High Yield Growth and Purification of Human Parainfluenza Type 3 Virus and Initial Analysis of Viral Structural Proteins

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SUMMARY

Structural proteins from a large-plaque variant (LPV) of human parainfluenza type 3 virus were analysed by electrophoresis on Laemmli-type polyacrylamide gels. High virus concentrations were obtained by growth in BS-C-1 cells cultivated on microcarrier beads. Purification of the virus in composite equilibrium gradients of potassium tartrate:glycerol resulted in 25% recovery of input infectivity and a preparation containing <0.08% of input host cell protein and RNA. Parainfluenza type 3 virus equilibrated at a density of 1.20 g/ml in these gradients. Analysis by polyacrylamide gel electrophoresis of 3H-glucosamine-labelled virus taken from peak gradient fractions revealed 8 or 9 major virion peptides, ranging in mol. wt. from $17 \times 10^3$ to $125 \times 10^3$ ($17K$ to $125K$), two of which were glycoproteins. The sum of the estimated mol. wt. of these peptides, $501.5K$ to $570.5K$, does not exceed the estimated genomic potential of other paramyxoviruses.

INTRODUCTION

Human parainfluenza 3 virus is the causative agent of serious lower respiratory tract infections in infants and less serious upper respiratory tract infections in adults (for review, see Gelzen et al., 1976). Despite its importance as a pathogenic agent, little is known of its molecular composition although recent progress has been made on antigenically related bovine parainfluenza 3 virus (Shibuta et al., 1979).

Parainfluenza viruses are members of the Paramyxoviridae family and, as such, have biological properties consistent with closely related viruses such as Sendai virus, SV-5 and Newcastle disease virus (NDV). These viruses contain 6 to 8 structural proteins (Mountcastle et al., 1971) two of which are glycoproteins (Klenk et al., 1970; Scheid & Choppin, 1973, 1974). The structural proteins responsible for the biological activities of these viruses have been identified (for review, see Choppin & Scheid, 1980). The fusion (F) and the haemagglutinin/neuraminidase (HN) glycoproteins of Sendai virus, SV-5 and NDV are responsible for initiation of infection and haemagglutination and neuraminidase activities respectively (Scheid et al., 1972; Tozawa et al., 1973; Homma & Ohuchi, 1973; Nagai et al., 1976a; Hsu et al., 1979). Although these activities have been demonstrated in human parainfluenza 3 virus (Chanock et al., 1963), the virion components responsible for them have not been identified.

This paper presents methods for obtaining parainfluenza 3 virus in relatively high concentrations, a means of isolating and purifying the virus and initial examination of the purified viral structural proteins by polyacrylamide gel electrophoresis (PAGE). Results indicate that human parainfluenza 3 has 8 or 9 structural proteins, two of which are glycoproteins.
METHODS

Cells and virus. BS-C-1 cells (obtained from Dr D. Nathans, Johns Hopkins University, Baltimore, Md., U.S.A.) were routinely cultured as monolayers in 32 oz prescription bottles with reinforced Eagle’s medium (REM; see Bablanian et al., 1965) also containing 100 μg gentamicin/ml (Sigma) and supplemented with 5% foetal bovine serum (FBS). These cells provided the seeding concentrations for Corning 60 mm plastic dishes (1 x 10^6 cells/dish) or for microcarrier (super) beads (Flow Laboratories) at 1 x 10^6 cells/0.5 g beads. Cells cultivated on microcarrier beads were enumerated by counting nuclei as described by Levine et al. (1977). Human epithelial (HEp-2) cells cultivated as monolayers were adapted to grow in suspension as shaker cultures using a New Brunswick G-25 gyratory shaker. Suspension cultures were routinely maintained at concentrations of 0.5 x 10^6 to 1 x 10^6 cells/ml in 250 or 500 ml screw-cap Erlenmeyer flasks in Waymouth’s MB 752/1 medium supplemented with 5% FBS.

Human parainfluenza 3 virus was kindly provided by Dr R. W. Simpson (Waksman Institute, Rutgers University, New Brunswick, N.J., U.S.A.). The identity of this virus was independently confirmed by Dr G. Sedmak (City Health Laboratories, Milwaukee, Wis., U.S.A.). A large-plaque variant (LPV) isolated and cloned from preparations of parainfluenza 3 virus was used for this study. Seed stocks of the LPV of parainfluenza 3 virus were grown in BS-C-1 cells in REM with 5% FBS. Virus for experiments was grown in BS-C-1 or HEp-2 cells. Before infection, cells were washed with Mandel’s (1958) modified Hanks’ balanced salt solution (BSS), and subsequently inoculated with virus at an m.o.i. of 1 p.f.u./cell. After a 90 min adsorption period at 36 °C, infected cells were resuspended in appropriate volumes of serum-free Waymouth 752/1 or REM media and incubated at 36 °C for 48 to 60 h. Before the onset of demonstrable cytopathic effect (c.p.e.), culture fluids were clarified of cellular debris and/or microcarrier beads by sedimentation at 2000 g for 20 min at 4 °C. Supernatant fluids from microcarrier bead cultures were further processed by filtration through three layers of sterile nylon hose. The clarified supernatant fluids were immediately processed for virus isolation and purification.

Preparation of labelled virus. Parainfluenza 3 virus was labelled by exposing BS-C-1 cells, infected with an m.o.i. of 10 p.f.u./cell, to 10 pCi 6-3H-glucosamine (ICN, Irvine, Calif., U.S.A.; lot no. 892373; 8.5 Ci/mmol) per ml serum-free minimum essential medium (MEM) containing 10 mM-fructose instead of glucose (Nagai et al., 1976b). After 60 h at 36 °C, culture fluids were harvested and virus isolated as described below.

Virus assays. Infectivity was determined by plaque assay in BS-C-1 cells. Virus suspensions were diluted in BSS containing 0.5% (w/v) gelatin (GBSS), and 0.2 ml from selected dilutions was added in duplicate to preformed BS-C-1 cell monolayers in 60 mm plates. After a 60 to 90 min adsorption period at 36 °C, cells were overlaid with 7 ml MEM containing 0.7% (w/v) agarose (Type V, Sigma). On the 5th or 6th day of incubation, plaques were visualized by adding 2 ml of a 0.035% (w/v) neutral red solution in 0.8% (w/v) NaCl, and counted after an additional 12 to 18 h of incubation at 36 °C.

Haemagglutination assays were performed in 13 x 100 mm tubes by the method of Dick & Mogabgab (1962) using human type O red blood cells and incubated at 4 °C for 2 h before taking readings.

Virus concentration and purification. To avoid potential inactivation, samples were not frozen before or during the following purification protocol which was performed within a 24 h period. Supernatant cell culture fluids were clarified by centrifugation at 2000 g for 20 min at 4 °C. When infected cell culture vol. exceeded 100 ml, virus was initially concentrated using an Amicon model DC 2 hollow fiber dialyzer/concentrator equipped with an HIP 100 hollow fiber cartridge (Amicon, Lexington, Mass., U.S.A.). The concentrate (approx. 100 ml) was layered over a 2 ml 50% (w/w) potassium tartrate cushion in TE
buffer (25 mM-tris pH 7.2, 10 mM-EDTA) and centrifuged at 131000 g for 90 min at 4 °C in a Beckman SW27 rotor. Cushioned virus was collected, diluted with 3 vol. TE buffer and subjected to equilibrium centrifugation in composite 50% (w/w) potassium tartrate:30% (w/w) glycerol (KT:GLY) gradients made in TE buffer (Obijeski et al., 1974). Centrifugation was performed in a Beckman SW41 rotor at 208000 g for 18 h at 4 °C. Gradient fractions of 0.75 ml were collected by displacement with an equal volume of mineral oil pumped on to the top of the gradient tube. Aliquots of each fraction were analysed for fraction density (by direct weight), infectivity, haemagglutination and radioactivity (if appropriate).

Labelling of host cell macromolecules. Monolayer cultures of BS-C-1 cells (9 x 10⁷ total cells) grown in 32 oz prescription bottles were labelled essentially as described by Klenk & Choppin (1969). Cells were grown until confluent in REM medium (5% FBS) containing 0.5 μCi of a 14C-amino acid mixture/ml (ICN; lot no. 874071; 1.87 mCi/mg) or 10 μCi 5-3H-uridine/ml (RPI, Elk Grove Village, Ill., U.S.A.; lot no. 379; 27 Ci/mmol). After 2 days, the growth media were replaced with serum-free reconstituted MEM containing either standard concentrations of amino acids and 10 μCi 5-3H-uridine/ml or 1/10 the standard concentration of amino acids and 0.5 μCi 14C-amino acid mixture/ml. After 3 more days at 36 °C, labelled media were removed from the cells and mixed with an equal volume of unlabelled, infected culture fluids before virus isolation, as described in the text.

Protein assay and gel electrophoresis. Protein concentrations of virus samples taken from stages of purification were estimated with the Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Richmond, Calif., U.S.A.), using bovine serum albumin as a standard. Purified LPV virus was disrupted in an SDS/β-mercaptoethanol sample buffer and processed for electrophoresis according to the method of Laemmli (1970). The running gels were 10% polyacrylamide. After electrophoresis, the gels were stained with Coomassie brilliant blue, destained and scanned for absorbance at 580 nm with an ISCO gel scanner/UA-5 absorbance monitor. Radioactively labelled gels were sliced into 1 mm segments in preparation for radioactive analysis. Mol. wt. of viral structural proteins were estimated from the SDS–polyacrylamide gels by the methods of Weber & Osborne (1969), using Dalton VI (Sigma) and SDS–PAGE 40 x 10⁴ to 250 x 10³ (40K to 250K) mol. wt. standards (Bio-Rad Laboratories) as markers.

Radioactivity analysis. The amount of 3H and 14C activities in single samples was determined by precipitating 100 μl amounts of sample in the presence of 100 μg quantities of bovine serum albumin with 5 ml 10% (w/v) trichloroacetic acid (TCA). The precipitate was developed overnight at 4 °C, and collected on 2.4 cm GF/C glass fibre filters (Whatman). Filters were washed three times with 5 ml vol. cold TCA followed by two washes with 5 ml ether. Filters were dried for 30 min in glass scintillation vials at 120 °C and counted in 10 ml Liquifluor (New England Nuclear) with a Beckman LS-150 liquid scintillation counter. Values were adjusted for 14C and 3H spillover into the 3H and 14C channels respectively.

To determine 3H-glucosamine incorporation, gel segments were transferred to glass vials, digested overnight at 60 °C in 0.5 ml of a 9:1:10 mixture of protosol:water:toluene, and counted in Liquifluor. Protosol was from New England Nuclear.

RESULTS

Replication of parainfluenza 3 virus in HEp-2 and BS-C-1 cells

An experiment was designed to examine the kinetics (efficiency) of parainfluenza 3 virus replication in suspension and monolayer cultures of HEp-2 cells or microcarrier bead cultures.
of BS-C-1 cells. Cells were infected as described above and cultivated in serum-free REM. Sample aliquots were removed at indicated intervals (Fig. 1), and analysed by plaque assay. Results indicated that the latent period was approx. 6 h in cells cultured as monolayers and somewhat less in HEp-2 cell shaker cultures. Maximum virus yields (850 p.f.u./cell) were obtained at approx. 30 h post-infection from microcarrier bead cultures of BS-C-1 cells.

**Degree of parainfluenza 3 purity from host cell components**

An experiment similar to that described by Klenk & Choppin (1969) was designed to determine the degree of potential cellular contamination in the final viral preparations. Uninfected BS-C-1 cells were continuously labelled for 5 days with either 14C-amino acids or 5-3H-uridine. Culture fluids from the labelled cells, containing unincorporated label as well as labelled cellular macromolecules, were mixed with an equal volume of unpurified parainfluenza 3 virus grown in BS-C-1 cells just before purification. Samples were removed at each purification step for analysis of infectivity, haemagglutination and amount of acid-precipitable 14C and 3H activities. Analysis of fractions from samples subjected to composite KT:GLY gradients is shown in Fig. 2. One visible homogeneous band was seen in these gradients. Peaks of infectivity and haemagglutination were coincident and equilibrated at a density of 1.20 g/ml. The amount of acid-precipitable, cellular-specific 14C (protein) and 3H (RNA) activity in each fraction was less than 0.08% of that in the mixed culture fluids before purification. Recovery of purified virus and its degree of purity at each purification step are indicated by the data in Table 1. The constant ratio of haemagglutinating units (HAU)/p.f.u. at each step in the purification indicates that (i) virus is not disproportionately inactivated at any step of purification, and (ii) there is no preferential concentration of defective or non-defective virus particles during the procedures.
Fig. 2. Parainfluenza 3 virus purification on a composite potassium tartrate:glycerol (KT:GLY) gradient. Virus concentrated on to potassium tartrate cushions was diluted and centrifuged to equilibrium in composite KT:GLY gradients as described in Methods. Gradients were fractionated, and density, infectivity (○) and haemagglutination (●) were determined for each fraction. Culture fluids from uninfected cells labelled with \(^{14}\)C-amino acids and \(^{3}\)H-uridine were mixed with an equal volume of infected BS-C-1 cell culture fluids. Residual \(^{14}\)C (□) and \(^{3}\)H (▲) counts in acid-precipitable aliquots from each fraction were determined and compared to acid precipitable counts in the mixed culture fluids before purification.

Table 1. Recovery of infectious parainfluenza 3 virus during the steps of purification

<table>
<thead>
<tr>
<th>Purification step</th>
<th>log(_{10}) Virus (HAU)</th>
<th>Total titre (p.f.u.)</th>
<th>Total protein (mg)</th>
<th>Sp. act. (log(_{10}) p.f.u./mg protein)</th>
<th>log(_{10}) Total ct/min</th>
<th>(^{3})H (RNA)</th>
<th>(^{14})C (protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purification input</td>
<td>4.59</td>
<td>10.88</td>
<td>31.76</td>
<td>9.38</td>
<td>7.14</td>
<td>6.57</td>
<td></td>
</tr>
<tr>
<td>Clarification</td>
<td>4.57</td>
<td>10.86</td>
<td>31.90</td>
<td>9.36</td>
<td>7.08</td>
<td>6.32</td>
<td></td>
</tr>
<tr>
<td>Hollow fibre concentration</td>
<td>4.62</td>
<td>10.64</td>
<td>8.04</td>
<td>9.73</td>
<td>6.51</td>
<td>5.80</td>
<td></td>
</tr>
<tr>
<td>K-tartrate cushion</td>
<td>4.61</td>
<td>10.77</td>
<td>1.47</td>
<td>10.60</td>
<td>5.27</td>
<td>4.57</td>
<td></td>
</tr>
<tr>
<td>Pooled gradient peaks</td>
<td>3.98</td>
<td>10.28</td>
<td>0.25</td>
<td>10.88*</td>
<td>3.50</td>
<td>3.43</td>
<td></td>
</tr>
</tbody>
</table>

* Represents a 32-fold purification.

Structural proteins of parainfluenza 3 virus

Virus was grown in BS-C-1 cells infected at an m.o.i. of 10 p.f.u./cell. Radioactive 6-\(^{3}\)H-glucosamine was included in the growth medium to detect newly synthesized viral structural glycoproteins. Purified virus, isolated from peak gradient fractions, was analysed
Fig. 3. Electrophoretic profile of Coomassie brilliant blue-stained and $^3$H-glucosamine-labelled structural proteins of parainfluenza 3 virus. A virus preparation labelled with $^3$H-glucosamine was purified as described in Methods. Purified virus was collected by centrifugation at 131 000 g for 90 min and the pellet resuspended in 9 mM-phosphate buffer. An aliquot was denatured and subjected to PAGE on 10% Laemmli-type gels. Mol. wt. markers were run simultaneously on a separate gel (inset). Protein bands were resolved by scanning the stained gels at 580 nm (---) and liquid scintillation counting of gel slices (○). The mol. wt. markers were: myosin, 200K; β-galactosidase, 130K; phosphorylase B, 94K; bovine serum albumin (BSA), 67K; ovalbumin, 44K; pepsin, 34.7K; trypsinogen, 24K; β-lactoglobulin, 18K; lysozyme, 14.3K. Arrows in the inset indicate the mol. wt. estimates of the major virion polypeptides. HMW, High mol. wt. material.

by electrophoresis on Laemmli-type polyacrylamide gels. Eight distinct peaks within the linear range of the mol. wt. markers were resolved by scanning gels stained with Coomassie brilliant blue (Fig. 3). The gels were subsequently sliced and the amount of $^3$H-glucosamine incorporated into each fraction was determined. This pattern is superimposed on the gel scan. Two major glycosylated proteins were resolved. One glycosylated protein appeared to co-migrate with the structural protein present in greatest amounts. By comparison to mol. wt. marker proteins (Fig. 3, inset) run simultaneously on a second gel, the estimated mol. wt. of parainfluenza 3 structural proteins were: 1, 125K; 2, 88K; 3, 69K; 4, 59.5K; 5, 55K; 6, 49K; 7, 39K; and 8, about 17K. Additional high mol. wt. (HMW) proteins or aggregates (>125K) were also seen on the gels. The two prominent glycoproteins migrate with the 69K and the 55K peaks stained by Coomassie brilliant blue. The possibility that there may be both a glycosylated and a non-glycosylated protein co-migrating at 69K is discussed below.
Parainfluenza 3 virus structural proteins

Table 2. Comparison of paramyxovirus structural proteins

<table>
<thead>
<tr>
<th>Virus</th>
<th>Number of structural proteins</th>
<th>Mol. wt. range (× 10^{-9})</th>
<th>Active glycoproteins</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-PI-3*</td>
<td>8</td>
<td>17-125†</td>
<td>2±</td>
<td>55; 69</td>
</tr>
<tr>
<td>B-PI-3§</td>
<td>5</td>
<td>55-79</td>
<td>2</td>
<td>55; 69</td>
</tr>
<tr>
<td>SV-5</td>
<td>7</td>
<td>41-76±</td>
<td>2</td>
<td>56; 67</td>
</tr>
<tr>
<td>NDV</td>
<td>8</td>
<td>41-74±</td>
<td>2</td>
<td>56; 74</td>
</tr>
<tr>
<td>Sendai</td>
<td>8-9</td>
<td>39-160</td>
<td>2</td>
<td>48; 72</td>
</tr>
</tbody>
</table>

* Human parainfluenza 3 virus.
† Exclusive of high mol. wt. components in excess of 130K.
‡ The activities of these glycoproteins have not been ascertained.
§ Bovine parainfluenza 3 virus.

DISCUSSION

We have successfully applied a growth and purification protocol for isolation of biologically active human parainfluenza virus. The methodology used provided sufficient material for resolution of virion-associated proteins. The success of this method is attributed to three factors: (i) the use of microcarrier bead cultures of BS-C-1 cells to provide high-titre virus preparations in reduced volumes of culture media, allowing for economy and higher specific activities when labelling virus with isotopic precursors; (ii) the use of composite KT:GLY gradients which proved superior to sucrose and diatrizoate gradients in maintaining virus infectivity (L. E. Guskey & G. Bergtrom, unpublished data); and (iii) the relative brevity of this protocol which enabled virus purification without intermittent freeze–thawing of the preparation. These factors reduced problems of low yield and extreme lability that are inherent in studies of human paramyxoviruses (Kingsbury, 1977). A modified protocol of this parainfluenza 3 virus purification scheme may be applicable to other closely related human viruses (e.g. parainfluenza types 1 and 2 and respiratory syncytial viruses).

The data on the structural protein composition of the LPV strain of parainfluenza 3 virus compared favourably with those collected for other paramyxoviruses (Table 2). The mol. wt. range of the virus structural proteins was similar to that of other paramyxoviruses. One prominent structural protein, the nucleocapsid (NP) protein found in bovine parainfluenza 3 virus, NDV, Sendai virus and SV-5 viruses ranged from 56K to 68K (Mountcastle et al., 1971; Shibuta et al., 1979) and was not glycosylated. The prominent protein in parainfluenza 3 virus had an estimated mol. wt. of 69K and appeared to co-migrate with a glycosylated protein. We suggest that there are two proteins with a mol wt. at or close to 69K, only one of which is glycosylated. While we have not identified the parainfluenza 3 NP protein, the NP protein of NDV has been shown to co-migrate with glycoprotein on denaturing gels under reduced conditions (Mountcastle et al., 1971). Like other paramyxoviruses, parainfluenza 3 virus appears to contain two major glycoproteins. The estimated mol. wt. of these glycoproteins were identical to or closely approached those reported for other paramyxoviruses.

The 17K protein (no. 8 in Fig. 3) migrated with or near a heterogeneous peak of glycosylated protein. Although similar material has been reported on gels of SV-5, NDV, Sendai virus and bovine parainfluenza 3 virus structural proteins, the authors did not include this material in their cumulative mol. wt. calculations (Scheid & Choppin, 1974; Nagai et al., 1976b; Shibuta et al., 1979). Recent evidence, however, indicates that this material results from reduction of disulphide bonds in the F₁₂ disulphide-linked complex of the
fusion glycoprotein (Scheid & Choppin, 1977). This cleavage results in separation of a heavy from a light polypeptide chain on PAGE. Since our gels were run under reducing conditions, the low mol. wt. heterogeneous glycoprotein material we resolved may represent a product of disulphide bond cleavage. Some high mol. wt. material (greater than 125K) appeared to be glycosylated and may result from non-specific aggregation (Mountcastle et al., 1971; Lamb & Mahy, 1975). Assuming such aggregation and the co-migration of a glycosylated and a non-glycosylated protein at 69K, parainfluenza 3 virus appears to contain 9 virion proteins with a combined mol. wt. of 570 500. This figure does not exceed the estimated genomic potential of other paramyxoviruses (600000 to 700000 mol. wt. protein).

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Parainfluenza 3 virus structural proteins


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