Presence in Tumor Tissue of a Mouse Mammary Cancer Accelerant.* (19047)

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In studies of the effect of tissue extracts on transplantable tumors, it is known that the preparation of the recipient host by the injection of extracts may result in an acceleration of the growth of homologous tumor tissue upon its transplantation into susceptible hosts (1-7), or that it may cause normally resistant hosts to become susceptible to a homoiotransplant tumor (13-15,17,20,21). These extracts were derived from frozen (1-7,13,17) or lyophilized tumor tissues (14,15,20,21), or even from normal tissues (14,15). Casey (1-9) designated the observed enhancement, or accelerative effect, the "XYZ phenomenon", and the factors derived from frozen tissue which elicit enhancement as the "XYZ factor." It is significant to the present report that the attempts to date to demonstrate conclusively enhancive, or accelerative, factors in fresh unfrozen tumor extracts as potent as those derived from frozen tumor tissue have been uniformly unsuccessful (8-11,16,18,19).

The present work was undertaken with the purpose of exploring by physical, chemical, and immunological methods the factors responsible for enhancement and for the retardation in growth of tumor cells. This report makes it known that accelerant factors for a transplantable mouse mammary carcinoma of inbred C_3H mice are equally effective whether derived from fresh (unfrozen) or frozen homologous cancerous tissues.

Materials and methods. Tumor. The accelerative test materials and the transplantable tumor tissue employed for challenge inoculation were derived from a mouse mammary adenocarcinoma, Z8352. This tumor had arisen spontaneously in a C_3H female possessing the milk agent. At the time this report was written, it had been maintained for 13 successive passages by transplantation in

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susceptible ZBC mice‡ known not to possess the milk agent. *Hosts.* The mice employed as test animals were from ZBC stock, assumed, in the absence of direct testing, to be free from the milk agent. This stock supports regularly the growth of Z8352 tumor cells on transplantation. *Preparation of accelerant factors.* The accelerant factors from fresh (unfrozen) tumor tissue were prepared as follows: A freshly excised Z8352 tumor was passed through a tissue press to yield a mince. This mince was triturated by means of a mortar and pestle, using a minimum of sterile alundum as an abrasive, and suspended in 0.85% saline solution in the ratio of 3 ml of saline per g of tissue mince. The suspension was homogenized by means of a Potter-Elvehjem homogenizer and then centrifuged horizontally at 1200 r.p.m. for 15 minutes. The supernatant fluid was subjected to four successive centrifugations, the sedimented material being discarded after each run. Following the fourth and final centrifugation, the supernatant fluid was employed as a source of "fresh accelerant." The method employed for the preparation of accelerant factors from frozen tumor tissue was essentially similar to that outlined by Casey in his preparation of the XYZ factor(1-7). Freshly excised Z8352 tumor tissue was placed in cotton-stoppered test tubes and stored at —18°C in a deep-freeze chest for from 63 to 118 days. On removal from storage, the tumor tissue was thawed at room temperature, minced, triturated, and finally, made up to a 3:1 suspension in 0.85% saline solution as outlined for the fresh accelerant. This suspension was homogenized by means of a Potter-Elvehjem homogenizer, and the homogenate was employed as the source of "frozen accelerant." The procedures for the preparation of accelerant factors from fresh unfrozen tissues and from frozen cells were carried out at room temperature. *Challenge inoculum.* The challenge inoculum consisted of a 5% suspension of viable Z8352 tumor cells which was prepared from freshly excised tumor by forcing the tumor tissue through a tissue press to yield a mince permeable for a 22-gauge needle, suspending this mince in a ratio of 1 g of minced tumor to 19 ml of 0.85% saline solution, filtering the suspension of tumor cells through 4 layers of 12-ply gauze and, finally, allowing the cellular filtrate in a 50 ml centrifuge tube to sediment for 10 minutes at room temperature. The resultant supernatant suspension of cells was transferred by pipette to another tube to be employed as the source of the challenge inoculum; the sediment was discarded.

Method of testing. The determination of the efficacy of each test material was carried out in two steps: (1) by the injection subcutaneously into the groin of a preparatory dose consisting of 0.2 ml of accelerant, whether frozen or fresh, and (2) by the challenge 10 days later of these test mice and of control mice that had not received a prior inoculation of either accelerant by the injection subcutaneously of 0.1 ml of a 5% suspension of viable Z8352 tumor cells into the tissues of the nape of the neck. The test and control recipient animals following challenge were observed every second day for the development of tumors and for the time of death; these findings were recorded.

Method of calculating results. Inasmuch as the transplantation of mouse mammary adenocarcinoma Z8352 cells into ZBC mice produces uniformly lethal results, the time required for the recipient animal to succumb to the challenge injection provided a precise and convenient reference point for the determination of acceleration of the neoplastic process. Accordingly, two methods were utilized for demonstrating the accelerative effect: (1) As a means of comparing the *relative longevity* of the control and test groups following challenge, a method for the determination of a "Percental Accelerant Index" was devised. This index was calculated from the formula:

‡ The ZBC strain employed was developed in the following manner:

Ax ♀ (A strain lacking the milk agent) X Zb ♂ (Z or C_3H lacking the milk agent)

↓

AxZb Fl ♀ X Zb ♂

↓

ZBC

$$\text{Percental accelerant index} = \frac{\left[\begin{array}{c}\text{Interval in days required for}\\ \text{death of 50\% of control group}\end{array}\right] - \left[\begin{array}{c}\text{Interval in days required for}\\ \text{death of 50\% of test group}\end{array}\right]}{\text{Interval in days required for death of 50\% of control group}} \times 100$$

(2) As a means of comparing the *relative mortality* of the control and test groups during the course of the experiment, the percental mortality due to cancer within the control group at the time 50% of the test group animals were dead of cancer was determined; a similar determination was made of the percental mortality from cancer within the test group at the time 50% of the control group animals had succumbed to the challenge. As an aid for the reader in the interpretation of the data, the results of Exp. 1 of Series A (Table I) are cited as an example: Of the 14 test animals that received a prior injection of the frozen accelerant, 50% had died of cancer in 31 days, while only 10% of 17 control animals were dead at this time. Of the 17 control animals, 50% had died of cancer in 39 days, while 82% of the test animals were dead at this time. The calculated *Percental Accelerant Index* for this experiment was $\frac{39 \text{ days} - 31 \text{ days}}{39 \text{ days}}$, or 20% acceleration, due to the prior injection of the frozen accelerant.

Results. Two series of experiments were carried out to determine whether derivatives prepared from fresh and frozen cancerous tissues, respectively, would result in an enhancement in the rate and the extent of proliferative response of a challenge inoculum of homologous tumor cells which was injected subcutaneously after an interval of 10 days. This influence of the "accelerant" was determined by the accelerant index method described above.

Series A consisted of 3 experiments involving a total of 102 animals. This series was planned to determine whether enhancive factors for tumor growth as described by Casey (1-7), and others(13,17) could be elicited by the storage in the frozen state of mouse mammary cancer Z8352. That such enhancing factors do exist in this tumor is evident from the data presented in Table I. It can be seen from the findings presented in Table I that the prior inoculation of frozen accelerant had an accelerative effect of 22.2% (Percental Accelerant Index), as determined from a compilation of the results of Exp. 1, 2, and 3. Furthermore, when the "2 x 2 table" Chi square method(12) is applied to the comparative percental mortality of the *compiled* opposing groups, the results of these calculations establish for each of the compiled groups a significant P value of less than 0.01 (50% of 47 test mice vs. 17.4% of 55 control animals, $X^2 = 12.2$ and $P = 0.0005$; 50% of 55 control mice vs. 86.6% of 47 test animals, $X^2 =$

TABLE I. Effect of Frozen Accelerant on Growth of a Transplantable Mouse Mammary Cancer, Z-8352.

Series	Exp. No.	No. of animals by groups Test	Control	Deaths from cancer by groups as % incidence Test	Control	Accelerant index determination: Interval in days to calculation of % accelerant index	% accelerant index
A	1	14	17	50	10	31	$\frac{39-31}{39} = 20$
				82	50	39	
	2	8	19	50	21	36	$\frac{47-36}{47} = 23$
				88	50	47	
	3	25	19	50	0	35	$\frac{51-35}{51} = 31$
				88	50	51	
	Compilation 1-3	47	55	50	17	35	$\frac{45-35}{45} = 22$
				87	50	45	

TABLE II. Effect of Fresh Unfrozen Accelerant on Growth of a Transplantable Mouse Mammary Cancer, Z-8352.

Series	Exp. No.	No. of animals by groups Test	Control	Deaths from cancer by groups as % incidence Test	Control	Accelerant index determination: Interval in days to calculation of % accelerant index	% accelerant index
B	4	21	9	50	0	37	$\frac{65-37}{65} = 43$
				93	50	65	
	5	12	17	50	6	30	$\frac{39-30}{39} = 23$
				75	50	39	
	6	22	19	50	16	42	$\frac{51-42}{51} = 17$
				86	50	51	
	Compilation 4-6	55	45	50	19	37	$\frac{49-37}{49} = 24$
				86	50	49	

15.3 and P = 0.0001).

Series B consisted of 3 experiments involving 100 animals. The purpose of this series was to determine whether an accelerating factor could be obtained from extracts of *fresh* (unfrozen) tumor tissue much as it had been derived from tumor tissue stored in the frozen state. That such an accelerant was obtained is evident from the data presented in Table II. The findings presented in Table II make it known that the prior inoculation of fresh (unfrozen) accelerant produced an accelerative effect of 24.5% (Percental Accelerant Index) upon the malignant process as determined from a compilation of the results of Exp. 4, 5, and 6. Again, when the "2 x 2 table" Chi square method is applied to the comparative percental mortality of the *compiled* opposing groups, the results of these calculations establish for each of the compiled groups a significant P value of less than 0.01 (50% of 55 test mice vs. 18.9% of 45 control animals, $X^2 = 10.4$ and P = 0.0013; 50% of 45 control mice vs. 86.6% of 55 test animals, $X^2 = 15.4$ and P = 0.001.

A graphic presentation of the *compiled* results of Exp. 1, 2, and 3 of Series A appears in Fig. 1, while a similar presentation of the *compiled* results of Exp. 4, 5, and 6 of Series B appears in Fig. 2.

Discussion. Studies were carried out which successfully demonstrated the presence of an accelerant factor in unfiltered supernatant fluid derived from fresh (unfrozen) mouse mammary carcinoma Z8352. This accelerant factor was made known in 3 experiments involving 100 ZBC mice which served as recipients for the transplantation of the tumor. Significantly, the results obtained with the accelerant factor derived from fresh tissue was corroborated by similar findings when tumor tissue which had been stored at —18°C for from 63 to 118 days was employed for the preparation of the accelerant or, "XYZ," factor.

The technic of an "accelerant index" for comparison of the test and control groups was introduced as a means for minimizing individual variations between animals within groups and at the same time to provide a convenient reference point for the comparison and compilation of replicate experiments. The validity of such a technic rests on the assumption that one of the most important criteria for assessing the extent of a malignant process is the rapidity with which a proliferating tumor produces death. It was recognized in this present study that the period of time required for the recipient animal to succumb to the transplantable tumor provided a precise and convenient reference point. This seemed preferable to the employment of periodic palpation, or volumetric measurements, of the growing tumor as an index of enhancement,

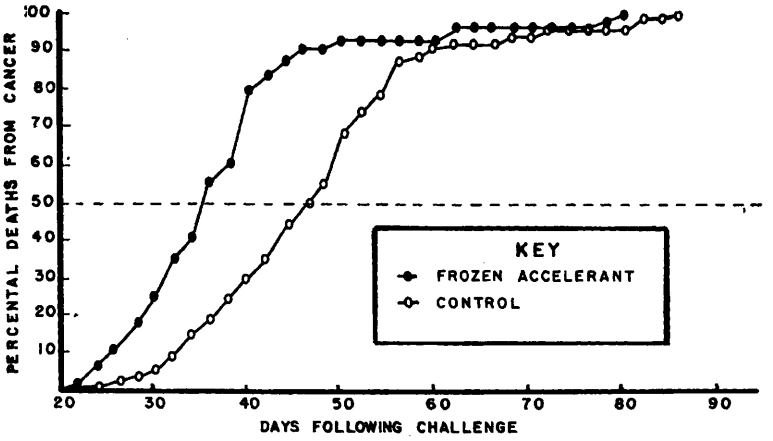

FIG. 1. The enhancement by frozen accelerant in the incidence and time to death: A graphic presentation of the compiled results of Experiments 1-3, Series A.

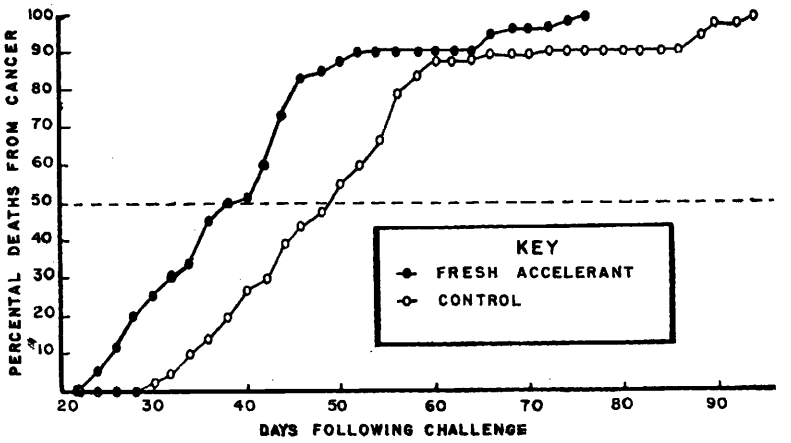

FIG. 2. The enhancement by fresh accelerant in the incidence and time to death: A graphic presentation of the compiled results of Experiments 4-6, Series B.

since these latter methods, regardless of the extreme care with which they may be conducted, are subject to interpretive differences when made by different observers, or even by the same person. Utilizing this technic, a significant acceleration in the onset of death (or a significant decrease in longevity) resulted when the tumor had been transplanted into test animals previously inoculated with either fresh (unfrozen) accelerant or frozen accelerant in comparison with control animals which had not been inoculated previously with either accelerant.

The striking similarity in the compiled results of the two series of experiments (Tables I and II and Fig. 1 and 2) strongly suggests that the two factors may in fact be a single entity derived from the tumor tissue by two different methods. In contrast to this evidence for acceleration, preliminary experiments indicate that the prior inoculation of mouse embryonic tissue, or fresh or frozen noncancerous lactating mouse mammary gland exerts little, if any, accelerating effect.

The possibility that the acceleration is not due to a specific phenomenon, but is merely the reflection of the summation effect of a double inoculum, may be discounted since in no instance did a tumor develop in the groin, the site which had been employed for the inoculation of the material under test. The only tumors arising occurred in the region of the nape, where the challenge inoculation of a 5% suspension of viable transplantable tumor

cells was made. Furthermore, 10 animals inoculated only with frozen accelerant and 10 animals inoculated only with fresh (unfrozen) accelerant and allowed to remain unchallenged failed to develop tumors during a 90-day period of observation.

The findings that fresh (unfrozen) cancerous tissues and frozen tumor tissues yielded equally effective accelerative effects upon the growth of the homologous mouse mammary tumor are interpreted as evidence to relate the demonstration by Casey(1-7), and others (13,17), of accelerative factors in frozen tumor tissue and the occurrence of similarly reactive materials in lyophilized tumor tissue, as reported by Snell and his associates (14,15,20,21). Evidently, the accelerative, or "XYZ", factors originate in the tumor cell. It would seem that it is incidental whether the technics for eliciting enhancement employ fresh unfrozen tissues, lyophilized tissues, or frozen cells. However, in contrast to the currently reported unequivocal findings which made it known that fresh and frozen cells were equally productive of accelerative factors, it has been reported for mouse mammary carcinoma 15091a on transfer to C57 mice that the employment of fresh tumor homogenate resulted in an enhancement in only 28 of 62 (45%) mice(16) as compared with evidence for enhancement in 141 of 183 (77%) of the recipients that had been prepared by injections of lyophilized tumor tissues(15). It is suggested that the technic herein described for the preparation of accelerative factors will be equally productive when it is applied to mouse mammary cancer 15091a, irrespective of whether the tissues are employed as fresh, unfrozen cells or after lyophilization.

The failure of various investigators(8-11, 16,18,19) to demonstrate unequivocally the presence of accelerants in *filtrates* of fresh tumor tissue may have resulted, as suggested by Casey *et al.*(9), because "the factors are attached to or parts of a macromolecule which is not filtrable through Berkefeld or Seitz filters." The immunological, chemical, and physical properties, including filtrability, of the fresh (unfrozen) and the frozen accelerants derived from the Z8352 tumor, are under investigation in our laboratories.

Summary and conclusions. (1) In a series of 3 experiments involving 100 ZBC mice an accelerating factor was demonstrated in the unfiltered supernatant fluid which had been prepared by centrifugation repeatedly of a saline extract of fresh (unfrozen) mouse mammary carcinoma Z8352. This tumor, which originated in a female C_3H mouse possessing the milk agent, is maintained by transplantation in ZBC mice not possessing the milk agent. (2) In a second series of 3 experiments involving 102 ZBC mice a similar accelerant was demonstrated in homogenates of Z8352 tumor tissue which had been stored in a deep freeze chest at —18°C for from 63 to 118 days. (3) It is probable that the two factors, the "fresh (unfrozen) accelerant" and the "frozen accelerant", are a single entity derived from the tumor tissue by two different methods.

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Rate of Urinary Calcium Excretion Following Its Intravenous Administration as an Indicator of Bone Metabolism.* (19048)

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The purpose of this study is an attempt to define the state of bone metabolism and some of the mechanisms that maintain calcium and phosphorus homeostasis. The principle of the

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