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Studies on the Mechanism of Localization of Gram-Negative Bacteria in Tumor

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Summary

As a part of the studies on the mechanisms of bacterial abortion, such as brucellosis of cattle, the role of endotoxin in the elective localization of Gram negative bacteria in tumor was studied and the following results were obtained.

1. Both *Sal. enteritidis* and *Sal. pullorum* proliferated more remarkably in implanted Ehrlich carcinoma of mouse than in the spleen which is generally considered to be an organ of predilection. However, *Dip. pneumoniae* did not show such an elective localization to the tumor tissue.
2. A very small dose of the endotoxin extracted from *Sal. pullorum* induced selectively marked hemorrhages in the tumor within a few hours. A similar effect was found by using killed cells of *E. coli*, while those of *Dip. pneumoniae* had almost no effect.
3. The elective proliferation of *Sal. pullorum* in the tumor was promoted by pretreatment with the endotoxin and depressed by daily injections of antitoxic immune serum.
4. Immunohistological observations revealed that the free endotoxin, released from *Sal. pullorum* grown in the spleen and other tissues, arrived at the small vessel wall of the tumor preceding the appearance of living bacteria in the tumor.
5. From these results, we conclude that one of the factors of elective proliferation of Gram negative bacteria in the tumor is the extreme sensitivity of the tumor tissue to the endotoxin.

Zahl and Bjerknes (1), and Takeda and Tsuchiya (2, 3) demonstrated the effect of endotoxin of Gram negative bacteria in inducing marked hemorrhage in the placenta of pregnant animals. Also, Zahl (4) and otheres (5–9) found that the endotoxin induced hemorrhage in tumor. On the other hand, it is known that certain kinds of gram-negative bacteria multiply vigorously in the placenta of pregnant animals, frequently to become a cause of abortion (10) .

If the injurious effect of the endotoxin is related with the intense prolifera-

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tion of Gram negative bacteria in the placenta, it may be that the bacteria also proliferate intensely in the tumor tissue. In a study to test this assumption, Toba and his coworkers (11) found that *Sal. cholerae suis* electively multiplied in implanted rhodamine sarcoma of rats and that the multiplication was selectively stimulated by the endotoxin and inhibited by antitoxic immune serum. Thus, they mentioned that the mechanism of the elective localization of the Gram negative bacteria in the tumor may be explained by the sensitivity of the tumor tissue to the endotoxin.

In this paper, the role of the endotoxin in the process of the elective proliferation of Gram negative bacteria in tumor tissue was further studied. These studies will be of use in the understanding of the mechanism of abortion caused by brucellosis or infection with *Sal. abortus equi* in domestic animals, for the sensitivities of the tumor to the living bacteria and to the endotoxin are thought to be very similar to those of the placenta.

Materials and Methods

Bacteria innoculated. *Sal. enteritidis* (strain 116–54), *Sal. pullorum* (strain 9–25) and *Dip. pneumoniae* (type III) were used. Cells of the first two strains harvested from 20 hour cultures on nutrient agar slant, were weighed and diluted appropriately for inoculation with physiological saline. The suspension of *Sal. enteritidis* contained 10^{-7} mg of cell per ml, and that of *Sal. pullorum*, 10^{-2} mg per ml. *Dip. pneumoniae* obtained from 18 hour culture in nutrient broth was diluted 1:10 with saline. These inocula, 0.1 ml, were injected subcutaneously into the left hind leg of tumor bearing mice.

Mice and tumor. The male dd mice, weighing 15–17 g at 4 weeks old were obtained from a commercial source. The mice were subcutaneously transplanted with two million Ehrlich ascitic carcinoma cells, into the back side of their right hind leg. Ten days after transplantation, when the tumor had grown as large as 10 mm in diameter, the experiments were undertaken.

Viable count of bacteria in tissue. Three infected mice were sacrificed daily. The mice were anaesthetised with ether and bled by heart puncture. The spleen, liver, lung, kidney and tumor of these mice were removed, weighed and homogenized with an appropriate volume of saline. The homogenates and blood were used for viable count according to the ordinal method. The medium for *Salmonella* was desoxycholate agar enriched with peptone, cystine and yeast extract and that for pneumococcus was blood agar. The number of the organisms was calculated per gram of the materials and the average was expressed as the geometrical mean.

Endotoxin of Sal. pullorum. Endotoxin of *Sal. pullorum* was prepared after the method of Boivin and Mesrobeanu (12), modified by Webster *et al.* (13). The LD_{50} of this endotoxin was estimated as 0.125 mg in 3 weeks old mice.

Killed cell powder. Cells of E. coli harvested from 24 hr. cultures on glucose

bouillon ager were suspended in saline containing 1 per cent of phenol. Broth containing the pneumococci cultured for 22 hours was added phenol of which the final concentration was 1 per cent. Then, these bacterial suspensions were centrifuged and washed. The packed cells obtained were dried with cold acetone.

Anti-endotoxic serum. The antiserum against endotoxin of *Sal. pullorum* was obtained from hyperimmunized rabbits or mice prepared by a series of injections of the endotoxin with Freund's adjuvant.

Immunofluorescense. The indirect immunofluorescent staining method (14) was used throughout the present studies as a rule. Conjugation of fluorescein isothiocyanate to sheep immunoglobulin against rabbit serum γ-globulin was performed by the slightly modified methods of Rigss *et al.* (15) and Marshall *et al.* (16). Tissues for the immunofluorescent studies were fixed with absolute ethanol at 4°C immediately after autopsy. Sections for staining were prepared according to the method of Kyogoku (17).

Results and discussion

I. Multiplication of Gram negative and Gram positive bacteria in tumor-bearing mice.

Toba *et al.* (11) found an elective localization of *Sal. cholerae suis* in the rhodamine sarcoma of rats. In order to study whether or not any relationship exists between this fact and the different pathogenicities of the bacteria, and to determine the behavior of the Gram positive bacteria, lacking O antigen, in tumor-bearing mice, the attitudes of *Sal. enteritidis*, *Sal. pullorum*, and *Dip. pneumoniae* in tumor-bearing mice were observed in this experiment.

Sal. enteritidis in tumor-bearing mice. Into mice transplanted with Ehrlich's carcinoma cells, virulent strain of *Sal. enteritidis* was inoculated. As shown in Table 1, four days after the inoculation, *Sal. enteritidis* invaded the spleen in considerable numbers which is an original site of parasitism, and then gradually multiplied. On the other hand, the invasion into the tumor started with only a few organisms but they proliferated remarkably there, soon far exceeding the number in the spleen. *Sal. enteritidis* thus appears to proliferate electively in the tumor.

Sal. pullorum in tumor-bearing mice. *Sal. pullorum* having oridinally rather weak pathogenicity to mice was inoculated into the tumor-bearing mice. The results are shown in Table 2.

A considerable number of *Sal. pullorum* were detected in the spleen in the first day but subsequent multiplication was very slow as compared with *Sal. enteritidis*. While, in the tumor, they multiplied with similar rapidity as the *Sal. enteritidis*. So the ratios of bacterial count in the tumor to that in the spleen gradually increased to reach nearly 10^7. Thus, it was found that *Sal. pullorum* also multiplied electively in the tumor.

Therefore, the property of the bacteria to favorably proliferate in the tumor

TABLE 1. *Multiplication of Sal. enteritidis in Tumor-bearing Mice.*

Days after inoculation[1]	Viable count per gram[2]			Ratio tumor/spleen
	Blood[3]	Spleen	Tumor	
1	0	0	0	—
2	0	0	0	—
3	0	0	0	—
4	9.65×10^{0}	1.22×10^{2}	2.71×10^{0}	0.022
5	4.93×10^{1}	6.19×10^{3}	8.44×10^{1}	0.014
6	1.46×10^{2}	9.31×10^{6}	1.50×10^{9}	160
7	4.12×10^{1}	2.64×10^{9}	6.42×10^{10}	24

1) 10^{-8} mg was inoculated per mouse. 2) Geometrical mean.
3) Heart blood.

TABLE 2. *Multiplication of Sal. pullorum in tumor-bearing Mice.*

Days after inoculation[1]	Viable count per gram[2]			Ratio tumor/spleen
	Blood[3]	Spleen	Tumor	
1	0	1.29×10^{3}	1.22×10^{4}	1.5
2	0	4.64×10^{0}	0	—
3	2.72×10^{0}	1.43×10^{3}	3.70×10^{2}	2.1
4	0	4.31×10^{0}	2.02×10^{1}	4.7
6	0	5.43×10^{0}	3.12×10^{1}	5.7
7	0	2.37×10^{2}	4.07×10^{3}	17
8	0	4.58×10^{3}	4.95×10^{5}	110
9	8.88×10^{0}	1.17×10^{2}	9.92×10^{8}	8500000

1) 10^{-3}mg was inoculated per mouse. 2) Geometrical mean.
3) Heart blood.

TABLE 3. *Multiplication of Dip. pneumoniae in Tumor-bearing Mice.*

Days after inoculation[1]	Viable count per gram[2]			Ratio tumor/spleen
	Blood[3]	Spleen	Tumor	
1	0	0	0	—
2	0	0	0	—
3	0	3.68×10^{1}	6.30×10^{0}	0.17
4	0	5.65×10^{1}	1.26×10^{1}	0.22
5	0	2.13×10^{2}	4.74×10^{1}	0.22
6	1.25×10^{3}	1.34×10^{5}	1.21×10^{6}	9.0
7	0	0	7.66×10^{2}	—
8	0	0	0	—

1) 0.01 ml of 18 hrs. nutrient broth culture was inoculated per mouse.
2) Geometrical mean. 3) Heart blood.

appears to be unrelated to the intensity of their pathogenicity.

That the considerable numbers of bacteria were detected in the spleen and tumor in the first stage of the infection may be due to the inoculation of a relatively large amount of the bacteria in view of their mild pathogenicity. That is, a

TABLE 4. *Effects of the Endotoxin of Sal. pullorum on Tumor-hemorrhage.*

Endotoxin injected (mg)	Hemorrhage[1]					
	in tumor			in liver		
1.0	++	++	++	−*	−*	−*
0.5	++	++	++	−*	−*	−*
0.25	++	++	++	−*	−*	−*
0.125	−*	++	++	−	−*	−*
0.063	+	++	++	−*	−*	−*
0.031	−*	+	−*	−	−	−
0.016	++	+	++	−	−	−
0.008	+	−	−	−	−	−
0.004	−	−	−	−	−	−
none	−	−	−	−	−	−

1) Marks, +∼++, indicate severity of hemorrhage in individual mice observed 24 hrs. after injection. Stars represent congestion-positive.

TABLE 5. *Relationship between Number of Tumor-hemorrhage mice and the Time after Injection of the Endotoxin from Sal. pullorum.*

Hours after injection[1]	0	0.5	1	1.5	2	6	24
Hemorrhage in tumor[2]	0/5	0/5	2/5	2/5	5/5	5/5	5/5

1) 0.2 mg of endotoxin was injected per mouse.
2) Positive/employed.

phenomenon resembling primary septicemia may have occured.

Dip. pneumoniae in the tumor-bearing mice. To give an example of Gram positive bacteria with relatively severe pathogenicity causing systemic infection in mice, *Dip. pneumoniae* was inoculated into tumor-bearing mice. As shown in Table 3, the pneumococci invaded and proliferated in the spleen and lung, from the third day of the experiment with simultaneous similar proliferation in the tumor. However, the numbers of bacteria in the tumor were generally less than those in the spleen and a marked increase as seen in Gram negative bacteria was not observed, even at the stage of marked bacteremia.

Exceptionally, on the 7th day when most of the bacteria disappeared from various organs including the spleen, a considerable number of bacteria were still found in the tumor. This was probably due to a less active clearance of the tumor, compared with other ordinary tissues.

From these results, no elective proliferation of *Dip. pneumoniae* in the tumor is evident.

2. *Influence of bacterial endotoxin on the tumor.*

Though the induction of hemorrhage in the tumor by endotoxin of Gram negative bacteria is generally known, it was tested to make sure that the *Sal.*

TABLE 6. *Effects of Killed Cells of E. coli and Dip. pneumoniae on Tumor-hemorrhage.*

Dose[1] in mg	Hemorrhage in tumor[2]					
	E. coli			Dip. pneumoniae		
100	++	++	++	+	−	−
10	++	++	++	−	−	−
1	++	++	++	−	−	−
0.1	+	−	−	−	−	−
none	−	−	−			

1) Aceton-dried cells.
2) Marks, +~++, indicate severity of hemorrhage in individual mice observed 24 hrs. after injection.

TABLE 7. *Effect of the Endotoxin on Multiplication of Sal. pullorum in Tumor-bearing mice.*

Group	Days after inoculation[1]	Viable count per gram[2]			Ratio tumor/spleen
		Blood[3]	Spleen	Tumor	
Untreated	1	0	9.89×10^2	2.44×10^3	2.5
	2	0	3.20×10^4	3.93×10^5	27
	3	5.46×10^2	6.10×10^3	2.02×10^5	33
Endotoxin-treated[4]	1	1.02×10^1	7.20×10^3	5.84×10^6	810
	2	1.02×10^3	3.99×10^4	6.32×10^8	16000
	3	2.62×10^2	7.86×10^4	7.24×10^9	92000

1) 10^{-3}mg was inoculated per mouse. 2) Geometrical mean.
3) Heart blood. 4) 0.1 mg per mouse was subcutaneously injected just before the inoculation.

pullorum used in the present studies had such an endotoxin, and on the contrary that the Gram positive bacteria had not similar substance.

Effects of endotoxin of Sal. pullorum on the tumor. Various doses of the endotoxin extracted from *Sal. pullorum* were injected into tumor-bearing mice. Autopsy performed 24 hours after the treatment gave a marked hemorrhage in the tumor tissue even with a dose of as low as 0.016 mg as shown in Table 4, while no hemorrhage was noted in other tissue even at 1 mg. The tumor hemorrhage began to appear at 1 hour after injection, spreading to include all the animals after 2 hours, as shown in Table 5.

Effects of killed cells of Gram positive and Gram negative bacteria on tumor. As shown Table 6, when the killed cells of *Dip. pneumoniae* or *E. coli* were injected into tumor-bearing mice, marked hemorrhage was seen on the treatment with 1 mg of E. coli cells, while hemorrhages were scarcely found even with an injection of 100 mg of cells of *Dip. pneumoniae.*

From these results, it was made clear that the endotoxin of Gram negative bacteria caused a marked hemorrhage of the tumor but such a response was not

TABLE 8. *Effects of Anti-endotoxic Mouse Serum on Multiplication of Sal. pullorum in Tumor-bearing Mice.*

Treatment[1]	Days after inoculation[2]	Viable count per gram[3]			Ratio tumor/spleen
		Blood[4]	Spleen	Tumor	
Normal serum	1	0	7.82×10^{0}	1.06×10^{2}	14
	2	0	1.36×10^{3}	6.18×10^{3}	4.5
	3	0	4.12×10^{1}	5.00×10^{3}	120
	4	0	7.96×10^{2}	1.22×10^{5}	150
	5	0	9.39×10^{5}	1.25×10^{7}	13
	6	0	2.05×10^{2}	1.52×10^{5}	740
	7	2.43×10^{2}	1.13×10^{5}	2.56×10^{8}	2300
Antitoxic serum[5]	1	0	$<2.15\times10^{0}$	2.15×10^{0}	>1.0
	2	0	3.02×10^{1}	2.71×10^{0}	0.09
	3	0	4.16×10^{1}	2.71×10^{0}	0.06
	4	0	1.71×10^{1}	6.69×10^{0}	0.39
	5	0	$<2.15\times10^{0}$	2.15×10^{0}	>1.0
	6	0	9.65×10^{0}	9.13×10^{0}	0.95
	7	1.41×10^{1}	7.14×10^{3}	8.73×10^{3}	1.2

1) 0.1 ml of sera was subcutaneously injected per mouse, daily.
2) 10^{-3} mg was inoculated. 3) Geometrical mean. 4) Heart blood.
5) Precipitine titer was 1:8.

present in *Dip. pneumoniae.* Based on this fact and the results previously described, it is concluded that viable Gram negative bacteria proliferate electively in the tumor and that their endotoxin easily causes tumor hemorrhage, though Gram positive bacteria and their cellular components do not. This suggests that there is a relationship between the elective proliferation of bacteria in the tumor and the action of their endotoxin.

3. Relationship between multiplication of Gram-negative bacteria in the tumor and endotoxin.

If there is really any relationship between the endotoxin and the bacterial proliferation localized in the tumor as above mentioned, the elective localization of the germ will become more remarkable in the mice injected with the endotoxin than in those not injected. While, the phenomenon will be depressed by a treatment which inhibits only the actions of the toxin in the infected mice. The next experiments were designed to confirm these presumptions.

Influence of endotoxin on the multiplication of Sal. pullorum in the tumor. Sal. pullorum was inoculated into tumor-bearing mice, some of which had been subcutaneously injected with its endotoxin, while others had not. The daily growth of the bacteria is shown in Table 7.

In the group pretreated with endotoxin, the multiplication of the germ was generally accelerated as compared with that in the untreated control, and this acceleration was remarkable in the tumor. The ratios of the bacterial number in the tumor to that in the spleen during the first 3 days were 810 to 92,000 in the endotoxin-injected group, though the ratio in the control group was 2.5 to 33.

This means that the endotoxin facilitates the selective proliferation of bacteria in the tumor.

Effects of antitoxic immune serum on the proliferation of Sal. pullorum in the tumor. After inoculation of *Sal. pullorum*, the tumor-bearing mice were divided into 2 groups. Into one group, a mouse-immune-serum for the endotoxin of *Sal. pullorum* was injected daily, while into the other group normal mouse serum was similarly injected as the control.

As shown in Table 8, the result indicates that the immune serum generally inhibited the proliferation of the invading bacteria and, especially, this action was remarkable in the tumor. The ratios of bacteria in the tumor to those in the spleen gave a high value in the control group but a very low one in the antiserum treated group. So it is said that the immune serum for the endotoxin selectively inhibits the proliferation of Gram negative bacteria in the tumor.

On the other hand, the bacteriocidal abilities of both sera, tested by Neiser and Wechsberg's method (15), were not different. Moreover, the inhibitory effect observed in the spleen and other organs was not so conspicuous as in the tumor. Therefore, it is not thought that the selective inhibition in the tumor was caused by only the bacteriocidal action of the immune serum. As the sera used were prepared from the mice in a strain, the only difference between them was the presence of the antitoxic antibody.

For these reasons, the inhibitory effect of the antitoxic serum on the bacteria in the tumor should be explained only by the neutralization of the endotoxin from the infecting bacteria. Based on the results described here, the elective proliferation of Gram negative bacteria in the tumor is probably caused by the action of the endotoxin of these bacteria.

4. Distribution of endotoxin in the tumor tissue.

In the above experiments, it was shown that the elective proliferation of Gram negative bacteria in the tumor is intimately related with their own endotoxin and that a very small amount of endotoxin rapidly caused hemorrhages in the tumor.

From these facts, it may be assumed that the elective proliferation in the tumor is probably due to the following mechanism. That is, the injury of the tumor tissue by the endotoxin released from the invading and proliferating bacteria in the original sites of the parasitism such as spleen and liver facilitates the multiplication of the bacteria which reach to the tumor, inducing an extreme proliferation.

To confirm this assumption, observation of the endotoxin in the tumor of the mice infected with *Sal. pullorum* was attempted by the fluorescent antibody technique. As shown in Fig. 1, specific fluorescence had become visibe in the vascular wall of the tumor before the inoculated bacteria appeared in it, though they were detected from the spleen or liver. This indicates that the released bacterial endotoxin had reached the vascular wall of the tumor via blood stream and fixed

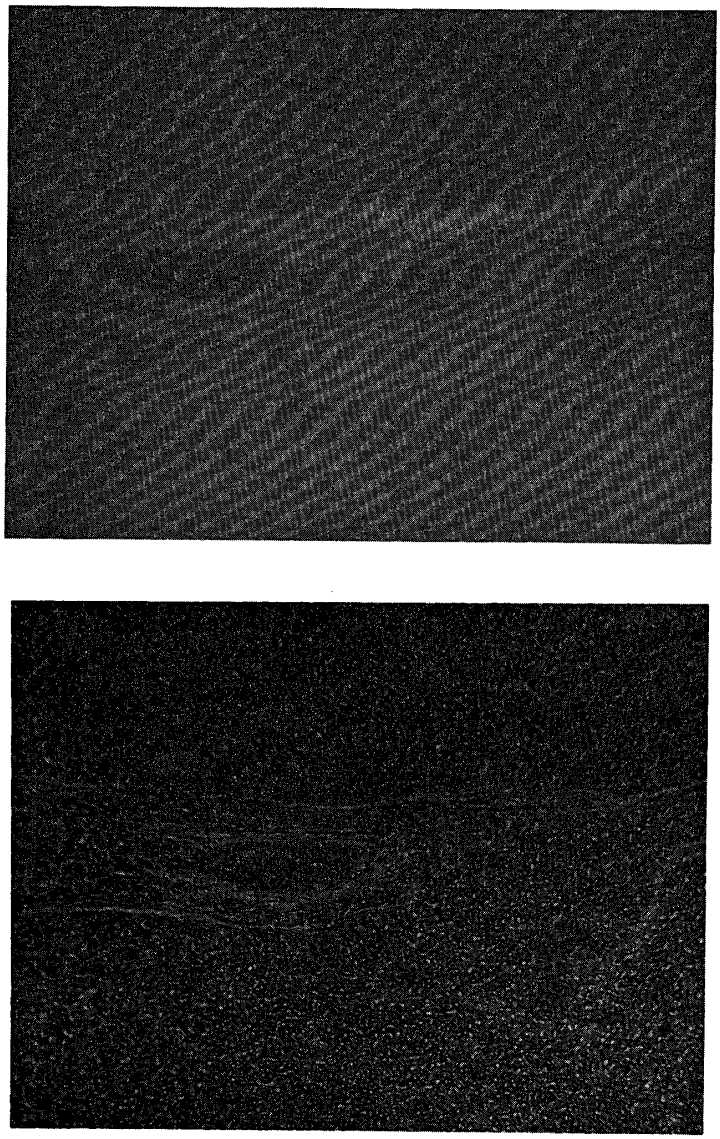

FIG 1. Endotoxin on the small vessele wall of tumor, stained by immunofluorescent technique.
Top: Fluorescent antibody staining (×400)
Bottom: Phase-contrast micrograph (×400)
Three days after inoculation of Sal. pullorum, the organisms were recovered from the spleen and liver but not from the tumor of this mouse.

itself there, preceding the invasion of the viable bacteria, and thus supports the above assumption. The bacterial abortion by *Br. abortus* or *Sal. abortus equi* may also involve a similar mechanism.

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