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Suppression of Tumorigenicity in Cybrids

We investigated cytoplasmic control of tumorigenicity in cybrids. Cytoplasts derived from nontumorigenic cells were fused to the highly tumorigenic 984 C1 10–15 cell line derived from a murine teratoma. The resultant cybrids did not retain the tumorigenicity of the original cell line. In addition, the majority demonstrated the ability to differentiate into skeletal muscle. The results of these experiments indicate a heritable suppression of the tumorigenic phenotype by nontumorigenic cytoplasm. These findings are in contrast to our previous experiments in which we used a different experimental system and demonstrated a nuclear control of tumorigenicity in cybrids.

Analyzing tumorigenicity using cell hybridization techniques has produced conflicting results. While tumorigenicity is dominant in a wide variety of hybrids formed between tumorigenic and nontumorigenic cells, many recent studies have reported the suppression of tumorigenicity in other hybrid combinations [1–5]. Studies by Stanbridge [1] and Marshall and Dave [2] suggested that tumorigenicity behaved as a recessive trait and that the suppression observed resulted from a complementation effect. Jonasson and Harris and co-workers [3–5] demonstrated that suppression of tumorigenicity existed when hybrids were produced between diploid human fibroblasts and tumorigenic murine melanomas. In these studies suppression was observed in hybrids containing only the single human X chromosome. In other studies, suppression was also observed in hybrids that contained no recognizable human chromosomes [4]. Experiments using gamma radiation suggested that suppression of tumorigenicity in hybrids between these cell lines may have involved the activity of extrachromosomal elements which were radiosensitive. These studies clearly illustrated the necessity of separating the various nuclear and cytoplasmic factors that may be important in suppressing the tumorigenic phenotype in whole cell hybrids, and suggested the need for a new approach to determine if normal cytoplasm alone can suppress the tumorigenic phenotype.

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Using cell enucleation techniques [6, 7] it is possible to separate the nucleus from the cytoplasm, and it has been demonstrated that it is feasible to fuse these anucleate cytoplasms (cytoplasts) with whole cells, resulting in a mononuclear, cytoplasmic hybrid termed a cybrid [8]. The construction of cybrids between tumorigenic cells and cytoplasts from nontumorigenic cells has recently been reported. Studies by Howell and Sager [9] and Shay et al [10] reported that the normal cell cytoplasm did not have any effect on the suppression of the tumorigenic state in cybrids. In these studies the tumorigenic cells were virally transformed. Therefore, it now appears to be a consistent phenomenon that the tumorigenic phenotype is dominant in experimental systems using virally transformed cells.

In another series of experiments using diploid spontaneously transformed cells, Howell and Sager [9] demonstrated that nontumorigenic cytoplasts could effect a suppression of tumorigenicity when cybrids were produced. In the present study, nondiploid cybrids were produced between nontumorigenic cytoplasts and spontaneously transformed cells derived from a teratoma. The results of our experiments clearly indicate a strong heritable suppression of tumorigenicity, and suggest that nontumorigenic cytoplasmic factors may suppress tumorigenicity.

MATERIALS AND METHODS

Cell Lines

A skeletal muscle cell line isolated from a pluripotential mouse teratocarcinoma has been previously described [11]. It undergoes normal terminal differentiation in cell culture and has been designated C17-S1-D-T984 (hereafter referred to as 984 C1 10). We have isolated a stable subclone of this line which is highly tumorigenic (984 C1 10–15) and has lost the ability to differentiate into skeletal muscle.

The nontumorigenic A-MT-BU-A1 cell line (hereafter designated AMT) was originally derived from MT-29240 in Dr. Hayden Coon's laboratory. The AMT cell line is contact-inhibited, contains intracisternal A virus particles, but is nontumorigenic as tested by lack of growth in soft agar and nude mice [12]. All cells utilized in these experiments, as well as the cybrids characterized, were found to be free from mycoplasma contamination by two different assays [13, 14]. Even though all murine cells contain virus sequences that are homologous to a family of viruses called murine leukemia virus, the AMT cells do not release viruses. However, the 984 C1 10–15 cells as well as cybrids shed C type virus particles.

Enucleation and Fusion Procedure

The AMT cells were enucleated in the presence of cytochalasin B with >95% efficiency by techniques previously described [12, 15]. The enucleated AMT cells were mixed with 984 C1 10–15 cells and fused using polyethylene glycol 400 with a fusion efficiency of 5% as previously described [16].

Selection of Cybrids

The tumorigenic 984 C1 10–15 cell line is resistant to HAT selection and sensitive to chloramphenicol (CAP^s). The AMT cell line [17] is sensitive to

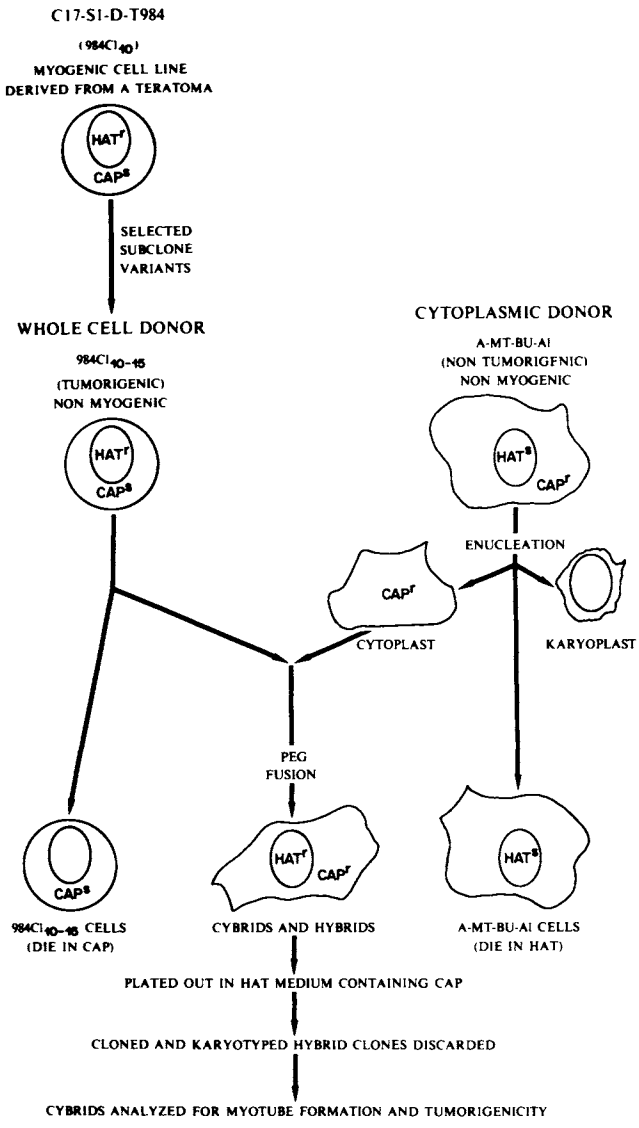


Fig. 1. Selection technique for isolating cybrid clones. See Methods for discussion.

HAT selection owing to absence of thymidine kinase (TK^-). It is also resistant to chloramphenicol growing in 50 μg/ml CAP, a concentration that will kill all 984 C1 10–15 cells.

Figure 1 illustrates the protocol for isolating cybrids between these cells. The CAP-reistant (CAP^r) AMT cytoplasts were fused to HAT-resistant (HAT^r) 984 C1 10–15 cells, and the fusion product was selected by culture in medium containing both HAT and CAP [18]. Unfused 984 C1 10–15 cells died in CAP, whereas intact AMT cells died in HAT. Clones were then isolated and karyotyped to identify and eliminate contaminating hybrid clones that also sur-

TABLE I. Differentiation and Tumorigenicity Properties

Differentiation	
No. of differentiating subclones / Total no. of 984 C1 10–15 subclones isolated	0 / 14
No. of differentiating cybrid clones / Total no. of cybrid clones isolated	17 / 27
Tumorigenicity	
No. of tumors formed / No. of mice injected with 984 C1 10–15 subclones	14 / 14
No. of tumors formed / No. of mice injected with differentiating cybrids	1 / 17
No. of tumors formed / No. of mice injected with nondifferentiating cybrids	1 / 10

For determining tumorigenicity, 10^6 cells of each subclone or cybrid clone were injected subcutaneously into nude mice. Animals were sacrificed when 1–2 cm diameter tumors were observed or 6 months elapsed, whichever occurred first. Differentiation was determined by placing subclones or cybrid clones in culture and observing if myotube formation occurred.

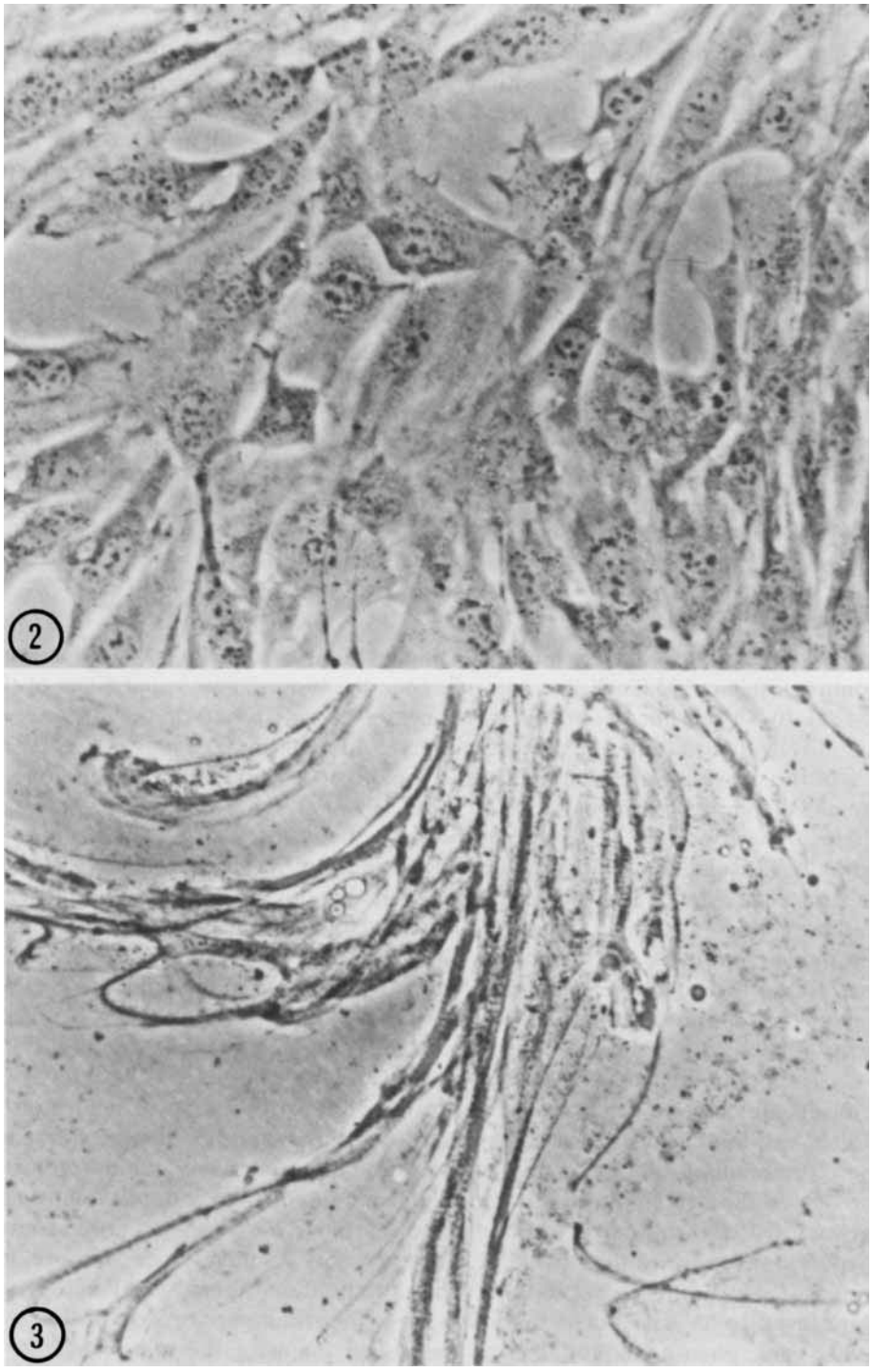
vived in the selection medium. It was estimated that the reversion frequency for the CAP^r mutation was less than 10^{-9} and for the TK^- mutation less than 10^{-8}. Using a combination of this selection technique and karyotyping, there was no evidence that there were hybrids or parental contaminants in the cybrid population.

Animals

Congenital athymic nude BALB/c male and female mice, 8–12 weeks old, homozygous for the nu/nu allele, were used in this study. The mice were obtained from the National Cancer Institute (Frederick, Maryland) and maintained in a pathogen-free environment. A colony of mice was established by breeding BALB/c–nu/nu homozygous males and BALB/c–+/nu heterozygous females.

Preparation of Cell Suspension for Injections

Cells were obtained from cultures in the log phase of growth. Cells to be injected were dispersed from the substrate using 0.5% trypsin, washed with sterile Dulbecco's Modified Eagle's Medium (GIBCO), and resuspended in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum (Kansas City Biologicals) at a concentration of 1.0×10^7 viable cells/ml. Using a 21-g needle, 10^6 cells were injected into the subcutaneous tissue of the back of nude mice. In this study the 984 C1 10–15 and AMT parentals as well as cybrid cells were injected within a 2-week period of each other into matched groups of nude mice in order to make meaningful comparisons. In this series of experiments, each of the 27 cybrid and 14 parental clones was tested twice for tumorigenicity, and Table I lists these as cumulative results. Because negative results could be due to the recipient animals, in each experiment both parentals and cybrids were injected into separate animals. Since the 984 C1 10–15 parental cells formed tumors in all instances and the AMT parental cells did not, it is unlikely that the negative results of the cybrids were due to the recipient animals.



Figs 2 and 3 Phase contrast photomicrographs of nondifferentiating 984 C1 10–15 cells (Fig 2) and differentiating cybrid cells (Fig 3) One hundred percent of the 984 C1 10–15 subclones were non differentiating, whereas 37% of the cybrids clones were nondifferentiating

RESULTS

We isolated and characterized 14 subclones of the highly tumorigenic 984 C1 10–15 cell, and demonstrated that all of these subclones were tumorigenic and produced undifferentiated sarcomas when injected into nude mice (Table I). When isolated colonies of these 14 subclones were examined for their ability to differentiate in cell culture, we observed that none of the clones examined were able to differentiate into myotubes (Fig. 2). Isolated subclones of the fibroblast AMT cell line were all observed to be nontumorigenic, and did not differentiate into myotubes in cell culture.

The cytoplasmic hybrids (cybrids) isolated by fusing AMT cytoplasts to the subclones of the 984 C1 10–15 cells yielded the following results: 17 of the 27 cybrid clones (63%) examined were capable of differentiating into myotubes in cell culture (Fig. 3). However, of all 27 cybrid clones isolated and injected into nude mice, only two clones developed undifferentiated sarcomas. One of the ten cybrid clones that were incapable of differentiation was still tumorigenic, and one of the 17 cybrid clones that were capable of differentiating into myotubes in cell culture was tumorigenic. However, nine of the ten nondifferentiating and 16 of the 17 differentiating cybrid clones lost the ability to form tumors.

DISCUSSION

The results of these experiments suggest that cytoplasmic factors in the nontumorigenic AMT cell line can influence the tumorigenic capabilities of the 984 C1 10–15 cell line. Fourteen subclones of the 984 C1 10–15 cell line were found to be 100% tumorigenic when injected in nude mice, whereas less than 8% of the cybrids were observed to be tumorigenic. In addition, while none of the cells from subclones of the 984 C1 10–15 cells were capable of forming clones that differentiated into myotubes in cell culture, nearly 63% of the cybrid clones isolated were capable of differentiation. Even though there was a general concordance between differentiation and suppression of tumorigenicity in the cybrids examined, there were cybrids isolated that were suppressed and incapable of terminal differentiation. These results support the conclusion that factor(s) in the nontumorigenic cell cytoplasm are able to induce long-term heritable suppression of tumorigenicity. In addition, this phenomenon is not necessarily related to the ability of the cells to differentiate terminally in culture.

The possibility exists that the "normal" cytoplasm fused to the tumorigenic cell line was active since it was derived from a permanent, nondiploid cell line. The state of differentiation of this nontumorigenic cell line may therefore be important in its influence on the other cell. Thus, the difference in the degree of differentiation of the cytoplasmic donor may result in failure of the cybrids to form tumors when injected into nude mice, perhaps because of a cytotoxic response to the host [19]. However, this is unlikely, since in previous experiments we have observed that when the nontumorigenic AMT cytoplasts were fused to SV_{40} 3T3 cells there was no evidence of suppression of the tumorigenic phenotype [10]. Thus it was surprising using the same AMT cytoplasm that we observed such a strong suppression of tumorigenicity in the 984 C1 10–15 cells. It has been suggested [20] that only transformed cells that have a normal diploid karyotype are capable of being suppressed. For example,

in a series of experiments by Howell and Sager [9] suppression of tumorigenicity in cybrids was observed using transformed whole cells containing a diploid karyotype. However, in the present study we observed suppression of tumorigenicity using transformed whole cells that were clearly abnormal in their karyotype (984 C1 10–15 chromosome range 60–70).

An important similarity between the experiments of Howell and Sager and those presented here is that in both instances the tumorigenic whole cells are spontaneously and not virally transformed. It could therefore be suggested that there is a basic and important difference between virally and spontaneously transformed cells and that factor(s) present in nontumorigenic cytoplasm may suppress the tumorigenic phenotype of spontaneously transformed cells but not virally transformed cells.

There are, however, several reports that tumorigenic cell lines that arose from spontaneous or chemically induced tumors are not suppressible by nontumorigenic cytoplasm [12, 20–22]. These data could thus be interpreted as indicating that the process of making cybrids between tumorigenic cells and nontumorigenic cytoplasms selects for non- or minimally tumorigenic cells, as suggested by Coon [20], or else the teratocarcinoma system is a special form of neoplasia. Experimental evidence suggesting that the latter is correct is that when murine teratoma cells are injected into a mouse blastocyst the tumorigenic cells may lose their malignant phenotype and differentiate normally [23–25]. Another experiment that supports this interpretation is the recent report of Litwack and Croce [26]. They demonstrated that hybrids between two tumorigenic cell lines, one a mouse teratocarcinoma and the other a rat hepatoma, were tumorigenic when injected into nude mice [26]. However, these same hybrids, which were not diploid, were able to differentiate normally when placed in a mouse blastocyst. These experiments and the ones reported here suggest that the teratocarcinoma system may be a valuable one in studying cell differentiation and perhaps certain aspects of neoplasia. We urge caution, however, in attempting to make any generalization concerning suppression of tumorigenicity based on these experiemnts.

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