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OF THE BLOOD SERUM IN MAN AND ANIMALS FOLLOWING
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**CHANGES IN THE PROTEOLYTIC AND ANTIPROTEOLYTIC ACTIVITY
OF THE BLOOD SERUM IN MAN AND ANIMALS FOLLOWING
EXPOSURE TO X-RAYS**

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reported a study of the blood of 15 patients undergoing x-ray therapy for malignant tumors. Using a nephelometric casein-digestion procedure for measurement of proteolytic and antiproteolytic activity he found a definite, sharp rise in the plasmin titer of the euglobulin fraction of the patient's sera within 1 hour after the treatment. This was followed in several cases by a second peak at 6 to 10 hours. The *antiprotease* titer showed an inverse relationship, decreasing at the time the proteolytic titer was high. He also noted that patients who had the greatest increase in protease titers were those whose tumors showed the most response to the radiation therapy.

An increase in activity of tissue cathepsins following irradiation has been reported (3). Kocholaty et al. (45) have described a drop in the plasmin-inhibitor content of rat blood in the first few days after total body x-irradiation, with a rising titer beginning on the 5th or 6th day. No measurements for plasmin activation were made.

The development by the present authors of improved techniques for the separation and titration of the components of the blood protease-antiprotease system (11, 12, 15, 16) made feasible a more extended study of the effects of x-irradiation, the first results of which are presented here. These newer methods have a number of advantages over procedures previously used. They have permitted us (1) to eliminate the antiproteolytic power of serum or plasma *in vitro* so that the active plasmin present could be accurately titrated in the complete absence of inhibitor; (2) to bring about the conversion of plasminogen to active plasmin *in vitro* under control conditions; (3) to titrate the plasmin content of the inhibitor-free samples by a simple, rapid test, avoiding the complications of other, widely used procedures; and (4) to measure with accuracy the protease-inhibiting capacity of the original, untreated specimen.

The techniques permitted, furthermore, the determination of the initial proteolytic activity of the serum samples *immediately after removal of the inhibitor*, and before any spontaneous activation of plasminogen present could take place, *as well as* the measurement of the total potential proteolytic capacity of the same specimens after all the plasminogen had been converted to active enzyme. Thus, an *initial* protease titer and a *total* protease titer was obtained with each blood sample. This is in contrast to most of the previously published work on plasmin activity, in which only a single titer has been reported.

MATERIALS AND METHODS

The material studied consisted of blood serum specimens (1) from normal guinea pigs, (2) from groups of guinea pigs before and after exposure to known intensities of whole body x-irradiation, (3) from normal human beings, and (4) from cancer patients before and after they had received therapeutic doses of x-rays. Included in the latter group were 28 patients under special treatment who were rayed over the whole body area. A total of 487 samples of guinea pig serum from 156 irradiated animals and more than 100 specimens from 36 human beings after x-ray therapy were each titrated for their proteolytic and antiproteolytic activity. Available for comparison were the results of tests of the same kind carried out on 345 normal guinea pig sera and on more than 500 normal human sera. Guinea pigs were chosen as the experimental animal for these studies because they could easily be bled repeatedly to furnish the desired number of blood samples, and because we were already acquainted with the levels of proteolytic and antiproteolytic activity exhibited by this species in health, and during anaphylactic reactions (10, 13). Guinea pigs have been shown to be entirely suitable for studies of reactions to x-irradiation (36).

Serum specimens

Blood samples from guinea pigs were obtained in dry, sterile syringes by cardiac puncture of the unanesthetized animals. The technique of bleeding from the heart had been utilized for a period of years prior to the beginning of this work, and was so well practiced that it did not in itself introduce a significant factor in the mortality of the control animals. A number of the normal guinea pigs were bled 14 times or more without illness or fatality. However, mortality among the irradiated animals *was* associated with the cardiac puncture, especially at certain periods after the x-ray exposure, when the guinea pigs had developed a hemorrhagic tendency. This is taken into account in the reports which follow.

Patients hospitalized at the Veterans Administration Hospital, or at the University of Texas M.D. Anderson Hospital for Cancer Research, in Houston, Texas, were the source of human serum specimens taken before and after therapeutic x-ray.* Samples of normal human serum were obtained in part from healthy medical students, but principally from young men at the time of examination for military service.**

* footnotes on page 3.

The blood samples were allowed to clot at room temperature; then a sterile applicator stick was introduced to free the clots from the walls of the tubes and left there while the tubes were refrigerated overnight. The next morning the serum was separated and stored in sterile, stoppered tubes at 4° to 10° C. All the tests on any one serum sample were carried out at the same time, usually within 24 to 36 hours after collection of the blood.

Irradiation of guinea pigs

Through the kindness of Dr. Lee D. Cady, manager, Veterans Administration Hospital, Houston, and the generous cooperation of the Radiological Service,* a 220 kv., deep therapy x-ray machine was made available for the irradiation of the experimental animals. The guinea pigs were held in a plywood box, without cover, holding 6 animals, each in a separate compartment. After the guinea pigs were in place they were covered by a muslin cloth, and the box was centered under the x-ray beam. The particular compartment occupied by each individual animal was always recorded, but there was no indication in any of the experiments that there was any difference in the amount of radiation each guinea pig received, regardless of its position in the box. A constant intensity of irradiation was used, after careful checking by a Victoreen dose meter, and the doses of radiation were controlled by varying the time of exposure.

Determination of proteolytic activity of serum samples

The methods used have been described in detail in previous reports (15, 16). A portion of each serum was first treated with a decalcifying agent—trisodium phosphate—to eliminate its natural content of plasmin inhibitor. A small amount of the serum, usually 0.3 ml., was mixed with an equal volume of 5 percent aqueous solution of trisodium phosphate and allowed to stand at room temperature for exactly 1 hour. The

Footnotes for preceding page.

*We are indebted to several persons for their help in securing these specimens. We wish particularly to thank Dr. Gilbert H. Fletcher, radiologist, M.D. Anderson Hospital for Cancer Research, and consultant in radiology, V.A. Hospital, for his invaluable assistance. The cooperation, throughout the work, of Dr. Lee D. Cady, manager, Dr. Bela Halpert, chief of laboratories, and Dr. Charles L. Spurt, chief, Medical Research Laboratory, V.A. Hospital, is greatly appreciated.

**For assistance in this connection we wish to thank Reuben D. Wende, director, City Health Department Laboratories, Houston. Many of the tests on these sera were performed by Iva Baggett, serologist, V.A. Hospital, Houston.

*Special thanks are due to Dr. Gilbert H. Fletcher, consultant in radiology, Dr. Harry L. Barton, acting chief, Radiological Service, and Mrs. Aline Brown, x-ray technician.

mixture was then diluted 1:30 with the stock borate-boric acid buffer, pH 7.5. This restored the pH to approximately 7.5, and eliminated by dilution any trace of plasmin-inhibitor that may have persisted in the original phosphate-serum mixture at the 1-hour time.

A portion of this 1:30 dilution was then titrated by the rennin-digestion method (16) for proteolytic activity at once (before there was opportunity for any spontaneous activation of the plasminogen present). The result was recorded as the *initial protease (IP) titer*. This titer may be assumed to represent the amount of *active plasmin* present in the original serum—that is, in vivo, at the time the sample was collected.

Another portion of the same 1:30 dilution of the original phosphate-serum mixture (now inhibitor-free) was treated with a 5 percent proteose peptone solution (0.05 ml. of the peptone solution for each 6.0 ml. of the diluted, decalcified serum), in order to bring about the conversion of its contained plasminogen to active plasmin, and thus permit determination of its *total potential proteolytic activity*. This peptone-activated portion was now titrated by the rennin digestion technique, in parallel with the IP test, to reveal the *total protease (TP) titer*.

The IP and TP titers were expressed (and are tabulated in this paper) as the reciprocal of the highest dilution of the serum under test which digested all of the rennin under the standardized conditions of this procedure. The range of actual titers obtained was from 56 to 450.

Titration of antiproteolytic power of serum specimens

Each of the original untreated sera was tested for antitryptic activity by our filmstrip gelatin digestion method (11, 12). This procedure had been fully standardized and had been continuously in use for two years previously.

Results were expressed, and are tabulated here, in terms of the *antitryptic index (ATI)*. To obtain this index the *difference* in the tryptic units (in terms of mg./ml. trypsin) required for the complete digestion of all the gelatin on the filmstrips in the presence of a particular serum, and that required in the trypsin control tubes, without serum, was first determined. This figure was then multiplied by 100 (the final dilution of the serum in the test) to give the antitryptic index.

In addition to antitrypsin tests a number of both human and guinea pig sera were titrated for their power to inhibit the homologous active *plasmin*.

The capacity of individual whole, untreated sera to inhibit the activity of plasmin derived from a decalcified, peptone-activated portion of the very same sera was tested in approximately 150 instances. The antiproteolytic measurements thus obtained were not significantly different from those revealed by the more precise antitrypsin tests, and in our opinion, by their very nature, could not be expected to be as sensitive or reliable (15, 16). Although the identity of antiplasmin and antitrypsin has been questioned (59) our experience clearly supports the prevalent view that measurement of antitrypsin serves to titrate accurately the natural antiprotease as well (16).

Clotting and bleeding time determinations—platelet counts

The coagulation time of the blood of the normal control guinea pigs and of the irradiated animals was determined at the same time that blood samples were secured for proteolytic and antiproteolytic titrations. The method used was the capillary tube method, McGowan's modification (8). Blood freely flowing from a small scalpel cut in a foot pad was allowed to enter capillary tubes. Clotting time was estimated by noting the time that elapsed before a fiber thread appeared when a capillary tube was broken apart.

Bleeding time determinations were made at the same time as clotting time tests by the method of Duke (8, p. 145). The blood flowing from the skin puncture made in the sole of the foot was blotted with filter paper every few seconds. The bleeding time was counted as that period elapsing from the time of the skin puncture until no blood appeared on the filter paper.

Platelet counts were performed by placing a small drop of brilliant cresyl blue stain on a slide and mixing a small drop of capillary blood with it. The

mixture was then covered with a cover glass and the ratio of platelets to red cells was determined.

EXPERIMENTAL RESULTS

1. Serum protease and antitrypsin levels

The mean values for initial and total protease (IP and TP) and for the antitryptic index (ATI), obtained in tests on normal human and guinea pig sera, are presented in table I. Included among the guinea pigs represented here are the same animals used in the irradiation experiments described below.

An unexpected finding was the almost identical average titers for both initial and total protease shown by the 512 samples of human serum and the 344 specimens of guinea pig serum. It appears that the amount of active plasmin, and also of plasminogen, normally present in the serum of healthy individuals of either species is about the same, and that the total potential proteolytic activity of the specimens, expressed by average TP titers of 184.9 to 188, was somewhat more than twice that exhibited by the same sera before full activation of their contained plasminogen, when the average IP titers were 82.6 to 82.7. The individual titers were distributed closely about the averages, as indicated by the low figures for the error of the means. The greatest amount of variation appeared in the total protease titers of human sera, where the range was from a titer of 113 to 375, but even so, the standard error of the mean value (188) was only 6.7.

In contrast to this virtually equal concentration of the blood protease and its precursor in the serum of human beings and of guinea pigs alike, the average antitryptic index of the normal guinea pig sera (162.9 ± 7.1) was found to be more than twice that of the human sera (76.0 ± 0.2). This especially high level of antitrypsin in guinea pig blood was first

TABLE I

Mean values for initial and total serum protease titers and for antitryptic index in normal human beings and guinea pigs.

Serum specimens		Initial protease (IP)			Total protease (TP)			Antitryptic index (ATI)		
Source	No.	Range of titers	Mean titer	S. E. of mean	Range of titers	Mean titer	S. E. of mean	Range of indices	Mean index	S. E. of mean
Normal human beings	512	56-150	82.7	0.6	113-375	188.0	6.7	37.5-137.5	76.0	0.2
	512									
	719									
Normal guinea pigs	344	56-150	82.6	1.0	90-450	184.9	2.8	75-300	162.9	7.1
	345									
	341									

able activation of the serum protease (i.e., a significant rise in initial protease titer) in irradiated guinea pigs within an hour or two after the x-ray exposure was dependent upon exposure to a sufficient intensity of radiation. In table VIII, a tabulation is presented, showing the amount of increase in the mean IP titers observed at the 1- to 2-hour interval after doses of external radiation, ranging from 25 r to 150 r. The 30 animals receiving the highest dose of x-ray had an average rise in their serum IP titers of 34 percent. This increase was twice that recorded among similar groups of guinea pigs receiving the relatively low doses of 25 to 50 r.

VII. The serum protease-antiprotease balance in human beings after exposure to x-rays

Specimens of blood were obtained from 36 cancer patients before and after exposure to therapeutic doses of x-rays. Of these, 8 received local irradiation only, while the remaining 28 patients were exposed to a whole body irradiation of 15 r to 50 r. The latter were voluntary participants in a special study being carried out under the direction of Dr. Gilbert H. Fletcher, radiologist, M.D. Anderson Hospital for Cancer Research, Houston.

Cancer patients receiving local therapeutic x-ray doses

The principal findings in tests for IP, TP, and ATI on this group of 8 patients are summarized in table IX. A blood specimen was obtained from each patient just before the treatment and also within approximately 1 hour afterward.

As shown in table IX a clear majority of the IP titers increased after the irradiation (eight out of a total of fourteen tests) while the TP titers also rose nine different times. The amount of increase in the IP titers ranged from 15 to 60 points, averaging 33 points. The post-exposure titers were lower in a few instances, however, and remained the same in a few others. Nevertheless, the tendency for an increase in the proteolytic activity of the serum from cancer patients after local x-ray therapy was clearly indicated. It should be noted that most of this group of cancer patients had already been given several previous courses of local x-ray therapy, which may have reduced the amount of response at the time of these tests.

In the case of one individual (No. 7), who had not previously received x-ray treatment, specimens were obtained not only at the 1-hour interval after each treatment, but also 4 or 5 hours afterwards, following each of a series of five successive local

therapeutic irradiations of 600 r. The changes in the serum IP and TP titers are charted in figure 14. Evidently an elevation in IP and TP titers did not occur after every exposure to local x-rays, yet an immediate steep rise, of obviously significant degree, was recorded after the first treatment, and again following the fifth treatment given 7 days later.

The serum antitryptic index of these patients remained virtually unchanged at the 1-hour post-irradiation interval. The average was above normal, however, to begin with, as was to be expected in cancer patients. Unfortunately, tests at later intervals were not made.

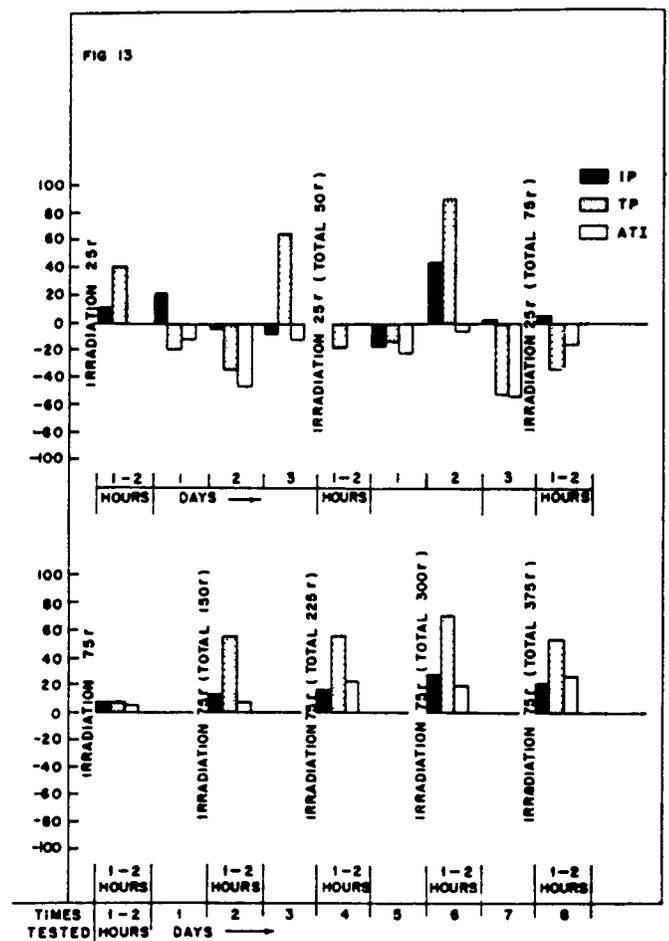


FIGURE 13

Differences between the mean titers of animals tested at different time intervals after exposure to repeated doses of whole body x-irradiation and the mean titers of the same guinea pigs before irradiation. Experiments IV and V.

Cancer patients receiving 15 to 50 r whole body irradiation. Blood samples were secured from these 28 patients just before the irradiation, and again 2 hours and 12 hours afterwards. The findings are summarized in table X. In order to facilitate discussion the patients are arbitrarily classified into several groups: 3 patients with carcinoma of the cervix; 4 with cancer of the thyroid or pharynx; 5 with leukemia; 8 with cancer of the lymphatic system; 4 with carcinoma of the breast; and 4 with miscellaneous types of skin cancers.

It will be noted that the average serum IP titer of the 28 patients before the x-ray treatment was 82, a figure almost identical to that obtained by us with the large group of 512 normal human sera (table I). The overall average was increased to 88 at the 2-hour post-irradiation interval, with a drop to 85 at 12 hours. Fifty-six specimens were tested after the treatments. Of these 25 (45 percent) showed an increased IP titer over that of the same patient's serum before irradiation, while 10 (18 percent) of the

citers were reduced, and 21 (38 percent) remained unchanged.

The average TP titers of these patients changed in a similar way, from 175 before treatment to 195 afterwards. A similar proportion (49 percent) of the TP titers were elevated at the 2-hour or 12-hour post-irradiation time.

In contrast, the direction of change in the figures for ATI after irradiation was downward, 32 (58 percent) out of the 55 tests showing a lower index than the average before the treatment, and only 16 (29 percent) showing an increased antitryptic power.

Thus, the tendency for the initial and potential proteolytic activity of the blood serum to rise directly after irradiation was again demonstrated, although the changes were not striking or constant. On the other hand, the antiproteolytic power of the same serum samples was not usually increased; instead somewhat lower averages for ATI were more often recorded. It would seem likely that the relatively slight changes of the protease-antiprotease system

TABLE VII

Changes in the average levels of serum IP, TP, and ATI in guinea pigs after exposure to repeated doses of whole body x-irradiation of 75 r every second day. Experiment V.

Number specimens	Times tested after irradiation	Initial protease			Total protease			Antitryptic index		
		Mean titers		Differences*	Mean titers		Differences*	Mean titers		Differences*
		Before irradiation	After irradiation		Before irradiation	After irradiation		Before irradiation	After irradiation	
48 (12 g.p.)		78 (±1.6)			186 (±4.6)			151 (±5.5)		
0 day		Irradiation 75 r								
12	1-2 hr.	78	87	+ 9	186	195	+ 9	151	156	+ 5
2 days		Irradiation 75 r (total 150 r)								
12	1-2 hr.	78	91	+13	186	243	+57	151	158	+ 7
4 days		Irradiation 75 r (total 225 r)								
12	1-2 hr.	78	95	+17	186	243	+57	151	173	+22
6 days		Irradiation 75 r (total 300 r)								
12	1-2 hr.	78	107	+29	186	256	+70	151	171	+20
8 days		Irradiation 75 r (total 375 r)								
12	1-2 hr.	78	99	+21	186	240	+54	151	179	+28
14 days		Irradiation 75 r (total 450 r)								
6	1-2 hr.	78	92	+14	186	208	+22	150	155	+ 5

*Differences between the mean titers of animals tested at different time intervals and the mean titers of the same animals before repeated irradiation.

following irradiation in these patients could be attributed to the fact that the doses of irradiation used were quite low.

DISCUSSION

The results of the present study confirm and extend the findings of Petersen and Saelhof (54, 55) and of Clifton (21). They show that a rather profound and prolonged disturbance in the normal content of proteolytic and antiproteolytic components of the blood serum occurs after exposure to x-rays in both human beings and guinea pigs. They indicate, further, that the degree of this disturbance is related to the intensity of the radiation received.

The most regularly observed change in the sera of irradiated individuals was a marked increase in what we have called the *initial protease (IP) titers*—that is, in the capacity of the sera to produce proteolysis as soon as the natural protease-inhibitor in the serum was eliminated. This rise in IP titers took place within the first hour or two after the irradiation, and relatively high titers often persisted for 2 or 3 days. The *total proteolytic capacity* of the serum at the same 1- to 2-hour post-irradiation interval (the *total protease (TP) titer*) was *not* usually increased to the same extent. Hence, it would appear that what happens in the blood directly after irradiation is primarily the conversion of an excessive amount of the inactive precursor, plasminogen, into active plasmin—a true activation, or mobilization, of the serum protease, rather than an increase in the total potential proteolytic power. An apparently simultaneous rise in both the initial and total protease titers immediately after irradiation was also observed, however, and in any case the curves for the two measurements soon came to follow an essentially parallel course.

In addition to this early enhancement of the serum proteolytic activity, increases in both the IP and TP titers above the normal, pre-irradiation levels, of an even greater degree, were recorded later, especially during a 2- to 4-day period beginning about the 19th post-irradiation day. The average guinea pig protease titers thus continued to fluctuate throughout at least 30 days after a single dose of whole body x-irradiation, to an extent not observed among nonirradiated control animals.

Although these data on serum protease are of unprecedented nature and depend upon techniques which have not as yet been critically tested by other investigators, the general consistency of the findings with the sera of both normal and irradiated

animals and human beings would seem to speak for their validity.

The process by which plasminogen is converted into active enzyme *in vivo* is not as yet fully understood, and further critical studies are needed. According to Astrup (2) and his associates (50, 51) the conversion of plasminogen to plasmin is not direct, but rather, it is mediated by an activator substance, which was shown to be present in spontaneously active human blood. We also have noticed that an increased proteolytic activity is produced in normal serum after addition of inhibitor-free serum containing active plasmin, or trypsin, and that this increase is out of proportion to the amount of protease added. Kocholaty et al. (44) have reported similar observations.

The studies described in this paper, as well as in earlier reports (15, 16) show conclusively that plasmin is always present in *normal* as well as *pathological serum*, together with plasminogen and protease-inhibitor. The presence of active fibrinolytic enzyme in the plasma of normal people has recently been confirmed by Fearnley and Tweed (31). Ionizing radiation seems to cause an immediate conversion of more of the plasminogen available in the blood to plasmin, thus mobilizing more active protease than is normally present. This may or may not result in a sufficient concentration to lead to excessive tissue proteolysis within the body, an effect which must depend upon the balance prevailing at any moment, locally or in the circulating blood, between the amount of active enzyme and the quantity of inhibitor.

The exceptionally high *total protease* titers found about the 6th, 11th, and especially the 20th to the 23d post-irradiation days, would seem to involve the

TABLE VIII

Effect of intensity of total body irradiation upon the amount of increase in the initial proteolytic activity of the serum (IP titers) within the first 2 hours after irradiation.

Dose of radiation (r)	Number of guinea pigs	Mean titers		Increase in titers	
		Before irradiation	1-2 hr. after irradiation	Amount	Percent
25	5	73	85	+13	17.8
50	12	76	87	+11	14.4
100	7	78	101	+23	29.4
150	30	79	106	+27	34.1