Preliminary Communication
reprinted from THE LANCET, *January* 27, 1968, *pp.* 173–176

INHIBITION OF ANTIPLASMIN, AND FIBRINOLYTIC EFFECT OF PROTEASE IN PATIENTS WITH CANCER

Summary Protease I (a fibrinolytic enzyme from *Aspergillus oryzæ*) inhibited thromboplastin and antiplasmin activity in vitro; and the antiplasmin inhibitory activity is potentiated in vitro by human thromboplastin. The in-vivo effects of this enzyme were examined in patients with cancer, since thromboplastin activity is raised in cancer tissue, and such patients have increased levels of antiplasmin. Thirteen patients with cancer and one with occlusive vascular disease were given one or more infusions of protease. Side-effects were mild and transient, except for pathological fibrinolysis (in three cases on a high dose) and a coagulative defect in one case. Subjective improvement was noted, but it is too early to judge the effects of therapy.

INTRODUCTION

THE induction of adequate anticoagulation or fibrinolysis by heparin, dicoumarol, or plasmin in animals decreases the metastatic spread of blood-borne cancer cells.[1–5] The lowering of prothrombin levels by warfarin sodium inhibits the locomotion and growth of V2 carcinoma cells in rabbit tissues.[6]

The thromboplastin activity of cancer tissue is many times greater than that of normal tissue,[7] and the inhibitors of fibrinolysis, the antiplasmins, are increased in patients with cancer.[8] We have been looking for an agent which would inhibit this thromboplastic activity of human cancer tissue, and decrease the antiplasmins and thus enhance the endogenous fibrinolytic system: a fibrinolytic enzyme from *Aspergillus oryzæ* fulfilled both these requirements. This enzyme has been called aspergillin O,[9] and protease I.[10]

MATERIALS AND METHODS

Materials

CA–7, protease I.—The fibrinolytic enzymes of *A. oryzæ* containing 1000 C_t (Connaught) units per mg. dry substance, as CA–7 (Connaught Laboratories, Toronto, lot 1016–2) or protease I (Astra, Sweden, lot 17047).

Plasmin.—' Kabi 1398 ', human plasmin grade " b " measured in casein units (Sgouris) lot LmD 3.

Reference serum.—Pooled sera from 20 healthy people stored at −20°C and diluted 1/8 in saline solution at time of use.

Bovine fibrinogen.—Kabi grade " B1 " lot QdX 19, 0·2%. Stored as 0·6% in 2·8% w/v sodium chloride at −20°C, thawed, and diluted 1/3 with tris(hydroxymethyl) aminomethane (" tris ") buffer pH 7·4.

Tirombin.—Thrombin, topical (bovine origin) (Parke Davis & Co.), 50 units per ml. In tris buffer at pH 7·4, stored at −20°C.

" *Normal saline* ".—0·9% w/v sodium-chloride solution (clinical, Antigen Ltd.).

Heparin.—' Pularin ' (Evans Ltd., batch G15840).

ε-*aminocaproic acid.*—' Epsikapron ' (Kabi, lot 84850).

' *Trasylol* '.—Protease inhibitor (FBA Pharmaceuticals), 25,000 kallikrein-inactivating units per ampoule.

Methods

Methods were as follows: antiplasmin activity,[8] dilute blood-clot lysis-time (B.L.T.),[11] and fibrinogen.[12]

Human thromboplastin was prepared from fresh frozen human chorion and titrated by the method of O'Meara and Thornes,[7] and has similar activity to the thromboplastin extracted from cancer tissue (cancer coagulative factor, C.C.F.).[13]

Bedside test.—Measured by Thornes' method.[14]

Protease resistance (*serum-level of inhibitor to protease*) was estimated by Roschlau's method [15] modified to use 0·1 ml. of whole blood instead of 1·0 ml. and 1/10 of the amount of protease (CA–7). To facilitate the reading of the end-point of titration, water is added to the tubes after 10 minutes incubation, and the percentage lysis can be easily assessed by inversion of the tubes. The results are expressed as " test tube requirement " (T.T.R.) units.

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Hæmatology and biochemistry.—The following tests were performed in all cases: Hb packed-cell volume, blood-cell counts (white, differential, red), mean corpuscular Hb concentration, urinalysis, blood-urea, total protein, total bilirubin, serum-alkaline-phosphatase, serum-glutamic-oxaloacetic transaminase, serum-glutamic-pyruvic-transaminase, clotting-time, prothrombin-time, platelet-count.

RESULTS

In Vitro

CA-7 was used for these experiments.

Protease in a concentration of 0·4 mg. per ml. inhibited 95% human *thromboplastin*: in a concentration of 0·04 mg. per ml. 50% of thromboplastic activity was inhibited. 100% inhibition of the *antiplasmin* activity in 1 ml. of serum is produced by 0·04 mg. of protease; and 20% is inhibited by 0·005 mg. of protease. When human thromboplastin, with a titre of 1/2000 but containing no plasminogen, was added to protease the inhibiting effect on *antiplasmin* was greatly enhanced (table I).

TABLE I—POTENTIATION OF PROTEASE (CA-7) BY HUMAN THROMBOPLASTIN

Concentration of protease CA-7 (mg. per ml.)	Antiplasmin inhibition (%)	
	Saline	Human thromboplastin
0·02	84	97
0·01	47	79
0·005	20	70

In-vivo Activity

The drug was given by intravenous infusion except in one case in which intra-arterial infusions were used. The dose was judged with reference to the protease resistance for that individual and this variable has been closely watched throughout therapy.

Fourteen patients have been treated with protease and a total of 42 infusions have been given. Infusions have been given repeatedly to nine cases, the maximum number given to one individual being 7. The dose of protease was diluted in normal saline for administration. The average duration of infusion was 2 hours but this varied from 20 minutes to 3 hours. The first seven cases treated were given doses below 40 mg. Thereafter the average dose in our series was 205 mg. The higher the dose the greater and more lasting was the effect produced. The doses given and the effect produced on protease resistance, antiplasmin, B.L.T., and fibrinogen are shown in table II.

In one case a dose of 600 mg. resulted in complete incoagulability of the patient's blood. In two other cases the degree of fibrinogenolysis was such that it was impossible to obtain clotting in the test system, and the tests for protease resistance, fibrinogen, B.L.T., and the prothrombin-time could not be performed.

Specific Effects

The effects produced on the variables measured are shown in fig. 1.

Protease resistance.—Comparison of the protease resistance in proven cancer patients and normal healthy individuals matched for age and sex shows that this level is considerably higher in the cancer cases. The protease resistance falls in all cases, after an infusion of over 200 mg. of protease, but returns to pretreatment levels within 24 hours (fig. 2).

Antiplasmin.—Infusion of protease lowers serum-antiplasmin levels (fig. 2). With doses over 200 mg., this effect is sustained for 48 hours. If no further infusions are

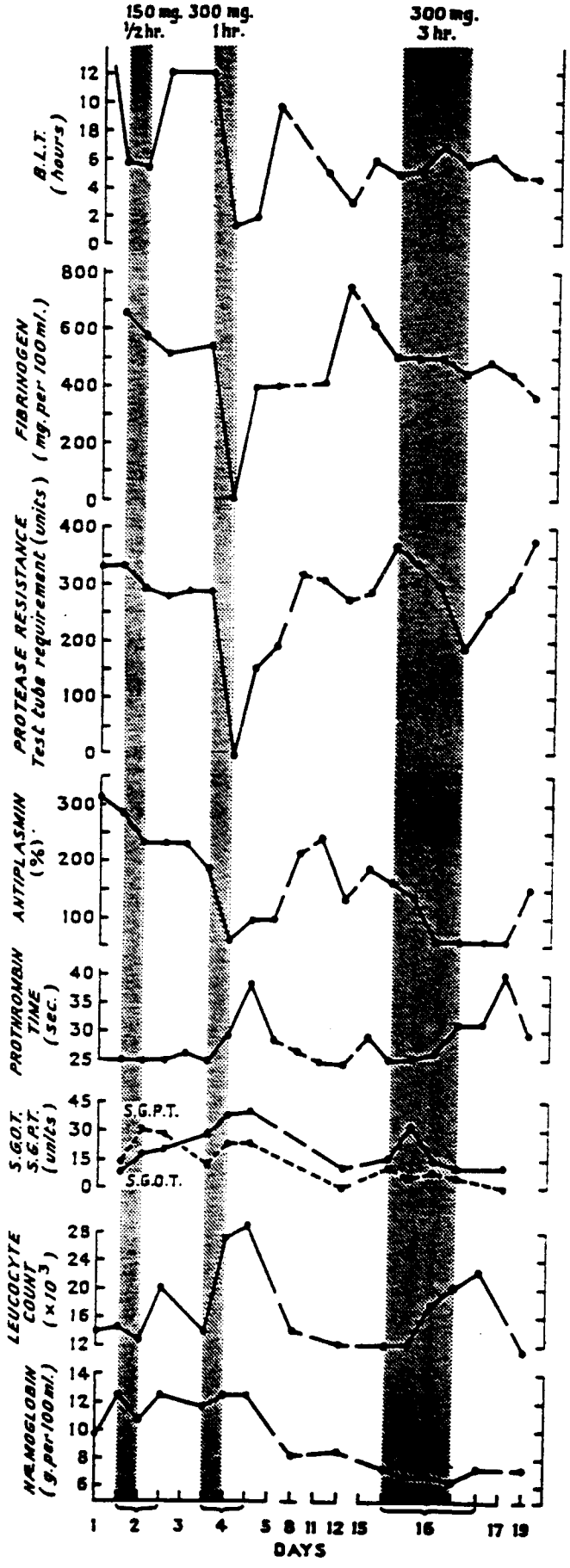

Fig. 1—Case 12: effect of repeated protease infusions.

TABLE II—MEAN VALUES AT VARYING DOSE LEVELS

Dose (mg.)	No. of estimations	Protease resistance (T.T.R. units)				Antiplasmin (%)				Dose (mg.)	No. of estimations	B.L.T. (hr.)				Fibrinogen (mg. per 100 ml.)			
		A	B	C	D	A	B	C	D			A	B	C	D	A	B	C	D
100	7	..	..	..	..	284	166	292	204	100	6	5¼	6¼	6¼	5	389	329	378	357
150	8	210	167	175	186	161	135	137	147	150	6	4½	3½	8	3¾	497	462	484	437
200	7	205	132	141	193	180	89	97	114	200	7	3	3¾	7¼	3	448	378	446	402
250	2	297	236	275	277	140	82	85	75	300	3	9	2½	3½	5¾	457	313	427	418
300	6	283	203	197	264	137	63	71	81	400	1	24	¼	1¼	24	525	0	414	375
400	1	320	0	130	265	220	53	86	..	600	1	24	0	0	18	638	0	0	262
600	1	375	0	0	285	280	0	60	95										

A = Immediately before infusion. B = Immediately after infusion.
C = 1 hour after infusion. D = 24 hours after infusion.
Zero values indicate incoagulable blood when test could not be performed.

given the antiplasmins tend to rise above pretreatment levels, but with repeated infusions every 3 days it is possible to keep them below the arbitrary 100% "normal" found in pooled serum. Antiplasmin levels, unlike protease resistance, vary from day to day.

B.L.T.—With doses over 200 mg. the lysis-time is shortened after infusion but returns to normal within 24 hours. A lysis-time of over 24 hours can be reduced to less than an hour by protease. With doses less than 200 mg. the effect is not constant, and lysis-time increases immediately after infusion (table II).

Fibrinogen.—Infusion of protease lowers the fibrinogen levels. With doses over 200 mg. the fibrinogen levels may remain depressed for as long as 3 days.

Prothrombin-time is increased after infusions of more than 200 mg. of protease. Patients already on anticoagulants of the coumarin type exhibited the greatest increase.

Clotting-time was not prolonged unless doses were excessive. In one case, the blood did not coagulate. In another case to which heparin was given simultaneously (10,000 units heparin, 200 mg. protease) clotting-time was prolonged to 60 minutes on two occasions, and returned to normal in 4 hours. Heparin, 10,000 units alone produced a clotting-time of 12 minutes.

Hæmatology and biochemistry.—A polymorphonuclear leucocytosis develops during and immediately after an infusion. This effect lasts for 24 hours. In two cases the increase was over 100%. The other *white blood-cells* were not apparently affected. The alteration in *platelet-counts* was too variable to assess. In one case a striking thrombocytosis of over 200% was observed after infusion. *Serum transaminases* were unaltered by infusion of protease, except in 1 patient with hepatic metastasis in whom a temporary rise was noted. In contrast, the *serum-alkaline-phosphatase* which was abnormally high in this case was reduced after two infusions from 60 to 40 King-Armstrong units. The other cancer cases also showed a slight fall. The *total bilirubin* was unaltered by infusion. A fall in the *Hb, packed-cell volume,* and *total protein* occurred in the majority of cases, suggesting hæmodilution. In four cases to whom high doses were given (over 250 mg.) a hæmoconcentration occurred. *Blood-urea* and *urinalysis* were unaltered by infusion of protease.

Side-effects

The commonest side-effect is flushing and a feeling of warmth of the face and extremities accompanied on occasions by paræsthesia. This is of short duration and if the infusion is started slowly its occurrence is reduced. Drowsiness has been observed on three occasions. Thrombosis at the site of infusion occurred once with a very low dosage and leakage of protease into the tissues causes a burning pain followed by œdema of the arm. Sloughing of tissue was not seen.

Pyrexia, tachycardia, sweating, nausea, diarrhœa, and chest discomfort occurred in three cases. Elek-Ouchterlony immunodiffusion tests in agar were carried out in one case 2 months after therapy, and precipitating antibodies to protease were shown.

Severe back pain occurred during infusion in two cases with vertebral metastasis. One patient complained of severe abdominal pain at the completion of infusion of 300 mg. of protease. This was relieved by 100 mg. of

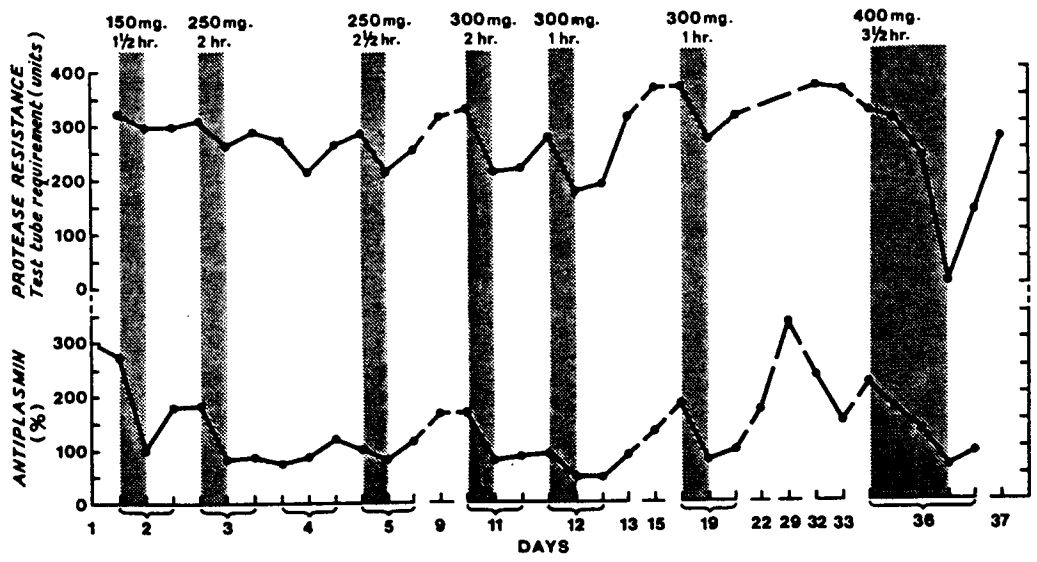

Fig. 2—Case 11: effect of repeated protease infusions on antiplasmin and on protease resistance.

pethidine. An infusion of 600 mg. of protease in one case resulted in depletion of protease resistance, epistaxis, bleeding from the gums, and hæmatemesis. The blood was incoagulable and the blood-pressure fell from 120/80 to 90/50 mm. Hg. The prothrombin-time and the clotting-time were notably prolonged, the B.L.T. was short and the plasma-fibrinogen was low. One unit of fresh frozen plasma was given initially followed by 4 g. of fibrinogen and resulted in control of hæmorrhage. Trasylol and ε-aminocaproic acid were without effect when examined by the "bedside test" and were not administered.

Effect of Therapy

No patient deteriorated as an immediate result of therapy. We have been encouraged by the improvement in a case of advancing myeloma, with paraplegia. Subjective improvement and diminution of pain was observed in the treated patients but we cannot yet draw any conclusion from this.

DISCUSSION

Proteolytic enzymes in extracts of *A. oryzæ* were discovered by Grassman.[16] Crewther and Lennox [17] crystallised a protease fraction from *A. oryzæ*, but this was found to be heterogenous.[18] The first report on fibrinolytic material produced by *A. oryzæ* was that of Stefanini and Marin.[19] Bergkvist [20] using the ion exchangers diethylaminoethyl cellulose and carboxymethyl cellulose isolated three different proteases, named protease I, II, and III. Protease I had the most fibrinolytic activity, and was the one used by us.

The fibrinolytic activity of protease is due to its direct proteolytic action on fibrin and its ability to deplete antiplasmin activity. Bergkvist and Svard [21] showed that at least part of the fibrinolysis induced by protease in cats is due to increased plasmin activity. They showed that the clotting-time, and fibrinogen and fibrinolytic activity are not affected by doses of protease which do not overcome the natural circulating inhibitors. These findings were similar to those of Roschlau.[15] He showed that complete exhaustion of protease resistance produced hypotension, incoagulability of the blood, elevated transaminases, and death in dogs.

The level of protease resistance is higher in cancer patients than in healthy people, and this is probably also true for a number of active disease processes. (In five healthy controls protease resistance was 195, 225, 235, 245, and 250 T.T.R. units; in five cancer patients the figures were 310, 315, 320, 325, and 410 T.T.R. units.) This high level of inhibitor to protease makes it necessary to give high doses to cancer patients. Roschlau [22] found the mean protease resistance in healthy people to be 131 T.T.R. units. The higher levels of protease resistance obtained in our controls is probably due to age differences, since our cases were over 45 years old.

The antiplasmins [23] and the inhibitors to protease [20] are in the α_1 and α_2 globulins. It seems that these inhibitors are similar but not identical with each other, since the antiplasmins are lowered for longer periods by protease infusion.

Side-effects with protease are, fortunately, mild and transient. In three instances high doses were given over a relatively short period of time and pathological fibrinolysis developed. In one case a coagulative defect was also present: antithrombin activity was greatly increased after infusion.

ε-aminocaproic acid and trasylol inhibit activators and plasmin, respectively, and are inactive against protease. A specific antidote is not available, and this is a serious drawback to the clinical use of protease. We are trying to isolate inhibitors by Cohn's ethanol fractionation, but at present fresh frozen plasma is probably the best inhibitor available. It has the added advantage of replacing clotting factors destroyed by protease. In cases of overdosage fibrinogen may be required to replace the loss caused by fibrinogenolysis.

Protease is almost non-antigenic.[24] Repeated infusion did not increase protease resistance, in contrast to streptokinase where the resistance (titre) is increased as antibodies form. In one case we found antibody to protease, but interestingly enough, protease resistance was not increased. This suggests that protease may not be inhibited by antibody in the same way as streptokinase.

The thromboplastic factor extracted from human carcinoma or human chorion has antiplasmin activity. We therefore expected that this factor would diminish the action of protease on antiplasmin in vitro. The opposite, in fact, happened, and should this effect occur in vivo, the action of protease within a tumour would be greatly enhanced. The fibrinolytic effect of protease should be of value in the removal of fibrin from malignant tumours. The motility of the cancer cell may then be inhibited, and the cell rendered more susceptible to cytotoxic therapy.

This study was supported by the Irish Cancer Society, Tenovus, and the Medical Research Council of Ireland. The following kindly gave permission to treat their cases: Prof. A. Thompson, Dr. H. Counihan, Prof. M. Abrahamson, Prof. W. McGowan, Mr. P. Carey, and Dr. B. Lemass. We thank Astra AB, Sweden, for supplies of protease and Connaught Laboratories, Toronto, for CA-7. Kabi AB, Sweden, kindly supplied plasmin, fibrinogen, and ε-aminocaproic acid.

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Printed in Great Britain

The Lancet Office, 7 Adam Street, Adelphi, London W.C.2