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Polyglutamic and Polyaspartic Acids: Synthetic Polypeptides with Predominantly Factor VIII-like Coagulant Activity[1] (39351)

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Several years ago we reported that proteins of small molecular weight with specific F. (factor) VIII-like activity are present in purified preparations of leucineaminopeptidase made from porcine and canine kidneys (1, 2). Later, we demonstrated that treatment with succinic anhydride of human plasma (3) or purified human albumin (4) produces a F. VIII-like activity of small molecular size. Since succinic anhydride irreversibly attaches carboxyl groups to exposed epsilon-amino groups of proteins, it occurred to us that the F. VIII-like activity of the succinylated proteins might be an expression of the increased number of carboxyl groups. If this idea were correct, then synthetic polyamino acids rich in carboxyl groups, such as polyglutamic and polyaspartic, should also have F. VIII coagulant activity. This paper describes our experiments with these synthetic polypeptides which do indeed show considerable F. VIII activity *ex vivo*. Quite unexpectedly they also have weak F. IX activity. Some of these findings were reported earlier (5).

Materials and methods. Poly-L-glutamic, poly-L-aspartic, and poly-D-glutamic acids of varying molecular weights were purchased from Schwartz-Mann and the Nutritional Biochemicals Corporation. Stock solutions of 1 mg/ml were prepared in distilled water and diluted, before testing, with 0.05 *M* Tris-HCl buffer, pH 6.8, containing 0.15 *M* NaCl. The partial thromboplastin used in our experiments was a crude phospholipid preparation (Thrombofax) distributed by Ortho Diagnostics. Bio-Gel A-15 M, 4% agarose, 200-400 mesh, was obtained from Bio-Rad Laboratories and used in a 2.5 × 45-cm Pharmacia column for separation of factor VIII from Hemophil, the partially purified plasma concentrate made by Hyland Laboratories. Citrated plasmas were collected and specific 1-stage assays were performed for coagulation factors V, VII, VIII, IX, X, and XII (using either the PT or PTT methods) by techniques described previously (1).

The transfusion studies were performed on dogs with the Chapel Hill strain of hemophilia A (6).

Results. Effect of the polypeptides in clotting factor assays. Polyglutamic acids of three different molecular weights, and a single sample of polyaspartic acid, were tested in clotting factor assay systems using plasmas deficient in specific factors. A representative experiment illustrating the effects of the polypeptides on the clotting times in seven different assay systems is shown in Table I. It is clear that none of the polypeptides shortened the clotting times of the F. V, VII, X, or XII deficient plasmas, and there was only a trace of activity in the F. IX assay. However, each of the peptides markedly shortened the clotting time of hemophilic plasma. In addition, polyaspartic acid was as effective against a F. VIII-deficient substrate from a subject with vWd as from a man with hemophilia A.

Specific requirements of kaolin-activation and phospholipid. Table II demonstrates that both kaolin activation of the hemophilic plasma and additional phospholipid are required for expression of the activity of the polypeptides. Hemophilic plasma was mixed with an equal part of buffer, polyglutamic acid (PGA), or normal plasma. One-tenth milliliter of the mixture was added to 0.1 ml of thrombofax and 0.1 ml of $CaCl_2$. Buffer was substituted for the phospholipid in columns 2 and 4. Hemophilic plasma in the first two columns had been activated for 10 min with kaolin before being tested. In the first column (kaolin-activated plasma and lipid present) PGA is almost as effective as normal plasma in correcting the pro-

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TABLE I. EFFECT OF POLYGLUTAMIC AND POLYASPARTIC ACIDS ON CLOTTING FACTOR ASSAYS: SPECIFIC SINGLE FACTOR-DEFICIENT PLASMAS.[a]

					VIII		
	V	X	XII	IX	HEM. A.	vWd	VII[b]
Test material	Clotting times, seconds/units of factor per ml						
Buffer	>250/0	228/0	>260/0	186/0	167/0	183/0	58/0
1% Normal plasma	218/.01	167/.01	256/.01	147/.01	139/.01	123/.01	36/.01
20% Normal plasma	122/.20	111/.20	163/.20	103/.20	99/.20	97/.20	17/.20
Poly-L-glutamic acid[c]							
4100 mol wt	>250/0	>230/0	>260/0	132/.06	97/.46	–	75/0
5000-15,000 mol wt	>250/0	216/<.01	>260/0	151/<.01	101/.32	–	78/0
75,000 mol wt	>250/0	>230/0	>260/0	144/.02	106/.24	–	85/0
Poly-L-aspartic acid[c]							
2500-6000 mol wt	>250/0	225/<.01	>260/0	148/.01	95/.52	92/.60	68/0

[a] Plasma obtained from individuals with hereditary, congenital factor deficiencies.
[b] Prothrombin-time assay. All other assays are of the PTT type, kaolin-activated. Each clotting time is the average of three determinations.
[c] Stock solutions of 1.0 mg of polypeptide/ml buffer were diluted from 20 to 1% and tested in the assays. These clotting times are of the 10% dilutions in each assay.

TABLE II. NECESSITY OF KAOLIN-ACTIVATION AND PHOSPHOLIPID FOR DEMONSTRATING THE F. VIII-LIKE ACTIVITY OF POLYGLUTAMIC ACID ON RECALCIFICATION TIMES.

	Clotting times, seconds			
Test material	Kaolin and thrombofax	Kaolin, no thrombofax	No kaolin, thrombofax	No kaolin, no thrombofax
Hemophilic plasma + buffer[a]	>250	>250	>250	>250
Hemophilic plasma + PGA[b]	101	>250	>250	>250
Hemophilic plasma + normal plasma[c]	89	172	107	>250

[a] Tris-HCl, 0.05 *M*, NaCl, 0.15 *M*, pH 6.8.
[b] Poly-L-glutamic acid, 175,000 mol wt, 0.1 mg/ml buffer.
[c] Undiluted.

TABLE III.

A. EFFECT OF POLY-L-GLUTAMIC ACID (PGA) OF 175,000 MOL WT ON PARTIAL THROMBOPLASTIN TIME (PTT) OF PLASMAS PREVIOUSLY ACTIVATED WITH KAOLIN

	Clotting times, seconds	
Type of plasma	Plasma + buffer[a]	Plasma + PGA[b]
Normal	63	59
Factor XII deficient	394	336
Factor X deficient	184	182
Factor IX deficient	260	118
Factor VIII deficient	204	90

B. EFFECT OF PGA OF 175,000 MOL WT ON THE PTT OF PLASMAS SUBSEQUENTLY ACTIVATED WITH KAOLIN

	Clotting times, seconds	
Type of plasma	Plasma + buffer[a]	Plasma + PGA[b]
Normal	62	68
Factor XII deficient	>400	>400
Factor X deficient	253	220
Factor IX deficient	241	252
Factor VIII deficient	221	213

[a] Tris-HCl, 0.05 *M*, pH 6.8.
[b] 0.1 mg/ml.

longed clotting time of the hemophilic plasma. Omission of the lipid alone (column 2) or the kaolin alone (column 3) abolishes the F. VIII corrective effect of PGA.

The previous experiment showed that PGA is effective in shortening the clotting time of F. VIII deficient plasmas in the presence of phospholipid when the hemophilic substrate plasma has been activated with kaolin. Table III shows that kaolin activation must occur before addition of the polypeptide. The bottom line of Table IIIA shows the corrective effect of PGA in prior-activated plasma. The bottom line of Table IIIB shows the absence of a corrective effect in subsequently activated plasma. (In each instance, the plasma was activated with kaolin for 10 min at 28° before addition of thrombofax and $CaCl_2$.)

The factor VIII "potentiating effect" of polyglutamic acid. When either polyglutamic or polyaspartic acid is added to normal plasma or to fractions of normal plasma containing F. VIII, more F. VIII activity is

observed than expected. (The same effect has been seen when kidney AHF or the small-molecular AHF_3 from succinylated albumin had been added to normal plasma and the mixture assayed for F. VIII). We referred to this effect as "potentiation" of plasma F. VIII (2). Table IV shows the degree of "potentiation" of plasma F. VIII when a sample of polyglutamic acid of 75,000 mol wt is added to normal plasma, to Fraction 1-0 (7) and to a highly purified F. VIII preparation. The table also shows the converse, i.e., the absence of "potentiation" when PGA is added to a hemophilic fraction. PGA enhanced the F. VIII activity of each test material that contained F. VIII activity derived from normal plasma by from two- to fivefold while the mixture with hemophilic I-0 was inhibitory. When the mixture of the purified F. VIII fraction and PGA was tested in assays based upon plasma defective in factors other than F. VIII, it lacked potentiating effect. Thus it is seen that the PGA specifically "potentiates" F. VIII.

Behavior of poly-L-glutamic acid in dogs, in vivo and ex vivo. Since polyglutamic acid is very active in F. VIII assays when kaolin-activated human hemophilic plasma is used as substrate, its effect was studied in F. VIII assays based on canine plasma. A variety of polyglutamic and polyaspartic acids of different molecular weights and a wide range of concentrations were studied in F. VIII assays using kaolin-activated plasmas obtained from hemophilic dogs of the Chapel Hill strain (6). No F. VIII-like activity was demonstrated under any of these conditions.

Despite these negative *ex vivo* results, a few *in vivo* experiments were performed. In one experiment, a mixture of 100 mg of poly-L-glutamic acid of 75,000 mol wt, 500 mg of canine albumin, and 2.0 ml of canine brain cephalin (1.5%), in a total volume of 10 ml of 0.15 *M* NaCl were injected into a 9.5-kg hemophilic dog. Blood was drawn at intervals over a 24-hr period following infusion and the following tests were performed: whole blood clotting time, thrombin clotting time, partial thromboplastin time, euglobulin lysis time, and assays for factors VIII, IX, and X. No significant change was observed in any of the tests. Negative results were also obtained when only polyglutamic acid was infused. These *in vivo* results are similar to those previously obtained on infusion into dogs of F. VIII activity of small mol wt isolated from canine kidneys or produced by succinylation of canine albumin (unpublished results). It should be noted, however, that both of these latter preparations had shown factor VIII activity *ex vivo* in canine assays.

TABLE IV. "POTENTIATION" OF F. VIII ACTIVITY BY PGA.

Test mixture	F. VIII, u/ml Obs.	Exp.	Ratio obs./ exp
(1) PGA[a] + buffer[b]	0.38		
(2) Normal plasma + buffer	0.56		
Normal plasma + PGA	4.65	0.94 (1 + 2)	4.9
(3) Normal fraction I-O + buffer	1.35		
Normal fraction I-O + PGA	4.40	1.73 (1 + 3)	2.5
(4) F. VIII fraction [c] + buffer	0.49		
F. VIII fraction + PGA	4.15	0.87 (1 + 4)	4.8
(5) Hemophilic fraction I-O + buffer	<.01		
Hemophilic fraction I-O + PGA	0.20	0.38 (1 + 5)	0.53

[a] Polyglutamic acid, mol wt 75,000, 0.1 mg/ml buffer.

[b] Tris-HCl, 0.05 *M*, NaCl, 0.15 *M*, pH 6.8.

[c] V_0 fraction of "Hemophil" filtered through Bio-Gel A-15 M.

Summary and discussion. We have shown that polyglutamic and polyaspartic acids are quite active in specific assays for F. VIII, much less so in specific assays for F. IX, and inactive in four other systems (Table I). The F. VIII and IX activities are demonstrable only when the deficient substrate plasmas have been activated previously with kaolin and when phospholipid is also present (Tables II and III). Furthermore, polyglutamic acid "potentiates" F. VIII activity, two- to fivefold, when it is added to normal human plasma or F. VIII-rich fractions of normal human plasma (Table IV).

"Potentiation" of F. VIII by PGA is apparently different from activation or en-

hancement of F. VIII activity by thrombin. Very low concentrations of thrombin are known to activate F. VIII in a time-dependent reaction in which maximum activation does not occur until several minutes after mixing (8). This increase in F. VIII activity is unstable, decreasing to the original value upon further incubation. In contrast, the "potentiating" effect upon plasma F. VIII of polyglutamic acids (as well as kidney AHF and AHF_3 prepared from albumin) is observed immediately upon mixing and remains stable for hours at room temperature (unpublished observations).

Our experiments suggest that polyglutamic acid does not decrease clotting times by activating factors XII, XI, or IX, but that, once these factors have been activated by kaolin, polyglutamic acid is able to "substitute" for F. VIII. The fact that the PGA shows a slight corrective effect in previously activated F. IX deficient plasma suggests a possibly even wider role, i.e., that polyglutamic acid may mimic the "intrinsic F. X activator."

Kaolin-activation of the F. VIII deficient substrate is an absolute requirement for expression of the activity of the polypeptides. In this respect the F. VIII-like activity of the synthetic polypeptides is similar to the small molecular F. VIII activity isolated from kidneys (1, 2) or obtained by succinylation of plasma or albumin (3, 4). This peculiarity suggests that F. IX_a may be required in the test system if the F. VIII activity of the polypeptides is to be detected. Phospholipid is also required for the measurement of the F. VIII-like activity of the polypeptides, indicating that they are not "thromboplastic" in nature.

Polyglutamic acids active in human F. VIII assays were found not to be active in F. VIII assays based upon hemophilic dog plasma, even when kaolin and phospholipid were present, nor to have any effect when transfused into hemophilic dogs. "Kidney AHF" and "albumin AHF_3", by contrast are active *ex vivo* in canine assays, although they too appear to have no effect *in vivo*.

There may be several explanations for the lack of an *in vivo* effect. Polyglutamic acid, kidney "AHF" and albumin "AHF_3" may be completely unrelated to the coagulation moiety recognized as plasma F. VIII. However, if it is assumed that they are related, at least three possibilities occur to us which might explain the negative *in vivo* results. (i) It may be necessary for each small molecular form of F. VIII to attach to a larger moiety in order to be active physiologically. Following transfusion in the dog, the coagulant-active component may be unable to combine with the F. VIII "carrier" protein (1, 9) due to abnormal binding sites on the latter. (ii) The "carrier protein" of the hemophilic dog might rapidly bind the coagulation-active peptide in a manner so sterically abnormal that it is rendered inacessible to the other reactants involved in normal coagulation (10). (iii) The small molecular entities may be so rapidly cleared from the circulation that an effect cannot be readily detected.

Some of these suggestions have been drawn from the experiments of Cooper *et al.* (11) who have demonstrated that when normal canine F. VIII is dissociated with 0.25 *M* $CaCl_2$ the active coagulant fraction does not recombine with the "carrier protein" of hemophilic dog plasma. This is, of course, the reverse of the results found with human fractions in which full recombination has been found to occur between dissociated normal F. VIII and the "carrier protein" purified from hemophilic plasma (11).

Our experiments with polyamino acids leave many questions unanswered. These include: (a) Why should PGA and PAA have *any* procoagulant activity? (b) Why should the procoagulant activities of PGA and PAA manifest themselves primarily as F. VIII, secondarily as F. IX, and not at all as F.'s V, VII, X, or XII? (c) Why is prior activation with kaolin an absolute requirement for assaying the activities of PGA and PAA? (d) Why is PGA active against the F. VIII defective plasma of man and not the F. VIII-defective plasma of dogs? (e) What is the significance of "potentiation."

Perhaps our experiments with the polypeptides are suggesting that F. VIII activity is a less specific phenomenon than is ordinarily thought, i.e., is less specific than the enzymatic activities of F. II, VII, IX, and X. If the "F. VIII effect" proves to be more complex and not as limited in specificity as

that of the serine proteases, our experiments suggest that it may be possible someday to prepare artificial F. VIII.

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