Effects of Chemotherapeutic Agents on Wounds Contaminated with Tumor Cells: * An Experimental Study

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Introduction

FOR MANY YEARS, it has been observed that implantation of tumor cells into surgical wounds may result in local recurrences.[1, 3-6, 9, 10, 12, 13, 21-24, 27] This observation has stimulated the study of prophylactic wound irrigation and local administration of tumoricidal agents into body cavities during cancer surgery. Several compounds have been tried in experimental systems or on patients.[2, 7, 8, 11, 14, 16, 18-20, 24, 25, 28]

Although the experimental data that we and others have obtained have indicated that tumor "takes" may be markedly decreased by various chemotherapeutic compounds, the concentration of drug which is effective against tumor cells often closely approximates the maximum dose that can be tolerated by local tissue. The purpose of the present study is to classify biologi-cally-active compounds by comparing the relationships of the minimal carcinostatic doses to local toxic effects on normal tissues as well as to the specific effects on tumor cells. Systemic effects of these locally-applied compounds have been insignificant.

The range of concentrations of each compound was screened in order to establish the minimal dose effective when directly applied to tumor cells *in vitro* and *in vivo*. The local toxicity on the normal tissues was evaluated by comparing the tensile strengths of treated and untreated surgical wounds made through the skin, the subcutaneous tissues, and the superficial fascia of the shivering muscle.

Methods

A. Determination of the Minimal Effective Dose

1. *In Vitro Mixing of Ehrlich Tumor Cells and Drug Solution:* To ascertain the effective dose of a compound, a series of concentrations ranging from low to high dosages was tested against 25×10^6 Ehrlich
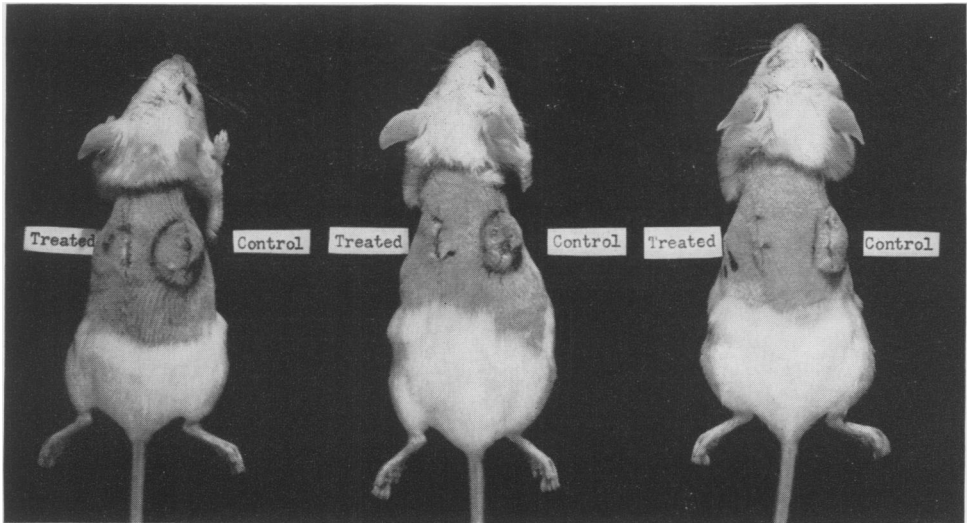
FIG. 1. Effects of Atabrine (quinacrine) on wounds contaminated with tumor cells. Each wound was inoculated with 500,000 Ehrlich ascites tumor cells. After a 10-minute incubation period, the wound on the left (experimental) side of each animal was treated with 0.05 ml. of a 2.5-mg./ml. solution of Atabrine. At the end of 5 minutes, the excess fluid was aspirated. The wound on the right (control) side was left without treatment.

cells. With a magnetic stirrer, 2 ml. of drug solution was mixed for five minutes with 1 ml. of the tumor-cell suspension. After one centrifugation, the mixture of drug and cells was washed by resuspension in mammalian Ringer's solution and was centrifuged again. An additional washing and centrifugation was followed by suspension of the solid material in 0.5 ml. of saline, and 0.1 ml. of this fluid was injected subcutaneously into C57 mice. The presence of tumor growth after 15 to 30 days indicated the effectiveness of the compound.

2. *In Vivo Wound Contamination with Ehrlich Tumor Cells and Application of Drug Solution:* Swiss mice were anesthetized with sodium pentobarbital, and the hair was clipped from the back. Two 2-cm. longitudinal incisions through the skin and the shivering muscle were made, one on each side of the vertebral column. A suspension of Ehrlich ascites cells in a concentration of 10×10^6 per ml. was prepared with isotonic saline, and 0.05 ml. (500,000 cells) was used for contaminating

the wounds. An incubation period of ten minutes was allowed. Excess fluid (approximately 0.025 ml.) was aspirated, and then 0.05 ml. of the drug solution was applied to the wound on the left side. Excess drug solution was aspirated after five minutes. The wound on the right side was inoculated with an equal number of tumor cells and was left untreated as a control.

The wounds were closed with Michel clips, and the animals were placed singly in cages for the first week. The clips were removed after three days. The length of time required to complete the initial surgery, cell inoculation, and treatment, using 15 or 20 mice in each group, was approximately two hours. This is important, since the viability of tumor cells in saline decreases over a period of hours. (For maximum viability, it is preferable to suspend the cells in tissue culture medium with 10% serum, keeping the mixture cool in an ice bath.) Fifteen days later, the animals were sacrificed and the results were recorded (Fig. 1).

3. In Vivo Wound Contamination with V-2 Tumor Cells and Application of Drug Solution: A large fragment of V-2 carcinoma was minced with scissors and forceps in mammalian Ringer's solution, and the tumor brei was filtered through a coarse wire-mesh screen. After a ten-minute interval to allow the larger particles to settle, the supernatant was aspirated through a No. 23 needle into a syringe. The suspension was flushed several times in and out of the syringe to eliminate cell clumps. Again the larger particles were allowed to settle, and the supernatant was aspirated through a No. 25 needle. This procedure gave a single-cell suspension with very few small clumps. The suspension was diluted to 12×10^6 cells per ml. before application.

In each test of a chemotherapeutic agent, eight separate transverse skin incisions 2 cm. in length (four on each side of the midline) were made in the abdominal wall of a shaved and anesthetized rabbit. These incisions were carried through the superficial fascia and into the muscle layers. Each wound was filled with 0.25 ml. of the cell suspension (3×10^6 cells). At the end of a 60-minute incubation period, the fluid remaining in the wounds was aspirated. Two of the contaminated wounds on each animal were left untreated as controls. In succession, 0.25-ml. samples of solutions containing various concentrations of the chemotherapeutic agent to be tested were applied to the remaining six wounds. After the carcinostatic agent had been in the wound for ten minutes, the excess fluid was aspirated, and the wounds were closed with silk sutures. The whole procedure took approximately three hours. The presence or absence of a tumor in the incisions was recorded in successive weeks (Fig. 2).

B. Determination of the Local Toxicity of Drugs

Measurement of Tensile Strength: Under sodium pentobarbital anesthesia, the back of a rabbit was clipped free of hair, and incisions 2 cm. long were made, perpendicular to the vertebral column and on each side of it. The space between incisions was 3 cm. Depending on the size of the animal, either four or five pairs of incisions were made. The shivering muscle was cut, creating a defect 2 cm. in length in all planes. On the treated (left) side, 0.1 ml. of drug solution was inoculated into the wound, allowed to remain in contact with its surface for ten minutes, and then aspirated. To the control (right) side, saline was applied and removed in the same manner as the drug solution. The wounds were closed in a standard fashion with interrupted sutures of 4–0 silk at 5-mm. intervals. A plaster collar was placed around the neck to keep the animal from biting the incisions.
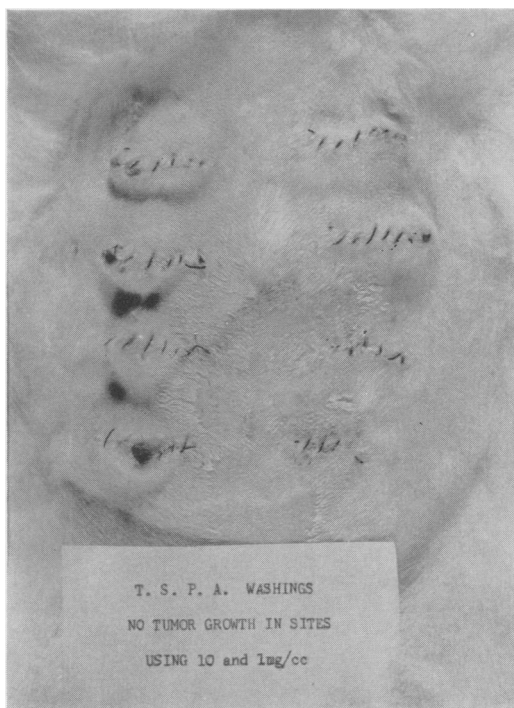

Fig. 2. Effects of TSPA solutions of various concentrations on wounds contaminated with V-2 tumor cells. Only two concentrations, 1 and 10 mg./ml., inhibited tumor growth completely. The less concentrated solutions were ineffective.

TABLE 1. *In Vitro Destruction of 25 × 10⁶ Ehrlich Cells by Carcinostatic Agents Assayed by Subcutaneous Injection into C57 Mice*

Compound	Concentration for 100% Prevention of Tumor Takes	No. of Mice in Experiment
Nitrogen mustard	0.015 mg./ml.	30
Mitomycin C	0.1 mg./ml.	30
Acriflavine	0.1 mg./ml.	30
Dakin's solution	⅛ full strength	25
Nitromin	5.0 mg./ml.	40
Rivanol	5.0 mg./ml.	40
TSPA	5.0 mg./ml.	50
5-FUDR	20.0 mg./ml.	25

Seven days after the operation, the animals were sacrificed and the sutures were removed. The healing incisions were excised and trimmed to 1 cm. in length, and the tensile strength of each was measured individually. After clamps had been applied to the skin parallel to the incision, a plastic vessel was attached to the clamp that was fastened to the skin on the opposite side of the wound, and water was gradually poured into the vessel until the healing incision ruptured. The total weight of water required to rupture the wound was recorded as the tensile strength of the healing incision. The percentage of decrease in the average tensile strength of the treated wounds, as compared with that of the controls, was considered to be a measure of local toxicity.

Comparative tensile strength measurements as a test of local drug toxicity may not be reliable unless certain precautions are taken: (1) treated and control incisions should be on the same animal and identical in all technical aspects; and (2) measurement of wound in rabbits should be done on the seventh day, since the tensile strength of the control wounds, after a three- or four-day lag period, reaches a maximum on the seventh day. Measurements taken after the seventh day may not reveal impaired wound healing, because the treated incisions will also have attained maximum wound strength. Measurements earlier than seven days are not advisable, since wound healing during the lag phase will be nearly the same. The standard deviation due to technical factors in our method for measuring tensile strength is ± 5 per cent.

Results

Table 1 shows the 100 per cent effective dose of the compounds in an *in vitro* system. Tables 2 and 3 show the minimal effective dose and the percentage of effective treatment produced by this dose in two different *in vivo* systems.

TABLE 2. *Minimal Effective Doses for Wounds Contaminated with Ehrlich Ascites Cells, in Mice*

Compound	Concentration (mg./ml.)	No. of Wounds Treated	No. of Tumor Takes	Percentage of Effective Treatment
Nitrogen mustard	0.0625	20	0	100
Acriflavine	2.5	11	2	82
Atabrine	2.5	20	4	80
Actinomycin D	0.05	16	4	75
TSPA	4.0	18	5	73
5-FUDR	10.0	10	3	70
Sodium hypochlorite (Dakin's solution)	5.0	40	12	70
Bichloride of mercury	4.0	16	6	63
Rivanol	2.5	16	8	50
Clorpactin XCB	20.0	38	19	50
Sodium chlorite	8.5	60	60	0

TABLE 3. *Minimal Effective Doses for Wounds Contaminated with V-2 Tumor Cells, in Rabbits*

Compound	Concentration (mg./ml.)	No. of Wounds Treated	No. of Tumor Takes	Percentage of Effective Treatment
Nitrogen mustard	0.05	30	2	93
TSPA	4.0	12	1	92
Sodium hypochlorite (Dakin's solution)	5.0	18	3	83
Atabrine	2.5	18	4	78
Rivanol	5.0	24	6	75
Actinomycin D	0.01	12	3	75
Acriflavine	2.5	12	3	75
Clorpactin XCB	20.0	24	11	54
Sodium Chlorite	8.5	28	28	0

The local toxicities of the compounds evaluated by decreased tensile strength of standard wounds are given in Table 4. In order to minimize variations due to technical factors, which are considerable for smaller doses, a dose three times the minimal effective dose of each compound was tested.

In Figure 3, the effectiveness and the local toxicity are compared. The specific effect (i.e., the ratio of chemotherapeutic effectiveness to local toxicity) of each compound is given in Table 5.

The data in this table are informative, but cannot be considered valid from a statistical standpoint. No satisfactory method exists for directly comparing effectiveness and relative toxicity. Table 5 and Figure 3 are meaningful only in relation to similar experiments with the same relative drug concentrations, fluid volume, and total amounts of material, as well as the same
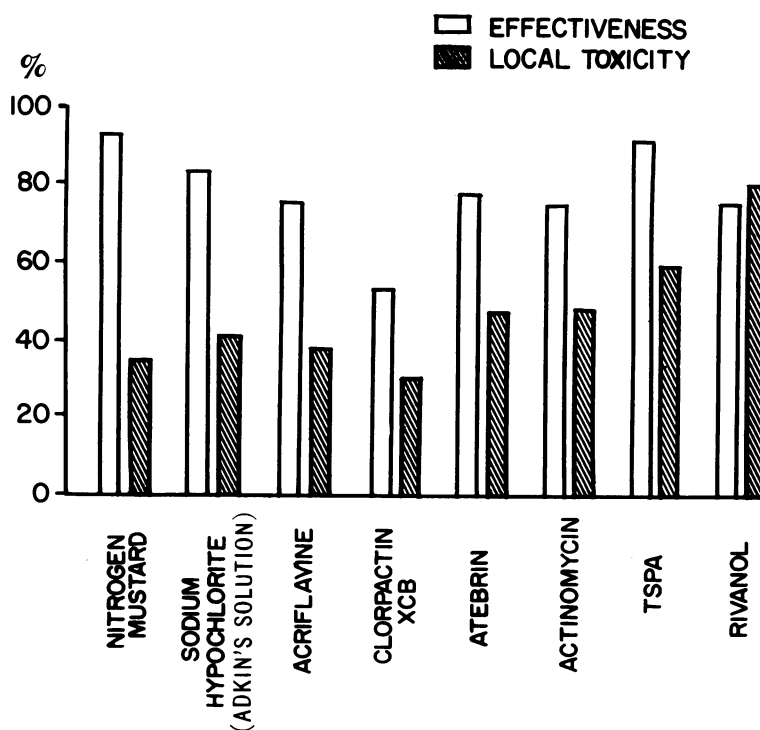

FIG. 3. Effectiveness and local toxicity of various chemotherapeutic agents.

TABLE 4. *Local Toxicity in Regard to Normal Healing*

Compound	Concentration (mg./ml.)	Local Toxicity*
Rivanol	15.0	80.0
TSPA	12.0	59.2
Actinomycin D	0.030	48.6
Atabrine	7.5	47.2
Sodium hypochlorite (Dakin's solution)	15.0	41.0
Acriflavine	7.5	37.7
Nitrogen mustard	0.15	35.0
Clorpactin XCB	60	29.8

* Percentage of decrease in average tensile strength of treated wounds, compared with strength of control wounds.

tumor cells. These results cannot be considered directly applicable to the treatment of patients.

Discussion

Since carcinostatic doses of chemotherapeutic agents can be administered locally without systemic toxicity, the destruction of tumor cells in experimental wounds may be easily accomplished by local administration of a wide variety of biologically active agents. Although direct clinical application of this sort of experimental data is not practicable, it is important to determine the comparative safety of these agents in animals. For example, the observation that either too low or too high a dose of these chemicals may *enhance* tumor growth must not be overlooked. Ship et al.,[26] Ketcham,[14] and Laszlo et al.[17] have re-

TABLE 5. *Ratio of Tumor-Cell Destruction to Impairment of Wound Healing at Optimal Drug Concentration*

Compound	Ratio
Nitrogen mustard	2.66
Sodium hypochlorite (Dakin's solution)	2.03
Acriflavine	1.98
Clorpactin XCB	1.81
Atabrine	1.64
Actinomycin D	1.54
TSPA	1.54
Rivanol	0.93

ported that chemotherapeutic agents may aggravate tumor growth in artificially seeded wounds. These observations parallel those of Kondo and Tsukui[15] and Moore and Kondo,[20] who reported that systemic chemotherapy, by decreasing host resistance, promotes the development of metastases if the tumor is insensitive to the drug.

One must also consider other manifestations of local toxicity, such as impaired wound healing, tissue necrosis, and infection. Clinicians have tried to be on the safe side by using minimal doses of carcinostatic agents, since greater concentrations

TABLE 6. *Relationship between Concentration and Therapeutic Effectiveness of Compounds on Wounds Contaminated with Ehrlich Ascites Cells, in Mice*

Compound	Concentration (mg./ml.)	Percentage of Effective Treatment
Acriflavine	0.156	23
Acriflavine	2.5	82
Acriflavine	5.0	100
Clorpactin XCB	10.0	0
Clorpactin XCB	20.0	50
Clorpactin XCB	25.0	80
Clorpactin XCB	30.0	90
Atabrine	2.5	80
Atabrine	5.0	100

of nitrogen mustard (McClure and Mengert[18]) and Mitomen (Blixenkrone-Møller[2]) have caused local complications which have discouraged further trials of the compounds. This conservatism not only tends to decrease the effectiveness of treatment, but also may bring about acceleration of local tumor growth. Under the circumstances, preliminary experimental models which reflect the biological activity of a compound with respect to both tumor cells and the local toxicity of the compound seem desirable.

In this study, the minimal effective dose and the percentage of effective treatment provided by this particular dose indicate

TABLE 7. *Relationship between Size of Tumor-Cell Inoculum and Effectiveness of Compound on Wounds Contaminated with Ehrlich Ascites Cells, in Mice*

Compound	Concentration (mg./ml.)	Size of Inoculum	Percentage of Effective Treatment
Dakin's Solution	5	0.5×10^6 cells	70
Dakin's Solution	5	1×10^6 cells	50
Dakin's Solution	5	3×10^6 cells	0
Clorpactin XCB	20	0.5×10^6 cells	50
Clorpactin XCB	20	3×10^6 cells	0

the biological activity of each compound. The mechanical factors which are frequently involved in the actual surgical "wound washing" and may themselves influence "tumor takes" have been eliminated. Therefore, the results given in Tables 1, 2, and 3 closely approximate the net biological effect of each compound in three different experimental systems. The tumors used in these experiments are relatively resistant to most kinds of systemic chemotherapy, and therefore simulate the refractoriness of most human solid tumors to carcinostatic agents.

Priority in biological activity is given to compounds on the basis of the percentage of effective treatment when the minimal effective doses are used. It is our observation that the percentage of effective treatment is proportional to the concentration of the chemotherapeutic agent. For example, Table 6 indicates that effectiveness increases with the strength of the solution. As is shown in Table 1, even 100 per cent effectiveness may be obtained by using sufficiently high concentrations of most of the compounds. On the other hand, the percentage of effective treatment decreases when the size of the inoculum of tumor cells increases (Table 7). A similar relationship between the concentration of drug and the local toxicity is indicated in Table 8.

Although there are differences among individual compounds, the local toxicity of a compound closely approximates its biological activity in regard to tumor cells. This has been demonstrated by evaluating effectiveness and local toxicity in the respective experimental systems (Tables 3 and 4 and Fig. 3). The effectiveness as compared with the local toxicity (Table 5) helps to classify each chemotherapeutic agent according to its actual clinical usefulness.

Summary

Because implantation of tumor cells into surgical wounds may be followed by local recurrence of the tumor, the use of chemotherapeutic agents for prophylactic wound irrigation is a subject of widespread interest. In the present study, the carcinostatic effects of various chemotherapeutic agents on tumor cells inoculated into ex-

TABLE 8. *Relationship between Concentration of Nitrogen Mustard and Local Toxicity, in Rabbits*

Concentration of Nitrogen Mustard (mg./ml.)	Local Toxicity*
0.05**	0
0.15	35
0.20	55
0.5	55
1	76
2	78.3

* Percentage of decrease in average tensile strength of treated wounds, compared with strength of control wounds.
** Minimal chemotherapeutic dose.

perimental wounds were compared *in vitro* and in two systems *in vivo,* and the local toxicities of the chemotherapeutic agents were measured by their effects on wound healing.

The ratio of the destruction of tumor cells to the impairment of wound healing was computed for each of eight chemotherapeutic agents, and was found to decrease in the following order: nitrogen mustard, sodium hypochlorite (Dakin's solution), acriflavine, Clorpactin XCB, Atabrine, actinomycin D, triethylenethiophosphoramide ("TSPA"), and Rivanol. Unfortunately, all of the compounds tested are of limited value, even nitrogen mustard.

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DISCUSSION

DR. DONALD M. GLOVER: This beautiful work of Dr. Moore's is obviously of very great importance. It is certainly proper to determine, as Dr. Moore has done, that the carcinostatic agents are effective and at the same time relatively nontoxic to normal tissues. Before embarking upon a program of introducing such carcinostatic agents into the operative wound, one should ask the question: "Just how frequently are implantation tumors occurring in operative wounds?"

It is our impression that well-documented instances are really a little bit difficult to come by. The finding of many tumor cells in the blood stream and in wound washings following cancer surgery are now well documented by several members of this Association and others, but so far there seems to be very little truly objective correlation between the finding of these cells and the incidence of local recurrence or of distant metastases.

It is obviously very difficult to distinguish implantation recurrences in soft tissue wounds, or in the peritoneal cavity, from growths that result from unrecognized residual tumor or lymph nodes left at the time of primary operation. It should be possible, however, to document clearly implantation tumors in the abdominal wall following surgery upon intra-abdominal neoplasms, where there is no direct connection with intraperitoneal recurrences. Reports of such instances are indeed rare in the literature.

In a review of several hundred fairly carefully followed personal operations for intra-abdominal carcinoma in the past 36 years, we have been able to find only one instance of an implantation neoplasm of the abdominal wall. This instance was the case of a 77-year-old woman who developed a massive local recurrence in the abdominal wall two and a half years after resection of a well differentiated papillary adenocarcinoma of the ampulla of vater arising in the duct of Wirsung. The cytology of the abdominal wall lesion was identical with that of the original neoplasm. At the time of resection of the abdominal wall thorough exploration of the abdominal cavity showed no evidence of residual tumor or metastasis; therefore, this must be considered an implantation tumor.

Why did it occur in this instance, when nothing comparable was observed in several hundred other procedures for intra-abdominal cancer? I believe we have no satisfactory answer to that question at the present time.

Perhaps before we employ anti-cancer agents to prevent such occurrences we need to know if the incidence of such implantation tumors is one in 500, one in 1,000, or perhaps greater or less in frequency. We believe that this information is not yet available.

DR. GEORGE E. MOORE (Closing): Yes, these remarks are pertinent; that is, the mere fact that you can isolate tumor cells from the wound does not mean that they are going to grow. As a matter of fact, very few of them grow. We know from our studies of tumor cells in the blood that perhaps less than 0.1 per cent ever grow.

We have tried to study this, as Dr. Cole knows. We recover cancer cells from the blood, and washings of the body cavities, and then try to grow them in tissue culture. We can show that the cells are viable.

At Roswell Park about nine patients of 100 having head and neck surgery for malignancy will have tumor cells in the wounds and nine of 100 will have local recurrence; the majority of them are the same patients.

Finally, I would point out that many of us have studied the deliberate inoculation of tumor cells into volunteer patients with far advanced cancer. In some instances, only a few million cells, and in some instances huge amounts of tumor are used. Only 13 per cent of the time do you get takes of such autotransplant inoculations. That is, if you have a patient with far-advanced carcinoma of the stomach and inoculate him with cells from his own cancer, subcutaneously, they will grow only 10 to 15 per cent of the time.