An Image Analysis Study of Adenovirus Type 5-induced Crystalline Inclusions

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SUMMARY

A combined electron microscopic and optical diffractometric study of Ad5-induced non-virion protein crystals found in the nuclei of infected KB cells at late times (48 to 72 h p.i.) after infection has been carried out. Data obtained have indicated that the unit cell of the crystal is rectangular and not hexagonal as previously presumed.

INTRODUCTION

Since first described by Morgan et al. (1957), the intranuclear non-virion crystal inclusions (paracrystals') induced by certain adenoviruses late after infection have been the subject of considerable interest. Several reports have been published concerning the chemical nature of the crystalline inclusions (Henry & Atchison, 1971; Marusyk, Marusyk & Lord, 1972; Marusyk, NorrbY & Marusyk, 1972), however, little information is available concerning the physical nature of the crystals. Weber & Liao (1969) implied that crystal morphology was related to the subgroup of the inducing adenovirus. Boulanger and co-workers (Boulanger, Torpier & Biserte, 1970) proposed a model of the crystal structure based on the presence of adenovirus structural components in crystal-containing extracts of adenovirus type 5-infected cells and have more recently (Lifchitz et al. 1975) constructed a three-dimensional model of the crystal based on crystallographic data.

The present study was undertaken to describe more adequately the crystal morphology using the combined techniques of electron microscopy and optical diffraction. The data presented support that of Lifchitz et al. (1975) in that the basic symmetry unit of the crystal has been shown to be rectangular in nature and not hexagonal as has been previously assumed by most investigators.

METHODS

Cell cultures and virus production. The prototype strain of human adenovirus type 5 (Ad5; ATCC VR-5) was propagated on monolayer cultures of KB cells (ATCC CCL-17; mycoplasma-free). Virus input multiplicities of approx. 10 p.f.u./cell were used in all experiments. The cell cultures were maintained in Eagles’s minimal essential medium containing 2 to 5 % inactivated calf serum. Virus-infected cell monolayers were incubated at 37 °C for 48 to 60 h prior to preparation for electron microscopy.

Electron microscopy. Virus-infected cell monolayers selected for microscopy were washed three times in phosphate-buffered saline (0.067 M, pH 7.2) followed by fixing and embedding

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using the technique of Doane et al. (1974). The washed cells were pelleted by centrifuging
at 800 g for 10 min in small glass tubes and fixed in a freshly prepared solution of 1 part
2.5% (v/v) glutaraldehyde to 2 parts 1% (w/v) osmium tetroxide in Millonig phosphate
buffer (Millonig, 1962) for 30 min at 4 °C. The fixed cells were then dehydrated with two
changes of 70% acetone for 10 min and three changes of 100% acetone for 15 min. After
10 min in a 1:1 mixture of 100% acetone and Epon 812 embedding material followed by a
further 10 min in 100% Epon 812, the cells were transferred to an embedding capsule,
covered with fresh Epon 812 and incubated at 85 °C for 2 h to achieve polymerization of
the embedding material. Thin sections (50 to 60 nm thickness) for electron microscopy were
prepared with a diamond knife mounted on a Reichert OM U2 ultra-microtome. Sections
were mounted on acid-cleaned copper grids (400 mesh) and stained for 10 min with 5%
(w/v) uranyl acetate/methanol followed by staining for 2 min with lead citrate (Reynolds,
1963). Micrographs were obtained on a Philips EM300 electron microscope operated at
80 kV. Magnification calibration was performed with a standard carbon grating (54,864
lines per inch; Ladd Research Industries, Burlington, Vermont, U.S.A.). The electron
microscope was fitted with a goniometer objective stage.

Optical diffractometry. Optical diffractometric studies were performed by examining
negatives of high magnification electron micrographs of transverse sections of the viral-
induced crystalline inclusions on a two metre Polaron optical bench (Dida Sciences, Inc.,
Montreal, Québec) equipped with a helium-neon laser light source (2 mW uniphase output).
Magnification calculation and alignment of the optical bench was performed with a
mounted negative image of the standard carbon grating. The diffraction patterns obtained
were photographed and the images obtained examined for symmetry elements.

RESULTS

Electron microscopy

Electron microscopic examination of Ad5-infected KB cells, 48 to 60 h post-infection
(p.i.), revealed the presence of typical adenovirus-induced inclusion bodies including the
protein crystalline arrays described by others (Fig. 1 (a); cf. Marusyk et al. 1972b). Depen-
dent upon the sectioning angle, transverse, diagonal or longitudinal sections were observed
(Fig. 1 b). The crystalline inclusions were generally 2 to 3 μm in width, with lengths of up
to 10 μm.

At early stages of crystal formation (36 to 48 h p.i.), the nucleoplasm surrounding the
crystals and numerous virus particles appeared to be disorganized or uncrystallized protein
material. At late stages of infection (48 to 72 h p.i.), the amorphous appearance was not
seen as virus and inclusions almost totally occupied the greatly enlarged nucleus.

Close scrutiny of electron micrographs of transverse sections through the crystal inclusions
revealed a lattice of faintly contrasted circular repeating units with electron dense centres
(Fig. 3 a). These electron dense centres seemed to be connected to neighbouring
centres by thin filaments. The diameter of this repeat unit was found to be approx. 47 nm
while the electron dense centre had a diameter of about 22 nm. It was observed that the
centre to centre spacing was 50 or 55 nm dependent upon the direction of measurement.
The crystals were usually six-sided in cross-section, but not necessarily hexagonal in outline.
Longitudinal sections revealed a lattice of long parallel electron dense bands, 22 nm across
(equivalent to the electron dense centre of the repeating unit). The inter-band spacings
varied from 0 to 35 nm in different preparations, also dependent upon the sectioning angle.

Electron micrographs obtained after tilting the specimen in different directions relative to
Fig. 1. (a) 'Normal' appearance of KB cell nucleus, 48 h after infection with Ad5. Note the presence of virions and virus-induced inclusions, including the large non-virion crystals. (b) High magnification micrograph of an infected KB cell nucleus, showing three different orientations of the non-virion crystal. Note the close proximity of virus particles to the borders of the crystals.
the electron beam (using the goniometer specimen stage) revealed that different longitudinal arrangements could be seen, simply by observing different planes (Fig. 2a–d).

Optical diffraction

Optical diffraction patterns (Fig. 3b) obtained from negatives of high magnification micrographs of crystal inclusion cross-sections were examined for symmetry elements. As
Fig. 3. (a) High magnification micrograph of transverse section of Ad5-induced non-virion crystal inclusion. Angular and distance measurements were carried out on over 100 such sections to obtain the average values presented in this study. (b) Representative optical diffraction pattern obtained following laser beam diffractometric analysis of thin-sections, such as illustrated in (a). (c) Diagrammatic representation of diffraction pattern, such as seen in (b) (not drawn to scale). The intensity spots were traced directly from a diffraction pattern and the reciprocal lattice lines were drawn. The unit cell in reciprocal space is indicated by a 'c', with the Y axis being perpendicular to the page.
Fig. 4. Schematic illustration of Ad5-induced non-virion crystal structure as proposed from data obtained in this study (not drawn to scale). X, Y and Z represent the unique axes of the crystal as well as the axes about which a transverse section through the crystal may be rotated and/or tilted in the goniometer specimen stage (see Fig. 2a to d). The enlarged area illustrates the structure of the crystal unit cell, where \( a = 50 \pm 2 \text{ nm} \), \( c = 94 \pm 3 \text{ nm} \), \( \beta = 95 \pm 2^\circ \), \( \alpha = \gamma = 90^\circ \).

shown schematically in Fig. 3(c), the angle formed between the two major axes, designated \( X^1 \) and \( Z^1 \) was found to be approx. \( 85^\circ \). As this is in reciprocal space, the corresponding angle in real space, i.e. in the actual crystal being examined, would be approx. \( 95^\circ \). As observed in Fig. 3(a) and as shown schematically in Fig. 4, an angle (\( \beta \)) of \( 95^\circ \) is formed between the two major rows of tubules designated \( X \) and \( Z \). These results would indicate that the two dimensional space group of the crystal is \( p2 \) based on an oblique unit cell with an 'a' distance along the \( X \) axis of \( 50 \pm 2 \text{ nm} \) and a 'c' distance along the \( Z \) axis of \( 94 \pm 4 \text{ nm} \) (Fig. 4). Angle \( \beta \) was \( 95 \pm 1^\circ \) while angle \( \gamma \) was defined as equal to \( 90^\circ \). For technical reasons, it was not possible to examine longitudinal sections in the optical diffractometer, thus the 'b' distance, the unit cell length along the \( Y \) axis, was not determined.

The data accumulated in this study would suggest that the two dimensional symmetry of the Ad5-induced crystal inclusions is either rectangular (two dimensional space group cm) or oblique (two dimensional space group p2; cf. Kasper & Lonsdale, 1952).

**DISCUSSION**

The results of electron microscopical and optical diffraction procedures in this study have resulted in clarification of the morphology of the intranuclear non-virion crystals formed during productive Ad5 infection of KB cells. By studying transverse thin sections, it was observed that the crystal consisted of repeating circular units arranged on an oblique lattice (Figs. 3a and 4). The asymmetric unit of the crystal was found to be \( 47 \pm 3 \text{ nm} \) in diam. with an electron-dense central ring, \( 22 \text{ nm} \) in diam., containing a central core approx. \( 7 \text{ nm} \) in diam. The electron-dense ring appeared to consist of small ring-like subunits but these
Adenovirus-induced crystalline inclusions could not be resolved sufficiently in the thin sections. The electron-dense circular units seemed to be connected to neighbouring units by thin, radiating filaments. Longitudinal sections revealed parallel rows of bands, corresponding in size to the electron-dense centres of the repeating unit seen in transverse sections. These sections also revealed thin filaments between and perpendicular to the parallel rows. The rings seen in transverse sections appeared much denser than the same structures in longitudinal sections of similar thickness (approx. 50 to 60 nm). This would indicate that the electron-dense rings represent long cylindrical columns.

In transverse sections, the asymmetric units were arranged on an oblique lattice as shown by electron microscopy and optical diffractometry of the same specimen. Y was designated as the unique axis, lying parallel to the long axis of the crystal and the crystal tubules. As a transverse section through the crystal would be perpendicular to this unique axis (by definition in this study), it was assumed that the angle thus formed between the Y axis and the XZ plane was 90°. A transverse section would, then, represent a two-dimensional lattice in the XZ plane (see Fig. 4). By measuring the angle formed between the X and Z axes on electron micrographs, and the angle formed between the X1 and Z1 axes in reciprocal space on diffraction patterns, a β angle of 95 ± 2° was determined. The unit cell dimensions were obtained by measuring the inter-tubule distances on electron micrographs and were found to be: a = 50 ± 2 nm, c = 94 ± 3 nm, β = 95 ± 2°, and α = γ = 90° (by definition). There were two asymmetric units per unit cell. It was concluded that the protein crystal most probably belonged to the monoclinic system with a space group of either p2 or p21, though a definitive statement to this effect is not valid unless it can be categorically shown that α = γ = 90°, β ≠ 90°, a ≠ b ≠ c.

Recently, Lifchitz et al. (1975) have reported data obtained from a highly detailed study of computer analysed Fourier transform analyses of digitized crystalline inclusion images. Several points of comparison and agreement may be made between the data of this study and that of Lifchitz et al., in that the repeating unit cell of the crystal was determined to be rectangular in nature (and not hexagonal as previously assumed) based on measurements of angle β ≅ 95°, with angle α = γ = 90° (assumed in this study, and experimentally determined by Lifchitz et al.).

Attempts in this laboratory to determine the chemical nature of the non-virion crystalline inclusions (Carstens & Marusyk, 1975a, b) have most recently shown that large amounts of hexon and fibre component polypeptide are found in extracted crystal material. In fact, it has been possible to crystallize the large amount of fibre protein present in the crystal extract (Carstens & Marusyk, 1975a) as well as to produce large two-dimensional hexon lattices similar to those described by Pereira & Wrigley (1974). These results, based on morphological and chemical studies, would, then, lend credence to the Lifchitz et al. (1975) model of packed hexon components within the crystal structure. A speculation as to the possible placement of the fibre component within the crystal cannot, at this time, be made.

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