Cellular Localization of Five Structural Proteins of Sendai Virus Studied with Peroxidase-labelled Fab Fragments of Monoclonal Antibodies

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SUMMARY

By the use of horseradish peroxidase-labelled Fab fragments of monoclonal antibodies, five major structural components of Sendai virus, namely the nucleocapsid (NP), polymerase (P), matrix (M), fusion (F), and haemagglutinin–neuraminidase (HN) proteins were localized in infected Vero cells. The P and NP proteins were found in association with large clusters of ribosome-like particles and nucleocapsids in the cell cytoplasm. They were not concentrated at the cytoplasmic membrane, except in nucleocapsids within budding viral particles. F and HN proteins, on the other hand, were found in connection with ribosomes and endoplasmic reticulum in the cell cytoplasm, but not in nucleocapsids. Both proteins were evenly distributed on the outer cytoplasmic membrane and appeared on the surface clearly before budding of viral particles. The M protein was seen in connection with both nucleocapsids and ribosomes. It was not found at the cell surface except in budding viral particles.

INTRODUCTION

Monospecific antisera to different viral structural antigens can be produced by the hybridoma technique (Köhler & Milstein, 1975). Such monoclonal antibodies have been used to demonstrate the topographical appearance of measles virus components in infected tissue culture cells by immunofluorescence (Norrby et al., 1982). In a recent study a large number of monoclonal antibodies against every major structural protein of another paramyxovirus, Sendai virus, were prepared and serologically characterized. By the use of these antibodies, the topographical appearance of the different Sendai virus proteins in infected Vero cells was examined by immunofluorescence (Örvell & Grandien, 1982).

We now report the cellular localization of five major structural components of this virus, namely the haemagglutinin–neuraminidase (HN), fusion (F), nucleocapsid (NP), matrix (M) and polymerase (P) proteins by immunostaining of infected cells with peroxidase-labelled Fab fragments of antibodies directed against these antigens. The labelling with peroxidase (horseradish peroxidase; HRPO) permits an ultrastructural localization of the proteins. In addition, the fragments should penetrate more readily into the cells than the whole immunoglobulin molecules previously used (Lin et al., 1975).

METHODS

Infection procedure. The Z strain of Sendai virus grown in embryonated eggs was used. Vero cells were grown on coverslips in Leighton tubes and maintained in Eagle's minimal essential medium with 2% foetal calf serum. The medium was removed and the cultures were infected with Sendai virus at a m.o.i. of 10 p.f.u./cell in a volume of 0.2 ml. The tubes were rocked at 37 °C for 60 min, after which 2 ml of the same medium was added to each tube. Cultures were fixed every 2 h up to 12 h and then at 16, 20 and 24 h post-infection.

Preparation of peroxidase-labelled Fab fragments. Specific monoclonal mouse antibodies directed against the five

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major antigens (HN, F, M, NP and P) were prepared as described in detail previously (Örvell & Grandien, 1982). For immunolabelling, the antibodies selected were those that gave the strongest reaction in immunofluorescence experiments. To prepare Fab fragments, the antibodies were precipitated with an equal volume of saturated ammonium sulphate. After centrifugation the precipitate was dissolved in and dialysed against 0.1 M-sodium acetate buffer pH 4.0. The antibodies were incubated with pepsin (Sigma) (125 μg/5 mg antibodies) for 16 h at 37 °C. The preparation was dialysed against 0.3 M-Tris-HCl pH 8.2 overnight at 4 °C, reduced with 0.01 M-2-mercaptoethanol for 1 h at room temperature, alkylated with iodoacetic acid (final concn. 0.012 M) and dialysed against 0.01 M-sodium carbonate buffer pH 9.5 (Wilson & Nakane, 1978). The different preparations of Fab fragments were analysed by SDS–polyacrylamide gel electrophoresis.

The method of Wilson & Nakane (1978) was used for HRPO-labelling of the Fab fragments. Four mg HRPO (Sigma) was dissolved in 1 ml distilled water, 0.2 ml freshly prepared 0.1 M-NaIO₃ was added and the solution was stirred for 20 min at room temperature. The solution was dialysed against 1 mM-sodium acetate buffer pH 4.4 overnight at 4 °C. The pH was then increased to between 9 and 9.5 by adding 0.2 M-sodium carbonate buffer pH 9.5. The Fab fragment solution (5 mg) was added and stirred for 2 h at room temperature after which 0.1 ml freshly prepared sodium borohydride solution (4 mg/ml) was added. The mixture was kept at 4 °C for 2 h and then dialysed against phosphate-buffered saline (PBS) pH 7.4. The immunological reactivity of the labelled preparations was confirmed by enzyme-linked immunosorbent assays with Sendai virus-coated plates as described previously (Örvell & Grandien, 1982).

**Immunohistochemical technique.** The cultures were fixed in a mixture of 4% formaldehyde and 0.2% glutaraldehyde in PBS for 20 min at room temperature and then washed four times and stored in PBS at 4 °C until use. In order to allow penetration of labelled antibodies into cells, some cultures were treated with 0.1% Triton X-100 for 30 min at room temperature. The cultures were then incubated with the HRPO-labelled Fab fragments diluted 1:10 in PBS for 30 min at 37 °C and washed thoroughly in more than 10 changes of PBS containing 5% sucrose for 4 h. The cultures were then reacted with a mixture of 5 mg 3,3'- diaminobenzidine (Sigma) in 10 ml 0.05 M-Tris-HCl pH 7.5, and 0.1 ml 1% H₂O₂ for 3-5 min and finally washed in PBS.

The cells were removed from the glass slides with razor blades and centrifuged in small embedding capsules at 4200 rev/min for 10 min. The pellets were treated with 1% osmic acid for 2 h, dehydrated and embedded in Epon. Unstained 1 μm-thick sections were examined and areas for electron microscopy selected. Ultrathin sections were examined both unstained and stained with 5% uranyl acetate and 3% lead citrate.

**RESULTS**

At the light microscopical level, in accordance with the previous immunofluorescence study (Örvell & Grandien, 1982), the HRPO-labelled Fab fragments against NP, P and M proteins stained cytoplasmic inclusions varying in size from small granules to more confluent masses, while the Fab fragments against the HN and F glycoproteins displayed fine granular material in the cytoplasm and a sharp margination of the cell surface membrane.

Most electron microscopic examinations were performed on samples taken 10 h and 20 to 24 h after infection. At 10 h, F and HN proteins were evenly distributed on the outer surface of the whole plasma membrane (Fig. 1a). In Triton-treated cells, positive reaction products were seen in connection with ribosome-like granules around dilated endoplasmic reticulum-like structures. No budding virions were observed. The NP, P and M proteins were associated with aggregates consisting of free ribosome clusters and nucleocapsid-like tubular structures in the cytoplasm. The latter structures were clearly observed 16 h after infection. There was no staining of the cytoplasmic membrane with these antibodies. After 20 to 24 h numerous budding virions, which varied considerably in size, were observed at the cell surface and in the cytoplasm large aggregates of nucleocapsids occurred. The HN and F antibodies stained the surface of the budding virions as well as the whole cytoplasmic surface between the budding particles (Fig. 1b). In Triton-treated cells, the reaction product was seen at this time in artificially dilated cisternae of Golgi apparatus-like structures and endoplasmic reticulum surrounded by ribosome-like particles. There was no staining of the nucleocapsids either in the virions or in the cytoplasmic aggregates (Fig. 2). Both NP and P antibodies stained nucleocapsids heavily both in the virions and in the large aggregates (Fig. 3). There was no staining of the outer surface of the cytoplasmic membrane. Reaction product was also seen associated with nucleocapsids, endoplasmic reticulum and ribosomes after incubation with the M antibodies. There was no association of the antibody with the cytoplasmic membrane except in connection with budding viral particles where reaction product occurred at the inner surface of the cytoplasmic
Localization of Sendai virus antigen

(a)

Localization at the outer cytoplasmic surface of the F antigen at (a) 10 and (b) 20 h post-infection. Note the even distribution of the antigen at the surface prior to virus budding (a), and the staining of virus envelopes (b). Bar markers represent 0.5 μm.

(b)

DISCUSSION

Several biochemical studies of myxo- and paramyxovirus-infected cells have shown that the different viral structural components are associated with different subcellular structures (Hay, 1974; Lamb & Choppin, 1977; Nagai et al., 1976; Schwalbe & Hightower, 1982). In Sendai virus infections the two viral surface glycoproteins, HN and F, have, in pulse–chase experiments, been found to migrate from rough to smooth endoplasmic reticulum and then to the plasma membrane where they are incorporated into the budding virions. The P and NP polypeptides have been associated with rough endoplasmic reticulum and free polyribosomes, while the M protein has been found in varying amounts in all cytoplasmic fractions (Bowen & Lyles, 1982; Lamb & Choppin, 1977). In the present study marked differences in the intracellular localization of the proteins were also found. The NP and P antigens were associated with large inclusions of nucleocapsids and clusters of ribosomes, while the HN and F antigens were found in connection with structures at least partly associated with the rough endoplasmic reticulum and with the Golgi apparatus. Our results therefore independently confirm conclusions which previously have rested principally on biochemical fractionation studies.

The HN and F antigens appeared early on the cell surface, prior to viral budding, and were later also included in the envelope of the budding virions. This contrasts with earlier studies...
using ferritin-labelled antisera against type 2 parainfluenza virus, where labelling of the surface was seen only in connection with budding virions of infected HeLa and amnion cells (Howe et al., 1967). The NP and P proteins, on the other hand, were never found on the outer cell surface, but stained in the nucleocapsids of the budding viruses. There was no association of M antigens with the plasma membrane except at areas of budding virions. If this suggests that virions are released shortly after appearance of M at the cell membrane, it may explain the failure to detect margination of the cell membrane with antibodies against this component (Portner & Kingsbury, 1976; Bowen & Lyles, 1982).

The topographical appearance of the different Sendai virus antigens differs in some respects from that of measles antigen in Vero cells. Monospecific antibodies to measles NP antigen stain
Localization of Sendai virus antigen

Fig. 4. Distribution of the M antigen in the cytoplasm at 24 h post-infection. No staining of the cell surface membrane. Virions (a) and a budding virus-like structure (b) show positive reactions. Bar markers represent (a) 0.5 μm and (b) 0.25 μm.

Material in the nucleus in addition to the cytoplasm and the anti-M antibodies stained both intracytoplasmic material and cytoplasmic membrane structures (Norrby et al., 1982). The present technique using labelled monospecific antibodies should be useful for detailed observations on asymmetric intracellular transport of different viral proteins in polarized, differentiated epithelial cells, where different viruses may be released from different areas of the cell membrane (Boulan & Pendergast, 1980).

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REFERENCES


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