Immunologic Relationships of Antihemophilic Factor of Different Species Detected by Specific Human and Rabbit Antibodies[1] (37395)

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(Introduced by Oscar D. Ratnoff)

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Patek and Taylor (1) demonstrated that sheep, rabbit, ox, and monkey plasma contained agents correcting the clotting defect of human hemophilic plasma. Antihemophilic factor (AHF, Factor VIII) has been demonstrated in the plasma of other mammalian species (2, 3), and Biggs and her colleagues (4, 5) have used heterologous AHF extensively in the treatment of hemophilic patients. Heterologous AHF is probably most useful in those patients whose plasmas contain circulating anticoagulants directed against AHF, both because larger quantities of AHF can be made available from animal sources and because animal AHF may be less susceptible than human AHF to inhibition by these human antibodies.

The present report deals with the relative susceptibility of the AHF of several animal species to inhibition by human circulating anticoagulants; among those tested, porcine plasma appeared to be most resistant to inhibition. Additionally, rabbit antiserum directed against human AHF was found to inhibit the AHF of all species tested except the rabbit. Using immuno-diffusion techniques, identity was demonstrated between human and primate AHF, and partial identity between human and nonprimate mammalian AHF.

Materials and Methods. Techniques for the purification of normal human AHF, by precipitation with ethanol at −3°, and with polyethylene glycol followed by filtration through columns of Sepharose 4B, and the preparation of antiserum to this AHF in white New Zealand rabbits, rendered monospecific by appropriate absorption, have been described (6). Rabbit sera were incubated at 60° for one hour and, after cooling, adsorbed with tricalcium phosphate (10 mg/ml plasma) to remove clot-promoting activity which might interfere with the clotting assays. The antigen detected by this antiserum is present in normal quantities in human hemophilic plasma and in reduced amounts in that of patients with von Willebrand's disease (6–10).

Human anticoagulants directed against AHF were obtained from seven patients. Five of these individuals (including patient A) developed the anticoagulant as a complication of classic hemophilia. The sixth (patient B) acquired the anticoagulant as a complication of an otherwise normal pregnancy. In the seventh patient, a 69-year-old man with rheumatic heart disease, the anticoagulant appeared after he had received penicillin daily for a period of two years.

Double diffusion studies were carried out on 3×2-inch glass slides in 0.9% agarose gels in barbital buffer (0.05 *M* sodium barbital, pH 8.4). Plasma samples were applied to the wells at a distance of 3 mm from the troughs which were filled with 0.2-ml aliquots of rabbit antiserum. Precipitin lines were allowed to develop for 36 hr at 4°.

Immunoelectrophoresis was carried out on 3×1-inch glass slides in 0.9% agarose gels

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in barbital buffer. Ten λ samples of ethanol concentrates of plasma were prepared (6) and placed in the wells; 225 V was applied to the slides for one hour, 0.2-ml volumes of the antiserum were placed in the troughs, 5 mm from the wells and the precipitin lines allowed to develop over a period of 36 hr at 4°.

Pooled normal human plasma was prepared from blood to which one-fiftieth volume of sodium citrate (pH 5.0, 0.5 *M* with respect to citrate) was added (6). Animal plasmas were obtained from a variety of sources, (Colorado Serum Co., Denver, Colorado; Pel-Freez Biologicals Inc., Rogers, Arkansas; or directly from some of the mammalian species studied), and had been separated from blood to which one-tenth volume of 0.13 *M* sodium citrate had been added.

Assays of AHF clot-promoting activity were performed by a modification of the partial thromboplastin time, using plasma from a known hemophilic patient as substrate (11). One unit of AHF clot-promoting activity is equivalent to that present in 1 ml normal pooled *human* plasma (11).

The rate of the reaction between the rabbit antiserum to human AHF was examined by incubating pooled normal human plasma at 37° with equal volumes of various dilutions of the antiserum in barbital–saline buffer (0.025 *M* sodium barbital, 0.125 *M* NaCl, pH 7.5). At intervals, residual AHF was measured. The influence of the quantity of antiserum on the inhibition produced was examined in a similar manner by incubating pooled normal human plasma with various dilutions of the antiserum for a standard period of 4 hr at 37° and measuring residual AHF. In both these experiments, control estimations were made of the activity of pooled normal human plasma incubated at 37° with treated normal rabbit serum for appropriate times, allowing correction for the natural decline in AHF clot-promoting activity during the experiment.

Results. 1. Levels of AHF clot-promoting activity in the plasmas of different species. The plasmas of all mammalian species tested contained agents correcting the defect in human hemophilic plasma (Table I). The units employed relate the activity found in the animal plasmas to that present in one ml of pooled normal human plasma as indicated in the Methods section. Almost no AHF-like material was found in the specimens of avian or reptilian plasma studied.

TABLE I. AHF Clot-Promoting Activity in the Plasmas of Various Species.

Species	AHF activity (units/ml)
Human[a]	1.0
Rhesus monkey	1.3
Chimpanzee	0.7
Baboon	1.1
Pig[a]	5.0
Cow[a]	1.7
Goat	6.0
Horse[a]	0.6
Sheep[a]	3.2
Dog[a]	3.2
Guinea pig[a]	4.5
Rabbit[a]	4.8
Hamster[a]	1.5
Porpoise	6.5
Killer whale	3.0
Chicken[a]	0.0
Pigeon[a]	0.0
Turtle[a]	0.0

[a] Pooled plasma specimens. All other values were derived from studies of a single animal.

2. Immunologic studies employing double diffusion. Lines of identity appeared on double diffusion against the rabbit antiserum between human and primate (monkey and baboon) plasmas (Fig. 1). The plasmas of the other mammalian species tested, except for the rabbit, produced precipitation lines against the rabbit antiserum, showing partial identity with human AHF. Typical spurring occurred between the precipitation lines of human and mammalian nonprimate plasmas. No precipitation lines were obtained when the avian or reptilian plasmas were tested against the rabbit antiserum to human AHF.

3. Immunoelectrophoresis of human and mammalian plasmas. Little difference was observed between the mobility of human, monkey, baboon, equine and bovine plasmas under the conditions employed.

4. Kinetics of inhibition of AHF clot-promoting activity by the rabbit antiserum to

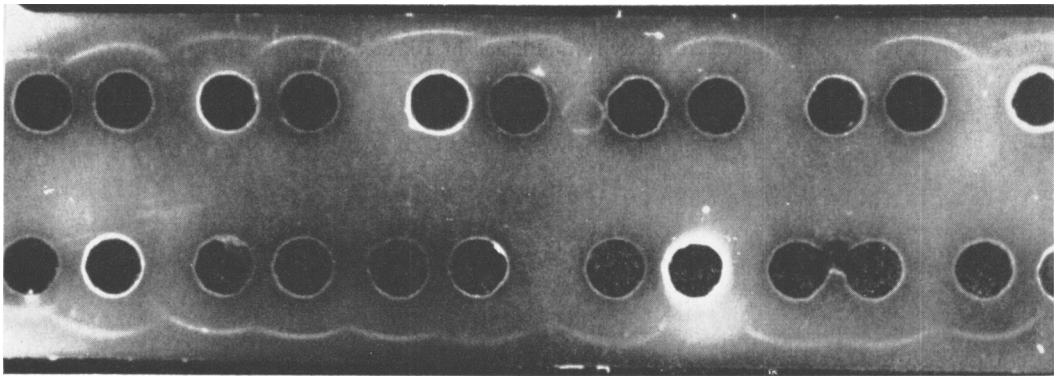

FIG. 1. Double diffusion of the plasmas of various species (wells) against the rabbit antiserum to AHF (troughs). Reading from left to right the upper row of wells contained the following plasmas: horse, human, cow, human, killer-whale, human, turtle, human, pigeon, human and porpoise. The lower row of wells contained plasmas of human, pig, human, baboon, human, monkey, human, guinea pig, human, human and human. Failure to demonstrate a precipitin line with the guinea pig plasma in this slide appeared to be an artifact as a precipitin line was observed between guinea pig plasma and the rabbit antiserum on other slides in which the distance between the wells and the troughs was greater than that normally used.

human AHF. Inhibition of AHF clot-promoting activity by rabbit antiserum was a progressive reaction, requiring 2–4 hr to reach completion (Fig. 2). Similar rates of reaction were observed between normal human AHF and the circulating human anticoagulants, as described earlier. The inhibition produced in 4 hr at 37° by the rabbit antiserum and two of the human anticoagulants studied was nonlinear (Table II). For this reason, susceptibility of the AHF of different species to inhibition by the antiserum or anticoagulants was measured by determining the dilution of antiserum which produced 50% inhibition of AHF activity in an equal volume of plasma after completion of their interaction, *i.e.*, after incubation at 37° for four hours. On six occasions such estimations were performed on a plasma specimen on two consecutive days; on each occasion, the amount of antiserum or anticoagulant producing 50% inhibition of the AHF activity of the plasma differed by no

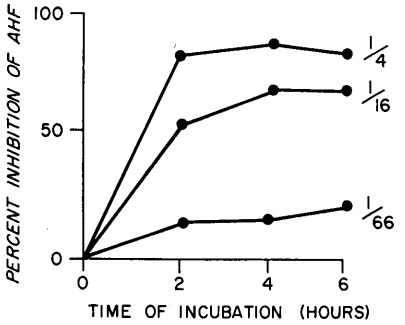

FIG. 2. Influence of the time of incubation of the rabbit antiserum to AHF with pooled normal human plasma on the inhibition of AHF clot-promoting activity. Antiserum used in this experiment came from a different rabbit from that used in the experiments in Tables II and III. The dilution of the antiserum used is indicated at the right end of each line. All values are corrected for deterioration of AHF activity in the control plasma during the incubation period.

TABLE II. Inhibition of Human AHF by Various Dilutions of Rabbit Antiserum to Human AHF and Human Anticoagulants to AHF.

Agent	Dilution of agent	% AHF inhibited in 4 hr
Rabbit Antiserum	1/40	80
	1/80	75
	1/160	60
	1/320	50
Human Anticoagulant A	1/40	60
	1/80	50
	1/160	30
Human Anticoagulant B	1/640	75
	1/1280	50
	1/2560	40

TABLE III. Dilution of Rabbit Antiserum or Human Anticoagulant to AHF Producing 50% Inhibition of the AHF Activity of Plasma of Different Species in 4 Hr.

Source of plasma	Rabbit antiserum	Human plasma A	Human plasma B	Precipitin line with rabbit antiserum
Mammal				
Human	1/320	1/80	1/2560	+
Rabbit	no inhibition at dilutions below 1/5	1/10	1/40	—
Pig	1/10	1/5	1/20	+
Cow	1/20	1/40	1/160	+
Horse	1/40	1/40	1/320	+
Sheep	1/20	1/10	1/160	+
Dog	1/10		1/640	+
Guinea pig	1/10	1/40	1/320	+
Monkey	1/10	1/40	1/640	+
Killer whale	1/5		1/640	+
Porpoise	1/10		1/320	+
Reptile				
Turtle	Only trace amounts of procoagulant activity present correcting the defect in human hemophilic plasma.			—
Bird				
Pigeon, Chicken	Only trace amounts of procoagulant activity present correcting the defect in human hemophilic plasma.			—

more than one doubling dilution.

5. Inhibition of the AHF clot-promoting activity of the animal plasmas by rabbit antiserum to AHF and by human anticoagulants to AHF. Human anticoagulants to AHF inhibited the clot-promoting activity present in the plasmas of all the mammalian species studied (Table III). Similar results were obtained using rabbit antiserum, with the exception of rabbit plasma. Each agent was a more efficient inhibitor of human plasma than of any of the animal plasmas tested. One human anticoagulant (B) was more powerful than the rabbit antiserum, and the other (A) was slightly more active against human AHF, but less active against some of the mammalian plasmas than was the rabbit antiserum. Porcine AHF was the most resistant of those tested to the inhibitory effect of all three agents in the test system used in these experiments; this observation was confirmed on three additional different specimens of swine plasma in experiments not included in the table.

6. Relative susceptibility of human, bovine and porcine AHF to inhibition. In order to obtain a more valid comparison of the susceptibility of human and animal plasmas to inhibition by human anticoagulants, their effect was tested upon plasmas diluted to the same AHF clot-promoting activity as normal pooled human plasma by appropriate dilution in barbital–saline buffer. The inhibition of these preparations by six different human anticoagulants to AHF was then measured by the technique described. Porcine AHF was more resistant to inhibition by each of the human anticoagulants than was bovine AHF clot-promoting activity (Table IV).

Discussion. The present study confirms the immunologic relationship between human AHF and that of other mammalian species, through the use of a specific precipitating rabbit antiserum directed against human AHF. Like human circulating anticoagulants, which do not precipitate AHF, the rabbit antiserum blocked the functional AHF activity in a wide variety of mammalian plasmas; rabbit AHF, as might be expected, was the exception.

Previous workers have shown that human

TABLE IV. Dilution of Six Different Human Anticoagulants to AHF Producing 50% Inhibition of AHF Activity of Human, Porcine, and Bovine Plasma in 4 Hr.

Patient no.	Dilution of anticoagulant plasma inducing 50% inhibition of plasma AHF activity in 4 hr at 37°		
	Swine plasma	Bovine plasma	Human plasma
1[a]	1/40	1/80	1/640
2[a]	undiluted plasma	1/2	1/20
3[a]	1/4	1/20	1/40
4[b]	1/40	1/160	1/2560
5[a]	1/4	1/20	1/80
6[a]	1/8	1/20	1/40

[a] Patients with classic hemophilia with a circulating anticoagulant.

[b] Patient with circulating anticoagulant post partum.

circulating anticoagulants directed against AHF vary in their capacity to inhibit human and animal AHF (13–25). The observations now reported confirm this variation and the similar variation of the inhibition of human and animal AHF by specific rabbit antiserum to human AHF (26). A constant proportionality of inhibition of the different animal plasmas did not exist between the three agents studied (Table III).

In situations in which powerful circulating anticoagulants have developed, administration of AHF concentrates is often withheld unless life-threatening bleeding occurs or surgery becomes essential, as infusion of these materials may stimulate the appearance of even greater amounts of anticoagulant in the blood. In such cases, concentrates of animal AHF, more resistant than that of humans to inhibition by the anticoagulants, have proved useful in critical situations. Although the plasmas of the mammals we have tested, with the exception of primates, contained AHF which differed antigenically from that of human plasma, as tested with rabbit antiserum, it seems likely that AHF preparations from all nonprimate mammals will prove antigenic in humans. Evidence in support of this is found in the clinical studies of Biggs and her group (13).

Bidwell (13) recorded that wide variability was observed in the ability of different human anticoagulants to AHF to inhibit the AHF activity derived from different species and described a patient whose anticoagulant inhibited bovine AHF more readily than porcine AHF. Denson (16) reported experience with anticoagulants which had developed in hemophilics treated with human AHF concentrates only; four had similar inhibitory activity against bovine and porcine AHF and two were more active against the bovine preparation; anticoagulants detected in four patients who had received human, bovine and porcine AHF concentrates were judged to have no selectivity for any particular type of AHF. In these and other reported studies, it is not clear whether the concentrations of AHF in the different animal preparations were equated. Though it is difficult to generalize from the small number of observations on record, the observations reported here would suggest that porcine preparations might be expected to enjoy an advantage over bovine in that they may prove more resistant to the inhibitory effect of human anticoagulants to AHF.

The presence of AHF, functional as a procoagulant and detectable as antigen, in mammalian plasma, is in keeping with the suggestion that mammalian hemostasis is dependent in part on a system similar to the human intrinsic clotting mechanism (27). The failure to demonstrate AHF-like antigen or AHF-like clot-promoting activity in the plasmas of reptilian or avian species is similarly in accord with the suggestion that the intrinsic clotting mechanism is poorly developed in these classes of animals (28, 29).

Summary. The plasmas of 14 nonhuman mammalian species corrected the specific clotting defect in human hemophilic plasma. The antihemophilic factor (AHF, Factor VIII) activity in all these plasmas was inhibited by human circulating anticoagulants directed against AHF and, except for rabbit AHF, by rabbit antiserum to purified normal human AHF, but human AHF was more readily inhibited than animal plasmas by both these agents. Porcine AHF was more resistant than bovine AHF to inhibition by all of 6 human circulating anticoagulants.

In immuno-precipitation studies using rabbit antiserum, primate plasmas gave reactions of identity with human plasma, while non-primate mammalian plasmas gave reactions of partial identity. Plasma from a reptile and from two avian species contained no detectable antigen or clot-promoting properties related to human AHF.

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