SELECTIVE TOXICITY OF A BACTERIAL PROTEASE PREPARATION FOR THE RAT BEARING ASCITES SARCOMA

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In recent years, some clinical observations and experimental studies with human subjects as well as animals have showed that a bacterial protease preparation "TSP", which is elaborated by a strain of Serratia, has anti-inflammatory activity (1).

In the course of our present work in which combination therapy of antitumor antibiotics and anti-inflammatory drugs were used, we observed that the rat bearing ascites tumor, especially MTK-sarcoma III, was particularly susceptible to intraperitoneal administration of TSP.

The exact reason for the selective toxicity of TSP for the rat bearing ascites sarcoma remains obscure. However, the results of the present report seem to indicate that the acute toxicity of TSP when administered intraperitoneally may not be the consequence of the formation of toxic metabolites by the interaction between TSP and tumor cells or ascitic fluid in the peritoneal cavity. Conceivably, further studies on this unexpected phenomenon will provide information concerning specific interactions between some protein and the peritoneal membrane and also alterations of the properties of plasma membranes resulting from this malignant tumor.

METHODS

Approximately 200 female Wistar rats weighing between 110 and 180 g were used as the experimental animals. The ascites tumor most commonly employed was MTK-sarcoma

| Table 1. Properties of various ascites sarcoma used. |
|-----------------------------------------------------|
| Tumor Properties | MTK-sarcoma III | Takeda sarcoma | Yoshida sarcoma |
| Form Stock Life span (days) | ascites serial transfer | ascites serial transfer | ascites serial transfer |
| 9 (8-12) | 6 | 8 |
| Method | artificial (p-Dimethylaminoazobenzene) | spontaneous | artificial (o-Aminoazotoluol) |
| Origin | Rat strain | Wistar/MK | Yamashita (albino rat) | -- |
| Region | peritoneal cavity | chest | scrotum |
| Date | 1952 | 1951 | 1943 |
III which was artificially produced originally in the peritoneal cavity of an inbred Wistar rat and has been carried in the Zoological Institute of the Hokkaido University for over 1,100 transfer generations. The average life span of the MTK-sarcoma III bearing rat was 9 days (Table 1). In experiment 2, each rat with the exception of control rats was inoculated with one of three types of ascites tumors, i.e. Takeda sarcoma, Yoshida sarcoma or MTK-sarcoma III.

The main drug used in this experiment was a highly purified bacterial protease preparation "TSP", isolated from a culture broth of Serratia sp. E15, which was manufactured by the Takeda Chemical Industries, Ltd., Osaka, Japan. The molecular weight of TSP was determined to be 60,000 by the Archibald method and its caseinolytic activity was 4,000 protease units/mg. Neither carbohydrate nor sulfur-containing amino acid was detected in the enzyme preparation. The enzyme showed maximal activity at pH 9.0 and at 40°C, and was stable under lower temperatures over a pH range from 5 to 10, whereas it was unstable at 37°C under alkaline conditions (2).

TSP was dissolved in warmed physiological saline solution immediately before use and administered to the ascites rat on the 3rd day after tumor transplantation in doses varying between 0.5 and 30 mg/kg. In experiment 1, where indicated, TSP was given intraperitoneally, intravenously, subcutaneously or orally to the rat bearing MTK-sarcoma III. Although intraperitoneal injection of 1.5 ml of saline appeared to have no toxic effects in the ascites rat, total volume injected to the rat never exceeded 1 ml. Injections were always done between 10 and 11 A.M. If the rat administered TSP died within 24 hours after the TSP administration, it was considered to be "death due to TSP poisoning".

In experiment 3, ascites was obtained from the rat bearing MTK-sarcoma III and the ascites cell fraction or the cell-free ascites fluid fraction prepared by gentle centrifugation was mixed with TSP in the tube and then incubated at 37°C for 30 minutes. The amount of TSP added to an incubation tube was sufficient to produce 100% death in the ascites rat but non-lethal in the normal rat when given intraperitoneally.

The ascites mixed with TSP was also administered to the normal rat. In one series of experiments, the ascites, including tumor cells, were pipetted out of the peritoneal cavity by inserting a fine glass pipette at 3 hour intervals after the TSP administration and smeared on a coverslip. Microscopic observation was made by the acetic dahlia squash method (3).

In experiment 4, the ascites fluid was removed as completely as possible from the peritoneal cavity of rats either by aspiration with a glass pipette or washout with a saline solution after laparotomy. After removal of the ascites fluid, TSP was injected into the ascites rat without ascites. In one series of experiments, some TSP preparations were inactivated of their caseinolytic activity by heating at 55°C for 15 minutes (pH 6.5), under this condition almost 100% of the caseinolytic activity of TSP was lost, and these "inactive" TSP preparations were administered to the rat.
RESULTS

1) **Enhanced susceptibility of the ascites rat to TSP**

The data presented in table 2 and figure 1 show that TSP was selectively toxic for the ascites rat when given intraperitoneally but was almost devoid of toxicity when given orally or subcutaneously.

| Table 2. Acute toxicity of TSP for rats. |
|----------------------------------------|
| Dose (mg/kg) | Route | No. of rats | Death* (%) | Survival time (hr) |
|--------------|-------|-------------|-------------|-------------------|
| 30           | i.p.  | 10          | 100         | 12                |
| 10           | i.p.  | 10          | 0           | -                 |
| 25           | i.v.  | 5           | 100         | 3                 |
| 8            | i.v.  | 5           | 0           | -                 |
| 15           | per os| 5           | 0           | -                 |
| 20           | s.c.  | 5           | 0           | -                 |
| 1            | i.p.  | 10          | 100         | 15                |
| 0.5          | i.p.  | 10          | 0           | -                 |
| 2            | i.v.  | 5           | 100         | 3                 |
| 1            | i.v.  | 5           | 0           | -                 |
| 15           | per os| 5           | 0           | -                 |
| 20           | s.c.  | 10          | 0           | -                 |

* Death due to TSP administration.

In normal rats the intraperitoneal administration of TSP in a dose of 10 mg/kg produced 0/10 death. When higher doses (20 mg/kg) of TSP was administered, death, if it occurred, took place after 24 hours, and thus it was not considered to be "death due to TSP". A 30 mg/kg dose of TSP was 100% fatal and the average time required for death was 12 hours.

In contrast, the intraperitoneal injection of TSP in doses of 10 mg/kg into the rat bearing MTK-sarcoma III resulted in death within 4 hours. The 1 mg/kg dose was also 100% fatal and when the 0.5 mg/kg dose was utilized, it was not fatal. Namely, MTK-sarcoma III bearing rats were about 20 times more sensitive to TSP, when administered intraperitoneally, as compared with normal rats. The effect of TSP was dose-dependent and the relationship between the survival and the dose was very steep (Fig. 1). On the other hand, subcutaneous or oral administrations of TSP appeared to have no specific effect in the ascites rat. It was also interesting that intraperitoneal administrations of TSP were more toxic for the ascites rat than TSP given intravenously. Death from the intraperitoneal administration of TSP was preceded by a state of prostration. We found the heart beating fairly well just before the death due to TSP. Therefore, these animals probably died of some cause other than cardiac failure. Autopsy findings of these animals, at gross examination, were limited to swollen black spleens, which was characteristic in the ascites rat.
FIG. 1. Lethal effects of TSP in ascites rats.

TABLE 3. Lethal effects of TSP in rats bearing various ascites sarcoma.

|                | Doses (mg/kg) (i.p.) | Death* (%) | Survival time after treatment (hr) | Increased body weight (g/day) |
|----------------|----------------------|------------|-----------------------------------|-----------------------------|
| Normal rats    | 30                   | 100        | 12                                |                             |
|                | 20                   | 70         | 20                                | 1                           |
|                | 10                   | 0          | --                                | --                          |
| Yoshida sarcoma| 30                   | 100        | 12                                |                             |
|                | 20                   | 60         | 18                                | 6                           |
|                | 10                   | 0          | --                                | --                          |
| Ascites rats** | 10                   | 100        | 14                                |                             |
| Takeda sarcoma | 5                    | 0          | --                                | 6                           |
|                | 1                    | 0          | --                                | --                          |
| MTK-sarcoma III| 20                   | 100        | 2                                 | 1.2                         |
|                | 10                   | 100        | 4                                 |                             |
|                | 0.5                  | 100        | 15                                |                             |
|                | 0                    | 0          | --                                | --                          |

*: Death due to TSP administration.
**: TSP was administered on the 3rd day after tumor inoculation.
2) Acute toxicity of TSP in rats bearing various tumors

It is evident from the results presented in figure 1 and table 3 that TSP was selectively toxic for the rat bearing MTK-sarcoma III. Although similar but lesser changes in sensitivity to the lethal effects of TSP were also observed in rats bearing Takeda sarcoma, there was no difference between rats bearing Yoshida sarcoma and normal rats.

3) Interactions between ascites and TSP in the peritoneal cavity

While the results obtained have suggested that rats bearing MTK-sarcoma III may be more sensitive to TSP than normal rats, there are several possible mechanisms which may be responsible for the difference observed. TSP with a dose of 1 mg/kg, which was not a lethal dose in the normal rat when given intravenously, was lethal in 100% of the cases of rats bearing MTK-sarcoma III when given intraperitoneally. Therefore, this phenomenon observed in the ascites rat might be explained as a formation of some highly toxic substances from TSP in the peritoneal cavity. If such toxic substances are responsible for the lethal effects of TSP, then ascites cells must be capable of producing such toxic substances from TSP in the peritoneal cavity or in a test tube. Thus, experiments with ascites were carried out extensively. However, it was difficult to obtain any evidence substantiating the theory of possible formation of any toxic substances from TSP by the interaction of ascites and TSP either in the peritoneal cavity or in the incubation tube. In addition, microscopic observations of ascites cells showed that TSP did not have any cytological effects on the MTK-sarcoma III cells when injected into the peritoneal cavity in doses of 10 mg/kg.

4) Analysis of the toxic effect of TSP

The purpose of these experiments was to study the mechanism if any which was responsible for the toxic effect of TSP. The selective toxicity of TSP for the ascites rat when TSP was given intraperitoneally can be explained by the formation of some highly toxic metabolites from TSP in the peritoneal cavity. In this experiment, however, it was demonstrated that the ascites rat without ascites, in which the ascitic fluid was removed as completely as possible from the peritoneal cavity, was also susceptible to the lethal effect of TSP.

It is a well-known fact that the peritoneal cavity offers a large absorbing surface from which drugs enter the circulation rapidly. In addition, it may be possible that the host responds to ascites cells in a nonspecific manner in the form of inflammatory response, i.e. changes in permeability, though no lesion was readily observed on the peritoneum membrane of the ascites rat. Thus, it was assumed that the peritoneal membrane of the ascites rat allow relatively large molecules, such as TSP, to pass rapidly. This possibility was checked. The lethal effect of TSP in the ascites rat was not prevented or modified by pretreatment with atropine sulfate (0.1 mg/kg) or by any anti-inflammatory drugs tested, such as acetylsalicylic acid (0.5 g/kg), cortisone acetate (2 mg/kg) or diphenhydramine hydrochloride (8 mg/kg). Therefore, it was unlikely that the rapid absorption of TSP from the peritoneal membrane was related to this phenomenon observed in the ascites rat.

In order to check whether the caseinolytic activity of TSP is related to this pheno-
menon directly, "inactive" TSP was injected into the ascites rat. The 1 mg/kg or 3 mg/kg dose of "inactive" TSP was not fatal but 10 mg/kg was 100% fatal. Thus, the caseinolytic activity of TSP per se did not appear to provide a sufficient explanation for the toxic effect of TSP in the ascites rat. Intraperitoneal administration of trypsin, in the dosage used, appeared to have no specific toxic effect in normal rats or ascites rats.

**DISCUSSION**

The results described above clearly demonstrated that the rat bearing MTK-sarcoma III was more sensitive to the lethal effect of a bacterial protease preparation “TSP” than normal or Yoshida sarcoma bearing rats, when TSP was given intraperitoneally. It was a point of considerable interest that subcutaneous or oral administration of TSP appeared to have no special effect on the ascites rat. While these results have suggested that TSP was selectively toxic for the rat bearing MTK-sarcoma III when given intraperitoneally, there are several possible mechanisms which may be responsible for the phenomenon observed.

Moriya et al. have demonstrated that TSP can travel across endothelial cells, although TSP has a molecular weight of 60,000 (4). Then, the most plausible hypothesis to explain this unexpected phenomenon is that TSP was rapidly and almost completely absorbed from the peritoneal cavity in the ascites rat when TSP was given intraperitoneally. However, while intravenous injections of TSP in doses of 8 mg/kg was well tolerated in the normal rat, this dose of TSP was 100% fatal in the ascites rat when given intraperitoneally (5). Furthermore, there is a basic question as to whether relatively large molecular substances such as TSP can be absorbed from the peritoneal cavity into the blood so at such a rapid rate, although changes in the properties of the peritoneum in the ascites rat can facilitate the TSP absorption from the peritoneal cavity (6).

Next, the observed toxicity of TSP for the ascites rat when TSP was injected intraperitoneally can be explained by the formation of some highly toxic substances from TSP injected into the peritoneal cavity by ascites cells. However, the data obtained showed no evidence of the formation of any toxic substance from TSP by the interaction of ascites cells and TSP in the peritoneal cavity. On the contrary, our results showed that the toxic effect of TSP for the ascites rat was not modified by the removal of ascites fluid. Therefore, a host component, probably the properties of peritoneal membrane, may be essential for this phenomenon observed.

The question that was raised in this experiment was whether the lethal effect of TSP in the ascites rat was related to its proteolytic activity or not. It was not possible to answer to this question conclusively, although our results showed that the caseinolytic activity of TSP was unlikely to provide a sufficient explanation for the observed effect of TSP. There was another question raised as to whether TSP actually enters the cell to carry out its enzymatic activity, or whether it merely acts at the level of the cell membrane of the peritoneum. In the present study we did not attempt to investigate these problems. It was also suggested from our data that some thermolabil substance or enzyme contaminated
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in a TSP preparation could be related to this phenomenon observed.

Lastly, it is known that the uptake of foreign proteins is usually followed by intracellular breakdown, hence the problem may be related to the function of ingested protein in the cells. Further work will be necessary to determine whether our findings with TSP constitute a special case or are generally applicable to other proteases, although intraperitoneal administration of trypsin appeared to have no toxic effect in normal or ascites rats.

In summary, it seems reasonable to conclude that the mechanisms responsible for the selective toxicity of a TSP preparation for the ascites rat may be of a complex nature and the specific interaction of the peritoneal membrane and the TSP preparation may play an essential role in this phenomenon observed.

SUMMARY

The rat bearing MTK-sarcoma III was more susceptible to the lethal effect of a Serratia protease preparation “TSP” than normal or Yoshida sarcoma bearing rats, when TSP was administered intraperitoneally. On the other hand, subcutaneous or oral administration of TSP appeared to have no specific effect in the ascites tumor rats.

While the results obtained suggest that TSP may be selectively toxic to the rat bearing MTK-sarcoma III, the exact reason for this phenomenon remains obscure. However, the results of the present report indicate that specific interactions between the TSP preparation and the peritoneal membrane might play an essential role in this phenomenon observed.

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