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Protocols

Comparison of hemagglutination inhibition assay, an ELISA-based micro-neutralization assay and colorimetric microneutralization assay to detect antibody responses to vaccination against influenza A H1N1 2009 virus

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The hemagglutination inhibition (HI) assay has been the main method used to investigate immune responses to vaccination against influenza H1N1 (2009) virus. However microneutralization tests (MNT) have been shown to be more sensitive and more specific. In this study, the three methods of choice: (i) the HI assay, (ii) an ELISA-based conventional MNT and (iii) a colorimetric MNT in terms of their ability to detect antibody responses in serum pairs collected from 43 healthy individuals before and 21 days after vaccination were compared. The colorimetric MNT was established yielding intra- and inter-run imprecisions of 7.5% and 12.4%, respectively. Testing of antisera to seasonal influenza viruses demonstrated the assay to be specific for antibodies to influenza H1N1 (2009) virus. A good correlation between the three methods was found, being highest for the ELISA-MNT and the colorimetric MNT ($r = 0.714$ for geometric mean titers (GMT) and $r = 0.695$ for titer increases). Similar rates of fourfold titer increases were detected: 95.3% in the ELISA-MNT vs. 93.0% in colorimetric MNT and 95.3% in HI assay. The ELISA-based MNT demonstrated the highest titer range leading to the highest postvaccination GMT and the highest titer increase (>50-fold). The lowest GMTs were measured with the HI assay, while the colormetric MNT detected the highest GMT in prevaccination sera. Taken together, similar seroconversion rates were obtained with the three assays. The ELISA-MNT appeared to be the best method to compare absolute pre- and postvaccination GMTs. The colorimetric MNT, being less labour-intensive than the ELISA-MNT, seems to be a suitable tool in vaccination studies.

1. Introduction

Clinical trials have investigated immune responses to the monovalent split-virus inactivated vaccine against influenza H1N1 (2009) virus (Liang et al., 2010; Plennevaux et al., 2010; Zhu et al., 2009). In these studies antibody responses were measured mainly by the hemagglutination inhibition (HI) assay because this method can be easily adapted to new influenza viruses. However, it has been shown for seasonal and for avian influenza that microneutralization tests (MNT) are more sensitive and more specific (Frank et al., 1980; Gitelman et al., 1986; Rowe et al., 1999). A previous study for seasonal influenza A indicated that strain-specific IgG antibodies with neutralizing capacity may be present, but these antibodies were not generally detected in homologous HI assays (Remarque et al., 1998). Conventional microneutralization tests are performed in different ways with regard to titer determination, cytopathic effect formation in cell cultures, red blood cells for detection of released virus or usage of the ELISA technique to identify virus infected cells. Especially the ELISA method is time-consuming, difficult to perform under biosafety-level 3 (BSL-3) conditions and requires the need for monoclonal antibodies that are not widely available for new viruses. Recently, a colorimetric MNT has been introduced for seasonal influenza (Lehtoranta et al., 2009). This assay is an adaptation of micro-cytotoxicity tests (van de Water et al., 1993) and is based on the cleavage of a tetrazolium salt by mitochondrial dehydrogenases reflecting the amount of viable and metabolically active cells.

In the present study, the three assays of choice: (i) HI assay, (ii) ELISA-MNT and (iii) colorimetric MNT were compared to detect immune responses in serum pairs collected from 43 healthy individuals before and 21 days after administration of a monovalent vaccine against pandemic influenza H1N1 (2009) virus. The colorimetric MNT was adapted and validated to detect antibodies to pandemic influenza and the method was modified to include less hands-on manipulations.

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2. Materials and methods

2.1. Cells

MDCK cells (obtained from BioWhittaker Europe, Verviers, Belgium) were grown in Dulbecco's modified essential medium (DMEM) supplemented with penicillin and streptomycin (500 U/ml each) and 2% fetal calf serum and were maintained in a 5% CO_2 humified atmosphere at 37 °C. Two to three times a week cells were subcultured at a split-ratio of 1:2–1:5.

2.2. Viruses

For the HI assay and the ELISA-MNT the pandemic influenza virus reference strain A/California/7/2009 H1N1 was used. The colorimetric MNT was performed with a pandemic influenza A H1N1 virus isolated from an infected patient in September 2009. This virus reacted well with a specific antiserum to the influenza virus A/California/7/2009 in HI assay. According to this close relatedness in the antigenic profile the virus was declared to be an influenza A/California/7/2009-like virus by the German national reference institute for influenza at the Robert-Koch-Institute in Berlin. Sequencing of the HA gene (984 nucleotides) yielded seven nucleotide exchanges compared to the reference strain A/California/7/2009 without any exchange in amino acid sequence. In comparison to the reference virus A/California/7/2009 a concentration of 500–1000 $TCID_{50}$ of this virus demonstrated a stronger cytopathic effect in MDCK cells leading to a cell destruction of roughly 90% after 72 h of incubation. Strong destruction of target cells has been described to be essential for a colorimetric MNT (Lehtoranta et al., 2009).

2.3. Human sera

In November 2009, 43 serum pairs were collected from healthy staff members of the Institute of Virology of the University of Düsseldorf before and 21 days after vaccination with an adjuvant monovalent split-virus inactivated vaccine against influenza H1N1 (2009) virus (Pandemrix®, GlaxoSmithKline, London, England). Informed consent was obtained from all individuals participating in the study. The mean age of subjects was 36.2 years (SD 10.4 years). Individuals with a history of infection with influenza H1N1 (2009) virus were excluded from the study.

2.4. Hemagglutination inhibition (HI) assay

The hemagglutination inhibition assay was performed as previously described (Lenette and Schmidt, 1979). Prior to testing, each serum was treated with receptor destroying enzyme (Cholera filtrate, Sigma, Germany) to inactivate non-specific inhibitors achieving a final serum dilution of 1:10. The sera were then diluted serially twofold in V-bottom microtiter plates. The virus was adjusted to 4 HA units/25 μl, which was verified by back titration, and 25 μl of this virus suspension was added to each of the 96 wells. After incubation at room temperature (RT) for 30 min freshly prepared 0.5% turkey red blood cells (RBCs) were added (obtained from the Bundesinstitut für Risikobewertung, Berlin, Germany). The plates were agitated briefly followed by a further incubation at RT for 30 min. Human sera serving as positive controls and negative controls were included on each plate. The determination of the HI titer was performed by calculation of the reciprocal of the last serum dilution which contained non-agglutinated RBCs. The sera were tested only once in HI assay because there was not sufficient sample material available for further assay runs for the majority of vaccines.

2.5. ELISA-based conventional microneutralization (ELISA-MNT) assay

The method of the ELISA-based microneutralization test was modified from an assay described previously (Rowe et al., 1999). All sera used were heat inactivated at 56 °C for 30 min and twofold serial dilutions were prepared (diluent: Eagle's minimal essential medium supplemented with 1% BSA and gentamycin 0.05 μg/ml). 50 μl of each dilution were added to a microtiter plate (flat-bottom, Corning Incorporated, Corning, USA) and mixed with 50 μl virus suspension (2×10^3 $TCID_{50}$/ml in diluent). The plates included four wells containing 50 μl of diluted virus and 50 μl diluent as positive controls, four wells containing 100 μl diluent as cell control and two wells each for back titration (a serial twofold dilution in diluent, to 1:16). The plates were incubated at 37 °C for 2 h and 5% CO_2. Afterwards 100 μl of MDCK-2 cells (1.5×10^5/ml) were added to each well. After overnight incubation (18–22 h) at 37 °C and 5% CO_2, the medium was removed from the wells and the monolayers were fixed with cold fixative (acetone 80% in PBS 1:5) for 10 min. The titer was calculated by performing an ELISA to detect virus-infected cells. The fixed plates were washed three times with the wash buffer (PBS, 0.1% Tween 20), the anti-influenza A NP mouse monoclonal antibody (WHO Influenza Reagent Kit, Centers for Disease Control and Prevention (CDC), Atlanta, USA) was diluted 1:4000 in blocking buffer (PBS, 1% bovine serum albumin and 0.1% Tween 20) and 100 μl were added to each well. After 1 h incubation the plates were washed four times with wash buffer and 100 μl of horseradish peroxidase-conjugated goat anti-mouse IgG (diluted 1:1000 in blocking buffer) was added to each well. The plates were incubated at room temperature for 1 h and then washed six times with wash buffer. 100 μl of freshly prepared substrate solution (hydrogen peroxide, 1 g/L, in sodium acetate buffer solution, 25 mmol/L [pH 4.1] with tetramethylbenzidine dihydrochloride, 5 g/L) were added to each well and the reaction was stopped after 5 min with 100 μl stop solution (0.5 N sulfuric acid) per well. The absorbance was measured at 450 nm (A_{450}) with Titertek Multiscan PLUS (Labsystems) and the virus neutralization endpoint titer of each serum was determined with the following equation: $X = [(\text{average } A_{450} \text{ of virus control wells}) - (\text{average } A_{450} \text{ of cell control wells})]/2 + (\text{average } A_{450} \text{ of cell control wells})$. X = 50% of specific signal and all values below this value are positive for neutralization activity. All sera were tested twice on different days and the final titer value was the average of two separate runs.

2.6. Colorimetric microneutralization (MNT) assay

To detect specific antibodies to influenza H1N1 (2009) virus a colorimetric MNT was established on the basis of different methods that had been introduced for seasonal influenza (Lehtoranta et al., 2009; van de Water et al., 1993). Sera were heat inactivated at 56 °C for 30 min, and twofold serial dilutions in 50 μl were prepared in flat-bottom 96-well tissue plates (TPP, Trasadingen, Switzerland) with Dulbecco's modified essential medium (DMEM) supplemented with penicillin and streptomycin (500 U/ml each), 2% fetal calf serum (FCS) and 1% trypsin. An equal volume of the same medium containing 500–1000 $TCID_{50}$of the challenge virus was added to each serum dilution, and plates were incubated at 37 °C for 1 h. All sera were tested in duplicate and the average absorption values were used for the calculation of neutralizing titers. Wells containing virus dilutions without serum, MDCK cells only and DMEM only were included on each plate serving as controls and system background, respectively. In each run four control sera yielding: (i) negative, (ii) low, (iii) middle and (iv) high reactive results in the HI assay were included to assess the between-run imprecision of the assay. After incubation 100 μl of a suspension containing 3×10^4/ml freshly trypsinized MDCK cells in DMEM