PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 503-507 (1976)

Studies on the Blood Clotting and Fibrinolytic System in the Plasma from a Sei (Baleen) Whale[1] (39427)

HIDEHIKO SAITO, MAN-CHIU POON[2], GEORGE H. GOLDSMITH[3], OSCAR D. RATNOFF[4], AND ÚLFUR ÁRNASON

Department of Medicine, Case Western Reserve University School of Medicine, and University Hospitals of Cleveland, Cleveland, Ohio 44106, and Institute of Genetics, University of Lund, Lund, Sweden

Sei whales are large deep-diving sea mammals that belong to the suborder baleen whales of the order Cetacea. They are said to be able to dive to depths of 300 m or more and to stay under the water for relatively long periods of time (1). As compared with terrestrial mammals, Cetaceans have many anatomical and functional deviations associated with their aquatic life. Although many comparative studies of blood coagulation in land animals have been published (2), little information is available concerning Cetacea. Robinson *et al.* (3) and Lewis *et al.* (4) reported that the plasmas of the Atlantic bottle-nosed dolphin and killer whale which belong to the other suborder of the Cetacea, the toothed whales, are deficient in Hageman factor (factor XII) activity, while we have demonstrated a deficiency of Fletcher factor (a plasma prekallikrein) activity as well (5). We have been unable to find a description of the blood coagulation system in baleen whales. The present report describes the studies of blood clotting and fibrinolytic systems in the plasma of one baleen whale species, the sei whale (*Balaenoptera borealis*).

Materials and methods. Blood was collected in Iceland waters from a male sei whale by heart puncture 10–15 min after the expiration of the animal. The blood was drawn to the deck of a whaling boat with the aid of a vacuum pump. The anticoagulant used was 1/10 vol of citrate buffer (pH 5.0, 0.13 *M* with respect to citrate). The plasma was separated by centrifugation of the blood (1000 rpm, 10 min) immediately after the sampling. The plasma was frozen aboard the whaling boat and shipped to Cleveland in dry ice.

Pooled normal human plasma and plasma from patients with congenital clotting factor deficiencies were prepared as described earlier (6). Fletcher trait plasma and Fitzgerald trait plasma were kindly supplied by Dr. Charles Abildgaard, University of California at Davis, and Dr. Robert Waldmann, Henry Ford Hospital, Detroit, Mich., respectively.

Crude bovine thrombin (Topical Thrombin) and *Bothrops atrox* venom (Reptilase) were obtained from Parke, Davis & Co., Detroit, MI and Abbott Laboratories, Chicago, Ill., respectively. Rabbit brain tissue thromboplastin (Permaplastin) was purchased from Alban & Co., St. Louis, Mo. and human brain tissue thromboplastin from Cappel Laboratories, Wayne, Pa. Rabbit antiserum against human clotting factors were prepared as described earlier (6–8). Goat antiserum against human kininogen and human C1 esterase ($C\bar{1}$) inhibitor were kindly supplied by Dr. Virginia Donaldson, University of Cincinnati College of Medicine, Cincinnati, Ohio and Dr. Jack Pensky, Case Western Reserve University, Cleveland, Ohio. The following antiserums were obtained from commercial sources: rabbit antiserum against human plasminogen, fibrin-stabilizing factor (factor XIII) and inter-α-trypsin inhibitor from Behringwerke AG, Marburg, Germany; goat antiserum against α_2-macroglobulin and α_1-trypsin inhibitor from Miles Laboratories, Elkhart, Ind.; rabbit antiserum

[1] This study was supported in part by grant HL 01661 from the National Institutes of Health, U.S. Public Health Service, and in part by grants from the American Heart Association, and Northeast Ohio Affiliate of the American Heart Association.

[2] Centennial Fellow of the Medical Research Council of Canada.

[3] Research Fellow of the American Heart Association.

[4] Career Investigator of the American Heart Association.

against fibrin from Hyland Laboratories, Costa Mesa, Calif.; and rabbit antiserum against antithrombin III from Nyegaard & Co. AS, Oslo, Norway.

Routine clotting tests and assays for individual clotting factor activity were performed as described previously (9, 10). One unit of clotting factor activity was arbitrarily defined as that present in 1 ml of pooled normal human plasma (6). Antithrombin activity (11), plasma inhibitor against activated PTA (12), and plasma inhibitor against activated Stuart factor (13) were measured as described. The inhibition of the clot-promoting activity of activated Fletcher factor (a plasma kallikrein) was tested by incubating purified plasma kallikrein (25 μM MeOH released/ml/hr in a TAME (*p*-toluene sulfonyl-L-arginine methyl ester) esterase assay (8), with equal amounts of sei whale plasma or bovine serum albumin (1 mg/ml in barbital–saline buffer, pH 7.4) at 37°. At intervals, aliquots were tested for residual activated Fletcher factor activity upon Fletcher trait plasma as a substrate. The results were expressed as percent of the initial clot-promoting activity.

A crude fibrinogen fraction (fraction I–0) was isolated from citrated plasma by the method of Blombäck (14).

Plasma plasminogen concentration was assayed by the method of Remmert and Cohen (15) after activation by streptokinase (high purity, the gift of Lederle Laboratories, American Cyanamide Co., Pearl River, N.Y.) or human urokinase (WHO Standard from Abbott Laboratories. a gift of Dr. A. Johnson, New York University Medical Center, N.Y.).

Double immunodiffusion studies were carried out on 7.5 × 2.5 cm glass slides in 0.9% agarose gel in barbital buffer (0.05 *M* sodium barbital, pH 8.4). Ten microliter samples were applied to the wells which were 3 mm apart. Precipitin lines were allowed to develop for 24 hr at room temperature. Immunoelectrophoresis was performed on glass slides of the same size at a constant voltage of 150 V for 60 min at room temperature.

Antibody neutralization tests for AHF were performed as recently reported (16).

Results. Blood clotting system. Routine clotting tests showed that, compared to human pooled plasma, the thrombin time, Reptilase time, and one-stage prothrombin time were moderately prolonged. The kaolin-activated partial thromboplastin time (kaolin PTT) was greatly prolonged (Table I). A crude fibrinogen fraction (fraction I–0), isolated from whale plasma, clotted more rapidly upon addition of thrombin than the corresponding fraction of human plasma. The one-stage prothrombin time of the whale plasma, using human tissue thromboplastin, was comparable to that in which rabbit tissue thromboplastin was used. The prolonged kaolin PTT (355 sec) was largely corrected by addition of an equal volume of human plasma (81.3 sec), suggesting that this prolongation was not due to the presence of a circulating anticoagulant in the whale plasma. Individual clotting factors were measured in comparison to pooled normal human plasma using human, congenitally deficient plasmas as substrates (Table II). Activities attributable to Hageman factor, Fletcher factor, and PTA were absent, whereas those of other clotting factors were comparable to those of human plasma. In immunologic studies employing double diffusion, whale plasma formed a precipitin line of nonidentity with human plasma against rabbit antihuman fibrin serum. Little difference was observed on immunoelectrophoresis between the mobility of the fibrinogen-related antigen of whale and human plasma. In contrast to fibrinogen, no precipitin lines formed between whale plasma and antiserums to human AHF, PTA, HF, Fletcher factor, Fitzgerald factor, and fibrin-stabilizing factor. AHF activity of whale plasma was also studied by antibody neutralization tests, using both rabbit anti-human AHF serum and a human circulating anticoagulant against AHF. Whale AHF was 7.5× more resistant to inactivation by rabbit antiserum and 7.6× more resistant to inactivation by the human circulating anticoagulant than was the AHF in human plasma.

Fibrinolytic system. When human urokinase was used as an activator, plasma plasminogen was detected at a concentration of 1.4 R and C units/ml, but not when streptokinase was used. Whale plasma formed a

single precipitin line of nonidentity with human plasma against rabbit anti-human plasminogen serum. A precipitin line formed upon immunoelectrophoresis of whale plasma against this antiserum with electrophoretic mobility similar to that of human plasma.

Inhibitors of blood coagulation and fibrinolysis. Functional assays of plasma antithrombin and plasma antiplasmin activity showed 200 and 50% activity relative to that of a standard pool of normal human plasma, respectively. The titers of the inhibitors against activated PTA and activated Stuart factor in whale plasma were 74 and 100% of normal human pooled plasma, respectively. When activated Fletcher factor (a plasma kallikrein) was incubated with baleen whale plasma, clot-promoting activity was decreased to 44% at 5 min and 26% at 10 min of the initial activity. There was, however, no loss of activity when activated Fletcher factor was incubated with a bovine serum albumin control. Immunodiffusion studies of whale plasma revealed single precipitin lines of partial identity with human plasma against the antiserums to human α_1-trypsin inhibitor, antithrombin III and $C\bar{1}$ inhibitor. Upon immunoelectrophoresis, little difference was observed between the mobility of human and whale plasma α_2-macroglobulin and inter-α-trypsin inhibitor-related antigen.

TABLE I. ROUTINE CLOTTING STUDIES OF SEI WHALE PLASMA

Tests	Clotting time (sec)		
	Baleen whale	Human control	Whale + human (1:1)
Thrombin time[a]	25.3	13.2	16.0
Reptilase time	24.1	18.4	–
Prothrombin time, brain tissue thromboplastin			
Human	16.6	10.7	10.7
Rabbit	19.6	13.3	–
Kaolin-activated PTT	355.0	62.2	81.3

[a] Five units per milliliter of bovine thrombin was used.

Discussion. Sei whale plasma showed a greatly prolonged kaolin PTT that was corrected by addition of normal human plasma. These experiments suggest that a deficiency of one or more clotting factors participating in the intrinsic pathway of thrombin formation was present. The most remarkable findings were the absence of detectable Hageman factor, Fletcher factor, and PTA activity. These results, in test systems comprising heterogeneous protein mixtures, should be interpreted carefully. A failure to correct the clotting defect in human Hageman trait, Fletcher trait, and PTA deficiency may have several causes. The factor may be absent in both plasmas, or it may function only in a

TABLE II. CLOTTING FACTOR ACTIVITY AND ANTIGEN IN SEI WHALE PLASMA

Clotting factors	Normal human range[a]	Activity (U/ml)	Precipitin line with heterologous anti-human clotting factor serum
Factor I (fibrinogen)[b]	177–415	141	+
Factor II (prothrombin)	0.65–1.50	0.90	NT[c]
Factor V (proaccelerin)	0.45–1.65	0.54	NT
Factor VII	0.55–1.85	0.17	NT
Factor VIII (AHF)	0.46–1.75	1.44	–
Factor IX (Christmas factor)	0.50–1.80	2.30	NT
Factor X (Stuart factor)	0.65–1.85	0.75	NT
Factor XI (PTA)	0.50–1.56	0.04	–
Factor XII (Hageman factor)	0.35–1.83	<0.01	–
Factor XIII (FSF)	Present	Present	–
Fletcher factor (plasma prekallikrein)	0.50–1.50	<0.01	–
Fitzgerald factor (high MW kininogen)	0.46–1.32	0.30	–

[a] Mean ± 2 SD.
[b] Expressed as milligrams of protein per 100 ml of plasma.
[c] Not tested.

species specific manner. Nonetheless, this combination of clotting factor deficiencies and the prolonged kaolin PTT suggest that the sei whale is truly deficient in these factors. In contrast, all other known clotting factor activities were present in whale plasma in amounts comparable to those present in human plasma. Although factor VII activity was moderately low, species specificity of tissue thromboplastin used to assay this factor may explain the low level (17). Immunological studies showed that the AHF, fibrin-stabilizing factor and Fitzgerald factor (high molecular weight kininogen) of whale plasma were antigenically different from those of human plasma. Whale fibrinogen, however, appeared to contain antigens partially similar to those of human fibrinogen.

• It is interesting to note that 3 out of 4 surface-mediated clotting factors (HF, PTA, Fletcher factor and Fitzgerald factor) were absent in the plasma of a deep-diving animal. The sei whale is said to dive as deep as 300 m or more (1). At these depths the water pressure is about 30 or more atmospheres. It has been shown that the blood circulation to some parts of the seal may be stopped or retarded during deep dives (18). The role of retarded blood flow in thrombosis is well appreciated (19). It is also said that blood pH falls during long dives (20). Acidosis may contribute to thrombogenesis (21). Yet these animals do not apparently undergo thrombosis. It is tempting to speculate that the deficiency of HF, Fletcher factor, and PTA may contribute to the lack of thrombosis in these deep-diving animals.

Normal human plasma possesses various protease inhibitors which may counterbalance the activation of blood clotting. Whale plasma appears to have similar inhibitors which can be detected functionally and immunologically. It is interesting to note that whale plasma is able to inactivate activated Fletcher factor and activated PTA, although both Fletcher factor and PTA are deficient in the plasma.

Studies on the fibrinolytic system in marine mammals are also sparse. The present study demonstrated that plasma plasminogen of a baleen whale was activated by human urokinase, but not by streptokinase. This is consistent with the previous report that plasma plasminogen in the killer whale and dolphins was not activated by streptokinase alone (3). The presence of plasma plasminogen in the sei whale was also demonstrated immunologically. Additionally, whale plasma had some antiplasmin activity.

Comparative study of blood coagulation and fibrinolytic systems in mammals may help not only to elucidate the evolution of these systems but also to understand the possible role of these systems in the pathophysiology of human disease.

Summary. Blood clotting and fibrinolytic systems were studied in the plasma of a sei whale (*Balaenoptera borealis*). The sei whale belongs to the suborder baleen whales of the order Cetacea. Whale plasma had a greatly prolonged kaolin-activated partial thromboplastin time and was deficient in Hageman factor (factor XII), Fletcher factor (a plasma prekallikrein), and PTA (factor XI). All other clotting factor activities were present in amounts comparable to that of normal human plasma. Whale plasminogen was activated by human urokinase, but not by streptokinase. Whale plasma contained inhibitory activities against thrombin, activated Stuart factor, activated PTA, activated Fletcher factor, and plasmin.

We are grateful to Dr. James Mead of the Smithsonian Institution, Washington, D. C., who imported the whale plasma. Ms. Ellen Strecker and Miss Marilyn Burke provided invaluable technical help.

1. Kooyman, G. L., and Andersen, H. T., *in* "The Biology of Marine Mammals" (H. T. Andersen, ed.), p. 65, Academic Press, New York (1969).
2. Archer, R. K., Symposia of the Zoological Society of London **27,** 121 (1970).
3. Robinson, A. J., Kropatkin, M., and Aggeler, P. M., Science **166,** 1420 (1969).
4. Lewis, J. H., Bayer, W. L., and Szeto, I. L. F., Comp. Biochem. Physiol. **31,** 667 (1969).
5. Saito, H., Goldsmith, G., and Ratnoff, O. D., Proc. Soc. Exp. Biol. Med. **147,** 519 (1974).
6. Zimmerman, T. S., Ratnoff, O. D., and Powell, A. E., J. Clin. Invest. **50,** 244 (1971).
7. Saito, H., Ratnoff, O. D., Marshall, J. S., and Pensky, J., J. Clin. Invest. **52,** 850 (1973).
8. Saito, H., and Ratnoff, O. D., Nature (London) **248,** 597 (1974).

9. Crum, E. D., Shainoff, J. R., Graham, R. C., and Ratnoff, O. D., J. Clin. Invest. **53,** 1308 (1974).
10. Saito, H., Ratnoff, O. D., Waldmann, R., and Abraham, J. P., J. Clin. Invest. **55,** 1082 (1975).
11. Monkhouse, F. C., Thromb. Diath. Haemorrh. **9,** 387 (1963).
12. Nossel, H. L., and Niemetz, J., Blood **25,** 712 (1965).
13. Yin, E. T., and Wessler, S., Thromb. Diath. Haemorrh. **21,** 398 (1969).
14. Blombäck, B., and Blombäck, M., Ark. Kem. **10,** 415 (1957).
15. Remmert, L. F., and Cohen, P. P., J. Biol. Chem. **181,** 431 (1949).
16. Poon, M. -C., Wine, A. C., Ratnoff, O. D., and Bernier, G. M., Blood **46,** 409 (1975).
17. Didisheim, P., Hattori, K., and Lewis, J. H., J. Lab. Clin. Med. **53,** 866 (1959).
18. Elsner, R., Franklin, D. L., Van Citters, R. L., and Kenney, D. W., Science **153,** 941 (1966).
19. Wessler, S. S., J. Clin. Invest. **34,** 647 (1955).
20. Elsner, R., *in* "The Biology of Marine Mammals" (H. T. Andersen, ed) p. 117. Academic Press, New York (1969).
21. Crowell, J. W., and Houston, B., Amer. J. Physiol. **201,** 379 (1961).

. Received March 1, 1976. P.S.E.B.M. 1976, Vol. 152.