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SIMULTANEOUS DETERMINATION OF THE LEVEL OF ANTIBODIES TO INFLUENZA VIRUS SURFACE AND INTERNAL PROTEINS BY ENZYME-LINKED IMMUNOSORBENT ASSAY

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Summary. — Enzyme-linked immunosorbent assay (ELISA) has been adopted for simultaneous determination of the levels of antibodies to different influenza virus proteins in human sera with known haemagglutination-inhibition (HI) titre. Whole virus of serotypes H1N1 and H3N2, haemagglutinin (HA), matrix (M) and nucleoprotein (NP) proteins have been used as antigens. For detection of antibodies bound to the antigen, peroxidase labelled *Staphylococcus* protein A conjugate has been used. Correlation of the ELISA and HI titres of anti-HA antibody has been demonstrated. The use of isolated HA as antigen increased the specificity of ELISA. The analysis of human reconvalescent sera has shown that increase in the titre of antibodies to internal proteins does not always coincide with the increase of antibody level to HA. Out of 8 sera with significant increase of the HI titre to the H3 subtype 5 specimens showed 4-fold increase of antibody titre to NP protein. The antibody titre to M protein was elevated in 2 sera only, while 1 serum showed no rise of antibody response to the tested viral proteins.

Key words: influenza virus; serodiagnosis; ELISA; haemagglutinin; internal proteins

Introduction

Over the last decade, enzyme-linked immunosorbent assay (ELISA) has been widely used for determination of antibody level in blood during a variety of viral diseases, influenza included (Kharitonenkov, Khristova, 1985). It has been demonstrated that ELISA was more sensitive than conventional serologic tests, such as haemagglutination inhibition (HI) or complement fixation (Bishai, Galli, 1978; Hammond *et al.*, 1980; Julkunen *et al.*, 1984). The specificity of ELISA is largely dependent on the purity of antigens. Recently available isolation methods of individual influenza virus proteins laid to the development of ELISA systems for determination of the level of antibodies to these proteins (Masihi, Lange, 1980; Murphy *et al.*, 1981; Khan *et al.*, 1982).

The rise of antibody level to influenza virus surface proteins is known to be directly related to antiviral resistance (Verelizier *et al.*, 1979). The role of antibodies to internal influenza virus proteins in antiviral immunity, however, is not evident yet. Although the internal proteins (M and NP) are most abundant in the influenza virion, studies on use of ELISA for detection of antibodies to these proteins are scarce. It has not been cleared whether or not the rise of antibody titre to surface proteins is correlated with the titre of antibodies to internal antigens. Upon natural exposure to the virus, Khan *et al.* (1982) demonstrated that the antibody response to M protein precedes, or coincides with that to haemagglutinin. However, these authors determinated the titre of M protein antibodies in ELISA while the titre of haemagglutinin antibodies in HI test. No comparative studies have been carried out with respect to NP protein.

The purpose of the present paper has been to develop an assay system for simultaneous determination of the level of antibodies to individual influenza virus proteins. With respect to internal type-specific antigens this system can be especially useful for diagnosis of influenza when a major shift had occured in influenza virus surface proteins.

Materials and Methods

Viruses. Influenza virus strains A/Kiev/59/79 (H1N1) and A/Leningrad/385/80 (H3N2) have been used. The viruses were grown in the allantoic cavity of 9-day-old chick embryos. They were concentrated and purified as described elsewhere (Kharitonenkov *et al.*, 1977).

Haemagglutinin was isolated by affinity chromatography on Sepharose-tyrosine-sulphanylic acid column; the total glycoproteins were solubilized from various influenza virus strains by octyl glucoside (Veselov *et al.*, 1984).

Internal proteins (M and NP) were isolated by preparative polyacrylamide gel electrophoresis (Zagidullin *et al.*, 1985).

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

Haemagglutination inhibition (HI) test was performed by a conventional technique (Hierholzer *et al.*, 1969) using the virus in allantoic fluid as antigen at a concentration of 4 to 8 HA units.

ELISA was performed using a modification of the method of Voler *et al.* (1977). The antigens were directly absorbed to polystyrene micropanels (Linbro, England). Optimal antigen concentrations were determined by titration curves with hyperimmune rabbit antisera. At the chosen concentration the further increase of sorbed protein was not followed by elevation of antibody titre. The antigen in phosphate buffer solution (PBS) pH 7.2 prepared according to the procedure of Voler *et al.* (1977) was added into micropanel wells (50 μl) and kept for 17 hr at 4 °C. The panels were then washed 3 times with PBS containing 0,05 % Tween-20 (PBS-Tween). 100 μl of PBS-Tween with 0.5 % bovine serum albumin (PBS-Tween-BSA) were then added into the wells and kept for 1 hr at 37 °C. The sensibilized panels could be kept after air-drying for as long as a few weeks and then used again for the analysis after 1hr treatment with PBS-Tween-BSA.

Titration of the sera tested (initial dilution 1 : 200, vol 50 μl) was carried out directly in the wells by double dilution in PBS-Tween-BSA containing 2 % allantoic fluid from uninfected chick embryos. The serum was incubated for 3 hr at 37 °C. After three washings with PBS-Tween, 50μl of protein A (Sigma, USA) conjugated with horseradish peroxidase (Biokhimreaktiv, Olaine, Latvian SSR, RZ-3.0) diluted 1 : 2000 in PBS-Tween-BSA were introduced into the wells.

Covalent linking of peroxidase with protein A was carried out according to the procedure of Nakane and Kawaci (1974). The incubation with the conjugate lasted for 1 hr at 37 °C. After washing of the panels, the enzyme activity was determined by addition into the wells of 100/μl of 0.05 % orthophenylene diamine solution (Sigma, USA) in 0.03 mmol/l. NA-phosphate buffer, pH 5.8 containing 0.003 % H_2O_2. The panels were kept at room temperature in the dark for 35 min. The reaction was stopped by addition of 50 μl of 4 mol/l H_2SO_4.

Optical density (OD) was measured by automatic colorimeter (Titertek Finland) at wavelength 490 nm. In control wells containing either no antigen or no serum or no conjugate, the OD_{490} was usually below 0.1. The highest control values were subtracted from the ODs of the sera tested. Antibody titre in the sera was defined as the highest dilution at which the ratio of OD_{490} of the serum tested to the OD_{490} of the negative serum was equal to 2.

Sera. Paired sera were taken during an outbreak of influenza in 1985 among military soldiers aged 18 to 22 years with sings of acute respiratory disease. The first sera were taken on day 1–3 of the disease, and the second after 10 days.

Results

Horseradish peroxidase labelled protein A in ELISA was shown to bind, in addition to IgG, also to IgM and IgA from human serum (Grangeot-Keros *et al.*, 1982). To asses the reproducibility and significance of the assay, we selected 6 serum pairs out of 26 reference sera. During the analysis, 50 μl of reference serum to the antigen was given at a 1:800 dilution into 2–4 wells of each panel with the adsorbed antigen. Table 1 shows mean OD_{490} values of these sera (averaged over different panels and days of observation) with standard mean deviations. It can be seen that OD_{490} deviations from the mean value generally do not exceed 10%. The spread od OD values of these sera obtained on the same day ranged within 5%. The chosen system and accurate observation of time parameters of EI-assay yielded statistically significant results.

One of the goals of ELISA application for serologic influenza diagnostic is to establish the correlation between antibody titre as determined by ELISA and by conventional HI. This correlation has been established by many authors (Bishai, Galli, 1978; Hammond *et al.*, 1980; Murphy *et al.*, 1981). In addition, this has been also confirmed in the present paper by means of protein A peroxidase conjugate. Fig. 1 shows such comparison of antibody titres to influenza virus A/Leningrad/385/80 in 26 paired sera by HI test and ELISA. It can be seen that even using intact virus as antigen in ELISA, the results correlated with HI (correlation coeficient $r=0.7 p < 0.001$). Absolute values of antibody titres in ELISA are much higher than those in HI due to higher sensitivity of the former method.

Table 1. Reproducibility of ELISA findings

Antigen and reference serum titre in HI	Mean value (OD_{mean})* ± standard deviation ($\triangle OD_{mean}$)	$\frac{\triangle OD_{mean}}{OD_{mean}}$ (percentage)
A/Kiev/59/79		
negative serum (< 10)	0.110 ± 0.006	5.5
positive serum (80)	0.395 ± 0.045	11.5
positive serum (160)	1.080 ± 0.115	10.8
A/Leningrad/385/80		
negative serum (< 10)	0.110 ± 0.005	4.5
positive serum (160)	0.370 ± 0.030	8.0
positive serum (640)	0.785 ± 0.062	8.0

*OD_{mean} – mean OD_{495} of 10 to 13 measurements for serum dilution 1 : 800.

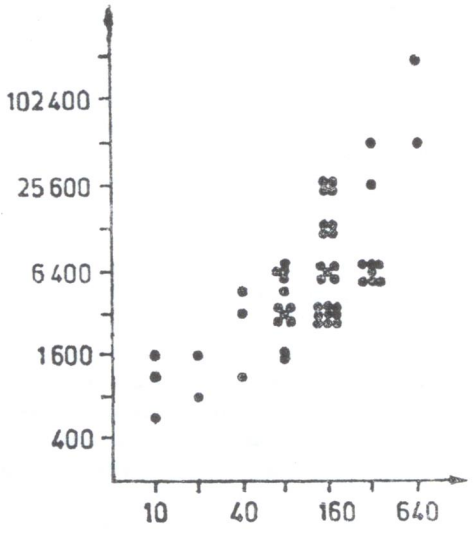

Fig. 1.
Comparison of antibody titres measured by ELISA and HI in 25 paired sera (antigen — influenza virus A/Leningrad/ /385/80).
Abscissa: antibody titre in HI, ordinate: antibody titre in ELISA

It has been demonstrated that specificity and sensitivity of ELISA largely depends on the purity of used antigens (Hammond *et al.*, 1980). When whole virus was used as antigen, determination of antibody titre in convalescent serum would entail artifacts. Since virus suspension always contains partially or totally destroyed virions, false positive results may occur. This is related to the fact that not only antibodies to surface virus proteins of the subtype in question are assayed, but also antibodies to internal antigens crossreacting with different strains of influenza virus. We have tested paired sera simultaneously against whole virus serotypes H1N1, H3N2, against haemagglutinins of both serotypes and against matrix nad NP proteins. The results are presented in Table 2.

Fig. 2 shows titration curves of serum pair No. 3 against each antigen. It can be seen that by titration against intact virus of both subtypes (H1N1, H3N2) the antibody titre increased 8- fold (Fig. 2 - I, II). When tested against the isolated HA the increase of antibodies appeared only for the serotype H3 (Fig. 2 - III, IV). Meanwhile, a significant rise of antibody titres to M and NP proteins has been observed (Fig. 2-V,VI). This caused an increase in antibody titre at titration of the serum against whole virus of H1N1 serotype. It can be seen from Table 2 that according to HI findings a significant increase of antibody titre was observed for 4 paired sera (Nos. 1, 3, 9, and 10). In ELISA, however, upon titration against the whole virus, also sera Nos. 7 and 8 appeared to be positive, and on titration against the isolated HA another 2 sera — Nos. 5 and 6 — also showed a 4-fold increased antibody titre. The fact that antibody titre to HA was not increased in HI, although it had been increased 4- to 8-fold by ELISA, can be probably explained by detection in ELISA of even those antibodies that had not reacted in HI. This agrees

Table 2. Titres in HI and ELISA of paired convalescent sera with individual viral proteins as antigens*

No. of serum		Virus		HA	Virus		HA	NP	M
		A/Len/385/80 (H3N2)		A/Len/385/80 (H3)	A/Kiev/59/79 (H1N1)		A/Kiev/59/79 (HI)	A/Kiev/59/79 (H1N1)	
		HI	ELISA	ELISA	HI	ELISA	ELISA	ELISA	ELISA
1	1st	20	800	800	20	1 600	1 600	1 600	1 600
	2nd	160	6 400	3 200	40	3 200	1 600	1 600	800
2	1st	80	1 600	3 200	80	6 400	3 200	800	800
	2nd	80	3 200	6 400	80	6 400	6 400	1 600	6 400
3	1st	40	3 200	3 200	40	3 200	3 200	2 400	600
	2nd	160	25 600	19 200	80	25 600	6 400	19 200	4 800
4	1st	10	1 200	1 200	40	800	400	1 680	300
	2nd	10	1 600	2 400	80	3 200	1 200	6 400	600
5	1st	160	12 800	12 800	320	25 600	6 400	4 800	800
	2nd	160	25 600	51 200	640	51 200	6 400	4 800	800
6	1st	320	6 400	3 200	80	9 600	1 600	400	200
	2nd	320	6 400	12 800	80	9 600	3 200	1 600	400
7	1st	320	51 200	102 400	160	51 200	6 400	6 400	800
	2nd	640	204 800	409 600	160	102 400	6 400	6 400	400
8	1st	160	3 200	1 600	160	3 200	1 600	2 400	2 200
	2nd	160	25 600	6 400	160	12 800	1 600	12 800	3 400
9	1st	20	1 600	800	80	1 600	800	800	00
	2nd	320	6 400	3 200	40	800	800	3 200	800
10	1st	40	1 200	1 600	80	1 200	800	6 400	800
	2nd	160	12 800	25 600	40	6 400	3 200	5 400	1 600

*Antibody titres are expressed as reciprocal values of serum dilutions.

with the data of Kida *et al.* (1982) who have detected antibodies to some HA epitopes that can inhibit the infectivity of influenza virus, but exert no effect in HI. The data obtained suggest that the employment of isolated HA in ELISA provides better conditions for the interaction of these antibodies with the HA molecule. This is the cause of antibody increase in sera Nos. 5 and 6.

During titration of the sera tested with the virus A/Kiev/59/79 no rise of antibodies has been observed in HI, whereas in ELISA 3 sera showed

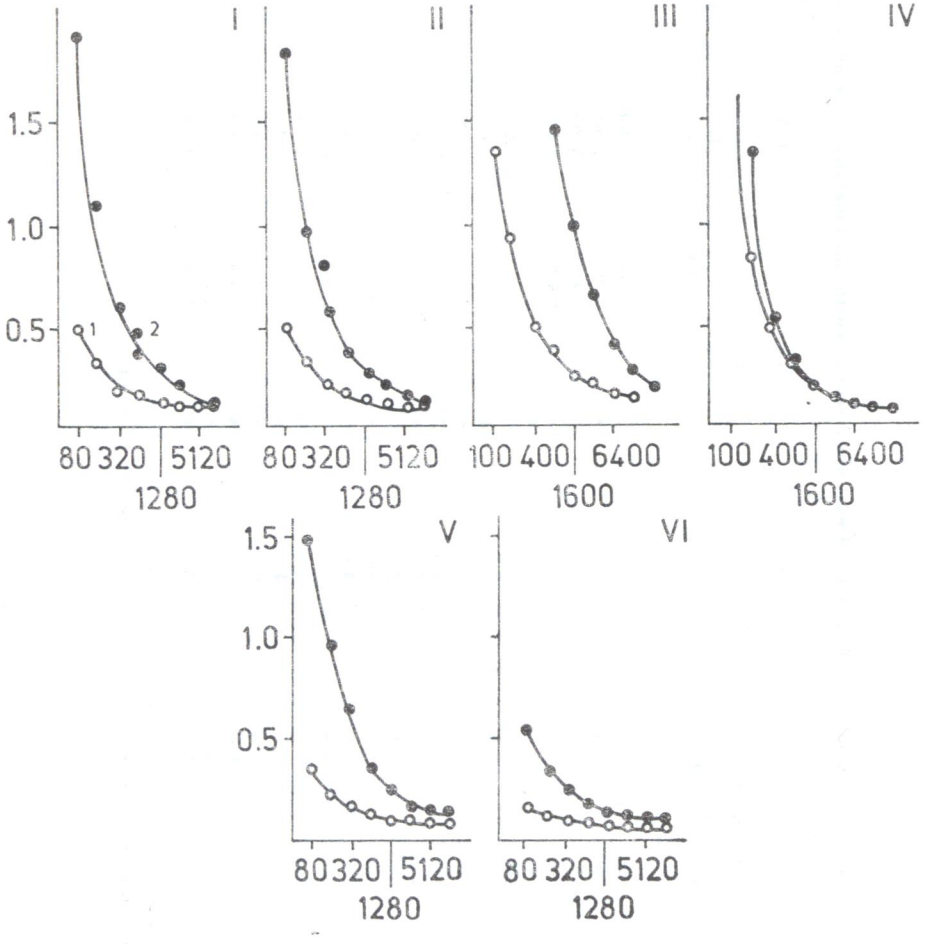

Fig. 2.
Titration of human paired sera (1st serum — 1, 2nd serum — 2) against different antigens.
I — influenza virus A/Leningrad/385/80, II — influenza virus A/Kiev/59/79, III — HA of influenza virus A/Leningrad/385/80, IV — HA of influenza virus A/Kiev/59/79, V — NP protein, VI — M protein.

a 4-fold increase (Nos. 3,4 and 8). However, only in case of one serum this was associated with a rise of antibody titre to HA of given serotype. Another two sera showed an increase of antibody titre to the whole virus related to the appearance of antibodies to internal proteins induced by the serotype H3N2.

Discussion

The rise of antibody titre to internal proteins does not always conincide with the rise of the antibody titre to HA. Thus, for example, only in 5 sera out of 8 in which a significant increase of antibody titre to HA has been registered, the same occurred for antibody to NP protein. The M protein antibody titre appeared to increase only in 2 cases. In one of these sera (No. 2) the titre of antibodies to other viral proteins remained unchanged.

Khan *et al.* (1982) used ELISA to study the antibody response during vaccination. They have found increased antibody titre to M protein in approximately 1/3 of the samples examined. In some cases antibodies to M protein appeared earlier than those to HA; the latter, however, were detected in HI.

At present, the role of antibodies to influenza virus internal proteins in the prevention of viral infection is unclear as well as in the specific course of the disease. With the use of the technique of radial immunodiffusion it was shown that antibodies to M protein generally appear in patients with a severe course of disease but have a low protective activity (Cretescu *et al.*, 1978). There are no data available on the role of antibodies to NP protein although it has been recently reported that this protein is important for the cellular immune response. Yewdell *et al.*, (1985) demonstrated that influenza virus NP protein is first expressed on the cell surface being the target for cytotoxic T-lymphocytes.

Hence the data in this paper indicate that employing pure antigens (especially HA) provides high sensivity, specificity and reliability of the ELISA procedure. The use of micropanels with a presorbed antigen and the choice of optimal assay conditions enables standardization of results. Furthermore, the proposed assay system will allow to study the possible relationship between clinical picture of the disease and the rise of antibodies to individual influenza virus proteins and also to determine the role of antibodies to internal proteins in the infection process. The employment of the assay for M and NP proteins can be of particular importance in the diagnosis, especially in cases when influenza virus surface proteins undergo dramatic changes.

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