CELLULAR STUDY OF DRUG ALTERATION OF PROTHROMBIN SYNTHESIS

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ESSENTIALLY nothing is known about the mechanism of action of dicoumerol or vitamin K_1 in the modification of prothrombin formation, even though an extensive literature exists on the experimental and clinical manipulations of prothrombin levels. This is because most methods are not sufficiently discriminating to permit definitive conclusions. For a cellular evaluation of the function of coumadin and vitamin K_1 an immunohistochemical procedure, the fluorescent antibody technique was employed. The dynamic aspects of prothrombin synthesis were studied in dogs using drugs which alter the rate of prothrombin production. Liver biopsies and blood samples were taken at intervals for a correlated study of liver cell function and circulating coagulation factors. These data support the interpretation that normally liver parenchymal cells synthesize prothrombin in a cyclic asynchronous manner. Dicoumarol derivatives halt prothrombin synthesis, while vitamin K_1 stimulates synchronized activity of all the liver parenchymal cells.

The cellular evaluation of liver function required the preparation of a specific fluorescent anti-prothrombin, so that only those cells containing prothrombin or a close relative would be marked. Accordingly prothrombin of high activity (30,000 U/mg tyrosine) was purified from canine plasma[1]. Rabbits were injected and produced potent anti-prothrombin sera. The specificity of these antisera was established using Ouchterlony agar gel diffusion plates[2]. A single precipitin line formed between the anti-prothrombin serum and several different concentrations of the purified dog prothrombin used to induce antibody formation. The precipitin band also identified with the prothrombin band of plasma (Fig. 1). From such a specific anti-dog prothrombin serum the gamma globulin or antibody fraction was isolated using Rivanol. Then the concentrated anti-dog prothrombin was conjugated with a fluorescent dye, either rhodamine sulfonyl chloride or fluorescein isothiocyanate[3]. The specificity of the product was established using various techniques of adsorption and blocking (Table I). This fluorescent antiprothrombin

TABLE I

Specificity of rhodamine anti-dog prothrombin
(dye-protein ratio 26×10^{-3}, 3 mg protein/ml., 80 μg dye/ml.)

Experimental condition	Staining intensity (relative)
R-antiprothrombin (dilute, O.D. = 0·150)	
I. Dog liver	++++
II. Dog pancreas	0
III. Dog bone marrow	0
IV. Dog spleen	0
V. Dog lymph node	0
VI. Beef liver	0
VII. Human liver	0
Adsorbed with	
I. Mouse liver powder 1×	+++
II. Dog liver powder 1×	+++
III. Dog prothrombin, purified (1-2 mg/ml)	+
Pre-treatment of dog liver	
I. Unconjugated specific antiserum	
(a). 5-10 min	+++
(b). 30 min	+
II. Unconjugated normal γ-globulin	++++
Other controls	
I. Free rhodamine (O.D. = ·150)	+
II. R-normal γ-globulin (O.D. = ·150)	+
III. Unconjugated antiserum	0

was used as the fluorescent marker of those cells containing adequate concentrations of prothrombin.

Application of fluorescent anti-prothrombin to either imprints, or frozen sections of normal dog liver resulted in fluorescence of about 10% of the liver parenchymal cell population (Fig. 2A) Kupffer cells and other cell types of the liver did not exhibit fluorescence different from autofluorescence. Specific staining did not occur when fluorescent anti-prothrombin was applied to bone marrow smears, or to spleen, lymph node or pancreas sections. These results clearly indicate that it is the liver parenchymal cell and not the reticulo-endothelial cell that is responsible for prothrombin synthesis under normal circumstances.

Knowing then that the cells actively engaged in prothrombin synthesis could be marked and identified, the fluorescent antibody technique was used to study how coumarin drugs and vitamin K affected prothrombin synthesis. One may speculate from information in the literature that dicoumarol and various relatives that decrease the circulating levels of prothrombin might act by halting biosynthesis of

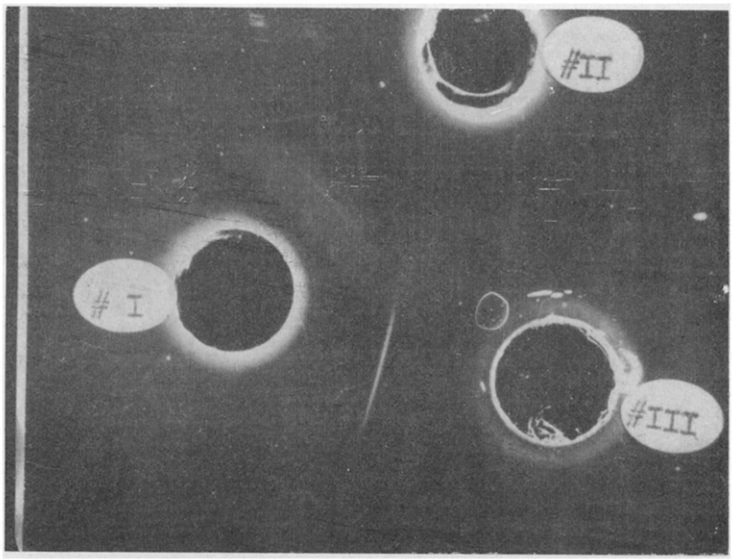

FIG. 1. Agar gel diffusion pattern of components of anti-dog prothrombin serum (I) reacted with fresh whole dog plasma (II) and purified dog prothrombin (III). Note the single antigenic component (prothrombin) indicated by the precipitin line between wells I and III, which identifies with the sharp line of the wide precipitin band between wells I and II. The other cross-reacting components of the plasma are probably circulating prothrombin derivatives.

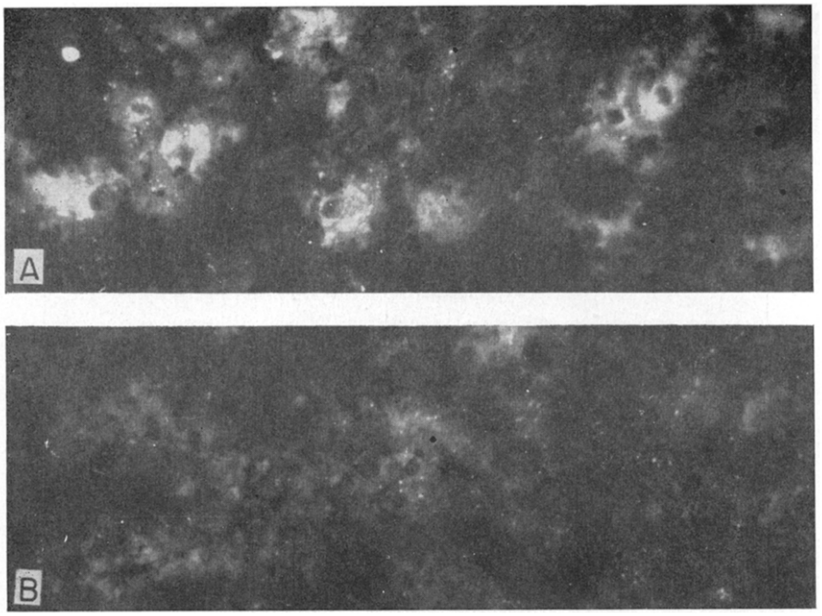

FIG. 2. Photomicrographs of frozen sections of dog liver treated with fluorescein isothiocyanate anti-dog prothrombin. The brightest, whitest parenchymal cells represent specific staining of prothrombin. The small light granules are naturally fluorescing lipofuscin. Mag. × 120. Tri-X film, exposure 3 min. A. Normal dog liver. B. Prothrombin deficient liver following coumadin treatment.

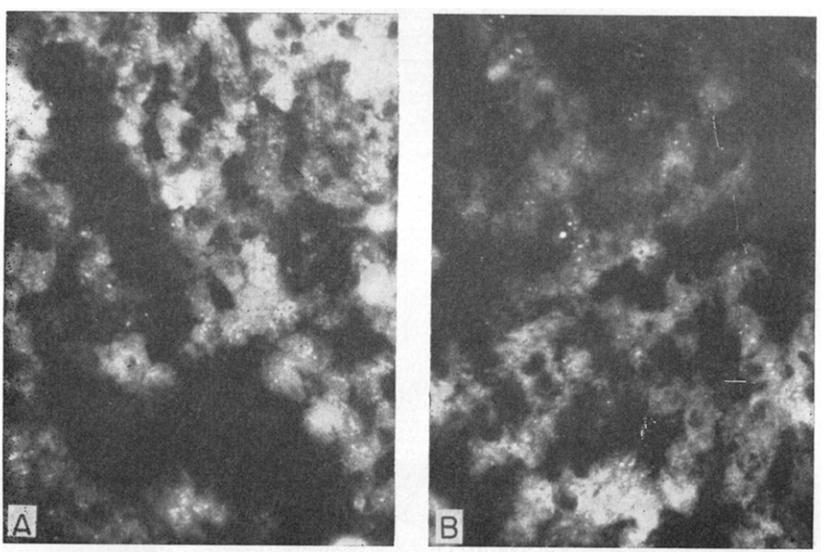

FIG. 3. Photomicrographs of frozen sections of dog liver treated with fluorescein isothiocyanate anti-dog prothrombin. Recovery from hypoprothrombinemia following coumadin. Mag. × 120. Tri-X film, exposure 3 min. A. Four hours after vitamin K_1 administration. About 70% of parenchymal cells fluoresce brilliantly. The circulating prothrombin had not increased significantly at this time. B. Ten hours post vitamin K_1. Note the decreased cellular fluorescence and patchy distribution of bright parenchymal cells. Prothrombin now released into circulation in significant amounts.

prothrombin. Or the mechanism of action might be to promote storage and interfere with the release of prothombin from liver parenchymal cells. When a prothrombin deficiency was produced in dogs by giving coumadin intravenously, in all cases, there was a decrease in specific fluorescence. The magnitude of the response correlated with the depression in circulating prothrombin. Liver parenchymal cells from severely hypoprothrombinemic dogs hardly differed from natural fluorescence (Fig. 2B). Quite obviously coumadin does not function by interfering with the release of prothrombin for if it did the liver cells would have fluoresced more brilliantly. Thus the mechanism of action of coumarin drugs appears to be the inhibition of some step in prothrombin synthesis within the parenchymal cell.

TABLE II

Influence of drugs on prothrombin synthesis. A correlation of liver cell function and circulating prothrombin

Samples	Drug	Prothrombin time (seconds)	Liver cell fluorescence (relative)*	Prothrombin (lowa units)
1 Control	None	14·6	10	100
2	Coumadin (2 doses of 5 mg/kg)	600·0	0	0
3	Mephyton 5 mg/kg			
4 (1st hour)	—	600	2	0
5 (2nd hour)	—	600	5	0
6 (3rd hour)	—	47	25	3
7 (4th hour)	—	16·2	70	5
8 (5th hour)	—	13·8	95	3
9 (6th hour)	—	13·8	100	4
10 (7th hour)	—	13·8	100	6
11 (8th hour)	—	13·8	80	10
12 (9th hour)	—	14·0	60	12
13 (10th hour)	—	13·8	40	25
14 (11th hour)	—	14·0	10	91

* Percentage of liver cell population fluorescing when rhodamine anti-dog prothrombin was applied to liver biopsies.

To study the cellular mechanism of action of vitamin K_1 the drug was given intravenously. Each dog served as his own control. As before, a liver biopsy was taken surgically as were blood samples, and the animal permitted to recover. About four weeks later a severe prothrombin deficiency was produced using coumadin. When the circulating prothrombin level, as determined by the modified 2-stage procedure of

Ware and Seegers[4], was essentially zero, vitamin K_1 was given. Blood samples and liver biopsies were taken at intervals from the beginning of the experiment until the normal circulating level of prothrombin was regained (Table II). Coincident with the coumadin depression of circulating prothrombin there was the decrease in liver parenchymal cell fluorescence noted before. Within 1 hr after the administration of vitamin K_1 the cellular fluorescence was brighter and continued to increase in intensity up to about four hours. Not only was the normal pattern of cellular fluorescence re-established, but after 3 hr greater numbers of parenchymal cells fluoresced brilliantly than seen in the normal control liver sections (Fig. 3A). By 4 hr essentially all of the parenchymal cells fluoresced more brilliantly than seen in the normal, while the Kupffer cells still did not exhibit specific fluorescence. At this time (4 hr) not very much prothrombin had been released to the circulation even though the prothrombin time, an index of circulating procoagulants, had returned to the normal range. Only after 5 hr was prothrombin released to the circulation. Coinciding with this event, the cellular fluorescence decreased as did the number of parenchymal cells fluorescing until the patchy distribution noted in the control state was seen (Fig. 3B). Normal circulating levels of prothrombin were achieved within 12 hr.

Vitamin K_1 also was given to normal dogs to see if the cyclic asynchronous production of prothrombin could be synchronized. This was the case and, soon after giving vitamin K_1, intense fluorescence of essentially all of the parenchymal cells was noted. However, only a small amount of prothrombin was released to the circulation to increase the circulating prothrombin level about 30%. A second dose of Mephyton (75 mg/kg) did not stimulate further release of prothrombin.

Thus, it appears that vitamin K_1 promotes prothrombin synthesis by stimulating all parenchymal cells to synthesize prothrombin. Some time is required to synthesize molecules that can react with the fluorescent antibody. Also, time is taken for either maturation of the molecule or transport intracellularly before prothrombin is released to the circulation. Some regulatory mechanism seems to monitor the circulating prothrombin and to maintain it within limits by promoting either storage, synthesis or release of prothrombin from liver parenchymal cells.

To summarize, the fluorescent antibody technique and quantitative assays of circulating prothrombin have provided information on the cellular mechanism of action of coumarin drugs and vitamin K_1 alteration of prothrombin synthesis. In addition, it is clearly established that liver parenchymal cells not reticuloendothelial cells normally synthesize prothrombin. All parenchymal cells are capable of this action, although they normally function out of phase with one another. (Aided by Michigan Heart Association and USPHS Grant H-4712).

REFERENCES

1. G. F. ANDERSON; Doctor of Philosophy Thesis, Wayne State University. June, 1961.
2. O. OUCHTERLONY; *Acta Pathol. Microbiol. Scand.* **32** 231 (1953).
3. M. I. BARNHART; *Amer. J. Physiol.* **199** 360 (1960).
4. A. G. WARE and W. H. SEEGERS; *Amer. J. Clin. Path.* **19** 471 (1949).