Polypeptide Synthesis of Dugbe Virus, a Member of the Nairovirus Genus of the Bunyaviridae

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SUMMARY

The replication of Dugbe (DUG) virus, a member of the Nairovirus genus of the Bunyaviridae, has been investigated. During the infection of BS-C-1 cells a virus-specific c.p.e. was initially observed followed by recovery of the cell monolayer but with continued production of infectious virus. Six DUG virus-induced polypeptides were identified with apparent molecular weights, determined by gel electrophoresis, of 92000 (p92), 82000 (p82), 77000 (p77), 52000 (p52), 48000 (p48) and 34000 (p34). The polypeptides p77 and p34 were detected in purified DUG virions but not in extracts of virus-infected cells pulse-labelled with [3H]leucine. Polypeptides p48 and p52 were found in both purified virus preparations and in extracts of infected cells. p82 and p92 were found only in lysates of infected cells. When two-dimensional gel electrophoresis was used to analyse infected cells, p48 was found to have a net positive charge.

INTRODUCTION

The Nairovirus genus is one of four genera classified in the Bunyaviridae family of arboviruses (Bishop et al., 1980; Casals & Tignor, 1980). Biochemical analyses have shown the nairoviruses to have some characteristics in common with other members of the Bunyaviridae, but they nevertheless form a distinct group (Clerx & Bishop, 1981; Clerx et al., 1981; Foulke et al., 1981). As for other Bunyaviridae the nairovirus genome consists of three unique RNA segments demonstrated by oligonucleotide fingerprinting (Clerx & Bishop, 1981; Clerx et al., 1981). Purified nairovirus virions contain three major structural polypeptides, a nucleoprotein (N, molecular weight 48 x 10^3 to 54 x 10^3) and two external glycoproteins (G1 and G2, molecular weights 72 x 10^3 to 84 x 10^3 and 30 x 10^3 to 40 x 10^3 respectively) (Clerx et al., 1981). Foulke et al. (1981) identified an additional glycoprotein of molecular weight 45 x 10^3 with purified virions of Hazara virus.

Analyses of intracellular, virus-induced polypeptide synthesis and processing have been limited to Qalyub virus. In Qalyub virus-infected cells, in addition to N and G1, two non-structural glycoproteins (gp115 and gp85) were detected and were considered to be precursors to the virion glycoproteins (Clerx & Bishop, 1981). The structural polypeptides, N and G1, have been identified by immune precipitation in cells infected with a range of nairoviruses (Clerx et al., 1981). In the work described below Dugbe (DUG) virus has been used as a representative member to investigate further the replication of nairoviruses. DUG virus was originally isolated from ticks collected in Nigeria but has since been isolated from vertebrates and arthropods from various locations throughout Africa (Berge, 1975). On serological criteria DUG virus is classified in the Nairobi sheep disease serogroup (Bishop et al., 1980). Biochemical analyses of purified DUG virions have shown them to have a polypeptide and RNA composition similar to other members of the Nairovirus genus. Only a single virus-induced polypeptide, N, has been previously detected in DUG virus-infected cells (David-West, 1974; Clerx et al., 1981). The following experiments present information on DUG virus replication in BS-C-1 cells and the identification of two non-structural polypeptides.
The virus was passaged, undiluted, three times in BS-C-1 cells before being cloned once in BS-C-1 cells. The virus stocks, used as inocula in the experiments described below, were passage 2 from the cloned material.

Two isolates of DUG virus have been used during the present study. One, DUG(KT281/75) virus, originally isolated in Kenya, E. Africa, was received, at passage number 7, as an infected suckling mouse brain suspension from Dr D. H. L. Bishop, University of Alabama in Birmingham, Ala., U.S.A. Stocks, used as inocula in the experiments described below, were passage 2 from the cloned material. DUG(IbAr 1792) virus, passage 12, was provided by Dr J. S. Porterfield, University of Oxford, U.K., as freeze-dried material from infected suckling mouse brain. This virus isolate was originally collected in Nigeria, W. Africa. Cloned stocks of DUG(IbAr 1792) virus were prepared as for DUG(KT281/75) virus.

**Assay for infectious virus.** Infectious virus was assayed by plaque formation in BS-C-1 cell monolayers. Virus samples were diluted in phosphate-buffered saline (PBS) containing 2% NCS, and 0·1 ml of virus was inoculated per 50 mm dish of cells (approx. 2 × 10⁶ cells). After 30 min adsorption at 18 °C the cells were overlaid with MEM containing 5% (v/v) NCS, DEAE-dextran (200 μg/ml) and solidified with 0·9% (w/v) agar Noble (Difco). After incubation at 37 °C for 7 days plaques were detected by staining with neutral red.

**Purification of DUG virus.** BS-C-1 cells (approx. 2 × 10⁶) were infected with DUG virus at a multiplicity of 0·03 p.f.u./cell. Infected cells were overlayed with virus growth medium (MEM containing 5% NCS) and incubated at 33 °C for 60 h. The culture medium was clarified at 3000 g for 45 min and the supernatant treated with polyethylene glycol 6000 and NaCl (final concentrations 70 g per litre and 23 g per litre respectively) at 4 °C for 4 h. The precipitated virus was collected at 3000 g for 45 min, resuspended in 0·01 M-Tris–HCl pH 7·4, and loaded onto a combination sodium potassium tartrate–glycerol gradient prepared in TE buffer (2 mM-EDTA, 2 mM-Tris–HCl pH 7·4) (Obijeski et al., 1974). Gradients were centrifuged at 175000 g for 90 min using an MSE 6 × 5·5 ml swing-out rotor.

Approximately 10% of the infectious virus originally present in the culture medium was recovered as a visible white band found two-thirds of the way into the gradient. This band was collected, diluted in 0·01 M-Tris–HCl pH 7·4, and virus particles concentrated by pelleting through a cushion of 30% (w/w) glycerol in TE buffer.

**Preparation of radiolabelled cell lysates.** BS-C-1 cell monolayers (10⁶ cells per 30 mm Petri dish) were infected with DUG virus at the multiplicities indicated in the text. After 30 min adsorption at 18 °C the cell monolayers were overlayed with virus growth medium and incubated at 37 °C. At 1·5 h post-infection the cell monolayers were washed to remove unadsorbed virus and incubation continued at 37 °C in fresh virus growth medium. At the times indicated, cell monolayers were washed twice with PBS and incubated in PBS containing 25 μCi/ml of L-[4,5-3H]-leucine (40 to 60 Ci/mmol, New England Nuclear). After the required period of labelling the cells were treated as described below.

**Polyacrylamide gel electrophoresis (SDS–PAGE).** Discontinuous SDS–PAGE in slab gels was performed as described previously (Cash et al., 1977) using a resolving gel of 10% polyacrylamide and a 3·6% polyacrylamide stacking gel. Samples for SDS–PAGE were treated with protein dissociation buffer (2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol in 0·15 M-Tris–HCl pH 6·7 (all final concentrations) plus bromophenol blue as a tracking dye) at 100 °C for 5 min. Radioactive polypeptides were detected by fluorography using PPO-DMSO (Laskey & Mills, 1975) and pre-exposed X-ray film. Non-radioactive polypeptides were detected using silver staining (Merril et al., 1981) (Bio-Rad reagents).

Molecular weights of the virus-induced polypeptides were determined by comparison with the electrophoretic mobilities of proteins of known molecular weights (14000 to 94000) electrophoresed on the same slab gel (Pharmacia).

**Electrofocusing and two-dimensional PAGE (2D-PAGE).** Samples for electrofocusing and 2D-PAGE were prepared using basically the method of King et al. (1982). Cell monolayers were lysed in 150 μl of TTE buffer (0·01 M Tris–HCl pH 7·4, 1% Triton X-100, 1 mM-EDTA) containing 10% (v/v) glycerol and RNase A (0·1 mg/ml) at 4 °C for 5 min. A cytoplasmic extract was prepared by removing the nuclei by centrifugation for 3 min on a Microfuge B (Beckman). The supernatant was incubated for 20 min at 37 °C and then adjusted to 9 M-urea (AnalaR, BDH), 3% Triton X-100, 6% 2-mercaptoethanol and 3% ampholytes (Pharmacia). The supernatants were reincubated for 20 min at 37 °C prior to loading onto gels.

Flat-bed gels (0·75 mm thick) for electrofocusing were cast for the Bio-Rad model 1405 horizontal gel apparatus. Gels were prepared with 5% acrylamide, 0·15% N,N'-methylenebisacrylamide, 9 M-urea, 2% Triton X-100 and 2% ampholytes (Pharmacia). The gels were photopolymerized using riboflavin-5'-phosphate. Ten μl of cell lysate was applied to the surface of the gel and focusing was carried out at 5 W for 3500 volt hours (anolyte = 1 M-H₂PO₄; catholyte = 1 M-NaOH). Gels were immediately fixed in 5% sulphosalicylic acid–10% trichloroacetic acid for 30 min and processed for fluorography using En³Hance (New England Nuclear).

For 2D-PAGE the first dimension gels for NEPHGE (non-equilibrium pH electrophoresis) were as described above for electrofocusing except that they were cast in glass tubes (2·7 mm internal diameter). Treated cell lysates were loaded at the anode and focused at 500 V for 3500 volt hours. The anolyte used was 0·01 M-iminodiacetic acid.
and the catholyte 0.01 M-ethylenediamine. The gels were expelled from the glass tubes and equilibrated for 10 min in equilibration buffer [10% (w/v) glycerol, 0.05% (v/v) 2-mercaptoethanol, 2.3% (w/v) SDS, 0.125 M-Tris-HCl pH 6.8]. The tube gels were laid over discontinuous slab gels, prepared as for SDS-PAGE, and embedded in 1% agarose. Electrophoresis was carried out under the standard conditions for SDS-PAGE.

RESULTS

Growth of Dugbe virus in BS-C-1 cells

The two DUG virus isolates grew in BS-C-1 cells at both 33 °C and 37 °C. Fig. 1 shows the release of virus during replication of DUG(KT281/75) virus at 37 °C with multiplicities of infection of 2 and 0.1 p.f.u. cell. Under these conditions, virus-specific cytopathic effect was observed by 24 h post infection and maximum titres of cell-released virus were attained by 30 to 36 h.

Although virus-specific c.p.e. was evident for DUG virus-infected BS-C-1 cells there remained a background of apparently uninfected cells. It was considered possible that these cells had become persistently infected with DUG virus. This was investigated further by infecting cells with DUG(KT281/75) virus at a m.o.i. of 0.5 p.f.u./cell. The cells were incubated at 37 °C and each day the culture medium was assayed for infectious virus and replaced with fresh growth medium. Infectious virus was detectable throughout the incubation period, reaching a peak of $3 \times 10^7$ p.f.u./2 x 10⁶ cells at 2 days before declining to $10^4$ p.f.u./2 x 10⁶ cells at 15 days. Virus-specific c.p.e. was first observed at 2 days but by 15 days a confluent cell monolayer had re-formed. These cells were similar in morphology to uninfected BS-C-1 cells by light microscopy. At this point the cells could be passaged as for uninfected BS-C-1 cells At the third passage the culture medium still contained $1.5 \times 10^3$ p.f.u. from 2 x 10⁶ cells. Despite virus replication no virus induced polypeptides (see below) were demonstrated by either SDS-PAGE or 2D-PAGE at the third passage (data not shown).

Identification of DUG virus-induced polypeptides

In parallel with the growth curve presented in Fig. 1(b), monolayers of BS-C-1 cells infected with DUG(KT281/75) virus were pulse-labelled for 1 h with [³H]leucine at the times indicated in Fig. 2. Cell lysates were prepared and analysed by SDS-PAGE. Three virus-induced polypeptides, designated p92, p82 and p48 according to their molecular weights, were identified in DUG virus-infected BS-C-1 cells. They differed in their patterns of synthesis during virus.
Fig. 2. DUG(KT281/75) virus-induced polypeptide synthesis. BS-C-1 cells, infected with DUG virus (m.o.i., 0.1 p.f.u./cell), were pulse-labelled with $[^3H]$leucine for 1 h at the times post-infection indicated. Cell lysates were prepared and analysed by SDS–PAGE. MI, mock-infected BS-C-1 cells.

replication. The first virus-induced polypeptide identified was p48, the rate of synthesis of which increased to a peak at 27 h post-infection before declining to undetectable levels by 48 h. Polypeptide p92 followed similar kinetics as p48. The third polypeptide, p82, differed from p48 and p92 in being synthesized later in infection and continuing to be synthesized at 48 h. A similar pattern of synthesis was observed for virus at a m.o.i. of 2 p.f.u./cell. Under the conditions of infection used there was no evidence for any significant inhibition of host protein synthesis. In experiments described below a m.o.i. of 1 to 2 p.f.u./cell was used routinely.

To improve the detection of virus-induced polypeptides, radiolabelling in hypertonic culture medium was employed. Nuss et al. (1975) demonstrated that increasing the NaCl concentration of the culture medium selectively inhibits the initiation of host cell proteins compared to the initiation of protein synthesis of either vesicular stomatitis virus or poliovirus. At 24 h after infection, DUG virus-infected and uninfected BS-C-1 cells were incubated for 1 h in MEM containing 320 mM-NaCl (final concentration). The cells were then pulse-labelled for 1 h with $[^3H]$leucine in PBS containing 320 mM-NaCl. Intracellular polypeptides were prepared and analysed by SDS–PAGE (Fig. 3). At both 120 mM-NaCl, the normal concentration in MEM, and 320 mM-NaCl, four virus-induced polypeptides (p92, p83, p52 and p48) were detected. The virus-induced polypeptide p52 was identified in extracts of infected cells in some, but not all, experiments for both DUG virus isolates.

For the identification of virion polypeptides, DUG(KT281/75) virus was grown in BS-C-1 cells and purified as described in Methods. The polypeptides were resolved by SDS–PAGE and stained by silver nitrate (Merril et al., 1981) (Fig. 4). A profile of the virus intracellular polypeptides is included in Fig. 4 for comparison. Both p52 and p48 were associated with purified virions together with two polypeptides, p77 and p34, not detectable in cell lysates radiolabelled with $[^3H]$leucine. However, when infected cells were labelled with $[^3H]$glucosamine a minor
Nairovirus polypeptides

Fig. 3. DUG(KT281/75) virus-induced polypeptides. (a) BS-C-1 cells infected with DUG virus (m.o.i., 2 p.f.u./cell) were pulse-labelled at 24 h post-infection with [3H]leucine for 1 h. Cell lysates were prepared and analysed by SDS–PAGE. DUG, DUG virus-infected BS-C-1 cells; MI, uninfected BS-C-1 cells. (b) Cell-released DUG virus was purified as described in the text and virion polypeptides analysed by SDS–PAGE and silver staining.

Fig. 4. DUG virus polypeptide synthesis in the presence of NaCl. DUG virus-infected and uninfected cells were incubated in MEM containing NaCl at the concentrations indicated (MEM normally contains 120 mM-NaCl), for 1 h prior to and during a 1 h pulse-label with [3H]leucine. Cell lysates were prepared and analysed by SDS–PAGE. DUG, DUG virus-infected cells; MI, mock infected BS-C-1 cells.

virus-induced glycoprotein of molecular weight 77 000 was detected (data not shown). Neither p92 nor p82 was identified in purified DUG virus. Identical polypeptide profiles were found for virus-infected cells and purified virions with the DUG(IbAr 1792) virus isolate.

Analysis of DUG virus intracellular polypeptides by electrofocusing and 2D-PAGE

To characterize the DUG virus intracellular polypeptides further, cell lysates from DUG virus-infected and uninfected BS-C-1 cells were analysed by electrofocusing and 2D-PAGE. Under the conditions of electrofocusing described in Methods a single virus-induced polypeptide was consistently detected against the background of host protein synthesis. Due to its migration towards the cathode this polypeptide appeared to have a net positive charge (data not shown). The same virus-induced polypeptide was identified for both DUG virus isolates using two independent cloned stocks for each virus isolate.

Two-dimensional PAGE was employed to correlate the polypeptide identified by electrofocusing with the polypeptides demonstrated by SDS–PAGE. When cell lysates from DUG virus-infected cells were loaded at the cathode for the first dimension, using a pH 3 to 10 gradient, and focused for 8000 to 9000 volt hours no virus-induced polypeptides were resolved in
Fig. 5. 2D-PAGE analysis of DUG virus-induced polypeptides. Radiolabelled cell lysates from virus-infected and uninfected BS-C-1 cells were compared by 2D-PAGE as described in the text. The location of p48 is indicated by the arrow. The direction of electrofocusing is left to right and SDS-PAGE from top to bottom.

the second dimension (data not shown). Consequently the cell lysates were loaded at the anode and focused under non-equilibrium conditions as described in Methods. With this protocol a virus-induced polypeptide with a net positive charge was demonstrated (arrowed in Fig. 5). When co-electrophoresis by 2D-PAGE with molecular weight markers was performed, this polypeptide had an apparent molecular weight of $45 \times 10^3$. Analysis of the same infected cell lysates by SDS-PAGE located only p92 and p48. From this it was concluded that p48 and the positively charged virus-induced polypeptide were equivalent.

Co-electrophoresis of lysates from DUG(KT281/75) and DUG(IbAr 1792) virus-infected cells showed p48 to be indistinguishable for the two virus isolates (Fig. 6) under the conditions
used. As shown in Fig. 6, minor polypeptides were observed on some occasions which trailed from the major p48 component.

**DISCUSSION**

The growth of DUG virus in BS-C-1 cells passed through two stages: a lytic phase, during which virus-specific c.p.e. and virus-induced polypeptide synthesis were detectable, followed by recovery of the cell monolayer but continued release of infectious virus, albeit at low levels. The recovery of the cell monolayer was paralleled by a decline in virus c.p.e. and virus-induced polypeptide synthesis. The continued production of infectious virus even after passage of the cells in culture suggested that a persistent infection was established by DUG virus in BS-C-1 cells. Gould et al. (1983) established a persistently infected monkey cell line with Hughes virus, a member of the Nairovirus genus, in which approximately 20 to 40% of the cells expressed virus antigen as determined by immunofluorescence. In the case of DUG virus, no virus-induced polypeptides were detectable by the electrophoretic methods employed but this may be due to a lack of sensitivity of the procedures. The recovery of BS-C-1 cells following DUG virus infection was also suggested from the virus plaque morphology. Both DUG virus isolates, but in particular the DUG(KT281/75) virus isolate, produced 'bull's-eye' plaques (Welsh & Pfau, 1972) under the assay conditions used for infectious virus. Plaques with this morphology could be explained by the incomplete destruction of the cell monolayer by DUG virus followed by the recovery of cells in the centre of the plaque, resulting in a clear plaque with a turbid centre. For lymphocytic choriomeningitis virus, an arenavirus, 'bull's eye' plaques were attributed to defective interfering virus in the virus stock (Welsh & Pfau, 1972). If this were the case for DUG virus, defective viruses were rapidly generated since virus plated directly from a plaque produced 'bull's-eye' plaques (unpublished data).
Six virus-induced polypeptides were identified in DUG virus-infected cells and purified virus. Three of these polypeptides (p92, p82 and p52) had no counterparts, based on molecular weight estimations, in previous descriptions of nairovirus polypeptides. Four polypeptides (p77, p52, p48 and p34) which were detected in preparations of purified virus were tentatively identified as virus structural polypeptides. Polypeptides p77 and p34 are similar in size to the glycoproteins of other nairoviruses (Clerx et al., 1981) although in the present investigation only p77 was labelled with [3H]glucosamine. Of the two other structural polypeptides p48 was found in all preparations of intracellular polypeptides whereas p52 was only sporadically observed but was identified for both DUG virus isolates. Polypeptide p48 was of the correct size for the nucleoprotein reported for nairoviruses (Clerx et al., 1981) and its association with RNA in the nucleocapsid would thus be consistent with its overall positive charge demonstrated by electrofocusing and 2D-PAGE. The heterogeneity observed for p48 by 2D-PAGE suggests that there may be intermediate processing steps in its synthesis, although the possible introduction of charge artefacts on p48 during sample preparation cannot at present be discarded and is currently being investigated. The remaining two virus-induced polypeptides (p92 and p82) were found only in virus-induced cell lysates and not in purified virus using silver nitrate staining as the detection method. These may represent precursors to the virus structural polypeptides. Preliminary experiments, however, using a pulse–chase protocol failed to demonstrate any processing for p92 (unpublished data).

The studies on DUG virus reported here, together with previously published analyses on other nairoviruses have demonstrated a number of differences in the structure and replication between these viruses and other members of the Bunyaviridae. The nairoviruses thus warrant further investigations on their mode of replication and pathogenesis.

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REFERENCES

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