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Comparative Studies of Hageman Factor (Factor XII) in Mammalian Plasmas by Immunological Techniques (40563)[1]

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Hageman factor (HF, factor XII) is an agent functionally deficient in plasmas from individuals with Hageman trait (1). Human HF is a plasma protein of an approximate MW of 80,000 that, under certain conditions, triggers several surface-mediated plasma reactions such as the intrinsic pathway of blood coagulation, surface-mediated fibrinolysis, and kinin generation (2).

All normal mammalian plasmas except those of certain cetaceans (3–5) contain an activity that correct the clotting defect of human HF-deficient plasma. HF has been isolated and characterized from human, bovine, and rabbit plasma (6–8). The present paper examines the immunological relationships of HF in 18 mammalian species using rabbit antiserums directed against human, bovine, or rat HF, and compares the titer and molecular weight of HF activity in these plasmas.

Materials and methods. Pooled normal human plasma, plasma from individuals with Hageman trait, and animal plasmas were prepared or obtained as described earlier (5, 9).

HF clot-promoting activity was measured using human HF-deficient (Hageman trait) plasma as a substrate (6). A standard pool of 24 normal male plasmas was prepared by an earlier method (5) and was used as the standard for measurement of HF activity. The pooled human plasma was arbitrarily said to contain 1.0 unit of HF activity per milliliter.

Monospecific rabbit antiserum to human HF was prepared and a crude immunoglobulin fraction of antiserum was separated as reported previously (10).

Purified bovine HF was isolated from 430 ml of bovine plasma thromboplastin antecedent (PTA, factor XI)-deficient plasma (11) by a method described for human HF (10). The specific activity of the purified bovine HF was 100 units per milligram of protein. Antiserum to this protein was raised in New Zealand albino rabbits, and the crude immunoglobulin fraction was separated. The anti-bovine HF serum, diluted eightfold, inactivated 99% of the HF activity of an equal volume of bovine plasma upon incubation at 37° for 1 hr, but did not significantly inactivate the titers of other clotting factors. Upon immunodiffusion, this antiserum formed at least two precipitin lines with bovine plasma. The antiserum was then absorbed with equal volume of bovine plasma that had been adsorbed with Celite 512 (40 mg/ml, Johns Manville Products Corp., Celite Div., Denver, Colo.). The absorbed antiserum formed a single precipitin line of complete identity with bovine plasma and purified bovine HF, and was used in immunodiffusion studies.

Purified rat HF was prepared from 700 ml of plasma from Sprague–Dawley rats by a method described for human HF (10). The purified preparation had a specific activity of 17 units per milligram of protein, and showed a sharp single band on polyacrylamide disc gel electrophoresis in the absence and presence of sodium dodecyl sulfate (SDS) (12). The antiserum to purified rat HF was raised in New Zealand albino rabbits and a crude immunoglobulin fraction was separated. The anti-rat HF serum, diluted eightfold, inactivated more than 99% of the HF activity of an equal volume of rat plasma upon incubation at 37° in 1 hr, but did not significantly inactivate the titers of other clotting factors. Upon immunodiffusion, this antiserum formed a

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single precipitin line of complete identity with rat plasma or purified rat HF. The crude immunoglobulin fraction of normal rabbit serum was used as a control in experiments involving antiserum.

Immunodiffusion was carried out on 2.5 × 7.5-cm glass slides in 1% agarose gels in barbital buffer (0.05 *M* sodium barbital, pH 8.4). Precipitin lines were allowed to develop for 48 hr at RT.

Gel filtration of animal plasmas was performed at 4° on a 1.5 × 80-cm column of Sephadex G150 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), equilibrated with barbital–saline buffer containing 0.0001 *M* EDTA. One milliliter of plasma containing, as an internal marker, 0.05 ml ^{125}I-labeled human HF (approximately 45,000 cpm) (13) was applied and 2-ml fractions were collected at a flow rate of 15 ml per hour. Each fraction was assayed for HF activity and absorbance at 280 nm, and was counted for radioactivity. The HF activity of ^{125}I-labeled human HF added was less than 0.001 U/ml and thus did not appreciably contribute to the HF activity of the effluent. Blue dextran 2000 (Pharmacia Fine Chemicals, Inc.), bovine γ-globulin (Armour Pharmaceutical Corp., Chicago, Ill.; MW: 160,000), bovine serum albumin (Pentex, Kankakee; MW 67,000), and ovalbumin (Schwarz/Mann, Orangebury, N. Y.; MW: 45,000) were used as markers.

The rate of inactivation of the HF activity of human, bovine, or rat plasma by its respective antiserum was studied by incubating each plasma (diluted appropriately to 0.5 U/ml in 1 mg/ml bovine serum albumin) with equal volume of various dilutions of the antiserum in barbital–saline buffer at 37°. At intervals, residual HF activity was measured. In other experiments, the effect of incubation temperature upon the rate of the inactivation of HF activity by the antiserum was tested at 37 and 0°.

Neutralization of the HF activity of animal plasmas by three antiserums was studied by incubating 0.05 ml test plasma with equal volumes of various dilutions of the antiserums at 37° for 30 min. Since different animal plasmas contained different titers of HF activity (Table I), the plasmas were diluted in 1 mg/ml bovine serum albumin to 0.5 U/ml human HF activity in order to provide the same antigen:antibody ratios.

TABLE I. HF CLOT-PROMOTING ACTIVITY IN PLASMAS OF VARIOUS SPECIES.

Species	HF activity (U/ml)
Man[a]	1.0
Apes	
Chimpanzee[b]	2.24
Baboon[c]	5.21
Black ape[b]	1.14
Gibbon[b]	2.48
Bovine[a] (hereford)[d]	1.24
Goat[b] (Mexican)[d]	2.8
Swine[b]	5.92
Sheep[a] (Merion)[d]	1.64
Horse[a] (palomino)[d]	0.98
Dog[a]	1.56
Cat[a]	3.76
Rabbit[a] (New Zealand albino)[d]	0.38
Guinea pig[a] (American short hair)[d]	7.04
Rat[a] (Sprague–Dawley)[d]	1.34
Hamster[b]	0.60
Mouse[a] (Swiss Webster)[d]	1.16
Gerbil[a]	3.00

[a] Pooled plasma specimens.
[b] A single animal was studied.
[c] Mean of five animals.
[d] Names in parentheses indicate the breed used.

Results. Titers of HF clot-promoting activity in plasmas of various species. As previously reported (2, 14), the plasmas of all mammalian species tested contained agents correcting the defect in human Hageman trait (factor XII deficiency) plasma (Table I). The units employed relate the activity found in the animal plasmas to that present in 1 ml of pooled normal human plasma as described in the Methods section. Plasmas from baboon, swine, and guinea pig contained much higher levels of the HF than that of human plasma.

Molecular weight determinations of HF activity in plasmas of various species by gel filtration. When each of the 16 mammalian plasmas was filtered through a column of Sephadex G150, the HF activity eluted at the same place as the radioactivity of ^{125}I-labeled human HF (apparent MW: 100,000). These experiments suggest that the size of HF in all mammalian plasmas tested is approximately that of human HF.

Kinetics of inhibition of HF activity of human, bovine, or rat plasma by respective antiserum. The rate of inhibition of human HF activity by anti-human HF serum was very rapid; inhibition was complete in 5 to 10 min at 37° (Fig. 1A). The same degree of inhibi-

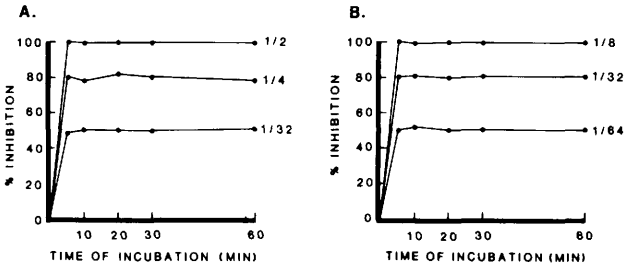

FIG. 1. (A) Kinetics of inhibition of human HF activity by anti-human HF serum. Normal human plasma containing 0.5 U/ml HF activity was incubated with equal volume of various dilutions (1/2, 1/4, or 1/32) of anti-human HF serum at 37°. At intervals, residual HF activity was measured after 20-fold dilution. Ordinate indicates percentage inhibition and abscissa the incubation time in min. (B) Kinetics of inhibition of rat HF activity by anti-rat HF serum. Rat plasma containing 0.5 U/ml HF activity was incubated with equal volume of various dilutions (1/8, 1/32, or 1/64) of anti-rat HF serum at 37°. At intervals, residual HF activity was measured after 20-fold dilution. The results were compared to control experiments run with normal rabbit serum. The ordinate indicates percentage inhibition and the abscissa the incubation time in min.

tion was obtained at 0°. The rate of inhibition of rat HF by anti-rat HF serum was also very rapid; the degree of inhibition by various dilution of antiserum was nonlinear (Fig. 1B). Similar results were obtained with bovine plasma and anti-bovine HF serum. For this reason, the susceptibility of the HF activity of different species to inhibition by these antiserums was measured by determining the dilution of antiserum which produced 50% inhibition of the HF activity in an equal volume of plasma in 30 min at 37°.

The relative susceptibility of HF activity in various mammalian plasmas to inhibition by anti-human HF serum. Anti-human HF serum inhibited the HF activity in human, chimpanzee, gibbon, baboon, and black ape plasma much more efficiently than that in plasmas from nonprimate mammalians (Fig. 2). For example, 50% of HF activity in human plasma was inactivated by a 1:32 dilution of the antiserum, whereas even undiluted antiserum did not inhibit 50% of the HF activity in swine, horse, rat, and hamster plasma. In experiments not illustrated using another lot of antiserum, HF activity in gerbil plasma was reduced approximately four-fifths upon incubation for 16 min in ice.

The relative susceptibility of HF activity in various mammalian plasmas to inhibition by anti-bovine HF antiserum. Fifty percent of HF activity in bovine plasma was neutralized by a 1:46 dilution of anti-bovine HF serum and that in goat, swine, and sheep plasmas was inhibited by 1:15 to 1:19 dilution (Fig. 3). In

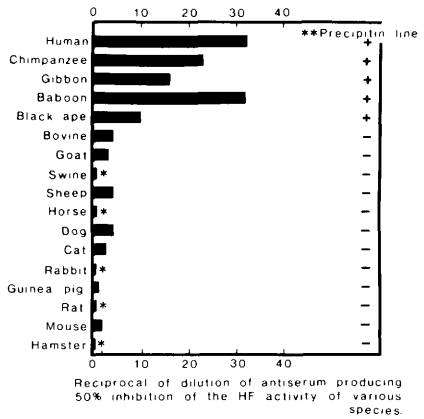

FIG. 2. Effect of anti-human HF upon HF activity in various mammalian plasmas. Each animal plasma was diluted in 1 mg/ml bovine serum albumin to provide 0.5 U/ml HF activity and then was incubated with an equal volume of various dilutions of anti-human HF serum at 37° for 30 min. The dilution of antiserum that produced 50% inhibition of HF activity was determined and the results were expressed as the reciprocal of this dilution. (*) Indicates that even undiluted antiserum did not produce 50% inhibition. (**) Indicates the presence (+) or absence (–) of a precipitin line in immunodiffusion studies.

contrast, HF activity in primates and other mammalian plasmas was much more resistant to inhibition by this antiserum.

The relative susceptibility of HF activity in various mammalian plasmas to inhibition by anti-rat HF serum. A 1:64 dilution of anti-rat HF serum inhibited 50% of HF activity in rat plasma and 1:32 dilution, in mouse plasma

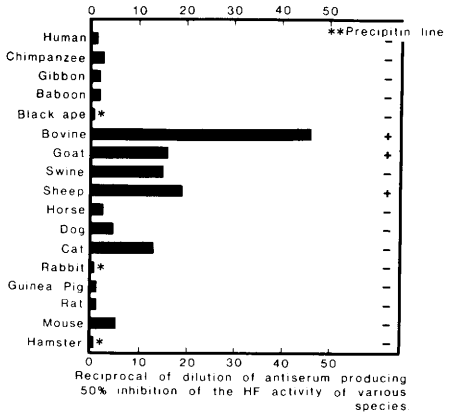

FIG. 3. Effect of anti-bovine HF upon HF activity in various mammalian plasmas. The procedure was identical to that described in Fig. 2, except that anti-bovine HF serum was used.

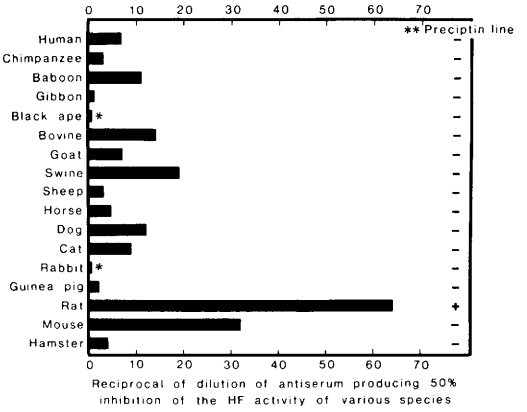

FIG. 4. Effect of anti-rat HF upon HF activity in various mammalian plasmas. The procedure was identical to that used in Fig. 2, except that anti-rat HF serum was used.

(Fig. 4). The HF activity in swine plasma was also relatively sensitive to anti-rat HF serum. The HF activity in other species was much more resistant to this antiserum.

Immunodiffusion studies. Four primate plasmas (chimpanzee, gibbon, baboon, and black ape) formed a precipitin line of complete identity with human plasma against anti-human HF serum. In contrast, plasma of most individuals with Hageman trait and those of all other species tested formed no precipitin line.

Anti-bovine HF serum formed a precipine line with bovine, sheep, and goat plasma, but did not form any line with other plasmas.

Anti-rat HF serum produced a precipitin line only with rat plasma.

Discussion. Blood coagulation involves the action of multiple plasma proteins and plays an important role in hemostasis in animals with closed circulatory systems. Studies in the past 30 years have elucidated the nature and mode of action of human blood-clotting proteins. Comparative studies of plasma coagulation factors in other species, however, are limited (15–17).

Amino acid sequence analysis is the most reliable means of phylogenetic study of proteins, but such analyses have been available for only a few clotting proteins. The evolution of fibrinopeptides regions of fibrinogen has been studied from amino acid sequence analyses (15). Immunological techniques are simple and sensitive methods of comparing the structural similarity of the proteins (18).

The present study compared the HF activity in 18 mammalian species. It is interesting that, upon gel filtration, all HFs tested had approximately the same MW as human HF. The susceptibility of HF activity of different species to inhibition by different antiserums suggested that HF in the same taxonomic order has similar antigenic determinants. Rodent plasmas contained HF activity comparable to that of human plasma, yet, such activity was not inactivated by anti-human HF serum and, upon immunodiffusion, no precipitin line was developed between rodent plasma and anti-human HF serum. These data suggest that HF protein in rodent plasma contains appreciably different amino acid sequences from that in human plasma, but that the functional sites required for the clot-promoting activity are similar. Thus, considerable evolution appears to have taken place in the mammalian HF molecule that is not directly related to its clotting function.

In this regard, it is interesting to note that CRM^+ (cross-reacting material positive) variants of Hageman trait plasmas contain nonfunctional, but immunologically indistinguishable HF from normal HF (19). Thus, the functional site may be altered in these cases, whereas the bulk of the molecule is similar to normal HF.

Wilson *et al.* (18) recently compared the evolutionary changes in amino acid sequences of many polypeptides, and postu-

lated that the rate of protein evolution is partly dependent on "dispensability." The degree of changes in amino acid sequence during evolution appears related to the ability of organisms to function in the absence of a particular protein. Histone, one of the most indispensable proteins, is a very slowly evolving protein in terms of amino acid sequence. This hypothesis may be applied to clotting factors. Immunological studies of antihemophilic factor (AHF, factor VIII) and factor XIII (fibrin-stabilizing factor) have shown that rabbit antiserums to human AHF and factor XIII formed a precipitin line with all mammalian plasmas tested (16, 17), suggesting that all mammalian AHF or factor XIII share many common antigenic determinants. These observations are in contrast to the present study of HF. Thus, HF seems to have evolved more rapidly than AHF and factor XIII. These data are consistent with the fact that deficiency of HF, unlike those of AHF or factor XIII, is not associated with bleeding tendency.

Summary. The HF (factor XII) activity in plasmas of 18 mammalian species has been studied by immunological means and gel filtration. All HFs had the same approximate size as human HF. Primate HF activity was most susceptible to inhibition by antihuman HF serum, ungulate HF activity by antibovine HF serum, and rodent HF activity by anti-rat HF serum. Immunodiffusion studies were consistent with these inhibition studies. These data suggest that mammalian HFs in the same taxonomic order share common antigenic sites.

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