Gradual Changes of Influenza Virions During Passage of Undiluted Material

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SUMMARY

Defective influenza virions undergo gradual changes during passage of undiluted material: the amount of RNA in virions diminishes, the equimolarity of the RNA segments is lost and the interfering activity of defective virions decreases. Defective virions of early passages have a small deficiency in genetic material and at a high m.o.i. induce the synthesis of the complete set of virus-specific proteins. Defective virions from later passages are characterized by a considerable deficiency in genetic material and a decrease in the amount of high mol. wt. RNA segments. They do not demonstrate complementation and at a high m.o.i. do not induce the synthesis of virus-specific proteins, thus revealing analogous defects in the genome of the majority of defective virions. The characteristics of protein synthesis in cells infected with these virions are similar to those in uninfected cells.

During undiluted passage of influenza virus the cyclic production of defective and infectious virions takes place.

INTRODUCTION

The nature of von Magnus incomplete influenza virus has been the subject of several recent studies. It has been shown that incomplete (defective) virions contain less of the large RNA fragments and more of the small ones than do normal virions (Duesberg, 1968; Pons & Hirst, 1969; Crumpton et al. 1978; Nayak et al. 1978; Janda et al. 1979). Small RNA fragments are apparently derived from large fragments of virus RNA (Nakayama et al. 1979). However, it is still unclear as to what is the structural basis of the functional defects of incomplete influenza virus and what processes it can induce in the cell.

A comparative study of changes in defective influenza virus during passage of undiluted material may elucidate some of these problems. This paper presents the results of such a study.

METHODS

Virus. Influenza virus HoN1 (WSN strain) was used. The standard virus was obtained after cloning a plaque obtained in MDCK monolayers five times. For accumulation of the standard virus, chick embryos were infected at 100 p.f.u./embryo. The standard virus had $5 \times 10^7$ p.f.u./ml and 16 HAU/ml. Incomplete virus was obtained after infection of chick embryos with undiluted inocula. The harvest was collected 18 h p.i.

Radioactive labelling. Labelling of virus RNA was performed in chick embryos. $^3$H-uridine was introduced at a dose of 250 $\mu$Ci/embryo (sp. act. 24 Ci/mmol) simultaneously with infection.
Table I. Plaque-forming and haemagglutinating titres of influenza virus during passage of undiluted material

<table>
<thead>
<tr>
<th>No. of passage</th>
<th>P.f.u./ml</th>
<th>HAU/ml</th>
<th>Ratio p.f.u.: HAU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard virus</td>
<td>$5 \times 10^7$</td>
<td>16</td>
<td>$3 \times 10^8$</td>
</tr>
<tr>
<td>2nd</td>
<td>$5 \times 10^6$</td>
<td>16</td>
<td>$10^6$</td>
</tr>
<tr>
<td>3rd</td>
<td>$5 \times 10^4$</td>
<td>16</td>
<td>$3 \times 10^3$</td>
</tr>
<tr>
<td>4th</td>
<td>$0.8 \times 10^4$</td>
<td>16</td>
<td>$0.5 \times 10^5$</td>
</tr>
<tr>
<td>5th</td>
<td>$3 \times 10^4$</td>
<td>16</td>
<td>$2 \times 10^4$</td>
</tr>
</tbody>
</table>

Purification of virus. Virus from clarified allantoic fluid was pelleted by centrifugation at 20,000 rev/min for 1 h. The pellet, after ultrasonic disintegration, was resuspended in TNE buffer (tris-HCl, 0.01 M, pH 7.4; NaCl, 0.1 M; EDTA, 0.001 M) and centrifuged in a sucrose gradient (15 to 30%) in a SW27.1 rotor of a Spinco L5-65 centrifuge at 20,000 rev/min for 30 min. The gradient fractions that contained haemagglutinin activity were collected and centrifuged in a sucrose gradient (15 to 60%) at 24,000 rev/min for 18 h. Virus was pelleted from the gradient fractions, resuspended in TNE buffer and used for further studies.

Radioactive labelling of newly synthesized virus proteins. Chick embryo cell or MDCK monolayers containing $1 \times 10^8$ to $5 \times 10^8$ cells were infected with virus from allantoic fluid. After adsorption at room temperature for 1 h, unadsorbed virus was washed off with saline and medium 199 was added for 5 h. It was replaced with medium that contained a $^{14}$C-amino acid-reconstituted mixture ($50 \mu$Ci/ml, sp. act. 100 mCi/mmol) or $^{35}$S-methionine ($20 \mu$Ci/ml, sp. act. 1200 Ci/mmol) and the cultures were incubated for 20 min. They were then washed with saline and treated with 0.5% SDS. Before electrophoresis β-mercaptoethanol was added to a final concentration of 2.5%, glyceral to 0.5% and bromophenol blue, and the mixture was boiled for 2 min.

Electrophoresis of proteins. This was carried out in 12% polyacrylamide slab gels. Before the electrophoresis 50 μg of each sample was dissociated in buffer containing 0.01 M-tris-HCl, pH 6.8, 2% SDS, 2% β-mercaptoethanol and applied to a slab gel prepared using the technique of Laemmli (1970). The gels were stained with 2% amido black, washed with 7% acetic acid/ethanol, dried and autoradiographed.

RNA extraction. A suspension of purified virus containing 0.01 M-tris-HCl (pH 7.4) 0.14 M-NaCl, 0.5% SDS was used. An equal volume of fresh distilled phenol was added and the mixture was shaken for 20 min at room temperature. The RNA in the aqueous phase was precipitated by 2.5 vol. of ethanol. After 24 h, the RNA was dissolved in a small volume of 5 mM tris-HCl buffer (pH 7.4), containing 1 mM-EDTA, 10 mM-NaCl and 0.05% SDS, re-precipitated by 2.5 vol. of ethanol and resuspended in a small volume of tris-EDTA–borate (TEB) electrophoresis buffer. All samples were stored at $-20$ °C and boiled before application to the gels.

Electrophoresis of RNA. This was carried out in $0.6 \times 14$ cm cylindrical gels containing 2.8% acrylamide, 0.15% bisacrylamide, 0.04 M-sodium acetate, 0.02 M-tris, pH 7.4, 0.01M-EDTA and 0.5% agarose. Electrophoresis was performed at 4 °C and 2.5 V/cm for 14 h. A TEB buffer system was used (Peacock & Dingman, 1967). After electrophoresis the gels were stained with methylene blue and longitudinal slices were scanned with a densitometer.

RESULTS

Changes in biological parameters of virus during passage of undiluted material

During serial passage of undiluted virus in eggs the plaque-forming and haemagglutinating titres of the virus changed (Table I). It can be seen that the decrease in the portion of infectious virus is maximal at the 4th passage and an increase occurs at the 5th passage. Since the
Properties of incomplete influenza virions

Fig. 1. Kinetics of u.v. inactivation of infectivity and interfering activity of incomplete influenza virus as measured by the plaque reduction test. ○——○, Interfering activity; •——•, infectivity. For measurement of interfering activity monolayers of MDCK cells (1 × 10⁶ to 5 × 10⁶ cells/flask) were infected with standard virus (approx. 150 p.f.u./flask) or co-infected with standard and incomplete viruses of the 2nd undiluted passage. After 40 min at room temperature the monolayers were washed three times with saline and 1.8 % Bacto-Difco agar overlay was added (Porterfield, 1960). Five days p.i. the plaques were counted. The interfering activity is \[\frac{\text{p.f.u.}_0 - \text{p.f.u.}_{\text{u.v.}}}{\text{p.f.u.}_0}\] × 100. Survival ability was analysed by plaque assay and is \[\frac{\text{p.f.u.}_{\text{u.v.}}}{\text{p.f.u.}_0}\] × 100.

Fig. 2. Electrophoresis in polyacrylamide gels of polypeptides of standard and incomplete influenza virus. Total virions electrophoresed in 15 % polyacrylamide gel: lane 0, standard virus in non-reducing conditions; lanes 1 to 5, reducing conditions. Lane 1, standard virus; lanes 2 to 5, viruses from 2 to 5 passages of undiluted material. The characteristics of the viruses are presented in Table 1.
Fig. 3. Content of RNA in virions with different p.f.u.: HAU ratios. • •, Percentage of d/min/mg protein (RNA content) in virions of the given passage; ○--○, percentage p.f.u./HAU ratio.

Table 2. Interfering activity of von Magnus virus* from various undiluted passages

<table>
<thead>
<tr>
<th>Virus</th>
<th>Interfering activity in Chick embryos†</th>
<th>Interfering activity in MDCK cells†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P.f.u./ml</td>
<td>HAU/ml</td>
</tr>
<tr>
<td>Standard only, 1:2</td>
<td>120</td>
<td>$1 \times 10^6$</td>
</tr>
<tr>
<td>Standard and 2nd, 1:1</td>
<td>0</td>
<td>$3 \times 10^5$</td>
</tr>
<tr>
<td>Standard and 3rd, 1:1</td>
<td>5</td>
<td>$2 \times 10^4$</td>
</tr>
<tr>
<td>Standard and 4th, 1:1</td>
<td>23</td>
<td>$1 \times 10^3$</td>
</tr>
</tbody>
</table>

* Incomplete viruses were u.v.-irradiated for 100 s.
† Interfering activity of incomplete virus assayed as in Fig. 1.
‡ Chick embryos were infected with standard virus or both viruses. The virus yield was determined by plaque assay after 15 h of infection.
§ P.f.u. were determined in monolayers under agar overlays. Similar results were obtained with unirradiated von Magnus viruses, which can be used in these experiments due to auto-interference at low dilutions.

haemagglutinating titres do not vary from passage to passage, it may be considered that the number of virions remains stable.

Experiments were conducted to establish the interfering activity of defective virus that accumulates during serial passage. In these experiments u.v.-inactivated incomplete virus was used. It was previously shown that the curves of u.v.-inactivation of infectivity are the same both in standard and in incomplete virus (Kantorovich-Prokudina et al. 1979). These data suggest that the infectivity of von Magnus virus is due to infectious virus rather than multiplicity dependent reactivation of incomplete virions. It can be seen from Fig. 1 that the interfering activity of incomplete virus is more resistant to u.v.-inactivation compared to the infectivity. These data are similar to those of Nayak et al. (1978). Consequently it is possible to investigate the interfering activity of u.v.-irradiated uninfectious von Magnus virus. Comparative data for the interfering activity of virus from different passages are presented in Table 2, which shows that interfering activity of incomplete virus gradually decreases from early to late passages, thus indicating that either the portion of defective interfering (DI) particles decreases while the total number of defective particles increases (cf Table 1) or the interfering activity of all virions is gradually decreased from 2nd to 4th passage.

The polypeptide composition of the purified standard and incomplete influenza virus was studied by electrophoresis in polyacrylamide gels. In Fig. 2 the polypeptide composition is the same both in early and in late passages.
Properties of incomplete influenza virions

We attempted to compare the amount of RNA in virions with the infectivity of the virus preparation, using \(^3\)H-uridine-labelled RNA (Fig. 3). There is a marked correlation between the content of RNA in virions and their infectivity as well as p.f.u.: HAU ratio. However, the dependence is not linear: the infectivity of virus of the 4th passage is 10,000 times less than that of the standard virus, whereas the amount of RNA is reduced twofold. Similar data were obtained using the absorbance analysis of influenza virus nucleic acid (Ada & Perry, 1956).

RNA from influenza virus produced during serial passage of undiluted material was studied by electrophoresis in polyacrylamide gels. It is seen from Fig. 4 that the standard virus contains all eight RNA fragments in certain relative amounts. During serial passage the amount of high mol. wt. RNA in virions sharply decreases and peaks of low mol. wt. RNA appear that are absent in the standard virus. These fragments are approx. 50 times shorter than P\(_1\) to P\(_3\) RNA fragments.

**Virus-induced protein synthesis in cells infected with incomplete virus**

This study was carried out in two cell systems: chick embryo primary cell cultures (CEC) which are not fully permissive for WSN influenza virus, and MDCK cells which are permissive. Both cell cultures were infected with undiluted inocula of the viruses. Fig. 5 shows
Fig. 5. Incorporation of $^{35}$S-methionine into (a) MDCK cells and (b) chick embryo cells infected with standard virus (st) and viruses of the 2nd to the 5th passages (2 to 5) as compared with non-infected cells (N). At 5 h p.i. $^{35}$S-methionine (1 µCi/culture) was added to duplicate infected and uninfected cell monolayers for 10 min and incorporation into trichloroacetic acid-insoluble material was measured.

that at 5 h p.i. with standard virus, a stimulation of total $^{35}$S-methionine incorporation was observed in CEC and inhibition took place in MDCK cells. Although during the first hours after infection of both cell types with standard virus a stimulation of total protein synthesis took place, in MDCK it was changed by a marked inhibition at 5 h (not shown). In CEC such inhibition occurs later than in MDCK cells and this explains the difference of total protein synthesis in both cell types 5 h after infection by standard virus. This is consistent with the kinetics of total protein synthesis in CEC (Skehel, 1972) and MDCK cells (Etchison et al. 1971) infected with infectious influenza virus. It is evident from Fig. 5 that during passage of undiluted virus the rate of total protein synthesis approaches that of non-infected cells. The rate of synthesis of virus-specific proteins decreases from the 2nd to the 4th passage and increases at the 5th passage (Fig. 6), indicating some correlation with infectivity and quantity of RNA in virus preparations. It is also shown in Fig. 6 that during passage of undiluted material the ability of influenza virus to inhibit cell synthesis in MDCK cells diminishes.

Since the standard and incomplete virus preparations, although containing the same number of virions, differ considerably in the content of infectious virus, experiments were conducted in which the cells were infected with the same amount of infectious virus from the standard and incomplete virus preparation of the 2nd passage. For this purpose the standard virus was diluted 100 times. It can be seen from Fig. 7 that in these conditions the synthesis of virus-specific proteins is considerably more intensive in cells infected with incomplete virus, thus indicating that protein synthesis in this case is realized predominantly by non-infectious virions.

Further studies were performed to determine the synthesis of virus-specific proteins in the case of simultaneous infection with standard virus and incomplete virus of the 3rd
Properties of incomplete influenza virions

Fig. 6. Electrophoretograms of $^35$S-methionine-labelled proteins (non-reducing conditions) from virus-infected (a) MDCK cells and (b) chick embryo cells. The cells were infected with the same amount (as measured in HAU/ml) of the standard virus (lane 1) and viruses of the 2nd to the 5th passages of undiluted material (lanes 2 to 5). Uninfected cells (lane 6) served as a control. $^35$S-methionine (20 µCi/ml) was added to monolayers at 5 h p.i. for 20 min and the cells were then washed and dissociated in buffer (0.01 M-Tris-Cl, pH 6.8, 2% SDS) at 100 °C. The labelled polypeptide components of the cell extracts were separated by electrophoresis. Autoradiograms of the dried gels are shown.

passage. Fig. 8 shows that the virus of the 3rd passage does not inhibit the ability of standard virus to direct the synthesis of virus proteins. These data indicate that standard virus is capable of being a helper for incomplete virus.

DISCUSSION

This work showed that during serial passages of undiluted influenza virus, gradual changes in the structure of virions occur that are reflected in the changes of their biological properties. Thus the virus of the 2nd undiluted passage, in spite of defectiveness (the loss of 2 logs of infectivity) and a partial loss of genetic material (about 20%), is characterized by a molar ratio of RNA segments similar to that of the standard virus. Defective virions of the 2nd passage at high m.o.i. are able to induce the synthesis of the full set of virion proteins which can be due to complementation of virions with different defects in their genomes. It is also probable that a certain proportion of virions of the 2nd passage, in spite of defectiveness,
is able to initiate a partial cycle of virus reproduction. This is indicated by the ability of incomplete virions to transcribe their genomes in vitro (Bean & Simpson, 1976; Kantorovich-Prokudina et al. 1979) and in vivo (Scholtissek & Rott, 1969) and to undergo a single cycle of replication (Barry, 1961).

Defective virions of later passages are characterized by a considerable deficiency of genetic material (about 50%) and a decrease in the amount of high mol. wt. RNA segments. In spite of a high m.o.i., they do not induce virus-specific syntheses and are not able to complement, thus indicating that the majority of virions have similar defects in their genomes. It is apparent that even a multiplicity greater than 10 virions/cell is insufficient to provide a single set of eight RNA segments/cell and therefore, both replication and complete transcription are impossible.

It may be assumed that defective virions of this type need a helper for their reproduction.
which, as apparently follows from our experiments, ensures the synthesis of virion proteins for incomplete virions in the case of simultaneous infection. The gradual increase in the degree of defectiveness of the incomplete virus is manifested also in a decrease of its interfering activity and in its influence upon cellular syntheses.

The data presented allow the conclusion that cyclic structural and functional changes occur in influenza virions during serial passages with undiluted material, as described for vesicular stomatitis virus (Huang, 1973). An increase in the production of infectious virus may be connected not only to a decrease of the synthesis of DI virus due to a decrease of helper virus, but also with a decrease of interfering activity of the defective virus.

Thus defective influenza viruses with various degrees of deletion of genetic material may be characterized by various degrees of structural and functional defectiveness. Hirst & Pons (1973) demonstrated deletion mutants with a random distribution of defects in their genomes, which, in conditions of high m.o.i., had a capacity for multiplicity reactivation and produced infectious progeny. We showed that, during serial passages with undiluted material, defective virions with defective genomes appear at first which interfere with infectious virus and lose the capacity for multiplicity reactivation but still are able to induce the full set of virus-specific proteins. Later virions are accumulated with high deficiency in their genomes which are not able to induce the synthesis of virus-specific proteins in spite of high m.o.i. and which have a decreased interfering activity.

REFERENCES

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