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Fletcher Factor Activity in Plasmas of Various Species[1] (38378)

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Fletcher trait is an asymptomatic, hereditary disorder of blood coagulation in which the partial thromboplastin time of the plasma of affected individuals is prolonged (1, 2). Hathaway *et al.* (1), who first recognized Fletcher trait, observed that the defect measured by the partial thromboplastin time was corrected by prolonged exposure of plasma to glass. They believed that the abnormal plasmas lacked a hitherto unrecognized procoagulant substance, "Fletcher factor." Recently, Wuepper (3) demonstrated that preparations of human plasma prekallikrein corrected the clotting defect of Fletcher trait plasma, and concluded that Fletcher factor was a plasma prekallikrein. This observation has been confirmed in this (4) and other (5) laboratories. In all these studies, the site of action of Fletcher factor has been localized to an early step of the intrinsic pathway of thrombin formation. Consistent with this view, treatment of normal human plasma with monospecific antibody directed against a human plasma kallikrein impaired the intrinsic pathway (6).

These results implied that a plasma prekallikrein (Fletcher factor) is necessary for normal *in vitro* coagulation in human plasma. The role of Fletcher factor in clotting of animal plasma is not known. This paper will report Fletcher factor activity in different species as assayed on human Fletcher trait plasma as a substrate. The generation of arginine esterase activity in these plasmas by kaolin, attributed primarily to activation of a plasma prekallikrein (7), was also studied and compared with Fletcher factor activity.

Materials and Methods. Pooled normal human plasma and *plasma from patients with Hageman factor* (HF, Factor XII) *or plasma thromboplastin antecedent* (PTA, Factor XI) *deficiency* were prepared from blood to which 1/50 vol of sodium citrate buffer (pH 5.0, 0.5 *M* with respect to citrate) was added (8). *Fletcher trait plasma* (2), prepared from blood to which 1/10 vol of citrate buffer (pH 5.0, 0.13 *M* with respect to citrate) was kindly supplied by Dr. Charles Abildgaard and Dr. Paul Hattersley, University of California at Davis. This plasma contained 0.86 units/ml of HF and 1 unit/ml of PTA, one unit in each case being arbitrarily defined as that amount found in 1 ml of a standard pool of normal citrated plasma. *Glass-adsorbed plasma* was prepared as previously described (9), and contained 0.98 units/ml of HF, but no detectable PTA activity. *Aluminum hydroxide gel-adsorbed plasma* was prepared by shaking citrated plasma with 1/10 vol of aluminum hydroxide gel (Amphojel without flavor, 4% aluminum oxide, Wyeth Laboratories, Philadelphia, PA.) at 37° for 10 min. After centrifugation, the supernatant plasma was separated. The procedure was repeated three times. The final supernatant plasma contained 0.5 units/ml of HF, but very low PTA activity (less than 0.01 units/ml) and no Vitamin K-dependent factors.

Animal plasmas were obtained from a variety of sources (Colorado Serum Co., Denver, CO; Pel-Freez Biologicals Inc., Rogers, AR or directly from some of the species studied) and had been separated from blood to which 1/10 vol of 0.13 *M* sodium citrate had been added. The plasmas were stored at −70° until used. Killer

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whale plasma in ACD anticoagulant was a gift of Dr. T. S. Zimmerman (Scripps Clinic and Research Foundation, La Jolla, CA). Killer whale plasma and bottlenosed dolphin plasma were kindly supplied by Dr. K. N. Gray (Sea-Arama Marineworld, Galveston, TX), and had been separated from blood to which 1/10 vol of 0.13 *M* sodium citrate had been added. Beluga whale plasma was obtained through the kindnessof Dr. J. A. Oliver, Dr. J. Hyman, and Mr. W. Flynn (The New York Aquarium, Brooklyn, NY), and had been separated from blood to which 1/50 vol of 0.5 *M* sodium citrate (pH 5) had been added.

Kaolin (Fisher Scientific Co., Fairlawn, NJ), chiefly hydrated aluminum silicate, *Centrolex "O"* (Central Soya Co., Chicago, IL), a crude preparation of soybean phosphatides, and *kaolin-Centrolex,* a mixture of 50 mg of kaolin suspended in 5 ml of 0.1% Centrolex "O", was prepared as described earlier (10).

Barbital-saline buffer, pH 7.4, contained 2.76 g of barbital, 7.3 g of sodium chloride and 2.06 g of sodium barbital per liter.

Assays for HF and PTA were performed as described earlier (11).

Assays of Fletcher factor clot-promoting activity were performed by the method described by Hathaway and his associates (1). In essence, 0.1 ml of a test sample, diluted 1/50 in barbital–saline buffer, was incubated with 0.1 ml kaolin-Centrolex and 0.1 ml Fletcher trait plasma for 1 min at 37° in a 10 × 75 mm glass tube. The mixture was then recalcified, and the clotting time was measured at 37°. The clotting time was converted to arbitrary units by comparison with the clotting activity of serial dilution of a standard pool of human plasma (10). A linear relationship existed between the logarithm of the clotting time and the logarithm of the concentration of Fletcher factor activity.

Techniques for the purification of normal human plasma kallikrein and the preparation of antiserum to human kallikrein in New Zealand albino rabbits have been described (12). *Double immunodiffusion studies* were carried out on 7.5 × 2.5 cm glass slides in 0.9% agarose gels in barbital buffer (0.05 *M* sodium barbital, pH 8.4). Precipitin lines were allowed to develop for 24 hr at room temerature.

*Kaolin-activated TAMe (p-*toluenesulfonyl-L-arginine methyl ester) *esterase activity of plasma* was tested as described by Colman *et al.* (13). TAMe was obtained from Nutritional Biochemical Co., Cleveland, OH.

Results. The ability to correct the clotting defect of Fletcher trait plasma was assayed in 16 mammalian and two avian species (Table I). The units employed relate the activity found in animal plasma to that present in 1 ml of pooled normal human plasma as indicated in the Methods section. Three human HF-deficient and 4 PTA-deficient plasmas contained normal amounts of Fletcher factor activity, but glass-adsorbed or aluminum hydroxide gel-adsorbed human plasma had almost no activity comparable to that found in human plasma. On the other hand, bovine, dog, cat and rabbit plasma as well as cetacean plasma contained little or no Fletcher factor. Nonetheless, the kaolin-activated partial thromboplastin time (kaolin-PTT) with 1 min preincubation of bovine, dog, cat and rabbit plasma was not prolonged compared to that of human plasma (58, 28, 68 and 76 sec, respectively). Two fowl plasmas also had no activity. Glass-adsorbed or aluminum hydroxide gel-adsorbed human plasma contained normal amounts of HF activity, but no PTA or Fletcher factor activity. Dolphin plasma had normal amounts of PTA, but no HF or Fletcher factor activity. Attempts to make an artificial substrate for Fletcher factor assay by mixing these two plasmas were unsuccessful. In immunologic studies employing double diffusion, only human and ape plasma showed a precipitin line of identity against rabbit anti-human kallikrein serum. No other animal plasmas made a precipitin line.

The generation of plasma TAMe esterase activity with kaolin was measured and compared with HF and Fletcher factor activity in plasmas of some species (Table II). Gibbbon, swine, guinea pig and mouse plasmas showed amounts of kaolin-activated TAMe esterase at 1 min incubation comparable to that of human plasma. In contrast, rabbit, dog, cat and calf plasmas showed no esterase generation at 1 min, although they contained appreciable amounts of HF activity assayed on human HF-deficient plasma as a substrate. When rabbit, dog or cat plasma was incubated with kaolin for a longer period of time, significant esterase activity was generated.

Discussion. There has been very little information on Fletcher factor activity of animal plasma in the literature. Hathaway and his associates (1) reported that chicken and duck

TABLE I. Fletcher Factor Clot-Promoting Activity of the Plasmas of Various Species.

Species	Fletcher factor activity (units/ml)	Precipitin line with rabbit anti-human kallikrein serum
Human: normal[a]	1.0	+
: glass adsorbed	<0.01	−
: aluminum hydroxide gel adsorbed	<0.01	−
: Hageman trait (3)[b]	0.76 (0.5–1.1)[c]	+
: PTA-deficiency (4)[b]	1.1 (0.9–1.25)[c]	+
Apes: chimpanzee (2)[d]	0.38, 0.6	+
: gibbon[d]	1.3	+
: black ape[d]	0.35	+
Swine[a]	1.4	−
Guinea pig[a] (English)[e]	1.7	−
Mouse[a] (Webster Swiss)[e]	0.94	−
Goat[a] (Mexican)[e]	0.27	−
Sheep[a]	0.22	−
Horse[a]	0.19	−
Dog[a]	0.02	−
Cat[a]	0.02	−
Calf[a] (Hereford)[e]	0.06	−
Rabbit[a] (New Zealand albino)[e]	<0.01	−
Killer whale[d]	<0.01	−
Beluga whale[d]	<0.01	−
Atlantic bottlenosed dolphin[d]	<0.01	−
Porpoise[d]	<0.01	−
Duck[a] (Muscovy)[e]	<0.01	−
Chicken[a] (White Rock)[e]	<0.01	−

[a] Pooled plasma specimens.
[b] Figure in parenthesis indicates numbers of individuals studied.
[c] Mean (range).
[d] A single animal was studied.
[e] Name in parenthesis indicates the breed used.

plasma lack this activity. Our results are consistent with theirs. Abildgaard (14) reported that nonhuman primates' plasma contained 63–206% of Fletcher factor activity relative to that in human plasma. The present study showed that rabbit, dog and cat as well as cetacean plasma contained almost no activity. The results in test systems comprising heterologous protein mixtures should be interpreted carefully. Failure to correct the clotting defect in Fletcher trait plasma may have several causes. The factor may be absent in both plasmas, or it may function in a species specific manner. Athough dog or rabbit plasma lacks Fletcher factor activity, their plasmas did not show the prolonged kaolin-PTT of Fletcher trait plasma. Probably, these animal plasmas do not require Fletcher factor for the intrinsic pathway of thrombin formation. Pig, guinea pig and mouse plasmas contained normal to high Fletcher factor activity, but did not make a precipitin line against anti-human kallikrein (Fletcher factor) serum. Their Fletcher factor might be antigenically different from that of human subjects.

Colman *et al.* (13) reported that kaolin-activated TAMe esterase activity in normal human plasma was due principally to a plasma kallikrein by activated HF. They developed a simple assay for plasma prekallikrein based on kaolin-activated TAMe esterase activity generated during incubation for 1 min. Since Fletcher factor was functionally and antigenically identified as a human plasma prekallikrein (3) and Fletcher trait plasma generated no esterase at 1 min incubation with kaolin (15), we measured the generation of TAMe esterase in plasmas of some species. There was good correlation between kaolin-activated esterase levels at 1 min and Fletcher factor activity in nine species tested (Table II). The kaolin-activated esterase activity

TABLE II. Comparison of HF Activity, Fletcher Factor Activity, and Kaolin-Activated TAMe Esterase Activity upon 1 min Incubation in Various Species.

Species	HF Activity[c] (units/ml)	Fletcher factor activity[c] (units/ml)	Kaolin-activated TAMe esterase at 1 min[d] (μM MeOH/ml/hr)
Human[a]	1.0	1.0	68.2
Gibbon[b]	1.5	1.3	53.7
Swine[a]	5.9	1.4	88.0
Guinea pig[a]	6.4	1.7	35.2
Mouse[a]	1.9	0.94	22.0
Rabbit[a]	0.29	<0.01	0
Dog[a]	0.96	0.02	0
Cat[a]	2.7	0.02	0.2
Calf[a]	0.65	0.06	0

[a] Pooled plasma specimen.
[b] A single animal was studied.
[c] Human plasma was used as a substrate.
[d] Tested on each species' own plasma.

tested on each species' own plasma was low in four species, all of which showed low Fletcher factor activity as assayed on a substrate of human plasma. The lack of esterase generation at 1 min in rabbit, dog, cat or calf plasmas was due to a deficiency of HF. All these data suggest that plasma of these species is deficient in a plasma prekallikrein.

Plasma prekallikrein is the precursor of the plasma proteolytic enzyme, kallikrein, which releases biologically active peptides, kinins from a precursor (kininogen). Exposure of human plasma to foreign surfaces such as glass or kaolin releases kinins in plasma through the activation of HF (16). Margolis (16) proposed that activated HF activates a "component A" (probably prekallikrein), which together with another factor ("component B") brings about the generation of plasma kinins. When human or guinea pig plasma is exposed to glass, kinins appeared in the plasmas. In contrast, Armstrong and her associates (17) reported that no kinins were generated when dog or rabbit plasmas are exposed to glass. The failure of dog or rabbit plasmas to generate kinins was thought to be due to a deficiency of "component A" (18, 19). This interpretation appears to be consistent with our present demonstration that these plasmas had little or no Fletcher factor activity or kaolin-activated TAMe esterase activity at 1 min.

In contrast to our observations, plasma prekallikrein has been isolated from rabbit (20) and bovine (21) plasmas. It is not clear if the defective kinin generation in rabbit plasma upon contact with glass is because such plasma has a relatively small amount of prekallikrein. It is also possible to assume that there may be more than one plasma kallikrein (19) and that Fletcher trait, rabbit or dog plasma lack only one of these. This assumption receives support from the recent experimental evidence that some kallikrein-like activity could be demonstrated in a fraction prepared from Fletcher trait plasma (15).

Summary. The plasmas of 18 species were assayed for Fletcher factor activity using human Fletcher trait plasma as a substrate. Ape, swine, guinea pig and mouse plasmas had Fletcher factor activity comparable to that of human plasma. In contrast, bovine, dog, cat, rabbit and cetacean plasmas contained almost no Fletcher factor. In immunodiffusion studies using rabbit anti-human kallikrein (Fletcher factor) serum, only human and ape plasmas gave a precipitin line. There is a good correlation between Fletcher factor activity tested on human substrates and kaolin-activated TAMe esterase levels of each species' own plasma at 1 min incubation.

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