J. Hyg., Camb. (1965), **63**, 99
With 3 plates
Printed in Great Britain

Histological study of adenovirus type 14 development in cell cultures

By E. MĂGUREANU, M. MUŞETESCU and M. GROBNICU
This investigation was carried out in the 'Dr I. Cantacuzino' Institute, Bucharest

(*Received* 23 *July* 1964)

Ever since the first papers concerning the detection of antigenic material in cells by means of antibodies coupled with fluorescein (Coons, Creech, Jones & Berliner, 1942; Coons & Kaplan, 1950) were published, the methods have been modified and improved, either by the use of new fluorochromes and of high-titre antisera, or by the application of more adequate procedures.

Acridine orange (AO) staining as used by Armstrong & Hopper (1959) and Bertalanffy, Masin & Masin (1956) in cell cultures or in smears, is based on the different types of fluorescence in ultraviolet light displayed by nucleic acids after staining.

The development of adenoviruses can be detected in infected cells before the occurrence of cytological changes, by means of fluorescent antibody and acridine orange staining.

So, immunofluorescence was used by Pereira, Allison & Balfour (1959) and Boyer, Denny & Ginsberg (1959), while AO staining was used by Armstrong & Hopper (1959), and Bartolomei Corsi & Harkevitch (1960). In 1964, Mayor applied these methods in an investigation of the changes undergone by ribonucleic acids in cells infected with adenovirus.

The present study is concerned with the changes caused in monkey kidney and KB cells by adenovirus type 14, using these methods.

MATERIALS AND METHODS

Cell cultures

KB cell cultures and secondary Macaccus rhesus kidney cell cultures (MK) on neutral glass cover-slips of 5×20 mm. in 16×160 mm. tubes were used. The culture fluid was Hanks's solution with lactalbumin hydrolysate and 2% or 10% calf serum for MK or KB cultures respectively. After appearance of the monolayer the culture fluid was removed and each cover-slip was inoculated with 0·1 ml. adenovirus type 14 (CPD 50/0·2 ml. = $10^{5.5}$). The same fluid containing 2% calf serum was used for maintenance of the cultures.

The controls consisted of non-inoculated KB or MK cell cultures. The cultures were incubated at 37° C., and samples were collected at definite intervals (6, 8, 12, 24, 36, 48, 72, 96 hr.).

Virus strains

Adenovirus strains grown on KB or MK cells and stored at −20° C. were used. The titres of the stock solutions of virus, computed according to the method of Reed & Muench (1938), are shown in Table 1.

Table 1. *Titres of stock solutions of strains of adenovirus*

Adenovirus type	Titre CPD 50/0·2 ml.
1	$10^{5\cdot5}$
4	$10^{6\cdot5}$
7	$10^{5\cdot0}$
14	$10^{5\cdot5}$
16	$10^{5\cdot3}$

Immune sera

For immunofluorescence we used antisera prepared in rabbits by six intravenous inoculations, at 3 days' intervals, of 1 ml. adenovirus types, 1, 4, 7, 14 or 16. The animals were bled 10 days after the last inoculation. Neutralizing titres average 1/256–1/512.

Conjugation with fluorescein isothiocyanate (ITC)

Immune rabbit sera were purified by precipitation with 60% saturated $(NH_4)_2SO_4$ solution, and the amount of protein obtained was determined by refractometry. For conjugation of the serum with ITC (FLUKA), 0·05 mg./mg. of protein were used, as in the method of Coons & Kaplan (1950), modified by Riggs *et al.* (1958) and by Marshall, Eveland & Smith (1958).

After dialysis for 4–5 days against 0·01 M phosphate buffer, pH 7·2, the conjugated globulin was passed through a Sephadex column. Sodium azide was added to make up a concentration of 0·08%, the sera were distributed in vials and maintained at 4° C.

Before use non-specific fluorescence was removed by absorption of the conjugated serum with human or monkey liver powder (two absorptions). An additional absorption was made with KB or MK cell cultures. The serum was used in a 1/40 dilution.

Fluorescent-antibody staining technique

Infected and control cell cultures were fixed in acetone for 10 min. at room temperature, at various intervals after inoculation. Preparations were air-dried and covered with anti-adenovirus serum conjugated with ITC and diluted 1/40. A number of preparations were covered with a mixture of conjugated anti-adenovirus serum and complement (fresh guinea-pig serum) in equal parts (final dilution of complement 1/20).

The culture was left in contact with the anti-adenovirus serum for 30 min. at 37° C. in the moist chamber. The preparation was carefully washed with phosphate buffer, pH 7·2, for 20 min., and finally rinsed with distilled water for the removal of phosphate crystals. The preparation was mounted in buffered glycerol, pH 7·2, and examined in ultra-violet light, using a monocular Zeiss microscope and Zeiss

L (HBO 50) mercury vapour lamp, with UG_1, 1·5 mm. screening filter and GG_9 protection filter.

Suitable controls were put up, which are described in detail under Results.

AO staining

For the demonstration of nucleic acid in the cell and of the changes resulting from adenovirus infection fluorochrome AO was used in a 1/40,000 aqueous solution.

Infected and control cultures were fixed in 96% ethanol for 1 min. and stained with AO for 40 sec. They were rinsed with tap water for 3 min., mounted in water and examined in ultra-violet light.

By this method ribonucleic acid (RNA) is stained orange-red and deoxyribonucleic acid (DNA) green.

RESULTS

Fluorescent-antibody staining technique

During the first 8 hr. following inoculation cells infected with adenovirus were not specifically stained by anti-adenovirus serum conjugated with ITC. The control culture displayed the same behaviour. In both cases the cytoplasm was weakly fluorescent, while the nucleus appeared as a dark central area (Fig. 1).

Between 8 and 16 hr. fluorescent granules of various sizes, forming conglomerates or rosettes, appeared in the nuclei of some cells. The nucleoli remained dark (Fig. 2).

After 16 to 24 hours the fluorescent material appeared either as lumps, as above, or filled the whole area of the nucleus. The nuclei at this stage were as a rule seen to be hypertrophic. Background fluorescence of the cytoplasm became more intense in certain cells (Fig. 3).

Between 24 and 48 hr. about 80% of the cells were displaying specifically stained nuclear material. The nuclei were large, deformed and contained conglomerates of fluorescent material (Figs. 4, 5). Some of the cells appeared as compact fluorescent masses. In the other cells, the fluorescent mass was placed like two central symmetrical blocks (Fig. 6). The unstained areas corresponding to the nucleoli, as seen in the preceding stage, had disappeared.

At the end of 72 hr. and later, advanced lesions were seen progressively to prevail.

The specificity of fluorescent staining was demonstrated by the negative results with the following controls:

KB and MK cultures infected with adenovirus type 14 + normal rabbit serum conjugated with ITC.

KB and MK cultures not infected with adenovirus + anti-adenovirus type 14 serum conjugated with ITC.

Serum inhibition control: KB and MK cultures infected with adenovirus type 14 + unconjugated homologous immune serum; incubation for 30 min. at 37° C.; addition of type 14 anti-serum conjugated with ITC; incubation for 15 min. at 37° C.

As controls for the detection of possibly existing type specificity, we used:

KB and MK cell cultures infected with adenovirus type 14+type 1, 4, 7 or 16 antiserum conjugated with ITC.

KB and MK cell cultures infected with adenovirus types 1, 4, 7 or 16+type 14 antiserum conjugated with ITC.

In both cases fluorescence was similar to that recorded in the specific reaction of the antigen with homologous type serum.

The addition of complement to the conjugated serum resulted in an increased intensity of fluorescence without any change in non-specific background fluorescence.

No differences in the appearance and course of adenovirus infection were found to exist between KB and MK cells.

AO staining

AO staining afforded the possibility of discriminating between the nucleic acids of the cell. In normal KB or MK cells the cytoplasm displayed a reticulum stained orange-red. The spaces within the reticulum were non-fluorescent. As a rule, the cytoplasm around the nucleus appeared to be condensed. The nucleus displayed a dull green fluorescence and contained varying numbers of nucleoli stained an intense orange-red (Fig. 7).

In infected KB or MK cells the first changes in the staining properties of the cell constituents appeared about 8 hr. after the infection. The first detectable change consisted in a slight increase in size of the nucleus which contained bright yellow-green granules. The nuclei displayed a normal pattern (Fig. 8).

After 24–48 hr. the number of cells with large nuclei had increased. The nuclei contained bright yellow-green lumps (DNA). At the same time orange threads and granules of RNA, or even peripheral or central nuclear areas with processes reaching the cytoplasm, could be seen in some nuclei. In other places the granules of DNA were surrounded by a fine orange zone. Some nuclei appeared to be separated from the rest of the cytoplasm by non-fluorescent perinuclear vacuolar spaces (Fig. 9).

After 48 hr. the whole mass of the nucleus was stained a bright green. No RNA staining was any longer detectable. The nucleoli had disappeared. At this stage a narrow zone of orange cytoplasm was still detectable at the periphery of large nuclei. Similar results were obtained with both cell lines used, i.e. KB and MK.

CONCLUSIONS

By means of the fluorescent antibody method and of acridine orange staining, the course of adenovirus infection in KB and MK cell cultures could be followed. Both methods appear to be specific and sensitive and permit the early detection of infection.

Thus, a latent period of about 8 hr. was found to exist in the multiplication of adenovirus type 14. The first detectable lesions involved the nucleus and probably corresponded to the appearance of a modified DNA.

The development of the adenovirus, which contains DNA, rapidily results in

important histochemical and morphological alterations of the cell. With the methods used these changes become demonstrable during the first 48 hr., i.e. before the appearance of a cytopathic effect.

The presence of a soluble group antigen could be demonstrated by immunofluorescence. The existence of this antigen results from the reaction of various adenovirus types with the same conjugated immune serum, and vice versa, from the reaction of adenovirus type 14 with various anti-adenovirus sera (types 1, 4, 7, 14 or 16) conjugated with ITC.

SUMMARY

By means of the fluorescent antibody method and of acridine orange staining experimental infection with adenovirus type 14 has been investigated in KB and MK cell cultures. Intracellular changes could be shown to precede the appearance of the cytopathic effect.

Thus, progressive stages in the development of adenovirus at this level could be separated both by the demonstration of the specific antigen and by the appearance of an altered DNA.

Both methods have proved to be equally useful for the detection of virus multiplication and of the histochemical changes induced in the cells.

No significant differences have been found to exist with respect to the cell line used.

REFERENCES

Armstrong, J. A. & Hopper, P. K. (1959). Fluorescence and phase contrast microscopy of human cell cultures infected with adenovirus. *Exp. Cell Res.* **16**, 584.

Bartolomei Corsi O. & Harkevitch, N. (1960). Modificazioni morfologiche di cellule Kb infette da adenovirus, tipo 5. *Sperimentale*, **110**, 250.

Bertalanffy, L. von, Masin F. & Masin, M. (1956). Use of acridin-orange fluorescence technique in exfoliative cytology. *Science*, **124**, 1024.

Boyer, G. S., Denny, F. W. Jr. & Ginsberg, H. S. (1959). Intracellular localization of type 4 adenovirus. *J. exp. Med.* **109**, 85.

Coons, A. H., Creech, H. J., Jones, R. N. & Berliner, E. (1942). The demonstration of pneumococcal antigens in tissues by the use of fluorescent antibody. *J. Immunol.* **45**, 159.

Coons, A. H. & Kaplan, M. H. (1950). Localization of antigen in tissue cells (II). *J. exp. Med.* **91**, 1.

Marshall, J. D., Eveland, W. C. & Smith, C. W. (1958). Superiority of fluorescein isothiocyanate for fluorescent antibody technic with a modification of its application. *Proc. Soc. exp. Biol., N.Y.*, **98**, 898.

Mayor, H. D. (1964). A labile intranuclear RNA associated with the development of adenoviruses. *J. exp. Med.* **119**, 433.

Pereira, H. G., Allison, A. C. & Balfour, B. (1959). Multiplication of adenovirus type 5 studied by infectivity titrations and by the fluorescent antibody technique. *Virology*, **7**, 300.

Reed, L. & Muench, H. A. (1938). A simple method of estimating fifty per cent end-point. *Amer. J. Hyg.* **27**, 493.

Riggs, J. L., Sriwald, R. J., Burckhalter, J., Downs, C. M. & Metcalf, T. G. (1958). Isothiocyanate compounds as fluorescent labelling agents for immune serum. *Amer. J. Path.* **34**, 1081.

EXPLANATION OF PLATES

PLATE 1

Fig. 1. Normal KB cells. Fluorescent antibody method. Weak cytoplasmic fluorescence. × 500

Fig. 2. KB cells, 16 hr. after infection with adenovirus type 14. Fluorescent antibody method. Small aggregates of viral antigen and granules are present in the nuclei. × 500.

Fig. 3. KB cells, 24 hr. after infection with adenovirus type 14. Fluorescent antibody method. Large aggregates of viral antigen and specific material taking up the whole cell. × 500.

PLATE 2

Fig. 4. KB cells, 48 hr. after infection with adenovirus type 14. Fluorescent antibody method. Large fluorescent aggregates of viral antigen are seen in the nucleus; increased background fluorescence of the cytoplasm. × 500.

Fig. 5. KB cells, 36 hr. after infection with adenovirus type 14. Fluorescent antibody method. In the left part of the figure a cell displaying granules of viral antigen in its nucleus; in the right part of the figure the nucleus of a cell is filled with a strongly fluorescent mass of viral antigen. × 1250.

Fig. 6. KB cells, 72 hr. after infection with adenovirus type 14. Fluorescent antibody method. A cell displaying two intensely fluorescent nuclear masses in a characteristic 'butterfly' pattern. × 500.

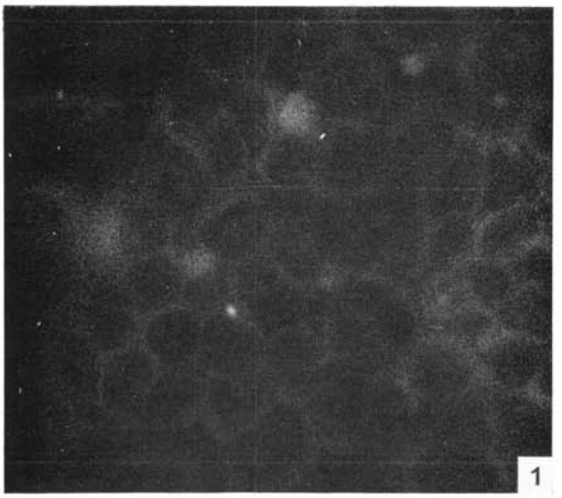
PLATE 3

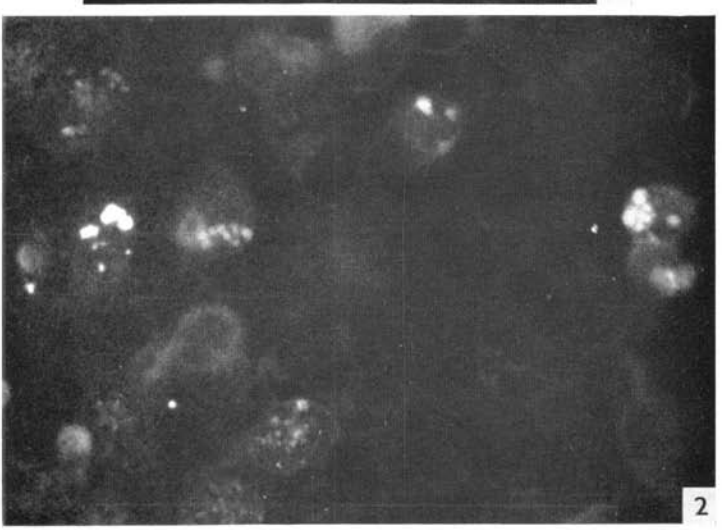
Fig. 7. Normal KB cells stained with acridine orange. Cytoplasm stains orange-red (RNA), nuclei green (DNA). Nucleoli display an intense orange-red stain.

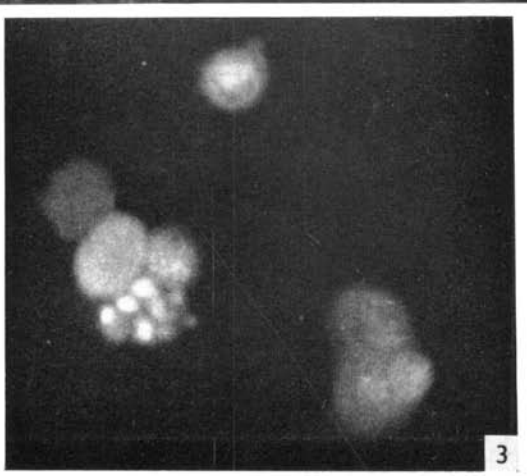
Fig. 8. KB cells 16 hr. after infection with adenovirus type 14. Acridine orange technique. Nuclei are enlarged. Green inclusions of DNA appeared in the infected nuclei.

Fig. 9. KB cells 24 hr. after infection with adenovirus type 14. Acridine orange technique. Nuclei contain bright yellow-green lumps. Nuclear RNA surrounds the viral inclusion and orange threads reach the cytoplasm. Some nuclei appear to be separated from the rest of the cytoplasm by non-fluorescent perinuclear vacuolar spaces.

Fig. 10. KB cells 48 hr. after infection with adenovirus type 14. Acridin orange technique. Bright yellow-green lumps are placed like two symmetrical blocks.



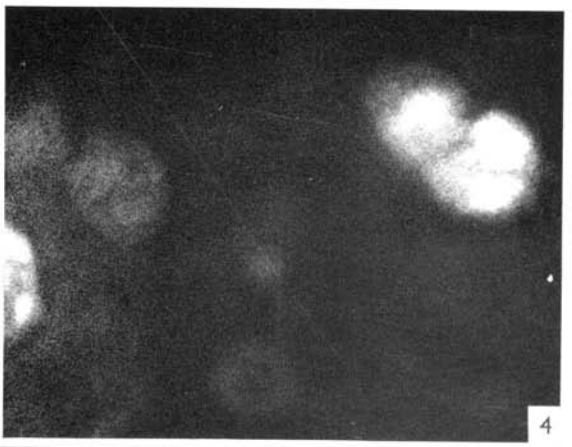


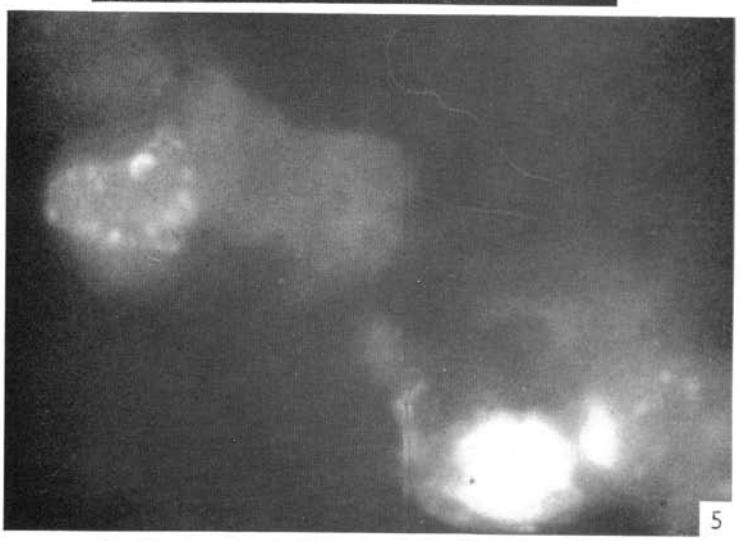


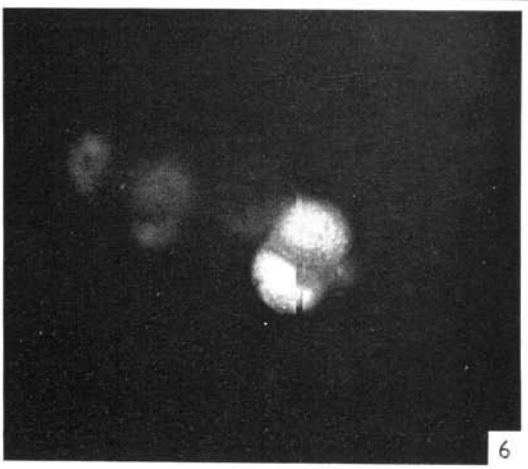
E. MĂGUREANU, M. MUŞETESCU AND M. GROBNICU

(*Facing p.* 104)

https://doi.org/10.1017/S0022172400045009 Published online by Cambridge University Press

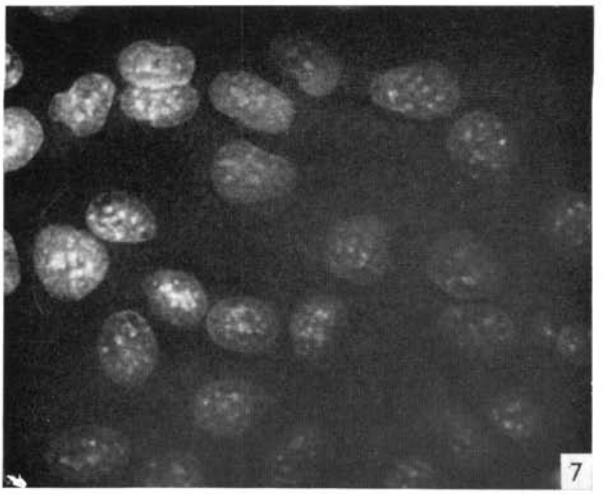


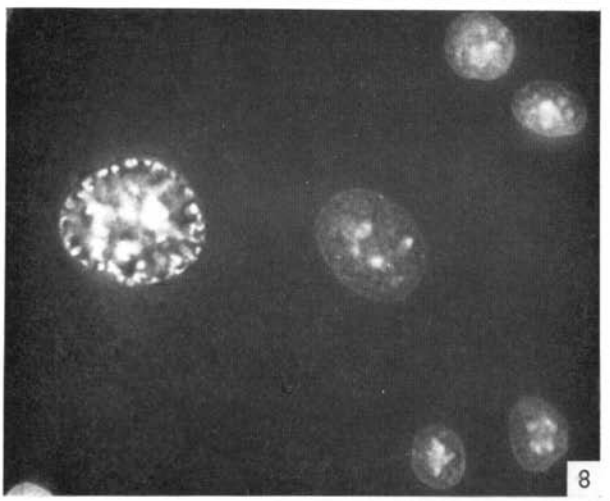


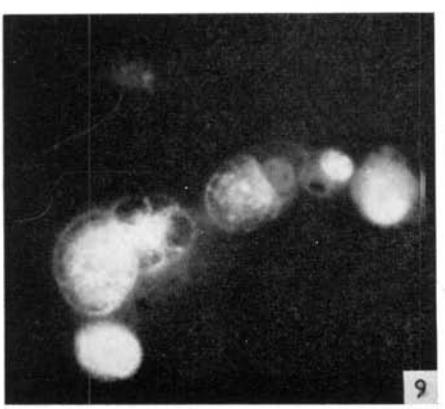


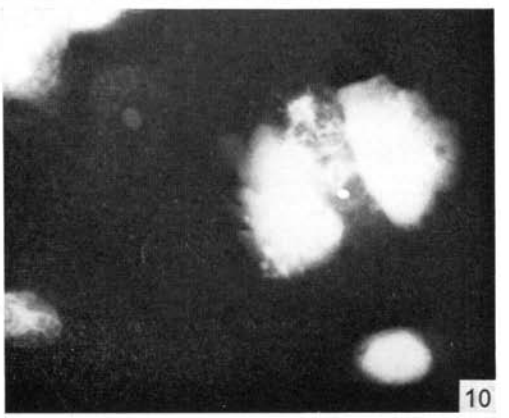
E. MĂGUREANU, M. MUŞETESCU AND M. GROBNICU

https://doi.org/10.1017/S0022172400045009 Published online by Cambridge University Press









https://doi.org/10.1017/S0022172400045009 Published online by Cambridge University Press