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Adenovirus Type 12-Rat Embryo Transformation System

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Adenovirus type 12 (Huie) inoculated into cultures of primary whole rat embryo produced foci of morphologically altered cells. The number and identification of these transformed areas was dependent upon the calcium concentration of the medium; more foci appeared in 0.1 mM than in 1.8 mM calcium. Cell lines derived from these inoculated cultures did not yield infectious virus, and also were similar to cell lines derived from adenovirus type 12-induced tumors with respect to morphology, presence of virus-specific tumor antigen, and oncogenicity. Dose-response curves revealed that transformation of rat embryo cells by adenovirus type 12 followed one-hit kinetics, and that approximately 7×10^5 infectious virus particles were required for one transformation event. Our results indicate that the transformation system described for adenovirus type 12 is reproducible, and that previous difficulties experienced in developing such a system may well be explained by the higher calcium concentration of the tissue culture media used.

There have been several reports of hamster cells being transformed in vitro from normal to malignant states by adenovirus type 12 (10, 13). Unlike the polyoma (11, 14) or simian virus 40 (1, 15) transformation systems, however, there has been little quantitation of adenovirus transformation. Not only has transformation been a relatively rare event, but the derivation of cell lines has been accomplished with difficulty. It has been suggested that, because of more consistent transformation and cell line derivation, rat cells may be superior to hamster or rabbit cells for adenovirus type 12 transformation studies (J. D. Levinthal and W. Peterson, Federation Proc. **24**:174, 1965).

It has been found that cell lines derived from in vivo adenovirus-induced tumors have a characteristic sensitivity to calcium at the 1.8 mM concentration found in tissue culture media based on Earle's balanced salt solution (4). At this calcium concentration, the tumor cells formed aggregates and came off the glass; initiation and propagation of adenovirus tumor cell lines could be more easily achieved in medium containing low concentrations of calcium (5, 6). The present study was undertaken to investigate whether a reliable adenovirus type 12 transformation system could be developed by use of rat cells grown in a medium containing an optimal calcium concentration for cells derived from adenovirus-induced tumors.

MATERIALS AND METHODS

Virus. Adenovirus type 12, strain Huie, was obtained from the American Type Culture Collection and was passed three times in KB cell cultures and once in human embryonic kidney (HEK) cultures. The HEK-grown pool was subdivided into ampoules and stored in the vapor phase above a liquid nitrogen reservoir. This pool, which was used for all quantitative experiments, was titered in HEK before and after the course of these experiments. The mean titer of $10^{8.2}$ $TCID_{50}$ per ml did not decrease during the storage period. A sample of this pool was found to be free from adenovirus-associated viruses types I, II, and III.

Cell cultures. Embryos, delivered by caesarian section from near-term inbred Fisher rats, were minced, washed, trypsinized, and planted at 2×10^5 cells per milliliter in Eagle's basal medium (2) with 10% fetal bovine serum, 2 mM glutamine, and penicillin and streptomycin in concentrations of 100 units and 100 μg/ml, respectively. These cultures, which were incubated at 37 C under 5% CO_2 and 95% air, were fed on the 3rd day and used when confluent, usually 5 days after seeding.

Media. Transformation studies were carried out in Eagle's minimal essential medium (3) formulated without calcium, and supplemented with 5% dialyzed calf serum, 2% fetal bovine serum, 2 mM glutamine, 0.1 mM nonessential amino acids (3), and antibiotics. From a 0.5 M stock of calcium chloride, calcium was added to the media at a final concentration of 0.1 or 1.8 mM.

Quantitation studies. Dilutions of the virus were made in the appropriate growth medium, and, when

confluent, cultures were drained and inoculated with 0.1 ml per tube or 0.4 ml per 4-oz flask. The virus was adsorbed at 37 C for 2 to 4 hr, with manipulation of the culture every 15 min to assure optimal virus contact with the cells. After the adsorption period, the cultures were fed by adding 1 ml per tube or 10 ml per flask of the appropriate growth medium. The cultures were maintained for periods up to 60 days by feeding the cells every other day with the growth medium. At 3- to 4-day intervals, the tubes were carefully scanned, and the number of foci was recorded. The count used for quantitation was taken approximately 7 weeks after infection.

Testing for virion. Transformed cell lines were tested for infectious virus by plating 2×10^5 viable cells on confluent tube cultures of HEK. In addition, a 20% extract of thrice frozen and thawed cells was prepared from each cell line. An equivalent of 2×10^6 cells was added in extract form to each of nine tube cultures of HEK. All tubes were examined for 21 days, at which time supernatant fluids were passed into new HEK cultures. These latter cultures were also observed for 21 days.

Oncogenicity. To determine the tumor-producing potential of the transformed cells, flask cultures were trypsinized and the viable cell count was determined with trypan blue as a vital stain. Weanling Fisher rats and weanling Syrian hamsters were inoculated subcutaneously with 10^5 to 10^8 cells per 0.5 ml.

Calcium sensitivity tests. Prescription bottles (4-oz) were seeded with 2×10^5 cells per milliliter in 10 ml of Eagle's minimal essential medium with 0.1 mM calcium, 5% dialyzed calf serum, 2% fetal bovine serum, 0.2 mM nonessential amino acids, and antibiotics. When confluent, replicate cultures were fed with media containing 0.1, 1.8, 5, or 7.5 mM calcium. The cultures were observed daily for 6 days, with a feeding on the 3rd day. Cultures were considered calcium-sensitive, or "positive," if retracting or clumping, or both, occurred at any calcium concentration as compared with the 0.1 mM control.

RESULTS

Response of rat cells to adenovirus type 12. Inoculation of rat embryo cells with as much as $10^{8.2}$ $TCID_{50}$ of adenovirus type 12 produced no cytopathogenic effect. From 18 to 36 days after the time of inoculation, there appeared morphologically altered, apparently transformed foci, composed of small, tightly packed epithelioid cells which were several layers thick.

Effect of calcium on morphological transformation and establishment of cell lines. The appearance of clearly identifiable morphologically transformed foci was greatly enhanced by using a medium with a low calcium concentration. As seen in Fig. 1A, control cells grown in Eagle's medium (1.8 mM calcium) were fully confluent after 48 days. If there were transformed foci in the inoculated cultures, they were difficult to identify (Fig. 1B). In a medium containing 0.1 mM calcium (Fig. 1C), however, the control cultures were not confluent, and in the inoculated cultures the transformed foci were readily identifiable (Fig. 1D). As described by McBride, the transformed foci looked very much like "sombreros" viewed from above (10). In the six cultures grown in medium containing 0.1 mM calcium, there were 56 foci, as compared with no foci in those cultures grown in the 1.8 mM calcium medium (Table 1). After 48 days, the calcium concentration in three flasks was increased from 0.1 to 1.8 mM. In those flasks kept in 0.1 mM calcium, the number of transformed foci continued to increase, whereas in those flasks changed to 1.8 mM calcium the number of foci decreased. After an additional 25 days, there were approximately 10-fold more foci in the 0.1 mM calcium cultures as compared with those with the increased calcium concentration. Similarly, three flasks from the 1.8 mM calcium group were changed to 0.1 mM calcium, 48 days after inoculation. Those cultures kept in the higher calcium concentration continued to contain no identifiable foci; those changed to 0.1 mM calcium developed six foci after an additional 25 days, which indicated that transformation had occurred but could not be expressed in a medium containing 1.8 mM calcium. In a second experiment, confluent cultures were exposed to 0.1 or 1.8 mM calcium during the 4-hr adsorption period (Table 1, B). Immediately thereafter, half of the tubes from each group were changed to the contrasting calcium concentration. Regardless of the calcium concentration during adsorption, clearly identifiable transformed foci appeared only in those cells maintained in 0.1 mM calcium. The results of this experiment indicate that adsorption occurs equally well in either calcium concentration.

Cultures containing transformed foci were subdivided in a 0.1 mM calcium medium. The daughter culture bottles appeared to be pocked with colonies of transformed cells (Fig. 2A). If kept in 0.1 mM calcium, the colonies grew (Fig. 2B), but, if changed to 1.8 mM calcium, the colonies appeared to become walled-in and no obvious growth occurred for weeks (Fig. 2C, D). Obviously, cell lines were derived much more readily from the 0.1 mM calcium cultures than the 1.8 mM calcium cultures. In each of 18 attempts, a cell line was derived by use of Eagle's minimal essential medium containing 0.1 mM calcium instead of the usual 1.8 mM calcium concentration.

Characteristics of the transformed cell lines. Each of the 18 transformed lines was made up of

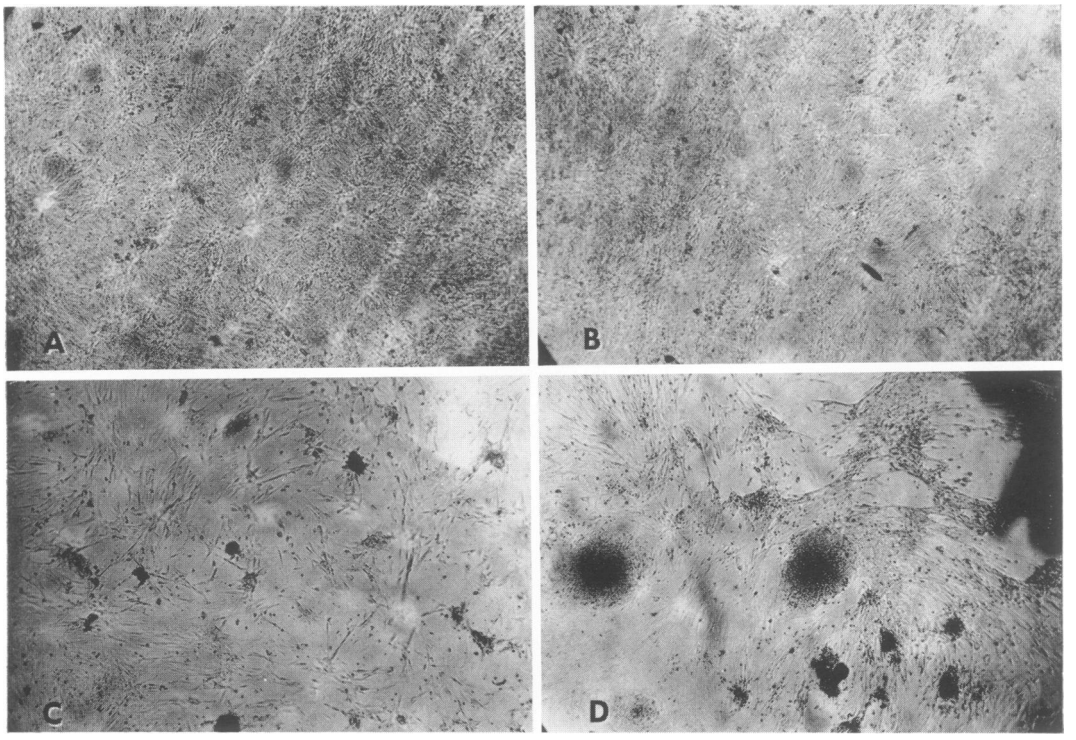

FIG. 1. *Effect of calcium concentration on the appearance of adenovirus type 12 transformed foci. (A) Uninoculated control, rat embryo cells after 48 days, 1.8 mM calcium. (B) Inoculated culture, rat embryo cells after 48 days, 1.8 mM calcium. (C) Uninoculated control, rat embryo cells after 48 days, 0.1 mM calcium. (D) Inoculated culture, rat embryo cells after 48 days, 0.1 mM calcium.* × *41.*

cells with an epithelioid morphology. There was loss of contact inhibition, in that the cells grew in multilayered foci. Of four lines tested for calcium sensitivity, each demonstrated the calcium effect at concentrations of 1.8 or 5 mM calcium, or at both concentrations. Of six cell lines tested for infectious adenovirus 12, all were negative. However, in these cell lines, approximately 95% of the cells were positive for adenovirus type 12 tumor antigen by the indirect fluorescent-antibody (FA) technique (13) when tested with a serum pool from hamsters bearing adenovirus 12-induced tumors. Each cell line was also positive for adenovirus type 12 tumor antigen by the complement-fixation (CF) test (7) when tested with 4 to 8 units of antibody from another serum pool similar to the one used for FA tests; the average CF titer at passage 20 to 24 was greater than 1:16. None of the lines tested had demonstrable simian virus 40 "T" antigen by either FA or CF test. One of the cell lines at passage 19 was compared by chromosome analysis with passage 2 normal rat cells. There appeared to be no obvious difference in the percentage of diploid cells. Of the metaphases from 111 transformed cells, 70% were diploid, as compared with 61% diploid in the 61 normal metaphases counted. Further, there appeared to be no chromosomal markers or aberrations in the transformed cells.

Tumorigenicity of cell lines derived from transformed cultures. Each of three of the cell lines was inoculated subcutaneously into 18 weanling Fisher rats, but only a single rat developed a tumor (10^6 cells, 170 days). This tumor had a characteristic adenovirus histology (12) and contained the adenovirus 12 "T" antigen by CF test. In addition, the serum from this animal had a titer of 1:80 when tested against another adenovirus 12 hamster tumor antigen. When inoculated subcutaneously into hamsters, three of four lines tested produced progressively growing (10^6 cells, 28 days) tumors. These tumors were pathologically and serologically similar to adenovirus-induced tumors, but analysis of the chromosomes indicated that the cells which were growing were rat, rather than hamster, cells.

Dose-response relationship. Attempts were

made to quantitate the transformation of whole rat embryo cultures by adenovirus type 12, by use of a medium containing 0.1 mM calcium. During the course of these experiments, the number of transformed foci continuously increased in cultures inoculated with $10^{6.2}$ or $10^{7.2}$ infectious units of virus. Approximately 7 weeks after the time of infection, however, the number

TABLE 1. *Effect of calcium on the number of identifiable transformed foci in the adenovirus type 12-rat embryo transformation system*

Expt	Virus dose ($TCID_{50}$)/ culture	Multiplicity of infection	Initial Ca^{++} concn of medium (mM)	Time at which some cultures were changed to a contrasting calcium concn	Distribution of foci at end of time period in initial calcium concn	New Ca^{++} concn (mM)	No. of foci after additional 25 days
(A)	$10^{6.2}$	0.1	0.1	48 days	31[a]	0.1	120[a]
					25[a]	1.8	15[a]
			1.8	48 days	0[a]	0.1	6[a]
					0[a]	1.8	0[a]
(B)	$10^{7.2}$	40	0.1	4 hr	0[b]	0.1	35[b,c]
					0[b]	1.8	1[b]
			1.8	4 hr	0[b]	0.1	24[b]
					0[b]	1.8	0[b]

[a] Total in three 4-oz prescription bottles.
[b] Total in nine tubes.
[c] Reading at 50 days = 194/8 tubes.

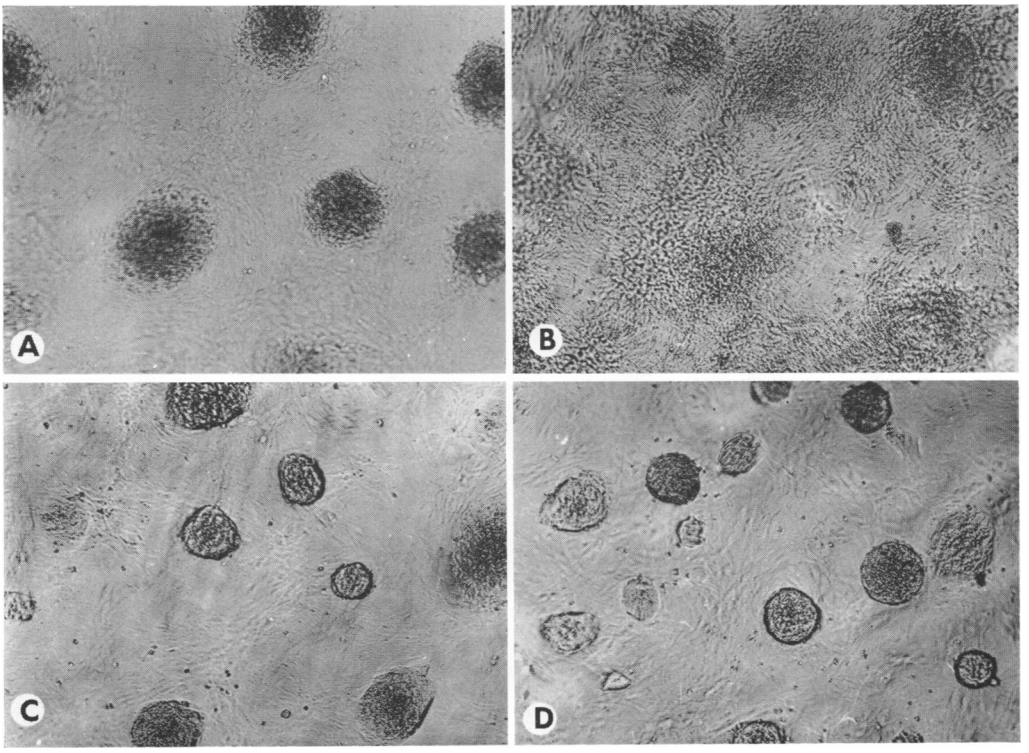

FIG. 2. *Calcium-dependent inhibition of transformed foci. (A) Adenovirus type 12 transformed foci in normal whole rat embryo cells grown in 0.1 mM calcium medium. (B) Replicate culture after 3 weeks in 0.1 mM calcium medium. (C) Replicate culture after 1 week in 1.8 mM calcium medium. (D) Replicate culture after 3 weeks in 1.8 mM calcium medium.* × 72.

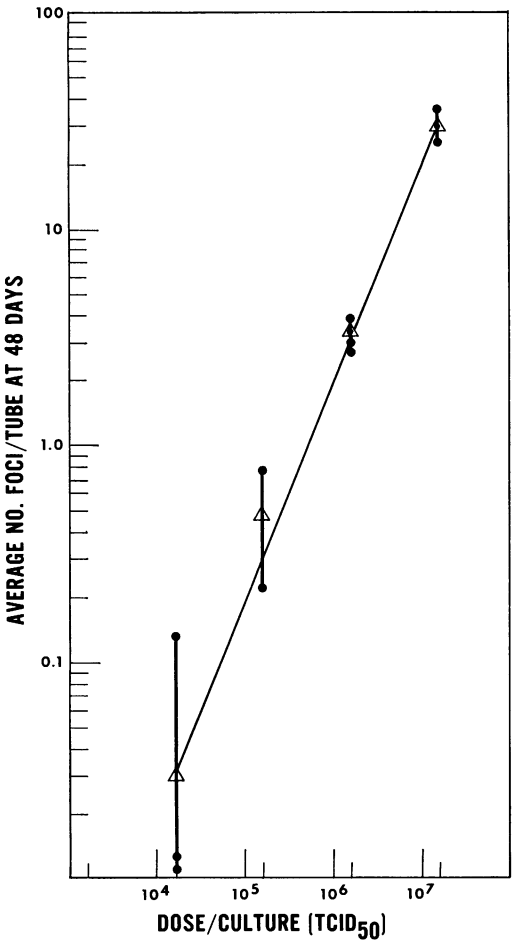

FIG. 3. *Relationship of dose of adenovirus type 12 to the number of transformed foci. Symbols: △, average of all experiments; ●, average of nine tubes representing one point in each individual dose-response experiment.*

of foci appeared to remain constant or decrease somewhat due to coalescence. Metastatic colony formation did not appear to influence these counts, since cultures inoculated with $10^{5.2}$ infectious units of virus developed single foci which remained localized for at least 3 to 4 weeks.

Results tabulated from four separate dose-response experiments indicated that there was a one-hit relationship between the number of transformed foci and the virus dose, with one transformation occurring for each 7×10^5 infectious units (Fig. 3). In cultures exposed to $10^{7.2}$ infectious units of adenovirus type 12, which was the most concentrated virus used, an average of 35 transformed foci per tube were present when counts were determined 48 days after inoculation. Since each tube culture contained approximately 4×10^5 cells when confluent, the transformation frequency was approximately 1 per 10^4 cells or 0.01%.

DISCUSSION

A number of cell lines derived from rat embryo cultures infected with adenovirus type 12 exhibited altered morphology, loss of contact inhibition, the presence of adenovirus type 12 specific tumor antigen, and oncogenicity in rats and hamsters. Since these cell lines derived from apparently transformed foci had the general characteristics of adenovirus-induced tumor cells (8), there appears to be justification for considering the morphological alterations as an indication of transformation.

By using the number of morphologically altered areas as an index of transformation, it became evident that a calcium concentration of 1.8 mM not only reduced the number of transformed foci, but also inhibited the growth of these foci once they were detectable. We consider that the effect of calcium on the rate of transformation depends upon the double selective property of the medium. The double selection can be broken down into (i) the selective force for the growth of normal cells and against the growth of adenovirus transformed cells in a medium containing 1.8 mM calcium and (ii) the selective force for the growth of adenovirus transformed cells and against the growth of normal cells in a medium containing 0.1 mM calcium. That transformation occurs, but cannot be expressed, in a medium containing 1.8 mM calcium was evident from the switch experiments (Table 1), and is consistent with previous reports (5, 6) regarding the sensitivity to calcium of cell lines derived from adenovirus-induced tumors. It should be stressed that 1.8 mM calcium is the concentration found in all tissue culture media formulated with Earle's balanced salt solution (4).

When the low calcium medium was used, transformation of primary whole rat embryo cells by adenovirus type 12 became so consistent that an attempt was made to quantitate the transformation event by counting the number of transformed foci. Such focus counting in a liquid medium is subject to error resulting from metastasis from the primary focus. Although it may have occurred, we do not believe metastasis significantly affected the increase in the number of foci which was observed in cultures inoculated with relatively high doses of virus, since dissemination from a single transformed focus was not observed in cultures inoculated with lower concentrations of virus. Further, if the increase in the number of foci was due to metastasis, the

ultimate number of foci should have been the same regardless of the virus input, but this was not observed. Rather, there was a linear dose-response relationship as shown in Fig. 3.

Although the dose-response curves were linear at the concentrations of virus used, it may be that, with a more concentrated virus inoculum, there would be a plateau in the transformation rate, similar to that reported for polyoma virus (14) and simian virus 40 (1). On the basis of the data presented here, however, adenovirus type 12 was a much less efficient transforming agent than either of these two viruses, since 7×10^5 infectious units were required for one transformation event, as compared with 10^4 and 10^3 infectious units for polyoma virus and simian virus 40, respectively. The frequency of transformation by adenovirus type 12 was 1 per 10^4 cells or 0.01%, as compared with the 1 per 10^4 to 1 per 10^5 transformation frequency in an adenovirus type 12-hamster embryo system reported by W. A. Strohl, H. C. Rouse, and R. W. Schlesinger (Bacteriol. Proc., p. 136, 1966). In the hamster system, however, the number of foci was determined 3 weeks after infection, and the dose-response curves were not linear. Furthermore, in the transformation system reported here, no end point was reached with the virus concentrations used and an increase in virus titer may well have increased the frequency of transformation.

A more recently derived adenovirus type 12 transformed rat embryo line caused tumors in 9 of 11 newborn Fisher rats in 30 to 33 days when 10^6 cells were inoculated subcutaneously.

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