Paracrystal Formation in Cell Cultures Infected with Adenovirus Type 2

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Large rod-shaped structures corresponding to paracrystals were seen in the nucleus, cytoplasm, or both of adenovirus type 2 (Ad2)-infected cells by immunofluorescence staining with antibody prepared against purified Ad2. In exception to this, Ad2-induced crystals did not stain with either hexon or fiber antibody. The crystalline structures were first observed in Ad2-infected Vero cells at 28 hr with a maximum number at 70 hr postinoculation. The kinetics of paracrystalline formation closely paralleled the experimental synthesis of infectious progeny virus. Acridine-orange staining revealed the lack of nucleic acids associated with the crystal. Also, the paracrystals stained intensely with phenanthrenequinone, suggesting that they are composed of basic proteins. Interferon induced by Newcastle disease virus from African green monkey kidney cell cultures was used to pretreat Vero cells prior to Ad2 infection. This resulted in inhibiting the formation of viral-induced paracrystals in 97% of the cells and reduced virus yields by 95%. The African green monkey kidney cell culture interferon did not reduce Ad2 yields in HeLa cell cultures or display any virus inhibitory activity in rabbit kidney cell cultures. Staining procedures, fluorescent-antibody tests with whole virus, hexon or fiber antibody, and interferon studies suggested that the paracrystals were viral-directed and composed of basic proteins (possibly core proteins).

The morphological changes which occur in cells infected with adenoviruses have been extensively studied, especially those provoked by human adenovirus type 2 (Ad2; reference 36), type 5 (Ad5; references 6, 12, 26), and the highly oncogenic adenovirus type 12 (Ad12; references 10, 16, 22). Electron and light microscopic observations have revealed paracrystalline structures in cells infected with certain strains of Ad5 (25), Ad2 (36), infectious canine hepatitis virus (ICHV; references 11, 23) and with Ad12 (21). The chemical nature and ultrastructure of the crystals have been previously reported although their immunological relationship to the virus was unknown. A recent report (5) suggests that the paracrystals associated with Ad5 were composed of viral antigens. The present study reports the observation of similarly shaped structures in Ad2-infected cells. It also provides direct evidence associating the paracrystals immunologically to the structural antigen(s) of the infecting virus. Interferon induced by Newcastle disease virus (NDV) from the primary African green monkey kidney (GMK) cell cultures was used in an attempt to inhibit the

replication of Ad2 and the formation of the crystals in Vero cells. Previous reports have shown that interferon does not interfere with the host cell's growth or protein synthesis (3, 17, 19) but does affect adenovirus replication (9). The significance of the viral-induced crystals and their similarity to paracrystalline formations found in association with other adenovirus infections are to be discussed.

MATERIALS AND METHODS

Cell cultures. All cell cultures were grown in either 3- or 32-oz. (ca. 90 or 960 ml) prescription bottles or in screw-cap culture tubes (16 by 125 mm) with or without cover slips (7 by 22 mm). Primary cell cultures of chick, hamster embryo (HE), and rabbit kidney were prepared by the method described by Bodain (4). All primary cells as well as the Vero cell line were grown in 0.5% lactalbumin hydrolysate in Hanks balanced salt solution (LAH) supplemented with final concentration of 2 to 7% newborn calf serum, 1% Eagle's minimal essential vitamin mixture, 0.04 to 0.12% sodium bicarbonate, 2 mM glutamine, and antibiotics. HeLa and a stable cell line of human amnion cells (RA, previously derived by R. W. Atchison) were grown in Eagle's basal medium (7) supple-

mented with 2 to 8% newborn calf serum, antibiotics, and sodium bicarbonate concentration as above. The Ad12-transformed line of hamster tumor cells (HT) was originally derived from an AD12-induced tumor and has since been subcultured 73 times in this laboratory. They were grown in a Joklik-modified Eagle's-Spinner minimal essential medium (Grand Island Biologicals, Grand Island, N.Y.) formulated without calcium but later supplemented with 0.12 mM CaCl₂ (8), 5% fetal calf serum, and 5% horse serum. All cell culture medium contained 100 units of penicillin and 40 μ g of streptomycin per ml.

Viruses. Ad2, originally obtained from J. A. Armstrong, University of Pittsburgh, was described previously (1) and represents a clinical isolate obtained from degenerating human kidney cell cultures (HuK). The Ad2 pool was prepared in HuK cell cultures grown in 32-oz. prescription bottles. The absence of contaminating adenovirus-associated virus (AAV; reference 1) from the Ad2 pool was determined with the electron microscope and the immunofluorescence staining technique. The latter method used fluorescein isothiocyanate-conjugated antiserum prepared against purified AAV types 1, 2, and 3. No simian virus 40 complement-fixing (CF) antigen could be detected in the adenovirus pool. The Indiana strain of vesicular stomatitis virus (VSV) used for assaying interferon titers and NDV (CG strain) used for induction of interferon were obtained from J. A. Armstrong.

Virus assays. A 0.1-ml amount of serial 10-fold virus dilutions was inoculated into HeLa cell culture tubes, four tubes per dilution. The cultures were examined periodically over a 21-day period for viral cytopathic effects, and medium was changed as needed. Viral infectivity was calculated by the tissue culture dose 50% endpoint (TCD₅₀) method of Reed and Muench (28).

Purification of virus. Ad2 used to hyperimmunize New Zealand White rabbits was purified and concentrated by a modification of the method of Green and Pina (13). Instead of RbCl, the virus was pelleted on a 55% (w/v) potassium tartrate cushion and CsCl gradients were used to band the virus. The visible band at a density of 1.34 g/ml contained the intact virus particles as observed by negative-staining electron microscopy. The virus fraction was dialyzed against 0.15 M NaCl at 4 C to remove CsCl.

Immune sera and preparation of fluorescein isothiocyanate-labeled antiserum. Equal parts of dialyzed, purified Ad2 and Freund's complete adjuvant were mixed and divided into three equal lots, each of which was injected intramuscularly into rabbits on days 0, 7, and 21. The animals were bled by cardiac puncture 7 to 10 days after the last injection, and the serum was collected and stored at -20 C. The antiserum was tested for its specific CF and virus-neutralization titer. A CF titer of 1:512 was shown against the purified Ad2, but no CF activity was observed when uninfected HuK cell extracts were used as an antigen. The antiserum was capable of neutralizing 3,000 TCD₅₀ of virus at a 1:512 dilution. The immunoglobulins were precipitated in the cold with ammonium sulfate at a final concentration of 34% and then labeled with fluorescein isothiocyanate as de-

scribed by Riggs et al. (30), and the conjugate was processed as described by Atchison et al. (2). Antibodies prepared against Ad5 hexons and Ad2 fibers were obtained from H. Ginsberg (University of Pennsylvania, Philadelphia, Pa.) and U. Pettersson (Uppsala University, Uppsala, Sweden), respectively.

Immunofluorescence procedures. All immunofluorescence studies were done with cell monolayers grown on cover slips in culture tubes. Cover slips were inoculated with 0.1 ml of the appropriate dilution of virus and incubated at 34 or 37 C. After the desired infection period, the cover slips were removed from the culture tubes and fixed in cold acetone for 10 min. The cover slips were air-dried and stored at -20 C or stained immediately by the indirect method with antihexon or fiber sera in combination with a goat antirabbit conjugate or by the direct method with the Ad2 conjugate as described elsewhere (34). Other cover slips were fixed in 100% ethanol for 10 min and stained with phenanthrenequinone by the method described by Russell et al. (32) for arginine.

Induction and assay of interferon. Interferon was induced by inoculating confluent monolayers of primary GMK cell cultures with approximately 10 plaque-forming units of NDV per cell. Twenty-four hours postinfection, the fluids were harvested from the cell sheets, pooled and brought to pH 2.5 with 1 N HCl, held for 48 hr at 4 C, and then neutralized with 1 N NaOH. This material was centrifuged at 10,000 $\times$ g for 2 hr and then checked for infectious NDV on chick embryo cell cultures. Such culture fluids free of viral infectivity as well as uninfected culture fluids were assayed for interferon activity on GMK, Vero, and rabbit kidney tube cultures as follows. Four replicate cell monolayers were treated 12 to 14 hr with 0.5 ml of serial twofold diluted interferon or control fluid, after which the monolayers were challenged with approximately 100 plaque-forming units of VSV per monolayer. The interferon titers were expressed as the reciprocal of the dilution inhibiting a cytopathic effect response in 50% of the tube cultures at 24 hr postinfection with VSV. Interferon prepared and assayed by this method had reciprocal titers ranging from 64 to 128 per ml on GMK cell cultures, 32 to 64 per ml on Vero cell cultures, and 2 to 4 per ml on rabbit kidney cell cultures. The interferon was nondialyzable and stable at pH 2.5 for 48 hr at 4 C (20).

RESULTS

Ad2 multiplication in Vero and HeLa cell lines and appearance of crystals. Large crystalline rod-shaped structures could be observed in the nucleus and cytoplasm of Ad2-infected Vero cells by immunofluorescence staining (Fig. 2). The paracrystalline structure fluoresced with a higher intensity than the nucleus of the cell, suggesting an accumulation of viral antigen(s) into such structures. It was essential to determine when the crystals first appeared in the infected cell and whether they coincided with the synthesis of early or late viral antigens. Monolayers of Vero

and HeLa cells grown in tubes or on cover slips were inoculated with 10 to 50 TCD₅₀ of virus per cell. After a 2-hr adsorption period at 37 C, the unadsorbed virus was removed from the cultures by washing the cell monolayers twice with 1.0 ml of LAH medium. After the addition of 1.0 ml of medium, the cultures were incubated at 37 C. At the designated time, duplicate samples were removed and disrupted with three cycles of quick-freezing in an alcohol-dry ice bath and quick thawing at 37 C. The harvests were stored at -70 C until titrated for virus content. For determining the kinetics of crystalline formation, duplicate cover slip cultures of Vero cells were removed at various time intervals postinfection and fixed in cold acetone for 10 min and stored at -20 C until stained with the Ad2 conjugate. Paracrystal formation in HeLa cell cultures was not determined because very few cells (Table 1) were observed by fluorescent-antibody staining to contain paracrystals. The results of such experiments are shown in Fig. 1. The formation of viral crystals in Vero cells closely paralleled the production of viral capsid antigens and infectious virus. The first crystals were seen in the cell monolayer at 28 hr, which was approximately 7 hr prior to the exponential rise in infectious adenovirus synthesis. Approximately 30% of the cells contained one or more crystals at 70 hr postinfection. The percentage of cells containing crystals was dependent upon the multiplicity of infection and the incubation temperature.

The formation of the paracrystalline structure is apparently very dependent upon the incubation temperature as crystals were formed in cell cultures incubated at 37 C but not at 34 C, even

FIG. 1. Multiplication of Ad2 in Vero (O) and HeLa cell cultures (●) and appearance of crystals in Vero cell cultures (■).

though positive nuclear fluorescence was present in cell cultures incubated at 34 C.

The replication (Fig. 1) of Ad2 in Vero and HeLa stable cell lines was compared. In HeLa cell cultures, Ad2 had a latent period of approximately 12 to 14 hr. There was a sharp increase in titer to 40 hr and then a somewhat gradual increase to 78 hr. The replication pattern in Vero cells was completely different with a longer latent period of 34 hr and an increase in the length of virus replication to 96 hr after inoculation. The titers produced in the HeLa cell cultures were always approximately 2- to 10-fold higher than those obtained from Vero cell cultures.

Vero cells infected with Ad2 for 70 hr and then stained with the Ad2 conjugate can be seen in Fig. 2A, B. A variety of different sized intranuclear and perinuclear crystals can be seen. Crystalline structure formed in the nucleus sometimes will severely extend the nuclear membrane (Fig. 2C). This phenomenon was also observed by Morgan et al. (25) in Ad5-infected cells. The length and width of the viral crystals vary; some have been seen that exceed 30 μ m in length and 5 μ m in width. Most of the crystals formed in Vero cell cultures appear to accumulate in the perinuclear area of the cell (Fig. 2A, B) but also several smaller crystals can be found in the nucleus of a single cell (Fig. 2D). Figures 3A and B show the result of staining infected cells with either hexon or fiber antisera. Specks and accumulation of hexon or fiber antigen were observed throughout the cytoplasm and nucleus of the cells. However, the paracrystals showed little fluorescence, indicating that small amounts, if any, of the two antigens are present in the paracrystals. The in-

TABLE 1. Paracrystalline formation and viral progeny yields in cells infected with adenovirus type 2 (Ad2)

Cell type	Ad2 inoculum (TCD ₅₀ /cell)	Ad2 yield (TCD ₅₀ /cell)	Per cent cells containing crystals ^a	Per cent cells with positive nuclear fluorescence
Vero	10	4,250.0	30.0 ^b	53.0
LLC-MK ₂	3	10.0	0.1	1.0
HE	50	330.0	7.0	30.0
HT	30	2.5	35.0	48.0
HeLa (S-3)	10	10,000.0	3.0	79.0
RA	10	8,500.0	0.0	65.0

^a Viral crystals observed by immunofluorescence staining.

^b Represent experiments when maximum number of cells containing crystals were observed, average number from 40 to 50 fields counted at $\times 250$.

FIG. 2. *Ad2*-infected Vero cells (70 hr postinfection) stained with fluorescein isothiocyanate-labeled *Ad2* antibody. $\times 450$. (A) Fluorescence of intranuclear and cytoplasmic crystals of varied sizes. (B) Nuclear fluorescence and presence of large cytoplasmic crystals. $\times 650$. (C) Fluorescence of nucleus and crystal that is extending the nuclear membrane; note lack of nucleolus fluorescence. $\times 1,300$. (D) Fluorescence of several smaller crystals in the nucleus. $\times 650$.

FIG. 3. *Ad2*-infected Vero cells. (A) Vero cells (48 hr postinfection), stained by the indirect method with hexon antiserum. Fluorescence of cytoplasm and nucleus is observed; note that paracrystals appear void of fluorescence (arrows). $\times 600$. (B) Vero cells (48 hr postinfection) stained with fiber antiserum. Paracrystals (arrows) show little fluorescence. $\times 350$. (C) Vero cells stained with phenanthrenequinone (48 hr postinfection), nuclear fluorescence and fluorescence of two paracrystals (arrows) located in the cytoplasm. $\times 650$.

tense fluorescence of the paracrystals observed with the Ad2 conjugate (Fig. 2A, B) was not noted with either of these sera.

Ad2-infected Vero, LLC-MK₂, HT, and HE cells stained with 0.01% acridine orange as described by Mayor and Diwan (24) showed bright yellow-green fluorescence associated with small areas in the nucleus. However, no fluorescence of the crystals was observed, suggesting the lack of nucleic acids in the crystals. When Ad2-infected Vero cells were stained with phenanthrenequinone, several cytoplasmic paracrystals fluoresced intensely (Fig. 3C). Infected nuclei also stained intensely for arginine, whereas the cytoplasm displayed a diffuse fluorescence.

Host cell dependence on virus-induced crystal formation. It has previously been reported that crystalline formation is dependent upon the virus strain and independent of the host cell (6, 26). The crystalline structures found in association with Ad2 used in these studies were generally independent of the host cell. These crystals were formed in a number of different cell types, e.g., LLC-MK₂ (a stable cell line of rhesus monkey kidney cells), Vero, HeLa, HT, and HE, but the number of cells containing crystal varied with each cell type. The crystals could not be found in RA cells by immunofluorescence staining (Table 1). Experiments utilizing various multiplicities of infection and incubation periods were performed; however, no rod-shaped structures could be seen in RA cells.

The percentage of cells containing crystals was different for each cell type as was the yield of infectious virus, even when given a similar dose of virus. Very little progeny virus was obtained from infected HT cell cultures (2.5 TCD₅₀), but a high percentage of the cells contained viral antigen and crystals. The reduced yield of Ad2 in HT cells in comparison to that produced from HE cells is in agreement with an earlier report by Rouse et al. (30). High yields of virus and a high percentage of cells fluorescing for Ad2 antigen were obtained from cell cultures of human origin, e.g., HeLa and RA; however, none or a low percentage of the cells contained crystals. The crystals were either present in these cells in low numbers or were present only in the nucleus and, therefore, masked by the intense nuclear fluorescence usually observed in these cell cultures.

Inhibition of viral crystal formation by interferon. Interferon, induced in GMK cells by NDV, was used to pretreat Vero cells in an attempt to inhibit the replication of Ad2 and reduce the number of cells producing the viral crystal. Vero cell monolayers grown on cover slips were pretreated overnight with 0.5 ml of GMK cell

culture interferon (titer of 1:128) or 0.5 ml of control fluid (LAH medium removed from uninfected GMK cell cultures) after which they were challenged with 20 TCD₅₀ of Ad2 per cell. At 30 and 72 hr postinfection, four cover slips were taken from each of the interferon- and control-treated tubes, fixed in cold acetone for 10 min, and stained with the Ad2 conjugate. The data presented in Table 2 indicate that interferon was effective in inhibiting the formation of the viral crystal. A 97% inhibition of crystalline formation in comparison to the control fluid was observed at 72 hr postinfection. A comparable reduction in yields of Ad2 (95%) was also observed in Vero cell cultures pretreated with GMK interferon (Table 3). However, the GMK cell culture interferon did not reduce the final harvest yields of Ad2 in HeLa cells.

TABLE 2. *Inhibition of viral crystalline formations by interferon*

Virus inoculum (20TCD ₅₀ /cell)	Duration of infection (hr)	Per cent cells ^a fluorescing for viral crystals when pretreated with		Per cent inhibition ^d
		Interferon ^b	Control fluid ^c	
Ad2	30	0.04	5.0	99
Ad2	72	1.0	31.0	97

^a Counted at $\times 250$: 80 to 90 fields, eight cover slips per evaluation.

^b Treatment 12 to 14 hr with 1 ml of Newcastle disease virus-induced African green monkey kidney interferon (titer 1:128).

^c Medium from uninfected African green monkey kidney cell cultures.

^d Values are expressed as a percentage of the inhibition of the control fluid pretreated cultures.

TABLE 3. *Inhibition of adenovirus 2 multiplication in Vero cells by African green monkey kidney cell culture interferon*

Cell type	Cells pretreated 12 to 14 hr with 0.5 ml of		Per cent inhibition
	Interferon ^a	Control fluid ^b	
Vero	5.7 ^c	7.0	95
HeLa	8.0	8.0	0

^a Titer 1:128.

^b Medium removed from uninfected African green monkey kidney cell cultures.

^c Log₁₀ TCD₅₀/0.1 ml, viral titer determined 96 hr after inoculation.

DISCUSSION

This study reports the findings of a new clinical isolate of Ad2 associated with crystals composed of viral antigen(s) (possibly core proteins) which are similar in shape and size to the crystals found with Ad5 (12). We obtained similar results in HeLa cells as those reported for the cellular site of crystals in Ad5- and Ad2-infected HeLa and HEp-2 cells; both serotypes induced crystals in the nucleus of the cell. In other cell types, e.g., Vero, LLC-MK₂, HE and HT, crystals were observed by fluorescent-antibody staining in both the nucleus and cytoplasm of the cells. Boyer et al. (6) reported the inability to detect specific fluorescence of protein crystals associated with Ad5-infected HeLa cells by using a conjugate prepared with antibody against purified Ad5. However, in this study, a low percentage of Ad2-infected HeLa cells (Table 1) demonstrated positive fluorescent staining for crystal formation. A recent report by Given et al. (11) showed similar crystals by electron microscopy associated with ICHV. They were observed in both the cytoplasmic and nuclear areas of a continuous line of dog kidney cells.

The strain-dependent formation of the protein crystals has been previously reported by Morgan et al. (26) who found them associated with two out of five strains of Ad5. Weber and Stitch (34) found crystals associated with only one of the two Ad2 strains they tested.

The crystals are apparently unrelated to the T (10, 18) antigens since T antigens appear earlier during an adenovirus lytic infection than do the crystals. They also show little fluorescence with a conjugate prepared against purified virus.

The reduction in crystalline formation in Vero cell cultures by pretreatment with GMK cell culture interferon is suggestive of the viral origin of these structures. Unpurified and partially purified interferon from various animal sources has been previously shown to have no effect on the host cell's growth, ribonucleic acid, or protein synthesis. Therefore, the reduction in crystal formation by interferon is most likely a direct inhibition of virus protein synthesis.

Since adenoviruses in general show little susceptibility to interferon (13), it appears unusual that such a large inhibition in Ad2 progeny yields by interferon should occur. Recently, Gallagher and Khoobyorian (9) reported that Ad2 was inhibited by both human and rabbit interferon; the extent of the inhibition was dependent upon the dose of interferon.

Human adenoviruses generally replicate poorly in cells derived from monkey tissue. The Vero cell line of green monkey has been reported to be

susceptible to human adenoviruses (29), which is apparently dependent upon the strain of adenovirus. For example, the V202 strain of Ad2 (research reference reagent branch, National Institutes of Health) replicates poorly in Vero cell cultures with little or no yields of progeny virus and no virus crystals produced (*unpublished data*). The Ad2 strain associated with the protein crystals multiplies well in Vero cell cultures producing relatively high titers of progeny virus (Fig. 1).

The relationship of paracrystal formation to the maturation and appearance of infectious virus is not clear. However, the appearance of paracrystals late in the growth cycle suggests that they may be composed of a protein(s) required for virus maturation. Since paracrystals showed little fluorescent-antibody staining with either hexon or fiber antisera, they are most likely composed of the internal viral core proteins. The core proteins of Ad2 have been reported to be extremely rich (22%) in arginine (27), whereas the capsid proteins contain little arginine. Positive fluorescence of paracrystals with phenanthrenequinone, a compound that forms a fluorescent condensate with arginine, suggests that they are composed of basic proteins, a characteristic common to core proteins. Originally, by ultrastructure observations, the Ad2-induced paracrystals (14) were thought to be composed of hexon, penton, and fiber antigens arranged in a small hexagonal structure as described previously by Boulanger et al. (5). However, in this study, hexon antiserum prepared against Ad5 failed to stain Ad2-induced paracrystals. This latter evidence suggests that little, if any, of the capsid antigens are incorporated into the paracrystal. Apparently, the antigenic composition of the paracrystals cannot be determined by their ultrastructural characteristics.

Why certain strains of adenoviruses are associated with crystals composed of basic viral proteins is unknown. These strains may produce an overabundance of viral protein which accumulates and crystallizes in the nucleus and perinuclear area of the cell as suggested by Russell et al. (33).

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