Fate and Effect of Endotoxin Derivatives in Tumor-Bearing Mice[1] (35194)

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The hemorrhagic and necrotic effects of bacterial culture filtrates and toxins on some malignant tumors are well documented (1–6).

The present investigations compare the tumor necrotizing effect of toxic endotoxin with its chemically detoxified derivative (endotoxoid) (7–11) and with an incomplete endotoxin which derives from a heptoseless rough mutant (12–14). The aim of these studies is to identify the site of the endotoxin molecule active in tumor hemorrhage and necrosis.

The question whether the endotoxin and its derivatives have a direct or a mediated effect on tumor tissues was also investigated. An attempt has been made in these laboratories to follow the fate of endotoxin and endotoxoid in tumor-bearing animals by using fluorescein-labeled rabbit antiendotoxin antibodies. In another series of experiments, vasoactive substances and other biologically active amines were tested for induction of hemorrhage in tumors.

Materials and Methods. Endotoxic materials. Toxic endotoxin was extracted with trichloroacetic acid by a modification of the Boivin method from *Serratia marcescens* 08 cells. Endotoxoid was prepared from the toxic endotoxin by deacylation with potassium methylate (7). Endotoxic glycolipids were extracted from cells of heptoseless mutants of *Salmonella minnesota* R595 strain by a procedure described earlier (13).

Spermine, putrescine, histamine, and cadaverine were obtained from K & K Laboratories, Plainview, N. Y. *Serotonin* was obtained from Sandoz Pharmaceuticals, Hanover, N. Y. These materials were dissolved in pyrogen-free saline for testing in the tumor hemorrhage assay.

Tumors. Ehrlich ascites tumor (EAT) was provided by Dr. R. Baserga of Temple University School of Medicine and maintained in the peritoneal cavity of C57 Bl/10 mice. Sarcoma 37 (S-37) was provided by Dr. H. F. Havas, and was also maintained interperitoneally in ICR mice. All mice used were males weighing 18-20 g at the time of tumor inoculation.

Tumor-hemorrhage assay was performed according to the method of Shear *et al.* (18) on S-37-bearing ICR mice. Inocula of 6.5×10^6 S-37 cells were implanted subcutaneously 7 days prior to the intravenous administration of the endotoxic material. Hemorrhage was observed 24 hr after challenge by intravenous injection of 0.2-ml quantities in the lateral tail vein. A minimum of 5 animals was tested at each dose.

50% End point in lethality and tumor-hemorrhage assays was determind by the Spearman-Karber method. Details of the procedure and calculation have been described (15). All lethality measurements were performed by intravenous injection.

Deposition of endotoxin and endotoxoid in mouse spleen, liver, and tumors was detected by direct fluorescent antibody (FA) assay of Coons and Kaplan (16). Tissues were snap-frozen and sectioned on a Lipshaw "cryotome" for FA testing. The procedure used has been described in detail in a previous publication (17).

Results. A. Tumor-hemorrhage experiments. The results of these experiments are summarized in Table I. The data clearly indicate that all three materials, *i.e.*, toxic endotoxin, endotoxoid, and endotoxic glycolip-

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TABLE I. Tumor Hemorrhagic Activity of Endotoxic Materials.

Materials	No. of mice injected	Lowest dose causing hemorrhage in 100% of animals (μg)	THD_{50} (μg) (calculated)
Endotoxin	42	25	10
Endotoxoid	48	100	22
Glycolipids	30	20	5
Saline controls	12	No hemorrhage induced	

ids, are active in the tumor-hemorrhage assay. When compared on the basis of the 50% end point dose (THD_{50}), there is no great difference in the activities of the three substances. The endotoxoid does appear to be somewhat less active than toxic endotoxin while the glycolipids show a higher activity than all other substances.

B. Lethality determination in normal and tumor-bearing mice. The initial experiments provided the determination of the lethal effects of endotoxin, endotoxoid, and glycolipids in normal and tumor-bearing mice.

The results of these experiments, summarized in Table II, indicate that the tumor-bearing condition enhances the sensitivity to endotoxin and endotoxoid. In the case of glycolipid, the same general trend can be seen, although a much greater sensitivity of both normal and EAT-bearing C57 Bl/10 mice was observed. Like endotoxin and endotoxoid, there seems to be a twofold difference in the sensitivity of the C57 Bl/10 mice compared with those bearing EAT. The effect of glycolipids on ICR mice is greatly enhanced when these animals bear S-37, *i.e.*, a thirteenfold increase in sensitivity compared with only fivefold for the endotoxin and endotoxoid.

C. Distribution of endotoxin and endotoxoid in tumor-bearing mice. In a previous report (17) it was demonstrated that normal BRVR mice display rapid phagocytosis of intravenously injected endotoxin in liver and spleen phagocytic cells. This was demonstrated by fluorescein antibody. However, the nontoxic endotoxoid was very poorly phagocytosed in these animals, and appeared to remain in the circulation significantly longer than did the endotoxin. In a subsequent publication, this observation was extended to other strains of mice, including C57 Bl/10 and ICR (19).

TABLE II. Lethality of Endotoxic Materials in Normal and Tumor-Bearing Mice.

Mouse strain and tumor	LD_{50}		
	Endotoxin	Endotoxoid	Glycolipids
C57 Bl/10 normal	230	>4000	70
C57 Bl/10 with EAT	100	~1600	35
ICR normal	400	>4000	1175
ICR with S-37	80	720	90

EAT-bearing C57 Bl/10 mice were injected intravenously with 100 μg of endotoxin; and the livers, spleens, and tumors were tested for the presence of O antigen after 4 hr. All animals tested showed the usual pattern of splenic and hepatic uptake of endotoxin. None of the animals displayed any evidence of endotoxin on or within the EAT cells. If mice of the same strain, bearing EAT for the same duration, were injected with 100 μg of endotoxin intraperitoneally, some tumor cells did exhibit positive immunofluorescence; however, the fluorescence observed appeared to be due to passive adsorption of endotoxin on the surface of the EAT cells. This experiment demonstrated that endotoxin could have been detected in association with the tumor cells if it had reached this site after intravenous injection. In the final experiment with C57 Bl/10 mice, EAT-bearing animals were injected with 100 μg of endotoxoid and assayed identically to the endotoxin-treated mice. No evidence for RES or tumor uptake of the detoxified material could be seen.

The S-37 bearing ICR mice were examined in a similar series of experiments. In this case, representative animals from the tumor-

hemorrhage experiment described in the previous section were tested for RES and tumor uptake of endotoxin or endotoxoid by FA assay. The tumor-bearing ICR mice displayed no major alterations in their pattern or degree of RES uptake of endotoxin.

No appreciable amounts of endotoxin or endotoxoid could be detected in the body of the tumors, regardless of their degree of hemorrhage. Also, the degree of hemorrhage or the sensitivity of the animals could not be correlated with degree or pattern of RES uptake of endotoxic materials. Presumably, endotoxin and endotoxoid do not exert their hemorrhagic activity by direct interaction at the site of malignancy.

D. Role of nonendotoxic components and some other vacocactive substances. High voltage electrophoresis of hydrolysates of crude endotoxin preparations as well as of endotoxic glycolipids revealed several fast-moving, ninhydrin-positive components. These highly basic compounds were identified as spermine and putrescine (Key and Nowotny, unpublished). In light of the biological activity of such amines, authentic samples of spermine and putrescine were tested in this assay. Cadaverine was also included in this study because of its chemical similarity. All three amines were quite inactive, even at dose levels of 100 μg/mouse. Presumably, then, these components are not solely responsible for the elicitation of tumor hemorrhage by endotoxin preparations.

Histamine and serotonin were also tested in this assay as examples of vasoactive agents. They were inactive in the tumor-hemorrhage assay even at doses of 500 μg/mouse. Although these experiments do not eliminate the release of endogenous vasoactive agents in the host animal as a factor in the hemorrhage of a tumor, they do suggest that these compounds, if they play a role in this phenomenon, are not solely responsible for these effects.

Discussion. Three endotoxic materials of different chemical nature have similar potency at the induction of tumor hemorrhage. The highly basic amines found associated with crude endotoxin and with the endotoxic glycolipids did not appear to have a significant role in the elicitation of the hemorrhagic reaction. These results indicate that some other portion of the endotoxic macromolecule, which was unaltered by detoxification and mutation, is present in all three materials, and must be responsible for tumor hemorrhage. This common constituent or property of the endotoxins, endotoxoid, or heptoseless endotoxic glycolipids provides activity in the tumor-hemorrhage assay. Neither the presence of the O-antigenic polysaccharide moiety nor the intactness of the lipid-rich zone is required for this action.

These experiments confirmed the conclusion of Seligman *et al.* (20) that endotoxin does not exert its hemorrhagic effect by direct interaction with the tumor cells. The hemorrhagic effect is probably mediated by factors released into the circulation after endotoxin injection. Although vasocative substances would seem to be prime candidates for such mediators, histamine and serotonin alone were found to be ineffective in this assay.

The lethality assays confirmed the findings of Havas *et al.* (21) that tumor-bearing mice are more sensitive to endotoxin lethality. However, there did not appear to be any parallelism between the activity of an endotoxic material in the tumor-hemorrhage assay and the enhanced susceptibility of the animal to the lethal action of the material. Although all three materials were more lethal in the tumor-bearing mice, endotoxoid was clearly much less toxic than endotoxin or glycolipids.

It is worth special note that the C57 Bl/10 strain was much more sensitive to the lethal effects of endotoxic glycolipids than any other mouse strains tested thus far. This extreme sensitivity is unexplained and deserves further study.

Summary. 1. A toxic endotoxin, its nontoxic chemical derivative called endotoxoid, and endotoxic glycolipids were tested in tumor-hemorrhage and lethality assays in tumor-bearing mice. All three materials were active in the tumor-hemorrhage assay. This indicates that neither toxicity nor the presence of O-antigenic polysaccharides is necessary to induce tumor hemorrhage.

2. No evidence of a direct action of endotoxin on tumors or of altered reticuloendothelial function could be obtained by the fluorescein antibody technique, which was used to detect the presence of endotoxin preparations in the different tissues.

3. Basic amines such as putrescine, spermine, and cadaverine, found in incompletely purified endotoxin preparations, and vasoactive agents such as histamine and serotonin could not duplicate the hemorrhagic activity of the endotoxic materials.

4. The tumor-bearing state increases the susceptibility of mice to the lethal action of all three materials.

5. Various inbred mouse strains, with or without tumor, showed a significantly different degree of endotoxin sensitivity.

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