THE ANTIGENICITY OF BOVINE THROMBIN:
CLINICAL EVALUATION

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THE RECENT RISE to importance of thrombin as a hemostatic agent has precipitated a real though largely unpublished debate on the subject of its antigenicity. Two incidental and unrelated factors have tended to promote division of opinion. On the one hand the tremendously rapid strides made in the purification of beef thrombin by the Iowa group under the leadership of Seegers, followed by proofs of its usefulness in surgery, have outdistanced research in the subsidiary problem of antigenicity. On the other, the collection of large quantities of human blood by the Red Cross for the benefit of the combat troops has afforded, in connection with its conversion to serum albumin, an abundant though temporary supply of human thrombin, the superiority of which has been championed by the Harvard group which sponsored the program of blood fractionation. The gulf between those who hold beef thrombin harmless in its surgical application and those who imply danger in the use of thrombin from any source except human blood remains unbridged by a connected line of evidence, and at best has been only sampled.

Antigenicity is a titanic problem handicapped by an interdiction against free transfer of animal evidence into human conclusions, and by a wide personal choice of values ranging from the scientifically absolute to the clinically tolerable. In this paper I shall do no more than to marshall the available evidence, add a new sampling, and reach an obviously temporary opinion. The question of the safety of animal thrombin, however, is of the moment: the cancellation in large part of the production of serum albumin resulted unavoidably in reduction of the by-products from which up to now human thrombin has been derived; the philanthropic nature of the blood collections by the Red Cross prevented the distribution by sale to civilian users; and surgeons therefore are giving increasing acceptance to the clotting agents derived from animal sources, such as bovine thrombin and rabbit clotting globulin, which have now been released by the National Institute of Health for commercial distribution. The case against bovine thrombin will be considered first.

POSITIVE RESULTS IN THE EXPERIMENTAL FIELD

1. EXPERIMENTS OF DR. BEATRICE CARRIER SEEGAL

There are no reports in the literature indicating that sensitization has been produced by injections of bovine thrombin, but immunologists affirm generally that thrombin, being a protein, under favorable experimental con-
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...should provoke antigenicity of some degree. A series of experiments done by Dr. Beatrice Carrier Seegal yielded successful sensitization of guinea pigs and rabbits to bovine thrombin. She has kindly communicated to me her experiments which may be summarized as follows:

**Sensitization of Guinea Pigs.** Eleven guinea pigs were injected subcutaneously with 0.5 or 1.0 mg. of alum precipitated bovine thrombin. Four weeks later, 6 of these animals were injected intravenously with 0.6 or 1.0 mg. of the thrombin in solution, given slowly over a period of 30 to 40 seconds in the foot vein. All 6 animals died within 3 to 4 minutes, showing the respiratory difficulty, the cyanosis, and the edema of the lungs typical of anaphylactic shock. The remaining 5 animals, after reactionless injections with human thrombin, likewise succumbed when bovine thrombin was injected. In addition to the 11 animals thus sensitized by subcutaneous injection of alum precipitated bovine thrombin, 3 animals (weight 350 to 400 gm.) were sensitized by intravenous injection of 1.4 to 2.0 mg. of thrombin in solution and tested for sensitivity 4 weeks later by the intravenous injection of 1.0 mg. of the antigen. Two of these animals died of anaphylactic shock, and the third showed symptoms of moderate shock with a drop in temperature of 1.8°F.

**Sensitization of Rabbits.** Three rabbits were injected intravenously with a total of 9 mg. each of alum precipitated bovine thrombin over a period of 24 days. Six days after the last injection a sample of blood was drawn from each animal. The serums were tested for precipitins with two lots of bovine thrombin and one lot of human thrombin, diluted 1–500 and 1–5000. All 3 serums strongly precipitated with both dilutions of the bovine thrombins. A minute precipitate occurred between human thrombin 1–500 and the serum of one rabbit. On the 7th day, the 3 sensitized rabbits were injected into the left cerebral area with bovine thrombin in amounts of 0.6, 0.4 and 0.4 mg. respectively in 0.3, 0.2 and 0.2 cc. physiological saline. Three normal rabbits were each similarly injected with 0.6 mg. of bovine thrombin in 0.3 cc. saline. The sensitized animal receiving the larger dose and 1 normal rabbit, 1½ to 2½ hours later, began circling to the right and falling to the right side, and both animals were dead the following morning. The other two sensitized animals showed clonic right-sided movements (and less active twitching on the left) beginning the day following injection and persisting until sacrifice at 3 days. At autopsy both of the rabbits that died at 20 hours showed slight gross increase in the size of the left cerebrum. One sensitized animal showed a centrally located area of hemorrhage and softening about 7 mm. in diameter, and this proved microscopically to contain nerve cells undergoing ischaemic necrosis, in addition to hemorrhage, oedema, and leucocytic infiltration—the “Arthus phenomenon.”

A second type of rabbit experiment was done by replacing the anterior chamber fluid of the eye with 0.4 mg. bovine thrombin dissolved in 0.2 cc. physiological saline. Four weeks later an intravenous injection of 3.5 to 4.0 mg. of bovine thrombin in solution brought death from anaphylactic shock to 1 out of 7 animals thus tested.

Important as these experiments are, certain conditions should be noted: a) The amount of material injected to produce shock was one-quarter to one-half the amount that had produced reactions in unsensitized animals; for example, a normal 298-gm. guinea pig died ten minutes after the rapid injection of 2.0 mg., while the remaining control animals, receiving 1.4 to 2.0 mg. by slow intravenous injection, showed temporary respiratory increase following injection. Likewise, a rabbit receiving 3.5 to 4.0 mg. of thrombin intravenously in the course of 4 minutes, died of intravascular clotting, and the injection time had to be prolonged to 10 to 20 minutes to permit survival by the rest. b) The potency of the solution of thrombin used intravenously is given as 100 Iowa units per mg., but the strength of the alum precipitated...
material is not recorded, nor is mention made of the nitrogen content. c) The potency of the human thrombin supplied by the Harvard Medical School that was used to detect cross-sensitization was unknown to Dr. Seegal. d) On the basis of maximum non-lethal doses of 2.0 mg. given to a 250-gm. normal guinea pig, the comparable dose in the 70,000-gm. human being would be over 500 mg. or 50,000 Iowa units of bovine thrombin.

2. EXPERIMENTS OF DAVIDOFF ET AL.

The significance of the experiments to neurosurgery is augmented by a group of studies done between 1931 and 1942 concerning the local sensitization of the brains of animals. Davidoff, Seegal and Seegal demonstrated that a local allergic reaction can be produced in the brain of the rabbit by repeated cerebral and intravenous sensitizing injections of an antigen (red blood cells, horse serum, egg albumen) followed by intracerebral re-injection of the same antigen. Clinical symptoms of varying degrees of severity appeared, referable to the site of injection, and were often followed by death of the animal. The pathological picture in the brain was interpreted as the homologue of the Arthus phenomenon seen in the skin.

To afford more prolonged and intimate contact of the antigen with a local area of the brain, Davidoff and Kopelof inserted in a cavity cut in the motor cortex a cube of agar jelly containing horse serum, and fortified this sensitizing implant by simultaneously injecting horse serum intravenously. Two to 4 weeks later a "shocking" dose of the antigen was given intravenously, which produced generalized anaphylaxis and, in addition, transient hemiparesis. The experiments were pursued with the addition of egg albumen as an antigen, and it was found possible to layer both antigens upon uninjured cortex, horse serum on the left and egg albumen on the right, and produce hemiparesis at will by intravenous injections of the separate antigens, the weakness occurring invariably on the side contralateral to the implanted substances. Some Jacksonian convulsions occurred and occasionally death.

On advancing another step in the animal kingdom, these investigators proved, first, that anaphylaxis can indeed be produced in the monkey (hitherto thought impossible) if sufficient quantities of antigen are employed, although fully sensitized animals showed no evidence of precipitins at the time of fatal shock; and, second, that in monkeys sensitized by the intravenous route the application of the antigen by repeated or continuous contact with the brain results in contralateral weakness, Jacksonian seizures, or death. Injected antigens produced severe Arthus-like reactions of the brain, with local swelling of the hemisphere and aseptic necrosis, the pathological details of which were given in a later report. It may be added that the rhesus monkey, sensitized by repeated injections, was later found to develop precipitins both to horse serum and to egg white. With refinement of technique, the immunological cortical stimulus became a successful experimental tool in the study of epilepsy.
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NEGATIVE EVIDENCE OF SURGICAL TRIALS

1. REPORTS OF TIDRICK, SEEGERS AND WARNER

The thrombins prepared from rabbit serum and from bovine serum (as well as from human blood) have been utilized as clotting agents in clinical surgery, and reports on these substances, totalling about a dozen, were consulted to learn if reactions of any sort have been detected. In only one series however, was attention paid to antigenicity, although absence of direct toxicity was mentioned by several.

In a series of 225 surgical cases¹⁴ beef thrombin was used as a hemostatic agent, and a portion of this series together with additions was restudied with special reference to the use of thrombin in skin transplantations in 122 operations done in 53 patients.¹⁵ The latter report is especially valuable, since the skin is an excellent proving ground for antigenicity, and for the further reasons that thrombin of high potency (1,000 Iowa units per cc.) was used, the material was spread upon relatively large areas, and applications were often repeated in the same patient at intervals of many weeks—a combination of circumstances conducive to sensitization. In no instance, however, was there any suggestion of the development of hypersensitization.

To this may be added my experiences, first in an animal experiment designed to simulate surgical conditions and, second, in a clinical series.

2. AN EXPERIMENT ATTEMPTING TO PRODUCE ANTIGENICITY IN MONKEYS BY REPEATED IMPLANTATIONS OF GELATIN SPONGE SOAKED IN BOVINE THROMBIN

Six *Macacus rhesus* monkeys were used in this experiment in which implantations of thrombin-soaked gelatin sponge were made,¹¹ just as in clinical surgery in the control of hemostasis. The animals were tested at later dates for the development of the precipitin reaction in the blood serum, and for the reaction of the brain to an injection of the antigen. To improve the chances of a positive result two alterations were made in customary surgical procedure: a) the concentration of the thrombin solution was made 1,500 units (Upjohn*) per cc. as against about 100 units believed optimum for hemostasis when using the absorbable matrix technique; and b) multiple implantations (2 or 3) were made upon the same tissue at intervals of 3 days.

An informal canvass was made among several immunologists to determine the most favorable interval for development of sensitization to bovine thrombin, but the answers varied and a graduated schedule, therefore, was set up covering the period between 15 and 45 days after the last surgical operation. Details of the experimental calendar are given in Table I.

Operative Procedure. General anesthesia was used (cyclopal, 100 mg. per kg. body weight, intraperitoneal). The scalp was clipped, shaved, and prepared with soap, ether and mer cresin.

* A single standard for the measurement of clotting activity has not yet been agreed on. The Upjohn unit is roughly one-third as potent as the Iowa unit. Henceforth in this paper the Upjohn unit is implied since the thrombin used was an Upjohn research preparation.
Midline incision made. Cranium perforated and rongeured away to expose the superior sagittal sinus. Sinus opened by an incision about 6 mm. long. A piece of gelatin sponge, cut 5 x 10 x 10 mm. and soaked in bovine thrombin, 1,500 units per cc., was laid across the opening in the sinus to secure hemostasis. It is estimated that 150 to 200 units of thrombin were embodied in the resulting clot. The wound was then closed in layers with silk. Three days later the wound was reopened, the pledget of gelatin sponge was peeled off the dura, bleeding was re-instigated, and a new piece of sponge laid down to effect hemostasis; the wound was then closed. Again 3 days later the wound was reopened, and the process repeated.

It was my hope to prepare 6 animals in this manner. However, one died soon after the conclusion of the third operation; another was considered strong enough only for two operations, and one developed a wound infection following the second operation which, though held in check by penicillin, prohibited further surgical exposures. Despite these inadequacies 5 animals contributed to the experiment, as indicated by Table I.

Bleeding for precipitin tests occurred just before the third surgical implant, just before the intracerebral injection, and at sacrifice. Six tubes, each containing 0.5 cc. serum, were layered with the antigen in dilutions 1-10, 1-100, 1-1,000, 1-10,000 and 1-100,000. The 1-10 failed to result in a good layer, but the rest were satisfactory. Tests were incubated at 37°C. for 1 hour, observed for ring formation, then refrigerated overnight and observed again.

Each animal was tied to a board for an hour in advance of the intracerebral injection and kept there for 3 hours following, record being made of the

TABLE I. Calendar in days showing the schedule of surgical operations, intracerebral injections of the antigen, and sacrifice for 5 Macacus rhesus monkeys.

<table>
<thead>
<tr>
<th>Monkey No.</th>
<th>Surgical Implants</th>
<th>Injection</th>
<th>Sacrifice (days after injection)</th>
</tr>
</thead>
<tbody>
<tr>
<td>56</td>
<td>0 3</td>
<td>15*</td>
<td>3*</td>
</tr>
<tr>
<td>57</td>
<td>0 3</td>
<td>15*</td>
<td>3*</td>
</tr>
<tr>
<td>58</td>
<td>0 3 6*</td>
<td>24*</td>
<td>3*</td>
</tr>
<tr>
<td>53</td>
<td>0 3 6*</td>
<td>32*</td>
<td>3*</td>
</tr>
</tbody>
</table>

* Blood serum tested for precipitation with bovine thrombin in dilutions 1/10, 1/100, 1/1,000, 1/10,000 and 1/100,000. No positive tests.

pulse, respiratory rate, and temperature. The injection consisted of 0.1 cc. of sterile distilled water containing 10 units of thrombin given through a 25 g. needle inserted 10 to 15 mm. into the right cerebral hemisphere. The amount of thrombin injected contained approximately 1 mg. of solids. The lot analyzed 4.96 per cent nitrogen.

Results. The precipitin tests were negative throughout. The injection of the antigen into the brain failed to cause any observable change in the behavior or appearance of the animals, or alteration of the trend of the pulse, respiratory rate, or temperature. The animals remained normal during the
three-day interval before sacrifice. The implantation sites failed to show re-
actions different from those produced by a single implant, and the reaction
in the brain to the injection was only that which would be expected from the
mechanical trauma of the needle insertion. The Arthus phenomenon was not
seen.

3. precipitin tests in 15 patients in whom bovine thrombin
was used for neurosurgical hemostasis

In a group of patients in whom bovine thrombin had been used in the
course of routine neurosurgical operations (see Light and Prentice for
types of procedures), precipitin tests were done on those willing to submit to

| TABLE II. Thirteen patients submitting to precipitin tests following surgical operation at which bovine thrombin was used as a hemostatic agent, implanted on pieces of gelatin sponge. |
|---|---|---|---|---|
| No. of Hemostatic Patches Laid Down | Thrombin: Units (Upjohn) per cc. | Post-oper. Interval in Days | Precipitin Tests |
| 1 | 300 | 194 | Negative |
| 3 | 100 | 130 | Negative |
| 2 | 300 | 128 | Negative |
| 6-8 | 50 | 121 | Negative |
| 8-10 | 50 | 100 | Negative |
| 5-6 | 100 | 79 | Negative |
| 2 | 100 | 77 | Negative |
| 6-8 | 100 | 70 | Negative |
| 1 | 100 | 54 | Negative |
| 1 | 100 | 42 | Negative |
| 4 | 100 | 37 | Negative |
| 10-12 | 100 | 11 | Negative |
| 3-4 | 100 | 10 | Negative |

<p>| TABLE III. Precipitin tests with bovine thrombin as the antigen, done on two patients who had undergone multiple operations at which bovine thrombin was used as a hemostatic agent. |
|---|---|---|---|---|---|---|</p>
<table>
<thead>
<tr>
<th>Sex</th>
<th>Age</th>
<th>Dates of Operations</th>
<th>Interval between Operations</th>
<th>No. Pieces Implanted</th>
<th>Units per cc.</th>
<th>Precipitin Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>45</td>
<td>2/2/45 2/21/45</td>
<td>19 days</td>
<td>1</td>
<td>100</td>
<td>7</td>
</tr>
<tr>
<td>M</td>
<td>26</td>
<td>12/27/44 1/11/45 2/26/45</td>
<td>15 days 46 days</td>
<td>3 4</td>
<td>100 100 100</td>
<td>2, 3, 5, 7, &amp; 1</td>
</tr>
</tbody>
</table>

collection of blood serum, using the laboratory procedure outlined above in
connection with monkeys. Tables II and III give the results.
Only crude estimates can be made of the total amount of thrombin permanently deposited in the body during hemostasis, but it appears to have approximated from 5 to 10 units for each piece of gelatin sponge implanted when the solution was made up to contain 100 units per cc. The values of implanted thrombin therefore range from 10 to 120 units.

There were no positive results of the precipitin tests.

**DISCUSSION**

While the brief experiences of clinical surgery do not constitute an adequate endorsement of bovine thrombin, nevertheless they offer assurance that the likelihood of sensitization is small in connection with the use of the material in routine surgery. That view gains support from an experiment of slightly increased severity done in monkeys, again representing, however, the surgical side of the case and not sound immunological technique. Whether in surgery the situation may ever arise in which the rigid sensitization procedures that have been applied successfully to guinea pigs and rabbits are duplicated, is a matter of conjecture. Presumably that will not occur.

One may speculate with interest on the position ultimately to be assigned to thrombin among the antigens. Pure thrombin has not yet been prepared and studies of antigenicity are confused to some extent by reactions to the contaminating proteins. The question naturally arises: when purity is achieved, will thrombin be found to possess species specificity or group specificity? The answer has already been determined with respect to its activity as a coagulant of the blood, for the thrombins of the mammalian series are alike, but differ from those of birds and those of amphibians. ²

In the case of insulin both hormonal activity and antigenicity are drawn upon the basis of group distinction rather than of species. ¹⁰ The danger of sensitization is from insulin itself, not from the parent animal substances. By comparable reasoning, the possibility exists that an animal can be sensitized to the thrombin derived from any species of the group including its own, a possibility made all the more valid by the presumption that thrombin is not a normal constituent of the blood. If this is true, the question of safety is one of quantity (modified by individual susceptibility), in which case experiments concerning actual surgical use become definitive.

**CONCLUSIONS**

1. After the surgical use of bovine thrombin in hemostasis, *Macacus rhesus* monkeys failed to develop either precipitins in their blood serum, or any local tissue reaction in the brain (Arthus phenomenon) upon injection of a small amount of the antigen.

2. In 15 patients precipitin tests done from 10 to 194 days following the neurosurgical use of bovine thrombin as a hemostatic agent yielded no positive reactions.

3. While bovine thrombin may be employed in surgical hemostasis with
relative safety from sensitization, much more precise knowledge is required
to establish the limits of safety and the basic position of thrombin among the
antigens.

The author wishes to express his deep appreciation to Dr. Hazel R. Prentice and her
laboratory staff for performance of the precipitin tests, and for the preparation and review of
the histological sections; to Dr. Beatrice Carrier Seegal for generous cooperation including
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its research staff.

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