Comments on Recent Experiments with Frozen and Dried Tissue as Evidence for the Virus Etiology of Tumors

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One of the tasks confronting investigators in any field is an occasional re-evaluation of the experimental work on which the basic theories in that field depend. A special impetus is given to such an endeavor when new data are collected and cited as conclusive evidence for or against any of these theories. The concept that the etiology of tumors involves the presence of viruses is one of the oldest theories on cancer: its popularity has varied from time to time and from investigator to investigator. With the unequivocal demonstration of the viral nature of the causative agents of a number of tumors, the main question has become whether all tumors are virus-induced. Ultrafiltration experiments have given an essentially negative answer, since it has not been possible to transplant most mammalian tumors by means of cell-free filtrates (16, 17).

The question, however, has been vigorously reopened by the recent work of Gye and his associates in London. These investigators have made extensive use of freezing and drying technics and have indicated that the successful transplantation of mammalian tumor tissue after these treatments, which are considered to have killed all the cells, is convincing evidence for the viral nature of the transmitting agent. These findings and conclusions have created widespread interest.

The present review brings together some of the available facts bearing on this problem. No position is taken on the merits of the virus theory in general. The central point of this review is to discuss the assumption of Gye and his collaborators that freezing and drying kill all the cells of a tissue and to inquire whether it can be safely concluded at this point that these technics provide unequivocal proof for the concept that the transplantation of mammalian tumors involves viruses and not intact cells. It is also hoped that this review may stimulate further investigations on this problem by indicating some of the approaches which may not yet have gained sufficient recognition.

Summary of the findings of Gye et al.—This work has been presented in a number of papers by Craigie, Gye, Mann, and others (9, 10, 14, 15, 22–25) and commented upon by Andrewes (1) and in two editorials in the British Medical Journal (2, 3). The conclusions of Gye and his associates may be briefly summarized as follows:

Normal embryonic tissue could not be successfully transplanted, and no normal cells could be found by histological examination after an hour's exposure to $-79^\circ$ C. Normal embryonic tissue was also killed when treated with glycerol and stored at $40^\circ$ C., while fine emulsions of frozen neoplastic tissue made with distilled water, 40 per cent glycerol or 40 per cent glucose caused cancer. A sarcoma suspension frozen in dextrose and 40 per cent glycerol was highly active after 253 days. Three mouse sarcomas, one induced chemically and the others spontaneous, were successfully propagated with tissues dried completely after freezing; some mouse sarcomas even retained their activity after drying without preliminary freezing. The continuing cause of these tumors was postulated to be a

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virus which was stable at \(-79^\circ\) C. Mann showed that mammary carcinomas whose etiology was associated with the Bittner virus could be transplanted after freezing to \(-79^\circ\) C. Under the usual conditions, when the virus is passed through the milk, an average of 9 months elapses before tumors appear; since tumors grew within a few weeks after freezing, she postulated that this treatment liberated an active form of the virus. The activity increased with the length of refrigeration, at least for the first 48 hours, and the cells which had been frozen induced tumors only in mammary tissue, in contrast to living tumor cells which grow in a variety of tissues. Repeated freezing and thawing of mouse mammary carcinoma tissue reduced its infective activity. Active Bittner virus obtained from this tissue by a single freezing could be dried in vacuo if no thawing occurred before the material was dry; such a preparation produced tumors in from 4 to 10 weeks in both male and female mice when injected into breast tissue.

The basic point conveyed by these papers is that tumor tissue, when transplanted following freezing and drying, is still capable of causing vigorous neoplastic growth in the new host, while normal cells cannot withstand these treatments. Gye et al. considered these facts to be proof of the viral nature of the causative agent of these transplantable mammalian tumors.

Survival of normal cells after freezing.—Perhaps the weakest link in the chain of evidence built up by Gye et al. is their readiness to assume that the lack of growth of normal embryonic tissue of two strains of mice after freezing is sufficient evidence for the death of all normal cells at extremely low temperatures; evidence to the contrary with other normal tissues, and under conditions different from those employed by Mann (29), is available. The following paragraphs do not purport to give an exhaustive survey of the pertinent literature but rather a summary of certain representative findings.

Breedis and Furth (7) showed that tracheal epithelium of chickens survived slow freezing to \(-70^\circ\) C. and storage at that temperature for 327 days. Many cilia were actively motile after this treatment, but the suitability of this criterion for life was later questioned by Breedis himself (6). According to Mider and Morton (26), normal adult rat skin could be frozen rapidly to \(-74^\circ\) C. and maintained at that temperature for 24 hours without losing its capacity to grow in the host, as determined by histologic examination. Thoenes (28) showed that muscle fibers from the sartorius of the frog, immersed in liquid air at \(-195^\circ\) C., for periods up to 1 hour and rewarmed rapidly by immersion in Ringer's solution at \(35^\circ\) C., responded to electrical stimulation in 91 per cent of the cases. Klinke (20) obtained evidence by vital staining for the survival of renal, testicular, and ovarian tissues of several animal species after storage at \(-196^\circ\) C. for several weeks. Tissue culture experiments by Klinke also showed that even short exposure to liquid nitrogen at \(-253^\circ\) C. occasionally permitted the survival of fragments of chick embryo liver, brain, and heart. Briggs and Jund (8) subjected the ventral or dorsal skin of three inbred strains of mice to slow freezing to \(-78.5^\circ\) C.; even when maintained at this temperature for from 1 to 24 hours, autologous grafts to dorsum or venter were successful in from 50 to 100 per cent of the cases. Quite recently, Blumenthal and his collaborators (4, 5) exposed the thyroid gland of guinea pigs to low temperatures and then autotransplanted it into a subcutaneous pocket in the anterior abdominal wall; 1 of 12 transplantation attempts was successful after exposure to \(-70^\circ\) C. for 24 hours, while 8 viable thyroid grafts were obtained in 12 attempts after immersion at \(-190^\circ\) C. for 10 minutes.

Not all attempts along these lines were successful. For example, Koo and Lemmel (21) reported that mouse skin exposed to liquid air for a short time appeared normal for 10–14 days after grafting but then always died. However, the majority of experiments in which normal tissue did not survive freezing involved embryonic tissue; the findings of Gaylord (13) with young mouse embryos, of Simonin (27) with rat, mouse, and ox embryos, and of Ferguson (19) with the smooth muscle of chick embryos may be cited. In view of the apparently greater sensitivity of embryonic tissue to freezing, it would seem particularly unfortunate that Mann (22) chose this type of normal tissue as the only control for the freezing procedures used in her recent investigations.

Growth of tumor tissue in tissue culture after freezing.—Tissue culture provides an especially useful tool for investigating the effect of low temperature on the survival of intact neoplastic cells. The successful culture of such cells following freezing is conclusive evidence that the cells were not destroyed by this treatment. The viability of cells following freezing has been determined in several experiments with variable results. Koo and Lemmel (21) were unable to find any evidence of growth attributable to intact cells when mouse carcinomata tissue was frozen in liquid air for 5 minutes. Cramer (11) was unable to cultivate normal, carcinoma, or sarcoma cells in tissue culture after repeated freezing and thawing.

On the other hand, positive results have been
obtained in the extensive studies of Klinke (18-20). Definite evidence for the presence of living cells in Ehrlich mouse sarcoma and Jensen sarcoma tissue after freezing in liquid nitrogen for 48 hours was obtained in this way. While not all attempts to culture such cells were successful, it would seem that the growth in tissue culture of even a few explants subsequent to freezing clearly indicates the survival of intact cells. Continued work along these lines is indicated.

Discussion.—A comparison of the findings of the various investigators is severely hampered by the diversity in experimental materials, technics of freezing and thawing, and criteria used to determine the presence or absence of intact cells. Nevertheless, every example of a successful survival of normal tissue to freezing and every demonstration of growth in culture of an explant of frozen tumor tissue weakens the arguments which have been advanced for the viral nature of the transmitting agent in this type of experiment.

Up to this point, the weaknesses of the position of the British investigators have been emphasized. It is also essential to discuss the more persuasive aspects of their work. These will be mentioned briefly, since many of the experiments which would permit an evaluation of these aspects from all points of view still remain to be done.

Gye and his associates rightly stress the fact that they have greatly strengthened their body of evidence by the subsequent drying of frozen cells. The experiments showing that tumor tissue suspensions can withstand treatment with 40 per cent glycerol without loss of activity also argue against the presence of intact cells in the material used for transplantation. Freezing-drying is of course widely used to preserve bacterial cultures, but no adequate information is available concerning the effect of such a treatment on normal mammalian cells of various types. Until such information becomes available, the drying of frozen tumor tissue appears to be the most promising technic to be used in these studies.

Answers to the following questions, then, should do much to establish the validity of the conclusions of Gye et al.: Is it possible to freeze-dry normal tissue with the technics described by the London group without abolishing their capacity to be successfully grafted into new hosts? Can tumor or normal tissue be subjected to the freezing-drying technic and subsequently grow in tissue culture? Can frozen tumor suspensions be passed, after thawing, through Berkefeld or similar filters to yield active but unequivocally cell-free filtrates? What is the effect of 40 per cent glycerol under the conditions of the British investigators on a wide variety of normal cells? Until some of these points have been elucidated, there is little hope of coming to a final decision on the correct interpretation of these findings. With the information now at hand, it would appear that this type of study has not yet proved or disproved the theory that all tumors are virus-induced.

Summary.—The data on the propagation of mammalian tumors in the absence of intact cells obtained by Gye et al. with freezing and drying technics are summarized and discussed. It is pointed out that a fair amount of evidence exists for the survival of normal cells after freezing. The occasional growth of tumor tissue in tissue culture after freezing further indicates that freezing technics alone are not suitable for the solution of this problem. The introduction of drying after freezing appears to provide stronger evidence against the presence of intact cells. Some questions are raised which must be answered before the data obtained by freezing-drying can be completely evaluated.

REFERENCES

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