BIKEN JOURNAL Vol. 11, 313-332, 1968

PROPERTIES OF HAMSTER EMBRYONIC CELLS TRANSFORMED BY 4-NITROQUINOLINE-1-OXIDE IN VITRO AND THEIR CORRELATIONS WITH THE MALIGNANT PROPERTIES OF THE CELLS

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(Received September 28, 1968)

SUMMARY The properties of malignantly transformed hamster embryonic cells by 4-nitroquinoline-1-oxide (4NQO) and their correlations with the malignant properties of the cells were investigated. The properties studied were cell morphology, focus formation, colony formation in soft agar medium, doubling time, length of each phase of the mitotic cycle, cloning efficiency, and resistance to the cytotoxic effect of 4NQO. Of these properties, the colony forming ability of cells in soft agar correlated well with the transplantability of the cells into host animals, while other properties did not always correlate so well. The colony forming ability of transformed cells in soft agar medium appeared to serve as the basis for an in vitro assay system of the malignancy of 4NQO-transformed hamster embryonic cells.

Various characteristics of 4NQO-transformed cells were also discussed with respect to their reliabilities as indicators of malignant transformation of cells in vitro.

INTRODUCTION

In previous reports we have demonstrated that treatment of the cell population of a primary culture of hamster embryos with 4-nitroquinoline-1-oxide (4NQO) induced malignant transformation of the cells within several culture passages (KAMAHORA and KAKUNAGA, 1966a, 1966b, 1967). The criteria for transformation which were used in these experiments were: alteration of cell morphology, irregular pattern of cell arrangement, indefinite life span in the culture and ability to grow and form tumors upon transplantation into host animals. These criteria have been also used in many experiments on transformation by tumor viruses. But they seem to be unsufficient as common criteria for assessing the malignant transformation of cells in vitro because there has been no clear demonstration of a correlation between these properties and the malignant properties of cells in vivo, as indicated by SANFORD (1965). Moreover, it is difficult to estimate quantitative alterations in these properties. The transformation system by 4NQO in vitro which we found, seems useful in studies on an

indicator of malignant transformation of cells in vitro, since it is possible to obtain cells of common origin with various degrees of malignancy. If a quantitative assay system for assessing transformation, directly reflecting changes associated with neoplasia of 4NQO-transformed cells were available, it would be very useful in studies on the mechanism of malignant transformation of cells by 4NQO in vitro.

This paper reports the properties of 4NQO-transformed cells and the correlation of these properties with the malignancy of the cells. Studies on a common indicator of malignant transformation of animal cells in vitro are also described.

MATERIALS AND METHODS

1. *Cell cultures*

Hamster embryonic cells were cultured and subcultured as described previously (KAMAHORA and KAKUNAGA, 1967). Unless otherwise specified, all culture was carried out in plastic dishes (Falcon Plastics, Los Angels, Calif. U.S.A.) at 37°C in a humidified incubator with 7% CO_2 in the air. The usual growth medium was Eagle's minimum essential medium (EMEM) supplemented with 10% calf serum. 4NTH-I~IV were cell lines which originated from hamster embryonic cells independently transformed by 4NQO and serially cultivated in bottles. SVTC was the cell line of hamster embryonic cells transformed by SV_{40} in vitro in our laboratory.

2. *Morphological experiments*

Microscopic studies were made on the morphology of clones formed in plastic dishes. Cultures were trypsinized and the cells were seeded sparsely so as to give rise to well separated colonies in 60 mm plastic dishes. Ten to twelve days after seeding, cultures were washed, fixed with methanol and stained with Giemsa.

3. *Assay of focus formation*

Hamster cells were well dispersed by pipetting or by filtering a cell suspension in the medium through several layers of gauze. Cultures were prepared in plastic dishes of 60 mm diameter in growth medium. The cell concentration was about 3×10^4 to 10^5 per cm^2 of growth surface. After 24 hr incubation the medium was replaced by 5 ml of agar medium with or without 4NQO. The agar medium consisted of EMEM, 5% calf serum and 10% tryptophosphate broth in 0.8% Noble agar (Difco). The final concentration of 4NQO was 5×10^{-7} to 1×10^{-8} M. The cultures received additional overlays of 2 ml of nutrient agar after 7 and 14 days. After 21 days incubation 2 ml of agar medium containing neutral red at a concentration of 1 : 8000 was added. The plate was scanned for foci at a low magnification with an inverted microscope on the 22nd or 25th day. For detailed histological observation, the agar was carefully removed and the cell sheet was fixed with methanol and stained with Giemsa.

4. *Colony formation in soft agar*

The procedure was based on that described by MACPHERSON and MONTAGNIER (1964), CRAWFORD *et al.*, (1964) and GOTO and SATO (1965). The assay procedure finally adopted was as follows. Agar medium was prepared by diluting melted stock agar, consisting of 5% Noble agar (Difco), 2% Bactopeptone, 0.5% NaCl and 0.25% $Na_2HPO_4\cdot12H_2O$ in water, with EMEM supplemented by 11% calf serum. Cultures of actively growing cells were harvested with 0.25% trypsin, centrifuged, suspended in EMEM with 11% calf serum, counted in a hematocytometer and diluted with the same medium in appropriate concentrations of cells. More than 96% of the cells in suspension were single. Melted stocked agar at 44°C was mixed with the cell suspension to give an appropriate final concentration of agar and cells. One ml of agar medium containing cells was poured on to a base layer of 5 ml of 0.5% agar medium. After the top layer containing the cells had set at room temperature, the cultures were incubated at 37°C. Colony counts were made on cultures with or without staining with neutral red (1 : 40000) with the aid of a low power microscope. In cultures containing many colonies an estimate of the total number was obtained by counting the number in a known area of the dish. The observed variation within each set of cultures was within the limits of sampling error.

5. *Measurement of doubling time*

The doubling time of the cells was calculated from the growth curve by measuring the cell concentration in the growing culture at various times with a hema-

tocytometer.

6. *Mitotic index and length of each stage of the mitotic cycle*

Mitotic indices were measured by counting the number of cells in mitosis among 1000 cells. In this experiment, cells showing a single group of clearly discernible chromosomes with no nuclear membrane were considered to be in metaphase.

Analysis of the durations of the various phases of the cell life cycle was carried out by pulse-labeling of H^3-thymidine and chasing methods (STANNERS and TILL, 1960; SISKEN, 1964; BASERGA, 1965). For this, cells were plated on glass coverslips in dishes, and cultures at the logarithmic phase of growth were exposed to H^3-thymidine in medium at 37°C for 30 min at a concentration of 0.05 μc/ml. Cells were then rinsed twice with cold phosphate-buffered saline, pH 7.0 (PBS), and reincubated in fresh prewarmed medium containing 10^{-5} M of unlabeled thymidine at 37°C. At intervals of 1 to 2 hr, the cells on coverslips were taken out, rinsed with cold PBS, fixed with methanol, and extracted with 2% perchloric acid at 4°C for 40 min. Dipping autoradiography was carried out with Kodak NTB-2 emulsion with an exposure period of 5 days. The cells were then stained with Giemsa solution. One hundred metaphase cells were examined to determine the percentage of metaphase cells labeled with 5 or more grains, the average background being about 1 grain/nucleus. Under the conditions employed, the grain background was usually negligible and very few labeled cells showed fewer than 5 grains. Accordingly, only cells with 5 or more grains were regarded as labeled in counting. The percentage of labeled mitosis was plotted against the time after exposure to the isotope. The total generation time and the length of each stage of mitotic cycle was estimated from this graph.

7. *Resistance to cytotoxic effects of 4NQO*

The resistance of cells to the cytotoxic effects of 4NQO was estimated by determining cloning efficiency in culture medium containing 4NQO as described previously (KAMAHORA and KAKUNAGA, 1967). For cloning of normal hamster cells and 4NQO-treated cells at early time in cultivation the X-irradiated (5000 r) rat embryonic cells were used as a feeder layer. 4NQO at a final concentration of 3×10^{-8} M was added 1 day after the cells were plated for cloning, and not removed until the plates were fixed with methanol and stained 11 to 14 days after plating.

8. *Transplantability of cells into animals*

The ability of cells to grow as tumors in vivo was estimated by determining the 50% tumor inducing dose of cells (TD_{50}). Cells were transplanted by subcutaneous injection of 0.5 ml of cell suspension into the back of 3–4 week old golden hamsters. Cell suspensions were prepared by trypsinization of cultures, washing, counting and resuspension in EMEM. Cells were generally diluted serially tenfold from 10^2 to 10^6. Groups of 2 to 4 animals of a local breed were inoculated with each dilution. The animals were palpated at a weekly intervals and were observed for at least 6 months for possible development of tumors.

RESULTS

1. *Morphology*

To examine the morphology of 4NQO-transformed hamster embryonic cells and to investigate the relationship between the morphology of the cells in culture and their transplantability in vivo, many clones of 4NQO-transformed and normal hamster embryonic cells were isolated and their clonal morphology and transplantability into host animals were examined.

Clones isolated from secondary cultures of normal hamster embryonic cells could be classified into two main morphological groups. One group consisted of fibroblastic cells (Fig. 1), and the second group of clones were epithelial-like cells (Fig. 2). Both groups of clones were thin and generally formed monolayers without marked overlapping or piling up. The cells of these clones did not produce tumors when implanted into adult hamsters.

4NQO-transformed cells also consisted of two morphological types, fibroblast and epithelial-like cells, and their clonal morphology was classified into 4 groups on differences in cell density and growth pattern. Clones of the first group were dense like those suggested by many authors to be typical of cells transformed by oncogenic viruses (SANFORD *et al.*,

FIGURE 1–9 Clonal morphology of normal and 4NQO-transformed (4NTH) hamster embryonic cells inculture. Fixed with methanol and stained with Giemsa.

FIGURE 1 and 2. Clones isolated from secondary cultures of normal cells growing in thin monolayers without overlapping and piling up of cells. Fig. 1 ; fibroblastic. Fig. 2 ; epithelial-like.

FIGURE 3 Clone 4NTH-II-6. Morphologically typical transformed cells with dense piling up of cells.

FIGURE 4 Subclone 4NTH-II-2-6 derived from clone 4NTH-II-2. Random growth pattern with frequent overlapping and piling up of cells.

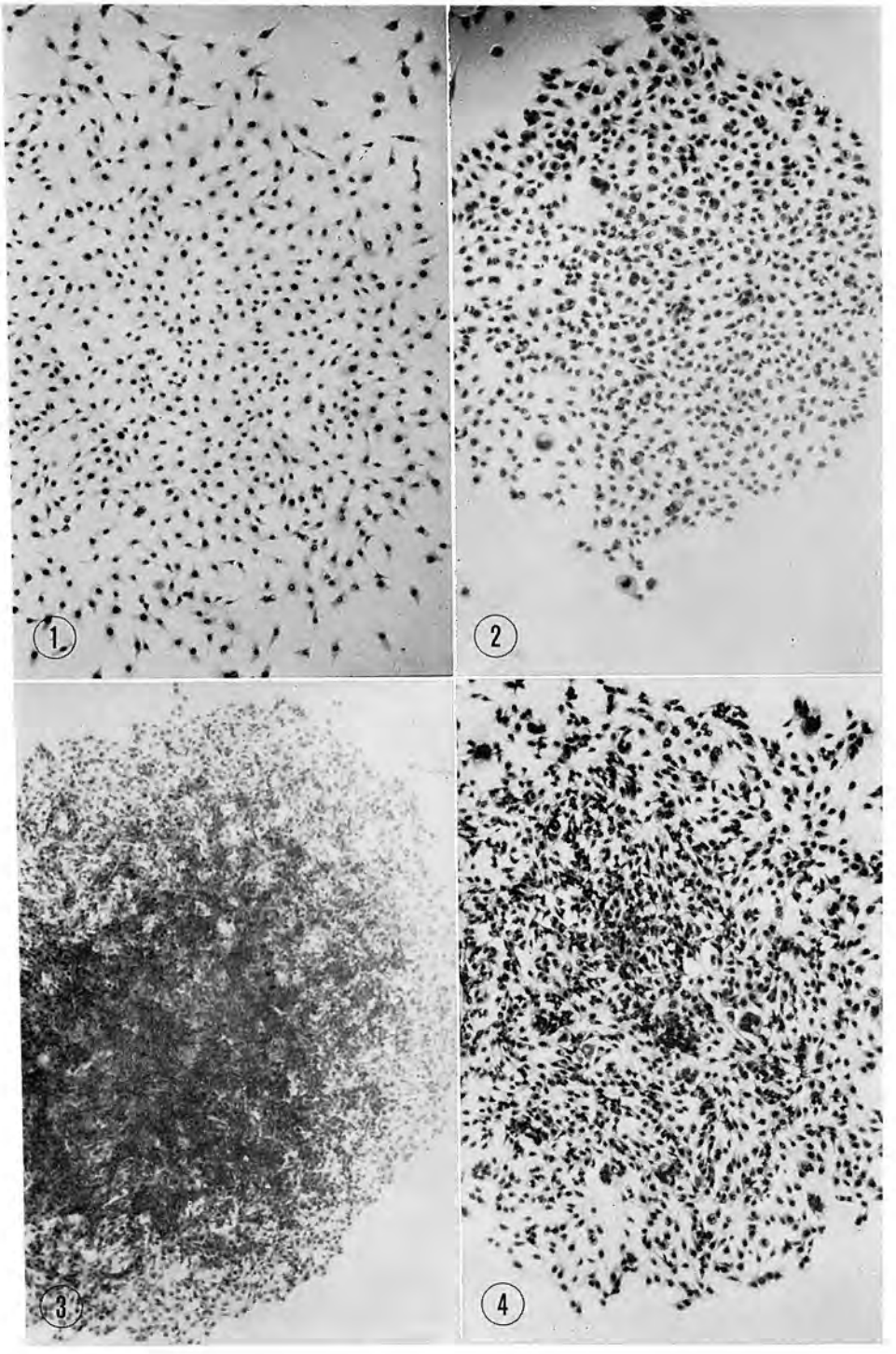
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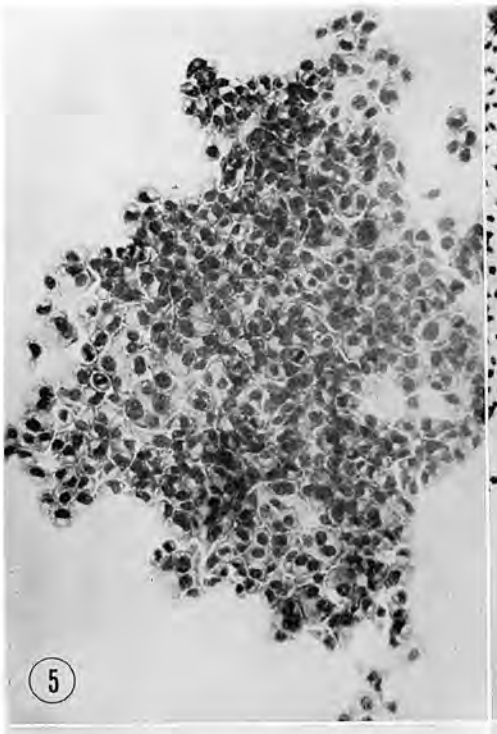
FIGURE 5 Clone 4NTH-I-6. Slowly growing epithelial-like cells with overlapping of cells and non-oriented growth pattern even in clones with few cells. Note large size of individual cells.
FIGURE 6 Clone 4NTH-III-26. Cells are randomly spread in thin monolayer with loose structure.

FIGURE 7 Clone 4NTH-II-2. Well-oriented compact bundles of cells with slight piling up of cells.
FIGURE 8 Clone 4NTH-I-3. Note morphological characteristics of normal cells; well-oriented bundles of cells in monolayers with no piling up of cells.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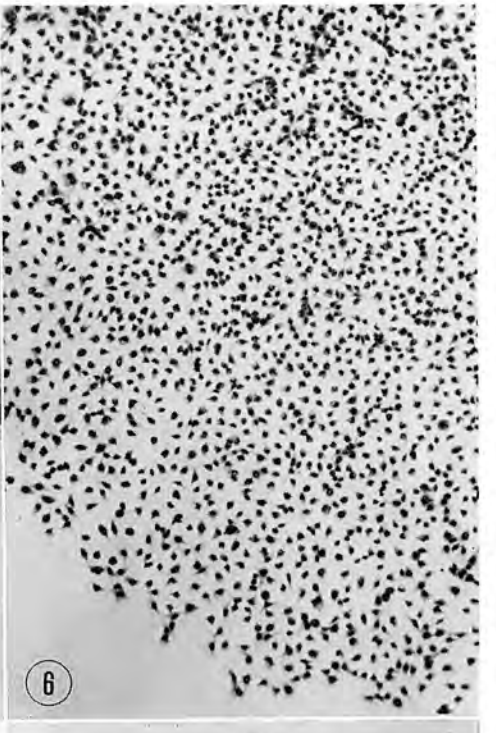
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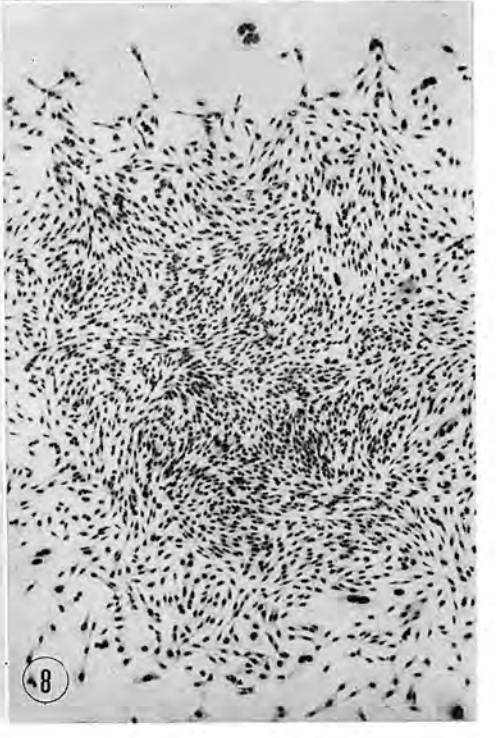
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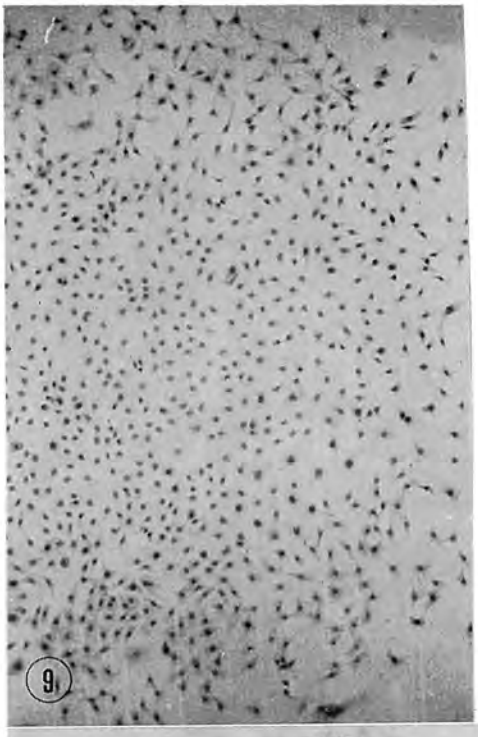
FIGURE 9 Clone 4NTH-III-3. Note similarity to normal cells showed in Fig. 1. Morphologically indistinguishable from normal cells.
FIGURE 10 Colonies of 4NQO-transformed cells formed in soft agar medium.



1961; JENSEN *et al.*, 1963; TODARO *et al.*, 1963; DEFENDI and LEHMAN, 1965; SANFORD, 1965). These clones showed a heavily piling up at the center, with dense strands or nodules. The clonal morphology of this group, i.e. that of 4NTH-II-6, 4NTH-II-2-6 and 4NTH-I-6 clones is showed in Figs. 3–5. In clones of the second group, e.g. 4NTH-III-26, cells mainly grew in monolayers but with random orientation, and loose structure (Fig. 6). Clones of the third group such as those of 4NTH-II-2 showed a compact well-oriented growth pattern with slight piling up (Fig. 7). Those of the fourth group, for example those of 4NTH-I-3 and 4NTH-III-3, were strictly monolayers without overlapping or piling up of cells. They were morphologically indistinguishable from normal cells (Figs. 8 and 9).

Table 1 shows representative experiments on the relation between clonal morphology and transplantability to hamsters. Transformed cells of the first morphological group such as 4NTH-II-6, 4NTH-II-2-6 and 4NTH-I-6

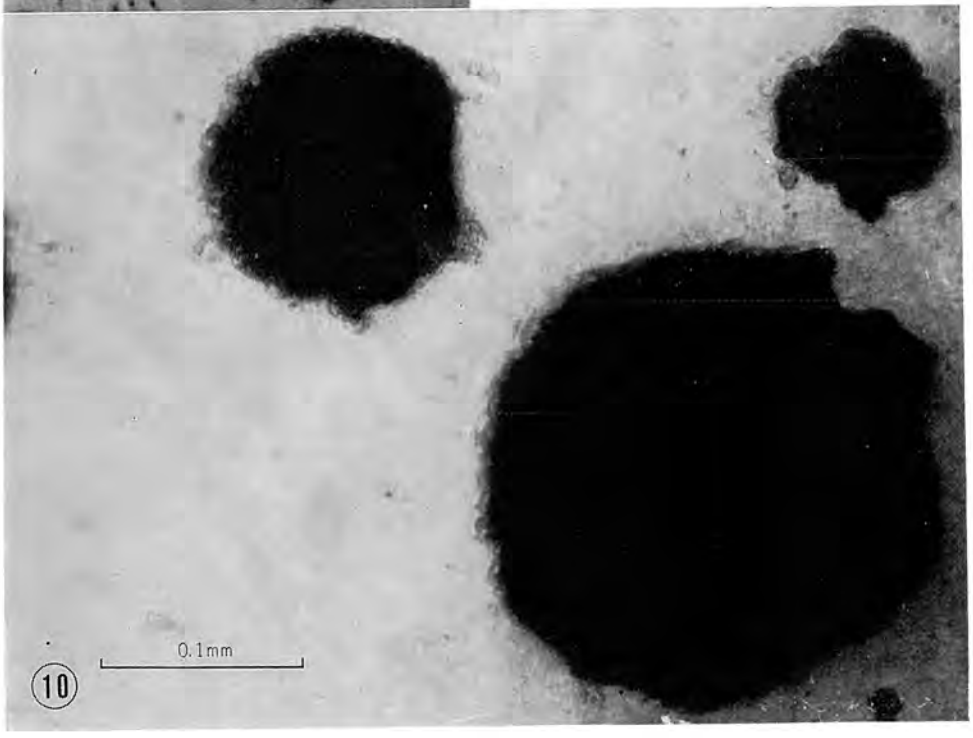


TABLE 1 *Clonal morphology and transplantability into animals of normal and 4NQO-transformed hamster cells*

Clones	Clonal morphology		Transplantability	
	Random orientation	Piling up	No. of cells inoculated per animal 10^6	10^4
Normal-I	−[a]	−	0/3[b]	ND[c]
Normal-II	−	−	0/3	ND
4NTH-II-6	2+	3+	ND	3/3
4NTH-II-2-6	2+	2+	ND	2/2
4NTH-I-6	2+	2+	ND	2/2
4NTH-III-26	+	−	3/3	2/2
4NTH-II-2	−	+	3/3	1/3
4NTH-I-3	−	−	3/3	1/2
4NTH-III-3	−	−	3/3	1/3

a − : no + : slight 2+ : moderate 3+ : marked
b No. of animals with tumors / No. of animals inoculated
c Not done

TABLE 2 *Number of 4NQO-transformed cells inoculated and number of foci formed*

Cells	No. of transformed cells inoculated per plate[a]	No. of foci formed per plate[b]	
		Days after inoculation 10^c	21^d
4NTH-I-2	10	3 (2)[e]	6 (3)
	50	17 (5)	26 (2)
	250	133 (14)	180 (12)
	1250	676 (15)	Confluent
4NTH-II-2	50	0 (3)	0 (3)
	250	0 (8)	0 (20)
	1250	0 (38)	0 (58)
4NTH-III-3	50	0 (4)	0 (7)
	250	0 (5)	0 (3)
	1250	0 (5)	0 (4)

a Appropriate numbers of normal hamster cells of a secondary culture were mixed with transformed cells to give a total number of 3×10^6 cells inoculated per plate.
b Average of 5 to 8 plates.
c Counted under an inverted phase-contrasted microscope without staining.
d Counted after staining with neutral red.
e Numbers in parentheses are the numbers of indistinct foci which were indistinguishable from nonspecific overlapping of normal cells.

clones showed high transplantability. It was surprising that not only the cells of the second and third morphological groups, i.e. 4NTH-III-26 and 4NTH-II-2 cells, but also those of the fourth group, 4NTH-I-3 and 4NTH-III-3 cells, produced tumors in animals at comparatively low cell doses. The morphological properties of transformed cells, such as the random orientation and piling up of cells in the culture, did not correlate directly with the malignant properties of the cells in vivo.

2. *Focus formation*

In parallel with the morphological experiments, the ability of 4NQO-transformed cells to form foci in cultures like Rous sarcoma cells (MANAKER and GROUPE, 1956; TEMIN and RUBIN, 1958) was examined. The transformed cells were trypsinized, suspended in medium at serial fivefold dilutions, mixed with a suspension of normal hamster embryonic cells, and plated in dishes. As shown in Table 2, the 4NQO-transformed cell clone, 4NTH-I-2, which had high transplantability into host animals and showed random orientation and piling up in clonal morphology, produced discrete, clear foci on a sheet of normal cells. The number of foci per plate was found to be nearly proportional to the number of transformed cells inoculated. On the other hand, 4NTH-II-2, which were transplantable and showed a clonal morphology of well-oriented growth with little piling up of cells, formed such indistinct foci that it was impossible to distinguish them from the non-specific overlapping of cells observed in cultures of normal cells only. 4NTH-III-3, which showed malignancy in vivo and showed no random orientation or piling up in clones, also failed to produce clear foci.

Next, the possibility of direct assay of transformation of cells by 4NQO was studied in the following two manners. First, 4NQO dissolved in liquid medium at final concentration ranging from 10^{-5} to 10^{-8} M was added on a sheet of primary hamster embryonic cells, and 1—2 hr later the agar was overlayed with or without removing the 4NQO. Second, primary cultures of hamster embryonic cells were treated with 4NQO at final concentrations of 5×10^{-7} M for 8 days. Previous experiments showed that this treatment induced malignant transformation of cells after several culture passages (KAMAHORA and KAKUNAGA, 1966a and b, 1967). Then the cultures were washed several times with PBS and agar was overlayed. No clear foci of transformed cells could be observed in either of these experiments.

3. *Colony formation in soft agar medium*

In preliminary experiments we found that the cells of one 4NQO-transformed cell line were able to grow and form colonies in soft agar medium. Thus we investigated whether this ability to form colonies in soft agar medium was a property common to all 4NQO-transformed cells and whether suitable culture conditions could be found so that on plating of a mixture of normal cells and transformed cells only the transformed cells formed colonies with high efficiency.

Table 3 shows the results of experiments in which normal cells and two lines of 4NQO-transformed cells were tested for colony formation in soft agar medium with agar concentrations in upper layer of 0.25 to 0.50%. Normal cells formed extremely small colonies and no colonies of more than 0.08 mm in diameter in soft agar medium when the agar concentration was higher than 0.275%. The cells of two 4NQO-transformed cell clones, on the other hand, formed large colonies of more than 0.08 mm diameter as well as small colonies (Fig. 10). It was remarkable that 4NTH-II-2, a clone with somewhat similar morphology to that of normal cells, formed distinct colonies. The number of colonies formed in agar increased with decrease in concentration of agar in the upper layer. Agar concentrations of less than 0.250%, were technically impossible due to the flow of the agar layer. Even with an agar concentration of 0.250%, breakage of colonies due to the flow of the agar layer was occasionally observed. The optimal con-

TABLE 3 *Concentration of agar in upper layer and colony formation of normal and 4NQO-transformed cells*

Cells	Colony diameter (mm)	No. of colony formed per plate[a, b]					
		Agar concentration in upper layer (%)					
		0.250	0.275	0.300	0.325	0.350	0.400
Normal cells	0.05≦ <0.08	295	66	26	24	30	17
	0.08≦ <0.15	3	0	0	0	0	0
	0.15≦	0	0	0	0	0	0
4NTH-I-6	0.05≦ <0.08	1604	1389	811	933	883	802
	0.08≦ <0.15	1940	1642	1565	1417	976	910
	0.15≦	1805	1859	1718	1386	1170	697
4NTH-II-2	0.05≦ <0.08	1895	1646	690	523	544	441
	0.08≦ <0.15	491	460	390	297	129	26
	0.15≦	64	61	49	23	25	9

a Counted 14 days after plating.
b Average of 4 to 5 plates.

TABLE 4 *Number of cells inoculated and plating efficiencies of 4NQO-transformed cells in soft agar medium*

Clone of transformed cells	No. of cells inoculated / plate		No. of colonies[a, b] per plate (Average)	Plating efficiency[c] of transformed cells (Average%)
	Transformed cells	Normal cells		
4NTH-I-2	10,000	0	4305.2	43.1
	2,000	0	874.3	43.7
	2,000	8,000	758.4	37.9
	400	0	65.0	16.3
	400	9,600	152.6	38.2
	80	0	7.2	9.0
	80	9,920	17.0	21.3
	8	0	0.5	6.3
	8	9,992	1.1	13.8
	0	10,000	0	0
4NTH-III-3	10,000	0	980.4	9.8
	2,000	8,000	171.8	8.6
	400	9,600	35.2	8.8
	80	9,920	4.2	5.3
	16	9,984	0.6	3.7

a and *b* As in table 3.
c Calculated as follows :

$$\frac{\text{Average number of colonies formed in soft agar medium / plate}}{\text{Average number of cells inoculated / plate}} \times 100$$

centration of agar in the upper layer was considered to be 0.300%, since with this concentration of agar flow of the agar layer was slight and colonies of more than 0.08 mm diameter were formed by 4NQO-transformed cells but not by normal cells. Extremely small colonies, of about 0.05–0.08 mm diameter, were formed by normal cells as well as by transformed cells, and the number of these colonies varied considerably in different dishes and different experiments, whereas the number of larger colonies was relatively constant. In following experiments, therefore, extremely small colonies were not counted, and the colonies formed were divided into 2 group according to their diameter to obtain a rough estimate of the size of the colonies formed. Thus, for convenience, colonies of 0.08–0.15 mm diameter were termed " small colonies " and those of more than 0.15 mm were named " large colonies ".

The effects of other culture conditions, such as the concentration of serum and the period of incubation on colony formation were also investigated. Colony forming efficiency was not greatly affected when the concentration of serum was varied from 7 to 15%, while at lower concentrations, plating efficiency increased with serum concentration. Colonies increased in number and size day by day and reached a maximum about 12 to 15 days after plating the cells into the agar.

Table 4 shows the results of experiments in which mixtures of normal cells and 4NQO-transformed cells were plated in soft agar medium. The presence of a large number of normal cells did not interfere the colony formation of transformed cells but tended to increase plating efficiency of transformed cells probably due to a feeder effect. When the transformed cells were plated at inoculum sizes ranging from 4×10^2 to 10^4 per plate with an appropriate number of normal cells to give 10^4 total cells per plate, the plating efficiency of the transformed cells was generally constant and a linear relationship was observed between the number of colonies formed and the number of transformed cells inoculated. The results also indicate that the soft agar method is sensitive enough to detect a fairly small number of transformed cells among a large number of normal cells. In the following experiments, the inoculum size of cells was 10^4 per plate. It should be noted that " morphologically normal " transformed cell, that is 4NTH-III-3 cells, formed colonies in soft agar medium, though these were relatively small.

To investigate the relation of the colony forming ability of transformed cells in soft agar to their transplantability in animals, the TD_{50} and colony formation in soft agar medium were simultaneously estimated with various 4NQO-transformed cell lines. As shown in Table 5, cells with a small TD_{50} tended to form many, large colonies. Generally the number of large colonies formed was inversely proportional to the TD_{50}. The 50% tumor inducing colony former dose was calculated using the number of colony forming cells as a unit of TD_{50}, i.e., multiplying TD_{50} by the colony forming efficiency. The 50% tumor inducing, large colony former dose were nearly constant ranging from 10^2 to 3×10^2, while the 50% tumor inducing, total colony former dose varied widely from 2×10^2 to 5×10^4. It is clear that a highly significant correlation exists between transplantability of the 4NQO-transformed cells in vivo and their ability to form colonies, especially large colonies, in soft agar, and that all strains of 4NQO-transformed cells so far tested are distinguishable from normal cells by the soft agar suspension method even if their morphology is similar to that of normal cells.

Since the size of the colonies formed in soft agar medium was found to be significant in relation with the malignancy of the cells, the nature of the " large " and " small " colonies formed in soft agar medium was investigated. Individual colonies were harvested from the soft agar layer of plates in which 4NTH-I cells had been seeded 12 days previously. Large colonies (termed 4NTH-I-L) were picked up by capillary pipette, while small

colonies (4NTH-I-S) were harvested by pipetting the upper agar layer, from which all large colonies had been removed, into PBS, and filtering the mixture through a layer of gauze. Colonies were broken up into single separate cells by treatment with trypsin or by pipetting in Ca- and Mg-free PBS. Then cells were suspended in medium, diluted or concentrated to give a definite concentration of cells, and their TD_{50} and colony formation in

TABLE 5 *Number and size of colonies formed in soft agar and fifty per cent tumor dose of transformed cells*

Cells	TD_{50}[a]	No. of colonies / plate[b, c]			$TD_{50}\times$large[d] size colony forming efficiency	$TD_{50}\times$total[e] colony forming efficiency
		Large size	Small size	Total		
4NTH-I	4.8×10^2	2781	1765	4546	134	218
4NTH-II	1.4×10^4	172	2353	3525	245	4935
4NTH-IIIa[f]	6.8×10^5	0	740	740	—	50320
4NTH-IIIb[g]	1.9×10^5	15	1567	1582	285	29596
4NTH-III / tc-I[h]	7.5×10^2	1686	2113	3799	127	912
4NTH-IV	1.0×10^6	0	65	65	—	6500
SVTC[i]	5.3×10^5	8	1820	1828	424	96884

a Fifty % tumor inducing cell dose.
b Counted 14 days after plating cells.
c Average of 8 to 10 plates.
d Represents 50% tumor inducing, large colony former dose.
e Represents 50% tumor inducing, total colony former dose.
f After 96 days culture.
g After 129 days culture.
h Derived from tumor produced by subcutaneous injection of 4NTH-III cells.
i Hamster embryonic cells transformed by SV_{40} in vitro.

TABLE 6 *Number and size of colonies formed in soft agar and fifty percent tumor inducing cell dose of 4NQO-transformed cells*

Cells[f]	TD_{50}[a]	No. of colonies formed per plate[b, c]			$TD_{50}\times$large[d] size colony forming efficiency	$TD_{50}\times$total[e] colony forming efficiency
		Large size	Small size	Total		
4NTH-I	4.8×10^2	2781	1765	4546	134	218
4NTH-I-L_1	1.6×10^2	3231	2125	5356	52	86
4NTH-I-S_1	9.8×10^2	1575	2964	4539	156	445
4NTH-I-L_1-L	2.0×10^2	3070	2881	5951	61	135
4NTH-I-L_1-S	ND[g]	2844	3112	5956	—	—
4NTH-I-S_1-L	2.5×10^2	3111	2305	5416	78	135
4NTH-I-S_1-S	2.4×10^3	672	2712	3384	185	812

a, *b*, *c*, *d* and *e* As in table 5.
f See text.
g Not done.

soft agar medium were reexamined. Then the TD_{50} and colony formation in soft agar medium of the large (4NTH-I-L-L) and small (4NTH-I-L-S) colonies formed in the cultures of 4NTH-I-L cells, and the large (4NTH-I-S-L) and small (4NTH-I-S-S) colonies formed in the cultures of 4NTH-I-S cells were retested in the same manner. The results are presented in Table 6. 4NTH-I-L, 4NTH-I-L-L, 4NTH-I-L-S and 4NTH-I-S-L cells showed almost similar TD_{50} values and colony formation in soft agar medium. However, 4NTH-I-S and 4NTH-I-S-S cells had less transplantability and colony forming ability than their parents, i.e. 4NTH-I and 4NTH-I-S cells respectively. These results indicate that the cells derived from large colonies all have the ability to form large colonies with a constant high efficiency while some cells derived from small colonies can form large colony and some cannot. Small colonies formed in cultures of cells derived from large colonies were not intrinsically small colony formers but were cells with the ability to form large colonies.

These conclusions are supported by the following cloning experiments. Many individual large and small colonies were picked up from cultures of 4NTH-I cells, and the cells separated from each colony were cultivated in dishes containing liquid medium, until the number of cells from each colony amounted to 5×10^4. Then the cells were replated into soft agar medium to observe their colony formation. Cell clones incapable of forming large colonies in soft agar medium were obtained from some small colonies but not from large colonies.

4. *Doubling time, mitotic index and length of each phase of the mitotic cycle*

Table 7 summarizes results on the doubling time, mitotic index and the length of each phase of the mitotic cycle in normal and 4NQO-transformed hamster cells. The mitotic index of all cells tested remained constant for 48 hr, so the cells seemed to proliferate asynchronously and exponentially under the experimental conditions.

The doubling time of transformed cells was almost equal to, or slightly shorter than that of normal cells in the early period after trans-

TABLE 7 *Doubling time, mitotic index and length of each stage of mitotic cycle in normal and 4NQO-transformed hamster cells*

Cells	Days in culture	Doubling[a] time (hr)	Mitotic[b] index (%)	Duration of each phase of mitotic cycle[c] (hr)				
				G_1	S	G_2	M	Total
Normal	10	24.4	2.8	4.4–4.7	9.0	3.0	0.7–1.0	17.4
Normal	51	46.0	1.6	9.4–9.9	10.3	4.0	0.6–1.1	24.8
4NTH-I	188	17.5	3.6	2.6–2.8	7.9	2.5	0.7–0.9	13.9
4NTH-II	127	21.0	3.0	3.7–3.9	9.5	3.0	0.7–0.9	17.1
4NTH-II-2	98	20.6	3.4	3.4–3.6	9.0	2.5	0.8–1.0	15.9
4NTH-II-2	158	20.4				——ND[d]——		
4NTH-II-2	363	14.6	3.8	2.5–2.6	6.8	1.5	0.6–0.8	11.6
4NTH-IIIa	64	23.8	3.2	5.0–5.2	9.3	3.0	0.9–1.1	18.4
4NTH-IIIb	91	21.8	3.0	4.1–4.4	9.0	2.5	0.7–1.0	16.6
SVTC	127	22.5	3.1	3.2–3.4	9.5	3.0	0.8–1.0	16.7

a Doubling time estimated from growth curve.

b Mitotic index determined on 1,000 cells.

c Length of each phase of mitotic cycle determined by measuring number of labeled metaphase cells after pulse labeling with H^3-thymidine.

d Not done.

formation and decreased with the period of cultivation in contrast to normal cells in which the doubling time after 51 days culture was twice that after 10 days culture.

On the other hand, the length of the mitotic cycle of transformed cells after some 4 months of cultivation was almost the same as that of normal cells after a short period of cultivation. On continuous cultivation of transformed cells, the length of the mitotic cycle decreased while that of normal cells increased. Since transformed cells in the early period of cultivation examined in this experiment showed colony formation in soft agar medium, as indicated in Table 8, it seems likely that variation in the length of the mitotic cycle does not reflect malignant transformation of the cells and that the decrease in the length of mitotic cycle of transformed cells after long cultivation is due to selection of cells with a shorter mitotic cycle which are dominant in growth under the conditions of cultivation.

The doubling time estimated on the basis of the growth curve differed from the length of mitotic cycle determined by autoradiographic studies. The difference was large in normal cells, especially after long cultivation and small in the transformed cells. This difference is probably attributable to the presence of undividing cells in the population of the size of the proliferating pool.

The mitotic cycle is subdivided into 4 phases; (1) a postmitotic phase called G_1; (2) an S phase, during which DNA is synthesized; (3) a premitotic phase called G_2; (4) mitosis extending from the beginning of prophase to completion of telophase. There were no differences in the lengths of each phase of the mitotic cycle in normal and transformed cells when the total length of the mitotic

TABLE 8 *Cloning efficiency, resistance to cytotoxic effect of 4NQO and colony forming ability in soft agar medium of normal and 4NQO-transformed cells*

Cells	Days in culture	Cloning efficiency (%)[a]		B/A[c]	Colony forming[d] efficiency in soft agar (%)
		— (A)	in presence of[b] 4NQO 3×10^{-8}M (B)		
Normal cells	11	0.42	0.008	0.02	0
»	35	0.50	0.02	0.04	0
»	59	0.19	0.002	0.01	0
4NTH-II	46	0.6	0.03	0.05	0.2
»	67	0.6	0.03	0.05	0.6
»	110	19.8	2.88	0.14	6.5
»	263	60.3	24.2	0.40	16.5
4NTH-II-2-4	263	31.9	10.6	0.33	25.4
4NTH-II-26-5	263	52.5	10.9	0.21	24.3
4NTH-II-9-3	263	61.2	7.5	0.12	47.0
4NTH-II-26-5	505	68.5	32.8	0.48	54.2
4NTH-II-9-3	505	88.9	40.5	0.45	52.7

a Fourteen days after seeding cells. Average of 6 to 8 plates.

b 4NQO added to culture medium 24hr after seeding cells.

c Ratio A/B represents the degree of resistance of cell to the cytotoxic effect of 4NQO.

d Calculated as follows :

$$\text{Average number of colonies formed in soft agar medium per plate} \times \frac{100}{10{,}000}$$

cycles were similar. Variation in the total length of the mitotic cycle depended upon variations in the length of the G_1 phase, i.e., during continuous cultivation the length of the G_1 phase of transformed cells decreased and that of normal cells increased, whereas the lengths of other phases did not change appreciably.

5. *Cloning efficiency and resistance to the cytotoxic effect of 4NQO*

Normal hamster embryonic cells had cloning efficiencies of 0.42, 0.5 and 0.19% after 11, 35 and 59 days culture, respectively. In the presence of 4NQO at a final concentration of 3×10^{-8} M, their cloning efficiencies were reduced to about 0.01—0.04 times normal. There was no increase in the degree of resistance to the cytotoxic effect of 4NQO in these cells within their life-span.

The cloning efficiency of the cells was very similar to that of normal cells 66 days after treatment with 4NQO, and treated cells were still as susceptible to the cytotoxic effect as normal cells, whereas they had the ability to form colonies in soft agar and to grow as tumors on subcutaneous inoculation into adult hamsters (KAMAHORA and KAKUNAGA, 1967). 4NQO-treated cells showed some degree of resistance after 110 days culture and this resistance increased with continued cell cultivation.

Next, the cloning efficiency, resistance to the cytotoxic effect of 4NQO, and colony forming ability in soft agar of the 4NQO-transformed clones, 4NTH-II-2-4, 4NTH-II-26-5 and 4NTH-II-9-3 derived from the same pool of hamster embryos were tested after 293 and 505 days culture. These clones showed various degrees of resistance to the cytotoxic effect of 4NQO and of colony forming ability in soft agar medium but high cloning efficiency. Thus, there seems to be no direct relation between the cloning efficiency, resistance to the cytotoxic effect of 4NQO and the colony forming ability in soft agar medium of 4NQO-transformed cells.

With further continued cultivation of clones, 4NTH-II-26-5 and 4NTH-II-9-3, their resistance to the cytotoxic effect increased with increase both in cloning efficiency and colony forming ability in soft agar.

DISCUSSION

The method of suspension of cells in agar was first reported by SANDERS and BURFORD (1964) and MACPHERSON and MONTAGNIER (1964) who found that hamster cells transformed by polyoma virus were capable of forming colonies in semi-solid agar medium whereas untransformed cells did not form colonies. Subsequently, this technique has been applied in various experiments (CRAWFORD *et al.*, 1964; STOKER and SUSSMAN, 1965; BORENFREUND *et al.*, 1966; ICHIKAWA *et al.*, 1966; TJÖTTA *et al.*, 1968). However, the precise relation of colony forming ability to the malignancy of the cells has not been established. In the present experiments it was found that 4NQO-transformed cells were able to form colonies in soft agar medium, and this property correlated well with their malignant properties in vivo. In addition, this property seems to be useful as a basis for a sensitive and quantitative assay of the number of cells transformed by 4NQO. A good method for assay of transformed cells should be as follows; 1) it should be based on properties which reflect changes directly associated with malignancy of cells, 2) it should exclude variable factors such as those affecting the development of tumors in animals, 3) it should be highly sensitive and quantitative, 4) it should be rapid. Two methods, i.e., the soft agar suspension method and the focus formation method, seem nearly to satisfy these conditions. The former, as indicated by the present results, seems suitable for assay of 4NQO-transformed cells. It should be noted that growth in soft agar medium is not a particular characteristic of 4NQO-transformed cells. SVTC, a line of hamster embryonic cells transformed by SV_{40} in vitro, also formed colonies in soft agar medium, though the ratio of colony forming ability to the TD_{50} differed

from that of 4NQO-transformed cells and it remains to be proved whether SVTC cells can be assayed quantitatively like 4NQO-transformed cells by the agar suspension method.

Another method available for quantitative assay of transformed cells in vitro, is the focus assay method, which has been generally used for measurement of transformation by Rous sarcoma virus and other avian leucosis viruses. It is based on morphological changes of transformed cells. 4NQO-transformed cells, such as 4NTH-II-2 cells and 4NTH-III-3 cells, which were morphologically indistinguishable from normal cells, did not produce clear foci, whereas 4NTH-I-2 cells, which differed in morphology from normal cells, produced distinct foci. The presence of the 4NQO-transformed cell line, which showed focus formation, prompted us to examine direct focus formation by hamster embryonic cells treated with 4NQO dissolved in agar medium or by cells pretreated with 4NQO for 8 days. No foci were formed by these cells. These findings suggest that culture passage may be required for the process of fixation or expression of the transformed state of cells.

4NQO-transformed cells showed various types of clonal morphology. The 4NQO-transformed cell clone, 4NTH-III-3, which has transplantability as demonstrated by simultaneous inoculation into animals, showed an apparently normal clonal morphology. Two transformed clones with high malignancy in vivo were also morphologically similar to normal clones, though it was possible to distinguish these transformed cells from normal cells by careful examination of their morphology. DEFENDI *et al.* (1963) also obtained cultured cells which grew as monolayer sheets but had malignancy in vivo. These findings indicate that the morphological changes of cells induced by 4NQO may not be truly representative of " malignant changes "; they may be additional or transitional phenomena, though morphological changes have been most widely employed as criteria of malignant transformation of cells in vitro. SANFORD *et al.* also reported that there was no correlation between morphological changes and malignant conversion of cells cultured in vitro (SANFORD *et al.*, 1961a, 1961b, 1963; SANFORD, 1965).

There are three possible causes of differences in morphology of transformed cells: (1) there may be differences in the cell types that are transformed, (2) there may be differences in the mode of interaction between chemical agents and each cell type, (3) cell variants may occur and become established during continuous cell cultivation. The finding that a subclone 4NTH-II-2-6, derived from the clone 4NTH-II-2, showed an appreciably different growth pattern from 4NTH-II-2 points to the last possibility.

The doubling time and length of the mitotic cycle of 4NQO-transformed cells soon after transformation were not greatly different from those of rapidly growing normal cells under experimental conditions in which cells were in the log phase of growth and were not affected by " contact inhibition ". This finding is in accord with earlier finding that the cycle time of tumor cells was comparable to that of their normal counterparts, that is the cells of the animal from which the tumors originated (BASERGA *et al.*, 1962; BASERGA and KISIELESKI, 1962; BERTALANFFY and LAU, 1962; KILLMANN *et al.*, 1963; POST and HOFFMAN, 1964; REISKIN and MENDELSOHN, 1964; BASERGA, 1965).

In some instances transformed cells may proliferate faster than normal cells, but it is likely that this is necessarily so, and that the growth rate of transformed cells is not due exclusively to an acceleration of the proliferative process but involves other parameters of cellular kinetics, such as the fraction of the cells participating in the proliferative pool, the amount of cell loss through death or migration, and the loss of contact inhibition of cell division. This concept was partially supported by the finding that the difference between the doubling time, estimated by cell counting, and the length of mitotic cycle, calculated from the labeled mitotic index, was larger in normal cells than in transformed cells even when both

cells had similar lengths of mitotic cycle.

Cloning efficiency and resistance to the cytotoxic effect of 4NQO were not closely correlated with colony forming ability in soft agar medium when these properties were examined with 4NQO-transformed cells soon after transformation. On continuous cultivation of transformed cells, the cloning efficiency and resistance increased. Similar phenomena were observed with clones of transformed cell. These results suggest that transformed cells give rise to more resistant cell variants with a selective advantage in culture as a secondary change after transformation. In previous papers, we reported that 4NQO-transformed cells were resistant to the cytotoxic effect of 3,4-benzpyrene (KAMAHORA and KAKUNAGA, 1967). From these findings, it is improbable that the resistance to the cytotoxic effects of carcinogens can be an indicator of malignant transformation of cells.

In conclusion, the alterations in the morphology, focus forming ability, length of the mitotic cycle, cloning efficiency and resistance to the cytotoxic effect of 4NQO seem to be unreliable as in vitro indicators of malignant transformation of cells by 4NQO. Colony forming ability of cells in soft agar medium appears to serve as a marker of malignancy of cells transformed by 4NQO. It is uncertain why only the colony forming property of 4NQO-transformed cells correlates well with the malignant properties of the cells in vivo while other properties of 4NQO-transformed cells tested did not. It is also unknown why 4NQO-transformed cells can grow in soft agar medium under the conditions employed. These problems require further study. In connection with the latter problem, it is interesting that chick embryo fibroblasts transformed by Rous sarcoma virus do not have a particularly high cloning efficiency, and cannot survive in culture, but they produce colonies in soft agar medium and tumors when implanted into host animals (TEMIN and RUBIN, 1958; RUBIN, 1966a and 1966b).

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