

CYTOPHOTOMETRIC STUDY OF DNA ACCUMULATION IN CELLS IN THE COURSE OF DEVELOPMENT OF INTRANUCLEAR INCLUSIONS CAUSED BY TYPE 1 ADENOVIRUS

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[Received May 21, 1968]

Summary. — In the course of reproduction of type 1 adenovirus in KB cells six types of characteristic DNA-containing intranuclear inclusions were distinguished: 1) “early” inclusions consisting of annulate structures; 2) friable fine-grained, 3) granular, and 4) coarsely granular inclusions; and 5) unformed and 6) formed central corpuscles. A significant increase in DNA content with respect to the control was found in nuclei with types 1 and 2 inclusions. The quantity of DNA remained constant at subsequent stages of inclusion formation. DNA synthesis was accompanied by an increase in the surface area of the nuclei of infected cells.

Introduction

In adenovirus-infected tissue culture cells characteristic intranuclear inclusions develop, which in the opinion of some authors are a site of synthesis and aggregation of the virus (Boyer *et al.*, 1959; Defendi, 1962; Morgan and Rose, 1959; Sharpless *et al.*, 1961). The initial stage of their formation is the forming of eosinophilic masses lacking nucleic acids, which on electron microscopic analysis correspond to the osmiophilic matrix consisting of filaments and granules (Bloch *et al.*, 1957; Godman *et al.*, 1960; Laris, 1965; Morgan and Rose, 1959). Virus particles are formed on the matrix, and they gradually form inclusions, frequently with crystalline structure (Laris, 1965; Morgan and Rose, 1959). A parallel histochemical analysis proved that basophilic DNA-containing masses are formed around or within the eosinophilic inclusions; these masses gradually increase and at late stages form a central corpuscle containing DNA and a specific antigen (Bloch *et al.*, 1957; Boyer *et al.*, 1957, 1959; Nász and Tóth, 1959).

We considered it expedient to study the intensity of DNA accumulation at various stages of the formation of adenovirus inclusions, since a study of the nature of the inclusions and of the processes occurring in the cell during their development is of great importance for the comprehension of the mechanism of adenovirus reproduction.

In view of the peculiarities of the development of adenovirus infection in tissue culture, due to the slow adsorption of the virus and the gradual involvement of cells in the infectious process, cytophotometry was applied

in the solution of this problem, since it permits the determination in an individual cell of the quantity of matter along with its morphological analysis. Cytophotometry proved to be useful in studying the mechanisms of reproduction of several viruses (Yerman *et al.*, 1966; Altera and Moulton, 1966; Kasten *et al.*, 1965).

Materials and Methods

Virus and tissue culture. Experiments were carried out on KB cell cultures with type 1 adenovirus (standard strain obtained from the Institute of Virology, U.S.S.R. Academy of Medical Sciences, Moscow). The cells were grown on cover glasses in a medium consisting of 45% of a 0.5% lactalbumin hydrolysate solution, 45% of medium 199 and 10% of bovine serum. After 24 hours the cultures were infected with virus at a multiplicity of 1 TCD₅₀ of virus per 75 to 80 cells. The preparations were fixed with Carnoy's fluid and stained by Feulgen's method. The percentage of infected cells according to inclusion type was determined per 1,000 cells investigated in each preparation, 6—8 preparations being examined at each interval. In studying the dynamics of infectious virus formation, the culture fluid was repeatedly frozen and thawed to destroy the cells and centrifuged at 3,000 rev/min for 10—15 minutes. The total titre of the intra- and extracellular virus was determined by the method of Reed and Muench.

Cytophotometry. The investigations were conducted on a MUF-5 apparatus in the visible region of the spectrum by the scanning method at a wavelength of 546 nm, with a probe of 1.17 nm. The amount of DNA was expressed in conventional units according to the formula recommended by Brödsky (1966): $Q = DS$, where Q is the quantity of matter, D is the mean optical density and S is the surface area of the nucleus. The examinations were carried out on 25—30 cells containing characteristic adenovirus inclusions of a definite type and, as a control, 60 cells from an uninfected culture 18 hours after change of the medium.

Biometric processing of the material. The quantity of DNA, the criteria characterizing the average value and the confidence of the difference in DNA contents by the t-test were determined. Similar indices were calculated in determining the surface area of the nuclei containing definite types of inclusions (Ashmarin and Vorobyov, 1960). The regression coefficient was calculated by Bailey's (1962) method.

Results

Changes in DNA contents and the surface area of cell nuclei with respect to the type of adenovirus inclusions

Characteristic adenovirus inclusions observed during reproduction of type 1 adenovirus were divided into six types for cytophotometric analysis (Fig. 1):

- 1) "early" inclusions; at this stage annulate structures consisting of small Feulgen-positive granules were observed in the nucleus;
- 2) friable fine-grained inclusions, occupying almost the entire nucleus; a light coloured zone began to appear between it and the nuclear membrane;
- 3) granular inclusion consisting of a large number (10—15) of coarser DNA-containing granules, joined to one another and sometimes forming intricate figures;
- 4) coarsely granular inclusions consisting of several, most often 3—4, large, dense Feulgen-positive granules;
- 5) not fully formed central corpuscle consisting of a DNA-containing mass of irregular shape with loose, weakly stained edges, within which foci of densification were sometimes observed; and

6) formed central corpuscle; the inclusion was more compact than in the previous stage, of rounded shape, intensely stained by Feulgen's method. In formation of inclusion types 5 and 6, a large light-coloured zone is observed between them and the nuclear membrane.

Table 1. Amount of DNA in nuclei of adenovirus-infected cells containing different inclusion types

Inclusion type	\bar{x}	σ	SE	Confidence interval	Criteria of confidence of difference	
					t	p
Control	0.24*	± 0.10	± 0.01	0.22 ÷ 0.26		
1	0.45	± 0.15	± 0.03	0.39 ÷ 0.57	6.2	< 0.1%
2	0.56	± 0.22	± 0.04	0.48 ÷ 0.64	2.2	< 5%
3	0.57	± 0.18	± 0.04	0.49 ÷ 0.65	1.7	> 5%
4	0.60	± 0.22	± 0.05	0.50 ÷ 0.70	0.66	> 5%
5	0.69	± 0.30	± 0.06	0.57 ÷ 0.81	1.66	> 5%
6	0.64	± 0.23	± 0.04	0.56 ÷ 0.72	0.7	> 5%

\bar{x} — arithmetical mean; σ — mean quadratic deviation; SE — standard error; t — criterion of confidence; p — significance level.

* The factor 10^{-6} is omitted in all cases for convenience.

The results of experiments on the determination of DNA are presented in Table 1. In nuclei containing "early" adenovirus inclusions consisting of annulate structures, the quantity of DNA equalled 0.45, which is 80% more than in the control cells ($p < 0.1\%$). A comparative analysis of the cells containing "early" adenovirus inclusions and friable fine-grained inclusions showed that the quantity of DNA in the latter increased to 0.56, this

Table 2. Surface area of nuclei containing different types of characteristic e adenovirus inclusions

Inclusion type	\bar{x}_{\log}	σ_{\log}	SE _{log}	\bar{x}_{geom}	Criteria of confidence of difference	
					t	p
Control	0.36	0.08	0.01	2.3*		
1	0.49	0.11	0.02	3.1	6.5	< 0.1%
2	0.52	0.16	0.03	3.3	2.11**	< 5%
3	0.56	0.13	0.03	3.6	1.0	> 5%
4	0.55	0.11	0.025	3.5	0.4	> 5%
5	0.44	0.14	0.02	2.75	2.75	< 1%
6	0.38	0.11	0.02	2.4	1.66	> 5%

\bar{x}_{\log} — "intermediate" arithmetical mean of logarithms of variations; σ_{\log} — mean quadratic deviation; SE_{log} — standard error; \bar{x}_{geom} — geometrical mean; t — criterion of confidence of difference; p — level of significance.

* The factor 10^{-6} is omitted in all cases.

** The F-coefficient was calculated as the criterion of confidence of the difference (Bailey, 1962).

rise being significant. At subsequent stages of the formation of inclusions, beginning with the granular ones, the DNA content in the nuclei underwent slight changes.

Table 2 presents data on the changes in the mean surface area of nuclei containing adenovirus inclusions in the process of their formation. Nuclei with type 1 inclusions had a mean geometric surface area exceeding that of the control nuclei by 37% ($p < 0.1\%$). During the formation of fine-grained inclusions the swelling of the nuclei continued ($p < 5\%$). An analysis of Tables 1 and 2 shows a parallelism between the increase in DNA and the increase in area of nuclei with inclusions consisting of annulate structures and friable fine-grained inclusions as compared with these indices for cells from an uninfected culture. The process of formation of granular and coarsely granular inclusions was not accompanied by an increase in the area of the nucleus. A characteristic feature of nuclei with inclusions of types 5 and 6 was a sharp significant decrease of the area with high DNA content. The mean size of nuclei with formed central corpuscles did not differ from that of the control cells ($p > 5\%$).

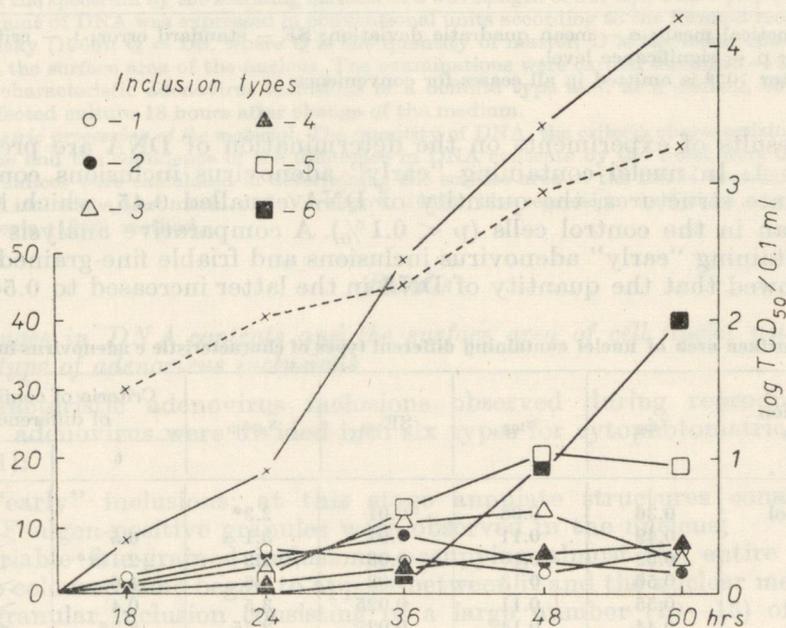


Fig. 2.

Dynamics of development of inclusions in KB cells infected with type 1 adenovirus. Left ordinate: number of cells according to inclusion types in %; right ordinate: virus titre; abscissa: hours after infection.

x — x total number of infected cells in %
 x - - - x virus titre

Dynamics of development of characteristic inclusions

The infectious process develops gradually and asynchronously; therefore affected cells at various stages of inclusion formation and normal cells are observed simultaneously in the cell monolayer.

As shown in Fig. 2, the first lesions in the form of inclusions consisting of annulate structures, appeared 18 hours after infection. A characteristic feature of the cell monolayer 24–36 hours after infection was the development of inclusion types 1–4. From 48–60 hours the great majority of cells contained unformed and formed central corpuscles, although earlier inclusions occurred in a certain percentage of the cells.

Fig. 2 also illustrates the dynamics of the formation and accumulation of the infectious virus. It appeared 18 hours after infection (the mean geometric titre was $10^{1.5}$ TCD₅₀/0.1 ml) and a significant increase in the virus titre to $10^{2.9}$ TCD₅₀/0.1 ml was observed 48 hours after infection.

Relationship between DNA contents and volume of the nuclei

The data obtained indicate that in the process of type 1 adenovirus reproduction an increase in the DNA content occurs in the cells. This increase may be due to two causes: a) synthesis of virus-induced DNA in the nuclei of the infected cells, and b) a change in the ratio between the number of diploid and polyploid cells in the investigated group tending toward an increase in the latter, which leads to an increase in the mean DNA value. To solve this problem we studied the relationship between DNA contents of the infected nuclei and their volume, since it is known that with increase in the ploidy of the nucleus its volume increases proportionally (Frankhauser, 1952). Two groups of cells were investigated: 1) the control group of cells from an uninfected culture 24 hours after change of the medium; and 2) the experimental group of cells with dense central corpuscles, since the cells of these two groups do not differ essentially with respect to size of the nucleus. The data on the volume of the nuclei (r^3) were obtained from the indices of their surface areas, determined by a planimeter, after arithmetical transformations. The results are presented graphically in Fig. 3.

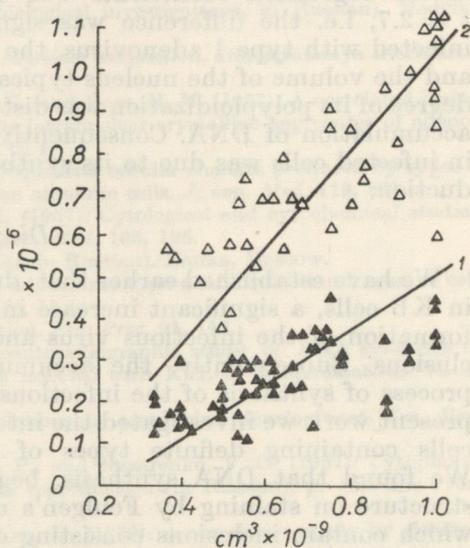


Fig. 3.

Correlation between amount of DNA and the volume of nuclei of KB cells, uninfected and infected with type 1 adenovirus

Ordinate: amount of DNA in conventional units; abscissa: volume of nuclei

▲ — Control cell nuclei

△ — Infected cell nuclei containing formed central corpuscles

1 — line of regression for control group of cells

2 — line of regression for experimental group of cells

The regression coefficient of the change in nuclear volume (v) depending on the DNA content of the nuclei (x) was calculated, and an equation of regression was derived for the control and experimental groups of cells. For the first group the equation had the form $y = 0.22 + 1.55 x$, where the regression coefficient equalled 1.55; for the second group $y = 0.11 + 0.85 x$. Thus, a correlation between the quantity of DNA and the size of the nucleus existed in both groups, but in the experimental group it differed quantitatively from that in the control group. The confidence of the difference in the regression coefficients was determined for the indicated samples, $t = 2.7$, i.e. the difference was significant ($p < 1\%$). Hence, in KB cells infected with type 1 adenovirus, the correlation between the DNA contents and the volume of the nucleus typical of a normal culture depending on the degree of its polyploidization was disturbed with a tendency of a more intense accumulation of DNA. Consequently, the increase in the quantity of DNA in infected cells was due to its synthesis in the process of adenovirus reproduction.

Discussion

We have established earlier that, during reproduction of type 1 adenovirus in KB cells, a significant increase in the mean DNA contents precedes the formation of the infectious virus and of the characteristic intranuclear inclusions. Subsequently, the accumulation of DNA continues during the process of synthesis of the infectious virus (Dyachenko *et al.*, 1967). In the present work we investigated the intensity of DNA accumulation in infected cells containing definite types of characteristic intranuclear inclusions. We found that DNA synthesis, beginning in cells with visibly unaltered structure on staining by Feulgen's method, continues only in those nuclei which contain inclusions consisting of annulate structures and friable fine-grained inclusions. In cells with inclusions formed at later stages, the amount of DNA does not increase. Processes of condensation of DNA-containing masses, the synthesis of structural protein and the formation of virus particles probably prevail in these cells.

The experiments of Defendi and Krichevsky (1960), conducted by autoradiography on chick adenovirus, also indicate that different processes occur in cells with inclusions developing at various stages of adenovirus reproduction. A similar conclusion follows from Carmichael's (1965) data on type 4 adenovirus multiplication in dog kidney cells. In this case infectious virus was not formed, although DNA and a specific antigen were synthesized; the morphological lesions were distinguished by development of friable fine-grained inclusions only. We disagree with Kiefer and Sandritter (1964) that there is no correlation between DNA content and adenovirus inclusion type, since they confined themselves to a cytophotometric analysis of cells with later inclusions, in which DNA synthesis does not occur.

The experimental data obtained make it possible to assume that the increase in DNA content in infected cells, observed during reproduction of type 1 adenovirus, is due to synthesis of virus-specific DNA. According to Bloch *et al.* (1957), Defendi (1962) and Laris (1965), intranuclear inclusions

formed in adenovirus infected-cells consist of virus-specific DNA and do not contain chromatin. The DNA of adenovirus virions differs by nucleotid composition from the cellular DNA and codes the synthesis of the corresponding RNA (Green *et al.*, 1964; Rose *et al.*, 1965).

References

- Altera, K. P., and Moulton, J. E. (1966): Effects of 5-fluorouracil on infectious canine hepatitis virus infection of dog kidney cells. *Proc. Soc. exp. Biol. (N. Y.)* **121**, 157.
- Ashmarin, I. P., and Vorobyov, A. A. (1960): *Statisticheskiye metody v mikrobiologicheskikh issledovaniakh* (Statistical methods in microbiological investigations, in Russian). Medgiz, Leningrad.
- Bailey, N. (1962): *Statistical methods in biology*, Russian translation. Innostrannaya literatura, Moscow.
- Bloch, D. P., Morgan, C., Godman, G. C., Howe, C., and Rose, H. M. (1957): A correlated histochemical and electron microscopic study of the intranuclear crystalline aggregates of adenovirus in HeLa cells. *J. biophys. biochem. Cytol.* **3**, 1.
- Boyer, G., Denny, F., and Ginsberg, H. (1959): Sequential cellular changes produced by types 5 and 7 adenoviruses in HeLa cells and in human amniotic cells. *J. exp. Med.* **110**, 827.
- Boyer, G., Leuchtenberger, C., and Ginsberg, H. (1957): Cytological and cytochemical studies of HeLa cells infected with adenoviruses. *J. exp. Med.* **105**, 195.
- Brodsky, V. Y. (1966): *Trofika kletki* (Cell trophies, in Russian). Nauka, Moscow.
- Carmichael, L. E. (1965): An incomplete cycle of adenovirus type 4 multiplication in canine cell culture and in dogs. *J. Amer. vet. Res.* **26** (110), 15.
- Defendi, V. (1962): Cytopathology of virus infection. *Fed. Proc.* **21**, 113.
- Defendi, V., and Krichevsky, D. (1960): A histoautoradiographic study of DNA synthesis in tissue cultures of chicken embryo liver cells infected with RPL-12 lymphomatosis virus. *J. biophys. biochem. Cytol.* **7**, 201.
- Frankhauser, G. (1952): Nucleo-cytoplasmic relations in amphibian development. *Int. Rev. Cytol.* **1**, 165.
- Dyachenko, N. S., Nosach, L. N., Gushcha, K. P., and Nazarenko, V. G. (1967): Cytophotometric study of DNA synthesis in adenovirus reproduction (in Russian), p. 122. In: Proc. of the Conf. on Cell Pathology, Moscow.
- Godman, G., Morgan, C., Breitenfeld, P., and Rose, H. (1960): A correlative study by electron and light microscopy of the development of type 5 adenovirus. II. Light microscopy. *J. exp. Med.* **112**, 383.
- Green, M., Pina, M., and Chagoya, V. (1964): Biochemical studies on adenovirus multiplication. V. Enzymes of DNA synthesis in cells infected by adenovirus and vaccinia virus. *J. biol. Chem.* **239**, 1188.
- Kasten, F. H., Vendrely, C., Tournier, F., and Wicker, R. (1965): DNA lesion induced in nuclei and nucleoli in tissue culture by simian vacuolating virus (SV-40). *J. cell. comp. Physiol.* **66**, 33.
- Kiefer, G., and Sandritter, W. (1964): Cytophotometrische Messungen des DNS-Gehaltes von Affen-nieren-Zellkulturen nach Infektion mit Adenovirus Typ 3. *Z. Naturforsch.* **19b**, 136.
- Laris, K. (1965): Electronmicroscopic examination of KB cell cultures infected with adenovirus type 12. *Acta microbiol. Acad. Sci. hung.* **12**, 241.
- Morgan, C., and Rose, H. (1959): Electron-microscopic observation on adenoviruses and viruses of influenza group, p. 256-271. *Virus growth and variation*, 9th Symp. of the Society for General Microbiology, Cambridge.
- Nász, I., and Tóth, M. (1959): Cytomorphological changes in human embryonic kidney tissue cultures infected with type 5 adenovirus strains. *Acta microbiol. Acad. Sci. hung.* **11**, 85.
- Rose, H., Reich, F., and Weissman, J. (1965): RNA production in adenovirus infected KB cells. *Virology* **27**, 571.
- Sharpless, G., Levine, S., Davies, M., and Engler, M. (1961): GAL virus: Its growth in tissue culture and some of its properties. *Virology* **13**, 315.
- Yerman, E. A., Essel, A. E., Bronitskaya, E. Yu., Shubina, S. V., and Myasnikova, A. T. (1966): Cytophotometric determination of the RNA content in HEP-2 cells infected with RNA-containing virus (in Russian), p. 136. In V. L. Ryzhkov (ed.): *O prirode virusov*, Moscow.