long as prothrombin and/or CRF is available and fibrinogen to fibrin conversion can proceed.

In vivo, defibrinogenation[3-8] by appropriate doses of coagulase imposes an obvious limitation on its use as a systemic hemostatic agent. However, the catastrophic potential of severe defibrinogenation is dose and rate dependent, and extensive experience[3-16] has established that lower levels of coagulase are well tolerated by the experimental animal, consistent with present findings. Recent investigations of the purified enzyme "Arvin," derived from the venom of the Malayan Pit-Viper, *Ancistrodon rhodostoma,*

have provided new insight into the phenomenon of defibrinogenation[19-28] Indeed, they indicate a considerable margin of tolerance by the host for significant defibrinogenation, as in the use of "Arvin" for the therapy of thrombosis. Thus, the defibrinogenating potential of coagulase does not *per se* preclude its trial as a hemostatic agent, provided the safeguards of proper dose, proper rate of administration, and of monitoring the plasma fibrinogen level are carried out.

At present, coagulase is not available commercially. Many techniques of purification have been described,[3,24-29] and there are no insuperable obstacles to large scale production. Coagulase is an antigen,[15-18] and conceivably anticoagulase may interfere with its action. However, it may be considered a relatively "poor" antigen, since experimentally adjuvants and hyperimmunization are generally required to elicit significant neutralizing antibodies. It seems probable that it would be feasible to overcome the effect of inhibitory antibodies by appropriate coagulase dosages.

Since coagulase is a protein derived from the ubiquitous staphylococcus, allergic sensitization must be considered as a potential limitation to its use. Preliminary studies in Yugoslavia[29] have given an indication of the extent of this problem. Partially purified coagulase was administered 39 times to 27 patients, at doses ranging from 0.013 mg. to 3.9 mg. per patient, by the subcutaneous, intramuscular, and intravenous routes. No reactions were encountered with 23 intravenous inoculations, with one exception. One individual, receiving the maximal dose of 3.9 mg. developed a light but reversible shock. No reactions occurred with six intramuscular injections. Phenomena of hypersensitivity, however, were noted following subcutaneous inoculation. While no local reactions occurred in ten patients receiving 0.013 mg., one of eleven subjects receiving 0.13 mg., and all four patients receiving 1.3 mg. of coagulase developed local induration. The local lesions were as large as 3 × 4 cm., and even greater, reached a maximum in 7-8 hours, and subsided in 24 hours. Occasionally, an elevation of temperature of 1° C. was noted, also subsiding within 24 hours.

At the present time, it is not possible to ascribe the reactions to coagulase or to co-precipitated impurities in the preparations. No improvement in bleeding manifestations among the patients was noted, but the dosages selected were principally designed to explore tolerance rather than to deliver effective amounts which might be anticipated to have significant hemostatic action.

SUMMARY

The intra-aortic administration of purified staphylocoagulase results in a statistically significant reduction in the whole blood clotting time of rats.

Following a single well tolerated dose of coagulase, the shortening of the clotting time persists for at least four hours. The level of coagulase reacting factor (CRF) of the plasma is not reduced at the time of maximal reduction of the clotting time. The intravenous administration of coagulase resulted in a statistically significant reduction in the amount and duration of blood loss from tongue lesions of rats. Blood loss was also reduced by 50 percent from lesions of the foot pads of rats.

REFERENCES

1. Tager, M. and Drummond, M. C.: Staphylocoagulase. *Ann. N. Y. Acad. Sci.*, 1965, *128*, 92-111.
2. Soulier, J. P. and Prou, O.: Staphylocoagulase and prothrombin: *Thrombos. Diathes. hemorrh.* (*Stuttg.*) *Suppl. Diffuse Intravascular Clotting.* Transactions of the Conference held under the auspices of the International Committee on Haemostasis and Thrombosis. St. Moritz, Switzerland, 1966, 255-267.
3. Tager, M.: Concentration, partial purification, properties and nature of staphylocoagulase. *Yale J. Biol. Med.*, 1948, *20*, 487-500.
4. Tager, M.: Staphylococcus coagulase. *Bull. N. Y. Acad. Med.*, 1954, *30*, 475-477.
5. Blobel, H. and Berman, D. T.: Further studies on the *in vivo* activity of staphylocoagulase, *J. infect. Dis.*, 1961, *108*, 63-67.
6. Smith, D. D. and Johnstone, J. M.: Staphylocoagulase activity *in vivo*. *Brit. J. exp. Pathol.*, 1958, *39*, 165-170.
7. Jeljaszewicz, J., Niewiarowski, S., Poplawski, A., Prokopowicz, J., and Worowski, K.: Intravascular coagulation and fibrinolysis by staphylocoagulase. Comparison with thrombin. *Thrombos. Diathes. haemorrh.* (*Stuttg.*), 1965, *13*, 457-469.
8. Soulier, J. P. et Prou-Wartelle, O.: Effets de l'injection de staphylocoagulase chez le lapin. Essais de prevention du syndrome de defibrination. *Nouvelle Revue Franc. d'Hematologie*, 1967, *7*, 779-800.
9. Popovic, V. and Popovic, P.: Permanent cannulation of aorta and vena cava in rats and ground squirrels. *J. appl. Phys.*, 1960, *15*, 727-728.
10. Biggs, R. and Macfarlane, R. G.: *Human Blood Coagulation and its Disorders.* 3rd Ed. Philadelphia, F. A. Davis Co., 1962, p. 342.
11. Botti, R. E. and Ratnoff, O. D.: Studies on the pathogenesis of thrombosis: An experimental "hypercoagulable" state induced by the intravenous injection of ellagic acid. *J. Lab. clin. Med.*, 1964, *64*, 385-398.
12. Cliffton, E. E., Agostino, D., and Girolami, A.: Prevention of traumatic bleeding by ellagic acid in rats. *Proc. Soc. Exp. Biol.* (*N.Y.*), 1965, *120*, 179-180.
13. Girolami, A., Agostino, D., and Cliffton, E. E.: The effect of ellagic acid on coagulation *in vivo*. *Blood*, 1966, *27*, 93-102.
14. Ratnoff, O. D. and Crum, J. D.: Activation of Hageman factor by solutions of ellagic acid. *J. Lab. clin. Med.*, 1964, *63*, 359-377.
15. Tager, M. and Hales, H. B.: The experimental production of antibodies to staphylocoagulase. *J. Immunol.*, 1948, *60*, 475-485.
16. Duthie, E. S. and Lorenz, L. L.: Staphylococcal coagulase: mode of action and antigenicity. *J. Gen. Microbiol.*, 1952, *6*, 95-107.
17. Rammelkamp, C. H., Hezebicks, M. M., and Dingle, J. H.: Specific coagulases of *Staphylococcus aureus*. *J. exp. Med.*, 1950, *91*, 295-307.
18. Lominski, I. D., Smith, A. C., Scott, J. P., Arburthnot, S., Gray, D., Muir, D., Turner G. H. and Hedges, C. K.: Immunization against experimental staphylococcal infection with coagulase-rich preparation. *Lancet*, 1962, *1*, 1315-1318.
19. Regoeczi, E., Gergely, J., and Macfarlane, A. S.: *In vivo* effects of *Agkistrodon rhodostoma* venom: studies with fibrinogen I^{131}. *J. clin. Invest.*, 1966, *45*, 1202-1212.

20. Ashford, A. and Ross, J. W.: Pharmacology and toxicology of a defibrinating substance from Malayan pit viper venom. *Lancet,* 1968, *1,* 486-489.
21. Bell, W. R., Pitney, W. R., and Goodwin, J. F.: Therapeutic defibrination in the treatment of thrombotic disease. *Lancet,* 1968, *1,* 490-493.
22. Reid, H. A., and Chan, K. E.: The paradox in therapeutic defibrination. *Lancet,* 1968, *1,* 485-486.
23. Esnouf, M. P. and Tunnah, G. W.: The isolation and properties of the thrombin-like activity from *Ancistrodon rhodostoma* venom. *Brit. J. Hematol.,* 1967, *13,* 581-590.
24. Duthie, E. S. and Haughton, G.: Purification of free staphylococcal coagulase. *Biochem. J.,* 1958, *70,* 125-134.
25. Blobel, H., Berman, D. T., and Simon, J.: Purification of staphylococcal coagulase. *J. Bacteriol.,* 1960, *79,* 807-815.
26. Murray, M., and Gohdes, P.: Purification of staphylococcal coagulase. *Biochim. Biophys. Acta,* 1960, *40,* 518-522.
27. Zolli, Jr., Z. and San Clemente, C. L.: Purification and characterization of staphylocoagulase. *J. Bacteriol.,* 1963, *86,* 527-535.
28. Tirunaryanan, M. L.: Investigations of the enzymes and toxins of Staphylococci. Stimulation of coagulase biosynthesis by glucose and bicarbonate. *Acta path. microbiol. scand.,* 1966, *68,* 273-280.
29. Mojovic, B. and Mojovic, N.: Unpublished studies.