Localization of an Arenavirus Protein in the Nuclei of Infected Cells

By P. R. Young,* A. C. Chanas,† S. R. Lee, E. A. Gould 1 and C. R. Howard

Department of Medical Microbiology and 1Arbovirus Unit, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, U.K.

(Accepted 18 May 1987)

SUMMARY

Host cell nuclear involvement in an arenavirus infection was examined by immunofluorescence. Both polyclonal antisera and monoclonal antibodies specific for the major nucleocapsid (N) polypeptide revealed virus-specific nuclear inclusions in Pichinde virus-infected Vero cells. Immunoprecipitation of infected cell extracts with the anti-N monoclonal antibodies and subsequent analysis by SDS-PAGE, identified two N-related proteins with mol. wt. of 36000 (p36) and 28000 (p28) in addition to the N polypeptide. Only those monoclonal antibodies which precipitated p28 as well as N and p36 were found to produce nuclear as well as cytoplasmic fluorescence. These findings suggest that either the p28 protein itself or a conformational variant of N was the nuclear antigen detected.

The family Arenaviridae comprises a group of enveloped viruses with a segmented, single-strand, ambisense RNA genome (Matthews, 1982; Auperin et al., 1984). These viruses replicate in the cytoplasm of a wide variety of cells and mature by budding through the host cell plasma membrane (Murphy & Whitfield, 1975). Cytopathic effects are often minimal and primarily involve modifications in cytoplasmic organization. Organelle destruction, membrane proliferation and subsequent condensation of the cytoplasm are characteristic features. In many cases however, cytopathology is absent and reflects the relative ease with which persistent infections can be established by arenaviruses (Pedersen, 1979). Although no specific alteration in nuclear morphology has been described, indirect evidence for the involvement of nuclear functions in arenavirus infections has been reported (Banerjee et al., 1976; Friedlander et al., 1984; López & Franze-Fernández, 1985). The requirement for a functioning cell nucleus was first demonstrated by the failure of Pichinde virus to replicate in BHK-21 cells enucleated with cytochalasin B (Banerjee et al., 1976) and in two recent studies (Friedlander et al., 1984; López & Franze-Fernández, 1985), the effect of infection on host cell DNA synthesis was also examined and a dose-dependent inhibition was found. In the present study, direct evidence for nuclear involvement of a virus-specific antigen in Pichinde virus-infected Vero cells is reported.

Pichinde virus (strain Coan 3739) was obtained from Dr J. Casals (Yale Arbovirus Research Unit, Yale University, New Haven, Conn., U.S.A.) after nine passages in suckling mouse brain. The virus was passaged twice more in mice and then plaque-purified three times in Vero cells. Working virus stocks were prepared by harvesting tissue culture fluids from infected BHK-21 cells 48 h post-infection.

Cells for immunofluorescence staining were grown on glass coverslips in multiwell plates. Prior to staining, the coverslips were washed in phosphate-buffered saline (PBS) for 10 min and fixed in ice-cold acetone for 5 min. Antisera were diluted in PBS and added to the substrates which were then incubated for 45 min at 37°C. The coverslips were washed in PBS for 10 min.

* Present address: Astra Clinical Research Unit, 10 York Place, Edinburgh EH1 3EP, U.K.
Fig. 1. Cytoplasmic inclusions in Pichinde virus-infected Vero cells. (a) Infected dividing cells showing virus-specific cytoplasmic inclusions by indirect immunofluorescence using a guinea-pig hyperimmune antiserum raised against Pichinde virus purified as reported previously (Young & Howard, 1983). (b) Thin section electron micrograph revealing large inclusions (arrowed) within the cytoplasm of infected Vero cells. Bar markers represent 5 μm (a) and 2 μm (b).

Table 1. Reactivity of various antibodies to Pichinde virus-infected Vero cells

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Immunofluorescence</th>
<th>Immunoprecipitation of p28</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytoplasmic</td>
<td>Nuclear</td>
</tr>
<tr>
<td></td>
<td>inclusions</td>
<td>inclusions</td>
</tr>
<tr>
<td>Immune serum*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gp antiserum†</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rb antiserum‡</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anti-infected Vero§</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Monoclonal antibodies¶</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PV2-14-19¶</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PV1-1-3¶</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PV1-1-7¶</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PV3B-35</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PV75-10b</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PV75-22</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

* Antisera were prepared as outlined in Chanas et al. (1980).
† Guinea-pig antiserum raised against purified Pichinde virus.
‡ Rabbit antiserum raised against purified Pichinde virus.
§ Rabbit antiserum raised against Pichinde virus-infected Vero cell extract (36 h post-infection).
¶ All monoclonal antibodies are against Pichinde virus nucleocapsid (N) protein.
‖ Monoclonal antibodies as outlined in Buchmeier et al. (1981).

and covered with fluorescein isothiocyanate-conjugated anti-species immunoglobulin (1:40 in PBS; Nordic Immunological Laboratories, Maidenhead, U.K.). After a further 45 min at 37 °C the coverslips were washed in PBS for 10 min, rinsed in distilled water and mounted in glycerol–PBS (9:1) on glass slides.

Vero cells infected with Pichinde virus at a multiplicity of 0-1 p.f.u./cell were examined daily by indirect immunofluorescence using a panel of antibodies (Table 1). After incubation for 24 h at 37 °C, viral antigens were seen in about 10% of the cells with each antibody preparation tested. In some cells the cytoplasm contained brightly stained inclusions (Fig. 1 a) analogous to those observed in thin section electron micrographs (arrowed in Fig. 1 b). The percentage of cells showing cytoplasmic inclusions and diffuse granular fluorescence increased until day 4 when approximately 70% of the cells were positive. No cytopathic effects were observed and even dividing cells showed the presence of viral antigen by fluorescence (Fig. 1 a). On about day 12 post-inoculation, small nuclear inclusions were seen in up to 10% of cells (Fig. 2). These were
Short communication

Fig. 2. Patterns of immunofluorescence staining seen between 10 and 20 days post-infection of Vero cells. (a) Small nuclear inclusions (arrowed) in association with other types of fluorescence. (b) Nuclear fluorescence (arrowed) in the absence of cytoplasmic antigens. (c) Indirect immunofluorescence and combined phase contrast. Note the presence of brightly stained inclusions in the nucleus (arrowed), but not nucleolus (arrowhead). (d and e) Indirect immunofluorescent staining of isolated nuclei. Antibodies were (a to d) guinea-pig hyperimmune antiserum raised against purified virus and (e) monoclonal antibody PV1-1-3 specific for the N polypeptide of Pichinde virus. Bar markers represent 5 μm in (a), (b) and (c) and 2 μm in (d) and (e).

usually associated with cytoplasmic types of fluorescence although cells with only nuclear inclusions were also seen (Fig. 2b). Phase-contrast microscopy showed that the inclusions were not located in the nucleolus (Fig. 2c). During the next 8 days, nuclear inclusions were observed in a small proportion of the cells, but after day 20 post-inoculation, nuclear staining was no longer detected.

In order to confirm the nuclear location of virus-specific antigen, nuclei were isolated from Vero cells 10 days post-infection and examined by indirect immunofluorescence. Infected cells were collected in TKM buffer (0-01 M-Tris–HCl pH 7-7, 0-01 M-KCl, 0-01 M-MgCl2) and homogenized with 15 strokes in a Ten Brook grinder. The nuclei were pelleted (1000 g for 10 min), washed twice in 0-25 M-sucrose in TKM with 3 mM-CaCl2, and finally resuspended in 5 ml of 0-25 M-sucrose in TKM buffer. This preparation was then layered onto a discontinuous sucrose gradient (7 ml of 2 M-sucrose and 7 ml of 1-6 M-sucrose in TKM buffer with 3 mM-CaCl2) and centrifuged at 60000 g in an SW40 rotor for 30 min. The banded nuclei were collected, air-fixed on glass slides and stained by indirect immunofluorescence. Both polyclonal antisera (Fig. 2d) and monoclonal antibodies specific for the N protein (Fig. 2e) revealed the presence of virus-specific inclusions within the isolated nuclei.

Although intranuclear inclusions were specifically stained with monoclonal antibodies against the major nucleocapsid (N) protein (Fig. 3), not all anti-N monoclonal antibodies produced positive fluorescence under similar conditions (Table 1). The specificities of the antibodies reactive for the nuclear inclusions were therefore characterized by SDS–PAGE of viral proteins immunoprecipitated from infected cell extracts metabolically labelled with [35S]methionine from 44 to 48 h post-infection. The cells were washed three times with PBS and
Fig. 3. Pattern of immunofluorescence obtained with monoclonal antibody PV1-1-3 in infected Vero cells 10 days post-infection. Nuclear inclusions are arrowed in (a) and (b). Bar markers represent 5 μm.

Fig. 4. SDS-PAGE analysis of (a) purified Pichinde virus metabolically labelled with $^{35}$S methionine (Young & Howard, 1983) and (b, c) products of $^3$S methionine pulse-labelled Vero cell extracts prepared 48 h post-infection which were immunoprecipitated with monoclonal antibodies specific for the N polypeptide of Pichinde virus: (b) PV2-14-19 and (c) PV1-1-3.

then lysed with 1 ml of RIP buffer (50 mM-Tris-HCl pH 7.2, 0.5 M-NaCl, 0.1% Nonidet-P40, 1 mM-PMSF) per $5 \times 10^6$ cells. Cell debris was removed by centrifugation at 5000 g for 10 min and the supernatants were either used immediately or stored at $-20^\circ$C. All subsequent procedures were carried out at 4°C. Five μl of either monoclonal antibody or hyperimmune antiserum were added to 50 μl of Immunobeads (Bio-Rad) in microfuge tubes and placed on a rotary shaker for 2 h. The coated beads were then pelleted (3000 g for 2 min), resuspended in 250 μl of the cytoplasmic extract and incubated with mixing for a further 2 h. The beads were pelleted, washed six times in modified RIP buffer (50 mM-Tris–HCl pH 7.2, 0.5 M-LiCl, 0.1% SDS, 0.1% Nonidet-P40, 0.5 M-NaCl, 1 mM-PMSF) and the final pellet was resuspended in SDS–PAGE buffer (0.1 M Tris–phosphate buffer pH 6.7, containing 2% SDS, 1% 2-mercaptoethanol and 10% glycerol). The samples were solubilized for 2 min at 100°C, the beads pelleted and the supernatants separated on a 7.5% to 15% gradient slab gel. Gels were fixed, dried and exposed to RP X-Omat film in intensifying cassettes at $-70^\circ$C.

All antisera were found to precipitate the N polypeptide together with a protein of mol. wt. 36000 (p36) (Fig. 4). In addition, an N-related species with a mol. wt. of 28000 (p28) was precipitated by the hyperimmune sera and three of the monoclonal antibodies tested (PV1-1-3, PV1-1-7 and PV75-10b; Table 1). These antibodies produced both cytoplasmic and nuclear
fluorescent staining in infected cells. The monoclonal antibodies that did not recognize p28 showed only cytoplasmic fluorescence (PV2-14-19, PV3B-3 and PV75-22; Table 1). The presence of these N-related cleavage products in Pichinde virus-infected cell extracts has been documented previously and were referred to as NP38 and NP28 (Harnish et al., 1981). These workers analysed two-dimensional tryptic peptide maps of the two proteins and demonstrated that they contain a number of peptides in common both with the N polypeptide and with each other (D. G. Harnish, personal communication), suggesting that they are overlapping fragments. This is supported by the finding in the present study that monoclonal antibodies that recognize the p28 protein also precipitate p36 (Fig. 4). Clegg & Lloyd (1983) have also identified similar protein species in Lassa virus-infected cells and suggested that cleavage fragments of N arose predominately after detergent lysis and may therefore simply be artefacts of the preparative techniques employed. In our hands however, both Western blot and metabolic labelling experiments revealed the presence of the two polypeptides in Pichinde virus-infected cells harvested directly into PAGE disruption buffer.

Regardless of the status of p36 and p28 as cell-associated viral products, the monoclonal antibodies clearly recognized distinct epitopes on the N protein and differentiated between N-related species in terms of their localization within the infected cell. Whether the N-related nuclear antigen observed in the fluorescence studies was p28 itself or a conformational variant of N which presents an epitope that is recognized on p28 and not normally exposed on the native molecule, remains to be determined. A possible candidate for such a conformational variant could be the phosphorylated form of N which has been described for both lymphocytic choriomeningitis virus (Howard & Buchmeier, 1983; Bruns et al., 1986) and Tacaribe virus (Gimenez et al., 1983). A more comprehensive analysis employing a larger panel of monoclonal antibodies and involving immune precipitations of isolated nuclei is needed to resolve this question conclusively.

Recent studies have shown that the uptake and accumulation of proteins in the cell nucleus is facilitated by defined amino acid signal sequences (Dingwall, 1985; Feldherr, 1986; Goldfarb et al., 1986). Although the signals so far identified possess little direct sequence homology, they share certain characteristics which may reflect an underlying structural similarity (Richardson et al., 1986). The finding that a particular subset of monoclonal antibodies identifies an N-related species that does not accumulate in the nucleus could be the consequence of a tertiary structure which conformationally constrains such a sequence (Richardson et al., 1986). Cleavage of the N protein or a conformational change in N which exposes a putative nuclear location signal would therefore explain the translocation of only this species across the nuclear membrane.

Although nuclear inclusions were only observed between 10 and 20 days after infection, the p28 peptide could be detected in labelling studies as early as 10 to 15 h after infection (data not shown). The transient appearance of nuclear antigen might therefore suggest compartmentalization late in infection. However, an earlier nuclear involvement cannot be excluded, as positive fluorescence in this study required the accumulation of sufficient antigen to form visible inclusions. Hence, both the time course of appearance and the observation that only a minority of cells were found to show nuclear fluorescence may simply reflect a threshold in antigen detection. The subsequent disappearance of nuclear inclusions from infected cells may in turn be the result of a lower rate of protein turnover.

The appearance of nuclear inclusions in Pichinde virus-infected Vero cells confirms the nuclear involvement of a virus-specific product in arenavirus infections for the first time since Mims (1966) reported nuclear fluorescence in the cells of lymphocytic choriomeningitis virus-carrier mice. Furthermore, the use of monoclonal antibodies specific for the major nucleocapsid (N) polypeptide has identified the recognized antigen. Although the significance of an accumulation of an N-related product in infected cell nuclei remains to be determined, a functional role for an N polypeptide cleavage product in the regulation of Pichinde virus infections has been suggested previously (Dimock et al., 1982; Young et al., 1986). In these studies it was proposed that either the N protein or a protease-resistant cleavage product of N may inhibit the expression of the viral genome. The possibility that the localization of these
species in the nuclei of infected cells plays a role in the observed regulation is presently under investigation.

We would like to thank Dr M. J. Buchmeier (Scripps Clinic and Research Foundation, La Jolla, Ca., U.S.A.) for kindly providing us with some of the monoclonal antibodies. This work was supported by a grant from the British Medical Research Council.

REFERENCES


(Received 19 February 1987)