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The Response of BHK21 Cells to Infection with Type 12 Adenovirus

III. Transformation and Restricted Replication of Superinfecting Type 2 Adenovirus[1]

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With 4 Figures

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Summary

Transformation of a clonal line of BHK21 cells by type 12 adenovirus (Ad12) is described. A transformation rate of approximately 2×10^{-5} per initially infected cell was observed after high multiplicity infection. A study of the fate of the abortively infected cells revealed, however, that only a small fraction survived the infection and initiated growth of a colony. Of these rare surviving colonies, nearly half contained transformed cells. Of three BHK21 sublines tested, one yielded transformed cells only from colonies growing in soft agar suspension, a second yielded transformants only as foci in monolayers, while the third did not yield detectable transformants by either method.

The adenovirus-transformed cells were distinguished from the parental BHK21 cells by the following characteristics:

1) a marked morphological alteration;

2) the synthesis of adenovirus tumor antigen;

3) growth to high cell density in the presence of a low concentration of serum;

4) induction, in hamsters, of tumors identical to those induced by inoculation of type 12 virus;

5) restriction in the ability to support the multiplication of type 2 adenovirus (Ad2).

The latter property is most likely due to blockage of a step late in the adenovirus replicative cycle, since 88% of the cells, which synthesized Ad2-specific structural antigens did not yield infectious virus. A similar shift from a productive to a largely abortive response to Ad2 was seen in BHK21 cells simultaneously infected with Ad12 and Ad2. This finding supports the view that the restriction of Ad2 replication in Ad12-transformed cells, like the other characteristics mentioned, is associated with continued activity of at least part of the Ad12 genome.

[1] Dedicated to Prof. Dr. Dr. C. Hallauer on the occasion of his 70th birthday.

1. Introduction

Early passage embryonic or newborn rodent cells have been used most commonly to demonstrate *in vitro* transformation by oncogenic adenoviruses. Despite the inherent variability of such cell populations (24), a practical, quantitative focus-forming assay has been described for hamster embryo cells (9). In all reports in which quantitative estimates could be made, a maximum of one transformed cell per 10^4 to 10^5 infected cells was noted (9, 16, 22, 24, 27, 37).

The heterogeneity of the cell population in cultures prepared from embryos is an obvious disadvantage in studies on the mechanisms of transformation, if only because the specific target cell is not known; therefore the transformed cells cannot be compared directly with their normal cell progenitors. The variable frequency of transformation from one set of embryos to another further complicates the situation (24). The use of clonally derived cell lines for *in vitro* studies would overcome these difficulties. The BHK21 line of hamster cells (31) offers certain advantages, including the large body of information already available about the cells and about their response to other oncogenic viruses (reviewed by 3). Attempts by others to achieve transformation of BHK21 cells with adenoviruses have been unsuccessful (22, 24, 44). On the other hand, another established line of hamster embryo cells (NIL-2, 11) has been reported to have been transformed by type 12 adenovirus (Ad12) (22).

By appropriately adjusting the conditions, transformation of certain sublines of BHK21 cells by Ad12 has been achieved, and is described in this paper. The isolated Ad12-transformed cells were found to have a number of properties in common with cells cultivated from *in vivo* induced Ad12 tumors, and these also are described.

2. Materials and Methods

2.1. Cells

From a stock culture of BHK21/13 cells, obtained from Dr. H. Koprowski, Wistar Institute, Philadelphia, Pa., U.S.A., a homogeneously fibroblastic subline was established by two successive clonal isolations. This subline will be designated as BI. A second culture of BHK21/13, 65 generations from cloning, was obtained from Dr. Ian Macpherson, Glasgow, Scotland. This subline was recloned and used in some of the later experiments, where it is referred to as BII. Repeated attempts to isolate *Mycoplasma* from these cell lines were negative. Human embryonic kidney (HEK) cells for both growth of stock virus and plaque assay of Ad12 were obtained commercially.

2.2. Virus

Ad12 stock virus was prepared from HEK cells infected with a plaque-purified isolate of Ad12 strain Huie. After disruption of the infected cells in a Raytheon 10 kc sonic oscillator, the suspension was clarified by low speed centrifugation and stored frozen at $-70°$C. Such stock virus preparations contained 1 to 5×10^9 p.f.u. per ml when assayed on HEK cell monolayers. Adeno-associated viruses could not be demonstrated by serological tests carried out through the kindness of Dr. D. Hoggan, National Institute of Health, Bethesda, Maryland, U.S.A. The plaque-purified strain of Ad2 was grown in KB cell suspension cultures, and is further described by ROUSE *et al.* (26).

2.3. Plaque Titrations

Ad2 was assayed on KB cell monolayers as described by ROUSE *et al.* (25), with the exception that adsorption was carried out for 2 hours in phosphate-buffered saline (PBS) without serum. The arginine concentration was 0.5 mM in all assays.

The Ad12 plaque assay procedure was a modification of that described for Ad2, and involved the use of HEK cells and an enriched overlay medium, *i.e.*, double the amino acid and vitamin concentrations, as well as the addition of Eagle's non-essential amino acid mixture (13) and 10% fetal calf serum.

2.4. Media

All cells were grown in an enriched basal medium of Eagle, designated HT medium, as described in ROUSE *et al.* (26), except that 10% fetal calf serum was used. For cloning, 20% serum was used. Cells to be removed from Petri dish cultures were suspended with a 0.025% solution of Viokase (Grand Island Biological Company, Grand Island, New York, U.S.A.) in Ca^{++}- and Mg^{++}-free PBS containing 0.02% EDTA.

2.5. Immunofluorescence Technique

Coverslip cultures of the cells to be tested for virus-specific tumor (T) antigen were fixed for 10 minutes in pre-chilled (−70°C) acetone at room temperature, and air dried. The indirect immunofluorescence technique was used. T antigen was detected with a pool of hamster sera from animals hyperimmunized with cultured Ad12-induced hamster tumor cells [HT2, (36)]. The fluorescein-conjugated caprine anti-hamster gamma globulin was the kind gift of Baltimore Biological Laboratories, Bethesda, Maryland, U.S.A. Appropriate controls with normal hamster serum and with untransformed B_1 cells were included. Staining of Ad2 structural antigens and the determination of the frequency of cells synthesizing any given antigen were done as described previously (38), except that the microscope fields were calibrated at each magnification, thus enabling the number of cells per culture to be calculated.

2.6. Cloning Techniques

Cloning in soft agar: Base layers of 5 ml HT medium with 10% fetal calf serum and 0.45% agar were first allowed to solidify in 60 mm plastic Petri dishes. Cells to be cloned were then plated in 1.5 ml of the same medium containing 0.33% agar. Such cultures were subsequently fed at 6—7 day intervals with an additional 5 ml of the 0.33% nutrient agar. Following the first two such feedings, the upper layer of agar was removed with suction before addition of each subsequent 5 ml feeding.

Cloning in fluid medium: Counted numbers of the cells to be cloned were serially diluted and seeded into Petri dishes containing HT medium supplemented with 20% fetal calf serum. Single colonies were isolated from the rest of the culture by attaching stainless steel cylinders to the surrounding area of the Petri dish. After suspending a colony with a drop of Viokase, the dispersed cells were transferred to another culture dish for further growth and, in some cases, for a second isolation by the same procedure.

2.7. Chromosome Preparations

Cultures of cells whose chromosomes were to be studied were incubated with 10 μg per ml of colchicine for 1.5 hours at 37°C, after which the loosely attached cells were harvested by pipetting, incubated in 1% sodium citrate for 10 minutes at room temperature, and finally fixed in 50% acetic acid. Metaphase chromosome preparations were spread by rapid drying of a drop of the fixed cell suspension with mild heat or flaming. Staining was done with Giemsa stain.

2.8. Neutralizing Antiserum

In superinfection experiments the uneclipsed Ad2 was neutralized with a hyperimmune rabbit serum prepared against gradient purified Ad2. This antiserum at a dilution of 1/500 could reduce the infectivity of Ad2 from 10^7 p.f.u. to 10^4 p.f.u. in 30 minutes, but had no neutralizing activity against Ad12. Anti-Ad12 antiserum was prepared in similar fashion, and exhibited approximately the same neutralization characteristics against Ad12, and no activity against Ad2.

3. Results

3.1. Establishment of Ad12-transformed Clones

In contrast to primary cell cultures, where the extent of normal cell multiplication is quite limited, the uninfected BHK21 cells have unlimited, rapid growth potential. Several attempts at seeding 200 to 2,000 infected cells per plate in fluid medium failed to yield any recognizably transformed colonies, presumably because of the overgrowth by untransformed cells. In order to obtain conditions suppressing the multiplication of untransformed cells, the soft agar suspension method, routinely used for transformation by polyoma virus, was employed (20).

Incomplete monolayer cultures of the B_I subline of BHK21 cells (see Materials and Methods) were infected with plaque-purified Ad12 at an input multiplicity of 350 p.f.u. per cell. After 2 hours adsorption at 37°C, the inoculum was removed, the cells were washed with PBS and fed with nutrient medium. After 24 hours, during which approximately 2 cell divisions had occurred in control cultures, the infected cultures were suspended with Viokase, and counted numbers of cells (from 10^3 to 10^6 cells per plate) were seeded in a nutrient soft agar suspension as described in Methods. Uninfected control B_I cells were treated in the same way.

Under these conditions approximately 0.2% of the infected cells underwent 2 to 4 divisions. However, by 3 to 4 weeks, only 0.003% to 0.01% had yielded colonies which were grossly visible and easily removed from the agar with a capillary pipette. The uninfected cells also formed grossly visible colonies in agar; in fact, 5% of the seeded cells grew to this size. Thus, the agar technique did not select against the B_I cells. The lower frequency of colony formation by infected cells is indicative of the cell killing produced by Ad12 infection (39, 45), a detailed study of which is described elsewhere (33, 34). Colonies were isolated from both infected and control plates, dispersed by incubation with 2 drops of Viokase and seeded with fluid medium into 35 mm Petri dishes. The resulting cultures were examined microscopically at frequent intervals during incubation at 37°C.

Two morphological types of cells could be recognized in the cultures derived from *uninfected* cell colonies: the typical fibroblastic BHK21 cell and its more epitheloid variant described by MONTAGNIER *et al.* (23). Cultures established from infected cell colonies contained a third, very distinctive, cell type: rounded and with very scant cytoplasm. This subsequently proved to be the characteristic type 12 adenovirus-transformed BHK cell hereafter referred to as B(Ad). Cells of this morphology were not observed in any of the 63 colonies isolated from uninfected cells.

In no case did the culture established from an infected cell colony consist entirely of transformed cells. The proportion was quite variable, and some Petri dishes contained only 1 to 2 foci of transformed cells among a sheet of normal appearing fibroblasts. The appearance of such a mixed culture, illustrating the characteristic morphology of the transformed cells and their tendency to pile up, in contrast to the untransformed cells, is shown in Fig. 1.

In order to estimate the frequency of agar colonies containing cells of transformed morphology, two experiments were done in which the 73 largest grossly visible colonies were isolated between 3 and 4 weeks after infection and characterized with respect to morphology. The data are presented in Table 1, where it may be seen

that roughly 40% of the colonies isolated contained transformed cells. In terms of the total number of cells seeded in these two experiments (column 2), this figure translates into an average transformation frequency of 1.5 to 3.2×10^{-5} per infected cell. However, if one considers that the reduced cloning efficiency of infected cells relative to control cells reflects the extensive killing effect of Ad12 on BHK21 cells (33), and furthermore that the soft agar suspension system does not select preferentially for Ad12-transformed cells (see section on growth and tumorigenicity), the observed figure of 40 to 50% colonies with transformed cells may in fact represent the actual frequency with which survivors of the infection acquired and transmitted to at least one descendent cell the information for eventual transformation.

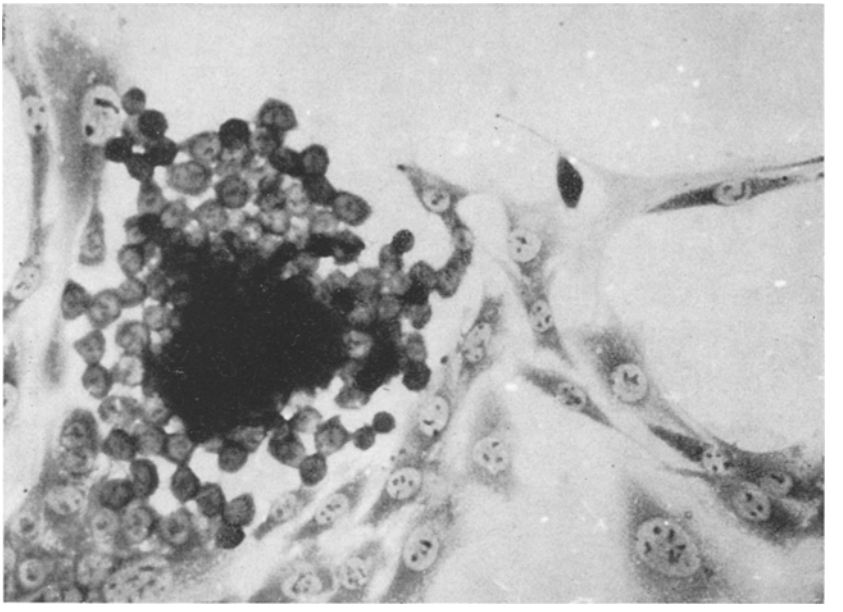

Fig. 1. Photomicrograph of mixed culture, showing piled colony of transformed cells [B_I(Ad)] at edge of untransformed BHK21 (B_I) cells. Stained with Giemsa stain. ×200

3.2. Variations in Transformability among Three Different BHK21 Sublines

The preceding results were obtained with the cells of one clonally isolated subline (B_I) of the parental BHK21 line. Since there have been reports indicating some genetic instability of the BHK21 line (10, 23), it seemed conceivable that other sublines may vary with respect to ease of Ad12 transformation under various conditions. That this is, in fact, the case was shown in transformation experiments done with two other BHK21 cell derivatives.

One derivative subline arose spontaneously from the B_I subline used in the preceding experiments. It was recognized by virtue of a high frequency of giant cells: chromosome preparations demonstrated that 25% to 50% of the cells were polyploid. When monolayer cultures of this subline were infected at multiplicities of 5—120 p.f.u. per cell and maintained in stationary phase condition under

medium containing 0.2% fetal calf serum (34), foci of morphologically altered cells of the type described above were observed 15 days after infection. Counts at 21 days indicated a transformation frequency of approximately 1 focus per 10^4 infected cells. However, none of about 100 colonies isolated from agar suspension cultures of the Ad12-infected polyploid subline contained transformed cells. Thus this subline showed a pattern of response which was, in terms of *detectable* transformation, the converse of that exhibited by subline B_I. The latter was readily transformed in soft agar cultures but not in monolayers.

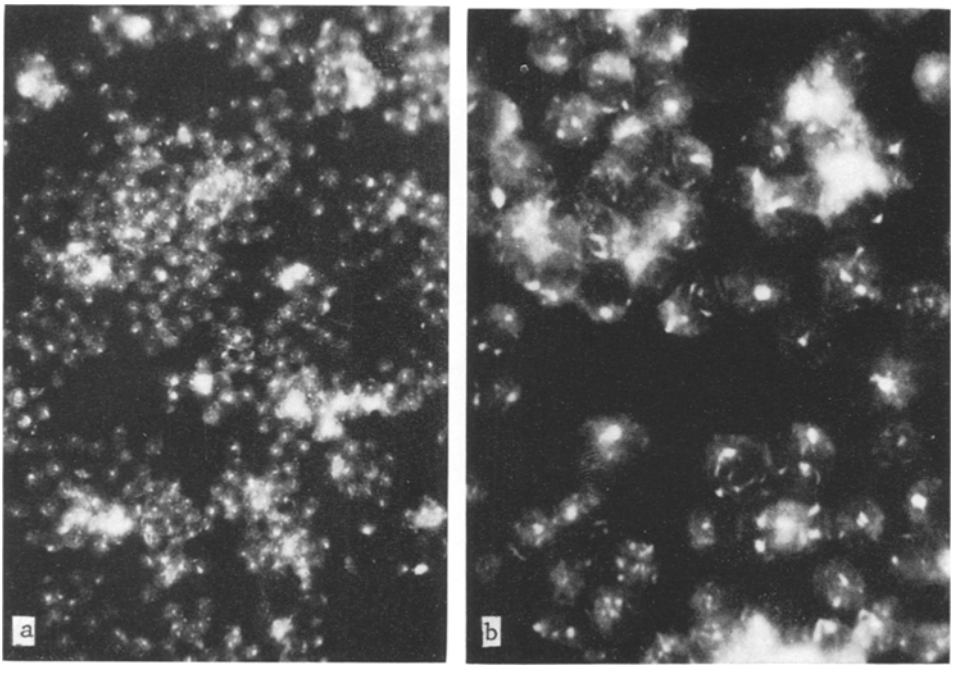

Fig. 2. Immunofluorescent staining of Ad12 T antigen in B_I(Ad) cells: (a) ×100; (b) ×400

Another subline (B_{II}, see Materials and Methods), representing a relatively early passage level of BHK21/13, has not yet been successfully transformed with either method, in agreement with the published reports of negative results from other laboratories (22, 44).

3.3. Characteristics of Adenovirus-transformed BHK21 Cells

For the experiments described in this section, cultures of transformed cells were purified by two successive clonal isolations under fluid medium.

3.3.1. T Antigen

A principal link recognized between an oncogenic virus and the transformed cells which result from infection with it is the continuing synthesis of a virus-specific tumor (T) antigen. Thirty-seven of the morphologically transformed B_I(Ad) cell isolates were tested for this antigen with the indirect immunofluorescence reaction. Thirty-three of these exhibited specific fluorescence in the form

of intranuclear splinters or flecks in at least some of the cells, and as a single fluorescent body or rod lying adjacent to the nuclear membrane in other cells (Fig. 2a and b). Some cells contained both. In four of the morphologically altered isolates T antigen was not detectable. These would require further study to prove the virus-related nature of the morphological change.

The morphologically transformed cells produced after infection of the polyploid BHK21 derivative described above were subcultured from the original foci and also were shown to contain T antigen.

The correlation between fluorescence and cell morphology was underlined by the observation that, in the original cell cultures isolated from agar suspension which contained both fibroblastic and morphologically altered cells, only the

Table 1. *Recovery of Colonies Containing Transformed Cells from Ad12-infected BHK21 Cells*[1]

Experiment number	Total number of cells seeded	Number of colonies		
		Total number of colonies recovered	With cells of transformed morphology	% of colonies with transformed cells
1	4.8×10^5	12	7	58%
2	6.5×10^5	61	21	34%

[1] BHK21 cells were infected with 350 p.f.u. of Ad12 per cell, and seeded into soft nutrient agar suspension 24 hours later. Colonies were picked with a capillary pipette 3—4 weeks later, dispersed with Viokase, and seeded into Petri dishes under fluid medium for determination of the cell types present.

latter exhibited T antigen fluorescence. Furthermore, among those colonies which were composed only of cells of typical BHK21 (untransformed) morphology, none of 22 tested contained detectable T antigen. Thus, most of the morphologically altered cell lines did express a presumed function of the Ad12 genome, while cells of unaltered morphology did not.

3.3.2. Growth and Tumorigenicity

The conversion of cells with limited growth potential, both in culture and *in vivo*, to cells with unlimited growth and considerable oncogenicity is a prime characteristic of the Ad12 effect in newborn hamsters and in hamster embryo tissue culture. Such a dramatic alteration cannot be demonstrated with BHK21 cells, for this is an established line of rapidly growing cells, certain sublines of which have been shown to be quite actively oncogenic (10). It was possible, however, to discern features of growth and tumorigenicity by which the transformed cells differed from the parental BHK21 cells.

The multiplication rate of one clonal transformed line [B_I(Ad), clone 508 No. 3] was compared with that of the parental B_I subline by periodic enumeration of the total cells per culture. Isolate 508 No. 3 was shown to have a doubling time of 11 to 12 hours, compared with that of the parental subline which was 8 to 10 hours. The cloning efficiency of the transformed line under fluid medium was found to be only 0.25%, compared with 30% for the parental B_I cells. Similarly,

in soft agar suspension the B_I (Ad) cells had no selective advantage, as they plated with approximately 1/5 the efficiency of B_I cells.

Oncogenicity of this transformed clone was compared with that of the parental B_I line by subcutaneous inoculation of 3-week-old Syrian hamsters with varying numbers of cells. The results are summarized in Table 2. It is clear that the B_I line produced tumors earlier and with fewer cells than did the B_I(Ad) line. This effect may have been due to the presence of a new "transplantation" antigen on the surface of the transformed cells which were therefore more effectively rejected by the host animal (29).

Table 2. *Tumor Formation by BHK 21 and Ad 12-transformed BHK Cells*[1]

Cell line inoculated	Number of cells inoculated	Time after inoculation		
		15 days	28 days	59 days
BHK 21 (B_I)	10^6	5/5[2]	5/5	5/5
	10^5	4/5	5/5	5/5
	10^4	3/5	5/5	5/5
	10^3	0/5	5/5	5/5
	10^2	0/5	4/5	5/5
Ad 12-transformed	10^6	0/5	5/5	5/5
[B_I (Ad), clone 508	10^5	0/5	1/5	3/5
No. 3]	10^4	0/5	0/5	2/5
	10^3	0/5	0/5	0/5
	10^2	0/5	0/5	0/5

[1] 3 week old Syrian hamsters were inoculated subcutaneously with the indicated number of cells in 0.5 ml PBS.

[2] Number of animals with a palpable tumor/number of animals inoculated.

The histology of tumors resulting from inoculation of the transformed BHK 21 cells has been described in detail (35). Of particular interest was the fact that tumors appeared to be similar in all respects to those arising after inoculation of the Ad 12 itself. When cells from such tumors were re-established in culture, they appeared identical with the original transformants.

3.3.3. Loss of Contact Inhibition of Cell Division

The loss of contact inhibition of cell division [or "density dependent inhibition" (32)] with accompanying multilayered growth of cells is a commonly used criterion for *in vitro* transformation of normal cells. With BHK 21 cells this criterion is more difficult to evaluate since multilayered growth is often observed with the non-transformed cells. When the serum concentration in the medium is reduced, however, a greater effect of cell density on multiplication of normal, as compared with transformed, cells can be observed (6, 18).

A differential effect of serum concentration was demonstrated with the parental B_I and transformed B_I(Ad) cell lines in the following manner. Petri dishes were seeded with 4×10^5 cells per plate in HT medium containing 10% fetal calf serum. After 24 hours, the plates were washed twice with HT medium containing no serum, then fed with HT medium supplemented with either 10%

or 0.2% fetal calf serum. The cultures were refed with fresh medium at two day intervals. The total number of cells per plate was determined at various intervals thereafter by hemocytometer counts on Viokase-suspended cells. The results are presented in Fig. 3, where the counts made immediately after the initial medium change are designated as 0 time.

Several points may be noted:

1) As already mentioned, the doubling time of parental B_I cells was 8 hours; that of B_I(Ad) cells 11 hours; similar differences were observed in many repeated experiments.

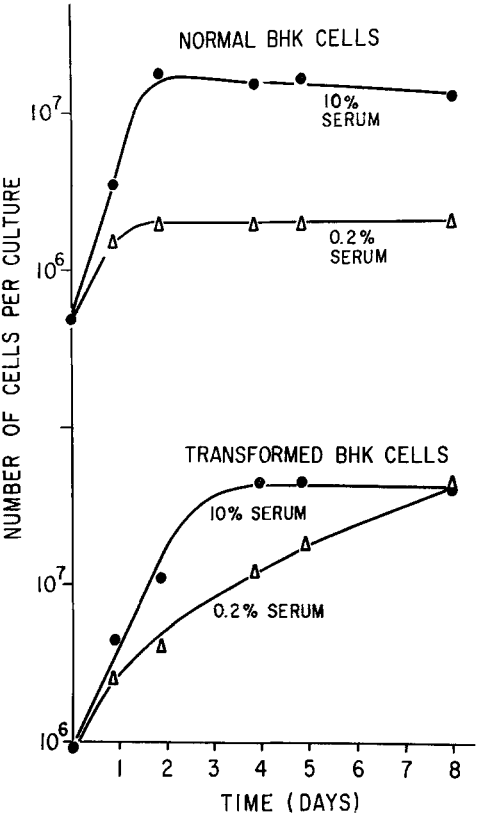

Fig. 3. Differential effect of serum concentration on growth of BHK 21 (B_I) and Ad12-transformed BHK 21 [B_I(Ad)-clone 508 No. 3] cells. Fifty mm Petri dishes were seeded with cells from actively growing cultures, allowed 24 hours to attach, the sheets washed twice with medium without serum, then fed with medium supplemented with the appropriate concentration of fetal calf serum. Hemocytometer cell counts were done on the Viokase-suspended cultures at the indicated times. Cultures were refed with the same medium at 2 day intervals

2) In low serum, B_I cells reached a maximum cell density of only 15% that reached in high serum, whereas the transformed cells reached the same maximum cell density in both serum concentrations.

3) Daily microscopic examination of the cultures, correlated with the cell counts, revealed that after reaching confluence, B_I cells in low serum showed no further increase in number, while in high serum they multiplied an additional 2- to 4-fold. The number of B_I(Ad) cells increased 2- to 4-fold beyond confluence in either serum concentration.

On the basis of these observations, we have concluded that the parental B_I cells exhibited a striking serum dependence for growth to high cell densities, while

the B_I(Ad) cells exhibited a greatly reduced serum dependence for such growth. The usefulness of this marker in the selection of transformed cells is under further study.

Increased calcium ion concentration (5 mM) has been shown to cause clumping and detachment of adenovirus-transformed cells which had been selected originally by growth in low calcium (0.2 mM) (16). This effect was not observed with the B_I(Ad) cells described above, possibly because they were selected and grown continuously in medium with the normal calcium ion concentration of 1.8 mM.

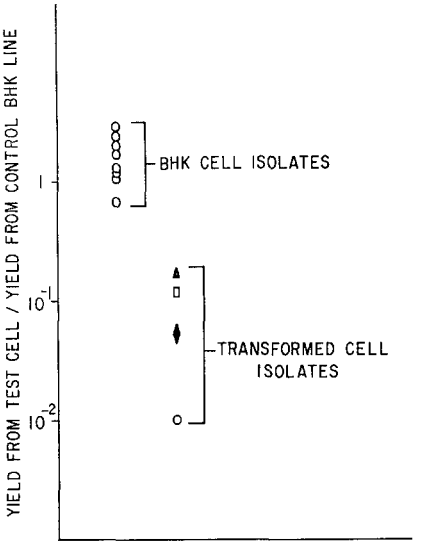

Fig. 4. Ad 2 yields from normal and Ad12-transformed clonal isolates of BHK 21 cells, relative to the parental B_I cell line. After infection of replicate cultures with 10 p.f.u. per cell, the inoculum was removed, and the cultures washed twice and fed with serum-free medium. At various times after infection the cultures were frozen and the virus content assayed. The maximum yields for each cell line were used in preparing this chart, and are expressed as a fraction of the yield from the parental B_I cell line. Each symbol represents the results for a different clonal isolate

3.3.4. Tumor-specific Determinant on the Cell Surface

A factor isolated from wheat germ, which selectively agglutinates neoplastic cells, was first reported by AUB *et al.* (2) and later defined chemically as a glycoprotein (5). The activity of this material (kindly supplied by Dr. MAX BURGER, Princeton University) in agglutinating normal and Ad12-transformed BHK21 cells was tested by the technique of BURGER and GOLDBERG (5). As shown in Table 3, the concentration of purified agglutinin required to agglutinate normal BHK21 cells was at least 5 times that needed for agglutination of transformed cells. This was a reproducible and significant difference which suggested that morphological transformation was accompanied by cell membrane changes thought to be typical of neoplastic cells in general (17). The agglutination observed with the untransformed BHK21 cells was not unexpected in view of the demonstrated capacity of these cells to induce tumors [see Table 2; (10)].

3.3.5. Karyotype

Chromosomes from colchicine-blocked metaphase figures of the parental B_I line and of transformed lines were prepared as described in Methods. All satisfactory figures were counted to determine a modal number for each cell line. Selected good figures were photographed, and the easily recognized chromosomes X, 16—19, 20, and 21 (as numbered by 19) were studied for aneuploidy. In ad-

dition, a complete karyotype analysis was carried out with a few figures from each cell line. The results are summarized in Table 4. The diploid number for the normal Syrian hamster cell is 44. It may be seen that there is a high frequency of cells, in all lines studied, of numbers differing from 44 by 1 or 2 chromosomes. Those with 44 generally proved to be pseudodiploid, *i.e.*, trisomy of one chromosome was balanced by monosomy of another.

Four complete karyotype analyses of the parental line were done, and several additional figures examined for aneuploidy of easily recognized chromosomes. No single anomaly appeared to be characteristic. Similarly, no characteristic change appeared to be associated with the fact of transformation. Each cloned

Table 3. *Agglutination of Ad12-transformed Cells by Purified Glycoprotein Agglutinin*[1]

Volume of agglutinin added	Degree of agglutination	
	BHK21 (B_1)	B_1 (Ad) [clone 508 no. 3]
0	0[2]	0
0.01 ml	0	+++
0.025 ml	0	+++
0.05 ml	+++	++++

[1] Cells, either untransformed (B_1) or Ad12-transformed [B_1 (Ad)] were suspended in EDTA at approximately 3×10^6 cells/ml. To 0.1 ml of cell suspension on a microscope slide was added the indicated volume of purified wheat germ agglutinin diluted 1/5 in EDTA. After a minute of agitation, the suspension was observed microscopically for the formation of aggregates.

[2] (0) indicates no noticeable agglutination; (4+) indicates all cells involved in very large aggregates.

subline was characterized by its modal chromosome number and by one or more anomalies in the number of the easily recognized chromosomes. None of the transformed lines exhibited an anomalous number in the 16—19 group. Whether the observed chromosomal irregularities resulted from the transformation process or were already present as minor fractions of the somewhat heterogeneous (although recloned) BHK21 cell population cannot be determined.

3.4. Restriction of Ad2 Replication in Ad12-transformed or Ad12-infected BHK21 Cells

3.4.1. Superinfection of Ad12-transformed Cells

Cells cultured from hamster tumors initiated *in vivo* by Ad12 were shown to be greatly restricted in their capacity to support the replication of type 2 adenovirus (26). The restriction manifested itself in variable but clonally stable degrees of reduction in yields of infectious Ad2 from tumor-derived cells compared with normal hamster cells. The yields in turn reflected the proportion of virus-producing infectious centers. Nevertheless, all tumor-derived cells in populations

Table 4. *Chromosome Patterns in Parental and Ad12-transformed BHK21 Cells*

Cell lines	Total number of figures examined	Incidence of figures with indicated numbers of chromosomes										Characteristic recognizable anomalies[1]
			40	41	42	43	44	45	46	47	48 to 80	>80	
BHK21 (B1)	56	Number	1	3	1	6	15	21	2	1	1	5	Variable, no characteristic
		Per cent	1.8	5.4	1.8	11	27	38	3.6	1.8	1.8	8.9	karyotype
B1 (Ad)	28	Number	1	2	3	11	6	0	0	0	2	3	47% with an additional long
clone 508 No. 1		Per cent	3.5	7.0	11	39	21	<3.5	<3.5	<3.5	7.0	11	metacentric
B1 (Ad)	61	Number	4	4	15	27	3	2	1	1	4	0	None recognized, other than
clone 508 No. 2		Per cent	6.4	6.4	24	43	4.8	3.2	1.6	1.6	6.4	<1.6	the low modal number
B1 (Ad)	53	Number	1	1	1	3	20	22	3	0	0	2	57% with an additional long
clone 508 No. 3		Per cent	1.9	1.9	1.9	5.7	38	42	5.7	<1.9	<1.9	3.8	metacentric, 70% with trisomy 20, and 100% with trisomy 21

[1] Specific chromosomes examined were the X (long metacentric), No. 16–19, No. 20 and No. 21, as defined in LEHMAN *et al.* (19).

infected with Ad2 at sufficiently high input multiplicity were shown to synthesize Ad2-specific structural antigens. The fact that this step was sensitive to inhibition by fluoro-deoxyuridine indicated its dependence on viral DNA synthesis (38). While this restriction to an abortive replication of Ad2 in most of the cells was considered to be a specific result of the persistence of the Ad12 genome, the uncertainty concerning the nature of the original normal target cell did not permit an unequivocal interpretation.

The present system, however, provided the means for simultaneously testing the starting (untransformed) cell line and the clonally isolated transformed cell lines derived from it. Accordingly, a number of transformed cell isolates were

Table 5. *Abortive Response of Ad12-transformed BHK21 Cells to Ad2 Infection*[1]

	Infectious centers per 10^6 cells	Cells synthesizing Ad2 structural antigens per 10^6 cells	Ratio of cells yielding infectious virus to cells synthesizing Ad2 structural antigens	Ad2 yield (p.f.u.) per 10^6 cells	Ad2 yield (p.f.u.) per infectious center
BHK21 (B_I)	8.5×10^3	2×10^4	0.43	3.3×10^7	3900
B_I (Ad) clone 508 No. 3	6.6×10^3	1.3×10^5	0.051	8.4×10^6	1300

[1] Average of two independent determinations. Parental B_I cells and B_I (Ad), clone 508 No. 3 cells were infected with 1.5 and 0.83 p.f.u. per cell respectively, and after neutralization of uneclipsed inoculum virus with anti-Ad2 antiserum, were suspended with Viokase and assayed for the number of cells yielding infectious virus (infectious centers) as described in the text. Virus yields and frequencies of cells synthesizing Ad2 structural antigens were determined as described in the text.

compared with regard to this characteristic with both the parental B_I cells and a number of untransformed clones derived from agar suspension colonies of uninfected B_I cells.

Petri dish cultures containing approximately 3×10^6 cells were exposed to infection with Ad2 at an input multiplicity of 10 p.f.u. per cell. After 2 hours adsorption at 37°C, the inoculum was removed, the cells were washed twice, and HT medium without serum was added. Samples were collected at appropriate times by freezing replicate whole cultures. These were assayed by the plaque method after several cycles of freezing and thawing to release intracellular virus. As seen in Fig. 4, the yields from the untransformed BHK isolates ranged from 0.68 to 2.9 times the yield from the parental B_I cells, while the yields from transformed cells were never higher than 0.2 and ranged as low as 0.01 of the parental cells. A given reduction in yield was characteristic of each isolate in repeated experiments. This clonal stability was similar to that reported for different lines of cells derived by long-term *in vitro* cultivation from Ad12-induced hamster tumors (26).

That the reduced yields were not due to altered efficiency of adsorption of Ad2 was demonstrated by immunofluorescence studies in which the number of cells synthesizing Ad2 structural antigens in each cell line was scored at 48 and 72 hours after infection with Ad2 at input multiplicities ranging from 0.1 to 100 p.f.u. per cell. The frequency of cells synthesizing structural proteins was found

to be proportional to the input virus dose and was approximately 5-fold higher at any given multiplicity for the B_I(Ad) than for the B_I cells. Furthermore, a maximum frequency of antigen-positive cells greater than 90% was achieved with both cell lines at sufficiently high multiplicities. The reduced yield of infectious Ad2 therefore was not due to transformation-related surface changes reducing the adsorptive capacity of the transformed cells.[2] Because viral structural proteins were used as the criterion for Ad2 infection, the results also implied that infection proceeded past the early steps in virus replication, including synthesis of viral DNA (14, 38). These results indicated that Ad12-transformed BHK21 cells were restricted in their response to superinfection with Ad2.

To determine whether the yield per cell or the frequency of yielding cells was reduced, the following experiment was done. Parental and transformed B_I cells were infected with Ad2 at multiplicities of 1.5 and 0.83 p.f.u. per cell, respectively,

Table 6. *Simultaneous Infection of BHK21 Cells with Ad12 and Ad2: Reduction of Ad2 Infectious Centers*[1]

Input MOI (p.f.u./cell)		Maximum virus yield (p.f.u./culture)	Infectious centers (% of total cells)	Virus yield per IC (p.f.u./IC)
Ad12	Ad2			
—	17	1.0×10^9	20.5	1.1×10^4
170[2]	17	2.3×10^6	0.2	2.3×10^3

[1] Monolayers of BHK21 cells (B_{II} subline) were infected with either Ad2 alone or mixtures of Ad2 and Ad12 at the indicated multiplicities. Total virus yields were determined at 48 and 72 hours after infection; infectious centers were assayed as in Table 5.

[2] This MOI of Ad12 was equal to approximately 20 T antigen inducing units (TAU) per cell, as defined in STROHL (33), and determined by immunofluorescence assay for T antigen in parallel cultures.

and the yields of infectious virus and frequencies of cells synthesizing Ad2 structural antigens were determined as in previous experiments. In addition, the infected cells were treated with Ad2-specific neutralizing antiserum to reduce the background of uneclipsed virus, suspended with Viokase, and plated onto KB cell assay monolayers in a 0.5 ml layer of nutrient agar, followed by 9.5 ml of additional nutrient agar. Typical results are given in Table 5. While 43% of the antigen-synthesizing B_I cells yielded infectious virus, only 5% of the antigen-positive transformed cells did. If 43% is taken to reflect the infectious center plating efficiency under these conditions, a maximum of 12% of infected transformed cells yielded infectious virus, and consequently 88% were abortively infected.

Table 5 also shows that the transformed cells produced about one-third as much virus per yielding cell as did the normal BHK21 cells. This moderate reduction in yield per cell was also a characteristic finding in the case of superinfected cultured tumor cells (38). Thus the *in vitro* transformed BHK21 cells exhibited

[2] These findings are consistent with those reported earlier, which showed that adsorption of Ad2 to Ad12-induced hamster tumor cells was more efficient than to normal hamster embryo cells (26).

the same characteristic restriction to Ad2 multiplication as did the *in vivo* induced hamster tumor cells, further supporting the view that the restriction is an expression of the presence of Ad12 genetic material in the tumor cells.

3.4.2. Ad2+Ad12 Mixed Infection as a Model of the Transformed or Tumor Cell

Infection of BHK21 cells by Ad12 produces an abortive response which includes synthesis of Ad12 T antigen and initiation of cellular DNA synthesis (34), but neither viral DNA (12) nor viral structural protein synthesis (STROHL and TEETS, unpublished observations). If transcription of early Ad12 gene functions in transformed and tumor cells were in fact responsible for the restriction of Ad2 replic-

Table 7. *Simultaneous Infection of BHK21 Cells with Ad12 and Ad2: Effect on Yield of Infectious Ad2 and Viral Capsid Antigens*[1]

Input MOI (p.f.u./cell)		Maximum virus yield (p.f.u./culture)	Maximum CF antigen titer[4]	CFU/p.f.u. ($\times 10^{-7}$)
Ad12	Ad2			
—	7.5	8.2×10^8	24	0.30
190[2]	7.5	1.3×10^7	8	6.20
190 (UV)[3]	7.5	6.7×10^8	24	0.36

[1] Monolayers of BHK21 (subline B_{II}) were infected with either Ad2 alone or mixtures of Ad2 and Ad12 at indicated multiplicities. Samples for virus yield were collected at 48 and 72 hours after infection.

[2] See footnote No. 2, Table 6.

[3] Ad12 diluted 1/5 in PBS and irradiated for 60 seconds at 15 cm from two 15 watt germicidal bulbs. Survival of p.f.u. $= 1.7 \times 10^{-3}$, and of T antigen inducing capacity $= 2.5 \times 10^{-2}$. Input refers to the infectivity before irradiation.

[4] Titer expressed as reciprocal of the endpoint dilution of sample; the microtechnique was that of SEVER (28), and employed anti-Ad2 antiserum, which would detect both Ad2 and Ad12 group reactive antigens. In similar experiments, homogenates from cells infected with Ad12 alone had no detectable CF titer.

ation, BHK21 cells abortively infected with Ad12 might be expected to exhibit the same characteristic restriction to Ad2 replication. The following experiments bore out this expectation.

Cultures of subline B_{II} were infected either with Ad2 alone or with mixtures of Ad2 and Ad12. The input multiplicity of Ad12 was sufficient to induce synthesis of T antigen in all of the cells, as demonstrated by immunofluorescence, while Ad12 structural antigens were never detected either by complement fixation or by immunofluorescence. In one type of experiment, infectious centers were assayed as described in the previous section, and total virus yields from parallel cultures were harvested at 48 and 72 hours. As seen in Table 6, the infectious centers in the mixedly infected cells were reduced to 1% of the Ad2-infected control cells, while the total virus yield was reduced to an even greater extent. The yield per infectious center was approximately 20% that of the control cells infected with Ad2 alone.

In the second type of experiment, yields of infectious virus and of viral structural antigens were determined. As seen in Table 7, structural antigen synthesis was reduced only to 33% of the control Ad2 infection, while the infectious virus yield was reduced to 1.5%. In this same experiment, the dependence of the restriction phenomenon on *functional* viral genomes was tested by using Ad12 which had been irradiated with UV to a survival of T antigen-inducing capacity of 2.5×10^{-2}. The data in Table 7 demonstrate that this treatment abolished the inhibitory effect.

While infection with Ad12 alone does not lead to synthesis of structural proteins, it was possible that in those mixedly infected cells which did yield Ad2, some synthesis of Ad12 capsid proteins might occur. The possibility of phenotypically mixed particles was tested by exposing the yields from a mixed infection to either anti-Ad2 or anti-Ad12 antiserum alone, or to the two antisera in combi-

Table 8. *Lack of Ad12-specific Virus in Yields from BHK21 Cells Mixedly Infected with Ad2 and Ad12*[1]

Serum added	Yield (PFU/ml) at 70 hours from BHK21 cells infected with		
	Ad2	Ad12	Ad2 + Ad12
None	1.8×10^8	$<10^2$	1.2×10^6
Anti-Ad2	1.0×10^6	$<10^2$	1.0×10^4
Anti-Ad12	1.1×10^8	$<10^2$	1.1×10^6
Anti-Ad2 + anti-Ad12	1.4×10^6	$<10^2$	1.5×10^4

[1] Virus yields were diluted 10^{-1} into an antiserum-PBS mixture and incubated at 37°C for 1 hour, after which they were diluted and assayed on KB cell monolayers under conditions which would detect Ad2, but not Ad12, plaque forming virus.

nation, and assaying for Ad2 plaque formers on KB cell monolayers. As seen in Table 8, no evidence for neutralization by anti-Ad12 antiserum, or enhanced neutralization by the combined antisera, could be demonstrated. This experiment thus failed to reveal the presence of Ad12-specific capsid proteins in yields from mixedly infected cells.

All of these results are entirely analogous to those reported for cultured adenovirus-induced tumor cells (26) and for the Ad12-transformed BHK21 cells described in this paper. They strongly imply that one or more Ad12 gene functions interfere with the successful replication of Ad2.

4. Discussion

The transformation by type 12 adenovirus of a cloned subline of BHK21 cells and the properties of the transformed cells have been described. Attempts to standardize the transformation system and to extend it to other BHK21 cell sublines have underlined the importance of the genetic nature of the cell to be transformed. The properties of the transformed cells described here leave no doubt as to the adenovirus-related nature of the alteration. Like cells derived by *in vitro* cultivation from Ad12-induced hamster tumors, they are characterized by

(a) unmistakable morphological features, (b) presence of adenovirus-specific T antigen, (c) decreased sensitivity to density dependent, or "contact" inhibition of multiplication, (d) restriction in their capacity to support the replication of type 2 adenovirus. On the other hand, the important criterion of Ad12-induced tumorigenicity cannot be approached with this system because of the highly neoplastic nature of the starting BHK21 cells themselves. Thus, while the mechanisms underlying oncogenicity cannot be explored with this system, the larger problem of the mechanisms by which viruses confer new genetic properties upon clonally derived cells can.

This problem of the oncogenicity of untransformed cells is particularly evident in the observations relating to tumorigenicity and *in vitro* growth of cells. The apparent loss of tumorigenic potential in the transformed cells may in part reflect the acquisition by them of new, virus-specified antigenic determinants on the cell surface. A similar reduced tumorigenicity has been found following the transformation of highly tumorigenic mouse embryo cells by SV_{40} (1).

The final saturation density to which cells will grow *in vitro* has been observed to be a function of the serum content of the medium, as well as of the frequency with which the medium is changed (6, 18, 41). In general, virus-transformed cells appear to have a reduced serum requirement for achieving their maximum saturation density as compared with the non-transformed cells from which they were derived. The morphological alteration and T antigen synthesis of the Ad12 transformed cells in the present study were clearly accompanied by this reduced serum requirement for growth to the maximum saturation density. The relationship of this change to the increased agglutinability by wheat germ phytoagglutinin is not clear. Agglutinability has been reported to be proportional to the lack of "contact inhibition" in various BHK21 cell sublines (17).

While the transformation frequency calculated on the basis of infected cells is very low (approximately 2×10^{-5} per infected cell) calculations allowing for cell killing and for cloning efficiency in agar suggest that a surprisingly high proportion (40%) of surviving cells may actually give rise to transformed cells. First, the cloning efficiency in agar of uninfected B_I cells was 5×10^{-2} and that of infected cells about 5×10^{-5}. Thus the relative clone survival in agar of infected cells was 10^{-3}. Among those cells surviving the infection, then, the transformation frequency can be calculated to be 2×10^{-2}, or 2%. Furthermore, it should be noted that, as used in the present experiments, the soft agar suspension method apparently did not selectively favor the adenovirus-transformed cells. In fact, they plated with lower efficiency in agar suspension than did the parental B_I cells. It therefore might not be unreasonable to assume that the observed frequency of clones with transformed cells (*i.e.*, 40%) does in fact reflect the actual frequency of transformation occurring among those cells surviving the infection.

This frequency determination was done only at high multiplicity, and therefore the effect of varying multiplicity on the efficiency of transformation cannot be evaluated. High multiplicity infection of BHK21 cells by polyoma virus resulted in a high proportion of mixed colonies, composed of normal and transformed sectors of cells (30). Under similar conditions, capsid antigen as well as intact polyoma virus was found to persist in some cells for several days after infection (4, 15). The recovery of Ad12-transformed cells only from mixed colonies suggests

that here, too, there may be a delay before the transforming event is completed. Asynchronous initiation of a productive adenovirus infection has previously been demonstrated by low multiplicity infection of single human tonsil cells with Ad2, in which delays as long as 5 days were observed (40). It is conceivable, then, that the transforming events are initiated by virus particles which for some reason do not begin to function immediately after infection. This seems to be particularly plausible in the adenovirus system, since the initial interaction results in the death of most of the cells (33, 39, 45). The prolonged intracellular period could provide time for random damages to inactivate that part of the viral genome responsible for cell death. On the other hand, in transformation with both simian adenovirus SA7 (9) and SV40 (43) the necessity for a critical cell function in the establishment of transformation was demonstrated. Such a critical period in transformation by adenoviruses might represent a portion of the cell cycle in which the adenovirus gene function leading to cell death is either repressed or its effect minimized.

The response of the Ad12-transformed BHK21 cells to superinfection with Ad2 was of particular interest, since the transformed cell could be compared directly with the untransformed parental cell. Imposition of a restriction to productive superinfection on the *in vitro* transformed, clonally derived cells strengthened the view that this transformation involved events which were basically similar to those occurring in tumor induction *in vivo* (26, 38). The fact that uninfected cell clones picked from agar suspension were all as susceptible as the parental BHK21 cell line eliminated the possibility that the restricted response to superinfection was correlated with ability to grow in agar.

That the Ad12 genome itself is involved is suggested by the experiments with simultaneous Ad2—Ad12 mixed infection of BHK21 cells. Since transformed cells represent a minute fraction of the total cell population initially exposed to Ad12, it was conceivable that they might be non-permissive for Ad2 even before transformation, and that the presence of the Ad12 genome was therefore unrelated to the restricted Ad2 replication. The results obtained after mixed infection make this possibility very unlikely inasmuch as 20% of the cells infected with Ad2 alone registered as infectious centers. The role of an Ad12 genome function acting to restrict Ad2 replication, as described for cultured tumor cells, transformed BHK cells, and mixedly infected BHK cells, is further supported by the results of MAK (21), who reported reduced synthesis of Ad2 DNA and RNA in the presence of (replicating) Ad12 in mixedly infected human cells. The existence of possible "repressor" activity in extracts of SV40-transformed or-infected cells has been reported by CASSINGENA and TOURNIER (7) and CASSINGENA *et al.* (8). The synthesis of such a "repressor" by Ad12 tumor and transformed cells would offer an attractive explanation for the observed superinfection restriction. Preliminary experimental evidence obtained in this laboratory is compatible with the existence of such a material.

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