16124

Effect of Histolyticus Infection and Toxin on Transplantable Mouse Tumors.*

RAYMOND C. PARKER, HELEN C. PLUMMER, CHARLES O. SIEBENMANN, AND MARION G. CHAPMAN.

From the Connaught Medical Research Laboratories, University of Toronto.

The treatment of experimental and human tumors with bacterial infections and bacterial products began with the work of Busch, almost 80 years ago.[1] Since then, the use of bacterial products in the treatment of clinical and experimental cancer has received the attention of many investigators, including Fehleisen,[2] Bruns,[3] Coley,[4] Gratia and Linz,[5] Duran-Reynals,[6] Andervont,[7] and Shear and his co-workers.[8] Brues and Shear[9] reported the treatment of 4 patients with intramuscular injections of a polysaccharide from culture filtrates of *Serratia marcescens* (*B. prodigiosus*), which had previously been shown to produce hemorrhage in and partial or complete destruction of sarcomas in mice.

In previous experiments carried out by two of the present authors, it was demonstrated that in experimental *Cl. welchii* infections in mice,[10] and to an even more marked degree in experimental infections with *Cl. histolyticum*,[11] the systematic administration of the specific antitoxin counteracts the toxemia without preventing the early development of the large local lesions produced by the lytic action of toxins of the gas gangrene group of anaerobes. Accordingly, it seemed reasonable to undertake an investigation designed (a) to destroy tumor tissue by infection with histolyticus spores, to control the resulting toxemia by means of a systemic administration of antitoxin and, finally, to control the infection itself by means of penicillin; (b), to destroy tumor tissue by repeated local injections of small doses of histolyticus toxin; and (c) to test the effect of histolyticus toxin on sarcoma, carcinoma and normal tissues cultivated *in vitro*. Although the results of the experiments that were carried out do not warrant the use of histolyticus infection, or of partially purified histolyticus toxin, in the treatment of human tumors, it is believed that they are of sufficient theoretical interest to justify the present report.

1. *Sarcoma treated with viable histolyticus spores, followed by antitoxin and penicillin. Exp. A.* The first experiment included thirty-three C57 black mice, 18 of which were inoculated with a rapidly growing, transplantable fibrosarcoma (Bar Harbor: L946AII). Of the sarcoma mice, 15 were infected with a spore suspension of *Cl. histolyticum* contained in 0.1 ml of a 5% calcium chloride solution that was injected directly into the developing tumors 8 days after tumor inoculation. Ten of these mice were subsequently treated either with antitoxin (Group 1, 5 animals) or with sodium-penicillin (Group 2, 5 animals) in order to control the infection. Five tumor mice infected with the spore suspension were otherwise untreated (Group 3). Three tumor mice received no treatment whatsoever (Group 4).

* This investigation was supported, in part, by a grant from the Ontario Cancer Treatment and Research Foundation.

1 Busch, W., *Berl. klin. Woch.*, 1868, **5**, 137.

2 Fehleisen, *Deutsch. med. Woch.*, 1882, **8**, 553.

3 Bruns, P., *Beitr. klin. Chir.*, 1888, **3**, 443.

4 Coley, W. B., *Ann. Surg.*, 1891, **14**, 199; *Glasgow Med. J.*, 1936, **126**, 49, 128.

5 Gratia, A., and Linz, R., *C. R. Soc. biol.*, 1931, **108**, 427.

6 Duran-Reynals, F., PROC. SOC. EXP. BIOL. AND MED., 1933, **31**, 341; 1935, **32**, 1517.

7 Andervont, H. B., *Am. J. Cancer*, 1936, **27**, 77.

8 Shear, M. J., *et al.*, *J. Nat. Cancer Inst.*, 1943, **4**, 81, 99, 107, 123; 1944, **4**, 461.

9 Brues, A. M., and Shear, M. J., *J. Nat. Cancer Inst.*, 1944, **5**, 195.

10 Siebenmann, C. O., and Plummer, H., *J. Pharm. and Exp. Therap.*, 1945, **83**, 71.

11 Siebenmann, C. O., and Plummer, H., unpublished experiments.

All treatments were started 24 hours after spore inoculation in order to give the histolyticus infection a chance to develop.

Each mouse treated with antitoxin received subcutaneously 12.5 units daily, for 7 days. The antitoxin consisted of Lederle's polyvalent gas gangrene antitoxin containing 150 units of histolyticus antitoxin per ml. Each mouse treated with penicillin received intramuscularly 250 units twice daily for 2 days, and subsequently, the same dose 4 times daily for 5 days.

The 15 control mice that did not receive tumor inoculations were all infected with spore suspensions. Subsequently, 5 of them were treated with antitoxin (Group 5), 5 were treated with penicillin (Group 6) and 5 were left untreated (Group 7).

In this and subsequent experiments, the tumor material was ground fine enough to be injected through a No. 24 needle. The usual inoculum consisted of 0.5 ml of a 20% suspension in Tyrode's solution made up without bicarbonate.

Results. (Table I). The sarcoma bearers that received no treatment following infection with spore material (Group 3) died early in the experiment, *i.e.,* between the 3rd and 10th day after infection. These early deaths resulted, apparently, from the acute histolyticus infection, for tumor tissue was either in small amount or non-existent. Spore-infected sarcoma bearers that received penicillin (Group 2) developed somewhat larger tumors than those without penicillin. One survivor of this group, which lived until the 16th day, developed a large tumor. But 4 of the 5 spore-infected sarcoma bearers that received antitoxin treatment (Group 1) died from the 15th to the 17th day with little or no tumor tissue evident on macroscopic examination. As in Group 3, these deaths were likely due to histolyticus infection. To the 5th mouse of the group, which survived the 17th day without showing a tumor, penicillin treatment was then given for 6 days in order to eradicate any residual histolyticus infection. The penicillin was given in 20 intramuscular injections of 250 units each. But on the 21st day after spore inoculation, this mouse showed

TABLE I.

Effect of Histolyticus Infection on Sarcoma in Mice (Exp. A.).

	Sarcoma bearing mice								Normal mice							
Group	1		2		3		4		5		6		7	
Infection	*Cl. histolyticum* Spores Histolyticus antitoxin (s.c.)		*Cl. histolyticum* Spores		*Cl. histolyticum* Spores		None		*Cl. histolyticum* Spores Histolyticus antitoxin (s.c.)		*Cl. histolyticum* Spores		*Cl. histolyticum* Spores	
Treatment*			Penicillin (i.m.)		None		None				Penicillin (i.m.)		None	
	Day of death†	Size of sarcoma	Day of death	Size of sarcoma	Day of death	Size of sarcoma	Day of death	Size of sarcoma	Day of death	Infected leg	Day of death	Infected leg	Day of death	Infected leg
Mouse 1	15	trace (L)‡	3	small (L)	3	trace (L)	13	medium	37§	lesion +‖ (healed)	8	lesion ++ (healed)	3	lesion +++
" 2	15	trace (L)	4	" (L)	3	" (L)	28	large	37§	"	37§	"	2	"
" 3	16	no trace (L)	5	" (L)	4	no trace (L)	37¶	"	37§	"	37§	"	3	"
" 4	17	" (L)	7	medium	6	" (L)			37§	"	37§	"	3	"
" 5	37	large	16	large	10	" (L)			37§	"	37§	"	3	"

* For dosage, see text.
† Number of days after infection.
‡ (L) denotes lysis of tumor.
§ Mouse apparently healthy when sacrificed.
‖ Small lesion +; medium lesion ++; large lesion +++.
¶ Mouse apparently near death when sacrificed.

TABLE II.
Effect of Histolyticus Infection on Sarcoma in Mice (Exp. B).

	Sarcoma bearing mice								Normal mice							
Group	1		2		3		4		5		6		7			
Infection Treatment*	*Cl. histolyticum* Spores Histolyticus antitoxin (s.c.)		*Cl. histolyticum* Spores Penicillin (i.m.)		*Cl. histolyticum* Spores None		None None		*Cl. histolyticum* Spores Histolyticus antitoxin (s.c.)		*Cl. histolyticum* Spores Penicillin (i.m.)		*Cl. histolyticum* Spores None			
Mouse No.	Day of death†	Size of sarcoma	Day of death	Size of sarcoma	Day of death	Size of sarcoma	Day of death	Size of sarcoma	Day of death	Infected leg	Day of death	Infected leg	Day of death	Infected leg		
1	3	small (L)‡	2	medium (L)	2	medium (L)	1	small	66§	normal	2	lesion +‖	1	lesion +		
2	4	medium (L)	6	large (L)	2	" (L)	1	"	66§	"	2	"	2	"	+++	
3	4	large	6	" (L)	2	small (L)	6	large	66§	"	47	" +++ (healed)	3	"	++	
4	5	"	7	" (L)	2	medium (L)	7	"	66§	"	66§	lesion + (healed)	3	"		
5	6	medium (L)	7	small (L)	3	small (L)	10	"	66§	"	66§	normal	3	"		
6	8	" (L)	8	" (L)	3	" (L)	10	"	66§	"	66§	"	4	"		
7	15	large	10	large (L)	3	" (L)	11	"	66§	lesion ++ (healed)	66§	lesion + (healed)	5	"		
8	22	medium (L)	11	large	5	" (L)	11	"	66§	normal	66§	"	5	"	+	
9	25	" (L)	12	"	8	large (L)	12	"	66§	"	66§	"	5	"	+++	
10	32	small (L)	12	"	12	"	12	"	66§	lesion ++ (healed)	66§	"	7	"	+	

* For dosage, see text.
† Number of days after spore infection.
‡ (L) denotes lysis of tumor.
§ Mouse apparently healthy when sacrificed.
‖ Small lesion +; medium lesion ++; large lesion +++.

a small tumor that grew to full size and resulted in death on the 37th day.

The 5 non-tumor mice that were infected with spore material and treated subsequently with antitoxin (Group 5) showed healed lesions at the time the experiment was terminated on the 37th day. Of the 5 non-tumor mice that were infected with spore material and treated subsequently with penicillin (Group 6), one showed a healed lesion at the time of death, from unknown causes, on the 8th day, whereas the others, also with healed lesions, survived until the termination of the experiment. None of the 5 non-tumor mice infected with spore material without further treatment (Group 7) survived more than 3 days. All showed extensive lysis of the muscles of the infected leg.

Exp. B. As a further means of studying the effect of histolyticus infection on mouse sarcoma, a second experiment was carried out. Except that the groups were larger, this experiment was similar to the previous one in all essential details.

Results (Table II). The sarcoma grew faster in this experiment and killed all the control mice (Group 4) by the 12th day. Of the 10 tumor mice that were infected with histolyticus spores and treated subsequently with antitoxin (Group 1), 4 survived the untreated tumor bearers. In order to suppress any residual histolyticus infection in these 4 survivors, penicillin treatments were begun on the 8th day after infection. The dosage (intramuscular injections of 250 units) ranged from 3,250 units (mouse No. 7) to 6,500 units (mouse No. 10) and depended upon the length of time each mouse survived. All 4 mice finally developed tumors, though in 3 the sarcoma showed definite signs of lysis at the time of death. The mouse that lived longest (mouse No. 10) outlived the last surviving sarcoma-control by 20 days.

II. *Carcinoma treated with viable spores, followed by antitoxin and penicillin.* This experiment, which was carried out simultaneously with Exp. A, and in essentially the same manner, included 33 first generation hybrid mice obtained by mating C57 black females with C3H males. The tumors consisted of transplantable adenocarcinomas (Bar Harbor: E-0771).

Results. Unlike the sarcomas treated in Exps. A and B, the carcinomas showed little evidence of tumor regression following the injection of spore material. With the exception of 2 mice in the group of tumor bearers that received spore material without subsequent treatment, all tumor-bearing mice survived for at least 13 days and developed tumors of considerable size.

III. *Sarcoma treated with toxin.* This experiment included thirty-five C57 black mice, 20 of which were inoculated with the fibrosarcoma (Bar Harbor: L946AII) already mentioned. Of the sarcoma mice, 15 were treated with the toxin of *Cl. histolyticum* administered on the 6th, 8th, 10th and 13th days following tumor inoculation. In 5 of these mice (Group 1), the toxin was introduced directly into the tumor in 4 injections of 1.5 mg each. In 5 mice (Group 2), the same amounts of toxin were injected subcutaneously around the base of the tumor. For the remaining 5 (Group 3), which also received subcutaneous injections around the base of the tumor, the initial injection of toxin consisted of twice the usual amount. Five sarcoma mice (Group 4) received no treatment whatsoever. The 15 control mice that did not receive tumor inoculations were divided into 3 groups of 5 mice each. These groups received the same amount of toxin, respectively, as Groups 1, 2 and 3 of the tumor-bearing mice.

The toxin consisted of a powdered, dry preparation provided by the National Institute of Health (Bethesda, Md.). It was dissolved in saline, distributed in small tubes and kept frozen in a CO_2-ice box until used.

Results. The 5 sarcoma bearers that received no treatment died between the 10th and the 13th day after tumor inoculation; and those that died after the 10th day showed large tumors. There were no deaths among the 15 non-tumor control mice that received toxin injections, although 10 of them developed lesions that eventually healed. Of the 5 tumor-bearing mice that were injected with toxin introduced directly into the devel-

oping tumors (Group 1), 2 died on the 7th day following tumor inoculation (1 day after the beginning of treatment) and the others survived for 13, 14 and 29 days, respectively. The 5 tumor bearers that were injected with toxin introduced about the base of the tumor (Group 2) died between the 14th and 31st days following tumor inoculation. Of the 5 tumor bearers that were injected with toxin introduced subcutaneously about the base of the tumor, but with a larger initial dose (Group 3), one died on the 7th day after tumor inoculation, and 4 survived from 32 to 36 days. Two mice of Group 2 and two mice of Group 3 showed tumors that were only about half the size of those observed in the untreated control mice, whereas the other mice of these groups developed large tumors. In Group 1, in which the toxin was injected directly into the tumors, the 3 mice that survived the treatment for 7 days or more showed an even greater reduction in tumor size.

IV. *Carcinoma treated with toxin.* Although a large series of experiments were made in an effort to test the effect of histolyticus toxin on carcinoma, only one will be reported in detail. This experiment included 16 first generation hybrid mice obtained by mating C57 black females with C3H males. Twelve of these mice were inoculated with a slow-growing, transplantable adenocarcinoma that had arisen spontaneously in a female of the C3H stock. Of the 12 tumor-bearing mice, 4 were treated with a total of 2.5 mg of histolyticus toxin that was introduced directly into the tumor in 8 injections over a period of 20 days, 4 were treated with the same amount of toxin that was introduced intramuscularly but into the leg opposite the one bearing the tumor, and 4 were left untreated. Four control mice without tumors were treated with a total of 4.0 mg of toxin given in 8 injections over 20 days. This was an amount of toxin in excess of that used in treating the tumor-bearers.

Results. On the 7th day following the beginning of toxin treatment (30 days after tumor inoculation), there were 3 deaths in the group that had been injected with toxin introduced directly into the tumors. At the time of death the tumors were bean-sized, 2 with slight lesions and one with a healed lesion. The remaining mouse of the group carried a small tumor that weighed 0.25 g at the termination of the experiment (Fig. 1A) 44 days after the tumor had been initiated and 21 days following the beginning of toxin treatment. The 4 tumor-bearing mice that had not been treated with toxin developed large tumors that averaged 7.25 g in weight at the termination of the experiment (Fig. 1B). The 4 tumor bearers that had been treated with toxin injected intramuscularly into the opposite leg also developed large tumors that averaged 6.56 g at the termination of the experiment (Fig. 1C).

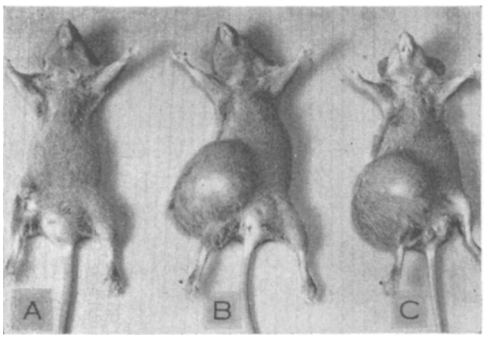

FIG. 1.
Hybrid mice (C57 black x C3H) inoculated with slow-growing, transplantable adenocarcinoma and subsequently treated with 2.5 mg of histolyticus toxin in 8 injections over period of 20 days. (A) Toxin injected directly into tumor, which weighed 0.25 g when photographed; (B) Control, untreated; (C) Toxin injected intramuscularly into leg opposite the one bearing the tumor.

V. *In vitro studies.* An effort was made to test the effect of histolyticus toxin on sarcoma, carcinoma and normal tissues cultivated *in vitro,* and also, by the same means, to demonstrate the protective action of antitoxin. Kidney was chosen as the normal tissue because it yields, in tissue culture, an abundant growth of both epithelium and connective tissue. The majority of the experiments were carried out in large Carrel flasks (5 cm in diameter) in which 3 or more fragments of sarcoma (Bar Harbor, L946-AII), of carcinoma (Bar Harbor, E-0771), and of young, adult mouse kidney were em-

bedded in 2.0 cc of medium consisting of 0.5 cc of chicken plasma, 0.2 cc of toxin, 0.4 cc of antitoxin and 0.9 cc of a mixture comprised of 40% horse serum (heated ½ hr. at 56°C), 40% Earle's solution, 20% chick embryo tissue juice and sufficient phenol red to make a final concentration of 0.005%. In the control cultures, the toxin and antitoxin were replaced by Earle's solution. The cultures were adjusted with gas mixtures to pH 7.2 and incubated without further treatment for 6 days.

When the medium contained both toxin and antitoxin, the toxin used in the various cultures of a typical series ranged in amount from 0.1 mg to 2.0 mg per cc. When the toxin was used alone, the amounts ranged from 0.01 mg to 1.0 mg per cc. Antitoxin, when present, was in the amount of 0.2 units per cc.

Results. In general, it may be said that the carcinoma was more resistant to the action of the toxin than sarcoma, just as normal epithelium seemed to be more resistant than stroma cells or fibroblasts. And normal and cancerous epithelium were equally resistant to concentrations of toxin that produced severe damage to the sarcoma cells.

In effective concentrations of toxin, the fibroblast-like sarcoma cells in the zone of outgrowth (or outward migration) rounded up, their nuclei and cytoplasm underwent a pronounced shrinkage, and they proceeded to disintegrate. But the cells of the explant were also affected. They too rounded up, underwent shrinkage and became dissociated into separate entities quite devoid of visible intercellular connections.

Discussion. With reference to the action of histolyticus infection on transplantable tumors, two possible effects were foreseen. The local infection of the tumor tissue might take the course of an acute anaerobic infection, which leads to toxemia and results in the early death of the animal host. Or, the local infection might, apart from any injurious effect on the animal, cause lysis of the tumor tissue and thereby prolong the life of the animal. And because earlier observations on histolyticus infections had shown that antitoxin counteracts the systemic effect of the toxin without eliminating, necessarily, its lytic action at the site of infection,[11] it seemed reasonable to study the effect of the antitoxin-treated infection on developing mouse tumors.

Both experiments in which sarcoma was treated with spores showed that the uncontrolled infection led more rapidly to the death of the animal than when the tumors were left untreated. The same held true for spore infected tumors treated with penicillin. The effect of the antitoxin treatment, however, was to prevent or postpone death from toxemia; and, as had been expected, it left enough of the local infection to cause a more or less marked lysis of the tumor tissue, thereby prolonging the life of some of the tumor bearers considerably beyond the life span of the non-infected tumor controls.

In order to determine whether, in such survivors, the histolyticus infection had completely destroyed all tumor tissue, the infection was finally eliminated by means of prolonged penicillin therapy. But none of the antitoxin treated mice, subsequently given penicillin for this purpose, survived the test; sooner or later they all developed sarcomas from which they died.

It has been noted that in the experiment in which carcinoma was treated with spore material followed by antitoxin and, finally, penicillin, these tumors, unlike the sarcomas, showed little or no regression. But it was impossible to determine, from the limited observations that were made, whether the greater resistance of the carcinoma was due to a greater resistance of the constituent cells or to the peculiar structure of the tumor itself. From the tissue culture experiments that were made with toxin, it would seem that the carcinoma is actually more resistant than the sarcoma, and that the greater susceptibility of the sarcoma may possibly be due to a greater looseness in the architecture of this type of tumor. In the cultures, the sheets of epithelial cells that grew out from the fragments of carcinoma survived concentrations of toxin that were definitely injurious to the sarcoma cells. But normal epithelium, growing side by side with the malignant epithelium,

was likewise resistant to concentrations of toxin that killed the sarcoma cells. The spindle-like sarcoma cells grow in an open meshwork, whereas carcinoma and normal epithelium grow in a compact mosaic in which the individual cells are far less vulnerable to outside influences.

In so far as the animal experiments with toxin are concerned, there is little that need be added to the observations already made. Just as the antitoxin-controlled histolyticus infection has, under certain conditions, a retarding influence on the development of the tumors, so also are the tumors susceptible to carefully regulated injections of toxin, the amount of which must be sufficient to produce lysis of the tumor but not enough to cause the death of the host. But regardless of how completely a tumor may seem to have regressed, it is almost certain to recur once the treatments have ceased. To be sure, the absorption of toxin does seem to be more rapid in tumor tissue than in normal tissue. Aside from the direct effect of the toxin on the tumor cells themselves, there is also damage to the young, actively proliferating vascular system of the tumor, and this results in hemorrhage and necrosis and the general destruction of the tumor mass. And as further evidence that the effect of histolyticus toxin on tumor cells is not a specific one, systemic injections of toxin at concentrations below the level that is injurious to the host have no effect on the tumors, just as toxin injected around the base of a developing tumor produces less lysis of the tumor than toxin injected directly into the tumor mass.

The main difficulty in attempting to evaluate the effect of histolyticus toxin on experimental tumors, in the present experiments, is the fact that the minimal effective dose approximated, very closely, the lethal dose. For further study, a more highly purified preparation of toxin would be required, and an attempt should be made to isolate, from the crude toxin, a factor that would be both highly histolytic and of low systemic toxicity.

Summary. When transplantable mouse sarcomas were infected experimentally with *Cl. histolyticum,* and the infection controlled by systemic injections of histolyticus antitoxin, the life span of some of the animals was prolonged for as long as 20 days beyond that of the non-infected tumor bearers. But in no instance did the infection completely destroy the tumor tissue. All surviving mice, treated eventually with penicillin in order to eradicate residual infection, developed large sarcomas. Under similar conditions, no such temporary regression was observed for transplantable mouse carcinomas.

When a study was made of the effect of histolyticus toxin on transplantable mouse tumors, it was found that systemic (intramuscular) injections of toxin were without effect, whereas repeated local injections of toxin, given either directly into the tumor mass or subcutaneously around the base of the tumor, resulted in marked regression of tumor tissue. But for the toxin preparations used, the effective dose approximated, very closely, the lethal dose, particularly when the toxin was injected directly into the tumor mass. In no instance was there a permanent regression of the tumor.

In tissue culture, carcinoma cells were more resistant to the action of histolyticus toxin than sarcoma cells, just as normal epithelium seemed to be more resistant that stroma cells or fibroblasts. But normal and cancerous epithelium were equally resistant to concentrations of toxin that produced severe damage to sarcoma cells.