

## Humoral Factor that Specifically Regulates Factor X Levels in Rabbits (Coagulopoietin-X)

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**ABSTRACT** A heat-stable humoral substance (coagulopoietin-X) is present in rabbits partially depleted of Factor X, which is capable of raising Factor X levels when injected into recipient rabbits. Rabbits were partially depleted of Factor X by slow infusion of a globulin fraction of goat anti-rabbit Factor X antibody. This resulted in the reduction of Factor X to 40–50% of normal at 1 h and 60–70% of normal at 6 h. No effect was noted on levels of Factors II, V, or VII.

Plasma from these animals, when injected into 10 recipients, specifically raised Factor X levels when measured by four different assays: one-stage assay with bovine VII- and X-deficient plasma and Russell's viper venom; one-stage assay with human X-deficient plasma and thromboplastin; chromogenic substrate assay with Russell's viper venom; and an immunologic assay (Laurell technique). No rise was noted in two control experiments in which normal plasma was injected into recipient rabbits from 2 rabbits injected with a globulin fraction of normal goat serum, nor in 12 rabbits injected with plasma from normal rabbits, nor in 5 rabbits injected with boiled plasma from normal rabbits. The rise in biologic activity of 120–150% of base line was significantly greater than the rise in immunologic activity of 114–117% of base line ( $P < 0.05$ ) on 3 different days, suggesting the production of a molecule with greater specific activity rather than increased protein synthesis.

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This work was presented in part at the Eighth Symposium on Vitamin K Metabolism and Vitamin K-dependent Proteins, University of Wisconsin, Madison, Wis., 13 June 1979.

Received for publication 2 July 1979 and in revised form 17 September 1979.

### INTRODUCTION

Little is known concerning the mechanisms that regulate constant levels of coagulation factors in a species as well as its individual members. Previous studies from our laboratory have suggested that humoral factors (coagulopoietins) participate in maintaining these levels. These studies demonstrated the presence of a humoral factor or factors in plasma from Coumadin (Endo Laboratories, Inc., Garden City, N. Y.)-treated rabbits that was capable of elevating vitamin K-dependent coagulation factors when injected into recipients (1, 2). A similar factor or factors was noted in the plasma of animals that had undergone disseminated intravascular coagulation (3). We have recently reported on the presence of a coagulopoietin-II, which specifically regulates a single coagulation factor, prothrombin (Factor II), by enhancing biologic activity (4–6). Coagulopoietin-II was shown to be heat stable, was retained on a G-50 Sephadex column, and stimulated a liver mince system to enhance prothrombin activity.

We now report on the presence of a heat-stable humoral factor that regulates the biologic activity of Factor X (coagulopoietin-X). Rabbits were depleted of Factor X with a goat anti-rabbit Factor X antibody. Plasma from these rabbits, when injected into recipients, specifically elevates Factor X levels.

### METHODS

*Collection of blood samples for factor assays.* Blood was collected from the lateral ear vein with a 23-gauge needle (0.9 ml in 0.1 ml anticoagulant containing 3.8% sodium citrate in molar epsilon aminocaproic acid). Platelet-poor plasma was obtained by centrifugation at 2,000 g for 15 min at 4°C and stored in portions at –30°C.

**Factor assays.** Factors II, V, VII, and X were measured by one-stage techniques as reported (2). In all assays at least four dilutions of each sample were tested and clotting times plotted against dilutions on double log graph paper. The mean value of three base-line samples was designated 100% for that animal and the ensuing samples expressed in relation to this value. The SD for the assays were  $\pm 9\%$  for Factor II,  $\pm 10\%$  for Factor V,  $\pm 9\%$  for Factor VII, and  $\pm 12\%$  for Factor X. Factor X was also measured with human X-deficient plasma and thromboplastin; a chromogenic substrate (7); and immunologically, by the Laurell technique (8). The synthetic substrate assay was performed in duplicate at two different dilutions, employing a Honeywell recorder (Honeywell Inc., Minneapolis, Minn.) coupled to a Gilford spectrophotometer (Gilford Instruments Laboratories Inc., Oberlin, Ohio) with cuvettes equilibrated at  $37^{\circ}\text{C}$ . The Laurell technique was employed at four different dilutions of each plasma sample. The SD was  $\pm 5\%$ .

**Preparation of goat anti-rabbit Factor X antibody.** Rabbit Factor X was purified by modifications of the method of Bajaj and Mann (19), employing barium citrate adsorption and elution, DE52-cellulose chromatography, and modified to include dextran sulfate-Sepharose affinity chromatography (10), and blue dextran-Sepharose affinity chromatography (11). The material gave one visible band when reduced on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. It contained 19,000 U/mg Factor X (1 ml of plasma contains 1,000 U) and  $<1\%$  Factors II, V, and VII. This material was employed to raise an antibody in a goat. This anti-rabbit Factor X antibody gave one line on immunodiffusion at a rabbit plasma dilution of 1:16. The antibody, when mixed 1:1 with rabbit plasma, removed Factor X activity at an antibody dilution of 1:20, whereas it had no effect on Factors II, V, or VII activity.

**Preparation of coagulopoietin-X.** A crude globulin fraction of heat-inactivated ( $56^{\circ}\text{C}$  for 30 min) antiserum was prepared by precipitation with 50% saturated ammonium sulfate, reconstitution of the precipitate in a volume of buffer equal to the original serum volume, and dialysis against the same buffer (0.01 M phosphate-buffered saline, pH 7.4). The globulin fraction was centrifuged at 100,000  $g$  for 1 h to remove immune complexes, filtered through a  $0.45\text{-}\mu\text{m}$  millipore filter (Nalge Co., Nalgene Labware Div., Rochester, N. Y.) and infused into the lateral ear vein of a rabbit (13 ml/kg) over a 1-h period. After 1 h the animal's Factor X level was  $\sim 40\text{--}50\%$  of base line, and at 6 h it was  $60\text{--}70\%$  of base line. Factors V, VII, and prothrombin were not significantly affected. These animals, as well as control animals, were injected with heparin, 100 U/kg and epsilon aminocaproic acid, 70 mg/kg, 15 min before exsanguination. The animals were exsanguinated by arterial puncture 6 h after injection and their plasma separated and stored as described (2).

**Injection of coagulopoietin-X.** Donor plasma from rabbits partially depleted of Factor X was injected into normal recipient rabbits (New Zealand white rabbits, 2–3 kg) to study its effect on Factor X levels. Base-line samples were collected from each recipient on 3 separate days, as described (2), and the plasma frozen and stored at  $-30^{\circ}\text{C}$ . Donor plasma (3 ml) was then injected twice daily (at 9 a.m. and 5 p.m.) for 4 d (days 1–4) and blood samples collected at 12 noon and stored as above on days 2–5).

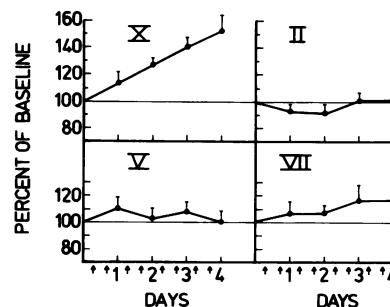
**Materials.** Rabbit plasma, for purification of Factor X, was obtained from Hobbs Pocono Rabbit Farm, Canadensis, Pa. DE52-cellulose was obtained from Whatman Chemicals, Div. W&R Balston, Maidstone, Kent, England. Sepharose CL4B, dextran sulfate, and blue dextran were obtained from Pharmacia Fine Chemicals, Piscataway, N. J. Cyanogen bromide was obtained from Pierce Chemical Co., Rockford, Ill. Factor-

deficient plasmas were obtained from George King Biologicals, Overland Park, Kans. Simplastin was obtained from General Diagnostics, Warner Lambert Co., Morris Plains, N. J. Russell's viper venom in cephalin, and bovine VII- and X-deficient plasma were obtained from Sigma Chemical Co., St. Louis, Mo. Chromogenic substrate S2222 was obtained from Ortho Pharmaceutical Corp, Raritan, N.J. Russell's viper venom was obtained from The Wellcome Foundation, Ltd., The Wellcome Research Laboratories, Beckenham, Kent, England. Sodium dodecyl sulfate, acrylamide,  $N,N'$ -methylene bisacrylamide,  $N,N,N',N'$ -tetramethylethylenediamine, and Coomassie Blue were obtained from Bio-Rad Laboratories, Richmond, Calif.

## RESULTS

Fig. 1 demonstrates the effect of injection of donor plasma, obtained from rabbits partially depleted of Factor X, into 10 recipient rabbits. Factor X rose linearly above the average of three base-line values to a maximum of 153% on day 4 (measured by Russell's viper venom and VII- and X-deficient plasma). Factors II, V, and VII did not rise significantly above base-line values (defined as an elevation of  $>2$  SD on 2 consecutive days [2]). Control experiments in which two donor rabbits were injected with a crude globulin fraction of normal goat serum; as well as 12 experiments in which normal plasma was injected into recipient rabbits (2–5) revealed no significant changes in Factors II, V, VII, or X.

Fig. 2 demonstrates the measurement of Factor X activity by four different methods. The assay employing human Factor X-deficient plasma and thromboplastin gave results that were almost identical with the assay employing the chromogenic substrate S2222. Activated Factor X could not be detected after incubation of various dilutions of rabbit plasma with the chromogenic substrate in the absence of Russell's viper



**FIGURE 1** Effect of donor plasma from a rabbit partially depleted of Factor X on coagulation factor levels in 10 recipient rabbits. Donor rabbits were infused with globulin fractions of goat anti-rabbit Factor X (13 ml/kg) over a 1-h period. The donor animal was bled at 6 h and the plasma separated and frozen. 3 ml of donor plasma was injected intravenously, twice daily (arrows) for 4 d and samples collected at midday on 4 different days. These were assayed for coagulation factor activity and were expressed as percentage of the average of three base-line samples collected on 3 or more days before infusion of the experimental plasma. SEM is given.

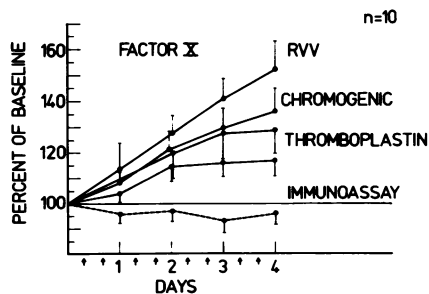


FIGURE 2 Assay of Factor X by four different methods in 10 different rabbits. The separated plasma samples were frozen in portions at  $-30^{\circ}\text{C}$ . These were assayed with bovine Factor VII- and X-deficient plasma and Russell's viper venom (RVV); human X-deficient plasma and thromboplastin; chromogenic substrate; and immunoassay (Laurell technique). The differences between the first three assays were not statistically significant. The differences between the immunoassay and these three assays were significant on days 2 ( $P < 0.05$ ), 3 ( $P < 0.01$ ), and 4 ( $P < 0.05$ ). Control samples for the immunoassay were obtained from 10 rabbits injected with normal plasma. Differences between control (---) and experimental immunoassays were statistically significant on days 2 ( $P < 0.05$ ), 3 ( $P < 0.01$ ), and 4 ( $P < 0.05$ ). SEM is given.

venom (two experiments). Results obtained with bovine VII- and X-deficient plasma and Russell's viper venom were not significantly greater than results obtained with either the thromboplastin or chromogenic assay ( $P > 0.1$ , Student's  $t$  test). The immunologic assay increased to 117% of base line on day 4. Differences between control samples (10 animals injected with a normal globulin fraction) and experimental samples were significant on day 2 ( $P < 0.05$ ), day 3 ( $P < 0.01$ ), and day 4 ( $P < 0.05$ ). Differences between the immunoassay and the three other assays were significant on day 2 ( $P < 0.05$ ), day 3 ( $P < 0.01$ ), and day 4 ( $P < 0.05$ ).

Fig. 3 demonstrates the effect of boiling (20 min at

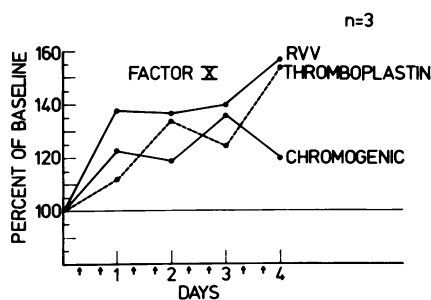


FIGURE 3 Effect of boiled experimental plasma on Factor X activity in recipient rabbits. Three different coagulopietin-X plasma samples were placed in a boiling water bath for 20 min. The denatured protein was discarded after centrifugation and the supernate injected, as in Fig. 1, into three different animals. The average values are given for the measurement of Factor X activity by three different methods: bovine Factor VII- and X-deficient plasma and Russell's viper venom (RVV); human X-deficient plasma and thromboplastin; and chromogenic substrate.

pH 6.8) of donor plasma followed by injection of the supernate into three different animals. Factor X activity was assayed by three different methods. Coagulopietin-X activity was stable to boiling. Four control experiments, in which normal boiled rabbit plasma was injected into recipients, showed no effect on Factors II, V, VII, or X activity (5). As an additional control, normal rabbit plasma was incubated with sufficient goat anti-rabbit Factor X antibody to lower biologic Factor X activity to 45% of base line. When this material was boiled, as above, and the supernate injected twice daily for 4 d, no change in Factor X level was noted.

## DISCUSSION

The present data extend our previous observations on the presence of humoral substances regulating the levels of coagulation factors (1-6). In our initial report (1-2), we noted the presence of a humoral factor or factors in the plasma of Coumadin-treated rabbits that was capable of elevating vitamin K-dependent coagulation factors when injected into recipients (1, 2). This could not have been caused by the injection of decarboxy precursors that require vitamin K for carboxylation (12) since the rabbit, unlike the human, does not have these precursors in the circulation (13). These observations have recently been confirmed by Shah and coworkers (14) who employed vitamin K-deficient rats (induced by diet).

Data obtained from the plasma of rabbits subjected to disseminated intravascular coagulation also suggested the presence of humoral substances capable of regulating coagulation factors (3). The increased activity of coagulation factors in recipient animals could not have been derived from activated coagulation factors generated in the donors, because activated factors are rapidly removed from the circulation, probably within minutes (15, 16); and the coagulation factors in the recipients were still elevated 3 d after the last injection of plasma.

Our present data demonstrate the presence of a heat-stable humoral factor that is capable of specifically raising Factor X biologic activity in recipient rabbits. The relatively smaller rise in immunologic activity suggests an enhancement of specific activity for Factor X.

Similar results have recently been reported for a coagulopietin-II that is also heat-stable and which specifically elevates the biologic activity of prothrombin (4, 5). Recent observations on immunologic prothrombin also reveal a relatively smaller rise in activity compared to biologic activity (6), suggesting an enhancement of specific activity for prothrombin. Other studies (4, 5) revealed that coagulopietin-II was capable of stimulating an *in vitro* liver mince system to enhance biologic activity (two-stage prothrombin technique) at 16 and 19 h of sterile incubation. Coag-

ulopoiectin-II did not appear to operate via protein synthesis (6) inasmuch as cycloheximide, which inhibited control prothrombin activity, did not inhibit the enhanced biologic activity induced by coagulopoiectin-II. Shah and coworkers (14) have recently reported similar observations on the enhancement of biologic prothrombin activity in the absence of enhanced immunologic activity.

The following working hypothesis is proposed for the mechanism of action of coagulopoiectins-II and -X: these humoral substances appear to enhance biologic activity by altering the coagulation factor molecule. This could result from complete carboxylation of a partially carboxylated molecule; or from some conformational change in the molecule which renders it more active. This could explain enhanced biologic activity in the absence of a concordant increase in immunologic activity. The small rise in immunologic activity could result from enhanced release of a fully carboxylated or "activated" molecule. This hypothesis is dependent upon the presence of more than one species of prothrombin or Factor X in the rabbit. Such a precedent has been noted in warfarin-treated humans, where the released vitamin K-dependent coagulation factors were heterogeneously carboxylated (17).

#### ACKNOWLEDGMENTS

The expert technical assistance of Karen Moroch and Ray-Jen Chang is gratefully acknowledged. We thank Bea Grofe for the illustrations.

Supported by grants National Heart, Lung, and Blood Institute 13336-10 and National Science Foundation PCM 7918282.

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