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THE INFLUENCE OF THE ISOLATION TECHNIQUE OF INFLUENZA VIRUS NUCLEOPROTEIN ON ITS ANTIGENIC PROPERTIES

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Summary. — ELISA has been used to study the antigenic properties 1. of influenza virus nucleoprotein (NP-1) isolated from virions with the help of preparative polyacrylamide gel electrophoresis (PAGE); 2. of virion ribonucleoprotein (NP-2), and 3. of NP structures prepared by dissociation of ribonucleoprotein into RNA and protein in sucrose gradient containing NaCl (NP-3). The investigation of immunologic cross-reactivity has shown complete identity of NP-2 and NP-3 and their striking difference from NP-1. In contrast to NP-2, NP-3 was not contaminated by other virus antigens, it was a good immunogen and could be used for preparation of monospecific antisera of high titre. NP-1 did not induce a high antibody response, however, like NP-2 and NP-3, it retained its capacity to react with antisera to native virus. Owing to its simple production and high yield, this protein can be used in serodiagnosis for testing the antibody level against NP-protein in convalescent sera.

Key words: ELISA; influenza virus; nucleoprotein

Introduction

More attention has recently been focused on the study of immunochemical properties of influenza virus isolated proteins. This is largely due to the development of new enzyme immunoassay (EIA) systems for rapid diagnosis and serodiagnosis of influenza and acute respiratory diseases (ARD), using individual polypeptides contained in the virion and monospecific antisera against these polypeptides (Vorkunova *et al.*, 1983; Shenderovich *et al.*, 1984; Masihi, Lange, 1980; Khan *et al.*, 1982*a*, *b*). As compared to the methods using intact virus antigen and polyclonal immunoglobulins to all virion components, these enzyme immunoassay systems exhibit high specificity in rapid diagnosis, and when applied for serodiagnosis provide more information about the spectrum of detected antibodies reacting with virus proteins.

The influenza virion consists of several antigenically and structurally different polypeptides. These are, in first instance, surface glycoproteins —

haemagglutinin and neuraminidase that undergo a slight, but constant changes in the course of antigenic drift. The internal proteins like matrix (M1) protein and nucleoprotein (NP) are characterized by relative antigenic stability. Since M1 and NP proteins are typespecific (significantly differ antigenically only in viruses of different types — A, B and C), it can be expected that diagnostic EIA system using these polypeptides as antigens will allow to differentiate influenza within the range of ARD agents.

Since the available methods of virus macromolecule isolation require the use of different solubilizing agents and physicochemical factors, it is very important to retain their immunologic activity during isolation. The purpose of this paper has been to investigate the influence of isolation method on antigenic and immunologic properties of influenza virus NP protein.

Materials and Methods

Viruses. Influenza virus strains A/Kiev/59/79 (H1N1), A/Bangkok/1/79 (H3N2), and recombinant strain MRC-11 (A/Port Chalmers/1/73 — A/PR/8/34) (H3N2) have been used. The viruses were grown in the allantoic cavity of 10-day-old chick embryos, purified and concentrated as described by Kharitonenkov *et al.* (1977).

Isolation of NP protein by preparative PAGE. Virus A/Kiev/59/79 was destroyed by 2.5% sodium dodecyl sulphate (SDS) at 37 °C for 1 hr, then it was applied on the 7.5% polyacrylamide gel (PAG) column 75 mm high and 16 mm in diameter. Electrophoresis was carried out under non-reducing conditions for 17 hr at 5 mA and then for 2 hr at 20 mA. The gel was removed from the tube and a band containing NP protein was cut out. Preliminary localization of NP-protein was determined by amido black staining of the analogous gel. The protein was removed from the gel by electroelution for 2 hr at 20 mA.

Virion ribonucleoprotein (RNP) was isolated from influenza virus MRC-11 subviral particles produced after bromelaine-treatment of virions according to Sokolova *et al.* (1982). Basic structural protein contained in this RNP will be further referred to as NP-2.

NP protein from virion RNP (NP-3) was isolated by dissociation of influenza virus MRC-11 RNP in linear (15—30%) sucrose concentration gradient prepared in 0.01 Tris-HCl buffer pH 7.4 containing 1.1 mol/l NaCl (Sokolova *et al.*, 1983). Since the above-described technique provides only a very low yield of NP protein (1—2% of initial virus material), we used the recombinant MRC-11 — a strain of higher yield for its isolation.

Protein concentration was measured according to Lowry *et al.* (1951).

Preparation of antisera against NP-proteins. 100 μg of each protein (NP-1, NP-2, or NP-3) in complete Freund's adjuvant was administered subcutaneously to rabbits at 28 days intervals. The third immunization was carried out with the same amount of the protein without adjuvant. Blood was collected on day 7 after the last immunization. Triton X-100 was added to NP-1 protein before immunization to a final concentration of 10%. Because of the low aggregation number and low molecular weight of SDS molecules (Berezin *et al.*, 1986), its micelles were removed from the protein preparation on a Sephadex G-100 column (1.5 × 30 cm). After the elution with saline, the NP-1 protein was diluted to a concentration of 100 μg/ml.

Monoclonal antibodies to haemagglutinin and neuraminidase of influenza virus A/MRC-11 were kindly provided by Dr. A. Douglas and Dr. J. J. Skehel (National Center of Medical Research, London, England). Monoclonal antibodies to NP protein were kindly supplied by S. S. Yamnikova (The D. I. Ivanovsky Institute of Virology, U.S.S.R. Academy of Medical Sciences).

Preparation of antiserum to M1 protein. M1 protein of influenza virus A/Kiev/59/79 was isolated by preparative PAG electrophoresis, as described by Zagidullin *et al.* (1985). About 100 mg of protein were taken for each immunization. Immunization procedure was the same as for NP proteins.

Enzyme-linked immunosorbent assay (ELISA) was made according to a somewhat modified method of Voller *et al.* (1976). Antigens were absorbed overnight at 4 °C from phosphate buffer solution (PBS) to polystyrene microplates Inotech-ELISA (U.S.A.). The panels were then washed

with PBS containing 0.05% Tween 20 (PBS-Tween); PBS-Tween with 0.5% bovine serum albumin (BSA) was added into each well (PBS-Tween-BSA) and kept for 1 hr at 37 °C. After the removal of PBS-Tween-BSA, 0.1 ml of immune antiserum was added into the wells with experimental samples, then normal serum was added to the control samples at equal dilutions prepared in PBS-Tween-BSA and incubated for 1 hr at 37 °C. After three washings, 0.1 ml of protein A conjugated with horseradish peroxidase (Olaine Chemicals plant, U.S.S.R.) prepared according to the method of Wilson and Nakane (1978) was added into the wells at a 1 : 5000 dilution and kept for 1 hr at 37 °C. Orthophenylenediamine solution (OPD) (Sigma, U.S.A.) in 0.06 mol/l Na-phosphate buffer pH 5.8 (50 μg of OPD per 100 ml of the solution + 10 μl of 30% H_2O_2) was used as substrate. After 20 min, the reaction was stopped by addition of 0.05 ml of 4 N H_2SO_4; the optic density (OD) was measured in Multiscan (Flow, England) at the wavelength of 490 nm.

Results

Fig. 1-I shows the results of 10 % PAG electrophoresis of influenza virus MRC-11 proteins (1), native RNP (2) and NP protein (3) prepared from RNP by dissociation. It can be seen that electrophoretic mobilities of free NP protein and the RNP complex were the same; the preparation was not contaminated by other proteins. Purity of NP protein was tested by ELISA technique using monoclonal antibodies to haemagglutinin and neuraminidase of influenza virus MRC-11 as well as a polyclonal monospecific antiserum

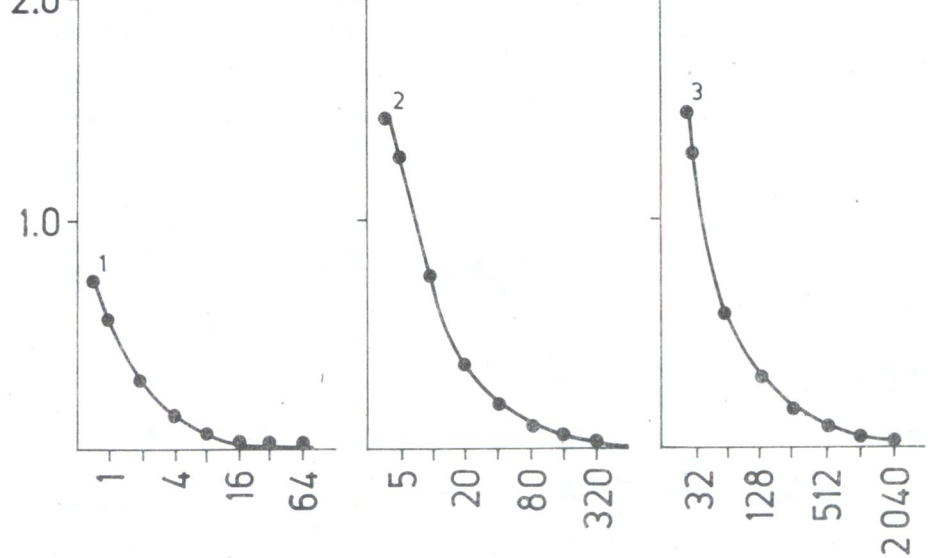

Fig. 2.

Titration curves of rabbit antisera I, II, and III against proteins NP-1 (1), NP-2 (2), and NP-3 (3), respectively.

Protein concentrations — 1 μg per well. Abscissa — serum dilutions: for I — $\times 10^2$; for II — $\times 10^4$; for III — $\times 10^3$; ordinate — OD_{490}.

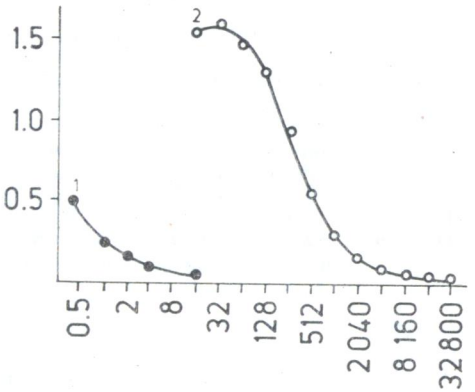

Fig. 3.
Titration curves of rabbit antisera I (1) and III (2) against the virus A/Bangkok/1/79 (1.5 μg per well) disrupted before adsorption by freezing and thawing.
Abscissa: serum dilution ($\times 10^2$); ordinate: OD_{490}.

to M1 protein of influenza virus A/Kiev/59/79. The results have shown the absence of any contamination with these proteins in the NP-3 preparation. Meanwhile, in spite of the absence of additional bands in analytical electrophoresis, the RNP preparation appeared to contain small amounts of haemagglutinin and M1 protein when tested by ELISA.

Fig. 1-II shows the electrophoregrams of influenza virus A/Kiev/59/79 (2) and NP protein (1) isolated from this virus by preparative PAG electrophoresis. Occasionally, NP-1 protein isolated by this method was slightly contaminated with M1 protein, which was often undetectable by PAG electrophoresis, but could be demonstrated in ELISA. The admixture of M1 protein was removed from NP-1 protein preparation with the help of anti-M1-serum immobilized on Protein-A Sepharose CL-4B, as described by

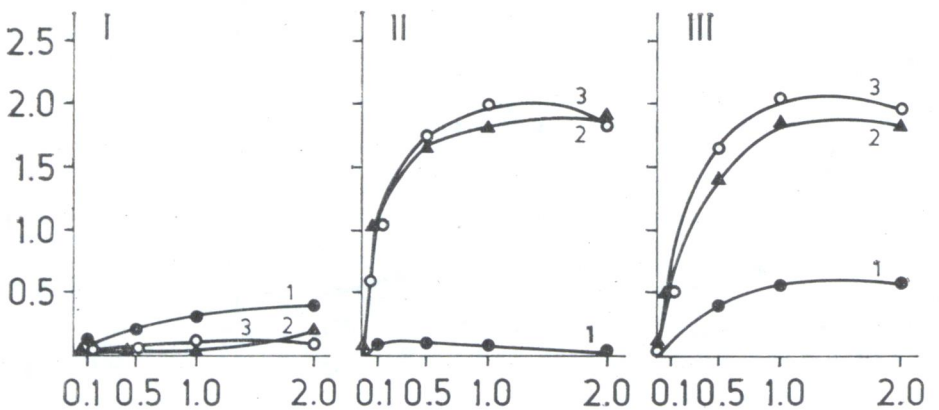

Fig. 4.
Immunologic cross-reactivity of proteins NP-1 (I), NP-2 (II), and NP-3 (III) and their titration with antisera (1 — 1 : 100; 2 — 1 : 16 000; 3 — 1 : 25 000).
Abscissa: protein concentration in μg per well; ordinate: OD_{490}.

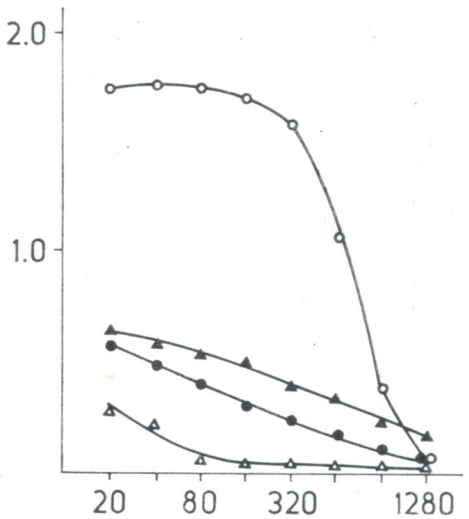

Fig. 5.
Titration curves of monoclonal antibodies to NP protein against different antigens
● — virus MRC-11; ▲ — virus A/Kiev/59/79; ○ — protein NP-3; △ — protein NP-1. Virus concentration — 2μg per well; NP protein concentration — 0.5 μg per well. Abscissa: monoclonal antiserum dilution ($\times 10^3$); ordinate: OD_{490}.

Zagidullin *et al.* (1985). The protein unadsorbed on the column was collected and tested by ELISA using anti-M1-serum. The results have shown that after additional affinity purification, the NP-1 preparation appeared free of any contaminating M1 protein.

Fig. 2 presents the titration plots of rabbit antisera I, II and III prepared against proteins NP-1, NP-2 and NP-3, respectively. This titration was carried out with homologous antigens. It can be seen that sera II and III have a very high titre of antibodies against NP protein (1 : 800,000 and 1 : : 1,024,000, respectively) indicating that the immunogenic properties of proteins NP-2 and NP-3 had been well preserved. Antisera against NP-1 had rather a low antibody titre (1 : 800), thus this protein had a much lower immunogenic activity, as compared to proteins NP-2 and NP-3. Low chromophore responses obtained during the titration of serum I are unlikely to result from lower sorption of NP-1 protein to polystyrene, as compared to proteins NP-2 and NP-3, for similar plots have been obtained during the titration of sera I and III against the whole virus previously destroyed by freezing and thawing (Fig. 3). Antisera I and III did not react with other viral proteins, whereas antiserum II contained a small amount of antibodies to haemagglutinin and M1 protein.

The findings obtained in the study of immunologic crossreactivity of proteins NP-1, NP-2 and NP-3 are given in Fig. 4. It shows that the isolated NP and the NP within the RNP are totally identical and react well with both, homologous and heterologous antiserum. It can also be seen from Fig. 4 that chromophore responses in ELISA for both proteins were dependent on the concentration of the sorbed antigen and that minimal detectable quantity of NP protein equaled to 5—10 ng. Within protein concentration range from 0 to 50 ng a linear correlation exists between the amount of the

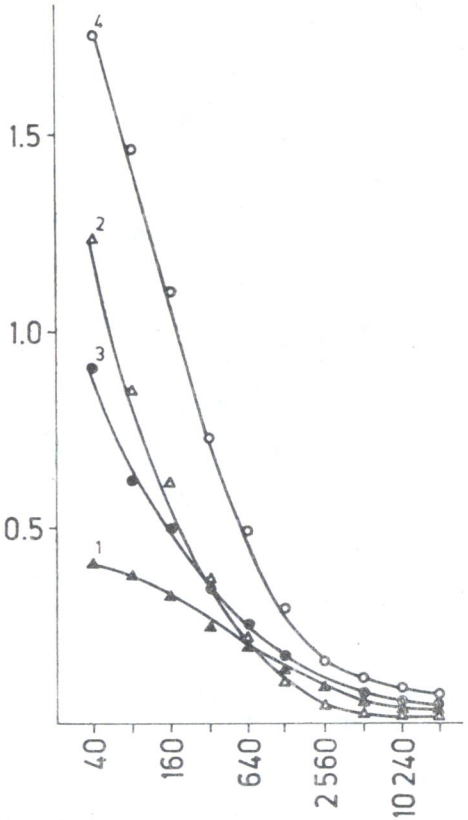

Fig. 6.
Titration of two sera taken from influenza convalescents against proteins NP-1 (1, 2) and NP-3 (3, 4).
Abscissa: serum dilution; ordinate: OD_{490}.

sorbed antigen and optical density (OD). At higher protein concentrations the plot of the OD against concentration reached the saturation point and then a plateau level. As evident from Fig. 4, protein NP-1 reacted only very poorly with antisera II and III, whereas serum I gave very low chromophore responses with proteins NP-2 and NP-3. This seems to indicate a significant change of the secondary-quaternary structure of NP-1 protein in the course of its isolation from SDS-PAG.

The differences in the secondary-quaternary structure of the isolated proteins have also been evident from the study of their interaction with monoclonal antibodies against NP of influenza virus A/Chaika/Kazakhstan/470/79 (H1N1). Fig. 5 shows the titration plots of monoclonal antibody F-81 with viruses MRC-11, A/Kiev/59/79 and with proteins NP-3 and NP-1 isolated from them. As it is seen from Fig. 5, the titration of monoclonal antibodies was virtually identical with both viruses; they reacted well with NP-3 and gave very low chromophore responses with NP-1.

The low level of cross-reactions between NP-1 and NP-3 when tested with "heterologous" antisera, as well as dramatic dissimilarities in their

Table 1. Chromophore responses obtained in ELISA during interaction of viruses A/Kiev/59/79, MRC-11 and A/Bangkok/1/79 with antisera prepared against proteins NP-1 and NP-3

Virus	Antiserum to protein NP-1 (diluted 1 : 100)	Antiserum to protein NP-3 (diluted 1 : 70,000)
A/Kiev/59/79	0.25+	0.65
MRC-11	0.53	0.90
A/Bangkok/1/79	0.34	0.67

+ OD_{490}

interactions with monoclonal antibodies are likely to indicate that the two isolation methods used damaged different antigenic determinants of the nucleocapsid protein.

Retention of the native structure and of immunologic activity is an important prerequisite for making a method of virus protein isolation advantageous over other methods. For the assessment of the immunologic activity of proteins NP-1 and NP-3 the sera taken from influenza convalescents were titrated against these proteins. Two sera with about the same amount of antibodies against haemagglutinin, i.e. having equal haemagglutination inhibition titres with the virus A/Bangkok/1/79 have been used. The results of titration are presented in Fig. 6. It can be seen that both proteins have rather a high immunologic activity, although it was better retained in NP-3.

We further tried to determine which of the protein preparations was structurally most similar to the native NP protein within the influenza virions. Therefore, we compared the chromophore responses upon interaction of whole virus with antisera I and III at appropriate dilutions chosen in correspondence with their titration plots (see Fig. 2), assuming that they contained equal amounts of antibodies when during titration on homologous antigens their dilutions gave equal ODs. Thus, for example, a 1 : 100 dilution of serum I was put in correspondence with a dilution 1 : 64,000 of serum III (OD = = 0.6 OU). It can be seen from Table 1 that the chromophore response obtained for antiserum III was about twice as high as the chromophore response of antiserum I, allowing to conclude that protein NP-3 was less altered than protein NP-1.

Discussion

It has been shown earlier that ELISA can be successfully used for determination of immunologic analogy of proteins differing in their amino acid sequence (Conroy, Esen, 1984; Hoffmann, Van Reygenmortel, 1984). Our investigations have shown that this method can be also used for testing of the conformational dissimilarities of proteins. It is also evident from this paper that the antigenic properties of influenza virus NP are closely dependent on the method of its isolation.

Ionic detergents, such as SDS, used for preparative isolation of the proteins from PAG, are potent denaturing agents. However, as we have previously shown, the application of SDS for isolation of influenza virus M1 protein from PAG is not followed by significant changes of its antigenic properties, so that it could be used for the production of hyperimmune monospecific antisera (Zagidullin *et al.*, 1985). The treatment of Sendai virus with 4 % SDS in the course of the isolation of M1 protein by reversed phase high-performance liquid chromatography (HPLC) was also without effect on its immunologic activity (Welling *et al.*, 1984). The antibody-producing capacity of influenza virus NP protein isolated in the presence of SDS was significantly decreased and it raised an antiserum showing low antibody titres (see Fig. 2, curve I). The method of NP protein isolation in which detergents are used only at the stage of RNP isolation seems to be milder and better preserves the native structure of the protein. Indeed, all the previously obtained electron microscopic findings concerning this protein indicated that it exists in the form of structures morphologically similar to native RNP (Sokolova *et al.*, 1983). According to the data presented here, the structure of NP protein isolated from RNP is essentially identical to that of the protein present within it (Fig. 3). Moreover, alike to RNP, NP-3 is highly antigenic producing an antiserum revealing antibody titre equal to 1 : 800,000 when tested in ELISA (Fig. 2). It should be emphasized that this antiserum was not contaminated with antibodies to other virus proteins.

Protein NP-1 isolated from SDS-PAG is significantly different from protein NP-3 in its secondary-quaternary structure, as evident from a low immunologic cross-reactivity between them, as well as from different character ot their interaction with monoclonal antibodies (Figs. 4 and 5). Although the presence of SDS causes a significant denaturation of the NP protein molecule, some of antigenic determinants seem to retain their native state. Antiserum produced against this protein interacts with NP protein present in the virion (Table 1). Furthermore, the sera of human influenza convalescents react with NP-1 and NP-3 at similar antibody titres (Fig. 6).

Our data suggest that the method of influenza virus NP protein isolation should depend on the purpose of the investigation. The isolation of NP protein from virion RNP by dissociation in sucrose gradient in the presence of NaCl is rather tedious and the protein yield is not higher than 5 % of the theoretically calculated value. However, unlike RNP, this protein is not contaminated by other virus antigens and can be successfully employed for the preparation of monospecific antisera of high titre, and also, for example, as antigen for monoclonal antibody testing in ELISA.

The procedure of influenza virus NP protein isolation by preparative PAG electrophoresis is very simple, the protein yield is about 50 %. Moreover, the matrix protein with unchanged antigenic properties can be simultaneously isolated from the same gel (Zagidullin *et al.*, 1985). Our data seem to indicate that such NP protein is inappropriate antigen for antiserum preparation, although, it can be utilized for determination by ELISA of antibodies to NP protein in convalescent serum.

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Legends to the figures (Plate XLVI):

Fig. 1. Electrophoregrams of virus-specific polypeptides in 10% PAG.
I — influenza virus MRC-11: intact virions (1), RNP (2), and protein NP-3 isolated from RNP (3); II — influenza virus A/Kiev/59/79: intact virions (2) and protein NP-1 isolated from them (1).