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INTERACTION BETWEEN ANTI-INFLUENZA VIRAL POLYMERASE ANTIBODIES AND RNP PARTICLES USING THE IN VITRO TRANSCRIPTION PROCESS AND AN IMMUNOGOLD LABELLING TECHNIQUE

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Summary. — Immunogold labelling and *in vitro* transcription of influenza virus vRNA have been used to analyse the interaction of anti-influenza polymerase antibodies with influenza-ribonucleoprotein (RNP) complexes. The polymerase proteins (P proteins) were localized exclusively at one end of the RNP segments. In the course of transcription the amount of P protein decreased significantly. The *in vitro* transcriptase activit y of influenza A virus RNP complexes in the presence of anti-polymerase antibodies to the strain A/PR/8/34 was inhibited by 60%. In contrast, RNP transcriptase activity of influenza B virus was not inhibited by these antibodies.

Key words: influenza virus, anti-polymerase antibodies, RNP complexes, electron microscopy, immunogold labelling

Introduction

Influenza virus, like other negative strand RNA viruses, has a virion associated RNA-dependent RNA polymerase responsible for transcription of the viral RNA (Chow and Simpson, 1971; Penhoet *et al.*, 1971; Skehel, 1971). *In vitro* transcription is accomplished by the virus ribonucleoprotein (RNP) complex, containing RNA, nucleoprotein (NP) and the three P proteins (PB1, PB2, PA). The biochemical function of the three P proteins was studied in detail by Ulmanen *et al.* (1981), Nichol *el al.* (1981), Blaas *et al.* (1982*a*, *b*) and Braam *et al.* (1983). Monospecific antibodies to influenza polymerase proteins seem useful in the *in vitro* analysis of the interaction of these antibodies with influenza virus RNP complexes. In a preceding paper we described the purification and immunoreactivity of anti-polymerase antibodies of influenza virus strain A/PR/8/34 (Schreier *et al.*, 1988*b*). Here we present the results of biochemical and electron microscopic investigations on the interaction of anti-polymerase antibodies with the RNP complex.

Materials and Methods

Viruses. The following human influenza virus strains were used: A/PR/8/34 (H1N1), A/Hongkong/1/68 (H3N2) and B/Greifswald/1/86. The viruses were grown in the allantoic cavity of 10-day-old embryonated eggs and purified by sucrose density gradient centrifugation.

Ribonucleoprotein (RNP) complexes. RNP complexes were prepared by treating purified influenza virions or spikeless influenza virus particles with Nonidet P-40 followed by sedimentation in glycerol gradients (Schreier *et al.*, 1988*a*; Rochovansky, 1976). Polyacrylamide gel electrophoresis of the proteins revealed the presence of P proteins and NP protein.

Monospecific anti-polymerase antibodies and transcriptase assay. Anti-polymerase antibodies against the three polymerase proteins (PB1, PB2, PA) of the influenza virus strain A/PR/8/34 were prepared as described by Schreier *et al.* (1988 *b*). The standard reaction mixture for the *in vitro* assay of virion-associated RNA-dependent RNA polymerase (transcriptase) activity of isolated RNP complexes contained 50 mmol/l Tris HCl (pH 7.9 at 30 °C), 5 mmol/l $MgCl_2$, 2.5 mmol/l DTT, 150 mmol/l NaCl, 0.5 mmol/l each of ATP, CTP, UTP, 0.2 mmol/l GTP, 0.1 mmol/l EDTA and (^{3}H) ATP (185 k Bq per 100 µl assay) and 0.25 mmol/l adenylyl (3′−5′) guanosine (ApG) (Schreier *et al.*, 1988 *a*). For testing the influence of anti-polymerase antibodies on the transcriptase activity the RNP fractions were preincubated for approximately 60 min at 20 °C with the IgG fraction. The transcriptase reaction was started by addition of the nucleosidtriphosphates and ApG at 30 °C.

Preparation of protein A-gold. Colloidal gold (3−5 nm in diameter) as well as its complex with protein A were prepared as described by Baschong *et al.* (1985). Tetrachloro auric acid and protein A were purchased from VEB Bergbau- und Hüttenkombinat, Edelmetallchemie Halsbrücke, G.D.R. and from Pharmacia, Sweden, respectively. The protein A-gold complex was stored at 4 °C in PBS containing 0.5 per cent BSA, 0.02 per cent sodium azide, and used up to 4 weeks after preparation.

Immunoelectron microscopy. The 3 µl droplets of RNP suspension were placed on microgrids for adsorption at room temperature and coated with a glow discharge-activated thin carbon film for 5−15 min. Then the grids were transferred twice to 20 µl drops of phosphate buffered saline (PBS) and then to 3 drops of 5 per cent bovine serum albumin (BSA) diluted in PBS for washing and surface saturation (5 to 10 min). The 3 µl droplets anti-polymerase antibodies (diluted 1 : 100 in 5 per cent BSA) were applied to the grids at room temperature. After 30 to 60 min, the grids were washed 3 times for 5 min in 1 per cent BSA followed by rinsing in PBS. Finally a 5 µl droplet of the protein A-gold conjugate was set up for reaction on the PBS-washed grids. The grids were then allowed to react for 30 min at room temperature, washed in the same manner as described in the application procedure of the antibody and subsequently fixed for 15 min in 2.5 per cent glutaraldehyde in 0.15 mol/l cacodylate buffer pH 7.4. After a short wash in distilled water the preparations were negatively stained by standard procedures with 1 per cent uranyl acetate or phosphotungstic acid dissolved in 50 per cent ethanol. Stained preparations were examined with a Siemens electron microscope, type Elmiskop 102.

Results

Influenza of anti-polymerase antibodies on the transcriptase activity of influenza virus RNP

The influence of rabbit serum IgG antibodies to the A/PR/8/34 influenza virus polymerase proteins (PB1, PB2, PA) on *in vitro* RNA synthesis of influenza virus strains A/PR/8/34, AHongkong 1/68 and B/Greifswald/1/86 has been examined. In all *in vitro* transcription experiments, purified RNP particles were used at a concentration of 5.5 µg protein per 50 µl assay in the presence of adenylyl-(3′-5′)- guanosine (ApG) as primer. The RNP particles were preincubated with the anti-polymerase antibodies (IgG

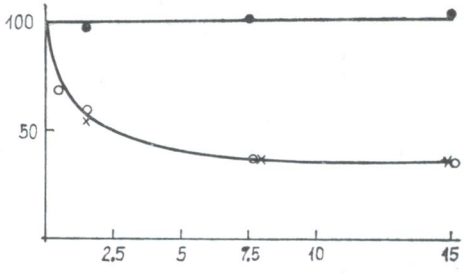

Fig. 1
Inhibition of influenza transcriptase activity of RNP complexes by monospecific antibodies to polymerase proteins. Protein A purified monospecific antibodies (IgG fraction) were added to 50 μl final reaction mixture containing 5.5 μg of RNP (see Material and Methods). Reactions were stopped by the addition of trichloroacetic acid after 2 min (for A strains) and RNA precipitates were collected on Whatman GF/C filters and radioactivity was quantitated with a Philips liquid scintillation counter.
A/PR/8/34, (x———x), A/Hongkong, (○———○). B/Greifswald/1/86. (●—●)
Abscissa: antibodies (μg): ordinate: enzyme activity (%)

fraction) for 1 hr at 20 °C. Fig. 1 shows that the *in vitro* RNA synthesis rate of both influenza A virus strains was reduced by approximately 60% in the range up to 1.5 μg IgG per 50 μl assay. In contrast, antibodies against the polymerase proteins of the influenza strain A/PR/8/34 did not inhibit the *in vitro* RNA synthesis of the influenza strain B/Greifswald/1/86.

Kinetic studies of *in vitro* RNA synthesis revealed that the IgG fraction of anti-polymerase antibodies of the influenza strain A/PR/8/34 influenced

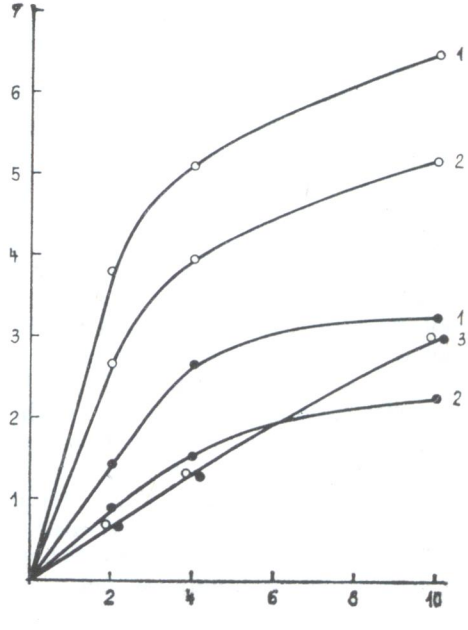

Fig. 2
Kinetics of in vitro influenza virus RNA transcription of RNPs in absence (○) and in presence (●) of monospecific anti-polymerase antibodies (IgG fraction). 50 μl reaction mixture (see Material and Methods) contained 5.5 μg of RNP and 7.6 μg of IgG fraction.
(1) A/PR/8/34
(2) A/Hongkong/1/68
(3) B/Greifswald/1/86
Abscissa: reaction time (min); ordinate: ^{3}H-cpm/min ($\times 10^{-3}$)

the kinetic of influenza A virus RNA transcription but not the kinetic of the influenza B virus RNP transcription (Fig. 2).

Immuno electron microscopy

Negative staining electron microscopy of RNP particles with uranyl acetate showed rods of variable length and a periodically arranged substructure (Fig. 3) as previously described (Pons *et al.*, 1969). It is obvious that the basic structure of RNPs remained preserved even during immunogold labelling procedure. But addition of BSA for prevention of unspecific background labelling results in a decreased fine structural resolution. Nevertheless, protein A-gold labelling is clearly visible (Fig. 4). Together 386 influenza-RNP particles from two labelling experiments have been analysed: 6 and 8 per cent of the particles carried gold label at one end of the rod, respectively. Labelling was found to be exclusively terminal. In the process of RNP transcription the conformation of RNPs appeared to change dramatically. In Fig. 5 transcriptional complexes are shown which had been run as described in Materials and Methods. The complexes appear as blown up highly loosened structures without any periodicity and regularity. The observed conformational change is accompanied by a nearly to zero level decreased affinity to the gold label.

Discussion

As reported previously, the largest influenza virus proteins (PB1, PB2, PA) could be isolated from purified influenza virus strain A/PR/8/34 or from the purified RNP particle fractions by gel chromatography in the presence of SDS or by polyacrylamide gel electrophoresis (Schreier *et al.*, 1988*b*). After their purification and characterization the IgG fractions of the anti-polymerase antibodies were used to study functional and structural properties of the influenza virus transcriptase complex.

In this paper we report that monospecific antisera to the three influenza polymerase proteins inhibit the *in vitro* enzyme activity of the homologous transcriptase to 60%. We don't know the reason for this incomplete inhibition It could be that the antiserum don't contain an effective concentration of antibodies directed to the three P proteins or the access of antibodies to P proteins in the RNP-polymerase complexes was hindered. Alternatively, anti-polymerase antibodies could inhibit *in vitro* RNA synthesis by steric hindrance. Further experiments are necessary to dicriminate between these possibilities. Antibodies against the A/PR/8/34 influenza polymerase proteins did not impair the *in vitro* RNA synthesis of influenza B RNP complexes. The results are consistent with the finding of the high degree of homology of the polymerase amino acid sequences among influenza A virus strains (Bishop *et al.*, 1982a; Bishop *et al.*, 1982b; Kaptein and Nayak, 1982; Sivasubramanian and Nayak, 1982; Winter and Fields, 1982; Robertson *et al.*, 1984; Roditi and Robertson, 1984; Schreier *et al.*, 1988c). On the other hand, amino acid sequence homology between influenza A and B virus PB1 pro-

teins was 61% (Kemdirim *et al.*, 1986) and for PA proteins 38% only (Akoto-Amanfu *et al.*, 1987).

In accordance to these results it was reported previously that monospecific antisera to the three influenza polymerase proteins of the strain A/PR/8/34 exhibit a high immunoreactivity with all influenza A virus strains of the subtypes H1N1, H2N2, H3N2, whereas only insignificant immunoreactivity was detected with influenza B virus strains (Schreier *et al.*, 1988*b*). In addition, the anti-influenza polymerase antibodies (IgG fractions) were used to study the structural properties of influenza RNP complexes. It has been shown that polymerase proteins were located at the end of RNP particles. After *in vitro* transcription structural changes observed in the RNP particles were associated with a decrease in the polymerase content.

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Legends to Figures (Plates LX–LXII):

Fig. 3. Electron micrograph of influenza RNPs (A/PR/8/34) negatively stained with uranyl acetate. Bar represents 100 nm.

Fig. 4. Electron micrograph of RNPs (A/PR/8/34) labelled with antibodies raised against the three influenza polymerase proteins PB1, PB2, PA of A/PR/8/34 and protein A colloidal gold conjugate. Negative staining with uranyl acetate. Bar represents 100 nm.

Fig. 5. Electron micrograph of influenza RNPs (A/PR/8/34) after transcription. Negative staining with phosphotungstic acid. Bar represents 100 nm.