Intranuclear Crystalline Zebra Structures Induced in Human Tumour Cells by Adenovirus Type 5

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SUMMARY

Intranuclear crystalline inclusions with leaf-like striated appearance, i.e. zebra structures, were observed by electron microscopy in adenovirus type 5-infected human cervical and bladder carcinoma cells at 4 to 6 days after infection.

Large amounts of excess virus structural components are produced in human adenovirus-infected permissive cells at different times after infection (Russell et al. 1967a). Adenovirus hexon and fibre capsid subunits derived from purified complete virus particles and from infected cell extracts have been crystallized (Pereira et al. 1968; Mautner & Pereira, 1971). More recently, crystallization and reaggregation of human adenovirus type 5 (Ad5) structural components derived from infected cell extracts have been reported (Carstens & Marusyk, 1977). In our previous studies of adenovirus replication in different histopathological types of human tumour cells (Winters et al. 1977), intranuclear striated crystalline inclusions were occasionally observed by electron microscopy in Ad5-infected human urinary bladder carcinoma cells. The results of our systematic attempts to consistently induce the formation of the crystalline inclusions in human tumour cells using Ad5 are reported here.

Human adenovirus type 5 (Ad5; strain Ad75) was propagated in KB cells, purified by serial caesium chloride density gradient sedimentations and assayed for infectious virus by previously described methods (Russell et al. 1967b; Winters et al. 1975). Replicate monolayer cultures (4 x 10^5 to 5 x 10^5 cells/flask) of human normal skin, bladder and kidney cells (Winters et al. 1977) and human urinary bladder (SCaBER; O'Toole et al. 1976) and cervical (ME 180; Sykes et al. 1970) carcinoma cells of the squamous cell type were grown in Dulbecco's minimal essential medium (DMEM) containing 15% heat-inactivated foetal bovine serum (FBS). Cells were infected with Ad5 input multiplicities of 25 and 50 p.f.u./cell in DMEM with 2% inactivated FBS. Cell cultures were incubated at 37 °C in the presence of 5% CO₂ and air.

Cultures of Ad5-infected and mock-infected normal and carcinoma cells were examined for evidence of virus-induced cytopathic effect (c.p.e.) and were collected daily for 10 days post infection (p.i.) or until 50% or more c.p.e. was observed in the individual infected cultures. At each time of collection, cells previously washed once with 5 ml of warm phosphate buffered saline (PBS; 0.01 M, pH 7.2) were scraped from the flask surfaces into 3 ml of PBS and were re-suspended by pipetting. Cells were counted and measured portions of the well-mixed cell suspensions were pelleted, fixed, processed and examined in the electron microscope according to previously described methods (Sykes et al. 1968). Other measured portions of each cell suspension were extracted by 6 cycles of freeze-thawing followed by three 1 min cycles of sonication at 50 W at 10°C. These cell extracts were assayed for Ad5 antigens by the solid bead radioimmunoassay (SBRIA) magnetic transfer technique (Smith & Gehle, 1977) and were analysed for adenovirus polypeptides by polyacrylamide gel electrophoresis (PAGE) according to methods described by Laemmli (1970).
Reference animal antisera capable of reacting specifically with Ad5 hexon, penton or fibre antigens (Russell et al. 1967a) and reference human convalescent serum containing neutralizing and precipitating antibodies against Ad5 were used in SBRIA tests.

In our present experiments, no c.p.e. was observed in Ad5-infected ME180 and SCaBER cell cultures at times when a majority of cells in these cultures were first shown by electron microscopy to have well-formed intranuclear zebra inclusions. Zebra structures were first observed at 4 to 5 days p.i. in nuclei of carcinoma cells originally inoculated with 25 to 50 p.f.u./cell of Ad5. At this time zebra structures were usually the only intranuclear inclusions seen in these cells. Rarely, however, an infected cell nucleus was observed to contain both zebra structures and a small linear paracrystalline inclusion. At 7 days p.i. and thereafter, zebra structures and adenovirus particles were found in infected cell nuclei. Intranuclear zebra structures in infected ME180 cells at 5 days p.i. (Fig. 1a) were identical in appearance to those observed in infected SCaBER cells at that time (Fig. 1b). The striated zebra inclusions were of various sizes, sometimes branching, leaf-like in general appearance and were composed of alternating regular repeating dark and light bands. Ten zebra structures measured at ×35000 and ×70000 magnifications had dark band widths of 24 to 25 nm and light band widths of 14 nm, while the centre to centre distances between repeating dark and light bands measured 34 to 36 nm. We did not observe intranuclear zebra structures in uninfected cells, in Ad5-infected normal cells or in any other types of human tumour cells infected with Ad5.

Levels of cell-bound Ad5 antigens detected by SBRIA in extracts of infected normal cells increased until 7 to 10 days p.i., when c.p.e. involved 25% or more of the individual cultures. In contrast, SBRIA tests of Ad5-infected ME180 cell extracts detected a slight decrease in Ad5 antigen levels at 5 days p.i. (Table 1). When portions of identical cell suspensions collected at 3 to 7 days were examined by electron microscopy and by SBRIA, the decrease in Ad5 antigen levels at 5 days p.i. coincided with observations of large numbers of zebra inclusions in the infected cell nuclei. These results strongly suggested the possibility that most Ad5 antigens were bound within the zebra inclusions at 5 days p.i., and decreased amounts of non-inclusion bound Ad5 antigens were being detected by the sensitive SBRIA tests. Levels of Ad5 antigens detected in extracts of Ad5-infected (m.o.i. = 50 p.f.u./cell) SCaBER, ME180 and KB cells at 4 days p.i. were similar, i.e. within 2000 ct/min in SBRIA tests. However, titres of Ad5 virions in the KB cells at this time were 2 to 3 log10 units higher than those detected in SCaBER and ME180 cells. Only occasional incomplete particles were seen in Ad5-infected SCaBER and ME180 cells, but they were frequently observed in Ad5-infected KB cells.

PAGE analysis confirmed that large amounts of hexon and fibre polypeptides were present in extracts of cells containing zebra inclusions alone and in those containing both zebra inclusions and virus particles. Attempts at isolation of striated inclusions have not been successful using freeze-thaw-sonication extraction methods. After our separation attempts, no leaf-like zebra structures were observed, although antigenic reactivity was retained.

Numerous types of intranuclear virion and non-virion crystalline inclusions and structures have previously been observed in several types of host cells infected by different human adenoviruses (Boulanger et al. 1970; Henry et al. 1971; Carstens & Marusyk, 1975). The zebra structures in the infected human cervical and bladder carcinoma cells did not resemble any of these previously described intranuclear structures. However, structures with a general appearance similar to those of the zebra inclusions have been described by Martinez-Palomo (1968) as striated protein fibres within type III inclusions in KB cells infected with
Fig. 1. Sections of human bladder (SCaBER) and cervical (ME180) carcinoma cells of the squamous cell type collected 5 days after inoculation with Ad5 at 50 p.f.u./cell. (a) Virus particles and striated structures in ME180 cell nucleus (× 60000). (b) Branching intranuclear leaf-like zebra inclusions in SCaBER cell (× 30000).
### Table 1. Comparison of adenovirus type 5 antigen levels detected by SBRIA in extracts of Ad5-infected human cervical carcinoma cells

<table>
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<th>Time p.i. (days)</th>
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</table>

* Average count of beads with virus-infected cells minus average count of beads with uninfected cells equals $^{125}$I, ct/min/bead at each time. Metal beads coated with resuspended cell extracts for 1 hr were suspended in human anti-Ad5 reference serum at 1:100 dilution as primary antibody for 18 hr at 4 °C and then in $^{125}$I-labelled goat anti-human IgG serum at 1:20 dilution as secondary antibody for 3 hr. All beads were washed 16 times between each step with the magnetic transfer carrier (Smith & Gehle, 1977) and were counted in a Beckman 310 gamma counter.

† Cell extracts (see text) were prepared from cultures that contained at time of virus (50 P.f.u./cell m.o.i.) or mock infection approx. $4 \times 10^6$ cells in experiments 1 and 2 and $5 \times 10^6$ cells in experiment 3.

Adenovirus type 12. The periodicity of repeating bands in Ad5-induced zebra structures was different from that observed in Ad5 hexon and fibre crystals and penton aggregates formed from Ad5 virus components extracted from infected cells (Pereira *et al.* 1975; Carstens & Marusyk, 1977). The zebra inclusions, like paracrystalline structures (Boulanger *et al.* 1970), did not inhibit the formation of adenovirus particles. Apparently, the zebra inclusions were composed of normal, and perhaps abnormal, adenovirus capsid proteins formed prior to virions in these two human carcinoma cell lines.

To our knowledge, this is the first report in which intranuclear crystalline inclusions with a striated leaf-like appearance have been observed and systematically induced in human carcinoma cells with human adenovirus type 5. The formation of the zebra inclusions during permissive infection appeared to depend on the initial multiplicity of infectious virus, the time after infection, and the human cell type. The possibility that similar intranuclear striated inclusions can be induced in human cancer cells by other serotypes of the human adenoviruses and by adenovirus mutants is presently being investigated.

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