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Effects of Route and Schedule of Administration of High-Molecular Levan on the Growth of AKR Lymphoma[1]

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SUMMARY

The route and schedule of treatment with high-molecular levan markedly influenced its inhibitory effect on the growth of transplanted AKR lymphoma. Injections of levan into the site of the primary tumor were more effective in inhibiting tumor growth and preventing tumor-associated weight loss and mortality than were i.p. injections. Local levan injections inhibited metastatic spread only in mice treated from Days 0 and 2. Levan i.p. was more effective in inhibiting metastases in animals started on treatment 7 to 13 days after tumor inoculation than in animals in which levanization was started earlier. Local injection of levan before inoculation of tumor enhanced tumor growth and shortened life-span in comparison to nonlevanized animals. In mice treated with levan for a short period only, the inhibitory effect on tumor growth slowly vanished within 2 weeks. Some animals treated for 5 to 8 months remained completely free of tumor.

The results indicate that the effect of levan on tumor development is mainly topical and depends on the concentration of the polysaccharide in the site. The tumor growth period from 0 to 5 days appears to differ from the following period in the reaction to levan treatment. The nature of this difference is not clear, but possible explanations are discussed.

INTRODUCTION

High-molecular levan has been previously shown to exert several biological activities, like preventing passage of cells and macromolecules from blood vessels to tissues (2, 6, 14). It also inhibited acute inflammatory response and wound healing and changed the constitution of connective tissue ground substance (17, 18). These activities can be partly explained by the coating of endothelial and other cell surfaces with levan. We have found that levan delays rejection of homologous skin grafts (9) and inhibits experimental allergic encephalomyelitis.[3]

Recently, high-molecular levan was shown by us to have an antitumor effect (10). The effect was exerted on both the primary and metastatic tumors. We found that the antitumor activity was related to the dose of levan and that levan had a direct effect on tumor cells. This showed that local administration of levan at the site of the tumor is more effective than i.p. injections in inhibiting tumor growth. This paper deals with the effects of levan injected at the site of the tumor inoculation in comparison to the effects of systemically administered levan.

In our previous experiments levan was administered prior to and during tumorigenesis and tumor dissemination. In the present study the effects of levan administration begun at different stages of tumor growth and spread have also been investigated. Furthermore, we studied the effects of interruption of levan administration after different periods of treatment.

MATERIALS AND METHODS

Mice. Six-week-old AKR/Cu male mice bred at the Weizmann Institute were used. The AKR lymphoma was maintained as before (14). Each experiment was done on groups of 4 to 10 mice.

Levan. Native *Aerobacter* levan (M. W. 20×10^6) prepared according to the method of Hestrin *et al.* (7) was purchased at the Department of Biological Chemistry, Technical Unit, The Hebrew University of Jerusalem. A 5% solution in 0.9% NaCl solution was prepared according to the method of Shilo *et al.* (14).

Levan was administered either i.p. or s.c. in the backs of the animals. The daily dose used throughout these experiments was 10 mg. Levan injections were started on the day preceding tumor inoculation (Day 0) or 2, 5, 7, 13, or 20 days after inoculation. In some experiments preliminary levanization, intended to decrease levan-related mortality, was administered on Days "−8," "−6," "−4," followed by daily injections as from Day "−1." In other experiments levan treatment was interrupted after varying periods of levanization.

Tumor Cell Inoculation. Tumor cell suspensions were prepared as described before (10). Approximately 10^5 cells were inoculated by 1 of 3 routes: s.c. in the back of animals, i.m. in the thigh, or i.p. Animals inoculated with tumor cells and not treated by levan served as controls.

Evaluation of Tumor Development. Tumor development was evaluated by studying the following parameters: incidence of primary and metastatic tumors, weight of mice, and their survival. The incidence was mostly determined by palpation and in dying animals by autopsy.

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RESULTS

Local *versus* Systemic Administration of Levan

Thirty mice were given s.c. injections of tumor cell suspensions in the back, and another 30 were treated i.m. in the thigh. Each group was subdivided into 3 subgroups: Group 1, 10 mice of each group receiving daily from the day of tumor inoculation s.c. levan injections in the back; Group 2, 10 mice receiving the levan i.p.; and Group 3, 10 mice not treated by levan. The results indicated that local administration of levan was the most effective way of inhibiting tumor growth. In fact, 19 days after inoculation of 10^5 tumor cells in the back only 1 of 10 mice treated with levan in the back had palpable tumors. Levan administration i.p. was less effective (3 of 10 animals with tumors), while without treatment 6 of 10 animals had tumors. In mice inoculated with tumor in the thigh, s.c. and i.p. levan had no effect on tumor growth. Comparable data were obtained in other experiments, but the rate of growth of the tumors varied in different experiments.

Effects of Length of Interval between Tumor Inoculation and Beginning of Treatment

Tumor Development. The effects of varying the interval between tumor inoculation and beginning of levan treatment were studied in the following way. Mice were inoculated with tumor cells in the back. About 50 animals were treated with levan i.p., and 50 were treated with local levan injections in the tumor site. In each experiment, groups of 6 to 10 mice were started on levan on the day of tumor inoculation and 2, 5, 7, 13, or 20 days later. The effect of the levan treatments on tumor development was studied in terms of incidence of primary tumors and metastases. The incidence of palpable primary and metastatic tumors was determined every few days.

The results of a typical experiment are presented in Table 1. Levan injections into the primary tumor site started at different times inhibited primary tumor growth. The findings were not unequivocal because the swelling caused by levan injections interfered with palpatory evaluation. Inhibition of metastatic spread was obvious only in mice started on levan on Days 0 and 2. A later start of levanization had little effect.

Levan injections i.p. had more inhibitory effect on the primary tumor growth in animals started on levan on the 7th and 13th days than in mice with earlier levanization. Beginning levan treatment on the 20th day was also ineffective. The inhibitory effect of i.p. levan injections on metastatic growth was obvious but variable. The data presented in the table refer to the 23rd day after tumor inoculation, because at later dates high mortality distorted the picture. In other experiments (see, for example, last lines in Tables 2 and 3), the effect of injecting levan into the site of the tumor on Days 8, 6, and 4 after and Day 1 before inoculation of tumor cells revealed the following. Locally pretreated animals developed more rapidly primary tumors than not pretreated mice, although both groups were maintained on the same schedule of levan after tumor inoculation. Pretreatment i.p. did not have a clear-cut effect on local tumor development.

Weight of Animals. The overall effect of tumor development could be gauged by changes in the weight of the animals. Although the average weights were determined in a selected population (including only mice that survived until the 57th day and probably did not have widespread tumors), levan treatment administered either locally or i.p. from Days 0, 2, 5, 7, or even 13 after tumor inoculation caused an obvious preservation of weight (28 to 34 g). In contrast the weight of nonlevanized mice was significantly reduced on that day (20 g). The weights of animals started on levan on the 20th day were intermediate between those with an early start of treatment and nonlevanized mice. The difference between groups of animals started on levan on different days was not statistically significant.

Survival. Chart 1 shows the effect of varying the starting date of topical levan treatment at the site of tumor inoculation on survival of mice. The chart shows that local administration of levan begun together with tumor inoculation, or 2 days later, prevented mortality due to spread of tumor. Levan treatment begun on later days was much less effective in prolonging life. In mice treated with levan beginning on the 5th day after tumor inoculation, death occurred more rapidly than in nonlevanized animals. The peculiar behavior

Table 1

Effect of length of interval between tumor inoculation and beginning of levan treatment on incidence of tumors

The mice were given injections of 10^5 tumor cells in the back. Metastatic tumors were estimated by palpation of inguinal lymph nodes.

	Levan injected i.p., tumor incidence on 23rd day				Levan injected in back, tumor incidence on 31st day			
Starting day of levan treatment	Primary	*p*	Metastatic	*p*	Primary	*p*	Metastatic	*p*
0	2/6[a]	0.05	1/6	0.05	0/06	0.005	0.06	0.005
2	4/6	0.05	2/6	0.05	0/10	0.005	1/10	0.005
5	4/6	0.05	2/6	0.05	0/6	0.005	5/6	0.05
7	0/6	0.005	1/6	0.05	0/6	0.005	3/6	0.05
13	0/6	0.005	5/6	0.05	0/6	0.005	4/6	0.05
20	4/6	0.05	1/6	0.05	0/6	0.005	4/6	0.05
Not treated	7/10		4/10	0.05	7/10		7/10	

[a] Tumor-bearing/tumor-injection-treated animals.

Table 2

Effect of duration of local levan treatment on the incidence of primary tumors

Each mouse was given an injection of 10^5 tumor cells on Day 0. Evaluation of significance according to a 1 degree of freedom continuity-corrected χ^2 (11).

Duration of treatment	With preliminary injections				Treatment from Day 0			
	Day of inspection				Day of inspection			
	15	18	20	*p*	15	18	20	*p*
0	8/10[a]	8/10	9/10		8/10	8/10	9/10	
2	2/10	5/10	9/10	0.05	1/4	1/4	2/4	0.05
5	2/10	1/10	1/10	0.01	0/4	0/4	0/4	0.02
7	0/10	0/10	0/10	0.01	0/4	0/4	0/4	0.02
13	0/10	1/10	4/10	0.01	0/4	0/4	0/4	0.02
Throughout the experiment	0/10	0/10	6/10	0.01	0/7	0/7	0/7	0.01

[a] Tumor-bearing/tumor-injection-treated animals.

Table 3

Effect of duration of i. p. levan treatment on incidence of primary tumors

Each mouse was given an injection of 10^5 tumor cells on Day 0. Evaluation of significance according to a 1 degree of freedom continuity-corrected χ^2 (11).

Duration of treatment	With preliminary injections				Treatment from Day 0			
	Day of inspection				Day of inspection			
	15	18	20	*p*	15	18	20	*p*
0	8/10[a]	8/10	9/10		8/10	8/10	9/10	
2	4/10	8/10	9/10	0.05	1/4	2/4	2/4	0.05
5	3/10	6/10	8/10	0.05	0/4	2/4	4/4	0.05
7	0/10	3/10	5/10	0.02	0/4	0/4	1/4	0.05
13	0/10	0/10	0/10	0.02	1/4	1/4	1/4	0.05
Throughout the experiment	0/10	4/10	5/10	0.02	2/8	2/8	3/8	0.02

[a] Tumor-bearing/tumor-injection-treated animals.

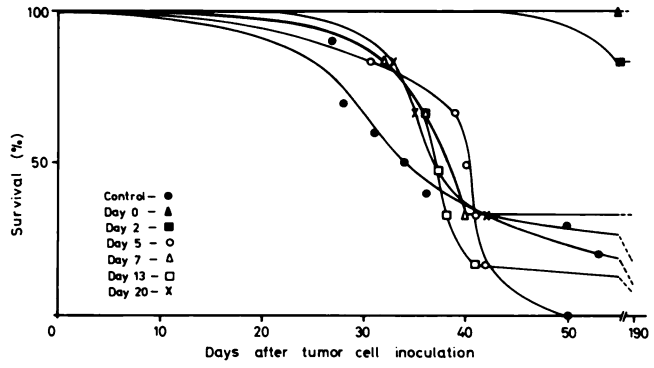

Chart 1. Effect of different starting times of topical injections of levan (10 mg daily) on survival of tumor-bearing mice (6/group); 10^5 tumor cells were inoculated in the back of each mouse on Day 0.

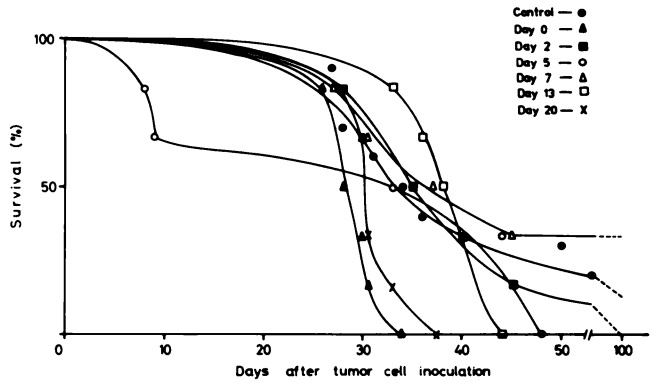

Chart 2. Effect of different starting times of i.p. injections of levan (10 mg daily) on survival of tumor-bearing mice (6/group); 10^5 tumor cells were inoculated in the back of each mouse on Day 0.

of animals in which treatment was begun on the 5th day recurred and will be discussed later. Levanized animals that remained alive for about 50 days had a high chance of living for 200 days and more. Thus, when 100% mortality is considered some mice treated beginning on Days 0, 2, 5, 7, 13, and 20 survived for over 210, 210, 50, 210, 148, and 81 days, respectively, while in nonlevanized animals 90 to 100% mortality was reached on the 69th day. In this particular experiment 1 mouse of 10 controls remained alive and tumorless for 180 days.

Chart 2 shows the effect of variations in the starting date of i.p. levan treatment. The effect of i.p. levan on survival, although present, was minimal in comparison to topical treatment. In the experiment shown in the chart and in other experiments, some delay in 50% mortality occurred in animals treated beginning on Days 7 or 13. Treatment i.p. started on earlier days either had no effect or shortened the life-span. The above findings have been consistently repeated through 15 transfers. At later times the injection of

tumor cells resulted in diffuse rather than well-defined primary tumors. This change was associated with increased mortality in levanized tumor-bearing animals in comparison to controls.

Effects of Varying the Duration and Continuity of Levan Treatment

Interruption of Levan Administration after a Few Days of Treatment. Tables 2 and 3 show the incidence of tumors at the site of injection in mice treated with levan for 2 to 13 days in comparison to nonlevanized animals and to animals with uninterrupted treatment. As in other experiments, injection of levan into the tumor site was more effective than i.p. treatment. It can be seen that even a 2-day period of levan treatment had some delaying effect on the appearance of palpable tumors. The degree of inhibitory effect on tumor development was a direct function of the length of treatment with levan. In this and in another similar experiment, levan treatment interrupted after less than 2 weeks had no effect on the life-span of the mice given tumor injections.

Pretreatment reduced the inhibitory effect on tumor incidence of levan administered by both routes (Tables 2 and 3). It has also been found that the pretreated animals died sooner than did comparable mice in which the treatment was started on the day of tumor inoculation.

Effect of Prolonged Treatment. Thirteen animals treated for 150 days were divided into 2 groups. Treatment was continued in 6 mice, while in 7 it was stopped. In both groups no palpable tumors were found on periodical examinations. Mice of both groups died apparently from intercurrent diseases, so that 12 months after tumor inoculation 1 animal of each group was alive and apparently free of tumor.

Four other animals were killed after 191 days of levan treatment. No palpable tumor masses were detected in them. Careful dissection failed to reveal gross changes in addition to hepatosplenomegaly, which is common in animals treated with massive doses of levan. Microscopic examination of the site of injection, spleen, liver, and various lymph nodes revealed no tumor in 3 mice. Amyloid deposition of the perifollicular type was found in their spleens and, in 1 mouse, also in the hepatic portal spaces.

In 1 mouse a microscopic nodule of lymphoma cells was found at the site of inoculation. The nodule consisted of cells with shrunken nuclei and debris. A moderate tumoral infiltration was also noted in the spleen. The tumoral invasion of the spleen was not widespread and did not mask the splenic architecture in the way we have usually observed in nonlevanized mice. In all animals the site of injection was filled with foamy macrophages.

DISCUSSION

These experiments indicate that the inhibitory effect of levan on the development of AKR lymphoma depends on the site and timing of levan administration. In all experiments local injections of levan into the area inoculated with tumor cells were much more effective than injections given by other routes. Topical administration raised the local concentration of levan to a greater extent than did i.p. or distant s.c. injections. In fact, persistence of levan at the injected site could be palpated during life and seen postmortem as a gelatinous mass. This persistence ensured high local concentrations of levan in the tumor cell-injected area. It is concluded that local levan concentration at the site of the tumor is of major importance in determining the fate of the primary growth. In most mice given local levan injections within the 1st 2 days after tumor inoculation, local tumor growth was never palpated or seen. Local levanization started 2 days or more after tumor inoculation had only a mild inhibitory effect on the primary tumor growth. The local effect can be explained by the results previously described (10), *i.e.*, levan caused destruction of malignant cells and inhibition of local spread.

Pretreatment with levan at the site of the tumor inoculation enhanced local tumor growth (Table 2, last 2 lines). This effect might have been caused by the action of levan on local macrophages. It has been reported, in fact (19), that local macrophages are important in the early defense of the host against tumors. Levanization of the site prior to the injections of tumor cells may be expected to paralyze the macrophages by blocking them and disturbing their immunological activity.

The effect of i.p. injections started 5 to 13 days after tumor inoculation on the incidence of primary tumors was more marked than that of treatment begun 2 days or less after tumor inoculation. Levanization started 20 days after tumor inoculation had, however, little effect on tumor growth. The effects of i.p. injections of levan started on Days 0 to 13 [which were more obvious at 23 days than at 31 days after tumor inoculation (Table 1)] indicate that levan acts not only by direct inhibition of tumor growth. In fact, direct inhibitory effect of levan on tumor cells may be expected to be more pronounced at early stages of tumor development when the number of malignant cells is small.

The inhibitory effect of levan was proportional to the length of treatment, presumably because of levan persistence in the tissues and the probable reduction in the number of surviving tumor cells.

The effect of local injections on metastatic spread of the tumor depended on the time when levanization was started. Local injections of levan started less than 5 days after tumor inoculation inhibited metastatic tumor growth (Table 1), while levanization started on the 5th day or later had only a slight inhibitory effect. These results may be correlated with the effects of levan on primary tumor growth. Local injections of levan had a maximal inhibitory effect on primary tumor growth when started within 5 days, while i.p. injections had a maximal effect only at later dates.

Tumor development has also been evaluated by changes in the weight of mice and in their life-span. In the surviving animals both local and i.p. injections of levan caused the mice to keep their normal weight in comparison to nonlevanized mice.

In most treated animals that survived for 191 days, the tumor was eradicated and did not recur after interruption of

treatment. In the 1 animal with a microscopic tumor at the site of injection and spread into the spleen, it is possible that the tumor was a spontaneous lymphoma, a possibility raised in the context of another treatment (13). Thus it appears that prolonged levan treatment started soon after the inoculation cured the animals from lymphoma.

The absence of growing tumors in mice continuously levanized over many months indicates that this treatment might prevent the development of spontaneous lymphoma. It is known (15) that 80 to 90% of AKR mice die of lymphoma by 1 year of age. The absence of palpable tumors (spontaneous or resulting from the inoculations) in the 17 mice treated for long periods seems to be of special significance in this respect.

It is possible that maintenance of adequate concentrations of levan at the metastatic sites might have prolonged the survival of animals with metastases. These considerations are consistent with the observation that i.p. injections started on the 5th day and later prolonged the life-span, while topical treatment begun on the 5th day or later and i.p. treatment begun before the 5th day had little effect in prolonging life. Later local treatment might be presumed to have been only partly effective because of the presence of metastases. Early i.p. treatment shortened the life-span, possibly in relation to its peculiar effect on the previously described tumor distribution in the liver (10) or because of its effect in inhibiting the function of the macrophage system (8, 16). The last-mentioned possibility corresponds to our observations on the effect of pretreatment by levan at the site of primary tumor inoculation. The harmful effect of levan pretreatment might be explained by inhibition of the afferent immunological path, *i.e.*, the uptake of antigenic material by macrophages.

The difference between the 1st 5 days after tumor inoculation and later periods might be related to various factors reported in the literature. Old *et al.* (12) observed increased phagocytic activity of the reticuloendothelial system as from the 5th day after tumor inoculation with a maximum at 7 to 12 days. Bansal and Sjögren (1) found that *Bacillus Calmette-Guérin* was active in inhibiting polyoma tumors when injected earlier than the 7th day after tumor inoculation and not at later days. Bruce and Mecker (4) found that after intravascular injection of AKR lymphoma cells no macroscopic evidence of metastatic spread was noted in the 1st 4 days. Brem and Folkman (3) observed that carcinomatous inocula in rabbits grew for 3 to 5 days without proliferation of blood vessels into the tumors. These studies indicate that (*a*) immunological factors, (*b*) beginning of metastatic spread and (*c*) growth of blood vessels may play a role in the change in behavior of the tumor around the 5th day. As levan is capable of (*a*) affecting immunological reactivity (5) and phagocytic activity (8, 20), (*b*) influencing metastatic spread (10), and (*c*) inhibiting capillary proliferation (18), we cannot at present explain the mechanism responsible.

The fact that tumors in our experiments began behaving differently after a certain period of time might have been caused by a modification in the tumor strain (progression with repeated transfers). We have found that the tumor that became more invasive and spreading and did not produce localized primary tumors responded differently to levan than the original strain. It is possible that rapid metastatic spread allowed tumor cells to escape the effects of local high levan concentrations. The increased mortality of the modified tumor might be related to the effect of levan on macrophages and the resulting inability of the reticuloendothelial system to clear the blood of harmful tumor-dependent agents.

REFERENCES

1. Bansal, S. C., and Sjögren, H. O. Effects of BCG on Various Facets of the Immune Response against Polyoma Tumors in Rats. Intern. J. Cancer, *11:* 162–171, 1973.
2. Behar, A., and Shilo, M. Effect of Native Levan on Membrane Permeability. I. Effect on the Vascular Membrane in Acute Non Specific Inflammation. A Study with the Light Microscope. Am. J. Pathol., *57:* 583–603, 1969.
3. Brem, H., and Folkman, J. Inhibition of Tumor Angiogenesis Mediated by Cartilage. J. Exptl. Med., *141:* 427–439, 1975.
4. Bruce, W. R., and Mecker, B. E. Dissemination and Growth of Transplanted Isologous Murine Lymphoma Cells. J. Natl. Cancer Inst., *32:* 1145–1159, 1964.
5. Coutinho, A., and Moller, G. B. Cell Mitogenic Properties of Thymus Independent Antigens. Nature New Biol., *245:* 12–14, 1973.
6. Davies, A. M., Shilo, M., and Hestrin, S. The Influence of *Aerobacter* Levan on the Permeability of the Blood Vessels of the Skin: Studies with Antibody Globulins and Trypan Blue. Brit. J. Exptl. Pathol., *36:* 500–506, 1955.
7. Hestrin, S., Shilo, M., and Feingold, D. S. Infection-Promoting Activity of Levan and Dextran as a Function of Degree of Polymerization. Brit. J. Exptl. Pathol., *35:* 107–111, 1954.
8. Howard, J. G., Courtenay, M., and Desaymard, C. Equivalent Responsiveness to Branched Polysaccharides and Their D-Nitrophenol Conjugates in the Biozzi High and Low Responder Lines of Mice. European J. Immunol., *4:* 453–457, 1974.
9. Leibovici, J., Bleiberg, I., and Wolman, M. Effect of Native Levan on Homograft Rejection in Mice. Proc. Soc. Exptl. Biol. Med., *149:* 348–350, 1975.
10. Leibovici, J., Sinai, Y., Wolman, M., and Davidai, G. Effect of High Molecular Levan on the Growth and Spread of AKR Lymphoma. Cancer Res., *35:* 1921–1925, 1975.
11. Mantel, N. Evaluation of Survival Data and Two New Rank Order Statistics Arriving in Each Consideration. Cancer Chemotherapy Rept., *50:* 163–170, 1966.
12. Old, L. J., Clark, D. A., Benacerаf, B., and Goldsmith, M. The Reticuloendothelial System and the Neoplastic Process. Ann. N. Y. Acad. Sci., *88:* 264–280, 1960.
13. Schabel, J., Skipper, H. E., Trader, M. W., Laster, W. R., Jr., and Simpson-Herren, L. Spontaneous A. K. Leukemia (Lymphoma) as a Model System. Cancer Chemotherapy Rept., *53:* 329–344, 1969.
14. Shilo, M., Wolman, M., and Wolman, B. Inhibition of Inflammatory Response of Skin to *Staphylococcus aureus* by High Polymer Levan. Brit. J. Exptl. Pathol., *37:* 219–221, 1956.
15. Strauss, M. Y., Choi, S. C., and Goldin, A. Increased Life Span in AKR Leukemia Mice Treated with Prophylactic Chemotherapy. Cancer Res., *33:* 1724–1728, 1973.
16. Wiener, E., and Bandieri, A. Difference in Antigen Handling by Peritoneal Macrophages from the Biozzi High and Low Responder Lines of Mice. European J. Immunol., *4:* 457–463, 1974.
17. Wolman, M. Histological Changes Produced by Injections of Polysaccharides. A. M. A. Arch. Pathol., *62:* 149–154, 1956.
18. Wolman, M., and Wolman, B. Effect of Polysaccharides on the Formation of Granulation Tissue. A. M. A. Arch. Pathol., *62:* 74–84, 1956.
19. Woodruff, M. F. A., and Dunbar, N. The Effect of Cell Dose and Distribution on the Development of a Transplanted Tumor. European J. Cancer, *10:* 533–537, 1974.