Effect of Radiation Plus Endotoxin on the Walker 256 Rat Carcinosarcoma.* (31893)

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The hemorrhage-producing and necrotizing action of endotoxin on tumors has been extensively investigated, yet the basic mechanism for this effect is not clear(1,2). The experiments reported here show in one tumor system a large potentiation of the endotoxin effect by a preliminary X-ray treatment directed at the tumor, and in addition give information on the nature of the endotoxin effect.

Materials and methods. Groups of Sprague-Dawley female rats, about 150 g weight, were inoculated subcutaneously in the right side, at the level of the lowest rib, with single 15 mg fragments of a line of the Walker carcinosarcoma 256 obtained originally from Dr. Florence Millar at National Institutes of Health. Eight to 10 days after inoculation, when the tumors had reached a weight of 4 to 8 g, the tumor-bearing animals were divided into groups equivalent in the distribution of tumor sizes. X-ray treatments of tumors were carried out with a 250 K.V.P. Picker Industrial unit with filtration giving a Cu half-value equivalent of 0.34 mm and an air dose of 1500 R, measured at a distance equivalent to the middle of the tumor, delivered in approximately 3 minutes. Animals were under Fluothane anesthesia and shielded by lead during treatment.

The source of endotoxin was Bacto-Lipopolysaccharide *S. marcescens* Lot 456047 obtained from Difco Laboratories. The LD_{50} for rats without tumors was greater than 4 mg/kilo, and for rats with 12-day-old Walker 256 tumors 2 mg/kilo when administered intravenously.

Purified I^{131}-labeled antibody to rat fibrin was prepared by methods previously described (3,4).

Radioautographs were prepared of 6-micron formalin fixed tissue sections using Kodak no-screen X-ray film separated by a layer of Saran wrap, prior to removal of the paraffin embedding material and conventional H and E staining.

To obtain semiquantitative measurments of the amount of viable tumor tissue present in control and treated tumors, drawings were made of stained tumor sections with a Wild M5 stereo-microscope equipped with drawing tube, the areas shaded that appear to contain viable tumor on the basis of criteria to be described later, the shaded areas cut out, and from weights of the paper outlining the whole tumor and the cut-out portions data obtained for calculating the "per cent of viable tumor" present in that particular tumor section.

Experimental studies and results. Observed grossly, young untreated Walker 256 tumors, up to a size of 0.7 cm diameter, on section routinely showed a pearly white cut surface with little visible evidence of blood or vascularization. Above this size evidence of central degeneration or necrosis was usually visible, in tumors up to 2 cm in greatest dimension usually granular white in appearance. In larger, older tumors, with increasing frequency with size there was evidence of internal hemorrhage or of a central cavity filled with pale or blood red fluid. Always, however, this area of central necrosis was surrounded with a rim of white, pearl-like tissue.

In several experiments endotoxin, given at levels up to 1 mg/kg, or a dose of 1500 R X-ray to tumor, seemed not greatly to alter the appearance of this tumor on section for the ensuing 48 to 96 hours.

In striking contrast was the appearance of cross sections of tumors that received 1500 R X-ray 4 days earlier and the host rats 0.5 mg endotoxin per kilogram 2 days after X-ray. These tumors on section were almost entirely of a brick-red, uniform granular appearance with occasional remnants at the edge of the specimen containing pearly white tissue.

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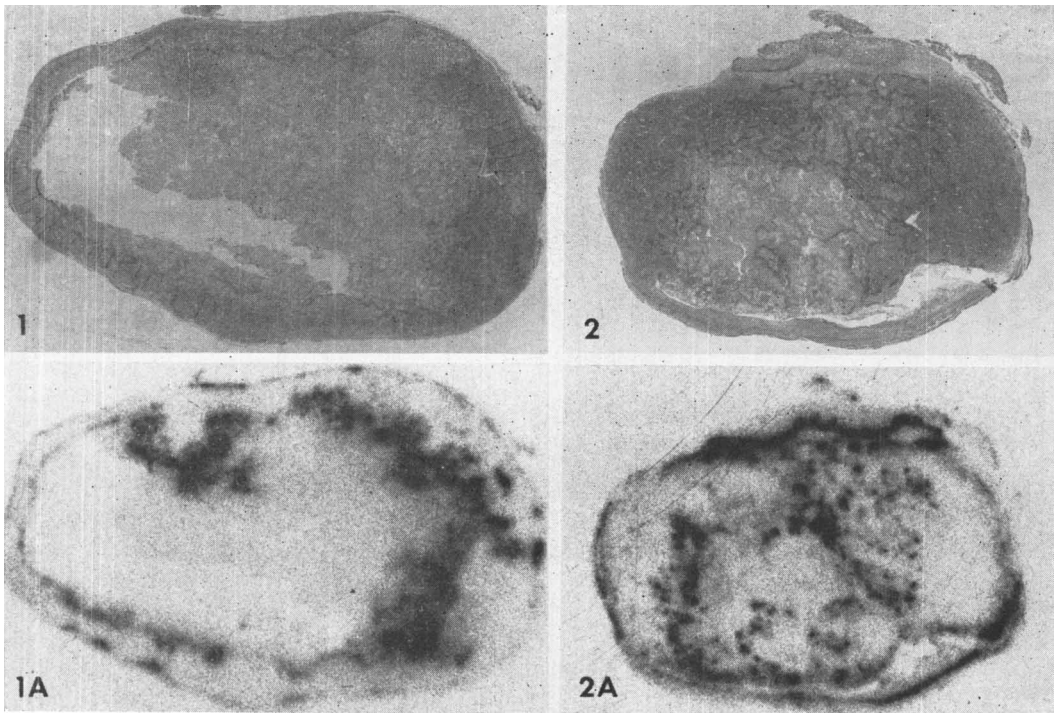


Fig. 1. Untreated Walker tumor 14 days after transplant. Haematoxylin-eosin X2.
Fig. 1A. Radioautograph showing distribution of I^{131} antibody to rat fibrin in tumor.
Fig. 2. 14-day Walker tumor given 1500 R X-ray 4 days earlier. H-E X2.
Fig. 2A. Radioautgraph showing antibody distribution.

An H and E stained cross section of untreated 14-day tumor is illustrated in Fig. 1. The dark stain of the border is due to hematoxylin and on higher magnification seems to be due predominantly to stained nuclei of typical Walker tumor cells. The central area of the tumor is mixed in character and consists of nests of tumor cells surrounded by areas of degeneration, invasion of inflammatory cells, and some hemorrhage. Fig. 2 is a section taken 14 days after transplant of a tumor irradiated with 1500 R 4 days earlier. Fig. 3 is from a tumor removed 2 days later from a rat that had received endotoxin at a level of 0.5 mg per kilo 10 days after transplant. It is difficult or impossible to distinguish by microscopic study tumors of these two classes from untreated tumors. Fig. 4, in contrast, is from a tumor 14 days after transplant that had received 1500 R at 10 days and the host rat endotoxin at 0.5 mg/kilo at 12 days after transplant. Here intact Walker tumor cells are not easily recognized. Instead the whole central area of tumor seems to be undergoing some type of complete necrosis, and the area comprising the border of the original tumor is a region of intense inflammatory reaction. Fig. 5 shows at a higher magnification such a reaction area, X, from Fig. 4.

In some tumors from rats treated with this combination of X-ray and endotoxin, this tumor altering effect was not entirely complete, and gross examination of the sectioned tumor showed areas remaining of pearly white tissue. Fig. 6 shows a section of such a tumor. Microscopic examination suggests that in this instance two areas of tumor had largely escaped the combined X-ray endotoxin effect that had altered the gross and microscopic appearance of the remainder of these tumor specimens.

To study the physiological basis of the combined X-ray endotoxin effect, one day before the rats were killed a tracer dose of I^{131}-labeled antibody to rat fibrin was administered to each rat in the experiment from which the illustrated examples of Fig.

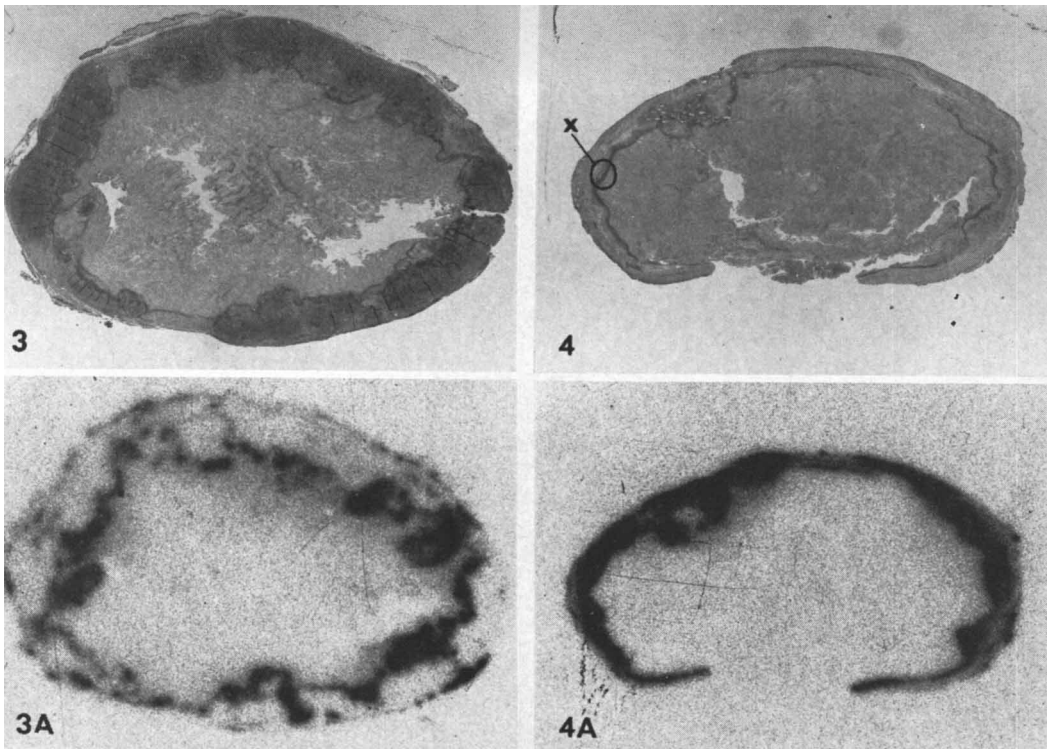


Fig. 3. Walker tumor given endotoxin 2 days earlier. H-E X4.
Fig. 3A. Radioautograph showing antibody distribution.
Fig. 4. Walker tumor 4 days after X-ray and 2 days after endotoxin treatment.
Fig. 4A. Radioautograph showing antibody distribution.

1 through 4 were taken. After rat sacrifice and tumor removal, per cent uptake of I^{131} per gram tumor was measured by gamma-ray counting, and radioautographs made of these tissue sections.

Fig. 1A, 2A, 3A, and 4A are the radioautographs prepared from the tissue sections pictured in Fig. 1 through 4. A comparison of Fig. 1 and Fig. 1A shows how antibody deposition has occurred in areas of tumor in which degeneration or necrosis is also evident, but that precisely in those areas of tissue with the richest abundance of tumor cells and no evidence of tumor degeneration are also the areas where no radioactivity is deposited. Thus the Walker rat tumor would not easily be cured by highly radioactive antibody, since even if the amount of I^{131} deposited in tumor were high, the distribution would be such that tumor cells would escape receiving any large amount of beta irradiation. This contrast with similar experiments with otherwise untreated Murphy-Sturm rat tumors where labeled antibody localization in tumors is uniform enough that treatment with highly radioactive antibody can routinely lead to cure of these tumors(3).

As Fig. 2A and 3A indicate, doses of X-ray or endotoxin, as given, do not greatly alter this pattern of I^{131} distribution in this tumor. In contrast, as shown in Fig. 4A, the combined effect of 1500 R X-ray, delivered to this Walker tumor, followed 2 days later by endotoxin, is completely to prevent deposition of radioactivity in the tumor except at the margin between tumor and normal tissue where a high level of radioactivity is found to correspond to a region of high concentration of inflammatory cells.

Description and data from one such experiment are given in some detail. Thirty rats were inoculated with Walker tumor 256; the tumors of 19 of these rats received a dose of 1500 R X-ray 8 days later. One day later

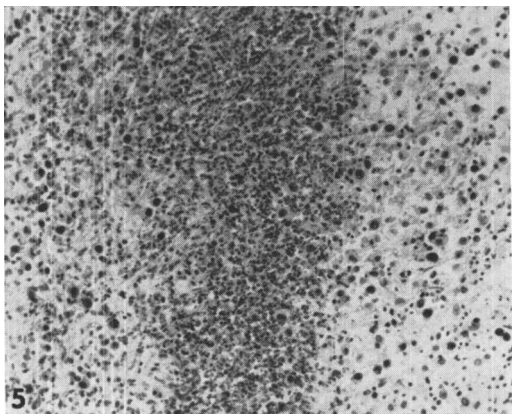
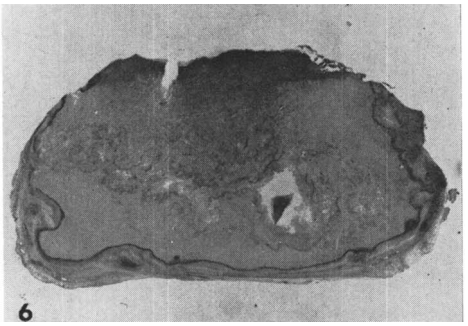


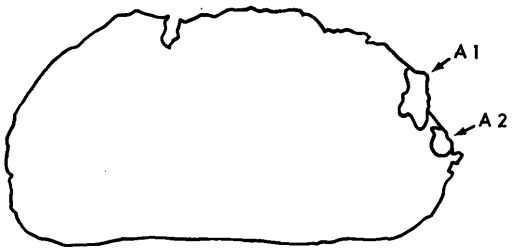
Fig. 5. Area of cellular reaction at (×) in Fig. 4. H-E ×120.

3 tumor dimensions were measured, the product of the 3 dimensions calculated, and the rats divided into 6 experimental groups in a manner that each group contained an equivalent range of tumor sizes. Groups I through III consisted of rats with X-ray treated tumors; in Groups IV through VI the tumors were untreated. The next day, 10 days after transplant, Group I, 10 rats, and Group V, 4 rats, received i.v. 0.5 mg endotoxin per kg. Group II, 6 rats, Group V, 4 rats, received 1.0 mg endotoxin per kg. Group III, 3 rats, and Group VI, 3 rats, were untreated. One day later each rat received an intravenous dose of 40 μcuries I^{131} as I^{131}-labeled antibody reacting with rat fibrin. One day later cardiac blood samples were taken and the rats killed with ether. Tumors were removed, weighed, radioactivity of tumor and remaining rat carcass measured as per cent of injected dose, the tumor sections examined grossly, and sections taken for histological study and radioautographs. In treated animals care was taken that these cross sections contained the most likely areas of surviving tumor. Experiments in which serial sections were made of complete tumors gave a basis for these solutions. Every 30th 6 μ section was stained and examined.

No rats died and all seemed in good condition at termination of the experiment. Weigh gains from the day preceding endotoxin injection until the end of the experiment occurred in all groups of rats except Groups IV and V where the average losses were 2.7 and 0.3% of the initial weight. Average tumor weights for Groups I through VI were 4.34 ± 1.77; 4.07 ± 0.54; 6.32 ± 0.31; 8.87 ± 5.21; 11.68 ± 3.46; and 15.20 ± 3.34 g where the variation indicated is that of standard deviation of individual weights from the average. The % of injected I^{131} found per unit mass tumor seemed not to vary significantly from group to group and averaged 2.42% of the injected I^{131} dose in a weight of tumor equal to 1% of the individual rat's weight at sacrifice, a value 4.6 times the average I^{131} content of other tissues. Blood I^{131} values were consistent with the finding in other experiments that nearly all I^{131} remaining in the rat outside tumor was present in blood or extracellular fluid. Whole body counts indicated that an average of 34% of injected I^{131} had been excreted.

In every rat treated with X-ray followed by endotoxin, the same profound change in gross appearance, histological character, and





6A

Fig. 6. Tumor from rat treated with X-ray followed by endotoxin showing 2 regions of apparently suriving tumor cells. Dark area at top of slide is due to extravasated blood. H-E ×2.

Fig. 6A. Drawing of Fig. 6. A1 and A2 contain apparently viable tumor. The index of surviving tumor is calculated as the % that areas A1 and A2 comprise of total tumor area shown.

TABLE I. Portions of Tumor Cross Sections Judged Free of Viable Tumor Cells by Histological Criteria.

Rat No.	Wt of tumor (g)	% free of tumor cells
	Group I, X-ray and endotoxin, .5 mg/k	
12	2.0	100
19	4.0	100
3	3.6	93
6	3.6	86.7
5	5.5	63.4
8	4.3	90
2	2.1	100
14	8.0	77.4
11	4.5	100
16	5.8	100
	Avg	91.1 ± 12.4
	Group II, X-ray and endotoxin, 1.0 mg/k	
9	3.2	100
13	4.6	98.4
15	4.6	100
17	3.3	98.8
18	4.3	99.2
1	4.0	100
	Avg	99.4 ± .7
	Group III, X-ray only	
4	5.3	10.8
10	6.4	13.1
7	7.3	9.2
	Avg	10.8 ± 2.0

In each group rats are arranged in order of increasing initial tumor size. Variation indicated is the standard deviation of individual measurements from their average. These results are based on one or two slides that contained a complete cross-section, including the longest dimension, of each tumor.

distribution of I^{131} in tumor as revealed by radioautographs was observed that are shown in Fig. 4, 4A, and 5. In contrast, in tumors treated by either X-ray or endotoxin alone large masses of apparently viable tumor remained.

The proportion of each tumor section that contained what could be recognized as intact and probably viable tumor cells was estimated by the procedure given under *Materials and methods*. Table I gives the results of this study for Groups I through III. The large effect of X-ray followed by endotoxin in reducing the portion of tumor containing intact tumor cells is evident. From gross inspection an effort had been made to take each tumor section from the region most likely to contain viable tumor. Nevertheless no firm conclusions can be made upon the number of viable tumor cells remaining in the tumor in which a single section showed "viable" tumor cells. No serial microscopic studies were made of the tumors in this experiment. On the other hand areas considered to contain viable tumor usually contained far fewer tumor cells per unit area than in control tumors treated with X-ray alone. Numerical values for Groups IV and V treated with endotoxin alone did not differ greatly from values for the untreated tumors of Group VI.

Other experiments showed that the omission of the tracer antibody dose had no effect on reduction of "viable" tumor by the combined treatment with X-ray and endotoxin.

Discussion. There is substantial evidence that malignant tumor growths, in man and in primary and transplanted tumors in animals, are often locations of a considerable conversion of fibrinogen to fibrin(5,6). When this conversion is extravascular, it may not retard tumor growth but may even provide an environment favorable for tumor growth (5). However, under conditions of slowing or stasis of the circulation, particularly with the blood in a hypercoagulable state, the same agents might diffuse into the blood vessels of the tumor and lead to massive intravascular coagulation and massive infarct formation in the tumor. There is evidence that endotoxin produces both a slowing or stasis of blood circulation in tumors(2), perhaps in association with a generalized hypotension, and a state of blood hypercoagulability, perhaps due to Factor XI or XII activation and a release of platelet-factor 3(7,8). Such intravascular clotting, proceeding from the venule end of the tumor blood supply, could first lead to extravasation of blood from engorged vessels more proximal to the arterial supply, the well-known "hemorrhagic" effect of endotoxin on tumors, and then to complete and prolonged stasis and infarct formation. The effect of X-ray may be to cause tumor cell and vascular damage that then promotes the release of coagulation-inducing factors from injured cells. Our consistent finding that, following X-ray and endotoxin, intravenously injected I^{131} antibody to rat fibrin localizes only in the periphery of tumor or in the junction of tumor with normal tissue, together

with histological studies, seems good evidence that most of the Walker tumor becomes an infarct without blood flow following this combined treatment. Long-term survival studies to be reported elsewhere show that a closely related type of combined treatment, with an X-ray dose that by itself has little effect on tumor growth, can be made consistently effective in producing permanent regression of the Walker tumor(9).

Summary. A dose of 1500 R X-ray to rat Walker carcinosarcoma 256, followed by a sublethal dose of endotoxin, produced routinely a condition that gross and histological study suggested was an infarct involving all or nearly all tumor tissue. Tracer studies with intravenously administered I^{131} antibody to rat fibrin showed a pronounced localization of radioactivity about the periphery of treated tumors with no radioactivity in the central portions of the tumor, a picture consistent with a lack of blood circulation there. X-ray or endotoxin, administered alone, at the same doses, produced no obvious effect on tumor histology or antibody localization.

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Influence of Na^+ on Synthesis of a Substrate Entry Mechanism in A Marine Bacterium.* (31894)

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Marine bacteria require Na^+ for growth (1,2) and for transport of oxidizable substrates(3) as well as a non-metabolizable amino acid analogue, α-amino isobutyric acid (AIB)(4). We noted(5) that an elevated concentration of K^+ (0.26 M) increased the rate of penetration into marine bacteria of two non-ionizing substrates, mannitol and L-arabinose, but not an ionizing compound, glucuronate. The only other set of ions or ionic substitutes in suspending media used in these studies that permitted entry of substrates was a combination of 0.25 M Na^+ and 0.01 M K^+. Working with animal cells, Kipnis and Parrish(6) observed that the presence of an elevated concentration of K^+ in suspending media for uptake experiments permitted entry of mannitol and other substrates into rat diaphragm cells, but the presence of Na^+ was required for the transport of AIB. They concluded that the elevated extracellular K^+ functioned to increase diffusion of the substrates into the cells, whereas those substances that were not able to enter in this way were transported by mechanisms influenced by Na^+. The present work was undertaken to investigate the possibility suggested by earlier work(5) that there is a function for Na^+ exerted during the *synthesis* of an induced entry mechanism, separate from the involvement of Na^+ in *operation* of the entry mechanism.

Experimental. Suspensions of resting cells of the marine bacterium *Pseudomonas natriegens,* harvested from sea water nutrient broth cultures and washed with 0.052 M $MgCl_2$,

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