Alterations of Actin-Containing Structures in BHK21 Cells Infected with Newcastle Disease Virus and Vesicular Stomatitis Virus

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SUMMARY

The distribution pattern of actin-containing structures in BHK21 cells and the changes which they undergo upon infection with Newcastle disease virus (NDV) and vesicular stomatitis virus (VSV) were studied by means of immunofluorescence. Double labelling with antibodies conjugated with fluorescein (for actin) and rhodamine (for virus antigens) has shown that the progressive cytopathic effects after virus infection are accompanied by extensive alterations of the structures demonstrable by antiactin antibodies. In NDV-infected BHK21 cells the number of actin filaments increases, some zones which contain virus antigens apparently being in close association with the actin structures. By contrast, infection with VSV results in a strong reduction of actin-containing fibres.

The results indicate that in the genesis of morphologically detectable alterations of a cell after virus infection – the ‘cytopathic changes’ – alterations of those structural elements are involved which are also probably responsible for maintenance of cell shape and motility.

INTRODUCTION

The isolation and characterization of actomyosin proteins from non-muscle tissues (for review, see Pollard & Weihing, 1974; Mannherz & Goody, 1976) have demonstrated the ubiquity of a highly organized system of potentially contractile cell structures. The production of immune sera against actin (Lazarides & Weber, 1974; Trenchev, Sneyd & Holborow, 1974), myosin (Weber & Groeschel-Stewart, 1974; Painter, Sheetz & Singer, 1975; Fujiwara & Pollard, 1976), tropomyosin (Lazarides, 1975), actinin (Lazarides, 1976) etc. has contributed to their structural localization and to the elucidation of their functions within the cell. It is assumed today that the contractile formations play a decisive role in many cell functions as, for example, in the preservation and control of the cell form, the cytokinesis and cell movement, endocytosis, exocytosis (for review, see Pollard & Weihing, 1974; Mannherz & Goody, 1976), and secretion (Vial & Garrido, 1976). A consequence of virus-host interactions during virus infection is the induction of the cytopathic effect (c.p.e.; Bablanian, 1975) observed during different virus infections in tissue cultures. These virus-induced alterations are readily recognized on a morphological basis due to the massive changes in the cell shape observable as c.p.e. in infected cultures.

In the light of new knowledge regarding the control of cell form and motility, one can assume that the morphological cell alterations during a virus infection could be caused by,
or associated with, changes of the structural elements in infected cells. With the aid of antibodies against actin, a main component of the actomyosin complex (Mannherz & Goody, 1976), we have endeavoured to study by immunofluorescence the relationship between actin localization and development of the c.p.e. during virus infection.

**METHODS**

**Cells.** BHK21 cells (Flow Labs, Scotland) were maintained in Eagle’s minimum essential medium with 10% (v/v) non-essential amino acids and 5% (v/v) inactivated calf serum. Passages were made by means of 0.25% (w/v) trypsin solution in phosphate buffered saline (PBS). For microscopic examination, the BHK21 cells at a density of 2.5 x 10^6 cells/ml were seeded on to glass coverslips (8 x 50 mm) and incubated for 24 h at 37 °C. L cells (Flow Labs) were cultivated in the same medium in the presence of 5% (v/v) horse serum.

**Chicken fibroblast cultures** were produced from 8-day-old chick embryos by trypsinization according to the method of Kondo (1965). They were seeded into Petri dishes of 50 mm diam. in an incubator with 5% CO₂ (v/v).

**Viruses.** Newcastle disease virus (NDV), strain Italia, was grown in 11-day-old chick embryos. After incubation for 36 h at 37 °C, the allantoic and amniotic fluids were collected, pooled and freed from cell and tissue debris by centrifugation for 30 min at 5000 g. Small portions were frozen at −70 °C. To produce anti-NDV immune sera, the virus was inoculated in 14-day-old duck embryos (Rutter & Mannweiler, 1976). The NDV pool was titrated for infectivity using a plaque test on chick fibroblast cultures (Gschwender, Rutter & Popescu, 1975).

**Vesicular stomatitis virus** (VSV), strain Indiana, was maintained in BHK21 cells. A virus pool was produced with highly diluted inoculum (0.01 p.f.u./cell) and stored in small samples at −70 °C. The infectivity titre was determined using a plaque test in BHK21 cells. For the production of antisera the virus was grown on horse serum-adapted L cells and concentrated approx. 100-fold by centrifugation for 1 h at 80000 g.

**Immune sera.** Antisera against virus antigens were produced by intramuscular inoculation of guinea pigs with virus emulsified in incomplete Freund’s adjuvant. Five weeks later the animals were boosted twice within a period of 1 week with aqueous antigen solution and bled after 7 days (Mannweiler & Rutter, 1975). The sera were inactivated for 30 min at 56 °C and frozen in small samples at −20 °C.

**Actin** was obtained from acetone-dried powder of pig or hamster striated muscle tissue according to the method of Iyengar & Weber (1964). The following solution was used as actin extraction buffer: ATP 0.2 mM, calcium chloride 0.2 mM, 2-mercaptoethanol 0.5 mM, tris/HCl buffer 5 mM, pH 8 (Spudich & Watt, 1971). Tissue powder (1 g) was extracted in 20 ml buffer for 30 min at 0 °C. After centrifugation for 15 min at 30000 g, a clear supernate was obtained. This crude extract (Fig. 1a) was used for further purification either by polyacrylamide gel electrophoresis (Lazarides & Weber, 1974) or by repeated salt-induced polymerization and depolymerization (Spudich & Watt, 1971).

**Antiactin sera.** A crude actin extract was treated with 1% (w/v) SDS and 1% (w/v) 2-mercaptoethanol, dissociated by boiling for 1 min and separated by electrophoresis (Lazarides & Weber, 1974). The actin band was cut out, minced and eluted in electrophoresis buffer. The eluate was dialysed by pressure to a protein concentration of 1 mg/ml, mixed with complete Freund’s adjuvant and injected intramuscularly into rabbits. Six weeks later the animals were boosted twice at short intervals with the same mixture and bled 10 days after the final inoculation. The serum was inactivated for 30 min at 60 °C and stored
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at $-20 \, ^\circ C$. In other experiments pure actin, obtained after two polymerization/depolymerization cycles (Spudich & Watt, 1971), served as the booster.

For the production of actin immunosorbents, a crude actin extract was brought to a protein concentration of 10 mg/ml by precipitation at pH 4.7. Four hundred mg of bovine serum albumin were dissolved in 10 ml of the concentrated actin solution and 2 ml of 2.5% glutaraldehyde in distilled water were added to this mixture. The reaction mixture was incubated for 3 h at room temperature. The resulting gel was subsequently washed and handled as described by Avrameas & Ternynck (1969). Sixty to eighty ml of antiactin serum were absorbed once with 10 ml of absorbent. After a thorough washing of the gel with PBS the bound antibodies were separated from the immunosorbent with 0.1 M-glycine buffer at pH 2.5 (Avrameas & Ternynck, 1969). The pure antibody solution was brought to neutral pH with 0.2 M-disodium phosphate and dialysed overnight against 200 volumes of PBS. After determining the protein concentration, small samples of the solution were frozen at $-20 \, ^\circ C$.

All immunological reagents, i.e. the anti-NDV and VSV sera as well as the antiactin antibody solution, were tested by seroneutralization against 100 p.f.u. VSV or NDV on chick fibroblast monolayers. In addition, all three reactants were tested by complement fixation, using a crude extract from hamster striated muscle powder as antigen. The tests were performed with the microtitre system, employing guinea pig serum as the complement source (Gschwender, Rutter & Lehmann-Grube, 1976). Purification of antiactin antibodies was also monitored by complement fixation.

**Immunofluorescence technique.** The cultures were infected as described (Rutter & Mannweiler, 1976) 24 h after seeding. The multiplicity of infection (m.o.i.) for NDV was 1 p.f.u./cell and about 0.6 p.f.u./cell for VSV. At the end of the desired incubation period the coverslips were washed three times in warm PBS and then fixed for 5 min in a mixture consisting of 0.7 M-formaldehyde and 0.1 mM-digitoxin (Levinthal, Dunnebacke & Williams, 1969) in 0.1 M-potassium phosphate buffer, pH 7.5.

Further staining of samples was carried out according to the following scheme: (a) rabbit-antiactin antibodies approx. 50 µg in 0.1 ml/coverslip; (b) fluorescein-labelled anti-rabbit gamma-globulin diluted 1:50; (c) guinea pig antiviral serum diluted 1:20; (d) rhodamine-conjugated anti-guinea pig gamma-globulin diluted 1:40. Each incubation step was executed for 30 min at room temperature and finished with washing the coverslips in several changes of PBS.

The stained coverslip cultures were placed on slides in buffered glycerin solution and examined in a Zetopan Reichert microscope. The microscope was provided with an HBO200 mercury lamp and darkfield condenser. The filter combinations required for immunofluorescence consisted of BG12/6mm (excitation) and OG1+GG4 (barrier filter) for fluorescein and of an interference band filter with a transmission maximum of 546 nm (as excitation) with an LP550 filter (as barrier filter) for rhodamine.

Infected coverslip cultures treated with normal rabbit gamma-globulin and normal guinea pig serum served as controls. Pre-immunization sera (or extracted gamma-globulins for actin staining) were also used as controls. Other specificity controls were carried out by means of cross absorptions, with virus concentrates or actin immunosorbents serving as antigens.

**Protein determinations** were carried out according to the method of Lowry et al. (1951). Bovine serum albumin solution served as the standard.

**SDS polyacrylamide gel electrophoresis** was carried out in 7.5% (w/v) gels. Gel and electrode buffer were according to Fairbanks, Steck & Wallach (1971) and contained 0.1% (w/v) SDS.
Glass tubes (100 mm in height, 6 mm in diam.) were used for analytical purposes. Slab gels (120 × 120 × 5 mm) were employed for actin purification.

The gels were stained with Coomassie brilliant blue (R.250) and destained according to the method of Fairbanks et al. (1971).

RESULTS

The method applied here to the production of antiactin antibodies in rabbits frequently yields low titres. Therefore, it was necessary to use concentrated gamma-globulins for immune staining (Lazarides & Weber, 1974; Wang, Ash & Singer, 1975) which in our hands produced preparations with high non-specific labelling. Using as booster pure actin obtained by two polymerization and depolymerization cycles (Fig. 1) without denaturation by SDS failed to improve the quality of the sera. Good results were obtained by immunological concentration and purification of specific antibodies (Fig. 2a). Although about 60% of the antibodies were lost during the procedure, the specificity of labelling increased to such an extent that normal rabbit gamma-globulin solutions of the same concentration as the antibody preparations caused a barely discernible non-specific labelling of the controls (Fig. 2b). This preparation failed to reveal any neutralizing activity against VSV or NDV, nor did anti-NDV and anti-VSV sera show any complement-fixing activity against crude actin extracts.

Normal BHK21 cells

These cells revealed in fluorescence staining a multitude of actin-containing filamentous structures. They spanned the interior of the cell and often ran parallel for several μm along the cellular main axis (Fig. 2a). In many cases the cell periphery was strongly defined by these fluorescing structures. The majority of these bundles seemed to be located in the immediate vicinity of the substrate because they were easy to bring into focus, even though the edges of the cell were sharply defined. Diffuse fluorescence, primarily around the nucleus, was seen in nearly every cell. Many cells on the same coverslip revealed a high degree of individuality, but the general patterns described here were always readily recognized (Fig. 2a).

Treatment of cell cultures for at least 6 h with cycloheximide (100 μg/ml, w/v) did not induce changes in the actin distribution (not shown).

NDV-infected BHK21 cells

As early as 5 h after infection masses of cytoplasmic virus antigens were demonstrable by rhodamine staining. The demonstrable amount of antigens increased consistently without registering changes in the actin pattern. After 8 h the first actin alterations appeared, i.e. discrete clumping of actin which in some cases produced aggregations of over 1 μm in size (Fig. 3b). They appeared as small distinct dots and were found in areas of the cell which also contained a large amount of virus proteins (Fig. 3a). On focusing, they appeared to be situated directly below the plasma membrane. At the same time a distinct decrease in the number of fluorescent bundles and a steady increase in the amount of thin actin-containing filamentous structures subjacent to the cell surface were observed (Fig. 3c, d). With the appearance of the first syncytia after approx. 12 h, the increased number of these filaments presented the most striking alteration. Fourteen h after infection, the sharply defined actin-containing regions at the cell periphery, which presumably served for adhesion of the cell to the substrate, began to be increasingly associated with virus antigens (Fig. 3e, f). At the same time large areas of the culture became detached from the glass surface.
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Fig. 1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of actin. (a) Crude extract of dried muscle powder, 40 µg; (b) after the first polymerization/depolymerization cycle, 30 µg of protein; (c) after the second polymerization/depolymerization cycle, 30 µg of protein.

VSV-infected cells

With the m.o.i. applied here, the first virus antigens were seen in the cytoplasm approx. 3 h after infection either as well defined dots or in diffuse form (Fig. 4a). At this time the structures demonstrable with antiactin antibodies revealed no changes compared with the controls (Fig. 4b). With the advancement of infection, there was a partial disappearance of the stainable actin filaments. This degradation took place mainly at sites in the cytoplasm where virus-induced antigens accumulated (Fig. 4c, d). Roughly 6 h after infection,
filamentous actin structures were no longer to be detected (Fig. 4e, f). At the same time many cells began to round up and to retract. The cell surfaces appeared extremely irregular with undefinable borders. Closely packed well-defined granules of different sizes were detectable in the cytoplasm. They contained virus antigens as well as actin (Fig. 4g, h).

Normal or infected cells labelled with normal sera revealed only weak diffuse staining. The absorption of immune sera with homologous antigens removed most of the specific fluorescence, but at least three consecutive absorptions were required for total disappearance. Cross absorption, i.e. antiactin antibodies mixed with virus concentrates or antiviral sera incubated with actin immunosorbents, failed to have any appreciable effect on the staining capacity of the immune reagents.

DISCUSSION

Since actin constitutes a large part of the total amount of cell proteins (Fine & Taylor, 1976), it probably represents a dominant constituent of the cytoskeleton in non-muscle cells and consequently appears to play a decisive role in many functions of the cell (Pollard & Weihing, 1974; Mannherz & Goody, 1976).

Compared to the striated muscle tissue, in which actin is organized in a stable structure, the actin structures in non-muscle cells responsible for other functions appear to be short-lived (Rubenstein & Spudich, 1977). The individual actin molecules participate in the formation of specialized structures which are built in response to environmental changes of the cell. These structures are readily visualized and characterized since the production of monospecific antiactin sera (Lazarides & Weber, 1974; Trenchev & Holborow, 1976) permitted the application of immune labelling methods (Lazarides & Weber, 1974; Trenchev et al. 1974).

The distribution of actin-containing structures in BHK21 cells, studied in immunofluorescence by means of pure antibody preparations, confirmed the findings previously
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Fig. 3. BHK21 cells infected with NDV at an m.o.i. of 1 p.f.u./cell. (a, c, e) Stained with guinea pig anti-NDV serum and rhodamine-labelled anti-guinea pig gamma-globulin from sheep. (b, d, f) Stained with rabbit antiactin antibodies and fluorescein-labelled anti-rabbit gamma-globulin from sheep. (a, b) 8 h after infection; (c, d) 12 h after infection; (e, f) 14 h after infection.

described by other authors (Lazarides & Weber, 1974; Goldman et al. 1975; Lazarides, 1976). Furthermore, our investigations have clearly shown that at least two different virus infections exert a strong influence on the organization of supramolecular, actin-containing structures. These alterations take different aspects, depending on the type of c.p.e. induced. The progress of the NDV-induced c.p.e. is accompanied by slow reorganization of the actin pattern which is detectable only in its final phase at the light microscope level (later than 8 h after infection). By contrast, the rapid development of the c.p.e. in VSV-infected cells is accompanied by a swift degradation of the actin-containing filament bundles. From these morphological differences it may be supposed that the two virus infections studied here influence the organization of actin in BHK cells probably in a specific manner.
Fig. 4. BHK21 cells infected with VSV at an m.o.i. of 0.6 p.f.u./cell. (a, c, e, g) Stained with guinea pig anti-VSV serum and rhodamine-labelled anti-guinea pig gamma-globulin from sheep. (b, d, f, h) Stained with rabbit antiactin antibodies and fluorescein-labelled anti-rabbit gamma-globulin from sheep. (a, b) 3 h after infection; (c, d) 4 h after infection; (e, f) 6 h after infection.
Some observations suggest that modifications at cell membranes may exert a strong restructuring effect on the cytoskeleton (Albertini & Clark, 1975; Sundqvist & Ehrnst, 1976; Vial & Garrido, 1976; Weber et al. 1976). It is obvious that the incorporation of virus components into the plasma membrane, a property both virus infections studied here have in common, cannot but have a strong influence on the structures located beneath the cell surface. These virus-induced proteins could trigger at the cell surface a mechanism responsible for the further alterations of actin-containing structures which consequently affect the cell shape. Such an explanation could, at least in part, be accepted for the development of described alterations induced by NDV. The marked and rapid degradation of filament bundles following infection with VSV, however, is more difficult to explain. It may be asserted that this phenomenon is not caused by blocking of cellular protein synthesis – an element of VSV infection (Bablanian, 1975) – because treatment of cultures with inhibiting doses of cycloheximide does not elicit alterations of a similar nature. It may also be possible that VSV-coded proteins have a direct influence on the aggregation of actin-containing structures. At least this was assumed to be the case for alterations of actin structures in Rous sarcoma virus transformed cells (Wang & Goldberg, 1976), since similar changes accompany the cell transformation after infection with some oncornaviruses (Pollack, Osborn & Weber, 1975; Edelman & Yahara, 1976; Wang & Goldberg, 1976).

Maintenance and control of the cell shape are visibly influenced after infection with VSV or NDV. The present investigations fail to elucidate whether these virus-induced morphological alterations are due entirely to the effect of virus multiplication on actin-containing structures or whether other elements of the cytoskeleton are also involved. Nevertheless the kind and degree of these virus-induced changes might play a decisive role in defining the morphology of the c.p.e.

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REFERENCES


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