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Cancer chemotherapy
fibrinolysis
fibrin stabilizing factor

The Effect of Cancer Chemotherapeutic Agents on Fibrin Formation and Stabilization *in Vitro*

DIANE M. KOMP,[32] ROBERT L. LYLES, JR., THOMAS H. BOYD, III, GLENN E. STONER, AND BETTY J. COX

Departments of Pediatrics and Materials Science, University of Virginia, Charlottesville, Virginia, USA

Extract

Functional and morphologic studies of fibrin formation in the presence of a variety of cancer chemotherapeutic agents *in vitro* showed that some of these drugs cause fibrinolysis, inactivation of fibrin-stabilizing factor (FSF), or a combination of both. The drugs with most potent activity are 5-[3,3-bis(2-chloroethyl)-1-triazeno]imidazole-4-carboxamide (TIC mustard), 5-fluorouracil, nitrogen mustard, adriamycin, and daunomycin. These changes were dose related.

Speculation

Interference with fibrin formation by anticoagulation has been associated with reduced implantability of tumor emboli in experimental systems. This study presents evidence that some cancer chemotherapeutic agents have chemical properties of an anticoagulant nature. In addition to any influence on cell multiplication, high pulse doses of adjuvant chemotherapy at the time of tumor manipulation such as surgery may interfere with fibrin formation around tumor emboli and discourage metastatic implantation.

Introduction

In a variety of animal tumor systems [5–7, 9, 16], successful implantation of metastases is related to fibrin formation and platelet aggregation around the embolized tumor cells. Inhibitors of fibrin formation (heparin [7] and coumadin [14]), inhibitors of platelet aggregation (aspirin [11]), inhibitors of fibrin stabilization (poly-L-lysine [12]), accelerators of fibrinolysis (plasmin [3]), and inducers of thrombocytopenia (neuraminidase [8]) have been reported to decrease the numbers of metastases in these tumor systems. Conversely, ϵ-aminocaproic acid (ϵ-ACA) and Trasylol, potent inhibitors of fibrinolysis, allow for more rapid fixation of tumor emboli [5]. In all of these systems, the inhibitor had to be present at the time tumor emboli were attempting to implant to exert its effect.

Remarkably similar inhibition of tumor metastases have been reported with the administration of nitrogen mustard [6] and triethylenethiophosphoramide [10]. It has generally been assumed that the role of adjuvant chemotherapy at the time of tumor manipulation is to reduce metastases by interfering with cell multiplication. Cliffton and Agostino [4] suggested that the protective effect might involve action of the coagulation system. It is the purpose of this paper to report *in vitro* studies of the influence of a variety of cancer chemotherapeutic agents on fibrin formation and stabilization.

Materials and Methods

Normal Plasma

The same six normal donors were used throughout the studies. Blood was anticoagulated using a ratio of

9 parts whole blood to 1 part 3.8% sodium citrate. After centrifugation at 4° for 15 min at 1,500 rpm, platelet-poor plasma was separated, pooled, and frozen in small aliquots in siliconized tubes. Aliquots were thawed just before use.

Chemotherapeutic Agents

The following drugs were dissolved with their appropriate solvent for intravenous administration: vincristine (Oncovin) [17], vinblastine (Velban) [17], cytosine arabinoside (Cytosar) [18], actinomycin D (Cosmogen) [19], methotrexate [20], cyclophosphamide (Cytoxan) [21], nitrogen mustard (Mustargen) [18], daunorubricin [22], adriamycin [22], L-asparaginase [19], 5-fluorouracil [23], and TIC mustard [24]. Further dilutions were made with sterile solvent just before use in testing. Table I lists the agent, its solvent, and the concentration of a 1/1 dilution. Test dilutions were chosen to approximate high concentrations anticipated with perfusion rather than plasma levels after infusion.

Serial Thrombin Time

Bovine thrombin [25] was dissolved with normal NaCl solution to give a concentration of 10 U/ml and was stored frozen in siliconized tubes until just before use. Thrombin solution was kept in a melting ice bath during use in testing.

Plasma (0.1 ml) and drug dilution (0.1 ml) were placed in Fibrometer [26] cups on a heating block. Thrombin time was determined at 0- and 60-min incubation at 37° by the addition of 0.1 ml thrombin solution. All determinations were performed in duplicate. Control tests were performed substituting the appropriate solvent for the drug. Drugs associated with progressive lengthening of thrombin time on incubation were restudied adding ϵ-ACA (Amicar) [20] to the drug solvent to give a concentration of 0.1 M ϵ-ACA.

Table I. Chemotherapeutic agent, solvent, and concentration

Drug	NSC no.	Solvent	1/1 Dilution, M
Vincristine	67574	Normal saline[1]	1.2×10^{-4}
Vinblastine	49842	Normal saline[1]	1.2×10^{-3}
Actinomycin D	3053	Sterile water	3.1×10^{-4}
Cytosine arabinoside	63878	Sterile water	6.7×10^{-2}
Methotrexate	740	Sterile water	1.1×10^{-2}
Cyclophosphamide	26271	Sterile water	1.8×10^{-1}
Nitrogen mustard	762	Sterile water	8.4×10^{-3}
Daunorubricin	82151	Sterile water	3.9×10^{-3}
Adriamycin	123127	Sterile water	1.8×10^{-3}
L-Asparaginase	109229	Sterile water	2,000[2]
5-Flurouracil	19896	Normal saline	7.8×10^{-3}
TIC mustard[3]	82196	0.01 N HCl	7.7×10^{-2}

[1] With benzyl alcohol preservative.
[2] Units per milliliter.
[3] 5-[3,3-Bis(2-chloroethyl)-1-triazeno]imidazole-4-carboxamide.

Urea Solubility

Equal volumes of normal plasma and 1/1 drug dilution (see Table I) were incubated at 37° for 60 min in glass tubes in a water bath. Clots were formed by recalcification of 1 ml mixture with 0.1 ml 0.25 M $CaCl_2$. The clots were suspended in a solution of 5 M urea and observed during the next 48 hr for dissolution. Saline and the appropriate drug solvents were substituted for drug in control determinations. Abnormal studies were repeated with the addition of ϵ-ACA to the drug solvent before mixing with plasma.

pH Studies

Equal parts of plasma were mixed with a 1/1 drug dilution and allowed to come to room temperature before the pH was determined by a Digicord pH meter [27]. Samples that were very alkaline were acidified drop-wise with 0.1 N HCl. Acid samples were alkalinized with 0.1 N NaOH. In studies with 5-fluorouracil it was necessary to acidify with 1 N HCl in order to accomplish decremental pH changes without excessive dilution. Thrombin time was performed as above on samples for each pH unit change of 0.1. Plasma-drug samples at pH 7.6 were recalcified with 0.025 M $CaCl_2$. Fibrin thus formed was placed in 5 M urea and observed for solubility.

Electrical Resistance Studies

Two milliliters of plasma were activated by mixing in a clean glass tube (12 by 75 mm) for 3 min. Two-tenths milliliter of a 1/2 drug dilution and 0.2 ml 0.25 M $CaCl_2$ were added sequentially with thorough mixing after each addition. The plasma was then placed in an aluminum incubation cell at 37°. The polymerization of the resulting fibrin was monitored by measuring the DC resistance of the plasma with an electrometer [28]. Optical density was monitored simultaneously. A pair of spherical microelectrodes melted and shaped from gold wire, 1.5 cm in length by 0.008 inch in diameter [29], were suspended in the tube of plasma with a pair of plain glass capillary tubes [30]. "Incubated" studies were performed by addition of drug to plasma 1 hr at 39° before glass activation and recalcification.

Electron Microscopy (Figs. 1 and 2)

Normal plasma was mixed with a 1/2 drug dilution in the ratio of 2 parts plasma to 1 part drug. All samples except for HN_2 and TIC mustard were incubated at 37° for 60 min. After incubation, 2 ml drug-plasma sample were activated in glass for 3 min and then recalcified by the addition of 0.2 ml 0.25 M $CaCl_2$. The plasma was pipetted into a 19 cm length of silicon rubber tubing with an internal diameter of 3 mm. The plasma was allowed to flow over the surface of a carbon plastic film mounted on an electron microscope grid on a substrate holder. Both ends of the tubing were inserted into the substrate holder to form a closed loop [3]. The circular tube was then secured on a turntable and rotated at 15 rpm until a fibrin clot formed in the moving plasma column. The specimens were removed from the substrate holder and fixed in 10% formalin for 12 hours.

In preparation for electron microscopy, the samples were stained for 2 min with uranyl acetate, washed in distilled water, and air-dried. The microscopy was performed on a JEM-6A transmission electron microscope at 100 kV.

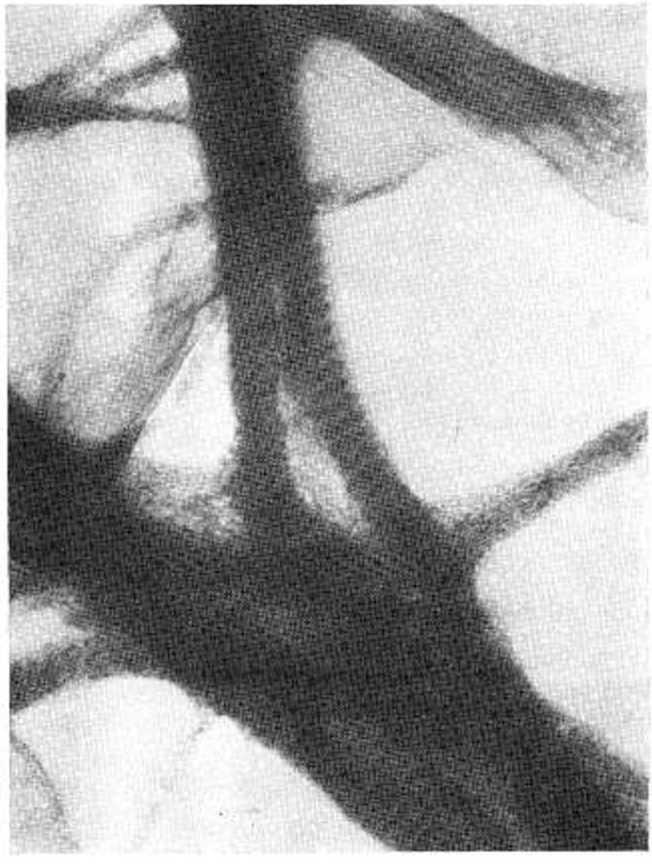

Fig. 1. Electron micrograph of fibrin formed from plasma containing vincristine (× 70,000), pH 7.9.

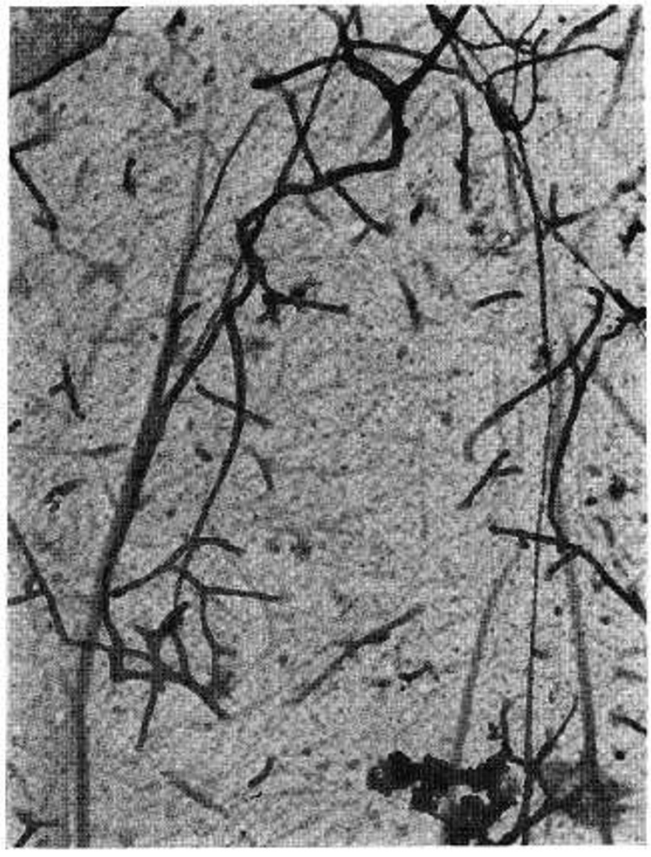

Fig. 2. Electron micrograph of fibrin formed from plasma containing 5-[3,3-bis(2-chloroethyl)-1-triazeno]imidazole-4-carboxamide (× 10,000) pH 3.7.

Results

Serial Thrombin Time

On the basis of serial thrombin times of mixtures of chemotherapeutic agents with plasma, drugs could be divided into three classes: *class I*, no influence on thrombin time; *class II*, abnormal incubated thrombin time corrected by premixture with ε-ACA; *class III*, markedly abnormal thrombin time with and without incubation; no correction by premixture of incubated specimen with ε-ACA.

Table II summarizes the thrombin times for each drug at three dilutions. Thrombin times that were abnormal were less so as the concentration of drug was decreased. Substitution of normal saline with benzyl alcohol, normal saline without preservative, distilled

Table II. Influence of drugs on serial thrombin time[1]

Dilution	Thrombin time, s		
	0 min	60 min	With ϵ-ACA
Class I			
Vincristine			
1/1	6.3	12.3	
1/2	6.8	11.3	
1/4	6.8	12.0	
Vinblastine			
1/1	5.8	11.0	
1/2	6.3	11.8	
1/4	6.8	12.8	
Cytosine arabinoside			
1/1	6.8	10.6	
1/2	6.4	12.6	
1/4	6.4	14.4	
L-Asparaginase			
1/4	7.3	10.5	
1/2	6.3	10.8	
1/4	7.3	11.0	
Adriamycin			
1/1	6.1	9.4	
1/2	6.4	10.9	
1/4	6.9	11.4	
Daunorubricin			
1/1	6.9	11.1	
1/2	6.9	14.0	
1/4	6.9	15.9	
Class II			
Actinomycin D			
1/1	7.3	23.3	11.1
1/2	6.8	19.0	
1/4	7.3	16.2	
Cyclophosphamide			
1/1	10.1	63.3	20.4
1/2	7.4	18.9	
1/4	6.9	11.9	
Nitrogen mustard			
1/1	7.4	105.9	21.4
1/2	6.4	42.3	
1/4	6.4	22.4	
Methotrexate			
1/1	10.3	39.5	23.0
1/2	7.8	21.0	
1/4	7.5	15.8	
Class III			
5-Fluorouracil			
1/1	180.4	>800	>800
1/2	68.8	115.3	
1/4	33.4	60.3	
TIC mustard			
1/1	>500	>500	>500
1/2	>500	>500	
1/4	8.9	7.9	
Normal range	6.1–7.9	8.7–17.5	

[1] ϵ-ACA: ϵ-aminocaproic acid; TIC mustard: 5-[3,3-bis(2-chloroethyl)-1-triazeno]imidazole-4-carboxamide.

water of 0.01 N HCl did not prolong control thrombin times (Table III).

Stepwise correction of pH towards 7.4 for *classes II* and *III* drugs reduced the magnitude of the thrombin time abnormality (Table IV). However, normal throm-

Table III. Influence of drug solvents on serial thrombin time and urea solubility

Solvent	Thrombin time, s		Urea solubility, hr
	0 min	60 min	
Sterile water (8)[1]	6.3–7.8	10.4–17.9	>24
Normal saline with benzyl alcohol (3)	6.4–7.3	12.0–12.8	>24
0.01 N HCl (1)	6.4	7.4	<24
Normal saline (12)	6.1–7.9	8.7–17.5	>24

[1] Number of duplicate determinations are indicated in parentheses.

Table IV. Influence of pH on serial thrombin time

Drug	*Class*	Thrombin time, s	
		0 min	60 min
pH 7.4			
Adriamycin	*I*	5.4	6.2
Daunorubricin	*I*	7.4	6.7
Cytosine arabinoside	*I*	7.4	11.2
Nitrogen mustard	*II*	7.9	80.4
Methotrexate	*II*	9.9	17.4
Cyclophosphamide	*II*	8.4	16.7
5-Fluorouracil	*III*	105.5	218.4
pH 7.5			
Adriamycin	*I*	4.9	6.9
L-Asparaginase	*I*	6.0	9.2
Nitrogen mustard	*II*	7.9	105.9
Cyclophosphamide	*II*	8.4	18.9
pH 7.6			
Daunomycin	*I*	5.4	7.7
Nitrogen mustard	*II*	7.9	129.4
5-Fluorouracil	*III*	134.9	230.5
pH 7.7			
Nitrogen mustard	*II*	8.1	131.4
Cyclophosphamide	*II*	8.4	22.4
pH 7.8			
Adriamycin	*I*	5.2	7.2
Cyclophosphamide	*II*	9.4	29.4
5-Fluorouracil	*III*	154.9	>400
pH 7.9			
Daunomycin	*I*	5.9	9.4
Methotrexate	*II*	9.9	17.4
pH 8.0			
Adriamycin	*I*	6.1	9.4
L-Asparaginase	*I*	6.0	11.7
5-Fluorouracil	*III*	155.9	>400
Normal range		6.1–7.9	8.7–17.5

[1] Nitrogen.

bin times were observed with *class I* agents at similar extremes of pH.

Urea Solubility

Plasma containing normal saline without preservative, distilled water without preservative, or normal saline with benzyl alcohol resisted solubility in 5 M urea during the 48 hr of observation. Plasma which contained 0.01 N HCl produced recalcified clots that dissolved in less than 24 hr in 5 M urea (Table III).

Four classes of agents could be discerned by the pattern of urea solubility. These classes corresponded strictly to those constructed on the basis of thrombin times except for a subdivision among the agents without influence on serial thrombin time (*class I*). *Subclass A*, exemplified by vincristine, vinblastine, actinomycin D, and cytosine arabinoside, showed normal resistance to solubility in 5 M urea. *Subclass B* agents, daunorubricin, adriamycin, and L-asparaginase, although without influence on the thrombin time, did influence urea solubility. This solubility was not influenced by prior incubation with ϵ-ACA. Adjustment of the pH to 7.6 before recalcification did prevent dissolution of clots formed in plasma containing L-asparaginase. Clots formed in the presence of daunorubricin and adriamycin remained soluble in 5 M urea despite adjustment of the pH to 7.6.

Class II agents such as methotrexate, cyclophosphamide, and HN_2 were associated with progressive prolongation of thrombin time and increased urea solubility. Prior incubation with ϵ-ACA prevented urea solubility. Adjustment of pH to 7.6 did not prevent urea solubility in the absence of ϵ-ACA.

The protective effect of incubation with ϵ-ACA on urea solubility was not seen with *class III* agents. Adjustment of pH to 7.6 for 5-fluorouracil did not prevent urea solubility.

Electrical Resistance Studies

During clot formation, a sigmoid curve with two inflections portrays the changes in electrical resistance in normal plasma. At the recalcification time, the electric resistance of the plasma increases markedly, seen by the first inflection (※*1*) in the curve (Fig. 3). Normally, the electrical resistance again shows another sharp increase displaying a broader inflection (※*2*). Abnormal curves were observed for all drugs in *classes I-B, II,* and *III*. The values for the time after recalcification for the inscription of the second inflection are summarized in Table V. Delay of the second inflection corresponded to solubility of fibrin in urea (Table VI).

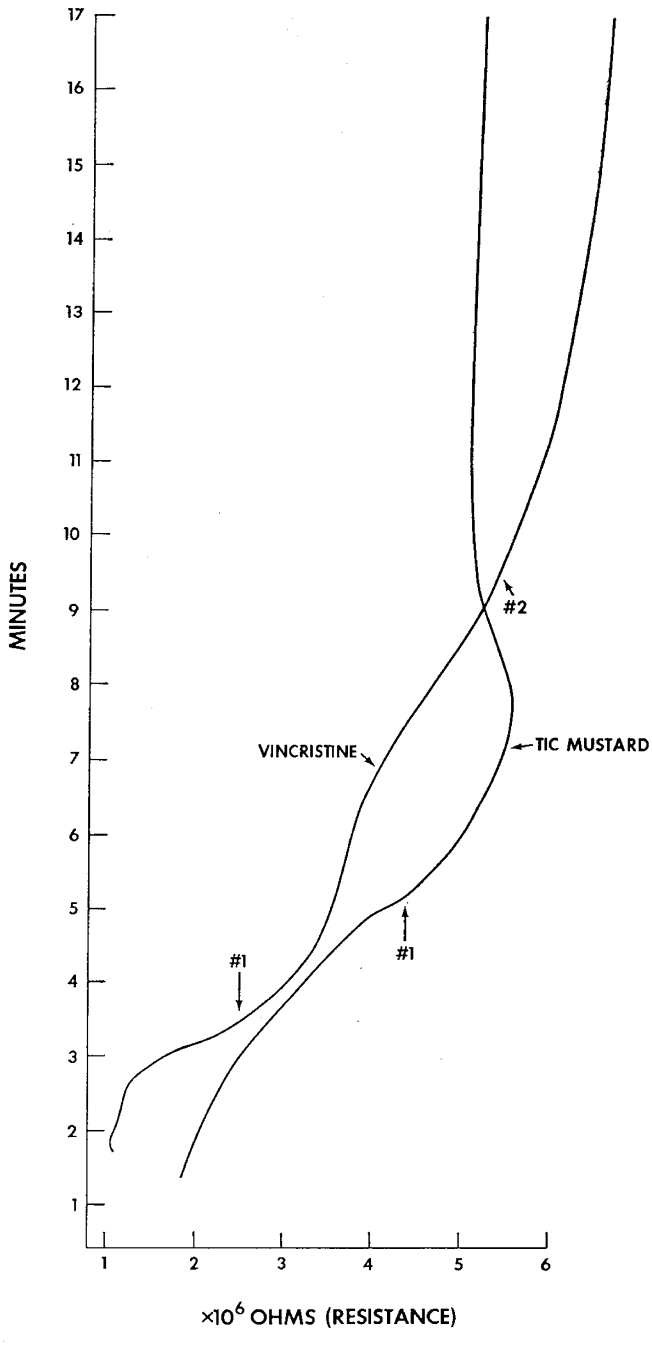

Fig. 3. Electrical resistance curves for 5-[3,3-bis(2-chloroethyl)-1-triazeno]imidazole-4-carboxamide (*TIC mustard*) and vincristine. A normal plasma curve is omitted since vincristine parallels it.

Electron Microscopy

Three types of abnormalities of fibrin formation were seen by transmission electron microscopy. The pattern for each drug is summarized in Table VII. Sparse fibrin formation, frayed rope appearance of the

strands, and loss of cross-striations were prominent in *class I-B, II,* and *III* agents.

Discussion

It is apparent from these data that high concentrations of some chemotherapeutic agents impede stable fibrin formation *in vitro*. Differences in functional and morphologic studies of the third stage of coagulation suggest that several mechanisms are operative.

Agents grouped together as *class I-B* do not alter the rate at which fibrin is formed although abnormal fibrin was visible by electron microscopy. Abnormal solubility in 5 M urea uncorrected by an antifibrinolytic agent and delayed inscription of the second inflection of the electric resistance curve were observed. These data suggest inactivation of FSF. The electron micrographs resemble those seen when fibrin formed in the absence of FSF is washed in urea [15]. Resistance of urea

Table V. Electrical resistance changes for plasma containing chemotherapeutic agents

Drug	Second inflection, min	
	Unincubated	Incubated
Class I-A		
Vincristine	8.7	9.2
Vinblastine	10.6	9.4
Actinomycine D	7.4	7.4
Cytosine arabinoside	8.8	9.0
Class I-B		
Daunorubricin	23	15
Adriamycin	11.8	11.8
L-Asparaginase	4.9	5.1
Class II		
Methotrexate	21	33
Cyclophosphamide	8.2	8.3
Nitrogen mustard	∞	
Class III		
5-Fluorouracil	13.0	∞
TIC mustard[1]	∞	
Normal range		
Mean ± 2 SD	8.7 ± 2.2	

[1] 5-[3,3-Bis(2-chloroethyl)-1-triazeno]imidazole-4-carboxamide.

Table VII. Summary of electron microscopic pattern of fibrin[1]

Drug	Thickness	Frayed rope	Cross-striations
Class I-A			
Vincristine	−	−	−
Vinblastine	−	−	−
Actinomycin D	−	−	−
Cytosine arabinoside	−	−	−
Class I-B			
Daunorubricin	±	+	+
Adriamycin	+	+	±
L-Asparaginase	+	+	−
Class II			
Methotrexate	+	+	+
Cyclophosphamide	+	+	+
Nitrogen mustard	+	+	+
Class III			
5-Fluorouracil	+	+	+
TIC mustard[2]	+	+	+

[1] −: normal; +: abnormal; ±: slightly abnormal.
[2] 5-[3,3-Bis(2-chloroethyl)-1-triazeno]imidazole-4-carboxamide

Table VI. Summary of studies of fibrin formation and stabilization in presence of chemotherapeutic agents[1]

Drug	Electron microscopy	Serial thrombin time			Electrical resistance		Urea solubility	
		Unincubated	Incubated	Incubated with ε-ACA[2]	Unincubated	Incubated	Without ε-ACA	With ε-ACA
Class I-A								
Vincristine	−	−	−	−	−	−	−	−
Vinblastine	−	−	−	−	−	−	−	−
Actinomycin D	−	−	±	±	−	−	−	−
Cytosine arabinoside	±	−	−	−	−	−	−	−
Class I-B								
Daunorubricin	+	−	−	−	+	+	+	+
Adriamycin	+	−	−	−	+	+	+	+
L-Asparaginase	+	−	−	−	−	−	+	+
Class II								
Methotrexate	+	±	+	−	+	+	+	−
Cyclophosphamide	+	±	+	−	−	−	+	−
Nitrogen mustard	+	−	+	−	+	+	+	−
Class III								
5-Fluorouracil	+	+	+	+	+	+	+	+
TIC mustard[3]	+	+	+	+	+	+	+	+

[1] −: normal; ±: slightly abnormal; +: abnormal.
[2] ε-ACA: ε-aminocaproic acid.
[3] 5- 3,3-Bis(2-chloroethyl)-1-triazeno]imidazole-4-carboxamide.

solubility was restored for plasma containing L-asparaginase when the pH was adjusted to 7.6. Loewy [13] has observed less activity of FSF at extremes of pH and optimal activity at 7.6. Adjustment of pH of plasma containing daunomycin and adriamycin to 7.6 did not prevent urea solubility, which suggests that, for these agents, the drug rather than the pH was responsible for loss of FSF activity.

The abnormalities seen with *class II* drugs are consistent with increased fibrinolysis. Incubation with ϵ-ACA corrected thrombin time prolongation and urea solubility. Since FSF can serve as a substrate for fibrinolysins [11], it is not surprising to find a deficit of FSF activity.

Class III agents, exemplified by 5-fluorouracil and TIC mustard, produced profound defects in all test systems. Although each represents an extreme of pH (8.0 and 3.7, respectively), neutralization of the plasma containing 5-fluorouracil to 7.4 only partially corrected the thrombin time abnormality. Similar studies were not possible for TIC mustard, because it is insoluble at more alkaline pH [2]. The properties of its solvent and its failure to remain soluble under less acid conditions prevent us from distinguishing from solvent and solute as cause for defective fibrin formation.

The relevance of these findings to clinical situations remains to be proven. Studies of blood from patients who receive these agents are necessary to determine whether fibrin formation is impeded *in vivo*.

Summary

When a variety of chemotherapeutic agents reconstituted as for intravenous administration were mixed with normal plasma, defective fibrin was formed. Mechanisms varied and included increased fibrinolysis and inactivation of fibrin-stabilizing factor. In one instance the abnormality was related to the pH of the reconstituted drug rather than specific action of the agent.

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32. Requests for reprints should be addressed to: Diane M. Komp, M.D., Department of Pediatrics, University of Virginia School of Medicine, Charlottesville, Va. 22901 (USA).
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