The Fate of Protein Subunits of Parainfluenza (Sendai) Virus after Adsorption to NIL8 Hamster Embryo Cells

By A. KOHN*

Imperial Cancer Research Fund, London WC2A 3PX, U.K.

(Accepted 16 July 1975)

SUMMARY

Adsorption of u.v.-inactivated Sendai virus on to NIL8 hamster cells causes fusion of the cells into polykaryocytes within 2 h. 'Infected' cells were incubated at 37 °C for periods of 10 min to 8 h and their surface proteins iodinated with [\(^{125}\)I] catalysed by peroxidase. Structural components of the viral envelope, such as haemagglutinin-neuraminidase (HN) and probably also the fusion protein (F) were detected in the cell membrane for periods up to 4 h post infection.

INTRODUCTION

Following the adsorption of some enveloped viruses to animal cells, the viral envelope is believed to fuse with the cellular membrane (Drzeniek, Saber & Rott, 1966; Morgan & Howe, 1968; Heine & Schnaitman, 1969; Zee & Talens, 1971; Apostolov & Poste, 1972; Armstrong, Metz & Young, 1973; Bach, Aguet & Howe, 1973). This incorporation can be demonstrated by haemadsorption tests (Kashiwazaki, Homma & Ishida, 1965; Watkins, 1965), or by labelling the 'infected' cells with antibodies, coupled either to ferritin (Dales, Gomatos & Hsu, 1965), fluorescein (Breitenfeld & Schaefer, 1957; Kashiwazaki et al., 1965), or peroxidase (Singer, 1959) which bind to the viral antigens in the cellular membrane. However, the fate of the viral antigens of u.v.-inactivated viruses following adsorption to animal cells has not been studied.

In this communication we show by peroxidase catalysed iodination (Stanley & Haslam, 1971; Hubbard & Cohn, 1972; Hynes, 1973; Owen, Knight & Thomas, 1973; Walter & Mudd, 1973) that some antigens in the envelope of u.v.-inactivated Sendai virus are incorporated into the cell membrane and persist there for at least 4 h following adsorption and incubation at 37 °C. The enzyme lactose peroxidase, which cannot permeate the plasma membrane, may be used to catalyse the radiiodination (\([^{125}\)I]) of tyrosine residues exposed on the outer cell surface of animal cells. When such cells, with or without viral antigens, are then dissolved in SDS and analysed by electrophoresis on polyacrylamide gels, the presence or absence of viral antigens in the cell membrane can be demonstrated.

METHODS

Cells. Cells from a clone of hamster fibroblasts NIL8 (obtained from Dr I. McPherson, Imperial Cancer Research Fund, London), were grown in monolayers in Eagle's medium (as modified by Dulbecco) with 10 % calf serum.

* Permanent address: Israel Institute for Biological Research, Ness Ziona, Israel.
Fig. 1. Purification of Sendai virus by zonal sedimentation in sucrose gradient. Virus was grown for 72 h in 10-day-old chick embryos and collected as allantoic fluid. Cell debris and membranes were removed by slow centrifuging (1000 g). Sucrose was added to 80 and 200 ml portions of the allantoic fluid to give 35 and 20 % solutions respectively. The solutions were fed into the rotor of zonal centrifuge MSE running at 2000 rev/min, as follows: 145 ml of sucrose 55 %, 80 + 200 ml of the 35 and 20 % sucrose-allantoic fluid, 100 ml of allantoic fluid without sucrose, and 100 ml PBS. Centrifuging was then continued at 45000 rev/min for 3-5 h. Thirty ml fractions were collected and monitored by measuring the extinction in a spectrophotometer at 260 nm, and by the haemagglutinin content. Fractions from two batches containing the virus were pooled (100 ml), diluted in PBS to 200 ml, and re-run in the zonal centrifuge as above in a step gradient consisting of 125 ml of 55 % sucrose, 250 ml of 30 % sucrose, 200 ml of 20 % sucrose+virus, and 50 ml PBS. The collected gradient is shown in the Figure. The thick line represents the extinction at 260 nm, the thin line the refractive index. The virus peak appears at 38 to 43 % sucrose.

Virus. Parainfluenza 1 (Sendai) virus (kindly supplied by Dr A. C. Allison, Clinical Research Centre, Harrow) was grown for 72 h in 10-day-old chick embryos and collected as allantoic fluid. After removal of cell debris and membranes by centrifuging at 1000 g, the virus was concentrated directly from the allantoic fluid by 2-step zonal centrifuging in a 20 to 55 % sucrose gradient, as shown in Fig. 1 (modified procedure of Chucholowius & Rott, 1972). Sucrose was then removed from the virus suspension, which was at the same time concentrated by vacuum dialysis against phosphate buffered saline (PBS), and stored at –70 °C until use. The final suspension contained 32000 H.A.U./ml. In the experiments 1 ml of the thawed virus suspension containing 5000 H.A.U. was irradiated by u.v. light at 3500 ergs/cm²/s for 1 min.

Virus iodination (Hynes, 1973). The purified virus was iodinated with [¹²⁵I] by adding 0.5 ml of Na[¹²⁵I] (200 µCi; Radiochemical Centre, Amersham, Buckinghamshire), 5 mM-glucose, 0.01 ml glucose oxidase and 20 µg/ml of lactoperoxidase to 1 ml of stock Sendai virus (total H.A.U. = 32 000). After 10 min at room temperature the reaction was stopped by the addition of cold phosphate buffered sodium iodide (PBI). The iodinated mixture was then placed in centrifuge tubes over a layer of suspension buffer and centrifuged at 11 000 rev/min in a Sorvall centrifuge (Model RC2-B) for 1 h. The supernatant fluid and the separating buffer were discarded and the pellet resuspended in 200 µl of suspension buffer and 25 µl of 20 % SDS.
**Sendai virus antigens on cell surface**

*Cell: virus interaction.* The medium was removed from almost confluent monolayers of NL8 cells and replaced by 0.3 ml of a suspension containing 1600 H.A.U. of u.v.-inactivated Sendai virus. In controls, 0.3 ml of PBS was added instead of the viral suspension. The virus-treated monolayers were incubated at 37 °C for 10 to 30 min; thereafter, the cells were washed with PBS to remove the unattached virus, and fresh, pre-heated medium was added to the dishes. The cells were then incubated for periods varying from 1 to 22 h. For tests with incubation times shorter than 30 min, the monolayers were processed without replacement of fresh medium. After appropriate incubation periods the cells were washed 3 times with PBS and divided into 3 groups. The cells in one group were labelled with $[^{125}I]$ as follows: after one wash with PBS + 5 mM-glucose, 0.5 ml of carrier free Na$[^{125}I]$ (400 µCi/ml) was added to monolayers and the reaction initiated by addition of 20 µg/ml of lactoperoxidase (Calbiochem, EC 1.11.1.7) and 0.1 units/ml of glucose oxidase (Worthington Chemical Corp., EC 1.1.3.4). The labelling was allowed to proceed for 10 min at room temperature after which it was stopped by addition of 5 ml of PBI and 2 mM-phenylmethylsulphonyl fluoride (PMSF). The medium was then removed and the cells washed twice with the same buffer (PBI + PMSF). The cells were scraped off into the same medium and centrifuged in 15 ml conical tubes through a layer of 10 % glycerol in buffer to separate the cells from the soluble enzyme and the unbound iodine. The pellet obtained was dissolved in 250 µl of a buffer containing 2 % SDS and PMSF. The radioactivity of 5 µl samples was measured in a gamma counter, and the protein content determined (Lowry *et al.* 1951). The samples were then reduced by boiling for 2 min in the presence of 0.1 µ-m-dithiothreitol. A suitable volume (normalized for radioactive count or for protein content) was diluted in suspension buffer containing bromphenolblue to required concentrations. Samples (5 to 50 µl) were deposited on top of slab polyacrylamide gels (7.5 ; Maizel, 1971), and run at 50 V for 1 h till the blue marker left the stacking gel. The current was then increased (at 10 V/cm) and the electrophoresis continued till the marker reached the bottom of the gel. The slabs were stained, destained, washed, dried on filter paper *in vacuo* and autoradiographed on Kodirex X-ray film for 5 to 10 days.

**Evaluation of autoradiographs.** Autoradiographs were scanned by a densitometer. The relevant peaks of the densitometric tracing were cut out and their weights used for construction of curves indicating changes in amount of viral antigen with time.

**Fusion control.** The cells in the second group were used for fusion control. After 30 min adsorption in PBS at 37 °C, the medium was replaced on the washed monolayers and the cells incubated for a further 2 h. The monolayers were washed, fixed in alcohol, dried and stained with Giemsa. The degree of fusion was assessed microscopically (Kohn, 1965). In some experiments, the presence of viral haemagglutinins on the cells was detected by haemadsorption tests (Marcus, 1962). Incubation in the cold with 3 ml of chick red blood cells (2 x 10⁸ cells/ml) for 20 min was followed by gentle rinsing 5 to 6 times with cold PBS (pH = 6) before microscopic examination.

**Electron microscopy.** The cells in the third group were processed for electron microscopy: After adsorption and incubation either at 4 °C (adsorption controls) or at 37 °C, the cells were washed with PBS to remove the unattached virus, and treated with rabbit anti-Sendai antiserum absorbed with chorioallantoic membranes. After 20 min incubation, the cells were washed 3 times in PBS and the monolayers covered with 0.3 ml of ferritin-conjugated anti-rabbit donkey serum for 20 min. Thereafter cells were washed again with PBS and fixed in 1.5 % glutaraldehyde in Soerensen buffer (pH = 7.73), scraped off the dish, pelleted by centrifuging and processed for electron microscopy.
Fig. 2. Electropherograms of $[^{125}I]$-iodinated Sendai virus on a 7.5% polyacrylamide-SDS gel (sample = 10 µl of virus containing 16000 H.A.U./ml). Strips A and C are photographs of gels stained with Coomassie blue, strip B is an autoradiograph of the same gel. A = BSA marker (69000 daltons); B and C = Sendai virus.

RESULTS

The electrophoretic pattern of iodinated virus is shown in Fig. 2. The polypeptide pattern in the electrophoretic gel (stained with Coomassie blue) is shown next to the autoradiographic pattern of the same gel. In stained gel the main identified viral polypeptides are clearly discernible (Content & Duesberg, 1970; Mountcastle, Compans & Choppin, 1971; Stanley & Haslam, 1971). Using the nomenclature of Scheid & Choppin (1974) they are: P, HN, NP, F and M. In addition two larger polypeptides of mol. wt. about 120000 and 140000 are present (the mol. wt. estimated by extrapolation from BSA and ovalbumin markers; Shapiro, Vinuela & Maizel, 1967) which might be aggregates of the smaller viral proteins. In autoradiographs, however, the band P (polymerase) is missing and the NP (nucleoprotein) band appears only faintly. Since iodination labels only proteins available on the surface of intact virions, the presence of labelled NP indicates that the envelopes of some of the purified virions broke and released NP. The most strongly labelled polypeptides are HN (haemagglutinin-neuraminidase) estimated to be 74000 daltons (Tozawa, Watanabe & Ishida, 1973; Scheid & Choppin, 1974), and the fusion glycoprotein F (47000 to 53000 daltons).
Sendai virus antigens on cell surface

[125I]-labelled polypeptides of Sendai virions were electrophorized in slab gels along with preparations of cell iodinated at various times after addition of u.v.-inactivated virus. A very prominent viral protein, HN, was labelled by iodine at various times in the cells that adsorbed the virus (Fig. 3 and 4). This antigen was also clearly discernible in cells adsorbing the virus in the cold (when virions remain adsorbed on the outside of the cells), but not in uninfected, control cells without the virus. Another viral protein which was available for iodination on the infected cells was the F protein. The HN and F bands were seen in autoradiographs of cells incubated with Sendai virus at 37 °C for 10 min to 4 h. The quantity of these antigens, iodinated on the surface of the cells (Fig. 4), decreased progressively to an almost undetectable level after 4 to 8 h (Fig. 5). Light microscopic observations showed that after 1-5 to 2 h of incubation at 37 °C most of such cells in the monolayers fused into giant, polynuclear cells. It seems that the presence of the HN band after incubation of ‘infected’ cells at 37 °C cannot be ascribed to the presence of free virions on the surface, since in electron micrographs of cell sections (labelled by ferritin-tagged gamma globulin antibody against Sendai) no such virions were observed. This result, however, might be due to insufficient sensitivity of this method. Moreover, haemadsorption tests on such cells were also negative. When infectious virions were used instead of the u.v.-inactivated virus, the iodinated band HN was observed on the cell surface 22 h after infection (data not shown) possibly indicating the reappearance of budding progeny virions on the cell surface.

**DISCUSSION**

During the adsorption of paramyxoviruses on to the host cell membranes, there is considerable membrane activity that may be observed by time-lapse cinematography. One might therefore expect that any antigen incorporated from the outside into the cell membrane...
Fig. 4. Time course of incorporation of u.v.-inactivated Sendai virus antigens into the membranes of NIL8 cells. NIL8 cells were iodinated with $^35$S at various times after addition of 1600 H.A.U. of Sendai virus. SDS digested samples of the infected cells were separated electrophoretically on a 7.5% polyacrylamide gel. (a) Gel stained with Coomassie blue only, showing fractionated proteins of uninfected cells (C), BSA marker (A) and Sendai virus alone (V). (b) Autoradiographs of the same gel. Abscissa represents time after adsorption. C = uninfected cell control; V = Sendai virus alone. Time 0 refers to addition of $[^35]$S to cells immediately after the virus (about 30 s).

would be either redistributed over the cell surface, or lost (to the inside of the cell by pinocytosis, or to the outside by shedding). If the fate of the antigen were redistribution only, one would not expect any gross changes in the amount of antigen available for iodination from without; in the event of pinocytosis or shedding, however, the antigen would gradually disappear from the surface. The results of the present experiments indicate that the second alternative is the more probable, since the amount of external proteins in the cell membranes, as determined by peroxidase iodination, decreased with time reaching undetectable levels after 4 to 8 h of incubation at 37 °C (Fig. 5). This finding corresponds with that of Okada et al. (1974) who showed that Sendai virus antigens, demonstrable by fluorescein-coupled antibody, move on the membranes of Ehrlich ascites cells treated with u.v.-irradiated virus to form a 'cap'. This movement is most pronounced for the first 4 h post infection, and the antigen is not detectable after 20 h.

The persistence of viral antigens detected by peroxidase iodination could, however, be due to virus particles which remain adsorbed on the surface of the cells. There is no conclusive evidence that this does not occur. The absence of virus particles in electron micrographs and lack of haemadsorption may be interpreted on the basis that these techniques are not sufficiently sensitive in comparison with radioiodination. A more conclusive proof
Sendai virus antigens on cell surface

Fig. 5. Disappearance of viral antigens HN and F from cell-virus complex during 8 h after adsorption of u.v.-inactivated Sendai virus. From densitometric tracings of autoradiographs of gels (such as in Fig. 4), peaks were cut out and weighed. Ordinate: arbitrary scale based on weight of paper tracings. Abscissa: time after adsorption of virus. □ = HA; ○ = F.

would be provided by analysing the membrane associated proteins after ‘infection’ with uniformly labelled virions (Heine & Schnaitman, 1970).

The iodination technique, as distinct from ferritin-antibody labelling, permits the detection not only of viral antigens in the cell membrane, but also the identification of those particular ones which remain on the surface. In our experiments, the main antigen detected by iodination and autoradiography of the gels was the HN ‘complex’. The persistence of the F antigen in membranes of cells which adsorbed virus is less clear because of the proximity in the gels of cellular membrane proteins of similar mol. wt. The evidence for the persistence of F protein is therefore mainly based on the change of intensity in the radioactive labelling with the progress of time, which parallels the curve of disappearance of the HN antigen (Fig. 5).

The period of persistence of the HN and the F proteins in the cell membrane (about 4 h, with maximum change occurring during the first 1 to 1.5 h) corresponds to some metabolic changes which have been observed in animal cells adsorbing u.v.-inactivated Sendai virus (or its subunits). Fuchs & Kohn (1971) found for example that 0.5 to 1.5 h after adsorption of such virus, there was an inhibition of methylation of dUMP to TMP, involving the tetrahydrofolic acid cycle, and inhibition of the synthesis of serine from glycine (Fuchs, Kohn & Neuhoff, 1974). The uptake of amino acids into Sendai-treated cells was also inhibited for a period of about 3 to 4 h (Negreanu, Reinhertz & Kohn, 1974). It is therefore suggestive of a correlation between the presence of the HN and F antigens on the surface of the cells and some of the membranal effects such as amino acid transport (Negreanu et al. 1974) and the ‘thymidine effect’ (Fuchs & Kohn, 1971).

I wish to thank Dr John Graham for his help in the purification of Sendai virus, Dr K. Apostolov for electron microscopy, and Dr R. O. Hynes for criticism and advice.
REFERENCES


*(Received 13 May 1975)*