ORAL ADMINISTRATION OF EARTHWORM POWDER AS A POSSIBLE THROMBOLYTIC THERAPY

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I. INTRODUCTION

The history of use of the earthworm as a therapeutic drug saurce for various diseases extends back for several thousand years in China and other parts of the Far East. However, practical pharmacological studies have not been performed except on lumbrofebrin as an antifebrile (1). In 1983, we reported that very strong and novel fibrinolytic enzymes could be extracted from the earthworm, *Lumbricus rubellus* (2). These enzymes were fractionated and purified as six novel fibrinolytic enzymes, and named collectively as lumbrokinase. We have also found that earthworm powder contains two kinds of inhibitory substances for the platelet aggregation induced by collagen and ADP. One of these inhibitors of platelet aggregation was identified with adenosine. However, the other was a novel substance of MW 260. The structure of this substance was decided on the basis of NMR, mass spectra and infrared spectra (3). This novel substance also displays a relaxation effect for the canine saphenous vein induced by prostaglandin F *in vitro* and an inhibitory effect on the active partial thromboplastin time (APTT). In veiw of the above-mentioned effects, the earthworm powder appeared to be a potentially very useful agent for thrombosis. We therefore undertook experiments on the oral administration effects against intravascular fibrin clots using earthworm powder in dogs and humans.

II. MATERIALS AND METHODS

A. Earthworm Powder

One kg of living earthworms, *Lumbricus rubellus*, which had been vermicultured at the Experimental Animal Center, Miyazaki Medical College, were thoroughly washed with tap water to remove attached mud, and were then left to evacuate the casts from their alimentary tract in distilled water over night. On the next day after washing the casts away, the living earthworms were homogenized in an ultra-homomixer (Nihon Seiki Co. Ltd., Japan) and lyophilized. The resultant earthworm powder was employed as the starting material for subsequent experiments.

B. Animal Experiments

As experimental animals, 9 beagle dogs were used. They were housed at the Experimental Animal Center, Miyazaki Medical College, under a controlled ambient temperature of 23 ± 1 ℃ with 50 ± 10% relative humidity. An experimental thrombus was produced in the saphenous vein of all 9 dogs by Sasaki's method (4) before administering the test materials. The dogs were separated into three groups of 3 dogs each. The first group was given earthworm powder extract solution as the earthworm powder group. The extract solution was prepared and administered as follows. One g of earthworm powder was dissolved in 5 ml of saline solution, and then incubated for 1 month at 37 ℃. After centrifugation for 30 min at 10,000 G, 5 ml of the supernatant was administered perorally into the dog's duodenum using a fiber-scopic instrument under general anesthesia with nitrous oxide. The second group was injected intravenously with commercial high molecular weight urokinase (M.W. 50,000, Mochida Seiyaku Co. Ltd., Japan) at a dose of 200,000 IU/5 ml saline/body as the urokinase group. The third group formed the control group, which was injected intravenously with 5 ml of physiological saline solution. After administration of the above test materials, the lysis of the thrombus was observed by X-ray angiography, and the lysed state was evaluated qualitatively.

C. Human Experiments

Seven normal men (aged 52-28) from our laboratory were orally administered single capsules containing 200 mg of specially treated earthworm powder three times after meals every day for 17 days. Blood was withdrawn once a day into 1/10 its volume of 3.8% citrate, before and at 1, 2, 4, 8, 11, and 17 days after commencing the administration. Plasma was

obtained by centrifugation at 3,000 rpm for 10 min, and fibrinolytic tests were performed on the plasma as follows. The whole blood clot lysis time (WBCLT) was measured by the method of Chohan et al. (5). The euglobulin lysis time (ELT) was measured by the method of Milstone (6) using a clot lysis time recorder (Riko Shoji Co. Ltd., Japan). The euglobulin fibrinolysis area (EFA) was determined by the plasminogen-rich fibrin plate method (7). The antigen value for tissue plasminogen activator (t-PA) was estimated by enzyme linked immuno-adsorbent assay using IMULYSE™ 5t-PA (Biopool AB, Sweden). Fibrinogen degradation products (FDP) were measured semiquantitatively by the latex aggregation test using an FDPL kit (Teikoku Zoki Co. Ltd., Japan). T-PA activity was assayed as follows. An equal volume of 1 M sodium acetate buffer, pH 3.9, was added to the plasma, and the mixture was frozen at -80 ℃. On the next day, 400 μl of thawed plasma was added with 300 μl of 0.1 M Tris buffer containing 0.2 M NaCl and 0.02% Triton X-100 and 400 μl of distilled water, and adjusted to pH 7.4. The plasma sample was diluted with 0.05 M Tris-HCl buffer containing 0.1 M NaCl and 0.01% Triton X-100 (pH 8.8), and serial dilutions were carried out. To prepare fibrin-coated microtiter plates, 100 μl of 0.156 mg/ml human fibrinogen (plasminogen-free, Kabivitrum AB, Sweden) was poured into each well of a microtiter plate and allowed to dry overnight at 45 ℃. On the next day, the fibrin-coated microtiter plate was washed three times with 0.05 M Tris-HCl buffer containing 0.1 M NaCl and 0.01% Triton X-100 (pH 8.8) and was then used to assay the t-PA activity. First, 100 μl of the diluted plasma was poured into each well of the fibrin-coated microtiter plate, and 100 μl of 0.05 CU/ml Lys-plasminogen solution (Kabivitrum AB, Sweden) and 100 μl of 0.6 mM S-2251 (Kabivitrum AB, Sweden) were added to each well. After incubation of the microtiter plate at 37 ℃ for 15 hours, the absorbance was measured at 405 nm and was recorded as absorbance A. On the other hand, the same procedure was performed using microtiter plates which had not been coated with fibrin, and the absorbance was measured as absorbance B. The t-PA activity was then calculated from the following equation: t-PA activity = absorbance A - absorbance B. As the standard for the t-PA activity, t-PA antigen, which was present in the IMULYSE™ 5t-PA (Biopool AB, Sweden) was employed.

III. RESULTS

A. Animal Experiments

As shown in Table I, the experimental thrombus was completely digested after 24 hours in the earthworm group. Two cases were digested at 4 hours and 8 hours after the administration. The other case was partially digested after 8 hours, and complete lysis was observed after 24 hours. In the urokinase group, complete lysis was observed in 2 cases after 8 hours

Table I. Thrombolytic Effect of Earthworm Extract and HMW-UK in Beagle Dogs with Experimental Saphenous Vein Thrombus

Route of administration	No.	Time after administration (hr) 0	4	8	12	16	20	24
1) Oral administration*	1	−	+	+	+	+	+	+
Earthworm Extract**	2	−	−	+	+	+	+	+
	3	−	−	±	±	±	±	+
2) Intravenous injection	1	−	−	−	−	−	−	−
HMW-UK***	2	−	−	+	+	+	+	+
	3	−	−	−	−	−	+	+
3) Control group	1	−	−	−	−	−	−	+
	2	−	−	−	−	−	−	−
	3	−	−	−	−	−	−	+

*; Administration into duodenum using the Fiber-scope instrument
**; 1g/5ml saline/body
***; 200,000 IU/5ml saline/body
−;non-recanalization, +;recanalization, ±;partially recanalization

and 20 hours. In the other case receiving the urokinase injection, no recanalization was observed. In the control group, no recanalization was observed.

B. Human Experiments

Fig. 1 summarizes the overall results for the fluctuations in fibrinolytic activities among 6 of the volunteers i.e. excluding one volunteer, who had been receiving earthworm powder for 3 years prior to the present experiments. In terms of the WBCLT, the fibrinolytic activity was increased on the next day after commencing the administration and this continued into the 2nd day. However, the activity decreased once on the 4th day. Subsequently, it increased until the 17th day, when the experiments were terminated. In terms of the EFA, the fibrinolytic activity continued to increase from the next day until the 17th day. Based on the ELT, the activity increased from the next day, decreased once slightly on the 4th day, but then continued to increase until the 17th day. These data clearly demonstrated that earthworm powder, following its oral administration, has the power to increase the fibrinolytic activity of the blood.

Fig. 2 summarizes the overall results for the antigen values of t-PA. T-PA increased from the next day to the 4th day after commencing the

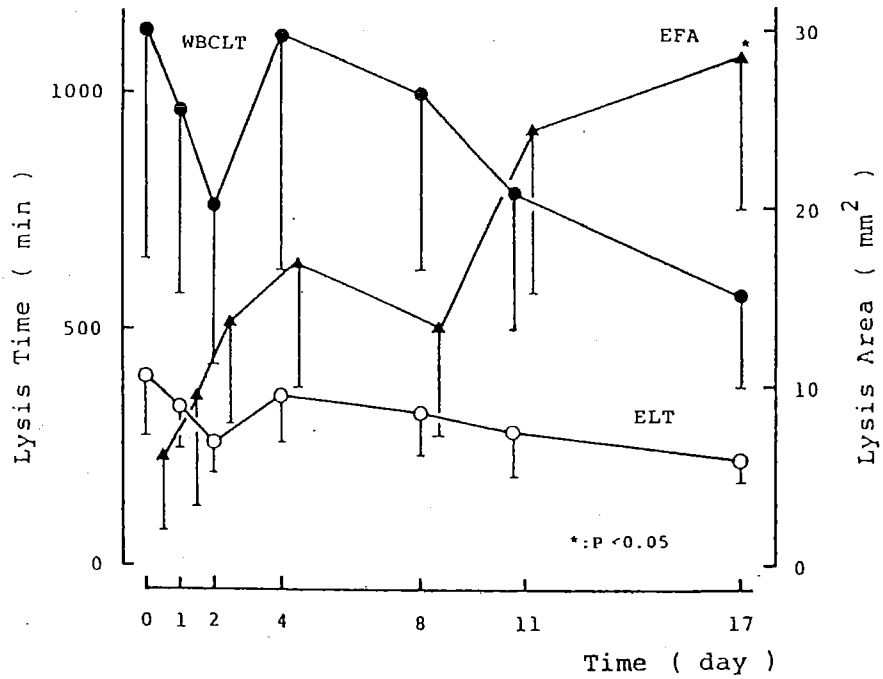

Fig. 1 Fluctuations of fibrinolytic activities based on WBCLT, ELT and EFA after oral administration of earthworm powder. The abscissa indicates the days after the administration. The left ordinate indicates the lysis time (min) of WBCLT and ELA. The right ordinate indicates the lysis area (mm^2) of EFA. — ● — *WBCLT,* — ○ — *ELT,* — ▲ — *EFA.*

administration of earthworm powder. Subsequently, the t-PA antigen value did not decrease until the last day of the experiment. However, a higher value than that before the experiment still continued until the last day of the experiment. The difference in t-PA antigen value between the day before the oral administration of earthworm powder and the 8th, 11th, and 17th days after commencing the administration was stastistically significant ($p < 0.05$).

Fig. 3 summarizes the results for FDP. As shown in Fig. 3, the levels of FDP increased very sharply on the next day after commencing the administration of earthworm powder and decreased considerably at the 2nd day and 4th day. The FDP values then decreased gradually until the last day of the experiment. The difference in FDP values between the day before the oral administration of earthworm powder and the next day and 2nd day after commencing the administration was statistically significant ($p < 0.01$). The

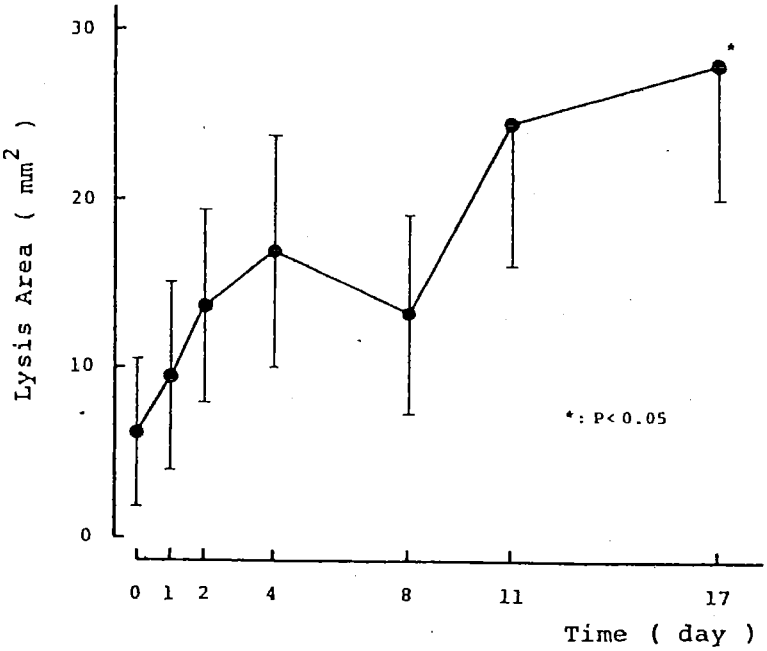

Fig. 2 Fluctuations of t-PA antigen value after oral administration of earthworm powder. The abscissa indicates the days after the administration. The ordinate indicates the t-PA antigen value (ng/ml).

difference between the day before and the 4th day after commencing the administration was also significant ($p < 0.05$).

The t-PA activities were found to be very different in each case, as shown below by the individual data. The overall results are therefore not summarized on a single graph.

To complement the above overall results following the administration of earthworm powder, each case is presented individually in Figs. 4 and 5. Fig. 4A illustrates the case of a 39-year-old and very healthy male. However, his FDP value increased on the 1st day after commencing the administration of earthworm powder. This meant that fibrin deposits were present within his vascular system despite the absence of any thrombotic symptoms. The FDP value then decreased from the 2nd day. It is assumed the fibrin deposits in the vascular system were entirely removed by the 17th day of administration of earthworm powder. The t-PA antigen value also increased from the 1st day after commencing the administration of earthworm powder. However, the t-PA activity was maintained at a low level. This could mean that t-PA was complexed with t-PA inhibitor.

Fig. 4B shows the case of a 32-year-old male. He was also very healthy. However, his FDP value increased on the 1st day after commencing

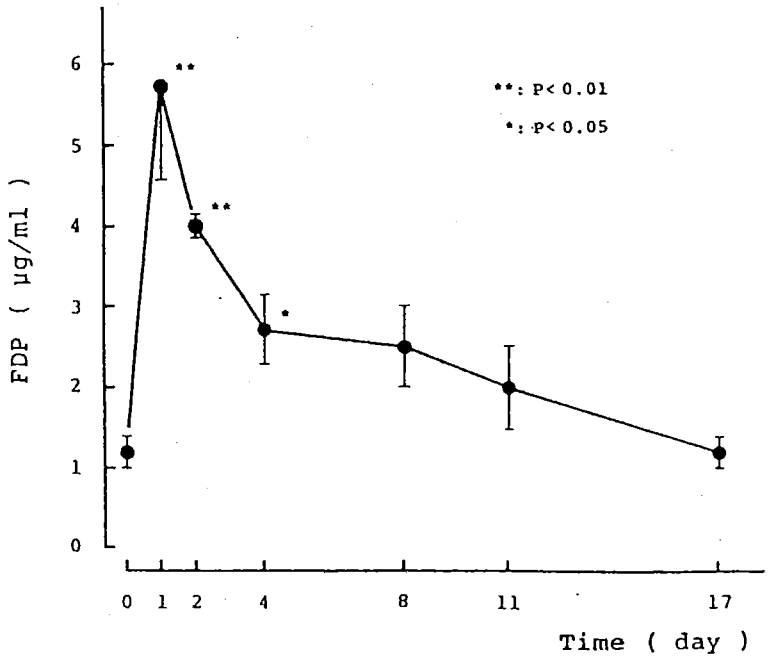

Fig. 3 Fluctuations of FDP value after administration of earthworm powder. The abscissa indicates the days after the administration. The ordinate indicates the FDP value (μg/ml).

the administration of earthworm powder and remained rather high for one week. Subsequently, the value decreased. It is assumed that the fibrin in the vascular system was completely removed by the 17th day. The t-PA antigen value did not increase as compared to case 4A. However, the t-PA activity was maintained at a higher level than that in case 4A.

Fig. 4C shows the case of a 30-year-old male. He was youthful and very healthy, and sometimes engaged in aquadiving. In this case, the FDP value increased on the 1st day and 2nd day, but then continued at a low level until the 8th day. The increase in FDP was less than in the above-mentioned cases. In this case, the t-PA antigen value was also maintained at a high level. However, t-PA activity was not observed.

Figs. 4D and 5A illustrate the cases of a 28-year-old and a 27-year-old male, respectively. Their FDP values were also somewhat increased at the next and 2nd days. The fluctuations in t-PA activity and antigen values were similar to those in case 4C.

Fig. 5B shows the case of another 28-year-old male. He was young, but was the only volunteer with thrombotic symptoms. His systolic blood pressure was 160 mmHg and he sometimes complained of headache. In this

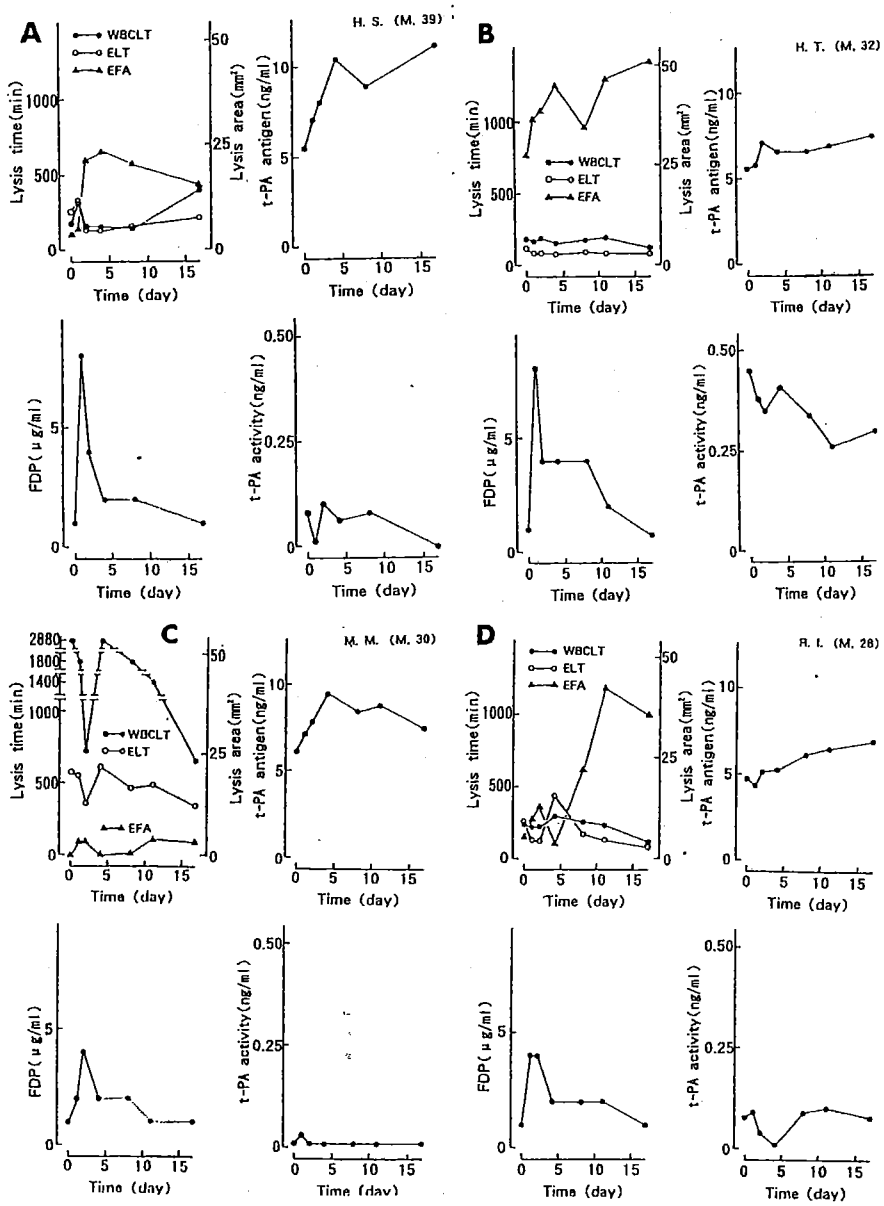

Fig. 4 Fluctuations of fibrinolytic activities. t-PA antigen values, FDP and t-PA activities after administration of earthworm powder in individual cases. For each case, the fibrinolytic activities are on the upper left, t-PA antigen values on the upper right, FDP values on the lower left, and t-PA activities on the lower right, respectively.

Fig. 4A: 39-year-old male case. Fig. 4B: 32-year-old male case. Fig. 4C: 30-year-old male case. Fig. 4D: 28-year-old male case.

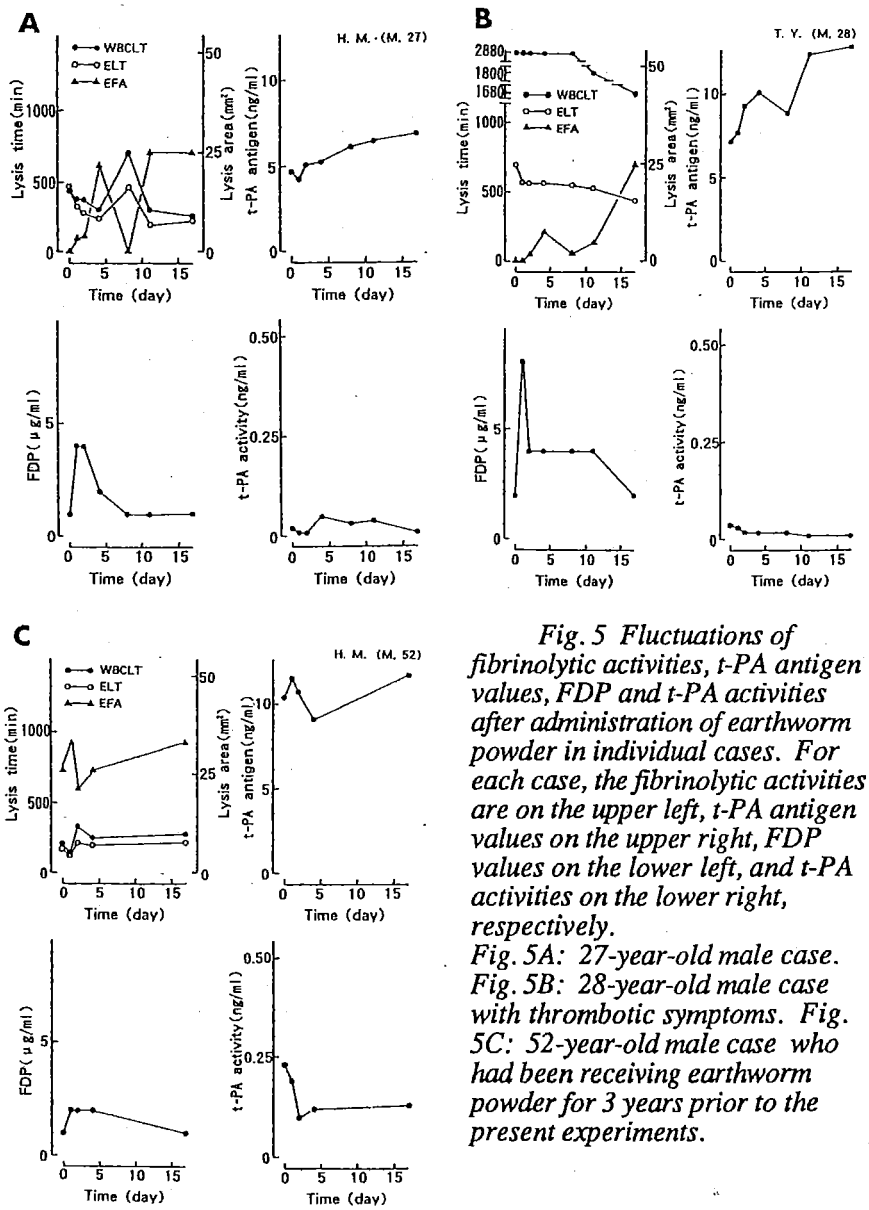

Fig. 5 Fluctuations of fibrinolytic activities, t-PA antigen values, FDP and t-PA activities after administration of earthworm powder in individual cases. For each case, the fibrinolytic activities are on the upper left, t-PA antigen values on the upper right, FDP values on the lower left, and t-PA activities on the lower right, respectively.
Fig. 5A: 27-year-old male case.
Fig. 5B: 28-year-old male case with thrombotic symptoms.
Fig. 5C: 52-year-old male case who had been receiving earthworm powder for 3 years prior to the present experiments.

case, the fibrinolytic activity was very low before the administration of earthworm powder and continued so after commencing the administration. His FDP value increased very sharply on the 1st day, and high values were observed for 2 weeks after commencing the administration. However, during the 3rd week of administration, the FDP value decreased and became normalized. His blood pressure also fell to a normal level at the 3rd week of

administration. The t-PA antigen value was increased from the 1st day until the last day. However, the t-PA activity was maintained at a very low level. These phenomena appeared to be similar to those in the older volunteer, case 4A.

Fig 5C shows the case of a 52-year-old healthy male, who had been receiving earthworm powder for 3 years prior to the present experiments. However, the dosage had been less than that in the present experiments. As demonstrated in Fig. 5C, the FDP value did not increase, and the t-PA antigen level was already high before commencement of the experiment. These findings may mean that no fibrin deposits were present in his body prior to the experiment and sufficient t-PA was circulating within his body.

The above data suggest that fibrin clots are usually present in the human vascular bed, and can be digested by the administration of earthworm powder. That is, earthworm powder exerts thrombolytic effects following its oral administration.

IV. DISCUSSION

An account of the earthworm being employed as a drug was given in the oldest known Chinese book, Shen Nong Ben Cao (神農本草経), which is thought to have been published between the first and third century. Chang Xui Chenghe Cheng Lei Ben Cao (重修政和証類本草), published in 1229, indicates that the earthworm had already been used for cerebral apoplexy before the book was actually published. This is a very important point, not least because, at the present time, more than 60% of cerebral apoplexy involves cerebral thrombosis. Clearly, this is very closely connected to the above report. However, despite these ancient descriptions in oriental medial books, detailed pharmacological studies have not yet been undertaken except on lumbrofebrin as an antifebrile (1).

We recently obtained novel fibrinolytic enzymes from the earthworm, *Lumbricus rubellus*, and named them collectively as lumbrokinase (2). We therefore attempted to utilize this earthworm as an oral thrombolytic agent. It is a problem as to whether functional proteins can be absorbed into the circulation from the intestine or not. However, many interesting investigations on the intestinal absorption of enzyme proteins have recently demonstrated that small amounts of enzyme proteins administered orally can be absorbed from the intestinal tract into the circulation (8, 9, 10). In fact, as shown in the present animal experiments, it is possible for orally administered earthworm powder to digest intravascular fibrin clots. Administration of earthworm powder orally to volunteers was therefore carried out. As clearly demonstrated by the present results, oral administration of earthworm powder was able to increase the fibrinolytic activity of the blood. From the data obtained, we believe that the increase in fibrinolytic activity may be due to t-PA-like activator, either that transported across the intestinal membrane or that newly synthesized by endothelial cells. The increase in FDP observed after 24 hours of administration also shows

that fibrinolysis occurred within the body.

It was unclear whether the increase of FDP demonstrated either fibrinolysis or fibrinogenolysis in the present experiments. However, when the levels of D-D-dimer were measured in some cases following administration of earthworm powder, an increase in D-D-dimer was observed like that for FDP (data not shown). The observed increase of FDP would thus appear to indicate that intravascular fibrin was digested by the administration of earthworm powder. In particular, the FDP levels were very sharply increased on the next day after commencing the administration of earthworm powder, and decreased a few days later. It is interesting to note that higher increases of FDP were observed in older volunteers in than younger volunteers. This suggests that fibrin clots are usually present to a greater or lesser extent in the vascular bed of healthy persons who are more than 30 years old, and can usually be digested by the administration of earthworm powder. In the one case who had been receiving earthworm powder for 3 years before the present experiments, the FDP did not increase and the t-PA antigen level was already high before the experiments. It is concluded that no fibrin deposits were present in this case prior to the experiments due to his long administration of earthworm powder.

One other interesting finding obtained in the present study was that t-PA antigen increased after the administration of earthworm powder. This implies that such administration can release endogenous plasminogen activator. The release of endogenous plasminogen activator seems to be very important in the treatment of patients with thrombosis. When we carried out treatment of patients with cerebral thrombosis by urokinase injection, cases in which the t-PA antigen value was increased after the injection of urokinase showed positive results, whereas cases in which no increase in t-PA antigen value had been observed showed no effect (11). We are now trying to extract a fraction, which is able to release endogenous plasminogen activator, from earthworm powder.

Streptokinase, urokinase and t-PA are currently in use as thrombolytic agents. Streptokinase is very effective for thrombosis, but it displays an antigenicity in humans, and cannot therefore be utilized repeatedly. Another known fibrinolytic agent is urokinase extracted from human urine. However, since only very small amounts of urokinase can be extracted in this way, urokinase is very expensive. In Japan, the use of urokinase has therefore been limited to 50,000 international units per person per day by the government. However, if more than 200,000 units of urokinase are needed, no appreciable effect can be expected. Recently, recombinant t-PA has been employed for thrombosis. However, even recombinant t-PA is rather expensive to produce. Also, streptokinase, urokinase and recombinant t-PA can be administered only by intravenous injection, so that fibrinolytic therapy with them needs to be undertaken in the hospital only. In contrast, earthworm powder can be given orally, which is very convenient for patient use. For this reason, earthworm powder has a potential application as a thrombolytic agent as demonstrated in this paper, and also exerts an inhibitory effect on platelet aggregation, an anticoagulation effect and a relaxation effect for the vascular system, which are all effective for

thrombotic therapy. We conclude therefore that earthworm powder represents a very promising agent for the treatment of thrombosis.

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