Basis for the Acquisition of Malignant Potential by Mouse Cells Cultivated in vitro

Abstract. *Balb/c mouse embryo lines maintained in culture for over 200 generations under conditions that minimize cell-cell contact do not become tumorigenic. Lines cultivated under conditions where there is extensive cell contact become tumor-producing within 30 generations. The tissue-culture property that correlates best with tumorigenicity is the loss of contact inhibition of cell division.*

The mouse cell line 3T3 is one that has been used extensively for studies of cell growth and viral transformation. It was originally derived from random-bred Swiss mouse embryo cultures (*1*) and therefore cannot be used to correlate directly the properties observed in culture with the tumor-producing capacity of the cells. Recently lines with properties that are virtually identical to those of 3T3 have been developed from inbred Balb/c mouse embryo cultures (*2*). Like the original 3T3, they are continuous, aneuploid cells that are extremely sensitive to contact inhibition of cell division (*3*, *4*) and grow to a very low saturation density (*5*). Lines that have acquired the ability to grow well at high cell density and are no longer sensitive to contact inhibition of cell division have also been derived from the same original pool of mouse embryo cells.

The purpose of the present experiments was to determine which in vitro properties are associated with tumorigenicity in the animal. The results show that lines that grow efficiently in the presence of extensive cell-cell contact readily produce tumors; those lines where cell division is arrested by neighboring cells in tissue culture are also unable to grow in the animal.

The cell lines used were all derived from a single pool of 14- to 17-day-old Balb/c mouse embryos. Briefly, by maintaining a schedule of cell transfer that minimizes cell-cell contact, lines have been developed that remain very sensitive to contact inhibition of cell division (Balb/3T3). From the same embryo cultures, Balb/3T12 lines have been established by using a transfer schedule in which cell-cell contact was extensive, thus selecting for cells better able to grow under crowded culture conditions.

A method which selects more rapidly for contact-insensitive cells in a heterogeneous population takes advantage of the ability of such cells to grow when inoculated onto monolayers of contact-inhibited cells such as 3T3 and Balb/3T3 (*2*). Balb/3T12 cells obtained by this method grew to very high saturation densities. Balb/3T3 and Balb/3T12 lines transformed by simian virus 40 (SV40) were obtained by infecting log phase cultures with a small plaque mutant of SV40 (*6*) and isolating clones by using the appropriate selective system (*2*). Dulbecco's modification of Eagle's medium supplemented with 10 percent calf serum (Colorado Serum Co.) was used in all experiments.

Both newborn and weanling Balb/c mice, the latter having been irradiated with 300 rads, were inoculated subcutaneously in the interscapular region. Rapidly dividing, subconfluent cultures of the lines to be tested were trypsinized, centrifuged at low speed, and resuspended in complete medium. Cell counts were performed in duplicate, and aliquots containing 10^5, 10^6, and 10^7 cells were injected. Animals were observed at weekly intervals for the appearance of progressively growing subcutaneous tumors at the site of inoculation. Though nodules of 1 to 2 mm could be detected, tumors were scored as positive only when they reached a size of 5 mm. The great majority of tumors, once having reached this size, continued to grow and eventually killed the animals.

The in vitro growth characteristics of the various cell lines tested included doubling time, cloning efficiency, saturation density, and colony-forming ability on confluent monolayers of contact-inhibited cells. Doubling times for all cultures used in the present experiments were 18 to 22 hours. By maintenance of cultures in such a way as to limit cell contact, the saturation density of Balb/3T3 cells has remained at 5×10^4 cells/cm^2 over a period of 10 months and through more than 200 cell generations since it was established. Balb/3T12 mass cultures after 30 cell generations in culture had saturation densities of 1.5 to 2.0 $\times$ 10^5 cells/cm^2. When less contact-inhibited clones were selected by growth on 3T3 monolayers, saturation densities of from 5 to 12 $\times$ 10^5 cells/cm^2 were obtained. Balb/3T12 sublines selected for their colony-forming ability on 3T3 monolayers were found to be much more efficient at forming colonies when reinoculated onto 3T3 monolayers.

The contact-sensitive Balb/3T3 line and the contact-insensitive Balb/3T12 lines were inoculated into animals after 30, 100, and 200 generations in culture. Table 1 shows that the inoculation of up to 10^7 Balb/3T3 cells per animal does not produce tumors. After 6 months and 100 cell generations in culture, the Balb/3T3 cells were found

Table 1. Tumor production by Balb/3T3 and Balb/3T12 cells after varying times in culture. NT, not tested.

Cell line	Time in culture (mo.)	Cell generations in culture*	Colony-forming ability (%)†		Saturation density ‡ (cells/cm^2, $\times$ 10^{-5})	No. of tumors/No. of animals §	
			Alone	On 3T3 monolayer		10^6 cells	10^7 cells
			Experiment 1				
Balb/3T3	3	30	30–50	0.0	0.5	NT	0/3
Balb/3T12	3	30	0.02–0.04	0.5–2	1.5–2.0	NT	4/6
			Experiment 2				
Balb/3T3	6	100	30–50	0.0	0.5	0/18	0/8
Balb/3T12	6	100	0.1–0.2	40–80	5.0–7.5	17/40	25/28
			Experiment 3				
Balb/3T3	9	200	30–50	0.0	0.5	0/8	0/8
Balb/3T12	9	200	5–10	80–100	10–12.5	8/10	10/10

* $\log_2$ of cumulative increment in cell number (see *1*). † The range of values obtained from two or more experiments where 10^2, 10^3, and 10^4 cells were inoculated both onto bare plastic and onto 3T3 monolayers. The medium was changed twice weekly and the colonies were counted at 14 days. ‡ The maximum cell number attained when 5×10^3 cells/cm^2 were inoculated onto 20-cm^2 petri dishes under conditions where the medium containing 10 percent calf serum was changed every 3 days. The saturation density was taken as that value where three successive cell counts at 2-day intervals showed no increase in cell number. § Observation period: experiment 1, 11 months; experiment 2, 8 months; experiment 3, 5 months.

to have a high colony-forming ability when inoculated onto the bare surface of a petri dish but no ability to form colonies when inoculated onto a confluent monolayer of 3T3 cells. The Balb/3T12 line had a poor colony-forming ability by itself but formed progressively growing colonies with high efficiency on monolayers of contact-inhibited cells. While no animals inoculated with Balb/3T3 cells produced tumors, 17 of 40 animals developed tumors with 10^6 Balb/3T12 cells. That the number of generations in culture need not be related to oncogenicity is shown by the fact that Balb/3T12 after only 30 cell generations was tumorigenic, while Balb/3T3 has not produced tumors even after 200 generations in culture.

The dose response in animals for a number of lines, including SV40-transformed Balb/3T3 and Balb/3T12 lines, is shown in Table 2. Transformation of Balb/3T3 by SV40 conferred on the cell the ability to produce tumors in one-half of the animals when 10^7 cells were used. In all of the animals there was progressive growth of nodules during the first 2 weeks. In four of the six animals these eventually regressed. In one, a tumor had grown even larger than 5 mm in diameter before regressing. At a higher cell inoculation (2×10^7 cells per animal) five out of nine animals developed progressively growing tumors which eventually killed them.

Balb/3T12 sublines, selected for their colony-forming ability on 3T3 monolayers, produced tumors in eight out of ten animals within 3 months after inoculation of 10^6 cells. An SV40-transformed Balb/3T12 line that had been through a comparable number of generations in culture and had a similar saturation density and cloning efficiency on 3T3 monolayers when compared to Balb/3T12 was found to be roughly two logs *less* efficient in tumor induction than the non-SV40-transformed tumor cells. As was seen with SV-Balb/3T3, the animals inoculated with 10^6 and 10^7 SV-Balb/3T12 cells all developed small nodules, most of which eventually regressed.

On the basis of the data presented, it appears that the tumor-producing potential of a given cell line correlates most closely with its saturation density (that is, its ability to continue growing in the presence of extensive cell-cell contact). This can be seen in Fig. 1,

Table 2. Tumor production by mouse cell lines in irradiated weanling Balb/c mice.

Cell line*	Tumor incidence † with various cell doses ‡		
	5	6	7
Balb/3T3	0/10	0/8	0/8
Balb/3T12	2/10	8/10	10/10
SV-Balb/3T3	0/10	0/9	3/6
SV-Balb/3T12	0/10	0/9	3/5
T-Balb/3T12	10/10	4/4	

* Except for T-Balb/3T12, which is a tumor cell line derived from mass culture 3T12, all lines had been through approximately an equal number of cell generations in culture (200) at the time of inoculation into animals. † Number of animals with tumors of 0.5 mm or greater per number of animals inoculated. Observation period, 3 months. ‡ The values (namely, 5, 6, 7) are $\log_{10}$ of the cell dose.

where the latent period for 50 percent tumor incidence is plotted against the saturation density for a series of Balb/3T12 cell lines. The latent period decreased from 6 months to 1 month as the saturation density rose from 1.5×10^5 cells/cm^2 to 12×10^5 cells/cm^2. The line with the highest saturation density, an SV40-transformed Balb/3T3 line that had been selected for its high plating efficiency on 3T3 monolayers, produced tumors in 50 percent of the animals within 2 weeks. The dashed line in Fig. 1 represents the saturation density of Balb/3T3. Though over 50 animals have been inoculated, no tumors have developed.

While more tumorigenic variants can be selected from a mixed population, as for example by growth on a contact-inhibited monolayer, the acquisition of the property of transplantability can be

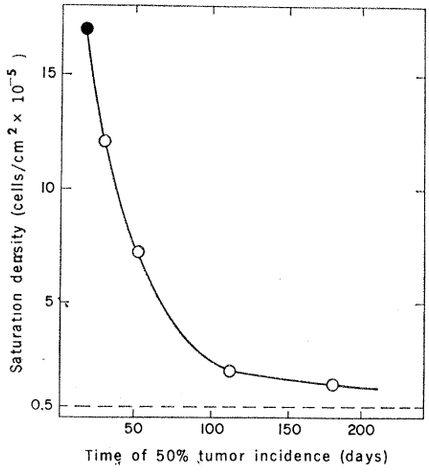

Fig. 1. Relationship between saturation density and tumor-forming ability. 10^7 cells were injected. ○, Balb/3T12 cells. ●, SV-Balb/3T3 cells. Dashed line represents saturation density of Balb/3T3 cells.

brought about directly by infection with a tumor virus such as SV40. Balb/3T3, a nontumorigenic mouse cell line, when transformed by SV40, loses contact inhibition of cell division, attains a high saturation density, and becomes malignant. The SV40-transformed cells initially grow well in vivo but are frequently found to regress. This failure of progressive tumor growth as well as the decrease in malignancy of SV-Balb/3T12 cells is most likely due to the increased antigenicity of the SV40-transformed cells. This may explain the apparent paradox between the inability of SV40 to produce tumors when injected directly in mice (*7*) and the high efficiency of SV40 transformation of mouse cells in tissue culture (*8*).

Although cells with increased saturation density and increased tumorigenicity are generally selected for by the standard procedures in tissue culture, the 3T3 transfer schedule avoids or at least minimizes this selective pressure. This regimen leads to continuous, aneuploid mouse cell lines that have not, even after hundreds of generations in culture, acquired detectable tumorigenicity. Selective conditions can also be employed to decrease the degree of malignancy of an already transplantable cell line. Pollock *et al.* (*9*) have shown that variants with reduced tumorigenicity can be obtained by using 5-fluorodeoxyuridine to select against cells that continue to divide in the presence of cell-cell contact. The less malignant variants are also found to have both a lower saturation density and a decreased plating efficiency on 3T3 monolayers (*9*).

Many long-established lines of spontaneously transformed mouse cells have been shown to contain viruses that are members of the mouse leukemia-sarcoma complex (*10*), while, on the other hand, primary cultures of Balb/c mouse embryo cells do not appear to contain these viruses (*11*). Kindig and Kirsten (*12*) have reported that 3T3 is unusual among permanent mouse lines in that it is apparently free of virus-like particles. Various Balb/3T3 and SV40-transformed Balb/3T3 lines and sublines are all, like 3T3, negative for mouse leukemia virus by complement-fixation (*11*). One subline of Balb/3T12, while negative at 30 generations, has been found to release mouse leukemia virus when tested after 100 cell generations. The relationship, if any, between the appearance of virus, the selection

of non-contact-inhibited cells, and the acquisition of transplantability in this line needs further study.

That there exists a strong correlation between the ability of cells to continue dividing in the presence of extensive cell-cell contact in vitro and the ability of the same cells to produce tumors in the animal has been demonstrated above. The nature of the cellular changes involved are as yet not understood. They may include, for example, surface alterations that decrease the requirement for adhesion to the plastic substrate (*13*), the loss of ability to produce and/or respond to short-range inhibitory molecules (*14*), or a decreased dependence under conditions of cell crowding for a factor present in normal serum (*3*, *15*). Whatever the basis for the relationship between the ability of the cells to grow in crowded cell cultures and their ability to produce tumors in the animal, the availability of simple means to select both for and against these properties by using cloned cell lines should permit a more systematic approach to the study of carcinogenesis.

STUART A. AARONSON
GEORGE J. TODARO
Viral Carcinogenesis Branch,
National Cancer Institute,
Bethesda, Maryland 20014

References and Notes

1. G. J. Todaro and H. Green, *J. Cell Biol.* **17**, 299 (1963).
2. S. A. Aaronson and G. J. Todaro, *J. Cell Physiol.* **72**, 141 (1968).
3. G. J. Todaro, Y. Matsuya, S. Bloom, A. Robbins, H. Green, *Wistar Inst. Symp. Monogr.* **7**, 89 (1967).
4. The term "density dependent inhibition" has been suggested as an alternative to "contact inhibition of cell division" [M. G. Stoker and H. Rubin, *Nature* **215**, 171 (1967)].
5. The saturation density or maximum cell number attained per unit of surface area depends both on the genetic properties of the cell being tested and on the amount of serum provided to the culture. The higher the serum concentration and/or the more frequently the medium is changed, the greater will be the saturation density. For a given set of culture conditions, however, the saturation density is a highly reproducible cellular property.
6. K. K. Takemoto, R. L. Kirschstein, K. Habel, *J. Bacteriol.* **92**, 990 (1966).
7. K. K. Takemoto, R. C. Ting, H. L. Ozer, P. Fabisch, *J. Nat. Cancer Inst.*, in press.
8. G. J. Todaro and H. Green, *Virology* **28**, 756 (1966); P. H. Black, *ibid.*, p. 758.
9. R. E. Pollack, H. Green, G. J. Todaro, *Proc. Nat. Acad. Sci. U.S.* **60**, 126 (1968).
10. W. T. Hall, W. F. Anderson, K. K. Sanford, V. J. Evans, J. W. Hartley, *Science* **156**, 85 (1967).
11. J. W. Hartley, personal communication.
12. D. A. Kindig and W. H. Kirsten, *Science* **155**, 1543 (1967).
13. K. K. Sanford, B. E. Barker, M. W. Woods, R. Parshad, L. W. Law, *J. Nat. Cancer Inst.* **39**, 271 (1967).
14. R. R. Burk, *Nature* **212**, 1261 (1966); *Wistar Inst. Symp. Monogr.* **7**, 39 (1967).
15. H. M. Temin, *J. Cell Physiol.* **69**, 377 (1967); *Wistar Inst. Symp. Monogr.* **7**, 103 (1967); R. W. Holley and J. A. Kiernan, *Proc. Nat. Acad. Sci. U.S.* **60**, 300 (1968).
16. This work is supported by National Cancer Institute contract No. PH 43-65-641. We thank Clinton Thompson, Lillian Killos, Elaine Rands, and Lillian Murphy for their valuable technical assistance.

27 September 1968

Pattern Perception: Integrating Information Presented in Two Modalities

Abstract. *Subjects were required to organize and identify temporal patterns composed of either (i) two stimuli in one modality; (ii) two stimuli in each of two modalities, with the pattern alternately presented in the two modalities; or (iii) one stimulus in each of two modalities. Patterns (i) and (iii) are organized as structured patterns, but (ii) is organized by modality, not by pattern structure. When elements of a pattern appear in two modalities, the auditory-tactual combination produces the poorest performance.*

Fraisse (*1*) suggested that elements in different modalities could not be organized into a single pattern, and that the elements in each modality are perceived separately. In his work, the individual elements in each modality were the important perceptual variables, not the pattern formed by the individual elements. However, it has been argued (*2*) that to study perception one must use stimuli in which the pattern formed by the individual elements is the important perceptual variable. This means that individual elements can produce only sensations, whereas the patterning among the elements creates perception. Our results demonstrated that (i) temporal patterns of two modalities were organized into a structured pattern, although temporal patterns of two elements in each of two modalities, alternately presented in the modalities, were organized by modalities, not by pattern structure; and (ii) the particular pair of modalities used to present the pattern affects the ease of identification of patterns.

The stimuli consisted of a sequence of dichotomous elements generated by a repeating pattern of eight dichotomous elements. Since a pattern is continuously repeated, it may be started at any one of the eight elements and still generate the same sequence (with the exception of the first few elements). Thus, for any given pattern, there are normally seven equivalent patterns.

It has been shown (*3*) that all such sequentially equivalent patterns are not perceptually equivalent; subjects generally identify and describe the sequence beginning at selected elements in the pattern (preferred start points) regardless of the actual element at which the pattern started. Also, pattern organization is essentially the same for the auditory (A), tactual (T), or visual (V) modalities (*3*, *4*). Thus, the pattern of the stimulus (rather than the specific elements of the pattern, or the particular arrangement used to start the pattern) determines perceptual organization.

In our study, equivalent patterns of eight elements were produced in the A, T, and V modalities by the use of identifiably different left and right stimuli as the dichotomous elements. The A stimuli were a 1200-hz tone and a 3000-hz tone equal in loudness. The T stimuli were a pair of vibrators (*5*), one held in each hand (6 volts, 30 hz and 12 volts, 60 hz, respectively). The V stimuli were a red and a green panel light.

Three different methods of pattern presentation were used. In the first method, patterns were presented in either the A, T, or V modalities. In the second method, the entire pattern of eight elements was presented first in one modality, then in another modality. The alternations were continued every eight elements. Three pairs of modalities were used: auditory-tactual (A-T), auditory-visual (A-V), tactual-visual (T-V). The stimuli of each modality were identical to the stimuli described above. In the third method, the dichotomous elements of a pattern were modalities, rather than a left- or right-stimulus element within a modality. Again, the three pairs of modalities were used. The tone (3000 hz), the vibrator (source, 12 volts, 60 hz), and the red panel light were the modality elements. The stimuli were placed directly in front of the subject.

To give an example of these methods, suppose a pattern of four elements was selected that is represented as 1100.