A Variant of Adenovirus 12 Producing Cytoplasmic Accumulation of Capsid Proteins

(Accepted 18 April 1978)

SUMMARY

A variant of adenovirus type 12 (R-Ad12) has been isolated by infecting a clonal line of normal rat kidney cells (NRK) with wild-type adenovirus 12. Although R-Ad12 failed to produce infectious progeny virions when cycled a second time through NRK cells, it nevertheless retained the ability to code for virus structural proteins that accumulated in the cytoplasm.

At the non-permissive temperature, some mutants of human adenoviruses as well as an avian adenovirus have been shown to exhibit the characteristics of accumulating virus capsid proteins in the cytoplasm (Ishibashi, 1970, 1971; Everitt et al. 1972; Russell et al. 1972). Whether or not these mutants are all defective in the same function is not known. Others have described an SV40 variant which accumulates T antigen in the cytoplasm (Butel et al. 1969). Also, spontaneous variants of polyoma virus with altered distribution of virus capsid proteins have been described (Hare, 1970; Betts et al. 1972). However, natural variants of human adenovirus have not yet been isolated or characterized.

In the present work, we have isolated a variant of human adenovirus 12 which arose in a cloned line of normal rat kidney cells (NRK) infected with wild-type adenovirus 12 (strain HUIE). This variant, hereafter referred to as R-Ad12, retained the ability to infect NRK cells but produced cytoplasmic instead of nuclear accumulation of virus capsid proteins and lost the ability to produce infectious progeny virions. This communication describes the analysis of various parameters of this virus-cell model.

A clonal subline of NRK cells isolated in this laboratory (E. Major, unpublished data) was grown in Eagle's MEM supplemented with 8 % foetal calf serum. For growth of wild-type Ad12 in NRK or HEp-2 cells, the monolayer cultures were infected with clonally purified virus at a multiplicity of 50 to 80 TCD50/cell and incubated at 37 °C for 3 h. After removal of unadsorbed virus, the cells were washed with medium lacking serum and with medium containing a 1:10 dilution of Ad12 antiserum. At various times after incubation, replicate cultures maintained in medium 199 without serum were frozen and thawed six times to release virus and the resulting supernatant fluids were centrifuged and then assayed for total virus on HEp-2 cells. During the growth phase there was approx. 1 log less wild-type virus in NRK than in parallel HEp-2 cultures, yet maximum yield of virus was 160 TCD50/cell in NRK cultures and 200 TCD50/cell in HEp-2 cultures 54 h post-infection. The virus which emerged from NRK cells was termed R-Ad12 and its identity was confirmed by Ouchterlony immunodiffusion using hyperimmune rabbit Ad12 antiserum. However, when purified R-Ad12 was used to infect fresh NRK cells at a multiplicity of 100 TCD50/cell, no infectious virions could be isolated by either direct or sequential blind passaging on HEp-2 cells. Also, when R-Ad12-infected NRK lysates were fractionated by equilibrium density centrifugation through CsCl gradients, no infectious virions could be found in any of the fractions obtained from these gradients, nor was there any peak of radioactivity
Fig. 1. CsCl profiles of Hirt extracts of Ad12-infected HEp-2, R-Ad12-infected NRK cells, and normal NRK cells. Monolayers of HEp-2 and NRK cells infected with parental Ad12 at a multiplicity of 50 TCD50/cell and R-Ad12 at a multiplicity of 80 TCD50/cell were incubated for 24 h at 37 °C. After this time, the cells were labelled with 10 μCi/ml of 3H-thymidine (28 μCi/mmol) followed by incubation at 37 °C for 48 h. Virus DNA was selectively separated from the infected cells as described by Hirt (1967). The 3H-thymidine labelled virus materials were centrifuged in a Spinco SW50 rotor at 39,000 rev/min at 4 °C for 60 h. All gradients were fractionated with an ISCO model D gradient fractionator (Instrumentation Specialty Corporation, Lincoln, NB) into approx. 3 drop fractions. Gradient fractions (20 μl) containing radioactive material were precipitated with 100 μg of bovine serum albumin and 10% trichloroacetic acid (TCA) at 4 °C overnight. The precipitates were collected on S and S membranes and washed with cold 5% TCA. The filters were dried and counted in a scintillation counter. (a) R-Ad12 DNA in NRK cells; (b) parental Ad12 in HEp-2 cells; (c) normal NRK cells.

corresponding to the density of either complete or incomplete particles detectable in these lysates. To find out whether changes in the physical property of R-Ad12 might occur, the banding patterns of 3H-leucine labelled wild-type Ad12 and R-Ad12 after equilibrium centrifugation in CsCl density gradients were compared. Both viruses banded at 1.34 g/ml, suggesting that no gross changes had occurred in R-Ad12 structure. Furthermore, adsorption kinetic studies did not reveal any apparent functional changes in the adsorbing capacity of
the virus capsid proteins, as approx. 65% of added R-Ad12 and 72% of wild-type Ad12 adsorbed to NRK cells within 3 h. Virus instability during this period was negligible.

To determine whether or not R-Ad12 existed in wild-type virus, plaque morphology studies were made. Wild-type virus produced predominantly large clear plaques and only a few small plaques on HEp-2 cells, while R-Ad12 produced only small pinpoint plaques. To obtain purified variant, the pinpoint plaques were plaque-purified and subsequently shown to have the appropriate phenotype on NRK cells (N. Khoobyarian, unpublished data). It thus appeared that R-Ad12 was present, though in small amounts, in the wild-type stock, but its selection was apparently enhanced after one round of replication of wild-type virus on NRK cells. Further properties of this variant will be reported in a separate communication.

To characterize the steps beyond the level of adsorption and perhaps penetration, it was of interest to determine whether virus DNA was indeed present in R-Ad12 infected NRK cells. Infected cell extracts prepared by the Hirt (1967) procedure were subjected to CsCl density gradient centrifugation. As shown in Fig. 1a, R-Ad12-infected NRK cells showed a major peak as did wild-type Ad12-infected HEp-2 cells (Fig. 1b) at 1.704 g/ml, which is characteristic of Ad12 DNA (Doerfler, 1968). No DNA peak of similar density could be identified in normal NRK cells (Fig. 1c). However, other experiments such as sucrose gradient centrifugation and hybridization are required before establishing whether or not the virus DNA is indeed normal. To find out whether R-Ad12 might code for major virus proteins, the NRK cells infected with R-Ad12 were examined for virus proteins by immunodiffusion assays. Cell extracts of NRK cells infected with 100 TCD50/cell of purified wild-type Ad12 or R-Ad12 were prepared by freeze-thawing the cells in 0.5 ml of MEM without serum. The infected cell extracts were clarified by centrifugation at 1000 rev/min for 20 min and the supernatant fluids were used as the source of antigens. Ad12 antiserum was prepared as described previously (Winters & Khoobyarian, 1970). Using Ad12 antiserum, monospecific antisera to hexon and fibre protein were prepared by immunoabsorbent chromatography (Axen et al. 1967). Briefly, 1 mg purified hexon or fibre protein prepared according to a previous method (Abid & Khoobyarian, 1976) was added to 10 ml of CNBr Sepharose 4B suspension (Pharmacia, Inc., New York, N.Y.). This mixture was allowed to interact for 24 h at 4 °C. Five mg of bovine serum albumin (BSA) per ml of Sepharose was added to bind any free sites on the activated Sepharose. A chromatography column was prepared and the antiserum was slowly added at a flow rate of 1 ml/min. The column was washed thoroughly with borate saline buffer (BSB), pH 9.0, to remove unbound protein. The antibodies bound to hexon or fibre were eluted with 0.2 M-glycine sulphate, pH 2.3, concentrated by vacuum and dialysed against BSB before use. Three immunoprecipitin lines with antiserum to purified Ad12 could be detected in extracts of NRK cells infected with R-Ad12. Two of the lines were further identified as hexon and fibre proteins. The third line which was presumably penton was not identified because of unavailability of antiserum to this protein. Based on these observations, it appeared that some adenovirus DNA must have been transcribed in these cells.

To study the distribution of adenovirus proteins in cells, NRK cells grown on coverslips and infected with purified R-Ad12 (at a multiplicity of 80 to 100 TCD50/cell) at 37 °C were removed at various times after infection, washed, and fixed in −70 °C acetone for 10 min. After this time, cells were treated with anti-fibre or anti-hexon antiserum and then reacted with fluorescein-conjugated goat antiserum to rabbit gamma globulin for 45 min at 37 °C. As shown in Fig. 2, treatment with these antisera revealed intracytoplasmic virus antigens 24 h post-infection. In subsequent experiments, using different batches of antisera, as much
as 60% of the cells contained intracytoplasmic virus antigens 72 h after infection, whereas treatment of NRK cells with wild-type virus only led to intranuclear accumulation of antigens. These results were considered unusual since cytoplasmic localization of adenovirus antigens have been found only in the cells infected with ts mutants at the non-permissive temperature (Ishibashi, 1970, 1971; Everitt et al. 1972; Russell et al. 1972). In the light of these findings, it would be interesting to investigate whether R-Ad12 might be defective in the same function, i.e. transportation, as are adenovirus mutants.

To find out whether or not the expression of adenovirus functions in NRK cells might inhibit cell growth, monolayers were infected with wild-type Ad12 or R-Ad12 at a multiplicity of 75 to 90 TCD<sub>50</sub>/cell for 24 h at 37 °C. The cells were then washed, trypsinized, diluted, and plated in 60 mm plastic dishes (1000 cells/dish) in MEM containing 10% foetal calf serum and 9·9 mM-NaHCO<sub>3</sub> and incubated at 37 °C in a 5% CO<sub>2</sub> incubator for 10 days. The colonies were then fixed, stained with Giemsa and counted. While wild-type Ad12 inhibited cell growth, no inhibition of cell growth was noted by R-Ad12, as plating efficiencies for control and R-Ad12-infected cells were 3·6 and 3·8%, respectively.

Although R-Ad12 failed to grow on NRK cells but grew well on HEp-2 cells, yielding a maximum of 140 TCD<sub>50</sub>/cell 54 h after infection, it is not known whether R-Ad12 is defective in the gene responsible for productive infection in rat cells despite the expression of some virus functions in these cells. On the other hand, it is possible too that the genetic
make-up of the rat cells might exert control over the synthesis of progeny virions. Whatever the mechanism, the block in virus synthesis is most likely at a later stage. Studies on factors that might be responsible for inhibiting the replication of R-Ad12 in NRK cells are now under way. We believe a cell-virus system of this sort in which adenovirus information is expressed without causing cytopathic effect as well as impairing cell growth can be of potential value in the analysis of various host cell factors that might exert control over virus synthesis.

This work was supported by an American Cancer Society grant (Illinois Division). K.A.B. was a recipient of an oncology training grant CA 05291 from the National Cancer Institute.

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REFERENCES

(Received 20 January 1978)

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