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The Oncogenic Potential of Three Different 7,12-Dimethylbenz(a)anthracene Transformed C3H/10T½ Cell Clones at Various Passages and the Importance of the Mode of Immunosuppression*

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Abstract—*The oncogenic potential of C3H/10T½ cells which were transformed* in vitro *with 7,12-dimethylbenz(a)anthracene is reported. The ability of the cells to grow as malignant tumors in syngeneic immunosuppressed mice was used as parameter for oncogenic potential. Cells of types* I, II *and* III *were assayed at several dosage levels, i.e.,* 10^4, 10^5 *or* 10^6 *cells per inoculum, with or without immunosuppression by antithymocyte serum globulin fraction. The studies were performed in several strains of host animals, i.e., male and female syngeneic C3H mice supplied by the National Cancer Institute, C3H mice supplied by Charles River and nude, athymic female mice. Morphological transformation preceded oncogenic transformation, and type* I *cells could not be established as tumors. Type* II *and type* III *cells developed oncogenic potential only after several passages in culture. Oncogenic potential was pronounced in the type* III *cells, and less strongly expressed in type* II *cells.*

Also tested were different methods of immunosuppression of the animal against the expression of the oncogenic potential of DMBA transformed C3H/10T½ cells from type II *and* III *clones. Immunosuppression by antithymocyte serum globulin fraction was an effective method of preparing the syngeneic host so that cells with a low oncogenic potential would grow as tumors, whereas total body irradiation was not effective. For cells with a high oncogenic potential both ways of immunosuppression were sufficient.*

Admixing lethally irradiated cells in the cell inoculum slightly enhanced the tumor development from cells with low oncogenic potential and such addition was clearly effective for cells with a higher oncogenic potential, both for the antibody-treated and for the irradiated series. The findings were reproducible. The study stresses the importance of immunosuppression by antithymocyte globulins for detecting in vitro *transformed weakly oncogenic cells.*

INTRODUCTION

CULTURED lines of mouse cells have proven useful in the study of oncogenic transformation [1]. A line of C3H mouse embryo cells designated C3H/10T½ shows strong postconfluence inhibition of cell division [2]. Transformation *in vitro* has traditionally been defined morphologically [3]. In the C3H/10T½ cell system, three types of morphological transformation were described after exposure to methylcholanthrene (MCA) or 7,12 dimethylbenz(a)anthracene (DMBA) [4]. Such transformed clones have individual tumor-specific transplantation antigens (TSTAs) [5].

Different *in vivo* assays for determining the oncogenic potential of transformed cells have been compared earlier [6], and it has been found that antithymocyte serum globulin (ATS) treated newborn hamsters, as well as monkeys, were more consistent in allowing the expression of tumors than were cortisonized adult hamsters. Stanbridge [7] reported that the optimal conditions for the growth of malignant cell populations were to be found in immunosuppressed mice exposed to thymectomy, followed by total body irradiation (TBI) and bone marrow reconstitution.

In these studies we have applied ATS and compared its effect on the expression of the

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oncogenic potential of the transformed cells with the method of total body sublethal X-irradiation of the host animal. An investigation was also undertaken to see whether addition of cells killed by radiation [8] would enhance the expression of the oncogenic potential of the inoculated cells.

The proof of real neoplastic conversion of *in vitro* transformed cells is that the cells, when implanted into suitable hosts, show progressive tumor growth. The ability of a cell line to grow as a transplanted malignant tumor is called oncogenic potential, and this parameter is quantitated as the inverse mean tumor development time.

Extensive studies of cells transformed *in vitro* by benzo(a)pyrene (BP), and other carcinogens have been reported [3, 4, 9–13]. Such studies are scanty in the case of cells transformed by DMBA. Studies of the oncogenic potential for the DMBA transformed cells have only been performed in few mice, and the results were inconclusive [4].

This communication shows the oncogenic potential of the DMBA transformed C3H/10T½ type II and type III cells at varying cell culture passages, and the lack of such potential in type I cells even after 46 *in vitro* passages.

MATERIALS AND METHODS

Cell and culture conditions

The origin and method of culture of the C3H/10T½ clone 8 cells have been described earlier [2]. All cultures were medium changed every 3½ days. The cells from the transformed clones were passaged every 7 days. At each passage the flasks (75 cm^2 Falcon flasks) were seeded with 0.25×10^6 cells and were grown for 3 cell doublings (to approximately 2×10^6 cells). The cultures then reached confluency, and the cells were passaged again. For large scale preparation, the cells were grown in roller bottles. These were seeded with 2.5×10^6 cells and harvested at 20×10^6 cells (1 passage or 3 cell doublings later). The cultures were routinely tested for mycoplasm infection [14]. These tests, as well as other tests performed by the State Laboratory of Hygiene at the University of Wisconsin, were negative for pleuropneumonia-like organisms (PPLO).

Transformation and scoring of morphological transformation

The transformation system, the scoring of the 3 types of transformed foci, and the isolation of clones were as reported elsewhere [4]. The DMBA was added at 2.5 μg/ml and the solvent acetone was added in the control series. Three types of DMBA-transformed foci could be found after 6–8 weeks, and were labeled I, II and III [4]. The type III foci constituted approximately 15% of the total number of transformed foci. The frequency of the type II foci was approximately 70% and the type I foci 10–15%.

Cell lineages

The passage number of the cells in culture is indicated after the Roman numeral indicating the type. Thus type III/7 signifies type III cells at the 7th passage. Photographs of the type I, II and III cells at the various passages in culture are shown in Fig. 1. These are representative pictures of the cell culture morphologies shown at one early (4th or 7th) and one late (32nd) passage. Throughout the experiments the cells were studied by phase contrast microscopy and no evidence was found for any spontaneous transition from type to type.

Mice

Syngeneic C3H mice, males and females, were supplied by the Frederick Cancer Research Center. These mice are labeled C3H NCI. Male C3H mice were also supplied from the animal farm at Charles River. These mice are labeled C3H CR. Athymic nude mice were supplied by the Frederick Cancer Research Center. The mice were four weeks old at the time of inoculation. The whole experiment involved 717 mice.

Cell inoculations

The cells were injected subcutaneously at the back in 0.5 ml of medium without serum with a 22 gauge 1½ inch hypodermic needle. The number of cells injected is indicated under each experiment.

Immunosuppression

The mice were immunosuppressed with anti mouse thymocyte globulins (Microbiological Associates, Bethesda, MD) essentially as reported by Arnstein *et al.* [15]. The activity of the serum was tested by the manufacturer, and the cytotoxicity reported as 1:6400, and the graft surviving time as 35.2 days. The ATS was dissolved in sterile water at 10 mg/ml, of which 0.25 ml was injected intraperitoneally with a 27 gauge needle at 25 hr before, 1 hr before, 24 hr after, 2 days after, 3 days after the cell inoculation and

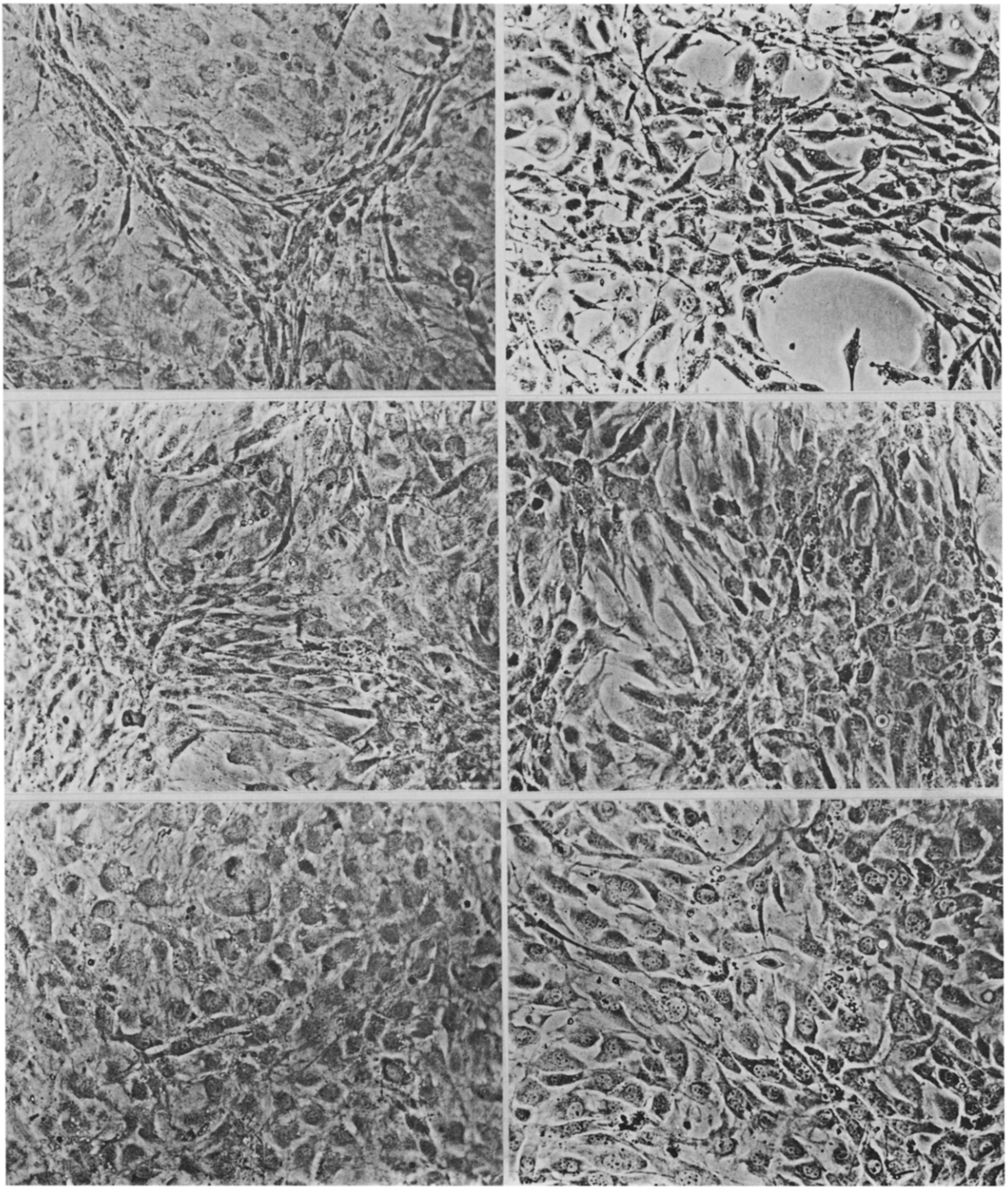

Fig. 1. *DMBA-transformed C3H/10T½ cells at various passages in culture. Phase contrast,* × 500. *Upper left: type* III/4 *cells* 6 weeks *postconfluence. Upper right: type* III/32 *cells* 1 week *postconfluence. Middle left: type* II/7 *cells* 6 weeks *postconfluence. Middle right: type* II/32 *cells* 1 week *postconfluence. Lower left: type* I/4 *cells* 6 weeks *postconfluence. Lower right: type* I/32 *cells* 1 week *postconfluence.*

subsequently every $3\frac{1}{2}$ days for 8 weeks. Alternatively, the mice were immunosuppressed by TBI with 600 rad from a ^{137}Cs source 4 hr prior to inoculation with the cells. The mice were palpated at least twice weekly. The tumor development time was defined as the number of days from the inoculation of the cells until the tumor was first palpable.

Preparation of lethally X-irradiated cells

The lethally X-irradiated cells were prepared as described by Peters and Hewitt [16]. The suspension of viable cells enclosed in a glass vial standing in an ice bath was exposed to 9 krad.

Tumors

The mice were palpated at least twice weekly for 12 months. The oncogenic potential of a cell type at a particular passage was determined by the number of injected animals that developed tumors, by the size of the inoculum necessary to start tumor growth and by the time from injection to palpable tumor could be observed. The tumors were removed when they reached 5 mm diameter and were studied histologically. All tumors were fibrosarcomas.

Chemicals and vessels

DMBA was purchased from Eastman Organic Chemicals, Rochester, N.Y. Acetone (Mallinckrodt Chemical Works, St. Louis, Mo) was purified by distillation. The Petri dishes and Falcon flasks were from Falcon Plastics, Oxnard, Ca. The medium and serum were obtained from Grand Island Biological Company, Grand Island, N.Y. ATS No. 59–210 was purchased from Microbiological Associates, Bethesda, Md.

RESULTS

The results are demonstrated in Tables 1–3 and illustrated diagrammatically in Fig. 2.

Modes of immunosuppression

The influence of different methods of immunosuppression on the oncogenic potential of the type III passage 20 cells (60 generations in culture) was first assayed. One series of host animals was immunosuppressed with ATS and another with TBI. Tumor development was observed for up to 360 days. All the mice developed tumors after an average of 57 days in the ATS-treated series. In the TBI group no tumors developed. Neither had any tumors developed after 360 days in the 6 control mice where immunosuppression had been omitted (Table 1).

The type III passage 38 cells (114 generations in culture) was also assayed. No tumors developed in the non-immunosuppressed mice, whereas all ATS-treated mice developed tumors after 30 days. All had tumors after 35 days in the TBI series, which is in contrast to the lack of tumor development in the TBI series of type III passage 20 cells (Table 1).

The type II cells at the 40th passage was tested under the same types of immunosuppression as above (Table 2).

To determine whether the conditions for expression of oncogenic potential of the cells could be further improved, the following method was examined. A number of 10^6 viable cells was mixed with a number of 10^6 lethally irradiated type III cells of the same passage, as described by Revesz [8]. For the type III passage 20 cells, a slight but consistent enhancement of tumor growth was observed (Table 1). For the passage 40 cells tumors appeared in all the mice at a substantially faster rate than for the cells injected without an addition of lethally X-irradiated cells (Table 1). The passage 38 and 40 cells are probably reasonably similar as regards the strengths of the oncogenic potential.

To see whether intramuscular cell deposit could further enhance the tumorigenicity in immunosuppressed animals, living cells with an admixture of dead cells were deposited in the hind leg. All the mice developed tumors after an average of 79 days (Table 1).

Type I cells

Type I/3, type I/8 and type I/13 cells were injected with 10^4, 10^5 and 10^6 cells per inoculum in immunosuppressed animals, and in non-immunosuppressed control animals with 10^6 cells. In none of the 12 series, each containing five C3H CR mice, did any tumor develop during 390 days of observation. The type I cells were also assayed at the 32nd and 46th passages in the C3H NCI male and female mice, in the C3H CR mice and in athymic mice. One million cells were injected per animal. In one series 10^7 cells were assayed. The experiments were undertaken with or without immunosuppression. In no cases did any tumors develop as shown in Table 3.

With a second type I clone, independently

Table 1. Tumor development by a clone of DMBA-transformed type III cells in C3H mice in the absence and presence of immunosuppression

Immunosuppression	Passage number of the 10^6 cells inoculum	Number of tumors/mice	Time of appearance	Average
ATS	20	2/2 2/2 2/2	50 days 57 days 65 days	57 days
TBI	20	0/6	360 days	
None	20	0/6	360 days	
ATS	38	5/5	30 days	30 days
TBI	38	4/4 1/1	30 days 56 days	35 days
None	38	0/6	300 days	
Viable cells admixed with 10^6 lethally X-rayed cells of the same passage:				
ATS	20	6/6	50 days	50 days
TBI	20	0/6	360 days	
None	20	0/6	360 days	
ATS	40	6/6	22 days	22 days
TBI	40	3/3 2/2 0/1	22 days 30 days 210 days	25 days
None	40	0/6	210 days	
Viable cells admixed with 10^6 lethally irradiated cells deposited intramuscularly:				
ATS	20	3/3 1/1 1/1	68 days 80 days 110 days	79 days

The expression of the oncogenic potential of DMBA-transformed C3H/10T½ type III cells was measured under different modes of immunosuppression. Antithymocyte serum globulin fraction (ATS) was compared to total body sublethal X-irradiation (TBI). Each inoculum contained 10^6 cells and each series contained six C3H mice 8 weeks old at time of inoculation. The cells were injected subcutaneously and deposited interscapularly (except where otherwise indicated). Cells were assayed at the 20th (60 cell doublings) passage and 38th respectively 40th (134 resp. 120 cell lethally X-irradiated cells (9 krad) in the inoculum and of intramuscular deposits (hind leg) are shown. All the tumors were removed when they reached 5 mm diameter, diagnosed histologically and were all fibrosarcomas. The tumor development time was defined as the number of days from inoculation of the cells until the tumor was first palpable.

derived 1 yr later, cell inoculations were performed at passages 6 and 15. In these series 10^5 and 10^6 cells were injected in immunosuppressed male CR and in male and female NCI mice. Control series were inoculated with 10^6 cells and immunosuppression was omitted. In none of the 9 series, each containing 5 mice when the observation period was terminated, had any tumors developed during 330 days of observation. Athymic nude mice were likewise inoculated with 10^6 type I/6 or type I/15 cells. The series contained 4 mice and were immunosuppressed. The mice survived for 40 days, but no tumors developed. Neither did any tumors develop in a non-ATS-treated series.

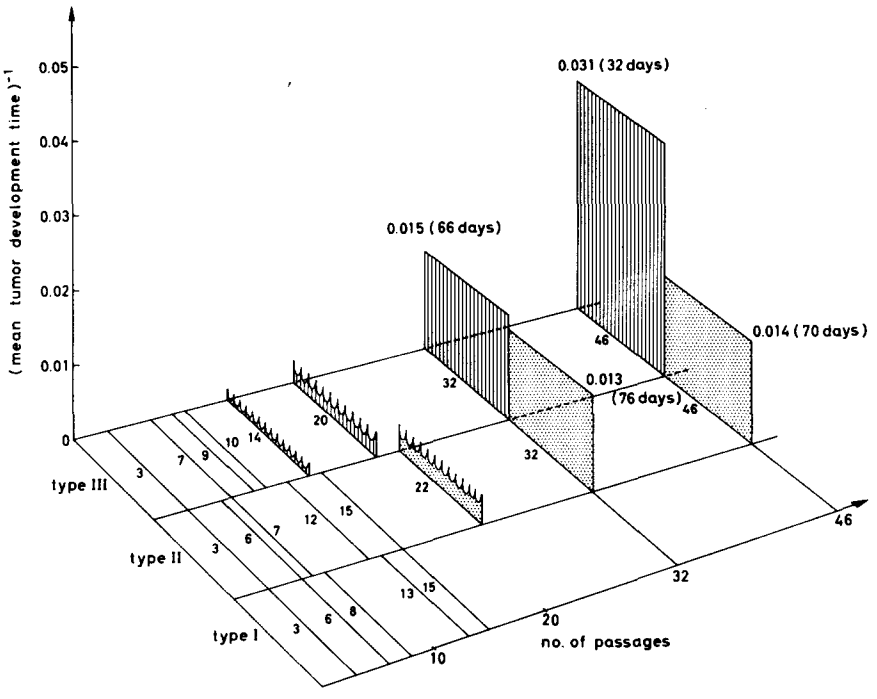

Fig. 2. Oncogenic potential of DMBA transformed C3H/10T½ cells (10^5) in ATS immunosuppressed syngeneic mice.

Table 2. Tumor development by a clone of DMBA-transformed type II *cells in C3H mice with or without immunosuppression*

Immunosuppression	Passage number of the 10^6 cells inoculum	Number of tumors/mice	Time of appearance	Average
ATS	40	2/2	54 days	59 days
		1/1	57 days	
		1/1	61 days	
		2/2	63 days	
TBI	40	1/1	50 days	
		1/1	53 days	
		1/1	123 days	
		0/3	240 days	
None	40	0/6	240 days	

The expression of the oncogenic potential of DMBA-transformed C3H/10T½ type II cells was compared under immunosuppression by ATS or TBI, as described under Table 1. Each inoculum contained 10^6 cells at the 40th culture passage.

Type II *cells*

The type II/3, type II/7 and type II/12 cells were injected with 10^4, 10^5 or 10^6 cells per inoculum in male CR mice with or without immunosuppression. One ATS-treated mouse injected with 10^6 type II/12 cells developed one tumor after 185 days. The 5 survivors after 380 days of observation in each of the 12 series did not develop any tumors.

The type II cells were then assayed at the 32nd and 46th culture passages with 10^6 or 10^5 cells per inoculum in the syngeneic immunosuppressed NCI male and female mice, in the CR strain and in athymic mice. In the non-immunosuppressed control series 5 mice were inoculated with 10^6 cells each. In case of the passage 46 cells with 10^6 cells per inoculum all the mice developed tumors. For the passage 32 cells the tumor development rates were slower, except in the CR and NCI male groups with 10^6 and 10^5 cells per inoculum respectively. None of the non-immunosuppressed C3H groups developed

Table 3. Tumor development from DMBA transformed type I, *type* II *and type* III *mouse embryo fibroblast cells at two different culture passages in several strains of mice*

Mice	Immunosuppression	Type I cells injected	Tumor/mice mean time of appearance, days		Type II cells injected	Tumor/mice mean time of appearance, days		Type III cells injected	Tumor/mice mean time of appearance, days	
			Passage 32 cells	Passage 46 cells		Passage 32 cells	Passage 46 cells		Passage 32 cells	Passage 46 cells
	ATS	10^6	0/3–270	0/1–334	10^6	5/5–58	4/5–46	10^5	4/4–75	4/4–33
C3H NCI	ATS				10^5	4/4–56	4/5–73	10^4	0/2–180	4/4–53
males	none	10^6	0/3–276	0/5–334	10^6	0/5–276	0/3–360	10^5	0/5–276	0/3–360
	ATS	10^7		0/3–258	10^6	4/4–51	4/4–32	10^5	3/3–66	4/4–32
C3H NCI	ATS	10^6	0/4–270	0/3–258	10^5	1/3–76	4/5–70	10^4	2/3–97	3/4–51
females	none	10^6	0/4–276	0/3–270	10^6	0/4–276	0/4–360	10^5	0/5–276	0/4–360
	ATS	10^6	0/4–260	0/1–334	10^6	5/5–43	5/5–45	10^5	4/4–60	5/5–43
C3H CR	ATS				10^5	5/5–59	4/5–47	10^4	3/4–69	2/3–44
males	none	10^6	0/5–270	0/4–334	10^6	0/5–276	0/4–360	10^5	1/5–139	1/4–160
									0/3–276	0/2–360
	ATS	10^6	0/3–30	0/3–38	10^6		2/2–19	10^5		4/4–23
Athymic	ATS				10^5		0/3–46	10^4		1/3–24
females	none	10^6	0/3–30	0/3–38	10^6	3/3–34	5/5–47	10^5	1/1–50	4/4–32
	none				10^5		1/1–44	10^4	1/1–50	

The DMBA-transformed C3H/10½ type I, type II and type III cells were assayed to determine whether they had any ability to grow to tumors at the 32nd or at the 46th culture passages. The cells were inoculated in male and female C3H NCI and C3H CR mice and in athymic mice. The cells were injected subcutaneously and deposited interscapularly at the titration levels indicated in absence or presence of immunosuppression. The mean tumor development time is given. The values ranged within $\pm 10\%$ of the mean. The tumors were diagnosed histologically, and all were found to be fibrosarcomas.

any tumors during the 360 days of observation. In the CR strain the tumor development rates were nearly the same with 10^5 and 10^6 cells per inoculum.

With a second type II clone that was independently derived one year later, passages 6 and 15 were tested for oncogenic potential with 10^5 and 10^6 cells per inoculum in the NCI male and female mice and in the CR mice. These series were immunosuppressed, and control series were inoculated with 10^6 cells per animal and immunosuppression was omitted. In none of the 9 series, each containing 5 mice after 360 days observation period, had any tumors developed. Athymic mice were likewise inoculated with type II/6 or type II/15 cells at the 10^5 or 10^6 titration level of cells per inoculum. Each of these series contained four mice. The four series were immunosuppressed, whereas immunosuppression was avoided in the 4 control series. In no case did any tumors develop during the 30 days of survival that the mice were observed.

Type III *cells*

Type III/3, type III/7 and type III/9 cells were injected at 10^6, 10^5 and 10^4 cells per inoculum in C3H CR mice in series that were immunosuppressed with ATS and in control series with 10^6 cells without immunosuppression. In none of the 12 series, each containing 5 mice, had any tumors developed during the 390 days of observation

Type III/32 and type III/46 cells were then assayed in syngeneic C3H NCI and C3H CR mice and in athymic mice. In each series 5 mice were injected, whereas the athymic series contained four mice. In the series that were immunosuppressed with ATS, 10^4 and 10^5 cells were injected per animal in separate series, vs 10^5 cells in the non-immunosuppressed control series. For the passage 46 cells with 10^5 cells per inoculum all the animals developed tumors. The immunosuppressed athymic mice showed the fastest tumor development. With 10^4 cells per inoculum the tumor development was faster in the C3H CR group than in the other syngeneic groups, and the same as at the 10^5 titration level of cells. In the non-immunosuppressed C3H groups tumors developed after 360 days in two cases as indicated in Table 3. The passage 32 cells developed tumors at a slower rate in all the series.

DISCUSSION

In transformation studies of hamster cells and of SWR mouse embryo cells in culture with BP, MCA and DMBA [9], tumor formation was reported for BP or MCA transformed cells only. Morphological transformation in culture with MCA, BP and DMBA was assayed in a mouse prostate system [17–19], and determination of the oncogenic potential was undertaken only for the MCA-transformed cells. Exposure of Syrian hamster embryo cells to BP, dibenzanthracene, MCA or DMBA gave rise to two types of colony morphology [10]. Cell lines from the BP-transformed clones [12] gave rise to fibrosarcomas in hamsters, but studies on the relative transplantability of the two varieties of the transformed colonies have not been reported. In the case of transformation of hamster embryo cells in culture by 4-nitroquinoline-1-oxide [13], the time between morphological transformation and neoplastic development was not consistent. The time which is required to obtain transformed cells that also give rise to tumors, was relatively long [20].

This study presents evidence that for DMBA-transformed C3H/10T½ mouse embryo cells, morphological transformation precedes oncogenic transformation in cell lines deriving from type II and type III clones. These cells develop their oncogenic potential after several passages in culture, and the acquisition of this potential thus appears to be gradual. The oncogenic potential was especially pronounced in the type III cells, less strongly expressed in the type II cells and never observed in the type I cells. Among the series that were assayed for oncogenic potential, the fastest tumor development arose from type III cells at the 46th passage. Tumors could only be found in immunosuppressed animals, except for two cases of type III cells at passage 46.

Other authors have previously shown oncogenic potential of DMBA-transformed type III/3 cells. A low tumor take was found when the cells were assayed in four animals, and a 50% tumor take was observed in eight mice assayed with the type II/3 cells [4]. The discrepancy with the observations in this paper is not easy to explain, but may be due to the different cell clones used.

The athymic mice represented a good source of hosts allowing tumor development from the type III/46 and the type II/46 cells after the same length of time as in the immunosuppressed syngeneic C3H mice. Since athymic mice possess thymus rudiments and are T cell

positive [21], the tumor development was alternatively assessed in athymic mice that were treated with ATS. The time period before tumors arose was then substantially reduced.

This suggests that the antigenicity of the cells and the immune system of the host are important for tumor development, since the more efficiently the immune response is suppressed, the more rapidly does the growth to tumors proceed.

One might argue that the increase in oncogenic potential of the type III and the type II cells with passage in culture might be due to a concomitant loss of antigenicity, making a rejection of the injected cells less and less likely. Under such circumstances the non-immunosuppressed series would be expected to allow some tumor growth. This was practically never observed, and the role of a decreased antigenicity as the main cause of an increased oncogenicity is less likely. This finds support in studies where [22] it appeared that new cell surface antigens were secondary characteristics of malignant cells, and the lack of cell surface antigenicity was not essential for malignancy. However, it cannot be ruled out that loss of antigenicity gives some contribution to the increased tumor development.

Some studies pointed out the lack of correlation between oncogenicity and expression of tumor-specific transplantation antigens [23–25]. Other studies demonstrated that the development of malignancy was not associated with the loss of TSTAs [26]. For some virus-induced tumors some evidence suggests that high oncogenic potential correlates with low antigenicity [24], but most evidence suggests that no such relationships exist [25–28]. Viral transformation studies of hamster embryo cells in culture have also presented evidence of oncogenic potential which was low or not detectable soon after morphological transformation, but which increased progressively as the cells were passaged in culture [25, 28, 29].

The growth pattern morphology of the cells at the various passages are shown in Fig. 1. Whereas the type III cells grow with a corded pattern at the early passages, these cells easily detach from the culture flask at the higher passages. Thus open areas may be seen in the postconfluent culture. This behavior may reflect the fibrinolytic activity displayed by tumorigenically transformed cells [30]. The type II cells appeared to retain the same culture morphology at the various passages. The tumorigenic type II cells showed a slighter tendency to detach than the type III cells. The type I cells retained the same growth pattern morphology and showed no tendency to detachment.

The fact that even the type III cells need several passages before they achieve real oncogenic potential, is difficult to elucidate. If each clone of the cells originally picked from the culture consisted of a variety of cells, one might expect a heterogeneity of karyotypes. Preliminary investigations of the DNA content of these cells by flow cytophotometry have indicated only one major, stable population for the cell lines from each type of clone. The non-transformed cells have no cells with higher DNA content than what is present during the G_2 phase of their cell cycle. The transformed cells contain very small subpopulations of cells with a higher ploidy which appears to remain stable. However, the DNA-content in the major population of the transformed cells decreases with increasing passage.

These observations render the possibility less likely that a selection of a subpopulation of cells (which originally had a fully expressed oncogenic potential) takes place through the passages. However, it does appear more likely that an intrinsic cell change becomes more and more expressed by the number of cell doublings in culture and thus the morphologically transformed type III and type II cells go through a process of tumor progression as described by Foulds [31]. The mechanism of such progression remains unknown.

In cell culture there is always the possibility of contamination with other malignant cells. For these cells, this possibility is probably nil, due to very strict culture conditions. The gradual development of the oncogenic potential in the type II and III cells, and its absence in the type I cells, which in this respect serve as internal control, makes the possibility that a random contamination with a malignant cell has taken place unlikely.

By scanning electron microscopy studies of these DMBA-transformed cells, it has been possible to find a positive correlation between the oncogenic potential and the appearance of microvilli on the cell surface [32]. The mechanism behind this increase in the fraction of cells with many microvilli at the surface, is not clear, but may be provisionally classified as a morphological marker of tumor progression.

The ATS-treated animals allowed the expression of the oncogenic potential of these cells more consistently than the TBI series (Table 2). Thus, for the weakly oncogenic type II cells, immunosuppression by ATS also proved more

efficient than TBI, similar to what was found for type III passage 20 cells.

The TBI method was only slightly less efficient than ATS in demonstrating the stronger oncogenic potential of the type III passage 38 cells, Our findings show that the passage 20 cells have a low oncogenic potential and the passage 38 a stronger one. Thus our present study suggests that in order to allow tumor growth from weakly oncogenic cells, immunosuppression by ATS is more effective. Thus the importance of using the ATS method for immunosuppression in assaying oncogenic potential of weakly oncogenic cells is clearly shown.

Furthermore, our findings indicate that the effect of an admixture of dead cells is of greater significance for tumor growth of the more oncogenic cells, than of the weakly oncogenic cells.

Intramuscular cell deposits were found to delay tumor development compared to subcutaneous inoculations.

The data suggest the usefulness of ATS immunosuppression in demonstrating the oncogenic potential of *in vitro* chemically transformed cells with low degree of malignancy. For more malignant cell variants (type III passage 38 cells) immunosuppression by TBI is also a good method. The enhancement of the tumorigenicity by adding dead cells described by Revesz [8], was of greater significance for the more oncogenic than for the less oncogenic cells.

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REFERENCES

1. C. Heidelberger, Chemical carcinogenesis. *Ann. Rev. Biochem.* **44,** 79 (1975).
2. C. A. Reznikoff, D. W. Brankow and C. Heidelberger, Establishment and characterization of a clonal line of C3H mouse embryo cells sensitive to postconfluence inhibition of division. *Cancer Res.* **33,** 3231 (1973).
3. J. A. Dipaolo, R. L. Nelson and P. J. Donovan, Morphological, oncogenic and karyological characteristics of Syrian hamster embryo cells transformed *in vitro* by carcinogenic polycyclic hydrocarbons. *Cancer Res.* **31,** 1118 (1971).
4. C. A. Reznikoff, J. S. Bertram, D. W. Brankow and C. Heidelberger, Quantitative and qualitative studies of chemical transformation of cloned C3H mouse embryo cells sensitive to postconfluence inhibition of cell division. *Cancer Res.* **33,** 3239 (1973).
5. M. J. Embleton and C. Heidelberger, Neoantigens on chemically transformed cloned C3H mouse embryo cells. *Cancer Res.* **35,** 2049 (1975).
6. J. C. Petricciani, K. E. Wallace and D. W. McCoy, Comparison of three *in vivo* assays for cell tumorigenicity. *Cancer Res.* **34,** 105 (1974).
7. E. J. Stanbridge, L. R. Boulger, C. R. Franks, J. A. Garrett, D. E. Reeson, D. Bishop and F. I. Perkins, Optimal conditions for the growth of malignant human and animal cell populations in immunosuppressed mice. *Cancer Res.* **35,** 2203 (1975).
8. L. Revesz, Effect of tumor cells killed by X-rays upon the growth of admixed viable cells. *Nature (Lond.)* **178,** 1391 (1956).
9. Y. Berwald and I. Sachs, *In vitro* transformation of normal cells to tumor cells by carcinogenic hydrocarbons. *J. nat. Cancer Inst.* **35,** 641 (1965).
10. J. A. Dipaolo, P. Donovan and R. Nelson, Quantitative studies of *in vitro* transformation by chemical carcinogens. *J. nat. Cancer Inst.* **42,** 867 (1969).
11. J. A. Dipaolo, P. J. Donovan and R. L. Nelson, *In vitro* transformation of hamster cells by polycyclic hydrocarbons: factors influencing the number of cells transformed. *Nature, New Biol.* **230,** 240 (1971).
12. J. A. Dipaolo, R. L. Nelson and P. J. Donovan, Sarcoma-producing cell lines derived from clones transformed *in vitro* by benzo(a)pyrene. *Science* **165,** 917 (1969).
13. T. Kuroki and H. Sato, Transformation and neoplastic development *in vitro* of hamster embryonic cells by 4-nitro-quinoline-1-oxide and its derivatives. *J. nat. Cancer Inst.* **41,** 53 (1968).
14. V. Allen, S. Sueltmann and C. Lawson, Laboratory diagnosis of mycoplasma pneumonia in public health laboratory. *Hlth. Lab. Sci.* **4,** 90 (1967).

15. P. Arnstein, D. O. N. Taylor, W. A. Nelson-Rees, R. H. Huebner and E. H. Lennette, Propagation of human tumors in antithymocyte serum treated mice. *J. nat. Cancer Inst.* **52,** 71 (1974).
16. L. J. Peters and H. B. Hewitt, The influence of fibrin formation on the transplantability of murine tumor cells: implications for the mechanism of the Revesz effect. *Brit. J. Cancer* **29,** 279 (1974).
17. T. T. Chen and C. Heidelberger, Cultivation *in vitro* of cells derived from adult C3H mouse ventral prostate. *J. nat. Cancer Inst.* **42,** 908 (1969).
18. T. T. Chen and C. Heidelberger, *In vitro* malignant transformation of cells derived from mouse prostate in the presence of 3-methylcholanthrene. *J. nat. Cancer Inst.* **42,** 915 (1969).
19. T. T. Chen and C. Heidelberger, Quantitative studies of the malignant transformation of mouse prostate cells by carcinogenic hydrocarbons *in vitro*. *Int. J. Cancer* **4,** 166 (1969).
20. J. A. Dipaolo and P. J. Donovan, Properties of Syrian hamster cells transformed in the presence of carcinogens. *J. nat. Cancer Inst.* **48,** 361 (1967).
21. M. Holub, R. Rossmann, H. Tlaskalova and H. Vidmarova, Thymus rudiment of the athymic mouse. *Nature (Lond.)* **256,** 491 (1975).
22. M. J. Embleton and C. Heidelberger, Antigenicity of clones of mouse prostate cells transformed *in vitro*. *Int. J. Cancer* **9,** 8 (1972).
23. L. M. J. Harwood and P. H. Gallimore, A study of the oncogenicity of adenovirus type 2 transformed rat embryo cells. *Int. J. Cancer* **16,** 498 (1975).
24. C. C. Ting, D. H. Lavrin, K. K. Takemoto, R. C. Ting and R. B. Herberman, Expression of various tumor-specific antigens in polyoma virus induced tumors. *Cancer Res.* **32,** 1 (1972).
25. T. Wesslen, SV-40 tumorigenesis in mouse. *Acta path. microbiol. scand.* B **78,** 479 (1970).
26. S. Kit, T. Kurimura and D. R. Dubbs, Transplantable mouse tumor line induced by injection of SV-40 transformed mouse kidney cells. *Int. J. Cancer* **4,** 384 (1969).
27. G. Klein and H. Harris, Expression of polyoma-induced transplantation antigen in hybrid cell lines. *Nature New Biol.* **237,** 163 (1972).
28. S. S. Tevethia and V. L. McMillan, Acquisition of malignant properties by SV-40 transformed mouse cells: Relationships to type C viral antigen expression. *Intervirology* **3,** 269 (1974).
29. V. Defendi and J. M. Lehman, Transformation of hamster embryo cells *in vitro* by polyoma virus: morphological, karyological, immunological, and transplantation characteristics. *J. cell. comp. Physiol.* **66,** 351 (1965).
30. W. E. Laug, P. A. Jones and W. F. Benedict, Relationship between fibrinolysis of cultured cells and malignancy. *J. nat. Cancer Inst.* **54,** 173 (1975).
31. L. Foulds, Multiple etiologic factors in neoplastic development. *Cancer Res.* **25,** 1339 (1965).
32. H. J. K. Saxholm, O. H. Iversen and A. Reith, Scanning electron microscopy of DMBA transformed C3H/10T½ cells of varying oncogenic potential. *Proc. Amer. Ass. Cancer Res.* **19,** 171 (1978).