Successful Growth of Tumor Allografts Following Explantation: Effect of Various Culture Conditions* (32844)

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A variety of murine tumors grow progressively or for prolonged periods of time when reimplanted into allogeneic recipients following short-term maintenance as organ culture explants in synthetic medium (1). These allografts retain their morphologic and functional characteristics in their foreign hosts. Return of the tumor to the strain of origin even after 20 serial passages in the foreign strain abrogates the ability of the tumor to grow as an allograft.

This paper describes various parameters of culture conditions in which this change has occurred including variations in media, incubation temperature, atmosphere, and length of time in culture, and discusses other conditions under which altered cell physiology may have induced changes in tumor specificity.

Methods. The transplantable BALB/c (C) testicular interstitial (Leydig) cell tumor C 4092 was used in these studies. The tumors were removed aseptically to medium 199, minced with scissors and transferred to fresh medium before being explanted onto agar supported by wire grid tables. The explants were incubated at 32°C in 5% CO_2 in air in medium 199 to which 50 units of penicillin–streptomycin mixture were added, unless otherwise noted. The time elapsing between the removal of the tumor from its host and placing the explanted fragments into the incubator was less than 20 min. The medium was changed every 4 days. The tumors were reimplanted subcutaneously through a small incision on the left side of the animal. DBA/1 (DBA) mice were used as the allogeneic recipients. Transplants of this tumor which have never been through culture do not grow in DBA recipients. This and further details on the general methodology and animal strains have been described in a previous publication (1).

Results. 1. Effect of temperature. Routinely, explants are incubated at 32° since many of the tumors we are studying originated from testicular tissue which is better maintained at lower than internal body temperature. In order to eliminate any possibility that maintenance at this temperature might have contributed to the changes we have described, duplicate cultures were set up at both 32° and 37° for 11 days. Tumors were reimplanted into 3 each DBA and C mice after incubation at each temperature. All C mice developed tumors and 2/3 and 1/3 DBA mice developed tumors after incubation at 32° and 37°, respectively. We therefore conclude that incubation temperature does not play a major contributory role in production of this phenomenon.

2. Effect of incubation atmosphere. Our observations originally had been made on tissues incubated in 5% CO_2 in air. Since 5% CO_2 in O_2 also is commonly employed in cell and organ culture studies, we explored the effect of this variable by setting up duplicate cultures in both gas mixtures, and after 12 days reimplanting the tissues from each incubation atmosphere into 6 each DBA and C recipients. All C and 1/6 DBA recipients from each group developed tumors. Therefore, it is apparent that the alteration occurs in both of the gas mixtures employed.

3. Effect of serum addition to the medium. Since 10% fetal calf serum commonly is added to various synthetic culture medium, we set up duplicate cultures with and without this additive to ascertain if the addition of serum might prevent the change. The data presented in Table I show that the change is not prevented by the addition of 10% fetal calf serum.

4. Effect of antibiotics. Routinely, we have added penicillin–streptomycin mixture to the culture media. To eliminate the possible influence of antibiotics on the alteration in

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TABLE I. Growth of Tumor C 4092 in Syngeneic and Allogeneic Recipients After Maintenance in Media with and without Serum Supplement.

	No. which grew/no. of recipients			
	Medium 199 alone		Medium 199 + serum	
Incubation period (days):	6	12	6	12
DBA/1	3/5	2/6	2/6	5/6
BALB/c	6/6	6/6	6/6	4/6

TABLE II. Growth of Tumor C 4092 in Syngeneic and Allogeneic Recipients Following 11 or 12 Days Maintenance in Hanks' Balanced Salt Solution and Waymouth Medium with and without the Addition of Antibiotics.

	No. which grew/no. of recipients					
	No antibiotic			Antibiotic added		
	Medium 199	Hanks' solution	Waymouth medium	Medium 199	Hanks' solution	Waymouth medium
Incubation period (days):	11	12	12	11	12	12
DBA/1	1/6	1/3	2/3	5/6	1/3	2/3
BALB/c	5/6	2/3	2/2	6/6	2/3	2/3

antigenicity, we set up 3 separate explant experiments under duplicate conditions except for the omission of penicillin–streptomycin from one group of explants in each experiment. The first experiment was terminated after 6-days incubation due to contamination of the cultures from which antibiotic had been omitted. Although the 6-day explants grew when reimplanted in the C recipients, they failed to grow in the DBA recipients. In the other 2 experiments, we obtained growth of the tumor in DBA mice whether or not the antibiotics were omitted (Table II). We therefore conclude that penicillin–streptomycin addition to the medium is not a major factor contributing to the inhibition of the antigenic expression of these explants.

5. Effect of length of time in vitro *and of the medium employed.* Having eliminated the possible effects of temperature, atmosphere, antibiotics, and serum additive, it still was possible that nutrition might play a role in producing the phenomenon. Therefore, we explored the effects of other media and varying the length of time in culture.

Triplicate cultures of the tumor C 4092 were set up in medium 199, Waymouth Medium 752 and Hanks' Balanced Salt Solution. The explants were maintained for 1, 2, and 6 days before being reimplanted into C and DBA recipients. The growth of the tumor in 105 of the 108 C recipients indicates that all of these media are adequate for maintaining sufficient cells to regrow in the syngeneic strain. However, Table III shows that significantly more DBA mice developed tumors after 6 days in Hanks' Solution and Waymouth medium than after 1 or 2 days ($p < 0.01$), and that significantly more developed

TABLE III. Growth of Tumor C 4092 After 1, 2, and 6 Day Maintenance in 3 Different Media and Reimplantation into DBA/1 Recipients.

	No. which grew/no. of recipients			
	Incubation period (days)			
Medium	1	2	6	Total
Hanks' Bal. Salt Soln.	2/12	5/12	8/12	15/36
Waymouth Med. 752	6/12	2/12	11/12	19/36
Med. 199	1/12	0/12	4/12	5/36
Total	9/36	7/36	23/36	

tumors after incubation in Hanks' or Waymouth medium than after incubation in medium 199 ($p<0.01$).

We therefore conclude that increasing the length of time in culture increases the frequency of growth of this tumor in allogeneic mice, and that the frequency also increases after maintenance in Hanks' and Waymouth media as compared to that in medium 199.

Discussion. Maintenance of antigenicity of cell cultures even after very long periods *in vitro* has been reported by the majority of investigators [see (2)]. However, most of these reports deal with *in vitro* identification of antigen rather than with the effect of reimplanting the tissues into allogeneic recipients. Furthermore, Franks has observed modulation of the presence of surface antigens on cells derived from clonal isolations (3). Both Rabotti *et al.* (4), and Foley *et al.* (5) have reimplanated long-term cell cultures of tumors into allogeneic hosts and noted that a loss of strain specificity had occurred. Long-term *in vitro* maintenance does not appear to be a prerequisite for this alteration since both we and Kaliss (6) have observed the change after only 24 hours *in vitro*. Furthermore, Morgan *et al.* have reported that an ascites lymphosarcoma and an ascites mammary carcinoma that had been preserved by freezing were able to grow as allografts (7).

Passage of tumors in chick chorioallantoic membrane (8) through fetal or newborn animals (9) or through syngeneic radiation chimeras (10), have been effective in producing a change in the cells which allows them to grow in foreign recipients. The relationship of these changes to those occurring *in vitro* and to the phenomenon of immunologic enhancement has not been investigated. It is possible that these various phenomena may be related to each other and to enhancement if one considers that all of these methods include the exposure of the cells to abnormal physiologic conditions which conceivably might alter or reduce their antigen content and/or availability. In this case it is possible that the antigens would evoke mostly humoral antibodies and that these might then combine with and protect the cells from cell bound antibodies which would be produced as the tumor continued to grow. Möhler and Möhler have proposed such a mechanism ("self-enhancement") to explain the growth of allografts in cases where specific antigens are known to be present (11).

Although this concept might explain allogeneic takes of cells exposed to nonphysiologic environments it gives no insight into the basic change which has occurred. In many of the cases the change is phenotypic, since the ability to grow in the foreign host may be reversed, sometimes by only a single passage through the strain of origin (1, 10, 12). This certainly would argue against a selective mechanism in the induction of the change, although selection might occur secondarily in some cases as the tumors are serially passaged in the allogeneic strain.

Summary. Alteration in the antigenicity of a Leydig cell tumor following short-term maintenance in organ culture and manifested by progressive growth of the tumor in allogeneic hosts occurs following incubation in media independent of the following factors: (i) presence or absence of antibiotics in the medium, (ii) incubation at 32 or 37°C, (iii) incubation in 5% CO_2 in air or in oxygen, and (iv) presence or absence of 10% fetal calf serum in the medium. The frequency of successful allografts is greater in tumors explanted for 6 days than for those cultured for 1 or 2 days. The alteration occurred more frequently after maintenance in Hanks' Balanced Salt Solution and Waymouth Medium than it did after maintenance in medium 199. The occurrence of *in vitro*-induced alteration in tumor specificity is discussed in relation to other conditions known to alter immunologic specificity and the possible relationship of these to the phenomenon of immunologic enhancement is suggested.

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Uridine Diphosphate Galactose Pyrophosphorylase from Calf Liver*† (32845)

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That nucleoside diphosphate sugars represent a metabolically important class of compounds is now well documented (1). Their importance lies not only in their intermediacy in monosaccharide formation, but also in their role in glycosyl transfer reaction. The pyrophosphorylase catalyzed synthesis of uridine diphosphate glucose (UDP-Glc) from uridine triphosphate (UTP) and glucose-1-phosphate (Glc-1-P) is of primary significance.

$$\text{UTP} + \text{Glc-1-P} \underset{}{\overset{\text{pyrophosphorylase}}{\rightleftharpoons}} \text{UDP-Glc} + \text{PP}_i \quad (1)$$

With crude extracts of mammalian tissues, a number of nucleoside diphosphate sugars (NPD-sugars) have been biosynthetically prepared, many of them novel, from the appropriate nucleoside triphosphate and the sugar-1-phosphate (2,3). These NDP-sugars have been prepared where the nucleoside is adenosine, guanosine, inosine, uridine, cytidine, or thymidine and the sugar is glucose, galactose, mannose, or xylose. From calf liver extracts it has been possible to identify and purify about 500-fold a guanosine diphosphate hexose pyrophosphorylase (4) and crystallize a UDP-Glc pyrophosphorylase (5). Neither enzyme is highly specific for substrate at this stage of purification.

Isselbacher (6) has implicated a UDP-galactose (UDP-gal) pyrophosphorylase from rat and bovine liver in an accessory pathway of galactose (Gal) metabolism as follows:

$$\text{Gal} + \text{ATP} \xrightarrow{\text{kinase}} \text{Gal-1-P} + \text{ADP}, \quad (2)$$

$$\text{Gal-1-P} + \text{UTP} \underset{}{\overset{\text{pyrophosphorylase}}{\rightleftharpoons}} \text{UDP-Gal} + \text{PP}_i, \quad (3)$$

$$\text{UDP-Gal} \underset{}{\overset{\text{epimerase}}{\rightleftharpoons}} \text{UDP-Glc}, \quad (4)$$

$$\text{UDP-Glc} + \text{PP}_i \underset{}{\overset{\text{pyrophosphorylase}}{\rightleftharpoons}} \text{UTP} + \text{Glc-1-P}, \quad (5)$$

Sum of Eqs. (3, 4, and 5) $\quad \text{Gal-1-P} \rightleftharpoons \text{Glc-1-P}$

Since the catalyst for Reaction (5) was obtained in cystalline form (5) and the epimerase for Reaction (4) is widely distributed in nature and well described, it became of interest to attempt to characterize the UDP-Gal pyrophosphorylase. Consequently, it appears that in calf liver all activity for UDP-Gal is inseparably associated with the UDP-Glc pyrophosphorylase. The evidence for the common identity of these enzymes together with some considerations of the specificity are the subject of this report.

Procedures. The chemicals and other materials have been described in detail elsewhere (5,7). For estimating enzyme activity in the direction of pyrophosphorolysis, the formation of Glc-1-P (8) or UTP (9) were measured. The chromatographic procedures for identifying the various substrates have been described (10). The extraction of calf liver and the detailed purification and crystallization procedures for UDP-Glc pyrophosphorylase are reported elsewhere (5).

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