THE EFFECT OF FREEZING IN VITRO ON SOME
TRANSPALNTABLE MAMMALIAN TUMORS
AND ON NORMAL RAT SKIN

G. BURROUGHS MIDER, M.D., AND JOHN J. MORTON, M.D.
(From the Department of Surgery, The University of Rochester School of Medicine and
Dentistry, Rochester, New York)

Isolated observations of the effect of freezing on various animal cells are
encountered in the literature. Bacteria resist freezing to a remarkable degree,
though Haines has shown that freezing of bacteria in aqueous suspension re-
results in the death of approximately 80 per cent of the living organisms (1).
Swift has been able to demonstrate that bacteria may be desiccated in the
frozen state, after which their cultural, biochemical, and immunological char-
acteristics are unaltered (2).

Unicellular microorganisms resist cold in different degree. Turner noted
that the treponemata of yaws and syphilis retain their normal motility and
virulence for rabbits after exposure to — 78°C for four months (3). Try-
panasoma gambiensc withstand freezing in liquid air for twenty minutes, but
not for forty minutes (4). Vegetative forms of amoebae were killed by ex-
posure to — 5°C for five minutes in vitro (5), while malarial schizonts sur-
vived for a longer period at the same temperature (6). Encysted Trichinella
spiralis failed to produce infection after exposure to — 33.9°C and viability
was reduced at — 27.6°C (7).

Chick embryo heart is killed by exposure to the temperature of solid carbon
dioxide (— 74°C.) for less than one minute but survives — 20°C for five
minutes (8).

Various observers have reported that specific mammalian tissues frozen
in vitro are killed by exposure to moderately low temperatures. Lake estab-
lished — 7°C as the critical point for rabbit tissue (9). Gaylord reported
that mouse embryo epithelium failed to grow on subcutaneous transplantation
after exposure to the temperature of liquid air (— 180°C.) (4). Simonin
subjected pieces of mouse, rat, and beef embryos to cold. At — 15°C the
cells were killed after a short period of exposure, although they grew in vitro
after five days at — 5°C. “as long as the tissues did not congeal” (10).
This investigator concluded that mammalian tissues are not equally sensitive
to cold, heart, lung and intestine being the most resistant.

Salvin-Moore exposed transplantable mouse sarcoma and carcinoma to the
temperature of liquid air for twenty to thirty minutes. The tumors grew on
transplantation (11, 12). Gaylord confirmed these findings (4). Both of
these investigators froze the cells en masse. Auler obtained different results
with saline emulsions. The Ehrlich mouse carcinoma grew after being frozen

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502
for twenty-five minutes at $-73^\circ$ C. and the Jensen sarcoma after five minutes' exposure; but the Flexner-Jobling rat carcinoma failed to produce tumors (13).

Cramer froze finely minced mammalian tumors with carbon dioxide snow. Sarcomata grew after freezing. Carcinomata did not. He found that mouse sarcoma 37 sometimes produced tumors after being frozen and thawed eight times in liquid air. The Crocker rat sarcoma did not produce tumors after four freezings. The reaction to freezing varied among histogenetic types and among different specimens of the same tumor (17).

Furth and his coworkers found that a transmissible leukemia of mice could be transplanted successfully after exposure to $-70^\circ$ C. for fifty-six days providing the induction of the lowest temperature occupied ten to twenty minutes. No growth was obtained from leukemic tissue attaining its minimum temperature within three or four minutes. A transplantable mouse sarcoma originally induced by benzpyrene gave similar results (14, 15).

Method

Three tumors have been studied. Sarcomata 37 and 180 were obtained from the Rockefeller Institute for Medical Research, and were inoculated in a Swiss albino strain of mice that is particularly susceptible to viruses. Tumors developed in more than 90 per cent of inoculations. The third tumor was the Walker rat carcinoma 256, which has been used in this laboratory for a number of years. It grows well in an inbred strain of Wistar Institute rats, giving 90 per cent takes.

Most of the tissues were frozen en masse. When a saline suspension of cells was used, ground tumor was diluted four times with a buffered saline-Ringer solution. The refrigerant was a mixture of methyl cellosolve and solid carbon dioxide attaining an average minimum temperature of $-74^\circ$ C. The temperatures of the bath and of the material under investigation were measured with calibrated thermocouples the constant temperature junctions of which were immersed in an ice bath. The tissue being frozen was impaled on the other junction of the thermocouple and sealed in a test tube.

Rapid freezing was accomplished by sudden immersion of the tube containing the tissue in the refrigerating bath. By this method the temperature of the material reached that of the methyl cellosolve-carbon dioxide mixture within three to five minutes. By placing the tube in the bath at room temperature and adding small pieces of dry ice at suitable intervals the rate of freezing could be prolonged. The induction of the minimum temperature in more than twenty minutes constituted slow freezing.

Thawing was done at room temperature, $+20^\circ$ C., or in a water bath at $+30^\circ$ C. It generally occupied eight to twelve minutes. The material that had been frozen was then cut into small pieces and inoculated subcutaneously in the inguinal region of appropriate animals by trocar or direct incision. A control inoculation of unfrozen tissue was made in the opposite inguinal region at the same time.

Tumors frozen more than once were removed from the refrigerating bath as soon as the minimum temperature was reached. They were allowed to return to room temperature before the freezing process was repeated.
Tumor and Rate of Freezing

Walker 256
- Rapid 45% 22% 0 40% 20% 0
- Slow 50% 0 0 0 0 0

Sarcoma 37
- Rapid 85% 50% 0 0 0 0
- Slow 85% 50% 0 0 0 0

Sarcoma 180
- Rapid 90% 50% 0 0 0 0
- Slow 80% 50% 0 0 0 0

Table I: Incidence of Tumor Growth after Freezing

RESULTS

When Walker rat carcinoma 256 was frozen en masse to $-74^\circ$ C. once, grafts of the frozen material produced progressively growing tumors in 40-50 per cent of subcutaneous transplants. The incidence did not vary significantly with the rate of freezing or the duration of the frozen state (five minutes or twenty-four hours). Saline suspensions of this tumor were more sensitive to the deleterious effects of cold. Rapidly frozen cells in suspension produced one tumor in 10 injections after remaining five minutes in the frozen state, but when they were maintained at $-74^\circ$ C. for twenty-four hours no tumors were produced. Slowly frozen cell suspensions gave rise to tumors in 40 per cent of the injections after five minutes at the minimum temperature but in only 10 per cent when the frozen state was prolonged for twenty-four hours.

The Walker tumor resisted successive freezings and thawings to a marked degree. When the minimum temperature was rapidly induced, four freezings and thawings did not alter the subsequent incidence of growth, which remained at 40 per cent; with six freezings and thawings 20 per cent of the grafts grew, but no growth was observed in tissues frozen eight and ten times. After two slowly induced freezings the incidence of growth of transplanted grafts was 20 per cent. No tumors were obtained from material that had been slowly frozen three times.

Mouse sarcomata 37 and 180 yielded almost as many tumors from tissue that was frozen once en masse as from normal, unfrozen cells. Variation of the rate of induction of freezing and duration in the frozen state up to twenty-four hours did not significantly alter the number of progressively growing tumors. Cells of these neoplasms when frozen in saline suspensions failed to grow on subcutaneous inoculation.

Four rapidly induced successive freezings en masse reduced the incidence of subsequent growth of sarcoma 37 to 10 per cent and of sarcoma 180 to 50 per cent. Neither tumor grew after being rapidly frozen five times. Again, these tumors showed an increased sensitivity to multiple slow freezings. After slow freezing twice, 50 per cent of the inoculations gave rise to progressively growing tumors, but none was obtained from material slowly frozen three times (Table I).
In every case the tumor arising from frozen material showed a longer latent period than did the control, but, once initiated, no difference in the rate of growth could be observed, nor could any significant difference be detected histologically between tumors arising from frozen and unfrozen inocula.

Grafts of the three tumors under consideration were desiccated in a lyophilic apparatus while frozen. No growth was obtained on subcutaneous inoculation of frozen and dried material. After soaking the dried tissue in a buffered saline-Ringer solution or in the liquid fraction obtained from low-pressure distillation of the corresponding tumor, grafts failed to show evidence of growth.

Rat skin was treated similarly to control the results obtained. Loeb has shown that homologous subcutaneous transplants of rat skin form cysts frequently filled with desquamated keratinized epithelium (16). The original transplanted epidermal tissue usually rests on the cutis and shows evidence of proliferation of the squamous epithelial cells. These often spread laterally to cover the newly formed granulation tissue of the host. Hair, follicular epithelium, and sebaceous glands may be seen (Fig. 1).

Normal rat skin frozen rapidly to \(-50^\circ\text{C.}\) and immediately thawed produced visible subcutaneous nodules comparable to those formed by unfrozen
skin on homologous transplantation. Histologic evidence of proliferation was obtained when the grafts were excised ten days after implantation. The stratified squamous epithelium of the dermis was deficient, but small islands of recognizable epithelial cells could be found along the surface of the grafts. Some of the hair follicles were lined by epithelial cells, especially in the region of the root bud. Occasional mitotic figures occurred among groups of distinctly epithelial cells. The connective tissue appeared to be hyalinized but otherwise normal. Round-cell infiltration, foreign-body reaction, and fibro-

Fig. 2. Homologous Subcutaneous Transplant of Normal Rat Skin Frozen Slowly to
—74° C. and Immediately Thawed; Excised after Ten Days

The upper part of the picture shows the granulation tissue of the host. Between this and the skin, in the lower part of the picture, lies a cyst filled with débris. × 100.

blastic proliferation were present in the peripheral parts of the grafts. The connective tissue of the host showed little inflammatory reaction.

Less evidence of cellular damage was seen in the skin grafts that were frozen slowly. Characteristic cyst formation was observed (Fig. 2). The skin epithelium was typically squamous and was thicker than the normal skin. Hair had grown into the cysts. The hair follicles were lined by squamous cells. On the side of the cysts formed by the host, young granulation tissue containing foreign-body giant cells was present. In some instances this was
partially covered by stratified squamous epithelium. Again, the periphery of the graft was infiltrated with round cells and fibroblastic proliferation was noted.

Other pieces of skin were frozen rapidly to $-74^\circ C.$ and kept at that temperature for twenty-four hours. These grafts were removed from the subcutaneous tissue of the host ten days later. Some of them showed definite histologic evidence of growth. This was seen most frequently at the periphery of the grafts, where optimal conditions for growth exist. The central portion usually appeared hyalinized, the epithelial cells being faintly outlined and failing to take the nuclear stain. Karyorrhexis was seen in some of the cells. Of ten grafts, one showed definite cyst formation with well preserved squamous epithelium lining the cyst and extending into the hair follicles (Fig. 3). Mitotic figures were present among the cells. The connective tissue stained characteristically. The usual foreign-body and inflammatory reactions were present. Three other grafts showed definite patches of squamous cells on the skin surface and morphologically characteristic cells lined the hair follicles at the periphery of the graft.

**DISCUSSION**

The tumors that were studied showed a marked variation in their reaction to freezing. Not only is this true of the two histogenetic types but the indi-
individual neoplasms reacted differently to the same experimental environment on different occasions. Biological variation in the behavior of a tumor has long been recognized.

The results of a single freezing en masse suggest that sarcoma is more resistant to cold than is carcinoma and that the rate of freezing and the duration of the tumor in the frozen state up to twenty-four hours are not significant in the tumors studied. The Walker tumor survived a greater number of repeated freezings than did either of the sarcomata. Slowly induced repeated freezings were more injurious than rapid inductions of the minimum temperature.

That saline suspensions of cells were more readily inactivated than were tumors frozen en masse is not surprising. In this state the cold acts on smaller particles. Apparently the structural relationship of the cells comprising the tumor is of some importance in protecting the tissue from the deleterious effects of low temperatures. The carcinoma cells in suspension were more resistant than those of the sarcomata.

From a review of the available literature the impression is gained that neoplastic cells resist cold to a far greater degree than do normal mammalian cells. It is possible that different tissues show a distinct variation in their reaction to cold as Simonin noted (10). The skin was chosen for this study because its two principal components, stratified squamous epithelium and connective tissue, are concerned primarily with growth rather than with absorption and secretion; also because it presented few technical difficulties. No recorded observations have been found on the freezing of adult skin in vitro. That it may survive freezing appears to be a new concept. Studies on transplanted normal tissues appear to be more sensitive, within certain limits, than studies in the artificial environment of tissue culture (15, 17). The entire work on the effect of low temperatures on tissues in vitro should be repeated.

The mechanism by which cold damages animal cells is incompletely understood. The prevailing opinions have been reviewed recently by Haines (1) and Turner (3). Much of the experimental work has been done on plant cells. That purely mechanical destruction accounts for cell death by freezing is improbable. If this were the sole explanation, slowly induced freezing should have produced larger ice crystals and, consequently, a more deleterious effect than rapid freezing. The reactions involved are probably of a complex physico-chemical nature beyond the scope of this investigation.

Salvin-Moore was probably the first to propound the hypothesis that the tumors resulting from frozen material are due to the action of a virus contained in the transplanted substance on the cells of the host (11). Indeed, the Rous chicken sarcoma and several rabbit tumors of virus origin are capable of transmission by cell-free material after many repeated freezings and thawings. After demonstration of growth in normal rat epithelium following exposure to low temperature no reason remains for postulating the action of a virus on the basis of direct evidence. Cramer (17), and Breedis, Barnes, and Furth (14) arrived at the same conclusion from different approaches to the problem. This, however, has no bearing on the moot point concerning the possible relationship of viruses to transplantable tumors.
TRANSPLANTABLE MAMMALIAN TUMORS AND NORMAL RAT SKIN 509

SUMMARY

Walker rat carcinoma 256 and mouse sarcomata 180 and 37 grew on subcutaneous transplantation after exposure to $-74^\circ$ C. in vitro. The effects of the rate of freezing, duration of the frozen state up to twenty-four hours, the number of repeated freezings and thawings, and the physical state of the tumor are discussed.

The squamous epithelial and connective-tissue cells of normal adult rat skin may grow after a single freezing to $-74^\circ$ C.

BIBLIOGRAPHY