Antithrombotic Effect of Malayan Pit Viper Venom on Experimental Thrombosis of the Inferior Vena Cava Produced by a New Method

By James L. Marsten, M.D., Chan Kok-Ewe, M.B., B.S., Ph.D., Jay L. Ankeney, M.D., and Robert E. Botti, M.D.

ABSTRACT
A reliable method of producing thrombosis of the inferior vena cava in dogs is described. This consists of placing a segment of umbilical tape across the lumen of the vein and sewing it in place without interfering with blood flow. In the control animals gross thrombi were not usually present in the immediate postoperative period but were demonstrated after 24 hours. By the 7th postoperative day large red clots completely obstructing the vessel were usually found. The venom of the Malayan pit viper proved to be highly effective in preventing experimental thrombosis. The antithrombotic effect of viper venom is believed to be due primarily to induced hypofibrinogenemia. Accelerated fibrinolysis may also be a factor. The experiments suggest that Malayan pit viper venom may be effective as an antithrombotic agent.

ADDITIONAL KEY WORDS anticoagulant snake venom hypofibrinogenemia fibrinolysis hemorrhagic complications Ancistrodon rhodostoma thrombocytopenia defibrination

Individuals who are bitten by the Malayan pit viper (Ancistrodon rhodostoma) may develop marked hypofibrinogenemia of prolonged duration.1,2 This hypocoagulable state is surprisingly uncomplicated by hemorrhage or mortality. In animals, the injection of this viper venom results in a similar hypocoagulable state, presumably caused by slow intravascular fibrin formation.3 Increased fibrinolysis is also seen but appears to be an associated phenomenon rather than a direct effect of the venom.4 The combination of prolonged hypofibrinogenemia, increased fibrinolysis, and few hemorrhagic complications seems to be a characteristic of an ideal anticoagulant.

This report describes a reliable, simple method for producing thrombosis in the inferior vena cava of dogs by the insertion and suture of a short segment of umbilical tape across the lumen. The intravenous injection of Malayan pit viper venom effectively prevented the formation of thrombi and perhaps lysed existing thrombi as well.

Materials and Methods
Male or nonpregnant female mongrel dogs, 15 to 25 kg in weight, were anesthetized by intravenous injections of 30 mg of pentobarbital sodium per kg (Diabutal, Diamond Laboratories, Inc., Des Moines, Iowa) with supplementation as necessary. With the animal in the left lateral recumbent position, the inferior vena cava was isolated through an extraperitoneal right flank incision, and the peritoneum was retracted anteriorly (Fig. 1). Usually one and occasionally two lumbar veins had to be divided between ligatures for adequate mobilization. An infrarenal segment of vena cava was then isolated between vascular clamps and a 3 to 4 mm venotomy was...
FIGURE 1
Steps A through E show the procedure for fixing the umbilical tape in the vena cava (see text).

made in the lateral caval wall (Fig. 1, A). An atraumatic suture of 5-0 vascular silk was then passed through the opposite caval wall to the venotomy into the caval lumen and then out through the venotomy (Fig. 1, B). The suture was tied or sewn to a short segment of 1/4 cm umbilical tape and passed back through the venotomy and out the opposite caval wall, 1 to 2 mm from its original point of entry. The tied or sutured umbilical tape was led through the venotomy into the caval lumen, and the suture was tied down to the caval wall, thus suturing one end of the umbilical tape to the vena cava (Fig. 1, C and D). The venotomy was closed with a second suture of 5-0 vascular silk, either as a purse string suture encircling the protruding end of the umbilical tape, or as a continuous suture (Fig. 1, E). Prior to final closure of the venotomy, the vascular clamps were released to expel any air from the caval lumen by back bleeding, followed by release of the caudal clamp to obtain maximal expansion of the caval lumen before fixing the remaining end of the umbilical tape. Thus, the vena cava was traversed by a bar of 1/4 cm umbilical tape fixed by sutures at each end, and with one protruding from the caval lumen.

The operated animals were divided into control and experimental groups; the latter were injected with venom. The venom used was vacuum-dried after being milked from the snakes. It was prepared for injection by dissolving the dried venom in physiological saline solution. The biochemistry of the venom has been previously studied.8 When it is kept in the dried state at −20°C the venom is stable for years. Solutions of venom kept at 2 to 4°C retain their coagulant activity for at least 2 weeks. The solutions are affected only slightly by acid pH and are non-dialysable. Ultraviolet spectroscopy studies have shown that the venom is mainly a protein material and that 1 mg per ml of crude venom contains 0.93 mg per ml protein. Although the venom used in the present experiments was the crude preparation, a fractionation procedure with DEAE-sephadex produced varying degrees of separation of proteolytic and clot-promoting components.

 Inferior vena cavagrams were performed by the manual injection of 30 cc of 50% Hypaque rapidly into a hind leg vein. A right posterior-oblique X-ray at the level of the incision was taken at the end of the injection. Venous blood was drawn from the external jugular or leg veins with an uncoated disposable 20-gauge needle and uncoated disposable plastic syringes (Monoject, Roehr Products Company, Inc., Waterbury, Conn.). The hematocrit was determined by a microtechnique. Direct platelet counts5 were performed on whole blood rendered incoagulable by the addition of 1 mg of dipotassium ethylenediamine-tetraacetic acid per ml.8 The concentration of fibrinogen was measured as previously described.7 Animals were killed by giving an excess amount of pentobarbital intravenously, immediately after heparin at a dose of 1 mg per kg was given intravenously to prevent postmortem clotting and to help differentiate premortem and postmortem intravascular clots.

Results
1. THE PRODUCTION OF THROMBI BY THE EXPERIMENTAL METHOD

Twenty-four dogs had a segment of umbilical tape inserted into the inferior vena cava, without further manipulation. These dogs served as controls. The results are summarized in Table 1. The animals were killed 30 min to 7 days postoperatively. In 6 of the 8 animals examined within 2 hours, the tape was covered only with small accretions. The other 2 had nonobstructing gross thrombi. In all 16 animals examined 1 to 7 days postoperatively, gross thrombi, varying from small red clots to large "currant jelly-like" masses, were found on the tape (Fig. 2). Complete obstruction of the vena cava was demonstrated by angiography in 6. The presence of thrombi with early fibroblastic proliferation was noted upon histologic examination. No serious bleeding
TABLE 1
Incidence of Experimental Thrombosis in Control Animals*

<table>
<thead>
<tr>
<th>Postoperative interval</th>
<th>No. of dogs</th>
<th>0</th>
<th>1+</th>
<th>2+</th>
<th>3+</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>45 min</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 hr</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2 hr</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1 day</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>2 days</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>5 days</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>7 days</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

*None of these animals bled.

10 = No gross thrombus; 1+ = small thrombus; 2+ = moderate thrombus; 3+ = large thrombus.

TABLE 2
Incidence of Experimental Thrombosis in Dogs Treated with Venom

<table>
<thead>
<tr>
<th>Postoperative day</th>
<th>No. of dogs</th>
<th>0</th>
<th>1+</th>
<th>2+</th>
<th>3+</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>6</td>
<td>1</td>
<td>1</td>
<td>0</td>
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</tr>
<tr>
<td>7</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

*Grades for thrombosis are listed in Table 1.

Inferior vena cava at autopsy of a control dog, demonstrating a large red clot propagated in both directions from the segment of umbilical tape (arrow) within the lumen.
ANTITHROMBOTIC EFFECT OF VIPER VENOM

FIGURE 3

Inferior vena cava at autopsy of two venom-treated dogs. The segments of umbilical tape do not show any gross clots.

pericaval abscess caused by a gauze sponge which had not been removed.

At this dosage level, in all animals, the injection of venom resulted in marked hypofibrinogenemia, a mild decrease in platelet count which always remained greater than 150,000 per mm$^3$ and little change in the hematocrit. Figure 4 illustrates the changes in fibrinogen in one of the animals. Despite the absence of serious hemorrhage, the animals bled easily after venepuncture, requiring prolonged pressure for hemostasis.

3. THE EFFECT OF VIPER VENOM ON ESTABLISHED THROMBI

The intravenous injection of viper venom is associated with a transient increase in fibrinolysis.$^1$ A series of experiments was designed

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Experimental Thrombosis in Dogs Treated with Venom$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Postoperative day</td>
<td>No. of dogs</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
</tr>
</tbody>
</table>

$^*$None of these animals bled.

$^+$Grades for thrombosis are listed in Table 1.

$^1$Retroperitoneal abscess.

completely obstructed the vena cava, was observed in a dog which had received two injections of venom. A vena cavaogram 48 hours postoperatively did not reveal a thrombus but complete obstruction became evident on the 9th postoperative day by both x-ray and post-mortem study. The result in this dog suggests that the thrombus had formed during recovery from the hypofibrinogenemic state.

Because of hemorrhage, the dosage regimen was modified in an additional 9 animals. Viper venom was given intravenously at a dose of 5 $\mu$g per kg approximately 1 hour postoperatively followed by 15 $\mu$g per kg on the 3rd postoperative day. There was no significant bleeding, yet this dosage regimen proved effective in preventing thrombosis (Table 3). Eight animals had no gross thrombus at autopsy 4 to 7 days after surgery. On the 5th postoperative day 1 animal was found to have a thrombus associated with a

Changes in plasma fibrinogen in an experimental dog. Fibrinogen concentration was measured. The animal was then operated on with insertion of a segment of umbilical tape into the inferior vena cava. One hour after surgery and again 72 hours later, viper venom was injected intravenously. Plasma fibrinogen became undetectable 4 hours after each injection and gradually rose over the next 48 to 72 hours. On the seventh postoperative day, the animal was killed. No gross thrombus was found at autopsy.

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to examine the effect of venom on clots which had already formed, thereby testing the importance of thrombolysis. Six dogs were untreated on the day of tape insertion. Fifteen μg per kg of viper venom was injected daily for the next two days into all 6 animals; they were killed on the 7th postoperative day. On the 1st postoperative day 3 revealed definite thrombi by angiographic study. No gross thrombi were found in 5 of the 6 dogs at autopsy, including 2 of the 3 which had evidence of thrombosis before venom administration. In 1 of the dogs a thrombus was seen on the tape. This was one in which the cavagram on the 7th postoperative day showed reduction of the size of the thrombus as compared to the earlier X-ray.

Discussion

To test the efficacy of a proposed antithrombotic agent in vivo requires a reliable thrombogenic procedure. A simple operation was devised to place a segment of umbilical tape into the abdominal portion of the inferior vena cava of dogs. Red thrombi were found attached to the tape in all animals killed serially 1 to 7 days postoperatively but in only 2 of 8 examined within 2 hours postoperatively. A week after surgery 4 of 5 dogs had large thrombi completely occluding the inferior vena cava.

This experimental model was used to test whether the venom of the Malayan pit viper (Ankistrodon rhodostoma) had an antithrombotic effect. The injection of the venom has been studied extensively1-4 both in human beings after snake bite and in dogs after its intravenous injection. In man a mild hemorrhagic syndrome occurs, predominantly at the site of the bite. This does not seem to be directly related to the severe generalized disorder,5 which is characterized by severe prolonged hypofibrinogenemia, thrombocytopenia, and transient accelerated fibrinolysis. The hypofibrinogenemia which may last for many days is thought to be due to disseminated intravascular clotting which results in defibrination. This then stimulates fibrinolysis, perhaps by release of an activator of plasminogen, in a nonspecific and as yet unexplained manner. In dogs,6 similar effects are noted. It has been previously reported that the intravenous injection of venom at doses similar to those used in the present study was found to cause severe hypofibrinogenemia within 4 hours and a fibrinogenemia and mild thrombocytopenia within 24 hours. We confirmed these results, which persisted for 48 to 72 hours. Extensive in vitro studies7 have shown that the venom at these low concentrations has no effect on clotting other than its action on fibrinogen. None of the known specific clotting factors was affected. No local or generalized hemorrhagic symptoms have been observed in dogs. As suggested earlier,8 the possible use of the hypofibrinogenemic action of the venom as a therapeutic anticoagulant seemed worthy of trial, especially since defibrination seemed to produce no ill effects and the venom also functions as a pharmacological fibrinolytic agent.

The infusion of venom immediately after surgery was found to prevent experimental clot formation in nearly all the animals. Protection was present as long as the seventh day postoperatively if second dose of venom was given 3 days after surgery. The mechanism by which Malayan pit viper venom prevented thrombosis appeared to make unavailable one needed element, fibrinogen. In the control animals gross thrombus formation did not occur in the majority of those examined postoperatively within 2 hours, which was the maximum delay before treatment with venom in most of the experimental dogs. In other words, no thrombi were present at the time of injection of the venom which then prevented thrombosis.

A complication of treatment was hemorrhage from the operative area, but this was minimized by a reduction in the initial dose. That this was not a manifestation of a more widespread hemorrhagic diathesis was evident by the local nature of the bleeding, confirmed clinically and at necropsy. Two animals did have thrombosis. One was associated with a pericaval abscess, a situation known to be inherently thrombogenic. The other dog may
have developed the thrombus after the hypo-
fibrinogenemia had subsided.

The effect of the venom was also tested up-
on established thrombi. In those animals in
whom angiograms suggested the presence of
thrombosis, the injection of viper venom
was followed by disappearance of the clots
in all but 1 dog. Even in this animal, X-ray
studies indicated that the thrombus had be-
come much smaller. The disappearance of
the thrombi probably resulted from the com-
bined effects of fibrinolysis and the preven-
tion of further clotting. Fibrinolysis may
occur spontaneously but may have been
enhanced by the venom, which is known to
induce this process.

The effect of further delay in treatment of
an experimental thrombus was not pursued
in detail. However, it appears that administer-
ing the venom on the 2nd instead of the 1st
postoperative day does not result in effective
thrombolysis.

There are several practical advantages to
the use of Ancistrodon rhodostoma venom as a therapeutic anticoagulant. In addition to
the low incidence of hemorrhage, administra-
tion is easy and requires only one injection
every 48 to 72 hours. An effective antivenom
is available which promptly arrests the hypo-
fibrinogenemia. A vast clinical experience
testifies to the benign course of individuals
who have been bitten by the Ancistrodon rhodostoma. Despite the protracted hypo-
fibrinogenemia, the victims of this snake’s bite
almost never have serious bleeding difficulties unless subjected to trauma. Although anti-
body production and inactivation have not
been studied in detail, preliminary data re-
vealed titers demonstrable only in the first
few weeks. Whether the venom might prove
superior to existing anticoagulants remains to
be proven.

Placing umbilical tape across the lumen of
the inferior vena cava provides a thrombo-
genic stimulus which may be greater than
that found in most clinical situations, even
those in which artificial heart valves or vas-
cular grafts are introduced into the circulation. Such valves are usually coated with si-
licone and grafts are preclotted to minimize
the effect of the foreign surface on the blood
clotting mechanism. It seems reasonable,
therefore, that the venom may prove effective
in preventing thrombosis in these clinical
situations, at least during the early postopera-
tive period.

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