Control of Radiation Hemorrhage with Splenic Extracts

By J. Philip Savitsky

The most significant alteration in the hemostatic mechanism following whole body irradiation appears at present to be the marked thrombocytopenia. Associated with the lack of platelets are defects in normal platelet activity such as poor prothrombin utilization, hypothyromboplastinemia, and absent clot retraction. Transfusion of normal platelets into these animals results in a temporary reversal of the clotting defect and a return towards normal of those hemostatic functions associated with the platelets.

It has been recently observed that prior to the development of the thrombocytopenia in the irradiated dog there is a change in the surface properties of the platelets as measured by the platelet adhesiveness and the clot retraction time. This suggested that changes in these properties of the platelets might play some role in the subsequent rapidly developing thrombocytopenia. Furthermore, a substance in the plasma of the irradiated animals was found which decreased the platelet adhesiveness and prolonged the clot retraction time of normal blood.

It seemed possible that in vivo neutralization of this plasma clot retraction time inhibitor in the irradiated animal might prevent the qualitative changes in the platelets and thereby modify the subsequent thrombocytopenia and hemorrhagic state.

The present article describes the finding of a tissue clot retraction accelerator (TCRA) which could neutralize in vitro and in vivo the plasma clot retraction time inhibitor present in irradiated dogs. Evidence will be presented that a protein extract of beef spleen containing TCRA can control the postirradiation hemorrhagic state but that more highly purified TCRA preparations are only partially effective.

Methods

Platelet Adhesiveness and Platelet Count

The platelet adhesive index was determined by the glass-wool method of Moolten and Vroman. The normal index in the dog varied from 1.15 to 1.35 and is reproducible with less than 10 percent error. Platelet counts were performed in the course of determination of the adhesive index.

Clot Retraction Time

The clot retraction time was determined by the method of recalcified venous blood in castor oil which is a modification of Hirschboeck's earlier method. This was modified to account for the more rapid clotting of recalcified dog's blood. 4.5 ml. of venous blood was

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Submitted April 12, 1954; accepted for publication July 21, 1954.

This investigation was done under Contract No. AT(30-1)-1544 with the U.S. Atomic Energy Commission.

The author wishes to thank Drs. Sol Sherry and Walter Troll for their valuable advice and Mr. Dwight W. Callaway for his excellent technical assistance. Dr. Lee Rosenberg of the Department of Radiology of the Jewish Hospital of Cincinnati kindly gave his assistance in the irradiation of the animals.
drawn into a syringe containing 0.5 ml. of 3.8 per cent sodium citrate. The blood was placed in a lusteroid tube and permitted to stand at room temperature for 10 minutes. 1 ml. of this blood was then placed in each of two glass tubes in a water bath at 18 C., and .06 ml. of 5 per cent CaCl₂·2H₂O solution was added to each tube. Immediately following the addition of the calcium, blood was drawn up in a siliconized capillary pipet and deposited as a single drop in castor oil. The tubes of castor oil were kept at 18 C. and the test was done in quadruplicate. The castor oil tubes were observed and the clot retraction time taken as the time from the clotting of the recalculated blood to the exudation of serum from the drop of castor oil. The calcium solution must be prepared on the day of use. The normal range, as performed on 15 dogs, was from 6 to 10 minutes with a mean value of 7.5 minutes.

Clotting Time

The clotting time was done in a glass clotting tube at room temperature. The blood was drawn using the two-syringe technic. The normal clotting time in the dogs varied from 3 to 6 minutes.

Assay of Tissue Clot Retraction Accelerator (TCRA)

A clot retraction accelerator can only be studied in a clotting system such as normal blood where there are sufficient normal platelets to produce good retraction. However, the initiation of clot retraction occurs so rapidly in normal dog's blood that it is difficult to study any acceleration. Therefore, the TCRA was assayed by determining its capacity to neutralize the clot retraction time inhibitor present in plasma from irradiated dogs. Plasma drawn from animals who were thrombocytopenic (10 to 14 days post radiation) was added to normal dog blood with and without dilutions of the TCRA. The amount of the TCRA present in the sample tested was found by determining the highest dilution of the TCRA preparation at which the delayed clot retraction time was returned to normal. A unit was defined as that amount of the TCRA which would fully neutralize the inhibiting effect of 2 cc. of plasma from an irradiated animal. All the plasmas from irradiated animals had the same clot retraction time inhibitor content (within 25 per cent), which was sufficiently uniform to standardize the assay. The technic of the procedure used is shown in the accompanying chart.

Control Inhibitor Accelerator

<table>
<thead>
<tr>
<th>Control</th>
<th>Inhibitor</th>
<th>Accelerator</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 ml. normal blood + 0.2 ml. citrate-saline + 0.1 ml. saline + 0.07 ml. 2/4% CaCl₂</td>
<td>0.5 ml. normal blood + 0.2 ml. plasma (irrad.) + 0.1 ml. saline + 0.07 ml. CaCl₂</td>
<td>0.5 ml. normal blood + 0.2 ml. plasma (irrad.) + 0.1 ml. TCRA in saline + 0.07 ml. CaCl₂</td>
</tr>
</tbody>
</table>

The glass tubes containing these mixtures were kept at 12 C. and recalcified simultaneously. Ten minutes following recalcification, the tubes were carefully and gently "ringed" with a thin steel wire. Ten minutes later the tubes were examined for evidence of retraction. The control tubes always showed retraction and the inhibitor tubes never showed retraction. The tubes containing sufficient TCRA to neutralize the inhibitor showed retraction, but those containing the dilute solutions of TCRA did not show retraction. The highest dilution of TCRA which had retracted at this time was considered the endpoint. All tests were done in duplicate.

Radiation

Dogs weighing from 20 to 30 pounds received 400 r., 250 kv. peak, 12 ma., dose rate 20 r/min. Target skin distance—100 cm. The dosage of 400 roentgens in these animals was LD₉₀. The dogs received nembutal for anesthesia.

The guinea pigs received 600 r., 250 kv. peak, 12 ma., dose rate 80 r/min. Target skin distance—50 cm. The dosage of 600 roentgens in these animals was LD₉₀.
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RESULTS

There is a substance present in saline extracts of all tissues which accelerates the initiation of clot retraction. To determine which organ of the body contains the largest quantity of the tissue clot retraction accelerator (TCRA) extracts of the various organs of the rat and mouse were tested on human blood using the methods described previously.\textsuperscript{10}

The animals were killed with ether and the lungs, heart, brain, liver, kidney, and spleen immediately removed, chopped, and washed several times with cold saline, to remove all blood. The material was then suspended as a 10 per cent solution in saline, homogenized in a cooled Waring Blender and centrifuged at 4,000 rpm. for 30 minutes in an angle centrifuge. The supernatant was then tested on human blood at various dilutions for its ability to accelerate the initiation of clot retraction. Typical results for immature and adult rats are shown in table 1. It is clear that the highest concentration of TCRA was found in the spleen and less in the other organs. Of interest was the consistent finding that all the tissues in immature rats and mice contained more TCRA than the same tissues in the adult. A high concentration of TCRA was present in the spleens of all mammalian species studied, including guinea pigs, hamsters, cats, dogs, and cows. Beef spleen was used as the starting material in all the following studies.

Saline extracts of beef spleen containing TCRA neutralized the plasma clot retraction time inhibitor present in irradiated dogs. Typical results are shown in table 2. This neutralization of the clot retraction time inhibitor was used as the assay for separation and purification of the TCRA. It was found that TCRA was not in the nuclear or mitochondria fractions but was present in the supernatant which did not sediment at 20,000 rpm.

A partially purified preparation from beef spleen was prepared in the following manner. Ten pounds of fresh spleen were ground, suspended as a 25 per cent solution in saline, homogenized in a cooled Waring Blender, and filtered through

<table>
<thead>
<tr>
<th>Table 1.—Amount of TCRA in Various Tissues</th>
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<tbody>
<tr>
<td>End point dilution</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Rat #3 (adult)</td>
</tr>
<tr>
<td>Rat #6 (adult)</td>
</tr>
<tr>
<td>Rat #12 (28 days)</td>
</tr>
<tr>
<td>Rat #13 (38 days)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2.—Neutralization of Plasma From Radiated Dogs by TCRA</th>
</tr>
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<tbody>
<tr>
<td>Clot retraction time</td>
</tr>
<tr>
<td>1 ml. normal blood plus 0.2 ml. citrate-saline</td>
</tr>
<tr>
<td>1 ml. normal blood plus 0.2 ml. citrate-saline plus 0.1 ml. saline</td>
</tr>
<tr>
<td>1 ml. normal blood plus 0.2 ml. plasma (irrad.)</td>
</tr>
<tr>
<td>1 ml. normal blood plus 0.2 ml. plasma (irrad.) plus 0.1 ml. saline</td>
</tr>
<tr>
<td>1 ml. normal blood plus 0.2 ml. plasma (irrad.) plus 0.1 ml. TCRA</td>
</tr>
<tr>
<td>1 ml. normal blood plus 0.2 ml. plasma (irrad.) plus 0.1 ml. TCRA</td>
</tr>
</tbody>
</table>
Crude spleen High speed centrifuged extract Fraction I Crude acetone extract Purified acetone extract

The filtrate was centrifuged at 4,000 rpm. and the supernatant spun at 18,000 rpm. in a refrigerated angle centrifuge. The pH of the clear supernatant fluid was brought to 5.2 and a heavy precipitate fell out in the cold. Following centrifugation in the cold, the precipitate was taken up in distilled water, the pH brought to 6.1 and the material again centrifuged. The clear colorless supernatant contained the TCRA. This was lyophilized and yielded 1.5 Gm. of a brownish-white powder which has been termed Spleen Fraction I. Spleen Fraction I contained a minute amount of thromboplastic material (less than 1 per cent of the TCRA content) and constituted a 50 fold purification of TCRA over the crude spleen (table 3).

Sixteen healthy dogs received 400 r of whole body irradiation and eight of them were given daily intramuscular injections starting 2 hours following irradiation of 20 mg. of Spleen Fraction I until the time of death. Daily determinations were made of the platelet count, platelet adhesiveness, clot retraction time and clotting time of the injected and the control animals and the results shown in figures 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14.

**Table 3.—Assay of TCRA Content in Preparations**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Units/mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude spleen</td>
<td>1.2</td>
</tr>
<tr>
<td>High speed centrifuged extract</td>
<td>3.2</td>
</tr>
<tr>
<td>Fraction I</td>
<td>64.0</td>
</tr>
<tr>
<td>Crude acetone extract</td>
<td>400</td>
</tr>
<tr>
<td>Purified acetone extract</td>
<td>3000</td>
</tr>
</tbody>
</table>

**Fig. 1.—** Clotting times of eight (8) irradiated control dogs and of eight (8) irradiated dogs receiving 20 mg. daily of Fraction I.
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Figure 2.—Platelet counts of eight (8) irradiated control dogs and of eight (8) irradiated dogs receiving 20 mg. daily of Fraction I.

3. Figure 1 shows the effectiveness of Spleen Fraction I in keeping the daily clotting times normal as compared to the controls. Figure 2 demonstrates the significant deceleration produced by Spleen Fraction I in the onset and extent of the radiation thrombocytopenia. Figure 3 shows that Spleen Fraction I was able to maintain a normal clot retraction time and a normal platelet adhesiveness in the irradiated animals. The animals which died were autopsied and examined for macroscopic evidence of hemorrhage. Table 4 demonstrates the control of hemorrhage in these dogs and in a group of irradiated guinea pigs. In the two injected dogs where hemorrhage was found, it was moderate and localized to single areas of gross infection. There was no difference whatsoever in survival between those animals injected with TCRA (Spleen Fraction I) and the controls.

Further studies of the TCRA demonstrated that it could be extracted from the lyophilized Spleen Fraction I by acetone. A preparation of TCRA which constituted a purification of 2,500 times over crude spleen was made in the following way. The lyophilized Spleen Fraction I was extracted with acetone in a soxhlet apparatus for 3 hours. The acetone solution was then evaporated to a small volume (50 cc.) and placed in the deep freeze overnight. The precipitate was removed by centrifugation, washed twice with ether, dried, taken up in ethanol, filtered to remove the material insoluble in ethanol at room temperature, dried and taken up in a large volume of distilled water. This was filtered, the filtrate lyophilized, and a white powder obtained containing 3,000 units of TCRA activity per milligram.

This “purified acetone extract” was then tested on 9 dogs who received 400 roentgens irradiation. Each animal was injected daily postirradiation with 2 mg. of the acetone TCRA extract, and daily studies of the platelet adhesiveness, platelet count, clot retraction time, and clotting time were performed. Similar observations were made on 4 control animals irradiated at the same time. The
FIG. 3.—Platelet adhesive indexes and clot retraction times of eight (8) irradiated control dogs and of eight (8) irradiated dogs receiving 20 mg. daily of Fraction I.

TABLE 4.—The Control of Hemorrhage in Irradiated Dogs and Guinea Pigs

<table>
<thead>
<tr>
<th></th>
<th>Number of animals</th>
<th>Gross hemorrhage</th>
<th>Moderate hemorrhage</th>
<th>No hemorrhage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Irradiated dogs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All controls*</td>
<td>15</td>
<td>12</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Fraction I†</td>
<td>8</td>
<td>0</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Acetone fraction</td>
<td>9</td>
<td>6</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Acetone plus alcohol fraction</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td><strong>Irradiated guinea pigs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All controls</td>
<td>36</td>
<td>26</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Fraction I†</td>
<td>12</td>
<td>0</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Acetone plus alcohol fraction</td>
<td>36</td>
<td>7</td>
<td>24</td>
<td>5</td>
</tr>
</tbody>
</table>

* One control animal was exsanguinated to obtain plasma for study.
† One dog survived.

effects of these injections on the clotting time is shown in figure 4. The acetone TCRA extract had only a slight effect in modifying the clotting defect, and in delaying the onset of the thrombocytopenia. These effects were in no way comparable to those observed with Spleen Fraction I. The purified TCRA, however, was able to keep the clot retraction time and platelet adhesiveness entirely
CONTROLS

**Figure 4.**—Clotting times of seven (7) irradiated control dogs and of irradiated dogs injected with the acetone extract (TCRA) alone and in conjunction with the ethanol extract.

normal throughout the postradiation period. At autopsy, all the dogs showed evidence of hemorrhage (table 4).

In the process of purification of TCRA the ability to control the hemorrhagic phenomena was lost indicating that Spleen Fraction I, which contained protein, had present other substances active in controlling the hemorrhage. Extraction of the acetone extracted residue with ethanol resulted in a preparation which in conjunction with the purified TCRA was capable of further modifying the clotting defect. This material was prepared by extracting the acetone residue in a Soxhlet apparatus for 3 hours with ethanol. The alcohol was then removed in vacuo and the material taken up in ether. The ether insoluble fraction was separated by centrifugation in the cold, dried, taken up in distilled water, filtered and the filtrate lyophilized.

Three irradiated dogs were injected daily with 2 mg. of the acetone-extracted TCRA and 10 mg. of the alcohol preparation. Daily determinations were made of the platelet count and clotting time on these animals and on 3 controls. It is evident from figure 4 that there was a marked modification but not complete control of the clotting time. The changes in the platelet count were similar to those found with Spleen Fraction I (fig. 2), where there was a marked delay in the onset of the thrombocytopenia. Autopsies on these animals showed only very moderate and localized hemorrhages (table 4). Three dozen guinea pigs which had received 600 roentgens irradiation were injected daily postradiation with 0.25 mg. of the purified TCRA and 0.5 mg. of the alcohol preparation and the extent of hemorrhage compared with controls. Table 4 shows that these injections markedly modified the hemorrhages although they did not prevent them as did Spleen Fraction I.

Studies of the nature of TCRA indicated that amino acids were present in the
most purified preparations but the TCRA was shown by chromatographic tech-
nics to be not an amino acid or peptide. It disappeared following acid hydrolysis
but was stable to mild alkaline hydrolysis (1N KOH at 37 C. for 24 hours). It
was dialyzable, heat stable, soluble in water and ethanol, only slightly soluble in
acetone, and totally insoluble in ether.

**Discussion**

The data presented indicate that there are substances present in beef spleen
which are capable of modifying the hemorrhage following whole body irradiation.
Almost total prevention of the hemorrhagic symptoms had no effect whatsoever
in prolonging survival of the irradiated animals, suggesting that the hemorrhage
probably plays no role in the ultimate mortality of the animals. The internal
hemorrhages might be only a preterminal phenomenon since thrombocytopenic
animals which had been exsanguinated at a time when they had a severe clotting
defect (12–15 days postradiation) showed little or no evidence of hemorrhage. In
addition, the seven animals in this laboratory that have survived the LD90 of
irradiation have developed the severe thrombocytopenia and clotting defect but
have never shown evidence of any purpura or abnormal bleeding.

The causes leading to radiation hemorrhage are quite complex. The thrombo-
cytopenia is apparently significant but the observation that those animals in-
jected with Spleen Fraction I developed no prolonged clotting time or evidence
of hemorrhage at autopsy despite thrombocytopenic levels indicated that only
small numbers of platelets are necessary for the hemostatic mechanism. These
observations are similar to those of Rosenthal and Benedek in the irradiated
rabbit where a marked thrombocytopenia is associated with a normal clotting
time and minimal hemorrhage. Similar too is the often noted observation that
some thrombocytopenic patients bleed and some do not although the platelet
levels may be equally depressed. The low platelet adhesiveness and prolonged
clot retraction time apparently do not contribute much to the clotting defect
because complete correction of these abnormalities with the injection of TCRA
(acetone extract) only slightly modified the clotting time, the thrombocytopenia
and the hemorrhage. There are several defects in addition, as shown by the observation
that the material extractable by alcohol partially controlled the clotting
time and the thrombocytopenia, in conjunction with TCRA, but not completely.
It is probable that the crude splenic preparation (Spleen Fraction I) which was
capable of controlling the clotting defect and the hemorrhage contained several
different materials, two of which have been separated.

Jacobson and his co-workers have made observations of the role of spleen
shielding and implantation in protection against radiation lethality. This work
has been extended by Cole et al. to the demonstration that splenic homoge-
nates, particularly the nuclear fraction, can protect irradiated animals. In con-
nection with this work, three questions have risen. First, are whole cells required
for the protection? Secondly, is there one specific substance or are many splenic
substances active in radiation protection? Thirdly, must the splenic extracts be
prepared from the same or closely related species to be effective? The evidence
presented here of the control of the hemorrhagic aspects of the acute radiation
syndrome can cast some light on these questions. It is obvious that there are no
intact cells in any of the preparations injected. Beef spleen extracts were equally
effective in the dog and the guinea pig in preventing hemorrhage, indicating no
species specificity. The finding that there are at least two and probably more sub-
stances necessary to merely control the hemorrhage without any prolongation
of survival suggests that probably many different substances are required to pre-
vent the manifold changes following whole body irradiation.

Moolten and his co-workers\textsuperscript{15, 14} have extracted a substance from spleen which
raises the platelet count and increases the platelet adhesiveness when injected
into rabbits. This “thrombocytosin” is a lipid with properties so similar to cho-
lesterol that it crystallizes with cholesterol and is difficult to separate from cho-
lesterol. Apparently thrombocytosin and TCRA are different substances since
their properties and physiological activities differ. The TCRA described here
accelerates the initiation of clot retraction time and raises the platelet ade-
hesiveness, but does not affect the platelet count. Thrombocytosin is ether soluble,
whereas TCRA is totally ether insoluble. Thrombocytosin is effective in vivo but
not in vitro in raising platelet adhesiveness,\textsuperscript{17} whereas, TCRA is effective both
in vivo and in vitro in controlling the platelet adhesiveness. It may be that throm-
bocytosin in vivo stimulates the production of platelets and of substances like
TCRA which control the qualitative changes in the platelets.

Conclusions

(1) There is a substance present in all mammalian tissues which accelerates
the initiation of clot retraction. This tissue clot retraction accelerator (TCRA) is
present in highest concentration in the spleen. The TCRA is capable of neutraliz-
ing in vitro and in vivo the plasma clot retraction time inhibitor present in irra-
diated dogs.

(2) A crude protein extract of beef spleen containing TCRA injected into irra-
diated dogs can prevent the hemorrhagic syndrome. Further purification of the
TCRA has indicated that it is only partly responsible for the control of the clot-
ting defect and that other substances are necessary.

Conclusiones in Interlingua

(1) Il existe in le textitos de omne mammala un substantia que accelerfa le
initiation del retraction del coagulo. Iste accelerator textital del retraction del
coagulo (ATRC) se trova le plus concentrate in le splen. ATRC es capace a
neutralisar in vitro e in vivo le inhibitor del tempore de retractiomi del coagulo
plasmatic que es presente in canes irradiate.

(2) Injectiones de un crude extracto proteinic ab splen bovin (que contine
ATRC) pote prevenir le syndrome hemorrhagic in canes irradiate. Le purificatiom
additional del extracto ha indicate que ATRC es solo partialmente responsabile
pro le inhibition del defecto coagulative e que su action es supplementate per
altere substantias.

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