Studies on Heterotypic Interference between Influenza A and B Viruses: A Differential Inhibition of the Synthesis of Viral Proteins and RNAs

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SUMMARY

Mixed infection of MDCK cells with influenza A and influenza B viruses leads to a reduction in the rate of synthesis of haemagglutinin (HA) and nucleoprotein (NP) as compared to their rate of synthesis in cells separately infected with these viruses. The reduction is much stronger for influenza A virus proteins. The synthesis of the non-structural NS1 protein of both viruses is relatively resistant to the heterotypic interference. The synthesis of virus-specific mRNAs exhibits the same pattern: the formation of the transcripts of HA and NP genes is much more drastically reduced than the synthesis of NS gene transcripts. The effect is strongly dependent on the multiplicity of infection and on the ratio of influenza A and B viruses in the inoculum. Primary transcription in the presence of cycloheximide is almost unchanged in doubly infected cells as compared to single infection, and no indication of differential inhibition has been observed. The results are discussed in connection with the mechanism of heterotypic interference and the regulation of influenza virus protein synthesis.

INTRODUCTION

Heterotypic interference between influenza A and B viruses was described as early as 1954 (Gotlieb & Hirst, 1954), but since then it has attracted only occasional attention. The interference is reciprocal and multiplicity-dependent (Gotlieb & Hirst, 1954; Tobita & Ohori, 1979). Although the degree of interference seems to depend on the strains or even clones used (Noronha-Blob & Schulze, 1976), influenza B virus is generally the stronger competitor. This relationship has been observed not only for virus yields (Gotlieb & Hirst, 1954; Noronha-Blob & Schulze, 1976) but also for the synthesis of viral proteins in the infected cells (Tobita & Ohori, 1979).

In the course of our studies on mixed influenza virus infections we observed that the accumulation of viral proteins in cells infected simultaneously with A/WSN/33 and B/Lee/40 at certain multiplicity ratios was affected in a selective fashion. This observation prompted us to study in more detail viral protein synthesis and RNA transcript accumulation in conditions of heterotypic interference, in order to establish whether the differential inhibition of protein synthesis operates at the translational level, or correlates with changes in the viral mRNA population.

METHODS

Viruses and cells. Influenza A/WSN/33(H1N1), B/HK/73 and B/Lee/40 viruses were propagated in the allantoic cavity of 10-day-old chicken eggs. The allantoic fluid was collected after 48 h at 37 °C for A/WSN and at 35 °C for B/Lee and B/HK/73. For infectivity measurements, haemadsorbing cell counts in Madin–Darby canine kidney
MDCK cells were obtained from the tissue culture department of the D. I. Ivanovsky Institute. Cells were grown in Eagle’s minimal essential medium (MEM) supplemented with 10% foetal calf serum for MDCK cells, or bovine serum for CEF. Cell monolayers were incubated with appropriate dilutions of the viruses, the effective multiplicity of infection (m.o.i.) being determined by concomitant haemadsorbing cell counting, the titration giving infectious units (i.u.). Infected cells were incubated under MEM at 37 °C.

For RNA labelling, [3H]uridine at a final concentration of 200 μCi/ml (sp. act. 24 Ci/mmol) was added to the maintenance medium. For labelling of protein, Hanks’ balanced salt solution (HBSS) or MEM containing 14C-labelled Chlorella hydrolysate (25 μCi/ml) was used. For the measurement of virus yield, cells were incubated under MEM containing 0.2% bovine serum albumin. The culture fluids were collected after incubation for 10 h at 37 °C, and haemagglutinating activity and infectivity were determined.

**RNA chromatography in poly(U)-Sepharose.** RNA from [3H]uridine-labelled cells was extracted as described by Bratt & Robinson (1967) and fractionated into polyadenylated and non-polyadenylated fractions using the technique of Lindberg & Persson (1972) as modified by Varich et al. (1976). RNA was dissolved in 0.1 ml of formamide, heated at 100 °C for 45 s, diluted with 0.7 M-NaCl, 0.05 M-Tris–HCl pH 7.5, 0.01 M-EDTA, 25% formamide and applied at a flow rate 13 ml/h to a poly(U)-Sepharose column equilibrated with the same buffer. The non-adsorbed material was re-applied to the column. Elution was performed with 90% formamide containing 0.01 M-NaCl, 0.01 M-Tris–HCl pH 7.5, 0.01 M-EDTA. Fractions containing the eluted RNA and the non-adsorbed RNA were pooled separately and precipitated twice with 2.5 vol. ethanol each time.

**RNA–RNA hybridization and the analysis of hybrid duplexes.** The procedure of Ito & Joklik (1972) as modified by Hay et al. (1977) was used with slight modifications. [3H]Uridine-labelled RNA extracted from the infected cells was mixed with an excess (25 μg/sample) of virion RNA and denatured at 100 °C for 45 s in 90% formamide. The sample was diluted with 0.1 M-NaCl, 0.04 M-Tris–HCl pH 7.5, 0.005 M-EDTA to make the final formamide concentration 50%, and then annealed at 37 °C for 15 h. RNA was ethanol-precipitated and purified by CF-11 cellulose chromatography (Franklin, 1966). The fractions eluted from the cellulose column with 35% and 15% ethanol were discarded. The material eluted with 0.1 M-NaCl, 0.05 M-Tris–HCl pH 6-9, 0.001 M-EDTA was ethanol-precipitated in the presence of carrier RNA and dissolved in 0.1 M-NaCl, 0.01 M-sodium acetate, 0.0005 M-ZnSO4, pH 4.5 for nuclease treatment. The samples were incubated with 1000 units of S1 nuclease at 37 °C for 5 h, extracted with phenol and analysed by polyacrylamide gel electrophoresis (acylamide concentration 7.5%) as described by Hay et al. (1977). Electrophoresis was performed at 110 V for 38 to 42 h at 20 °C in 25 cm slabs. The slabs were subjected to fluorography as described by Bonner & Laskey (1974).

**Analysis of 14C-labelled proteins.** Labelled cells were solubilized in 2% sodium lauryl sulphate, 5% 2-mercapto-ethanol, 0.05 M-Tris–HCl pH 7.0, 3% glycerol, heated at 100 °C for 3 min and analysed by polyacrylamide slab gel electrophoresis using the buffer system described by Laemmli (1970) as modified by Spear & Roizman (1972). The gels contained 10, 12 or 15% acrylamide or a 10 to 16% gradient of acrylamide concentration. Either methylenebisacrylamide or diallyltartardiamide (DATD) were used as linkers (Spear & Roizman, 1972).

**Virus purification and virion RNA extraction.** The allantoic virus was purified by ultracentrifugation in potassium tartrate solutions as described in an earlier publication (Kaverin et al., 1973).

Labelled virus was purified from culture fluids by adsorption to and elution from chicken red blood cells and pelleted through a layer of 10% sucrose in the SW27 rotor of a Spinco L-50 ultracentrifuge at 19000 rev/min. RNA extraction was performed with the use of the Pronase–phenol–detergent technique as described by Kolakofsky et al. (1974).

**Reagents.** Sources of chemicals were as follows: acrylamide (Bio-Rad), DATD (Bio-Rad), sodium lauryl sulphate (BDH), S1 nuclease (Calbiochem), poly(U)–Sepharose (Pharmacia), formamide (Merck) and cellulose CF-11 (Whatman).

**RESULTS**

The polypeptides of influenza A and B viruses are readily revealed in infected MDCK cells by 14C-amino acid labelling and polyacrylamide gel electrophoresis. The concomitant analysis of purified labelled viruses in parallel lanes identified the major virus-specific proteins. The migration rate of the matrix (M) protein of influenza B virus was somewhat higher than that of influenza A virus. The behaviour of influenza A/WSN virus non-structural protein NS1 differed in DATD-linked and methylenebisacrylamide-linked gels: in the former it moved faster than protein M, whereas in the latter both proteins moved with almost equal velocity. The nucleoprotein (NP) and NS1 polypeptides of influenza B virus differed sharply in their migration rates from those of influenza A virus, and their identification presented no difficulties (Racaniello & Palese, 1979). The pattern of virus-specific protein synthesis in doubly infected cells is strongly
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Fig. 1. Time course of virus-specific protein synthesis in MDCK cells doubly or singly infected with influenza A and influenza B viruses. 

(a) Cells infected with A/WSN and B/HK/73 viruses, m.o.i. for each virus 25 i.u./cell. Lanes 1, 4, 7 and 10, cells infected with B/HK/73; lanes 2, 5, 8 and 11, cells infected with both viruses simultaneously; lanes 3, 6, 9 and 12, cells infected with A/WSN. Cells labelled from 4 to 5, 6 to 7, 7 to 8 and 8 to 9 h post-infection are represented in lanes 1 to 3, 4 to 6, 7 to 9 and 10 to 12 respectively. Electrophoresis was in a DATD-linked gel, acrylamide concentration 12%.

(b) Cells infected with A/WSN and B/Lee viruses, m.o.i. 50 i.u./cell for A/WSN and 25 i.u./cell for B/Lee. Lanes 1, 4 and 7, cells infected with A/WSN; lanes 2, 5 and 8, cells infected with both viruses; lanes 3, 6 and 9, cells infected with B/Lee. Cells were labelled with 14C-amino acids in HBSS from 2.5 to 3 h (lanes 1 to 3), 3.5 to 4 h (lanes 4 to 6) and 4.5 to 5 h post-infection (lanes 7 to 9). Lane 10, mock-infected cells. Methylenebisacrylamide-linked, 10% acrylamide.
dependent both on the multiplicity of infection and on the ratio of the multiplicities of influenza A and B viruses. The synthesis of NP proteins of both viruses was reduced in comparison to synthesis under conditions of separate infection. A differential pattern of inhibition was quite evident: in contrast to the NP proteins, the intensity of the NS1 bands of both viruses was not reduced. In fact the NS1 band of influenza B virus was sometimes slightly enhanced. The general pattern of the differential inhibition was conserved throughout the multiplication cycle (Fig. 1), although there were some variations, such as a relative decrease in the synthesis of influenza B virus NP protein in the doubly infected cells at a later stage of infection. The predominant inhibition of HA and NP synthesis as compared to NS1 synthesis was conserved throughout the infection cycle, as well as the stronger inhibition of influenza A (as compared to influenza B) viral protein synthesis. A complete block of influenza B protein synthesis has never been registered at any ratio of multiplicities within the range used in these studies, whereas inhibition of influenza A virus protein accumulation was readily achieved (Fig. 2, lane 11) at a 1:4 ratio of A/WSN to B/HK/73. At this ratio of multiplicities, a strong inhibition of influenza A virus protein synthesis was also registered under conditions of superinfection of the cells with B/HK/73 virus 1 or 2 h after infection with A/WSN virus (Fig. 2). This result suggests that the inhibitory effect of the interfering virus is exerted after penetration and uncoating, and probably after the stage of primary transcription (Hay et al., 1977). For a further insight into the mechanisms of the suppression of viral protein synthesis in the course of double infection we turned to studies of virus-specific RNA synthesis.

In MDCK cells infected separately with influenza A or influenza B viruses, positive RNA strands are easily revealed by hybridization of the labelled RNA extracted from the cells with an excess of unlabelled virion RNA (vRNA) and polyacrylamide gel electrophoresis of the hybrid duplexes. For influenza A viruses, the gel pattern is quite characteristic (Hay et al., 1977), so that identification of the bands presents no difficulties, with the exception of the exact
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Fig. 3. Primary transcription of A/WSN and B/HK/73 viral genomes in doubly or singly infected MDCK cells. The cells were pretreated with cycloheximide (150 µg/ml), infected and incubated with the same concentration of cycloheximide. The cells were labelled with [3H]uridine from 0 to 4 h post-infection. RNA was extracted and hybridized with either A/WSN or B/HK/73 vRNA. The hybrid duplexes were analysed as described in Methods. Lane 1, RNA from cells infected with A/WSN; lanes 2 and 3, RNA from cells infected with both viruses; lane 4, RNA from cells infected with B/HK/73. Multiplicities were 100 i.u./cell. Lanes 1 and 2, hybridization with A/WSN vRNA; lanes 3 and 4, hybridization with B/Lee vRNA.

determination of the RNA polymerase protein genes (P1 to P3) which has not been attempted in these studies. The bands formed by influenza B virus-specific RNA duplexes have been identified in accordance with data on the migration rates of denatured viral RNA (Racaniello & Palese, 1979).

In order to register primary transcription, MDCK cells were pre-incubated with cycloheximide and infected with A/WSN and B/Lee at an equally high m.o.i. (Fig. 3). A barely detectable reduction (if any) of A/WSN transcription was observed. This result differs from the data presented by Mikheeva & Ghendon (1982) (see Discussion). The primary transcription pattern provided no explanation for the changes in viral protein synthesis in doubly infected cells.

In several preliminary experiments on secondary transcription, RNA extracted from doubly infected cells were annealed separately with an excess of unlabelled influenza A and influenza B virion RNA, in the same way as it was performed for the analysis of the primary transcripts. However, for the analysis of the stage of secondary transcription, such separate annealing was not quite appropriate, since interpretation of the electrophoretic pattern was somewhat complicated by the presence of bands arising as a result of autohybridization between labelled positive and negative strands. For this reason, in the studies on secondary transcription, we used
Fig. 4. Non-polyadenylated and polyadenylated virus-specific RNA transcripts accumulated in MDCK cells doubly or singly infected with A/WSN and B/Lee influenza viruses. Multiplicities were 50 i.u./cell for A/WSN, 25 i.u./cell for B/Lee. Cells were labelled from 0 to 5 h post-infection with \( ^{3}H \)uridine, RNA was extracted, fractionated in poly(U)-Sepharose, and the poly(A)-containing and poly(A)-deficient fractions were hybridized separately with an excess of unlabelled vRNA. A mixture of equal amounts of A/WSN and B/Lee vRNAs was used for the hybridization in all samples. Lanes 1 and 6, RNA transcripts from the cells infected with B/Lee; lanes 2 and 5, RNA transcripts from the cells infected with both viruses; lanes 3 and 4, RNA transcripts from the cells infected with A/WSN. Lanes 1, 2 and 3, non-polyadenylated RNA; lanes 4, 5 and 6, polyadenylated RNA.

A mixture of equal amounts of unlabelled influenza A and influenza B vRNAs in order to suppress the formation of the 'autohybridization' duplexes. There exists a partial homology between influenza A and B genomes (Scholtissek et al., 1977): this might be a source of bias in the hybridization results. However, in the experiments with RNA from separately infected cells we have never observed any difference between the results obtained using homologous virion RNA alone and the results of annealing with a virion RNA mixture (not shown). Probably the formation of homologous duplexes prevails, and the competition between influenza A and B RNAs in the hybridization mixture is of little importance. We assume, therefore, that the
relative amounts of hybrid duplexes registered in our experiments reflect the relationships in the population of virus-specific transcripts. The amounts of virion RNA per sample are several times higher than the saturation level (Varich et al., 1981).

Total RNA extracted from \(^{3}H\)uridine-labelled cells was separated into polyadenylated and non-polyadenylated fractions and hybridized with an excess of virion RNA. RNA duplexes were purified by chromatography in CF-11 cellulose, treated with nuclease and analysed by polyacrylamide gel electrophoresis. A differential inhibition of the accumulation of A/WSN mRNAs was quite evident: NP gene transcription was sharply suppressed, neuraminidase (NA) gene transcription was also strongly inhibited, whereas the formation of NS gene transcripts was only slightly affected (Fig. 4). The transcription of B/Lee genome segments 4 and 5 was also somewhat diminished; this correlates with the protein synthesis data at a ratio of multiplicities of 2:1 (Fig. 1b). The analysis of non-polyadenylated transcripts reveals a predominant suppression of A/WSN RNA synthesis, but the differential character of inhibition with respect to particular genes cannot be established with certainty.

**DISCUSSION**

The data presented in this paper confirm that competitive interference between influenza A and B viruses operates through the inhibition of macromolecular synthesis (Tobita & Ohori, 1979) and extend this conclusion from protein synthesis to the accumulation of virus-specific RNA. A novel feature is the unequal extent of inhibition for different proteins and RNAs. The synthesis of viral proteins in the course of influenza virus replication is strongly regulated at the transcriptional level (Hay et al., 1977). Translational mechanisms may also be involved, since the translational efficiency for transcripts of different genes may be unequal (Tekamp & Penhoet, 1980), but generally the changes in the relative amounts of newly formed viral polypeptides, both in the course of normal infection and under the action of inhibitors is determined mainly by the accumulation of gene transcripts (Hay et al., 1977; Inglis & Mahy, 1979). It seems of interest to investigate whether this rule holds for heterotypic interference. The results presented in this paper indicate that this is indeed the case: a correlation (at least qualitative) between the changes in the relative rates of the formation of proteins and the changes in the relative amounts of gene transcripts in double as compared to single infection supports the idea that the decrease in synthesis of viral proteins is due to the lack of the corresponding viral mRNAs. However, the actual quantitative relationships among the synthesis rates of different proteins may differ (due to differences in the translation efficiency) from the relationships among the mRNAs.

There are several possible ways to explain the competitive inhibition of virus-specific synthesis in the course of heterotypic influenza virus mixed infection. A competition for cellular primers (capped 5'-ends of cell mRNAs) may be involved; however, the differential or selective inhibition of transcription of specific genes is not easily explained by competition for primers. A more likely possibility is the formation of heterologous transcription complexes: alien NP and/or P1, P2 and P3 proteins in the complexes might hamper efficient transcription and its regulation. To verify this hypothesis, an experimental approach involving transcription in vitro with the use of phenotypically mixed virions might be of value.

Recently, a report dealing with protein and RNA synthesis in mixed influenza A and B virus infection was presented by Mikheeva & Ghendon (1982), who demonstrated complete suppression of influenza B primary transcription at very high (and equal for both viruses) multiplicities of infection. However, the authors also registered, in the same experimental conditions, a clear-cut reduction in A/FPV protein synthesis together with virtually unchanged synthesis of B/Japan proteins. This makes an interpretation difficult, since obviously the inhibition of influenza A protein synthesis cannot be explained by suppression of influenza B primary transcription. However, the authors stated that sometimes in the same conditions influenza B virus primary transcription was suppressed. Such variation of results might be due to a rapid loss of infectivity of partially purified and concentrated influenza B virus, the latter being rather unstable when purified. However, these considerations do not explain the inhibition of primary transcription observed in the experiments of these authors and never
observed in ours. It is conceivable that at very high multiplicities of infection some additional factors of interference are operative, such as, for example, an early competition for capped cell RNAs as primers for viral transcription. One might speculate that a high concentration of viral transcription complexes in the nucleus, which is achieved at an early stage of infection under conditions of very high multiplicity infection (e.g. Mikheeva & Ghendon, 1982), can only be achieved at the stage of secondary transcription if the m.o.i. is not too high. This, however, still does not explain the differential character of the inhibition observed in our experiments. It is possible that more than one factor is involved in the mechanism of suppression of influenza virus-specific synthesis by heterotypic interference.

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