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Neutralization Enzyme Immunoassay for Influenza Virus

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A neutralization enzyme immunoassay (N-EIA) was developed for the detection of antibody titer rises in sera of patients infected with influenza A (H3N2) virus. In this N-EIA, a selected strain of influenza A (H3N2) virus was added to monolayers of LLC-MK2 cells in microtiter plates. After 24 h, the replicated virus could be demonstrated with a virus-specific enzyme-labeled monoclonal antibody. Preincubation of the influenza virus with convalescent-phase sera of patients infected with influenza A (H3N2) virus resulted 1 day later in decreased absorbance values that could be used for calculation of neutralization titers. From use of paired serum samples from 10 patients with a history of flu-like symptoms, the results obtained with N-EIA correlated well (r = 0.83) with those of the standard hemagglutination inhibition test.

Hemagglutination inhibition (HAI) and complement fixation assays are the methods most commonly used for serodiagnosis of influenza virus infections. Neutralization tests, however, have been reported to be more specific and more sensitive (1, 3–5, 13–15). The classical neutralization methods based on plaque reduction or hemadsorption inhibition are laborious and take several days to complete. Therefore, they are less suitable for screening large numbers of serum samples. Recently, we have developed neutralization tests based on an enzyme immunoassay for mumps virus, encephalomyocarditis virus, and Semliki Forest virus in cell culture with horseradish peroxidase (HRPO)-labeled virus-specific monoclonal antibodies (MAbs), which can be applied for rapid and objective titration of virus infectivity and virus-neutralizing antibodies (8, 16–18).

In the present paper, we describe such an assay for the assessment of neutralizing activities of human serum samples against influenza A (H3N2) virus.

MATERIALS AND METHODS

Cells, media, and influenza virus. LLC monkey kidney cells (LLC-MK2D, hereafter called LLC cells) obtained from Flow Laboratories, Irvine, Scotland, were grown and maintained in roller bottles at 37°C in Dulbecco's minimal essential medium (DMEM) supplemented with 0.2% tryptose, antibiotics, and 10% heat-inactivated fetal calf serum (FCS). The medium was buffered with 0.01 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and 0.024 M sodium bicarbonate. For the purpose of growing stock virus, the cells were washed once gently with phosphate-buffered saline (PBS), pH 8.0, and kept overnight in DMEM without serum. In the experiments themselves, cells were washed with PBS (pH 8.0) and, after treatment with trypsin (0.25% solution; Difco Laboratories, Detroit, Mich.), were washed once by centrifugation in DMEM without serum before use.

Influenza A/Delft/2696/90 (H3N2) virus, passage history LLC2-tMK3, was passed in LLC cells in the presence of 0.001% trypsin in 1-liter roller bottles at 37°C. After 4 to 5 days of incubation, the medium was changed and the trypsin concentration was increased to 0.0014%. Then, the medium was changed every 6 to 8 h until the cells began to detach from the bottle. To separate the virus from the cell debris, the supernatant fluids were centrifuged at 800 × *g* for 15 min. Next, the samples of supernatant fluids were tested for the presence of influenza virus by hemagglutination. For the hemagglutination assay, 0.1 ml of a 0.5% suspension of chicken erythrocytes in buffer consisting of 150 mM NaCl, 3.8 mM Na_2HPO_4, and 0.2 mM citric acid (pH 7.2; Hirst buffer [9]) was added to 0.1 ml of twofold serial dilutions of supernatant fluids in 96-well round-bottom microtiter plates (Greiner, Frickenhausen, Germany) and incubated for 1 h at 4°C. The virus preparation with the highest hemagglutination titer was frozen in 1-ml aliquots at −70°C until use. In all experiments, only fresh aliquots of virus were used.

Development of MAbs. Female BALB/c mice (8 to 10 weeks old) were infected intranasally with 8.6×10^6 PFU of influenza A/Delft/2696/90 (H3N2) virus. Two months later, the mice were injected intravenously with 1.9×10^7 PFU of influenza A/Delft/2696/90 virus in 0.3 ml of supernatant fluid. After the second immunization, spleen cells from the mice were fused with SP2/0 myeloma cells. The supernatants of the hybridomas were tested for influenza virus-neutralizing activity by the neutralization enzyme immunoassay (N-EIA) as described below. Positive hybridoma cells were injected intraperitoneally into Pristane-primed BALB/c mice for ascites production. MAb subclasses were determined by enzyme-linked immunosorbent assay using a subclass-specific goat anti-mouse HRPO-labeled antibody (affinity purified; Boehringer, Mannheim, Germany). Throughout this study we used the MAb designated UM 11-52 (immunoglobulin G2a [IgG2a]), for detection of influenza virus antigen in cell culture by direct EIA.

Enzyme labeling of MAbs. HRPO was conjugated to MAbs in ascitic fluid by the periodate method (12). The conjugate, diluted 1:5 in PBS (pH 8.0) with a crystal of thymol (Sigma, St. Louis, Mo.), was stored at +4°C. Immediately before use, the conjugate was finally diluted to 1:20,000 in PBS supplemented with 0.05% Tween 20.

Human sera. Acute-phase and convalescent-phase serum samples from 10 adult patients with respiratory symptoms in the winter season of 1989 to 1990 were obtained from the diagnostic virus department of one of our laboratories (Department of Virology, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands). The

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paired serum samples were selected to represent high, low, and intermediate rises of HAI antibody titers against the epidemic influenza A (H3N2) virus strain.

HAI assay. HAI tests were performed with chicken erythrocytes following standard procedures (7) with slight modifications as described elsewhere (2). To prevent nonspecific inhibition, human sera were pretreated with in-house-prepared receptor-destroying enzyme.

Detection of viral antigen on infected cells by direct EIA. Influenza virus was added together with 1.5×10^5 LLC cells, resulting in a multiplicity of infection (MOI) of 0.1, to 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark) and incubated at 37°C. Wells containing grown monolayers were fixed at selected time intervals with 0.05 ml of 0.15% glutaraldehyde (Merck, Darmstadt, Germany) per well. After 32 h of incubation, all monolayers had been fixed. Subsequently, the plates were washed with tap water and incubated with HRPO-labeled MAb UM 11-52 for 1 h at 37°C. The plates were washed again with tap water, and bound peroxidase was visualized by incubation with 0.05 ml of substrate solution per well (TMB and H_2O_2 in 0.11 M sodium acetate buffer, pH 5.5). After 20 min of incubation at room temperature, the enzyme reaction was stopped with 0.1 ml of 0.18 N H_2SO_4 per well and the reaction was quantified by measuring the A_{450} with a Titertek Multiskan spectrophotometer (Flow Laboratories).

N-EIA. For the assessment of the influenza virus-neutralizing activities of patient serum samples, the preparations, which had been heat inactivated at 56°C for 30 min, were serially diluted in twofold steps in DMEM supplemented with 2% FCS in 96-well microtiter plates at volumes of 0.025 ml per well. Then, 0.025 ml of influenza A virus in DMEM containing 2% FCS was added to each well and the plates were incubated for 1 h at 37°C. Subsequently, from each mixture three aliquots of 0.01 ml were transferred to three wells of other 96-well microtiter plates and 1.5×10^4 LLC cells in DMEM containing 2% FCS were added. The plates were incubated at 37°C for 22 h, fixed with 0.05 ml of 0.15% glutaraldehyde per well, and washed with tap water. Finally, a direct EIA was performed as described above, using HRPO-labeled MAb UM 11-52. Virus controls (virus and cells only) and cell controls were each determined in fourfold in every microtiter plate.

For the calculation of the MOI used in N-EIA, the number of PFU of influenza strain A/Delft/2696/90 virus was assessed by using an immunoplaque assay slightly modified from that described by Harder et al. (6). The number of PFU was determined as 8.6×10^7 per ml.

Calculation of data. For each well, the inhibition of virus multiplication by neutralizing antibodies was calculated as the percentage of reduction of the absorbance value in respect of the virus control with a spreadsheet computer program by the following formula: percent inhibition = $100 - [(A_{450}$ of antibody dilution $- A_{450}$ of cell control)/(A_{450} of virus control $- A_{450}$ of cell control)] $\times$ 100. The neutralizing antibody titer was arbitrarily defined as the reciprocal dilution that caused 50% reduction of the absorbance value of the virus control (50% A_{450} reduction).

RESULTS

Detection of cell-associated influenza virus antigen by direct EIA. LLC cells were inoculated with influenza A/Delft/2696/90 (H3N2) virus and incubated without or in the presence of trypsin in the medium. After various time intervals, fixation of the monolayers with glutaraldehyde was followed by direct EIA of cell-associated viral antigen. The absorbance values measured are presented as virus multiplication curves in Fig. 1.

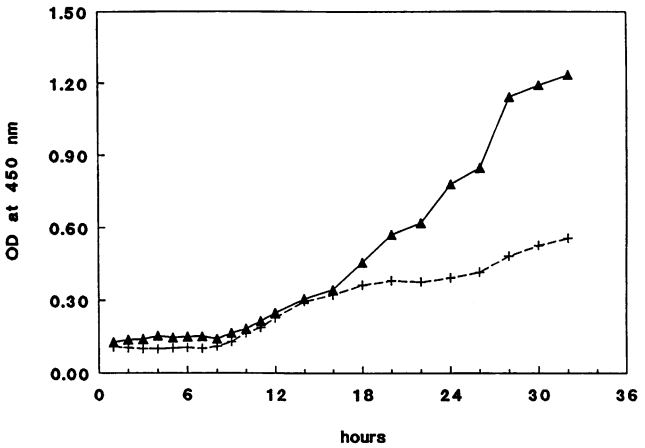

FIG. 1. Multiplication curves of influenza A/Delft/2696/90 (H3N2) virus. Cells were infected with the virus at an MOI of 0.1 with (——) or without (– – – –) addition of trypsin. Influenza virus-infected cells were fixed with glutaraldehyde at 1-h time intervals. Optical densities (OD) at each timepoint are given as means of threefold determinations.

At an MOI of 0.1, viral antigen became detectable within 12 h after influenza virus inoculation as indicated by a rise in absorbance values. A few hours later, the infectivity-enhancing effect of trypsin became obvious by the divergence of the two multiplication curves and finally resulted in a higher level of the asymptote of the curve. When cells were infected at a higher MOI, virus multiplication could be detected as early as 6 h after inoculation (results not shown). The height of the absorbance values measured at certain time points of infection is dependent on the viral input, as shown in Fig. 2. It appears

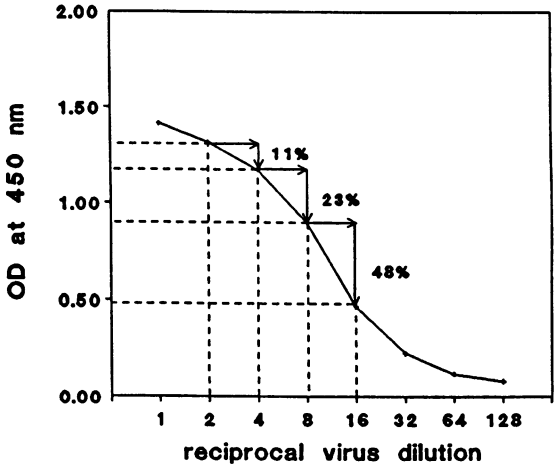

FIG. 2. Virus titration curve. LLC-MK2 cells were infected with twofold serial dilutions of influenza A/Delft/2696/90 (H3N2) virus without addition of trypsin. After incubation for 22 h at 37°C, cell monolayers were fixed with glutaraldehyde and EIA was performed with HRPO-labeled MAb UM 11-52 (1:20,000). Twofold serial dilutions of the virus are plotted against the optical densities (OD) measured at 450 nm. The horizontal arrows of equal length indicate a 50% reduction of the virus input (by dilution or neutralization). The vertical arrows of unequal length indicate the percent reduction of the absorbance values as a consequence of the 50% reduction of the virus input. Vertical and horizontal dashed lines indicate ODs and twofold dilutions, respectively.

TABLE 1. Comparison of N-EIA with HAI for detection of seroconversion in patients with flu-like symptoms

Patient no.	Serum antibody titers[a] as determined by:			
	HAI		N-EIA	
	Acute phase	Convalescent phase	Acute phase	Convalescent phase
1	<6	<6	20	12
2	<6	24	7	78
3	<6	24	20	126
4	<6	96	23	160
5	<6	96	22	487
6	<6	384	19	699
7	<6	384	8	774
8	48	768	65	738
9	<6	3,072	14	2,890
10	96	3,072	74	964

[a] Antibody titers were determined against influenza A/Delft/2696/90 (H3N2) virus. N-EIA titers are defined as the reciprocal of the dilution that causes a 50% reduction of A_{450}.

from this figure that a 50% reduction of the viral input by twofold dilution (or otherwise by neutralization) corresponds to a percentage reduction of the resulting absorbance value which depends on the absolute viral input. Therefore, the virus dilution to be used in neutralization experiments was chosen to correspond to the upper straight part of the curve yielding optical densities at 450 nm in the range of 0.8 to 1.2. At this part of the curve, linear regression can be applied for calculation of neutralization titers and the highest percentage reductions in absorbance values are observed with a 50% reduction of the viral input.

Determination of neutralizing antibody titers by N-EIA. Paired human serum samples taken in the winter season of 1989 to 1990 and showing a rise in HAI titer against influenza A/Delft/2696/90 (H3N2) virus were tested by the newly developed N-EIA. The resulting neutralizing antibody titers at 50% A_{450} reduction corresponded well with the HAI titers (Table 1). The overall correlation coefficient was 0.83. With eight patients, the antibody titers in the acute-phase serum samples were below the detection level (a titer of 6) when determined in HAI tests. When N-EIA was used, however, neutralizing activity could be demonstrated in all acute-phase serum samples. Figure 3 illustrates the method by displaying the curves for three paired serum samples with undetectable, low, and high rises in antibody titer.

DISCUSSION

The multiplication of influenza virus could be monitored by EIA (Fig. 1). Our results correspond very well with those found by Watanabe and Mackenzie (19). A rise in absorbance value could be prevented or diminished by preincubation of the virus inoculum with neutralizing antibody (N-EIA). This technique enabled titration of the neutralizing activities of human serum samples within 24 h (Table 1 and Fig. 3). Similar results were obtained when neutralizing activities of MAbs directed against influenza A (H3N2) virus were assessed (data not shown). Furthermore, it would be interesting to compare N-EIA with HAI for characterization of panels of MAbs directed against influenza viruses (unpublished data).

To become infective, the hemagglutinin (HA) of newly synthesized influenza virus has to be cleaved proteolytically into the subunits HA1 and HA2 (11). In vitro, this can be achieved by addition of trypsin (10). In our assay, this effect of trypsin is reflected by the divergence of the rise in absorbance values that resulted in different levels of the asymptote, as measured on virus-infected cells with and without the presence of the enzyme (Fig. 1). This assay may therefore be used to assess the effect of other proteolytic enzymes on influenza virus multiplication.

Because in the N-EIA only one cycle of multiplication of influenza virus is needed, addition of trypsin is not essential.

The choice of a suitable endpoint reading can be a problem in the N-EIA. The virus titration curve (Fig. 2) shows that every 50% reduction of the virus concentration yields a percentage of reduction of the absorbance value which depends on the virus input. This necessitates the prior construction of a titration curve to establish the optimal virus dose.

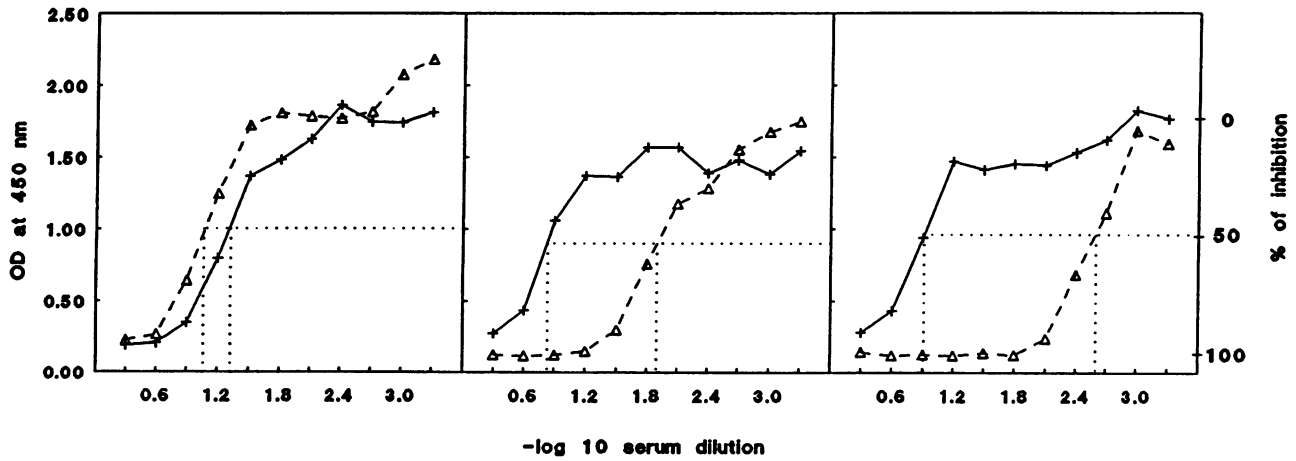

FIG. 3. N-EIA titration curves of three paired patient serum samples (left to right panels, respectively) with no rise, intermediate rise, and high rise in neutralizing antibody titers against influenza A (H3N2) virus. Optical density (OD) at 450 nm is given on the left vertical axis, and the percent inhibition (as determined by the formula given in Materials and Methods) is given on the right vertical axis. ——, acute-phase serum; – – – –, convalescent-phase serum. Dotted lines denote serum dilutions ($-\log_{10}$ values) yielding 50% reduction of absorbance values. In the figure, 100% neutralization corresponds to the mean (n = 4) absorbance value of the cell control and 0% neutralization corresponds to the mean (n = 4) absorbance value of the virus control. All other absorbance values are the means of three determinations.

Even then, some inaccuracy is introduced because of the small variations in titration between different experiments. However, when the aim is to measure rises in neutralizing antibody titers between serum samples the accuracy is not affected, because the samples are tested simultaneously by using the same MOI of influenza virus. Actually, the newly developed N-EIA demonstrated such rises in neutralizing antibody titers in close correspondence with the standard HAI (Table 1).

According to the prevailing theory that one antibody molecule might neutralize one virus particle (single hit), higher titers would be expected in N-EIA than in HAI. Actually, about 70 antibody molecules are necessary to neutralize one influenza virus particle (14). This could explain why antibody titers measured in convalescent-phase serum samples by N-EIA are of the same order of magnitude as those measured by HAI. A close correlation between HAI and neutralization titers in serum samples of volunteers after vaccination when tested against the vaccine strain has also been demonstrated by Okuno et al. (13).

The nature of the neutralizing activities measured in acute-phase serum samples that were determined as negative by HAI assay is not known. They could be due to a sensitivity of the N-EIA higher than that of the HAI or, alternatively, to non-immune factors that are present in some human serum samples and that become inactive when treated with receptor-destroying enzyme as is done in the HAI test.

A disadvantage of the N-EIA is the need for the development of suitable MAbs. Especially with the highly variable influenza virus (2), there is a considerable risk that previously suitable MAbs do not react with newly appearing variants of the virus.

Determination of neutralizing antibody titers may have special relevance in relation to the assessment of the immune response after influenza vaccination because it is not certain that all HAI antibodies can also accomplish neutralization. The N-EIA is suitable for the accurate titration of neutralizing antibodies in a large number of serum samples using small amounts of serum, as has been demonstrated in a recent study on the immune status of children after mumps vaccination (8).

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