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Induction of Neoplasms in Salivary Gland Rudiments Recovered from Frozen Storage. (29185)

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Preservation of semen, cell cultures, and neoplasms in the frozen state is now widely practiced by application of well standardized procedures(1,2,3,4). The frozen preservation and storage of more highly organized biologic materials such as tissues, organs, or whole organisms has had more limited application, partly because of technical difficulties(1).

We recently found it possible to freeze and store the embryonic rudiments of mouse submandibular salivary glands by the same technique that is used to freeze and store cell cultures. It has, furthermore, been shown that such frozen rudiments are capable of responding qualitatively to infection with polyoma virus (PV) in the same way that fresh rudiments do; *i.e.*, neoplasms of charactertistic morphology and biologic behavior develop in the rudiments when they are thawed, infected with PV, and grafted to isologous newborn mice.

Procedures. Submandibular salivary gland rudiments were dissected from 13- and 14-day embryo mice of strain C3H/Bi. They were kept for 2 hours at room temperature in a medium of adult human serum (30%), Morgan, Morton, and Parker's mixture 199 (65%), glycerol (5%), penicillin in concentration of 100 U/ml, and phenol red in final concentration of 0.001%.

Dishes containing rudiments in the medium were kept in an atmosphere of 95% air and 5% CO_2 to maintain a pH 7.0 to 7.4. After 2 hours, 6 to 12 rudiments in 1.0 ml of medium were transferred by capillary pipette into individual ampoules. The ampoules were sealed and placed in a refrigerator and cooled to 3 to 5°C. They were then slowly frozen under alcohol at a temperature decline rate of one degree per minute or less by placing in a vermiculite-insulated double-walled container immersed in 95% ethyl alcohol and CO_2 ice, pre-cooled to the starting temperature of 4°C. When a temperature below —20°C was achieved, the frozen ampoules were rapidly transferred to canes and placed immediately in a liquid nitrogen refrigerator, above the liquid level, at a temperature of approximately —150°C.

At intervals varying upwards from one hour after placing in the N_2 refrigerator, ampoules were withdrawn and rapidly thawed by agitating in a running water bath held at 38°C.

Rudiments were removed from the glycerol medium, washed once in 1.0 ml of similar medium lacking glycerol, and then transferred to a dish of virus suspension or to an equal volume of virus-free control medium. They were held then at room temperature in a 95% air-5% CO_2 atmosphere for 2 hours, and their condition was evaluated by: (1) histologic examination of serial sections; (2) observation of development in culture; (3) transfer (with and without PV) to subcutaneous tissue of new-born C3H/Bi mice.

Results. Histologically the rudiments showed little alteration as a result of the freezing and thawing procedure. The basic form and the relationships between epithelium and the capsular mesenchyme were retained, but in some preparations it was noted

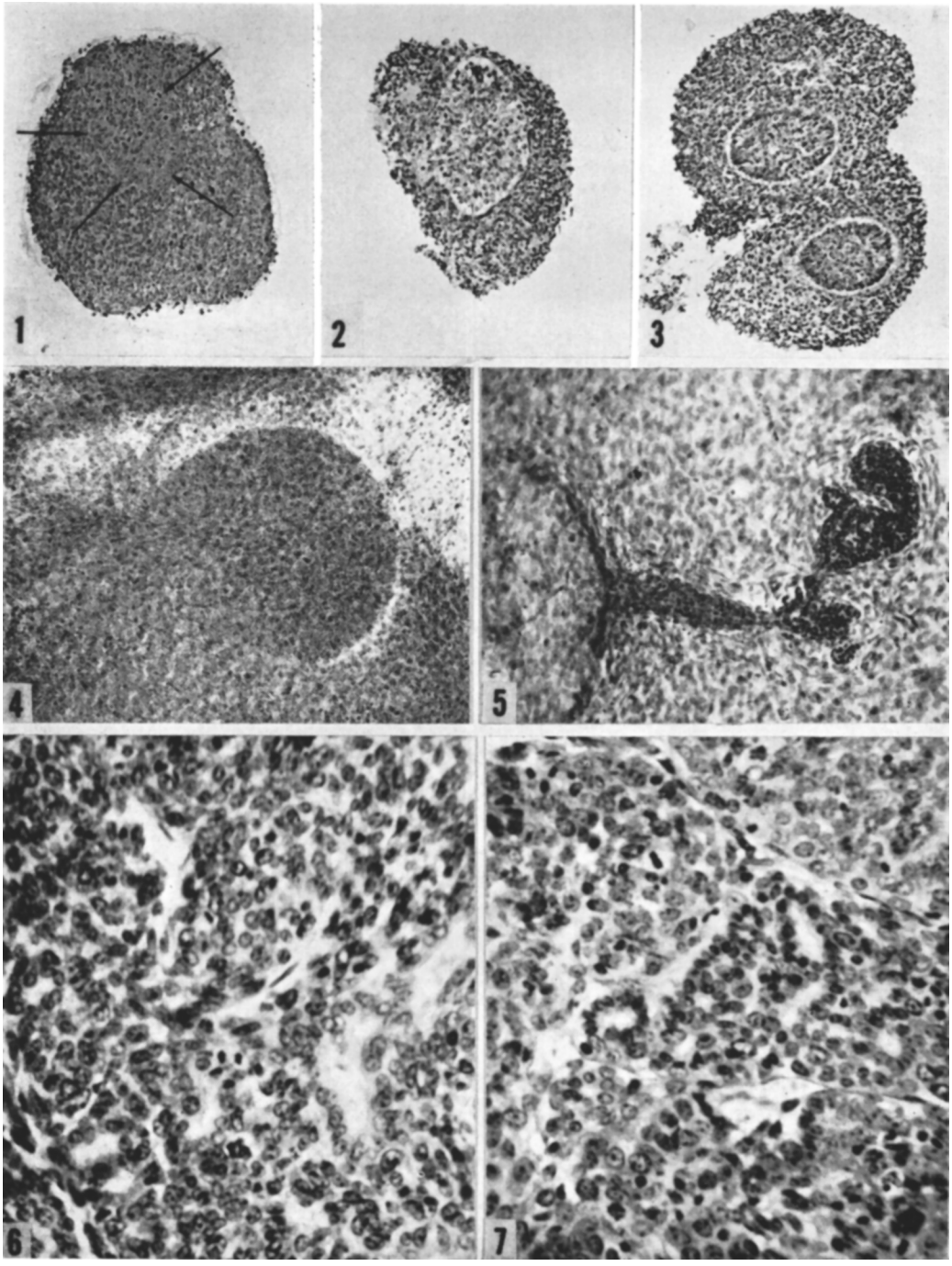

FIG. 1. Section of a freshly excised submandibular rudiment from a 13-day mouse embryo. Arrows indicate junction of epithelium (central) and mesenchyme (peripheral). H & E ×125.

FIG. 2 and 3. Sections of submandibular rudiments from 13-day mouse embryos, immediately after recovery from frozen state in glycerine medium. The rudiment in Fig. 2 shows "hydropic" appearance of epithelium and a more obvious demarcation between epithelium and mesenchyme than is present in Fig. 1. The 2 rudiments in Fig. 3 show still greater degree of separation of epithelium from mesenchyme. H & E ×125.

FIG. 4. Stained whole-mount of a submandibular rudiment from a 13-day mouse embryo.

that the line of demarcation between epithelium and mesenchyme was more evident than in the unfrozen controls (Fig. 1, 2, and 3). There was a tendency in the frozen rudiments for a slight separation to occur between epithelium and mesenchyme. In unfrozen rudiments, the two types of cellular components were more tightly apposed to one another, with interposition only of a very delicate layer of PAS-positive material. A second difference between frozen and unfrozen rudiments was a nearly complete loss of intact erythrocytes, both nucleated and nonnucleated, from the blood vessels.

Organ cultures on the surface of chicken plasma clot confirmed the existence of the differences noted histologically. In some rudiments, the separation of mesenchyme from epithelium was exaggerated in cultures to the point where a crescent-shaped gap of as much as 50 μ width developed between the epithelium and the capsular mesenchyme. In such examples, morphogenesis of the gland was either markedly retarded or completely halted. Where the separation of epithelium and mesenchyme was less marked, apposition of the 2 components was reestablished and morphogenesis proceeded, although not so rapidly and not to the same extent ultimately reached by unfrozen rudiments (Fig. 4 and 5). These observations are consistent with Grobstein's findings in experiments demonstrating the dependence of morphogenesis on maintenance of close epithelio-mesenchymal relationships(5).

In cultures it was also evident that some cell death resulted from freezing. This was apparent from small collections of cell debris that were shed from the peripheries of rudiments during the first day or two of culture. However, the viability of the majority of both the epithelial and the mesenchymal components became evident with passage of time as cell outgrowth and morphogenesis progressed. Macrophages appeared highly sensitive to the freezing procedure, being extremely reduced in number in cultures of frozen rudiments, but abundant in unfrozen controls.

Preliminary data from this laboratory have shown that maintenance of epithelio-mesenchymal relationships is necessary for tumor genesis to occur in mouse submandibular gland rudiments infected with polyoma virus, either in organ culture or in transplanted rudiments *in vivo*(6,7). Therefore, a functional test of the viability and integrity of both epithelial and mesenchymal components consists of infecting the rudiments recovered from the frozen state with polyoma virus, and observing these rudiments for development of tumors after transplanting them to isologous hosts.

Table I shows that when this was done, salivary gland tumors developed in 8 of 10 transplanted sets of rudiments (total of 20 rudiments) recovered from the frozen state. In comparison, tumors developed in 10 of 12 transplanted sets of rudiments that had not been frozen, and were infected with the same dose of virus. Uninfected controls of both types failed to produce tumors in any instance. From these data it is evident that freezing the rudiments does not appreciably impair their ability to respond to polyoma virus oncogenically. Further, histologic examination of the neoplasms that evolved from frozen rudiments showed that they were morphologically identical with those arising either from unfrozen rudiments or from salivary

The rudiment has been flattened somewhat by pressure from the coverslip, causing mesenchyme to break away and reveal the circular margin of the epithelial bud. H & E ×125.

FIG. 5. Stained whole-mount of a 10-day organ culture started from a rudiment like that shown in Fig. 4. Rudiment had been recovered from frozen state in glycerine-containing medium. Morphogenesis has occurred, with formation of a main duct, secondary and tertiary branches. Asymmetry and the limited degree of adenomere development reflect some damage. H & E ×125.

FIG. 6. Section of neoplasm that originated in a polyoma virus-infected fresh submandibular rudiment, transplanted subcutaneously, to a newborn host of the same strain. H & E ×340.

FIG. 7. Section of neoplasm that originated in a submandibular rudiment treated exactly as that which gave rise to neoplasm shown in Fig. 6, except that it was frozen and stored at −150°C for 20 hr before infection with polyoma virus. The histology of the neoplasm arising from frozen and unfrozen rudiments is identical. H & E ×340.

TABLE I. Effects of Frozen Storage on Survival of and Response to Polyoma Virus by Submandibular Gland Rudiments.

Method of evaluation	No. of rudiments		Time at −150°C	Result	
	Frozen	Fresh		Frozen	Fresh
Microscopy	27	10	2 hr	See text	See text
Organ culture	68	24	1 hr to 19 days	Survival++ Devel+	Survival+++ Devel++
Transplant + PV	22 (10 recipient mice)	21 (12 recipient mice)	20 hr to 19 days	Tumors in 8/10 transplants	Tumors in 10/12 transplants
Transplant − PV	38 (19 recipient mice)	16 (8 recipient mice)	15 min* to 19 days	Tumors in 0/19 transplants	Tumors in 0/8 transplants
Total	155	71			

* Eight of the 38 frozen rudiments in this group were held at −150°C for only 15 minutes before grafting. The other 30 were held at −150°C for 1 hour to 19 days.

glands undisturbed in their native hosts (Fig. 6 and 7).

The ability of salivary gland rudiments to survive the frozen storage procedure and retain their potential for oncogenic response should prove advantageous for several reasons. It has been noted that some cells (macrophages and red cells) are to a considerable degree selectively destroyed by the procedure. This makes it possible to assess more closely the possible parts played in oncogenesis by the several types of mesenchymal cells present in the rudiments. Because the frozen-thawed rudiments have already been shown capable of producing tumors in response to PV, it seems improbable that autologous macrophages play a significant part in PV oncogenesis in this organ, unless only a few surviving cells are adequate. It cannot be excluded, of course, that macrophages might migrate in from the secondary host and replace those killed by freezing.

Secondly, the method makes it possible to determine the response to PV by rudiments at less advanced stages of development than has heretofore been possible. This is so because one of the effects of freezing, as noted, is to retard the rate and diminish the final degree of development reached by rudiments. Without this suppressive effect, even the earliest rudiments develop to a fairly advanced stage by the time PV infection becomes generalized in the components of the rudiments. Judging from the results of these experiments, a rather limited degree of development is sufficient to render the rudiments capable of a neoplastic response to PV that is identical with that shown by fully-developed glands.

For certain types of experiments, storage of rudiments in the frozen state would have the advantage of making it possible to accumulate large numbers of them and thus to eliminate technical variables that occur in multiple runs. However, as indicated above, rudiments recovered from the frozen state would not be strictly equivalent to fresh rudiments for all experimental purposes, because freezing does cause some cell damage which is moreover of a selective sort and which results in some impairment of development. Improvements in technique may ultimately remove this restriction.

Summary. Viability of submandibular salivary gland rudiments of mice was demonstrated after slow-freezing and storing in a 5% glycerol medium at —150°C. Development of the rudiments after thawing was less rapid and less extensive than with fresh rudiments, but the capacity of the organ to develop morphologically characteristic neoplasms in response to polyoma virus was not appreciably impaired.

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Cyst Formation in the Parathyroids and Muscular Dystrophy Induced by Dihydrotachysterol and Calcium Acetate.* (29186)

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The formation of cavities in the parathyroids has frequently been noted as an accidental finding in man and other vertebrates. Its many variants have been described under such names as "cystic degeneration," "colloid formation," "follicular transformation," "vesicle formation," etc. On purely theoretic grounds, this change has been variously ascribed to degeneration, increased secretion, hormone storage, invasion of thyroid tissue into the parathyroid, or malformation of the bronchial-cleft system(1-3). However, the causative factors could not be analyzed experimentally since it has never been possible to reproduce this singular lesion by any type of intervention. There is a voluminous literature on induction of simple parathyroid hypertrophy, hyperplasia or atrophy by variations in dietary intake of calcium, phosphorus and vitamin-D compounds(4-11), but no studies have apparently been made on the morphology of the parathyroids following simultaneous overdosage with vitamin-D derivatives and calcium compounds. It was of some interest, therefore, when recently we observed, during experiments on calciphylaxis, that rats concurrently treated with dihydrotachysterol (DHT) and calcium acetate regularly develop cystic parathyroids and a singular form of muscular dystrophy.

Experimental. Forty female Sprague-Dawley rats of the Holtzman strain, with a mean initial body weight of 98 g (range 95-103 g) were subdivided into 4 equal groups: Group 1 acted as untreated controls. Group 2 received 30 μg of dihydrotachysterol "DHT" (Dr. A. Wander S. A., Bern, Switzerland) in 0.5 ml corn oil by stomach tube, once daily. Group 3 was given 1 mM of calcium acetate in 2 ml of water, also by stomach tube, twice daily. Group 4 received both DHT and calcium acetate as Groups 2 and 3 respectively.

The animals were kept exclusively on Purina Lab Chow (Purina Co. of Canada) and tap water. The experiment was terminated on the sixteenth day by killing the survivors with chloroform. Specimens of the thyroparathyroid apparatus and of the quadriceps muscle were fixed in alcohol-formol (1 part of 10% neutral formalin, 4 parts of 80% alcohol) for subsequent embedding in paraffin and staining with von Kóssa and PAS techniques.

Results. No mortality or clinical evidence of motor impairment occurred among the animals of the first 3 groups, but by the tenth day those given DHT + calcium acetate began to exhibit signs of muscular weakness as judged by their hesitating gait. Subsequently, mortality began and 80% of the animals were lost in this group by the sixteenth day when the experiment was terminated. At autopsy, all the rats of Group 4 which succumbed, as well as those that were killed at the end of the experiment, exhibited slightly enlarged parathyroids and widespread, light patches throughout the skeletal musculature. No such anatomic changes were observed in any of the other groups. Combined treatment with DHT + calcium acetate also led to severe cardiac and renal calcification but minor degrees of the same

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