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The human population is constantly exposed to multiple influenza A subtypes due to zoonotic spillover and rapid viral evolution driven by intrinsic error-prone replication and immunological pressure. In this context, antibody responses directed against the HA protein are of importance since they have been shown to correlate with protective immunity. Serological techniques, detecting these responses, play a critical role for influenza surveillance, vaccine development, and assessment. As the recent human pandemics and avian influenza outbreaks have demonstrated, there is an urgent need to better prepare to assess the contribution of the antibody response to protection against newly emerged viruses and to evaluate the extent of pre-existing heterosubtypic immunity in populations. In this study, 68 serum samples collected from the Italian population between 1992 and 2007 were found to be positive for antibodies against HSN1 as determined by single radial hemolysis (SRH), but most were negative when evaluated using haemagglutination inhibition (HI) and microneutralisation (MN) assays. As a result of these discordant serological findings, the increased sensitivity of lentiviral pseudotypes was exploited in pseudotype-based neutralization (pp-NT) assays and the results obtained provide further insight into the complex nature of humoral immunity against influenza A viruses.

MATERIALS AND METHODS
Serum Samples: A panel of human sera was provided by the Department of Molecular and Developmental Medicine, University of Siena. It consisted of 68 sera collected from Italian subjects (age between 6m and 92yr) from 1992 to 2007 that were previously found to be positive against HPAI H5N1 A/Vietnam/1194/2004 as determined by the SRH assay. The seropositivity of these sera was also confirmed by removing non-specific antibodies by their adsorption with a 1:1 volume mixture of A/New Caledonia/10/1999 H3N1 and A/California/7/2004 H3N2 viruses. The positive controls used in pp-NT assays were reference sheep sera against A/England/427/1988 (H3N2) and NIBRG-14 (H1N1) provided by the National Institute for Biological Standards and Control (NIHSC, United Kingdom), avian sera against A/African swine/England/983/1979 (H7N1) provided by the Animal Health and Veterinary Laboratories Agency (AHVLA), and monoclonal anti-humainfluenza A (H1N1, H2N2) antibody (mAb C179) (Takara, Clonotech).

Pseudotype Construction and Firefly Luciferase pp-NT Assays: Lentiviral pseudotypes with HA glycoproteins derived from HPAI virus A/Vietnam/1194/2004 (H5N1), A/Indonesia/5/2005 (H5N1), and HPAI virus A/chicken/Italy/1347/1999 (H7N1) were produced by cotransfection of 293FF/17 cells with the respective HA plasmid (pL1 backbone), the HIV gag-pol plasmid p8.91, and the reporter plasmid pC5FL (expressing Firefly luciferase) using the Fugene-6 transfection reagent. Neuraminidase activity was provided by exogenous bacterial NA addition for the release of pseudotype from producer cells. Serum samples (2.5 μl) were twofold serially diluted in culture medium and mixed with pseudotype virus (106.5 RLU luciferase input) at a 1:1 v/v ratio. After incubation at 37°C for 1 hr, 1:4 dilution of 293FF/17 cells were added to each well of a white 96-well flat bottomed tissue culture plate. RLU were evaluated 48 hr later by luminometry using the Bright-Glo assay system (Promega, UK). Endpoint neutralizing titres were calculated using GraphPad Prism 6. IC50 pseudotype neutralization titres are expressed as the reciprocal of the serum dilution that results in a 50% inhibition of pseudotype virus entry.

DISCUSSION

1. The population is constantly exposed to multiple influenza A subtypes due to zoonotic spillover and rapid viral evolution driven by intrinsic error-prone replication and immunological pressure. In this context, antibody responses directed against the HA protein are of importance since they have been shown to correlate with protective immunity. Serological techniques, detecting these responses, play a critical role for influenza surveillance, vaccine development, and assessment. As the recent human pandemics and avian influenza outbreaks have demonstrated, there is an urgent need to better prepare to assess the contribution of the antibody response to protection against newly emerged viruses and to evaluate the extent of pre-existing heterosubtypic immunity in populations. In this study, 68 serum samples collected from the Italian population between 1992 and 2007 were found to be positive for antibodies against HSN1 as determined by single radial hemolysis (SRH), but most were negative when evaluated using haemagglutination inhibition (HI) and microneutralisation (MN) assays. As a result of these discordant serological findings, the increased sensitivity of lentiviral pseudotypes was exploited in pseudotype-based neutralization (pp-NT) assays and the results obtained provide further insight into the complex nature of humoral immunity against influenza A viruses.

RESULTS

(a) Comparison of SRH assay versus pp-NT assay for antibody responses against the A/Vietnam/1194/2004 antigen. SRH titres expressed as diameter of haemolysis (in mm) are plotted on the x-axis. SRH titres > 25mm2 are considered to be “serum-positive” and this cutoff is represented on the plot by a vertical dotted line. IC50 pseudotype neutralization titres expressed as the reciprocal of the serum dilution that results in a 50% inhibition of pseudotype virus entry are plotted on the y-axis. (b) Comparison of HI assay versus pp-NT assay for antibody responses against the A/Vietnam/1194/2004 antigen. HI titres expressed as the reciprocal of the highest dilution causing complete inhibition of haemagglutination are plotted on the x-axis. IC50 pseudotype neutralization titres expressed as the reciprocal of the serum dilution that results in a 50% inhibition of pseudotype virus entry are plotted on the y-axis. (c) Comparison of MN assay versus pseudotype based neutralization assay for antibody responses against the A/Vietnam/1194/2004 antigen. The vertical dashed lines indicate the value of MN log 10 titre = 1.9 (corresponding to a titre of 1:80) and the proposed threshold of protective antibodies; horizontal dashed line indicates the corresponding value of pp-NT log 10 titre = 2.25 (corresponding to a titre of 1:357 as calculated by Alberini et al., 2009).

CONCLUSIONS

1. It is postulated that this highly sensitive pseudotype neutralization assay is measuring broadly specific anti-stalk antibodies that have been elicited via exposure to seasonal H1 and H3 viruses and/or antibodies that recognize highly conserved sequences located underneath the RBS of individual subtypes.

2. The HI data shows that the antibody responses measured in these individual subtypes are unlikely to be directed against the globular head.

3. No wild-type virus culture is necessary as the serological assay is entirely synthetic.

4. The influenza pseudotype assay is useful as an adjunct to the EMA/FDA approved serology assays, HI and SRH. It is a functional assay and quantifies neutralizing antibody responses against both the HA head (HAS) and stalk (H2).

5. Pseudotype neutralization titres are expressed as the reciprocal of the serum dilution that results in a 50% inhibition of pseudotype virus entry. It is postulated that pseudotype neutralization assay is measuring broadly specific anti-stalk antibodies that have been elicited via exposure to seasonal H1 and H3 viruses and/or antibodies that recognize highly conserved sequences located underneath the RBS of individual subtypes.

6. The HI data shows that the antibody responses measured in these individual subtypes are unlikely to be directed against the globular head.

7. No wild-type virus culture is necessary as the serological assay is entirely synthetic.

8. The influenza pseudotype assay is useful as an adjunct to the EMA/FDA approved serology assays, HI and SRH. It is a functional assay and quantifies neutralizing antibody responses against both the HA head (HAS) and stalk (H2).

9. Pseudotype neutralization titres are expressed as the reciprocal of the serum dilution that results in a 50% inhibition of pseudotype virus entry. It is postulated that pseudotype neutralization assay is measuring broadly specific anti-stalk antibodies that have been elicited via exposure to seasonal H1 and H3 viruses and/or antibodies that recognize highly conserved sequences located underneath the RBS of individual subtypes.

REFERENCES


