Localization of the influenza virus nucleoprotein: cell-associated and extracellular non-virion forms

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Both the supernatant of influenza virus-infected chick embryo cells and allantoic fluid containing influenza virus were shown to contain non-virion nucleoprotein (NP), which reacted readily with anti-NP monoclonal antibodies. Adsorption onto erythrocytes and centrifugation at 70000 g for 2 h resulted in the removal of about 20% of the extracellular NP, whereas centrifugation at 100000 g for 4 h eliminated about 50%, and practically all [3H]uridine-labelled virions. These results suggest that of the extracellular NP about 30% exists in the form of ribonucleoprotein, about 20% is precipitated with virions and about 50% occurs as free molecules. Comparative analysis of the kinetics of the accumulation of NP in the supernatant of infected cells, on the cell surface and inside the cells in relation to virus production, showed that there is a significant correlation between them.

The classical model of the structure of the influenza virion and its assembly implies that only glycosylated virus proteins may be present on the surface of infected cells (Choppin & Compans, 1975). However, this assumption has been challenged by a number of reports showing the localization of both glycosylated virus proteins and the phosphorylated nucleoprotein (NP) of influenza virus on the surface of infected cells. This has been demonstrated specifically by binding studies using monoclonal antibodies (MAbs) (Virelizier et al., 1977; Yewdell et al., 1981; Prokudina et al., 1987; Prokudina & Semenova, 1990; Stitz et al., 1990); the NP of influenza virus also was shown to be present on the cell surface in an electron microscopic study (Patterson et al., 1988).

It has long been known that allantoic fluid contains non-virion ribonucleoprotein (RNP), the so-called complement-fixation antigen (Hoyle & Fairbrother, 1937; Wiener et al., 1946) and, more recently, it has been shown that NP can readily be obtained from the supernatant of cultured cells after influenza virus has been removed (Flawith, 1978). The present study was undertaken to investigate the relationship between cell surface and extracellular NP in chick embryo cells.

Most experiments were done using A/WSN/33 (H1N1) (A/WSN) influenza virus, but some were carried out using A/PR/8/34 (H1N1), A/USSR/090/77 (H1N1), A/New Jersey/8/76 (H1N1), A/Aichi/2/68 (H3N2), A/Hong Kong/1/68 (H3N2) and A/gull/Kazakhstan/470/79 (H1N1) influenza viruses. Chick embryo cells were obtained after primary trypsin digestion and used throughout the study. The MAbs against the NP of A/WSN virus were a generous gift from Dr R. C. Webster (Van Wyke et al., 1980) and were iodinated with $^{125}$I to a specific activity of approximately $10^8$ d.p.m./µg protein as described by Hunter & Greenwood (1962).

A radioimmunoassay (RIA) was performed using a polyethylene film (Prokudina et al., 1986) coated with pooled anti-NP MAbs as a flat adsorbent, prior to plating of the material to be analysed. Immune complexes were identified using $^{125}$I-labelled anti-NP MAbs.

To investigate the dynamics of the capacity of anti-NP MAbs to bind to the infected cell surface, chick embryo cell cultures were infected with 5 p.f.u./cell A/WSN virus, unadsorbed virus was removed by washing and the cultures were incubated at 37 °C for various lengths of time. The cells were then put in a solution containing $^{125}$I-labelled anti-NP MAbs, washed to remove unbound antibodies and cell-bound radioactivity was measured. As seen in Fig. 1 (line i), virions adsorbed to the cell bind $^{125}$I-labelled MAbs immediately after adsorption, after which the level of binding decreases, possibly due to virion desorption and penetration. At 4 h post-infection (p.i.) the capacity of the cell surface to bind anti-NP MAbs increases, which allows newly synthesized NP to be distinguished from that derived from the inoculum, and to determine that the increase in the accumulation of NP on the cell surface is due to synthesis de novo. Additional evidence for this is given by the absence of NP accumulation on the cell surface in the presence of
Fig. 1. Comparative analysis of the kinetics of the accumulation of A/WSN virus NP on the cell surface, inside the cell and in the culture medium, in relation to virus production and cell degradation. ■, NP accumulation on the cell surface. Infected cells were incubated with 125I-labelled anti-NP MAb for various lengths of time p.i., washed and radioactivity was counted. □, As above but cells were preincubated with actinomycin D (1 μg/ml) before infection. ●, 125I-labelled rabbit anti-rat antibodies binding to infected cells. ○, Estimation of intracellular NP. At different times p.i. [35S]methionine-labelled cytoplasmic fractions of infected cells were plated on an adsorbent precoated with anti-NP MAb and bound radioactivity was counted. △, NP content of the culture medium. Aliquots of culture medium were plated on immobilized anti-NP MAb; immune complexes were identified using 125I-labelled anti-NP MAb. ▲, Acid-insoluble radioactivity. ×, HA titre.

actinomycin D (Fig. 1, line ii). The specificity of the method was supported by the fact that 125I-labelled rabbit anti-rat antibodies did not bind to infected cells (Fig. 1, line iii). Fig. 1 shows that the accumulation of NP on the cell surface (line i) closely parallels the synthesis of intracellular NP (line iv), as determined by the binding of the [35S]methionine-labelled cytoplasmic fraction to immobilized anti-NP MAb.

Subsequently, the dynamics of the accumulation of anti-NP MAb-reactive NP in the cell culture supernatant were investigated. Aliquots of the supernatant collected at various times after infection were pipetted onto an adsorbent precoated with anti-NP MAb and immune complexes were identified using 125I-labelled anti-NP MAb. Fig. 1 shows that there is a significant correlation between the amount of extracellular NP available to react with antibodies (line v), NP accumulation on the cell surface (line i) and intracellular NP synthesis (line iv). Fig. 1 also shows that the time course of cell surface and extracellular NP accumulation coincides approximately with that of haemagglutinin (HA) accumulation (line vii). However, the kinetics of HA accumulation (expressed on a linear scale) are different from those of cell surface and extracellular NP accumulation.

Experiments to estimate the dynamics of degradation of virus-infected cells were carried out to investigate the possibility that destroyed cells or virions were the origin of cell-associated and extracellular NP. [35S]Methionine (about 100 μCi/ml) was introduced into chick embryo cell cultures 2 h before infection and removed immediately prior to infection. At different times after infection, acid-insoluble radioactivity was determined in the extracellular medium. Fig. 1 (line vi) shows that there was no increase in the amount of acid-insoluble material in the culture medium during NP accumulation on the cell surface or in the culture medium until 9 h p.i.; radioactivity did increase much later. We conclude that degraded cells do not serve as a source of the anti-NP MAb-reactive NP identified on the cell surface and in the culture medium up to 9 h p.i.

It has been suggested that virions are subject to degradation when incubated for 9 h at 37 °C, which would result in the release and accumulation of anti-NP MAb-reactive NP in the culture medium. To examine this possibility, culture medium containing virus was incubated at 37 °C for 9 h. The results indicated (Fig. 2 and 6) that virus is partially inactivated during this period without any appreciable increase in the level of anti-NP MAb-reactive NP in the culture medium, a finding which contradicts the theory that NP accumulation in the culture medium can be attributed to virion degradation. Similar results were obtained when virions were disrupted by prolonged heating, or treatment with 0.5% NP40 for 1 h at 20 °C, (Fig. 2, bars 3, 4, 7 and 8). In the latter case, the additional NP exposed by disrupted virions may not be MAb-reactive owing to partial inactivation and the abundance of free non-virion NP in the culture medium.

To investigate the relationship between the extracellular anti-NP MAb-reactive NP and virions, chick embryo cell cultures were infected with virus and incubated for 5 h in maintenance medium containing [35S]methionine. Radiolabel was removed by washing and replaced with fresh medium, and aliquots were tested 10 h p.i. for labelled protein (by PAGE), infectivity, HA activity, and the ability to bind anti-NP MAb. These parameters
were analysed both before removal of virus, and after its removal using either adsorption onto erythrocytes, or centrifugation at 70000 g for 2 h or 100000 g for 4 h (Table 1). The amount of viral NP detected by PAGE in the medium after the removal of virus (centrifugation at 70000 g for 2 h) only decreased by about 20% although the virus had been removed almost completely (Table 1). A similar result was obtained after virions had been allowed to absorb onto erythrocytes (Table 1), which failed to change significantly the NP content, although haemagglutinating infectious virus was virtually absent. Centrifugation at 100000 g for 4 h (Table 1) resulted in a decrease in the NP content of the culture medium of 50%. These data were supported by RIA (not shown).

Similar results were obtained when culture medium was analysed by RIA (Table 1). The table shows that the removal of virions from the medium by adsorption onto erythrocytes or centrifugation resulted in a 1000-fold or greater reduction in the biological parameters used to determine the amount of virus present, whereas the amount of NP bound to immobilized MABs decreased by approximately 50% only after centrifugation at 100 000 g for 4 h. The other 50% of the anti-NP MAB-reactive NP could not be removed from the culture medium by centrifugation. The same technique yielded similar results (not shown) when used to test chick allantoic fluid and any of the viruses listed above.

The determination of whether the extravirion anti-NP MAB-reactive NP was present in the culture medium in a free form or as RNP was considered to be of special interest. Cells infected with A/WSN virus were labelled with [35S]methionine and [3H]uridine for 5 h, after which the radiolabel was removed and replaced with fresh medium containing no radioactivity. Aliquots of the medium were tested for the presence of acid-insoluble, labelled macromolecules. Table 1 shows that 3H-labelled structures were removed from the culture medium much more readily by adsorption onto erythrocytes or centrifugation than was 35S-labelled material; centrifugation at 100 000 g precipitated 50% of the NP and virtually all the 3H-labelled material present in the aliquots. This suggests that the centrifugation precipitated most of the viral RNP, whereas RNA-free material (presumably free NP molecules) remained in the supernatant. We conclude that both culture medium and allantoic fluid contain viral NP which can bind to specific antibodies and be detected by PAGE. About 20% of this NP is precipitated with virions, approximately 30% is bound within RNP complexes and about 50% occurs as free molecules. Based on these findings it seems appropriate to assume that cells infected with influenza virus release both mature virus particles and subviral structures, i.e. RNP and free NP, into the culture medium; a similar hypothesis was suggested by Flawith (1978). In vivo, it has been postulated that NP is secreted into the nasal discharge of animals infected with influenza virus, or into the allantoic fluid of similarly infected chick embryos (Cook et al., 1988; Siebinga & de Boer, 1988).

![Fig. 2. Effect on virus infectivity and binding to 125I-labelled anti-NP MABs of incubation of culture medium containing virus in conditions which destroy virus, as determined by RIA. 1 to 4, Infectivity; 5 to 8, binding to 125I-labelled anti-NP MAbs. 1 and 5, Initial values; 2 and 6, incubation at 37 °C for 9 h p.i.; 3 and 7, incubation at 37 °C for 30 h p.i.; 4 and 8, incubation with 0.5% NP40 for 1 h p.i. Anti-NP MAB binding was calculated using for formulae (c.p.m. of sample - c.p.m, of control)/(c.p.m, of control).](image)

| Table 1. Approximate amount of viral NP in cell culture medium after removal of virus |
|-----------------|-----------------|-----------------|-----------------|
| Virus removed by | 35S-labelled NP detected by PAGE (RIA) | Labelled acid-insoluble material (%) | Virus C02† |
| Sequential centrifugation at | | | |
| 70000 g | 80 | 15 | 0-01 | <1 |
| 100000 g | 50 | 2 | 0-001 | <1 |
| Adsorption onto erythrocytes | 80 | 30 | 0-1 | <1 |
| * Estimated on the basis of densitometric values. |
| † These values represent the percentage of virus in the culture medium after removal by the methods shown. Figures were based on the following results. P.f.u./ml values: initial, 5 x 108; supernatant, 7 x 104; pellet (diluted to initial volume), 2 x 108. HA/ml values: initial, 256; supernatant, <2; pellet (diluted to initial volume), 256. |
The present paper also demonstrates that chick embryo cells infected with influenza virus have NP exposed on their surface, which is in agreement with the findings of Virelizier et al. (1977), Yewdell et al. (1981) and Stitz et al. (1990). The similar dynamics of cell surface and extracellular influenza virus NP accumulation suggests that they are interconnected, and that NP synthesized inside the cell is secreted partially into the extracellular culture medium after a temporary but necessary step in which it is presented at the cell surface. The role of this mechanism in the pathogenesis of influenza virus infection is not known. Cell-associated NP does not induce a cellular immune response because cytotoxic T lymphocytes only recognize NP peptides (Townsend et al., 1985, 1986); the humoral immune response induced by cell-associated NP was shown to be unable to protect against infection (Stitz et al., 1990). The significance of extracellular NP also is unknown and is under current study.

We thank Drs R. G. Webster and S. S. Yamnikova for kindly providing MAb, and Dr N. V. Kaverin for critical reading of the manuscript.

References


(Received 8 January 1991; Accepted 29 March 1991)