

Cell transformation as aberrant differentiation: Environmentally dependent spontaneous transformation of NIH 3T3 cells

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ABSTRACT

NIH 3T3 cells, a mouse fibroblast cell line used as routine target cells for transfection experiments, undergo sponitaneous transformation in our experiments after they form a confluent sheet in medium containing fetal bovine serum (FBS) or lower concentration of calf serum (CS). The transformation takes the form of foci of multiplying cells among the surrounding cells which have stopped cell division. However, no focus of transformed cells could be seen in medium containing high concentration (10 %) of CS. Further experiments indicated that tho frequency of transformation is highly dependent on the concentration of serum and the transformation in CS is changeable when the cells are passaged in FBS. ³H-thymidine autoradiography has been proved to be a sensitive measurement indicadtor for focus formation. Our results suggest that the high frequency of transformation and its dependence on confluency as well as on medium composition are characteristics of cell differentiation rather than mutation. The role of the NIH 3T3 cell line as a eaneer-initiated cell population and its accelerated transformation by *ras* oneogene might be considered as a form of tumor promotion is discussed.

Key words: *Gell transformation, aberrant differentiation, NIH 3T3 cells.*

INTRODUCTION

The NIH 3T3 line of mouse embryo cells is the preferred target for transfection by presumptive cellular oncogenes [1], presumably because it readily becomes transformed when the oncogene is integrated into the cell's genome. However, we have found that spontaneous transformation occurred readily *in* these cells when they become confluent.

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Even the non-transformed NIH 3T3 cells would produce sarcomas in nude mice when inoculated in large numbers, although the latent period for the detection of tumors would be longer than that by morphologically transformed NIH 3T3 cells [2, 3]. The phenomenon of spontaneous transformation in culture was first encountered in cell strains derived directly from mouse embryos where it occurs only after 4 months to 4 years of culture [4–6]. The rare and sporadic occurrence suggested that spontaneous transformation arose from a mutation-like event among cells of the cultures [6].

Despite the sporadic nature of spontaneous transformation in Balb/c 3T3 cells, we have noticed over the years that it occurs more frequently after seeding the cells in agar, where their multiplication is greatly restricted, than when attached to a solid substratum, where their multiplication is maximized [7, 8]. The low frequency of spontaneous transformation in the Balb/c 3T3 cells prevented further systematic study of the phenomenon in these cells. However, the present study shows that the spontaneous transformation of NIH 3T3 cells was highly dependent on culture conditions, i.e., it occurred when the cells were cultured in growth limiting concentration of serum and maintained as confluent, non-growing cultures without passage for one or more weeks. These growth limiting conditions evoked an analogy to the conditions of cell suspension which favored transformation of Balb/c 3T3 cells. It was possible, however, that the confluent state was only needed as a background to display the unregulated growth of the transformed cells rather than to induce the transformation. The experiments reported here were done to determine whether confluency is a prerequisite for transformation, or is merely required for distinguishing the already transformed cells as primary center of expansively growing foci set off against a flat monolayer of density-inhibited nontransformed cells. The evidence presented here supports an inductive role of confluency for the morphological transformation of NIH 3T3 cells. It also reveals that the serum requirements for transformation change with repeated passage of the NIH 3T3 cells.

MATERIALS AND METHODS

Cells and culture methods

NIH 3T3 cells were originally derived from NIH Swiss mouse embryo cultures by selecting clones with a high plating efficiency and low saturation density [9]. They were kindly supplied by Dr. S. A. Aaronson, and cryopreserved after one month of twice-weekly low density passages in Dulbecco's Modified Eagle's medium plus 20% FBS and 10% DMSO. A vial was thawed 3 months later, and cultured in molecular, cellular and developmental biology medium 402 (MODB 402) [10] plus 10% FBS. The first experiment was started one week after thawing a cryopreserved vial of cells, and the second experiment two months after further passaging the cells weekly at 10^5 per 60 mm plastic dish cultured in 10% FBS MODB 402, cells were trypsinized and counted electronically.

Cells of the 03H 10T1/2 clone 8 subclone 2 lines were obtained from Dr. A. Feinberg were used as background cells in the first experiment for assaying focus formation by transformed NIH 3T3 cells. 10^5 C3H 10T1/2 cells were seeded together with 10^3 NIH 3T3 cells, incubated for 11, 14 and 17 days in MCDB 402 medium plus 10% FBS, then fixed in methanol, stained with Giemsa stain, scored the number of transformed foci per dish for

two dishes in each group every time and averaged the number of foci from each group respectively.

Foci were recognized as discrete groups of cells which were more elongated and refractile than the flat, isometric cells around them; they were also more compact and had more mitoses than the surrounding cells. They could be easily identified by Giemsa staining, or by labeling the cells with ^3H -thymidine ($1\mu\text{ Ci per ml}$) for one hour and processing for autoradiograph [11]. The cells in the foci stained darker than the surrounding cells and could be distinguished from interfocal areas by the high frequency of labeled cells.

Calf serum (CS) and fetal bovine serum (FLUS) were obtained from Hyclone Laboratories, Logan, Utah. MCDB 402 medium was prepared in the laboratory.

All experiments were repeated twice with basically the same results.

RESULTS

Autoradiographic study of spontaneous transformation

NIH 3T3 cells became transformed morphologically when they were cultured in 10% FBS and in 2% or 5% but not in 10% of CS. Since cells grew to such a high density in 10% GS that no foci of transformed cells would be discriminated from the dense background cells, if there were any. Therefore, we used a functional rather than a strictly morphological criterion to discriminate whether cells were transformed or not.

Cells from 10% FBS original culture were seeded in 2% CS, 10% CS or in 10% FBS, counted every 5 days for 30 days and also pulse labeled in situ with ^3H -thymidine. The proportions of labeled cells in identified foci and background were determined. In addition, the proportion of labeled cells in 10% CS was determined in randomly chosen fields.

The cultures multiplied faster and reached a higher population density in 10% CS than in either 2% CS, or 10% FBS (Fig. 1). All cultures were confluent at day 5. Exponential growth did not occur beyond day 5, but the cell numbers in all cultures continued to rise up slowly. Transformed foci first became visible in 10% FBS at day 10 and at day 15 in 2% CS, and their number increased continuously until day 30 (Fig. 2).

The number of foci remained much higher throughout in 10% FBS than in 2% CS. At no time there were any foci in 10% CS.

The percentage of ^3H -thymidine labeled cells in the foci and in the background between day 15 and day 25 is shown in Fig. 3. There were 30% to 43% labeled cells in the foci of cultures in 2% CS and 10% FBS respectively, compared with 2% to 7% labeled cells in the monolayer areas surrounding the foci. Twelve random fields were counted at each time point in the 10% CS cultures; the highest fraction of labeled cells in these fields was 14%. We conclude therefore that there were few labeled cells in 10% CS cultures which had the unregulated growth characteristic of transformed cells, but did not form foci. This is the main difference of 10% CS group from those of other groups in morphology.

To determine whether the transformed characteristic of cells in the foci was stable, 10^3 cells of the 20th day cultures in each category were added to 10^5 of The C3H 10T1/2 line. The mixed cultures were maintained in 10% FBS and foci enumerated between day 11 and day 17 later. In the group of NIH 3T3 cells originally cultured in 10% FBS, about 3% of the transferred cells would grow into foci on day 17 (Fig. 4). About 1% of the NIH 3T3

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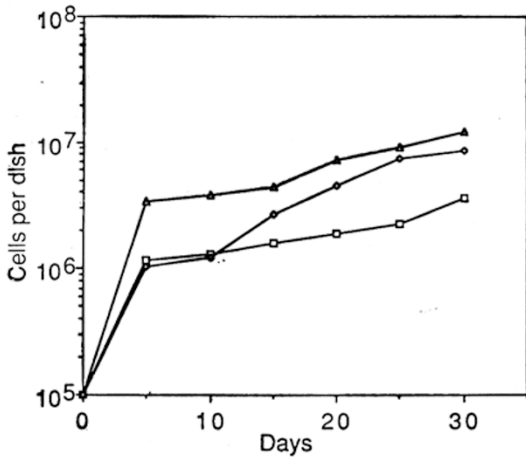


Fig. 1 Growth of one-week post-thaw NIH 3T3 cells in FBS. One week after thawing, 10^5 cells were seeded in MCDB 402 plus 2% 08, 10% CS or in 10% FBS. The medium was changed every other day after fourth day. The cells were trypsinized and counted every fifth day for 30 days. □, 2% CS; △, 10% CS; ○, 10% FBS.

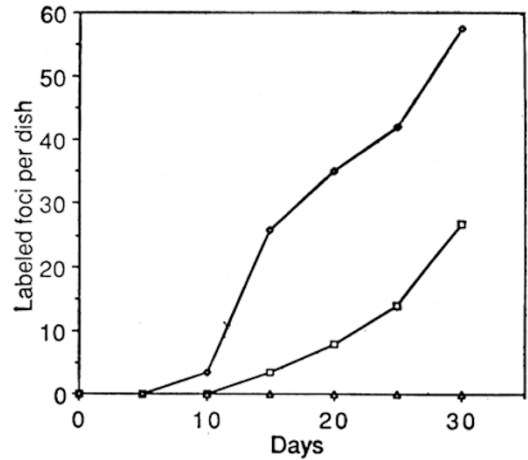


Fig. 2. Counting foci with high percentages of ^3H -thymidine labeled nuclei. Sister cultures to those of Fig. 1 were labeled for one hour with ^3H -thymidine at 5 day intervals and prepared for autoradiography. Counts were made of areas which had a much higher percentage of labeled nuclei than did the surrounding areas (see Fig. 3). □, 2% CS; △, 10% CS; ○, 10% FBS.

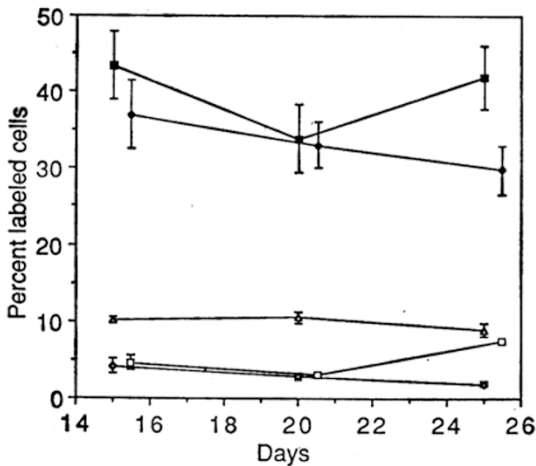


Fig. 3 Percentage of labeled nuclei in transformed foci and in the surrounding areas. This represents a count of number of nuclei labeled autoradiographically with ^3H -thymidine in Fig. 2. Cells in foci were identified by their elongated shape and dark staining nuclei. Cells in interfocal areas identified by their flat, pale-staining nuclei. The error bars are standard error of the mean. Foci: ■, 10% FBS; ●, 2% CS; Interfocal areas: □, 10% FBS; ◇, 2% CS.

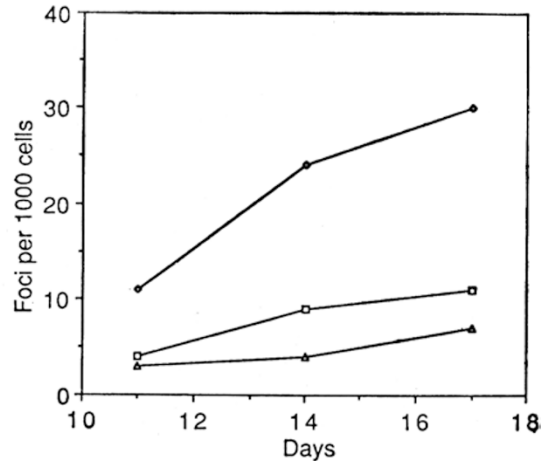


Fig. 4 Focus-forming capacity of transformed and non-transformed NIH 3T3 cells transferred on a background of G3H 10T1/2 cells. Cells of Fig. 1 which had been incubated for 20 days in various sera were trypsinized and 1000 of them were mixed with 10^5 C3H 10T1/2 cells in 10% FBS. At 11, 14 and 17 days, 2 of the dishes were fixed, stained with Giemsa and the number of foci counted. Original dishes in □, 2% CS; △, 10% CS; ○, 10% FBS.

cells at most would develop into foci in cultures transferred from 2 % CS, and still fewer in the case of 10% CS group. The results show that the different extent of transformation which occurred in the original culture with different serum variations was more or less maintained in their next passage after being transferred into medium containing 10% FBS. In addition, the result that the 10% CS cells acquired the ability to form some morphologically transformed foci after their passage to 10% FBS implies that the spontaneous transformation in this case is serum or environmentally dependent.

Change in transformation response with repeated passage of the non-transformed cell

The same experiment was repeated two months after the first experiment, using the same subline of cells, which had been passaged at weekly interval in 10 % FBS medium. The intent was to determine how reproducible the transformation under various conditions, and to observe more carefully the morphology of the foci produced under those conditions as well as the stability of that morphology upon transfer. A set of cultures grown in 5 % CS instead of 2% CS was used, because 5% CS was better for cell transformation. 1×10^5 cells per dish (60 mm) were seeded in 10% CS or 10% FBS and multiplied to higher densities on day 10 (Fig. 5) than they had in the previous experiment (Fig. 1). However, they remained saturated up to day 20, like the first experiment, in which their initial growth rate increased quickly till confluency (day 5) but later increased only slowly. The effects of the medium on focus formation on those cells were totally different from the first experiment, since 10 % CS medium which had elicited no foci previously now elicited many foci, while 10% FBS which had given the most abundant foci, now gave very few, and

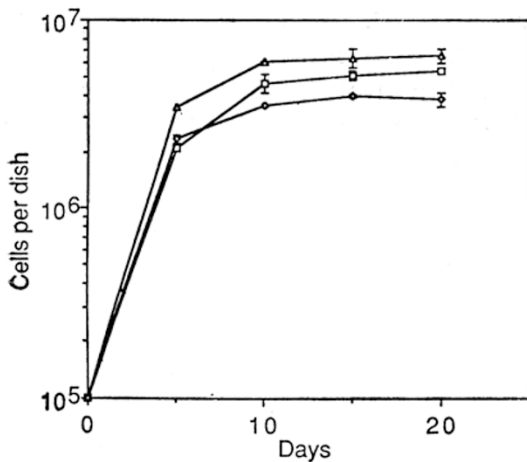


Fig. 5 Growth of two-month post-thaw NIH 3T3 cells in FBS. Two months after thawing and weekly passaging in MCDB 402 containing 10% FBS, 10^5 NIH 3T3 cells were seeded in MCDB 402 plus 5% and 10% aS or 10% FBS. The medium was changed twice a week. The cells were trypsinized every fifth day for cell counting. □, 5% CS; △, 10% CS; ○, 10% FBS.

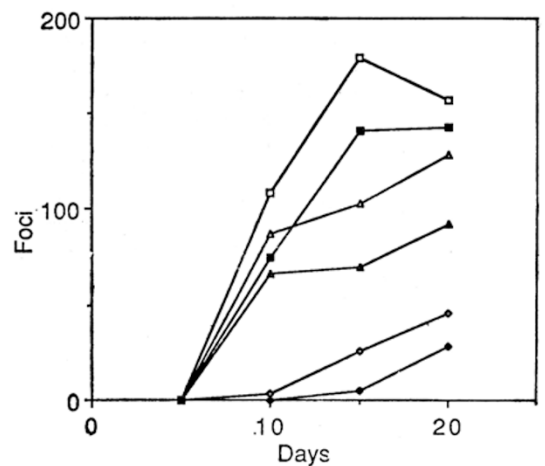


Fig. 6 Transformed foci identified by Giemsa staining and by ^3H -thymidine labeling of nuclei. Sister cultures to those of Fig. 5 were either fixed and stained with Giemsa (filled symbols) or were labeled with ^3H -thymidine (open symbols) at 5 day intervals, and the foci counted. ■□, 5% CS; ▲△, 10% CS; ●○ 10% FBS.

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appeared rather late (Fig. 6). By ^3H - thymidine autoradiography, a more sensitive assay for focus formation, the maximum number of foci detected in cultures of 5% CS on day 20 was about 0.2 % of the number of cells seeded (Fig. 6). The percent of labeled cells in the foci was much higher than that in the surrounding areas (data not shown), as in the first experiment (Fig. 3).

At each time point, 10^4 cells per dish were transferred from the cultures in each serum variation and maintained in 10% FBS for 20 days. The number of loci appeared in the secondary cultures was highest in those derived from cultures originally in 5% CS, followed by those in 10% CS and in 10% FBS respectively. (Fig. 7), reflecting the rank order of focus formation in original cultures (Fig. 6).

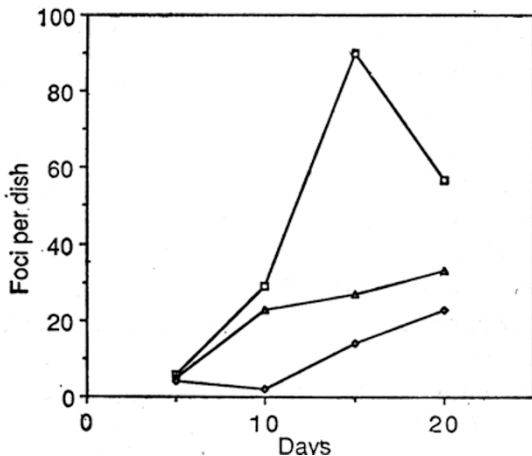


Fig. 7 Persistence of the transformed phenotype in progeny of transformed cells. At 5 day intervals the cells depicted in Fig. 5 were trypsinized, counted (Fig. 5) and transferred at a concentration of 10^4 per dish. All transferred cultures received MCDB 402 plus 10% FBS regardless of the serum of original dish. The transferred cultures were fixed 5 to 20 days after their transfer, stained with Giemsa and counted. Original cultures in: □, 5% CS; △, 10% CS; ○, 10% FBS.

In the first experiment, the frequency and morphology of transformed loci in the original cultures of 10% FBS, 2% CS or 10% CS groups virtually depended on the type and concentration of serum during the development of foci as shown by autoradiographic assay, giving no focus formation in 10% CS group (Figs. 2, 8).

In the second experiment, the same subline of cells, as in the first experiment, had been repeatedly passaged in 10% FBS medium for two months, then transferred into medium containing different type or concentration of serum (5% CS, 10% JS or 10% FBS). Again all groups were transferred and maintained in 10% FBS medium, and assayed every 5 days for the change of spontaneous transformation response. The main effect of longer culture conditions in 10% FBS MCDB 402 was its impact on the distinctive cell morphology and transformation potentials of NIH 3T3 cells,

by eliciting many foci in 10% CS medium group (Figs. 6, 7, 8), which had elicited no focus previously. Such change in transformation response was also stably retained upon the transfer of the cells back to 10% FBS, as evidenced from the results presented in Fig. 7.

DISCUSSION

A major criterion for a genetic event is that the altered phenotype be derived from its parental form with a spontaneous frequency of less than 10^{-6} [12]. The spontaneous transformation of NIH 3T3 cells occurs under optimal condition in our experiments has a frequency about 100 times higher than 10^{-6} , in case the number of cells at confluency is the basis for calculation, or more than 1000 times higher, if the number of cells initially

seeded is taken as the basis. The frequency of spontaneous mutation would not be expected to depend on the type and concentration of serum in the medium, but the frequency of spontaneous transformation of NIH 3T3 cells is strongly dependent on these conditions. Indeed, in the first experiment 10% CS had completely blocked transforming focus formation, and in both experiments, transfer of the original cultures showed that transformation occurred only after the cultures became confluent. The fact that the characteristic expression of transformation differed depending on whether it was elicited in CS or FBS, and that the expression was maintained upon transfer of the cells into a new medium is further evidence that transformation is not the result of a mutational event, since the later should be qualitatively independent of the medium in which it originated.

The appearance of foci was delayed in high serum concentrations especially in early-passage cultures of 10 % CS. This also occurred in the early-passage cultures when the CS concentration was raised to 20% (data not shown). Our results also showed that the late-passage cells were more sensitive than early-passage cells to transformation in low concentration of CS, and even in higher concentrations of FBS, which has more promoting effect on transformation of NIH 3T3 cells. There seems to be a negative correlation between cell division and transformation. The environmental dependence of spontaneous transformation of NIH 3T3 cells seems to indicate that serial passage of cells in growth-limiting concentration of serum gives rise to and maintains a certain population of cells with an improved capacity to multiply at certain concentration of serum, among them the density-inhibited nontransformed cells. This means that transformation may be a part of an adaptive response to the constraint of cell multiplication.

The enhancing effect of confluency on transformation in the present experiment is similar to that described for adipocyte differentiation in other mouse cell lines [13]. Epidermal cells in culture also differentiate into keratinocytes at confluency [14]. Keratinocyte differentiation is accelerated by lowering the serum concentration of epidermal cultures [14], just as spontaneous transformation of NIH 3T3 cells is enhanced by lowering the concentration of CS from 10% to 5% or 2%. These findings suggest that the inverse relationship between growth rate and differentiation which has been a shibboleth of developmental biology extends also to spontaneous transformation. By definition, of course, transformation is not differentiation, but it can be considered as an example of aberrant differentiation. In fact, defective terminal differentiation has been proposed as a consistent character of malignant human keratinocytes [15]. While defective differentiation has been recognized as characteristic of many, if not all cancers, our results indicate additionally that the defect may be triggered by the very condition which promotes differentiation in normal cells.

There is evidence that chemical and physical carcinogenesis is brought about in a manner like that of spontaneous transformation. Transformation of C3H 10T1/2 cells by chemicals or X-rays did not occur until several weeks after the cultures became confluent [16, 17] and did not occur if the cells were incubated in high serum concentrations [18]. A similar suppressive effect of high serum concentration was first reported for the transformation of chicken embryo cells infected with *Rous sarcoma* virus [19].

It is not surprising that the environmental requirements for spontaneous transformation changed in the two experiments reported here. The NIH 3T3 cells were

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originally selected for their low saturation density [9], but saturation density changed with repeated transfer as did the appearance of the cells. However, it means that each experiment with these cells has to be done with a range of serum concentrations.

The high frequency of spontaneous transformation of NIH 3T3 cells raises questions of interpretation about the oncogene-induced transformation of the same cells, since they are the routine target for transfection, particularly by *ras* oncogene [1]. The transformation by the *ras* oncogene can be viewed as an amplification and acceleration of their tendency to undergo spontaneous transformation. The NIH 3T3 cells should be considered as an Initiated cells in conventional cancer parlance, similar to the clone isolated from carcinogen-treated C3H 10T1/2 cells prior to its final transformation. It is known that the NIH 3T3 cells produce tumors in nude mice when large numbers are inoculated, although transformation lowered the number and time needed for tumor formation [2, 3]. In that sense the *ras* oncogene may be considered as a promoting, rather than an initiating agent.

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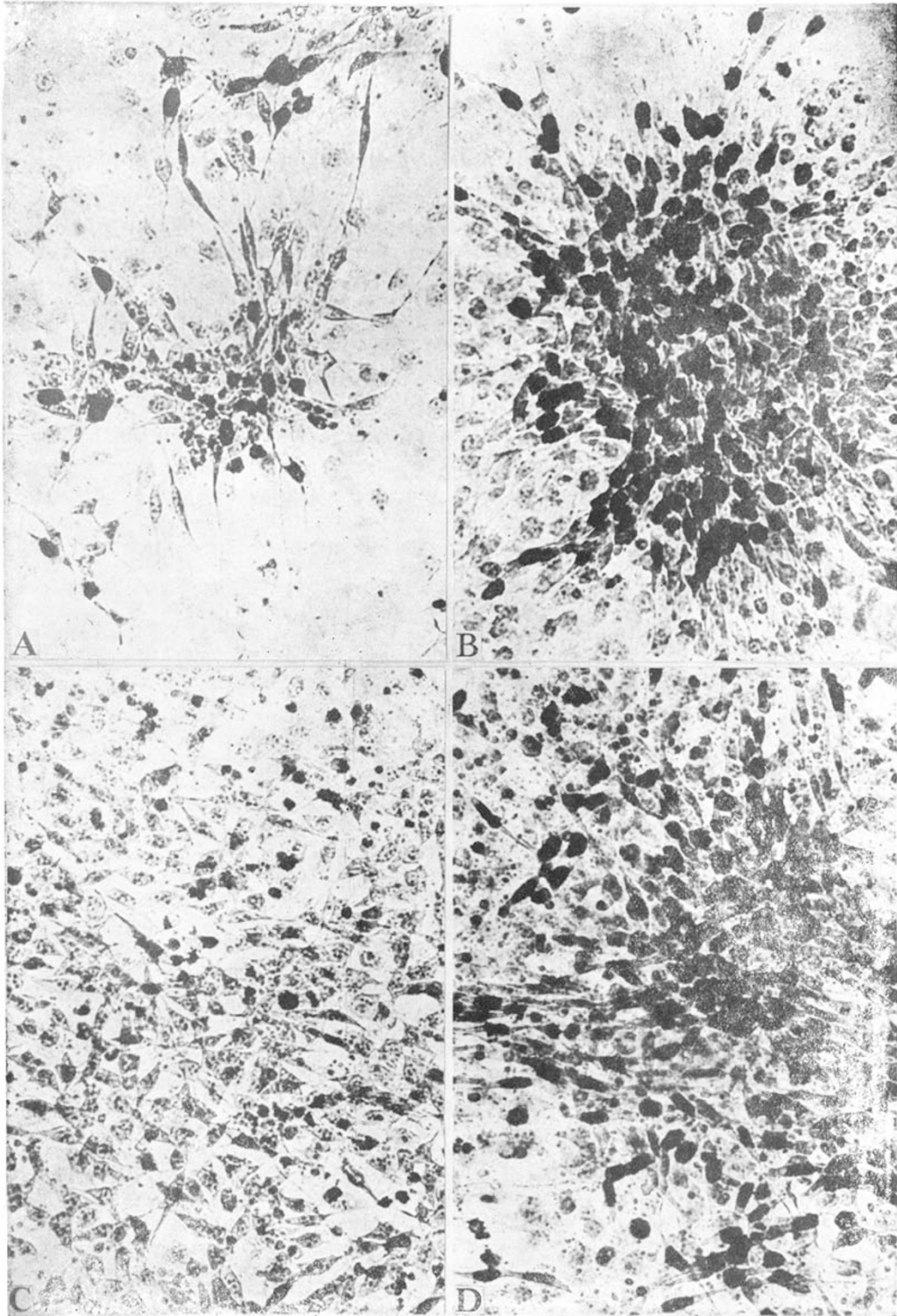


Fig. 8 The morphology of transformed foci identified by ^3H -thymidine labeled nuclei. In the original cultures of 2% CS (A) or 10% FBS(B) groups, the foci was shown clearly and there was no focus formed in 10% (AS group (G) in the first experiment (Fig. 2); but in the second experiment, many transformed foci did appear in various culture conditions from the original, 10% CS group (D) (Figs. 6, 7). All cultures were fixd on day 15 after their transfer. $\times 100$