Inhibitory Effect of Some Plasma Protein Fractions When Added to Hemostatic Arterial Blood

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IT WAS shown that the perfusion of the dog hind leg preparation adapted for studies on hemostasis[2] with citrated venous blood rearterialized *in vitro* was unable to induce hemostasis of skin cuts in the inner thigh side of this preparation. Perfusion with citrated arterial blood, on the other hand, induced hemostasis within 6 to 10 minutes. Venous blood is not only nonhemostatic but highly inhibitory, since the addition of venous plasma to normal arterial blood suppresses the hemostatic properties of arterial blood.[1] After this inhibitory ability of venous blood or plasma when mixed with arterial blood had been thoroughly studied,[1] it was found of interest to know, by plasma fractionation, which constituents were able to show this effect. The high inhibitory activity of some fractions not only from venous plasma but also from arterial plasma and lymph, as well as the inability of other fractions to interfere with the hemostatic capacity or normal arterial blood, is shown in this paper.

Methods

All blood samples employed for plasma fractionation or as nutrient for the hind leg preparation were taken from mongrel dogs, obtained from the city pound. The hemostatic capacity of blood samples was tested in dog hind leg preparation adapted for hemostasis observations.[2] In each determination a normal arterial blood was used before the sample under study to test the preparation for normal bleeding control, and again after the sample to confirm the result found in the sample, since it was shown[2] that the irrigation of the leg with nonhemostatic blood sample renders the preparation unable to induces hemostasis, even if normal arterial blood is used afterwards.

Blood for the hind leg perfusion was taken from a donor dog, care being taken to avoid contact with air and by keeping the preparation under vaseline throughout the perfusion. *In vitro* rearterialization of venous blood was carried out by shaking the samples in alveolar air, to avoid CO_2 liberation. The addition of fractions to rearterialized venous blood was done just previous to the perfusion by a pipette going through the vaseline layer, followed by gentle stirring of the blood.

Lymph was taken in citrate solution by cannulation of the thoraxic duct of an open chest dog under artificial respiration.

Method 10 of Cohn et al.,[3] i.e., precipitation of protein by buffered alcoholic mixtures of known ionic strength at −5 C. was used, the precipitate being spun down in an adapted refrigerated centrifuge. The saline resuspended protein precipitate was tested as fresh as possible, despite the fact that fractions could be kept active in the refrigerator for more than a week. Each fraction was resuspended in an amount of saline equal to the volume of plasma used for fractionation. The mixing of the fraction with arterial blood kept under vaseline was carried out just before the test, so as to avoid possible deterioration

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with time. Fraction concentration expressed in the tables correspond to the amount of fraction contained in the volume of plasma used for fractionation. For instance, one per cent corresponds to the fraction obtained from 1 ml. of fractionated plasma, added to 99 ml. of venous blood.

Results

Table 1 shows how striking the difference of behavior is between fractions I-III and fraction II when added to arterial blood and, on the other hand, the addition of fraction IV-V. In low concentration (2 ml. or less per 100 ml. of arterial blood) the inhibitory effect of fraction IV-V was observed in 34 determinations, only three determinations, with the lowest concentration of 0.1 ml./100 ml., giving normal results; fractions I-III and II at the highest concentration of this group (2 ml./100 ml. blood) gave normal results in 27 determinations, only four determinations of fraction from venous plasma giving inhibitory results. The noninhibitory fractions I-III and II when added at high concentrations (10 ml. per 100 ml. blood) still gave normal results in six determinations, whereas inhibitory effects were revealed in 19 determinations.

Further fractionation was carried out in fraction IV-V to obtain the separate fractions IV and V to ascertain which one was active in the observed inhibition of arterial blood. Table 2 shows inhibitory effect of fraction IV in 12 determinations (only in the lowest concentration of 0.25 ml./100 ml. blood was one determination negative, but even at this low concentration two other determinations showed inhibitory effect). Fraction V showed no inhibition in nine determinations with fractions from arterial plasma and from lymph, but three determinations of fraction from venous plasma gave positive inhibitory results.

Still further fractionation of fraction IV to obtain that which Cohn and collaborators called fractions IV_1 and fraction $IV_{6,7}$ in fractionation of human serum did not show any significant discrimination, since inhibitory results were observed in both of these fractions (table 3).

To test relationship in potency between the inhibitory fraction IV_1 and the fraction (fraction II) able to convert venous blood into hemostatic blood,[4] a mixture of different proportions of these two fractions was added to arterial and to venous blood (table 4). The results show clearly the increased potency of fraction IV_1 over fraction II, since in 13 determinations arterial blood fraction II was able to neutralize the inhibitory effect of fraction IV_1 only in two instances. In venous blood a mixture of a 90 per cent fraction II against 10 per cent fraction IV_1 was able to counteract the activity of fraction IV_1, converting venous blood into hemostatic blood. In five determinations, when fraction II was present in the mixture at lower proportions, only once was it able to show hemostatic conversion of venous blood.

As contamination with zinc cations in fraction IV is the rule, some experiments were carried out to test, as a control, the activity of this cation in normal arterial blood. Inhibitory fraction IV was heated to 100 C. for 10 minutes, the protein precipitate spun down and the supernatant containing the zinc cation added, at one per cent concentration, to arterial blood. Two experi-

Table 1.—*Hemostatic Behavior of Fractions I-III, II and IV-V of Lymph, Venous and Arterial Plasmas (Cohn, Method 10), when Mixed with Arterial Blood*

Fractions	Body fluid used	Fraction concentration in 100 ml. of arterial blood (ml.)	Number of determinations	Bleeding time (minutes)		
				Previous normal arterial blood	Arterial blood plus fraction	Posterior normal arterial blood
		Fraction at low concentration				
I-III	Arterial plasma	2	6	7 to 10	6 to 9	7 to 8
	Venous plasma	2	4	7 to 8	8 to 12	7 to 10
			4	7 to 8	+30	+30
	Citrated lymph	2	2	6 to 9	8	7 to 8
II	Arterial plasma	2	6	7 to 10	7 to 12	6 to 8
	Venous plasma	2	3	5 to 10	7 to 12	7 to 10
	Citrated lymph	2	3	7 to 8	5 to 8	6 to 7
IV-V	Arterial plasma	0.1	1	6	+30	+15
		0.5	2	7	+30	+20
		1	4	6 to 10	+30	+30
		2	5	7	+30	+30
	Venous plasma	0.1	3	6 to 10	8 to 14	7 to 12
		0.5	4	7 to 12	+30	+20
		1	4	6 to 12	+30	+30
		2	4	6 to 10	+30	+30
	Citrated lymph	0.1	2	9	+30	+30
		0.5	3	8	+30	+20
		1	2	5	+30	+20
		2	3	5 to 9	+30	+30
		Fraction at high concentration				
I-III	Arterial plasma	10	5	6 to 10	+30	+20
	Venous plasma	10	2	7 to 9	8 to 9	9
			5	7 to 10	+30	+30
	Citrated lymph	10	2	8	8	9
II	Arterial plasma	10	6	6 to 8	+30	+30
	Venous plasma	10	2	8	20	19
			1	8	+20	+20
	Citrated lymph	10	2	7	11 to 14	8

ments to test the eventual inhibitory activity of zinc cations were negative, i.e., when added to arterial blood they did not show any inhibitory effect.

Discussion

Plasma fractionation shows that most of the inhibitory component of venous plasma which when added to arterial blood converts it into non-

Table 2.—*Hemostatic Behavior of Fractions IV and V of Lymph, Venous and Arterial Plasmas (Cohn, Method 10), when Mixed with Arterial Blood*

Fractions	Body fluid used	Fraction concentration in 100 ml. of arterial blood (ml.)	Number of determinations	Bleeding time (minutes)		
				Previous normal arterial blood	Arterial blood plus fraction	Posterior normal arterial blood
IV	Arterial plasma	0.25	2	7	+30	+20
			1	7	7	—
		1	4	7 to 8	+30	+20
	Venous plasma	1	3	7 to 9	+30	+20
	Lymph	1	3	6 to 8	+30	+20
V	Arterial plasma	1	6	5 to 8	6 to 9	7 to 8
	Venous plasma	1	3	7 to 10	+30	+20
	Lymph	1	3	7 to 8	9 to 10	7 to 9

Table 3.—*Hemostatic Behavior of Fractions IV_1 and $IV_{6,7}$ of Lymph, Venous and Arterial Plasmas (Cohn, Method 10), when Mixed with Arterial Blood*

Fractions	Body fluid used	Fraction concentration in 100 ml. of arterial blood (ml.)	Number of determinations	Bleeding time (minutes)		
				Previous normal arterial blood	Arterial blood plus fraction	Posterior normal arterial blood
IV_1	Arterial plasma	1	1	6	8	8
			3	6 to 10	+30	+30
	Venous plasma	0.01	1	8	+30	+30
		1	3	6 to 8	+30	+20
		0.1	3	7 to 9	+30	+30
	Citrated lymph	1	5	6 to 9	+30	+30
		2	1	8	+30	+30
$IV_{6,7}$	Arterial plasma	1	3	7 to 8	8 to 12	7
	Venous plasma	1	3	7 to 8	+30	+20
	Lymph	1	3	7 to 9	+30	+30

hemostatic blood[1] is contained in fraction IV. Besides this observation, it becomes clear that fraction IV, even taken from arterial plasma or from lymph, is as inhibitory as that coming from venous plasma. Studies on fractionation and electrophoresis of dog plasma[5,6] show, like the Cohn observations in human serum, that alpha globulins are the main constituent of this fraction. That this fraction contains lipoproteins is inferred by the amount of cholesterol and phospholipids found in it, about 10 to 15 per cent.[7] The inhibitory effect could, under the above considerations, be related to plasma alpha-lipoproteins. As the noninhibitory fractions I-III and II (at low concentrations) have some alpha-globulin impurities,[6] this could perhaps explain the positive results found at largest concentrations.

That further discrimination could not be found by subfractionation of fraction IV might perhaps be explained by the differences found between frac-

Table 4.—*Hemostatic Behavior of Mixtures of Fractions II and IV$_1$ of Lymph and Arterial Plasma (Cohn Method 10) when Added to Arterial or Venous Blood*

Blood sample used in the leg preparation	Blood fluid used in fractionation	Fraction concentration in 100 ml. of blood (ml.)		Number of determinations	Bleeding time (minutes)		
		F II	F IV$_1$		Previous normal arterial blood	Blood plus fraction	Posterior normal arterial blood
Arterial blood	Arterial plasma	1.8	0.2	1	—	+30	+30
		1	0.1	1	—	12	9
		1	0.3	1	9	+30	+30
		2	0.3	1	8	+30	+30
		4	0.3	1	8	+30	+30
		7	0.3	1	9	+30	+30
	Citrated lymph	1	1	2	6	+30	+30
		1.8	0.2	1	7	+30	+30
		1	1	1	8	8	—
		1.5	0.5	1	9	+30	+30
		2	1	1	8	+17	+30
		1.9	0.1	1	8	+30	+30
Venous blood	Citrated lymph	1.4	0.6	1	7	+30	+30
		1.4	0.6	1	8	12	9
		1.4	1	1	9	+30	+30
		1	1	1	9	+30	+30
		1.8	0.2	1	8	8	9

tionation of human and dog plasma with Cohn method 10.[6] The use of reagents adjusted for fractionation of human plasma can hardly be used, with comparable efficiency, to fractionate dog plasma constituents. It is possible that by new adjustments of Cohn reagents a further purification of the inhibitory substances may be achieved.

The behavior of the mixtures of inhibitory fraction IV and the hemostatic fraction II throws light on the mechanism of normal hemostasis. It was shown that the inhibitory effect of fraction IV in arterial blood was much more pronounced than the hemostatic effect of fraction II showed in the conversion of venous blood into hemostatic blood, whereas results obtained by mixing these two fractions showed a poor neutralizing activity of fraction II over the inhibitory effect of fraction IV, when both fractions were added to arterial blood.

The results presented in this paper as well as the findings already published[4] showing the possibility, by simple addition of a small amount of plasma fraction (around one per cent) to convert nonhemostatic venous blood into hemostatic blood, and also to convert hemostatic arterial blood into nonhemostatic blood, point out the paramount significance of plasma protein balance in the mechanism of spontaneous hemostasis control. We believe that the findings connected with the function of antihemophilic globulin in hemophilia and the experimental studies *in vivo* employing the isolated dog hind leg, are typical contributions stressing the all important properties of plasma, rather than blood cellular components, in the intimate mechanism of control of small skin hemorrhages or intra-articular bleedings so characteristic of hemophilia. These results seem to indicate that future elucidative studies on

this mechanism should, therefore, come more from biochemical fields than purely hematologic ones.

Summary

Dog arterial and venous plasma and lymph were fractionated by Cohn method 10 and the fractions tested for inhibitory activity when added to normal arterial blood in the dog hind leg preparation adapted for studies on hemostasis.

Cohn fraction IV from arterial or venous plasma and from lymph was the only fraction, at low concentrations, able to inhibit the hemostatic ability of normal arterial plasma.

Some facts suggest that alpha-lipoproteins are the plasma constituent of fraction IV with inhibitory effect on normal arterial blood. Biochemical changes in blood plasma seem more important or more directly connected with the mechanism of normal hemostasis control than the participation of blood cellular elements.

Summario in Interlingua

Le methodo 10 de Cohn esseva empleate pro fractionar canin plasma arterial e venose e lympha, e le fractiones obtenite esseva essayate pro lor activitate inhibitori quando illos esseva addite a normal sanguine arterial in le preparato del gamba posterior canin adaptate pro studios de hemostase.

Le fraction IV de Cohn, obtenite ab plasma arterial o venose o ab lympha, esseva le sol fraction capace de inhibir a basse concentrationes le capacitate hemostatic de normal plasma arterial.

Varie factos suggere que lipoproteinas alpha es le constituente de plasma in fraction IV que ha un effecto inhibitori super normal sanguine arterial. Il pare que le alterationes biochimic in le plasma sanguines es plus importante o plus directemente connectite con le mechanismo de normal regulation hemostatic que le participation del elementos cellular del sanguine.

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