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ANTIBODIES TO POLYMERASE PROTEINS OF INFLUENZA VIRUS A/PR/8/34 (H1N1): COMPARISON ON THE IMMUNOREACTIVITY WITH POLYMERASE PROTEINS OF OTHER INFLUENZA A AND B VIRUS STRAINS

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Summary. — The polymerase proteins (PB1, PB2, PA) of the influenza virus strain A/PR/8/34 (H1N1) were isolated from whole virion or ribonucleoprotein (RNP) fractions by electrophoresis on polyacrylamide gel and electroelution or Sepharose CL-6B chromatography in the presence of SDS. Antisera to polymerase proteins (P proteins) were raised in rabbits; the immunoglobulins (Ig) were purified by affinity chromatography. Characterization of the antibody fraction by Western blot analysis showed a highly monospecific reaction with the three polymerase proteins. Spot immunobinding assay was used to compare the immunoreactivity of the monospecific polymerase antibodies with the P proteins of other influenza A subtypes and influenza B strains, revealing high immunoreactivity with the components of all influenza A strains and only insignificant reactivity with the components of the influenza B strains tested.

Key words: influenza virus; RNA-dependent RNA polymerase; anti-polymerase antibodies; dot immunobinding assay

Introduction

The RNA-dependent RNA polymerase of influenza virus is composed of three virion associated proteins PB1, PB2 and PA encoded by the three largest RNA segments of the virion (reviewed by Lamb and Choppin, 1983; McCauley and Mahy, 1983). The use of transcriptionally active influenza ribonucleoprotein (RNP) particles has shown that PB1 plays a role in both initiation and elongation of viral mRNA synthesis, PB2 is involved in the recognition and binding of capped host mRNA transcripts serving as primers for transcription. The function of PA is not yet clear but it has been assumed that PA links PB1 and PB2 together to form the polymerase complex (Ulmanen *et al.*, 1981; Nichol *et al.*, 1981; Blaas *et al.*, 1982*a*, *b*; Braam *et al.*, 1983).

The purification of RNA polymerase proteins in a functionally active form

is difficult because the amount of the three P proteins per virion is generally less than 1—3 % of total protein. Attempts to purify the influenza RNA polymerase were undertaken by Kawakami and Ishihama (1983) and Kato *et al.* (1985). They described the purification of a protein-RNA complex with the ability to cleave the capped mRNA and with the both initiation and elongation activities in mRNA synthesis.

Monospecific or monoclonal antibodies to influenza polymerase proteins should be useful probes in the analysis of the polymerase action. In a recent report the preparation of antisera to a specific fragment of each influenza polymerase protein was described. Rabbits were immunized with the polymerase fragment -β-galactosidase fusion protein synthesized in *E. coli* (Jones *et al.*, 1986). Detjen *et al.* (1987) prepared for each P protein an antiserum reacting with the hydrophilic region of the P protein.

In the present paper we describe the isolation of polymerase proteins of influenza virus A/PR/8/34 (H1N1) by electrophoresis either of dissociated virus or of RNP on polyacrylamide gel, followed by electroelution and Sepharose CL-6B chromatography. Monospecific antibodies were produced in rabbits and purified by affinity chromatography. The immunoreactivity of these antibodies was tested with P proteins of other influenza A subtypes and of influenza B virus strains.

Materials and Methods

Virus strains. Following strains of influenza virus were used: A/PR/8/34 (H1N1), A/Chile/1/83 (H1N1), A/Singapore/6/86 (H1N1), A/Krasnodar/101/59 (H2N2), A/Hongkong/1/68 (H3N2), A/Philippines/2/82 (H3N2), B/Hongkong/5/72, B/Singapore/222/79, B/USSR/100/83, B/Ann Arbor/1/86, and B/Greifswald/1/86. The virus strains were grown in 10-day-old fertile eggs purified by sucrose density gradient centrifugation and stored at 4 °C.

Preparation of ribonucleoprotein (RNP) complexes. Viral RNP complexes were prepared by treating purified influenza virions (A/PR/8/34) with the non-ionic detergent Nonidet P-40 (NP-40) followed by sedimentation in linear glycerol gradients according to Rochovansky (1976) and Schreier *et al.* (1988*b*). The three P proteins, NP protein, and a small amount of viral matrix protein and haemagglutinin were shown to be present in the RNP preparations.

Isolation of influenza polymerase proteins. (1) Polyacrylamide gel electrophoresis and electroelution: About 10 mg of whole virus or 2 mg of RNP of the influenza strain A/PR/8/34 were disrupted in 0.0625 mol/l Tris-HCl (pH 6.8) containing 1 % SDS, 10 % glycerol (w/w), 5 % 2-mercaptoethanol in a boiling waterbath for 10 min, and electrophoresed on a 1—2 mm thick preparative 7.5 % SDS-polyacrylamide slab gel according to Laemmli (1970). Electrophoresis was performed at room temperature for 8 hr at 120 V. Protein bands were visualised by cutting two thin strips from the sides of the gel and treating these with Coomassie brillant blue R250. The polymerase protein bands were cut from the gel and electroeluted using an ISCO electrophoretic concentrator, model 1750 (ISCO, Inc., USA) in 10 mmol/l Tris-HCl pH 9.2 and stored at 4 °C until immunization. (2) Sepharose CL-6B column chromatography: 10 mg of RNP of the influenza strain A/PR/8/34 were dissociated in 0.025 mol/l Tris-HCl pH 7.8, containing 0.2 % SDS, for 30 min at 37 °C. The disrupted RNP particles were applied to a Sepharose CL-6B (Pharmacia, Uppsala, Sweden) column (1.5 × 100 cm) in 0.025 mol/l Tris-HCl pH 7.8, containing 0.1 % SDS at 20 °C; 1 ml fractions were collected. The protein concentration was determined continuously by passing the effluent through a flow-through-photometer UVICORD S and registering on a 2 channel recorder 2210 (LKB producter, Sweden).

Preparation of antibodies. Rabbits (2 kg body weight) were immunized intramuscularly with 0.2 mg influenza polymerase protein mixture (PB1, PB2 and PA), emulsified in complete Freund's adjuvant, followed by two intravenous booster injections after 28 and 40 days with 0.1 mg polymerase protein. The serum was obtained by bleeding at day 56. The antiserum was purified by

affinity chromatography. The influenza proteins of the strain A/PR/8/34 with the exception of the polymerase proteins were coupled to CNBr-activated Sepharose 4B in coupling buffer ($NaHCO_3$/NaCl, pH 8.0) at 20 °C for 3 hr. The remaining active groups were blocked with 0.2 mol/l glycin, pH 8.0 and the excess of protein and the blocking agent was removed by washing with the coupling and acetate buffers (0.1 mol/l, pH 4.0, containing 0.5 mol/l NaCl). The antiserum was gently agitated with this influenza protein-Sepharose conjugate batchwise. The monospecific antiserum was further purified by protein-A-Sepharose (IgG fraction). CNBr-activated Sepharose 4B and protein-A-Sepharose were purchased from Pharmacia, Uppsala, Sweden.

Western blotting. Influenza proteins were electrophoresed on a 10 % SDS-polyacrylamide gel and transferred electrophoretically to nitrocellulose sheets (0.2 µm pore size, Satorius, BRD) using transfer buffer (0.2 mol/l Tris-HCl pH 8.8 with 20 % methanol). Electrotransfer was conducted for 16 hr at 250 mA at 12 °C. Proteins transferred to nitrocellulose sheets were stained with amido black 6B. The nitrocellulose sheet was blocked for 2 hr at 20 °C with blocking buffer (5 % (w/v) nonfat dry milk in phosphate buffered saline (PBS) containing 0.15 mol/l NaCl). Nitrocellulose strips were incubated with antiserum of the IgG fraction (2000-fold dilution) to influenza polymerase proteins in 3 % dry milk in PBS containing 0.3 mol/l NaCl and 0.1 % Tween 20 for 3 hr at 25 °C. After three washes with 0.3 mol/l NaCl, 0.1 % Tween 20 in PBS, the strips were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG. After washing, the strips were reacted with diaminobenzidine-H_2O_2.

Dot immunobinding assay. Antigens were obtained by detergent treatment of purified influenza A and B viruses (0.2 % SDS or 0.5 % NP-40 in PBS for 5 min at 37 °C. Antigen preparations containing 2 µg/µl total virus proteins were diluted in PBS (4—640-fold) and 1 µl was spotted onto nitrocellulose strips (0.2 µm). The strips were dried, saturated with blocking buffer (see Western blotting) for 2 hr and incubated with diluted IgG fraction (500-fold) to influenza polymerase proteins for 3 hr at 20 °C. The next steps were carried out as described for Western blotting.

Results

Isolation of polymerase proteins

Treatment of influenza virions with non-ionic detergents leads to solubilization of the lipid envelope without disruption of ribonucleoprotein (RNP) complexes. These RNP complexes which contain the viral RNA, the NP and the polymerase proteins (Bishop *et al.*, 1972) are important intermediates during the isolation of influenza polymerase proteins (PB1, PB2, PA). We used the influenza strain A/PR/8/34, which contains high amounts of RNA polymerase of human influenza A virus strains (Kawakami *et al.*, 1981). The procedure used to isolate the virion RNP complex was a modification of that described by Rochovansky (1976) (Schreier *et al.*, 1988*b*).

Sepharose CL-6B chromatography

RNP particles were dissociated with SDS, applied to a column of Sepharose CL-6B and eluted under denaturing conditions as described in Materials and Methods. As shown in Fig. 1, the polymerase protein eluted in a single peak before NP. The polyacrylamide gel electrophoregram indicates that the fractions 85—95 contain the polymerase proteins and a small amount of NP. The fractions 97—105 contain NP contaminated with a small amount of HA (Fig. 2). The RNA was eluted in the void volume. Attempts to separate the RNA and protein components in the presence of 1.2 mol/l NaCl did not yield good results. After incubation of RNP with 1.2 mol/l NaCl followed by Sepharose CL-6B column chromatography in the presence of 1.2 mol/l

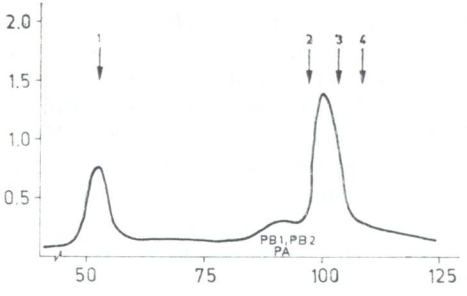

Fig. 1.
Sepharose CL-6B chromatography of dissociated RNP
The positions of molecular weight standards are indicated by arrows. (1) blue dextran; (2) bovine serum albumin 66 kDa; (3) egg albumin, 45 kDa; (4) chymotrypsinogen, 24 kDa; elution conditions are described in Materials and Methods.
Abscissa: fraction number; ordinate: arb. units at 280 nm

NaCl, the fraction of RNP was eluted in the void volume. Dissociation of RNP by glycerol gradient centrifugation in presence of high concentrations of NaCl was also unsuccessful.

SDS-polyacrylamide gel electrophoresis and electroelution

RNP particles or whole virus were disrupted by SDS under reducing conditions and electrophoresed on a 1—2 mm thick preparative SDS-polyacrylamide gel. The bands of the polymerase proteins PB1, PB2 and PA were cut from the gel in sections 5 × 150 mm. The proteins were electroeluted and concentrated using an ISCO electrophoretic concentrator. Co-electrophoresis of isolated polymerase proteins showed, that these were associated with small amounts of nucleoprotein, haemagglutinin and matrix protein.

Characterization of polymerase antibodies

Antisera to polymerase proteins were raised in rabbits. We immunized with PB1, PB2, PA in mixture according to a schedule described in Materials and Methods. The antisera were purified by affinity chromatography. Fig. 3 shows the Western blot analysis of the polymerase antibodies.

In order to compare the immunoreactivity of the monospecific antibodies (IgG fraction) to polymerase proteins of the influenza strain A/PR/8/34 with the polymerase proteins of other influenza virus strains dot immunobinding analysis was carried out. Purified influenza A virions of the subtypes H1N1, H2N2, H3N2 and influenza B virions were spotted onto nitrocellulose strips and incubated with anti-polymerase antibodies. The polymerase proteins of all influenza A subtypes reacted with the anti-polymerase antibodies to influenza virus A/PR/8/34. We were able to detect at least 0.1 ng of polymerase antigen. At this sensitivity of the assay no immunoreactivity was detected with any influenza virus B strain tested (Fig. 4).

Discussion

We report that monospecific antisera to all three polymerase proteins of the influenza strain A/PR/8/34 exhibit a high immunoreactivity with all

influenza A virus strains of the subtypes H1N1, H2N2, H3N2 while only insignificant immunoreactivity was detected with the influenza B strains. These data extend earlier reports about nucleotide sequences of the cloned cDNA copy of RNA segments which encode the polymerase proteins of influenza A virus strains (Bishop *et al.*, 1982*a*; Sivasubramanian and Nayak, 1982; Winter and Fields, 1982; Kaptein and Nayak, 1982; Roditi and Robertson, 1984; Bishop *et al.*, 1982*b*; Robertson *et al.*, 1984). A high degree of homology in polymerase primary structure was found among different influenza A virus strains. In a recent report the sequence of a cDNA clone which represented the full influenza B/Lee/40 virus genome RNA segment coding for PB1 protein has been published (Kemdirim *et al.*, 1986). This is the first complete sequence which has been obtained for any of the influenza B virus RNA segments which encode the polymerase proteins. Sequence comparison between the influenza A and B virus PB1 proteins revealed that there was a 61 % amino acid homology. The absence of immunologic reactivity of our antisera to the three polymerase proteins of an influenza A strain with those of influenza B strains corelates with the relative low sequence homology data of the PB1 proteins of influenza A and B strains. Recently we noted that cDNAs of RNA segments which encode the polymerase proteins of an influenza A subtype (A/Chile/1/83 H1N1) are also suitable as DNA probes for distinguishing between influenza A and B strains (manuscript submitted for publication).

Investigations of the inhibition of transcriptase (polymerase) activity of isolated RNP particles from influenza A and B strains by our anti-polymerase antibodies (IgG fraction) are in progress. First results suggest an inhibition of the transcriptase activity of the RNP particles of influenza A strains by the homologous IgG fraction. In contrast, RNP fractions of influenza B strains were not inhibited by the IgG fraction to polymerase of the influenza strain A/PR/8/34 (Schreier *et al.*, 1988*a*).

Our data about immunoreactivity suggest that the homology of the other P proteins (PB2 and PA) on influenza A and B strains may be in the same range or lower as PB1.

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Legend to Figures (Plates XXXV–XXXVII):

Fig. 2. PAGE of the polypeptides of the A/PR/8/34 strain of influenza virus on a 11 % slab gel stained with Coomassie brillant blue. Lane: (1) molecular weight standards: (1) phosphorylase, 96 kDa; (2) bovine serum albumin, 66 kDa; (3) egg albumin, 45 kDa; (4) chymotrypsinogen, 24 kDa; lane (2) virion; lane (3) RNP; lane (4) nucleoprotein and lane (5) polymerase proteins.

Fig. 3. Characterization of antibody to influenza polymerase protein by Western immunoblotting with SDS-PAGE separated proteins of the influenza strain A/PR/8/34, transfer: (1) +SDS, (2) −SDS.

Fig 4. Binding specifity of anti-A/PR/8/34 polymerase antibodies to lysates from various influenza virus strains in dot immunobinding assay. Virus strains: A/PR/8/34 (I 1—4), A/Chile/1/83 (II 1—4), A/Singapore/6/86 (III 1—4), A/Krasnodar/101/59 (IV 1—4), A/Hongkong/1/68 (V 1—4), A/Philippines/2/82 (VI 1—4), B/Hongkong/5/72 (I 5—8), B/Singapore/222/79 (II 5—8), B/USSR/100/83 (III 5—8), B/Ann Arbor/1/86 (IV 5—8), B/Greifswald/1/86 (V 5—8), Sendai (VI 5—8); the total virus protein amounts (lysis of virus with 0.5 % NP-40) were for each strain per spot 500 ng, 125 ng, 31 ng and 8 ng.