Acta virol. 33 : 1—7, 1989

ANTIGENIC REACTIVITY OF MATRIX PROTEIN AND NUCLEOPROTEIN OF INFLUENZA VIRUS AS DETECTED BY EIA AFTER DISSOCIATION WITH DIFFERENT DETERGENTS

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Received March 6, 1986

Summary. — Solid phase enzyme-immunoassay (EIA) was employed to assess the antigenic reactivity of matrix protein (M) and nucleoprotein (NP) of influenza A virus adsorbed to polystyrene in the presence of different detergents such as β-octaglucoside (OG), Triton X-100, Tween-20, sodium dodecylsulphate (SDS), sodium deoxycholate (Doch-Na), Nonidet P-40 (NP-40), and sarcosyl at concentrations ranging from 0 to 2 %. The antigenic reactivity of NP was the highest in the absence of detergents. For M protein, Doch-Na, SDS, NP-40 and sarcosyl of 0.05—0.1 % enhanced the chromatophoric response in EIA 1.5—2 times. In contrast, the antigenic reactivity of M protein remained unchanged after OG or Triton X-100 treatments, and it decreased in the presence of Tween-20.

Key words: *EIA*; *influenza A virus*; *matrix protein*; *nucleoprotein*; *detergents*

Introduction

Solid phase EIA is most frequently used for diagnosis of virus diseases, especially of influenza. Special attention has been paid to developing of EIA systems for testing the presence of type specific M and NP components of influenza virions in the clinical specimens (Vokrunova *et al.*, 1983; Shenderovich *et al.*, 1984). One of the simplest procedures for solid phase EIA employed direct sorption of viral antigens to the polystyrene plate. In such case, however, the conditions of virion destruction are of special importance; they allow optimal detection of internal proteins without influencing their sorption. Solid phase EIA may reveal the M protein of influenza virions after direct sorption of purified virus to polystyrene (Kharitonenkov, 1981), but such procedure does not allow the monitoring of virion destruction during

sorption and probably does not provide standard conditions for effective uncoating of virions to assure maximal sensitivity of the technique.

We followed the effect of different detergents used for dissociation of influenza virions on the antigenic reactivity of M and NP in solid phase RIA.

Materials and Methods

Viruses. The following strains of influenza virus were used: A/Bangkok/1/79(H3N2), A/Philippines/2/83(H3N2), A/Chili/1/83(H1N1), recombinant strains MRC-11 /A/Port Chalmers/1/73 (H3N2) – A/PR/8/34/, X-31 (H3N2), A/Aichi/2/68 – A/PR/8/34/, and Sendai virus strain 960. The viruses were grown in the allantoic cavity of 10-day-old chick embryos, concen trated and purified as described by Kharintonenkov *et al.* (1977).

M protein purification. M protein was isolated by preparative electrophoresis in polyacrylamide gel (PAG) as described by Zagidullin *et al.* (1985). Briefly, the virions were dissociated with SDS at 37 °C for 1 hr, and the material was applied on the tube of the 7.5 % gel 80 mm high and 16 mm diameter. The electrophoresis was performed under nonreducing conditions for 17 hr, at 6 mA current and then for 3 hr at 30 mA. In the absence of substances regenerating bisulphidic bonds, the mass of M protein was considerably lower than that of other virion proteins so that it left the gel earlier than other virion polypeptides, i.e. within 2–3 hr after the starting dye. The escaped M protein was collected into a dialysis bag fastened at the end of the gel-containing cylinder. The M protein obtained was not contaminated with other viral polypeptides.

The RNP of influenza virus was isolated according to Sokolova *et al.* (1983) from subviral particles obtained after treating the virions with bromeline for 17 hr at 37 °C (1 mg enzyme per 2 mg virion protein). The virus was resuspended in 0.1 mol/l Tris-HCl buffer pH 7.4, containing 1 mmol/l EDTA. The subvirion components were sedimented for 1 hr at 30 000 rev/min in SW-65 rotor (Beckman L5-65 centrifuge). The pellet was resuspended in 0.01 mol/l Tris-HCl pH 7.4 containing 0.1 mol/l NaCl to a final concentration of 1 mg/ml protein and treated with the mixture containing Doch-Na (0.1 %), NP-40 (1 %), 0.2 mol/l urea and 0.01 mol/l mercaptoethanol for 5 min at 37 °C. The mixture was cooled and immediately layered over a continuous glycerol (15–30 %) gradient prepared in 10 mmol/l Tris-HCl buffer pH 7.4 containing 0.1 mol/l NaCl, 0.02 % Doch-Na, 0.2 mol/l urea and 10 mmol/l 2-mercaptoethanol. Centrifugation was made on L5-65 centrifuge in SW-27.1 rotor at 18 000 rev/min for 17 hr at 4 °C. The fractions containing ribonucleoprotein were pooled, dialysed for 48 hr against 10 mmol/l Tris-HCl buffer pH 7.4.

NP was isolated by separation of virion RNP in a continuous gradient of 15–30 % sucrose concentration prepared in 0.01 mol/l Tris-HCl buffer pH 7.4 containing 1.1 mol/l NaCl. Within 4–6 hr before layering the sample over the gradient, NaCl was added to final concentration of 1.1 mol/l. After centrifugation in L5-65 ultracentrifuge rotor SW-27.1 at 24 000 rev/min for 17 hr at 4 °C the fractions were collected using Ultrorac (LKB) collector. In parallel, RNP separation has run from virions labelled with ^{3}H-uridine and ^{14}C-amino acid hydrolysate in order to determine the position of fractions containing protein and RNA. The fractions corresponding to NP were collected and dialysed against 0.05 mol/l phosphate buffer pH 7.2. No contaminating proteins were found in the NP preparation subjected to PAGE (Sokolova *et al.*, 1983).

Protein concentration was determined according to Lowry *et al.* (1951). Bovine serum albumin (Sigma) was used as standard.

Sera against influenza strain MRC-11 M and NP proteins were raised in rabbits. Each rabbit was given 30 μg M and 100 μg NP at 30-day-intervals with complete Freund's adjuvant. On day 7 after second immunizing dose the same amount of antigen was inoculated without adjuvant. Blood was drawn 7 days after last immunization. The immune sera contained no antibodies to other virus proteins.

Solid phase EIA was modified according to the procedure of Voller *et al.* (1977), using polystyrene microplates (Inotech ELISA, U.S.A.). Antigen was sorbed at concentration of 1 μg per well in phosphate buffered saline pH 7.4 (Voller *et al.*, 1977) overnight at 4 °C. The wells were washed 3 times in phosphate buffer containing 0.05 % Tween-20 (PBS-Tween) and filled with 200 μl of PBS-Tween containing 0.5 % bovine serum albumin (BSA) for 1 hr at 37 °C. After removing PBS-Tween-BSA, 200 μl of immune or control serum was added in parallel dilutions prepared

Table 1. Determination by solid phase EIA of the antigenic reactivity of M protein within influenza virions after sorption to the polystyrene carrier at different temperatures

Sorption temperature	OD_{490}	
	A/Bangkok/1/79	A/Chili/1/83
4 °C	0.39 ± 0.04	0.6 ± 0.08
37 °C	0.38 ± 0.04	0.52 ± 0.08

Notice: OD_{490} from 7 determinations, mean values and their standard deviations

in PBS-Tween-BSA. After 3-times washing with PBS-Tween anti-rabbit conjugate diluted 1 : 4000 (peroxidase labelled, olaine chemicals, U.S.S.R) or 1 : 1000 (alkaline phosphatase labelled) were added and incubated at 37 °C for 1 hr. After 3-times washing each well was given 200 µl of orthophenylendiamine solution (Sigma) in 0.06 mol/l PBS pH 5.8 (50 µg per 100 ml plus 10 µl 30 % H_2O_2) or 2-nitrophenylphosphate (1 µg/ml) in 10 % dietanolamine buffer. Within 30 min incubation at 37 °C in the dark the reaction was stopped by adding either 100 µl of 4 mol/l H_2SO_4 or 50 µl 3 mol/l NaOH (when alkaline phosphatase labelling had been performed). The OD was measured at 490 nm or at 405 nm.

Reagents. SDS "Sigma", Sarcosyl NL-97 "Ciba Geigy", Doch-Na "Calbiochem"; nonionic detergents: Triton X-100 "Sigma", NP-40 from "BDH", Tween-20 "Ferak", β-octylglucoside (U.S.S.R.). Before application, equal amounts of virus were incubated with corresponding detergent concentrations for 30 min at room temperature.

Results and Discussion

Recently we have shown (Busse *et al.*, 1986) that during the adsorption to polystyrene virus particles were not destroyed as it was suggested by some authors (van Wyke *et al.*, 1984). The accessibility of influenza virus internal antigens to the antibodies occurs due to the presence of partly or completly

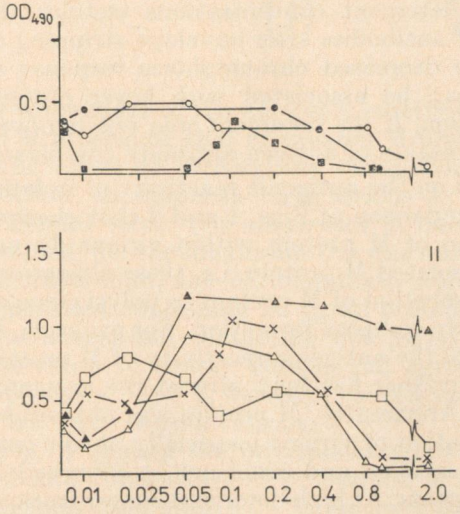

Fig. 1.
Relationship of chromophoric responses of MP protein in EIA using influenza virus A/Bangkok/1/79 with anti-M serum
Detergents: β — (octaglucoside (●——●), Triton X-100 (○——○), Tween-20 (■——■) on the part above; Doch-Na (▲——▲), SDC (□——□), Nonidet P-40 (×——×), and sarkosyl (△——△) on part II.
Abscissae: detergent concentrations (%); ordinates: OD values at 490 nm.
Virus concentration: 1.5 µg/well; antiserum dilution 1 : 2,500.

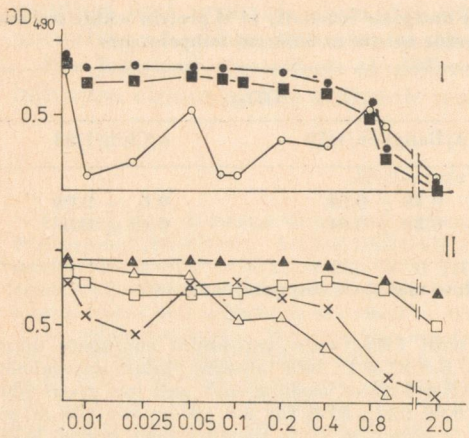

Fig. 2.
Relationship between M protein antigen reactivity and detergent concentration
Detergents: β-octaglucoside (●———●) Triton X-100 (○———○), Tween-20 (■———■) on part I; Doch-Na (▲———▲) NP-40 (×———×), sarkosyl (△———△), and SDS (□———□) on part II. Protein concentration 0.5 μg/well. For other explanations see Legend to Fig. 1.

destroyed virions in virus suspension. Table 1 shows the results of solid phase EIA employing influenza virus M protein and anti-M serum after virus sorption for 17 hr at either 4 or 37 °C. The adsorption temperature had no effect on the chromophoric response in EIA.

Fig. 1 shows the graphical relationship between antigenic reactivity of influenza A/Bangkok/1/79 M protein and detergent concentrations with various detergents used for virion dissociation. β-octoglycoside and Tween-20 had no effect on the chromophoric response in EIA at any concentration used, while Triton X-100 at concentrations 0.01—0.05 % revealed OD values close zero. Other detergents shown in Fig. 1 caused 1.5—2 times increased M reactivity. Increased OD at higher detergent concentrations testifies the enhanced availability of M protein for antibodies after envelope stripping or perhaps the solubilization of M. The decreased chromophoric response at increased detergent concentration may be associated with lower antigen adsorption in their presence; in addition, if the detergent was not removed by washing it might influence the formation of antigen-antibody complexes.

Fig. 2 shows the effect of detergents on the antigenic reactivity of isolated M protein. It can be seen from the comparison of Figs. 1 and 2 that changes in chromophoric response at detection of M protein within virions do not always correlate with the changes of isolated M protein, i.e. these alterations cannot be explained only by lower adsorption of M protein to polystyrene or by interfering with the antigen-antibody complex formation. For example, in the presence of 0.01—0.05 % Tween-20 the antigenic reactivity of M protein has remained practically unchanged, neither has been altered the antigenic reactivity of virion bound M protein. Apparently, M protein was not solubilized during detergent-induced dissociation of virions (especially at low concentrations), thus it remained lipid-associated and could not be adsorbed to the polystyrene in the presence of detergents. At high detergent concentrations

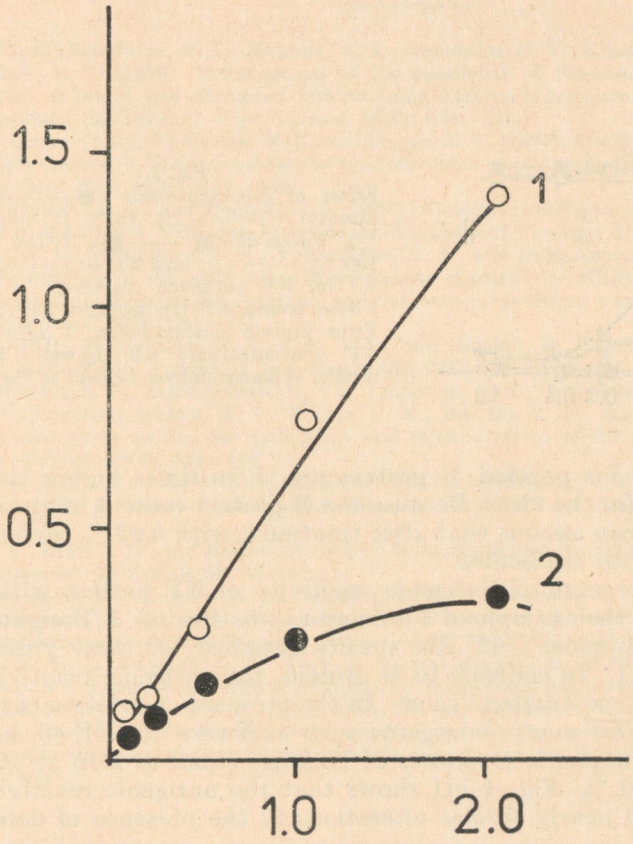

Fig. 3.
Relationship of chromophoric responses in EIA from M protein concentration (1) and virus concentration (2) The virus was treated with 0.05 % sarcosyl.
Abscissa: M-protein (1) or virus (2) concentrations, respectively (in μg); ordinates: OD values at 490 nm.

the alterations of the antigenic reactivity of virion bound and then dissociated M protein or that of isolated M protein were completely similar.

The effect of sarcosyl on virion bound M protein was investigated, in addition, on influenza virus strains A/MRG-11 and A/Chili/1/83 as well as on Sendai virus. For all viruses in question practically similar curves were obtained, i.e. the maximum OD was observed at concentrations 0.05—0.1 %.

Furthermore, the chromophoric responses obtained when treating virus suspensions containing 30 μg/ml protein with 0.05 % sarcosyl as well as the virus suspension containing 600 μg/ml protein with 1 % sarcosyl (diluted afterwards 20 times) were in full agreement (0.71 and 0.65, respectively). Apparently, 0.05 % sarcosyl concentration seems sufficient for virion lysis at low virus concentrations.

Fig. 3 depicts the interrelationships between chromophoric responses of EIA and concentrations of purified M protein (curve 1) as well as of virus concentrations after 0.05 % sarcosyl treatment (curve 2). As seen from this

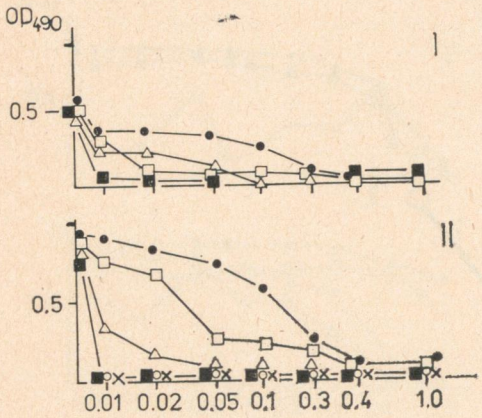

Fig. 4.
Effect of β-octaglucoside (●———●), sarcosyl (△———△), SDS (□———□), Tween-20 (■———■), Triton X-100 (○———○), and NP-40 (×———×) on NP antigenic reactivity
Virion bound NP (I); isolated NP (II). Virus protein concentration: 1 μg/well; NP concentration: 0.5 μg/well. For further explanations see Legend to Fig. 1.

Figure, the OD values for purified M protein are three times higher than the OD value obtained for the virus. Because the M protein content in virions amounts 25—30 % we can assume that after treatment with 0.05 % sarcosyl this protein interacts with antibodies.

The influence of detergents on antigenic reactivity of NP protein within virions was followed on the example of 3 influenza virus strains: A/Bangkok/1/79, X-31, and A/Philippines/2/82. The results obtained for these viruses were identical (Fig. 4—I). In contrast to M protein, the antigenic reactivity of NP is the highest for non-treated virions. In the presence of detergents the OD decreases, whereas for many detergents such as Tween-20, NP-40, and Triton X-100 the chromophoric responses of EIA are equal to zero at concentrations already 0.01 %. Fig. 4—II shows that the antigenic reactivity of purified NP revealed nearly similar alterations in the presence of detergents.

Following the effect of detergents on antigenic reactivity of internal influenza virus proteins we found that the reactivity of NP was higher in the absence of detergents in contrast to that of M protein. The possible increase of NP amounts in the presence of detergents is apparently compensated by its lower adsorption capacity to polystyrene.

M protein is likely to remain virion associated after virion dissociation, therefore, introduction of detergent and M protein solubilization enhances its reactivity. Detergents such as sarcosyl, Doch-Na, and NP-40 increased the chromophoric response nearly two times. Virus sorption at 37 °C, as shown in Table 1, did not enhance the M protein antigenic reactivity. Thus the determination of M protein, especially with monoclonal antibodies (van Wyke *et al.*, 1984), by means of whole virions absorbed to the polystyrene carrier needs additional dissociation of the lipid membrane to achieve better solubilization of M. The high antigenic reactivity of M and NP proteins after direct virus sorption to the carrier may be used for evaluation of virus presence in nasopharyngeal secretions, i.e. in rapid virus typization.

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