Heterosubtypic Protection Conferred by the Human Monoclonal Antibody PN-SIA28 against Influenza A Virus Lethal Infections in Mice

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Previous in vitro studies demonstrated PN-SIA28 neutralizing activities against phylogenetically divergent influenza A subtypes. In this study, the protective activity of PN-SIA28 was evaluated in mice inoculated with lethal influenza A/WSN/33 (H1N1), A/Quebec/144147/09 (H1N1)pdm09, and A/Victoria/3/75 (H3N2) viruses. At 24 h postinoculation (p.i.), animals received PN-SIA28 intraperitoneally (1 or 10 mg/kg of body weight) or 10 mg/kg of unrelated Hu-MAb (mock). Body weight loss and mortality rate (MR) were recorded for 14 days postinfection (p.i.). Lung viral titers (LVT) were determined at day 5 p.i. In A/WSN/33 (H1N1)-infected groups, all untreated and mock-receiving mice died, whereas MRs of 87.5% and 25% were observed in mice that received PN-SIA28 1 and 10 mg/kg, respectively. In influenza A(H1N1) pdm09-infected groups, an MR of 75% was recorded for untreated and mock-treated groups, whereas the PN-SIA28 1-mg/kg and 10-mg/kg groups had rates of 62.5% and 0%, respectively. In A/Victoria/3/75 (H3N2)-infected animals, untreated and mock-treated animals had MRs of 37.5% and 25%, respectively, and no mortalities were recorded after PN-SIA28 treatments. Accordingly, PN-SIA28 treatments significantly reduced weight losses and resulted in a ≥1-log reduction in LVT compared to the control in all infection groups. This study confirms that antibodies targeting highly conserved epitopes in the influenza HA stem region, like PN-SIA28, not only neutralize influenza A viruses of clinically relevant subtypes in vitro but also, more importantly, protect from a lethal influenza virus challenge in vivo.

Influenza viruses possess two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), which determine strain classification into several subtypes. Although there are 16 known subtypes of HA (H1 to H16) and 9 of NA (N1 to N9) (1), the influenza A viruses responsible for pandemics and epidemics in the last century belonged to H1N1, H2N2, and H3N2 subtypes (2, 3). Notably, influenza A(H1N1) and A(H3N2) viruses are responsible for recent seasonal influenza epidemics (4). Trivalent or quadrivalent vaccines usually elicit a potent neutralizing activity against specific influenza strains. However, influenza viruses undergo rapid antigenic drifts, which may result in antigenic mismatches between the vaccine and the circulating strains (5). Moreover, when a pandemic strikes, no vaccine is available for some months, as seen during the latest 2009 pandemic (6). Two classes of antivirals are available against influenza infections. These include the M2 ionic channel blockers (adamantanes: amantadine and rimantadine) and the NA inhibitors (NAI), such as oseltamivir and zanamivir. However, reports have shown that resistance to adamantanes in influenza A(H3N2) viruses isolated after 2005 exceeds 95% and that all influenza A(H1N1) pdm09 viruses recovered after 2009 contain the S31N M2 substitution conferring adamantanes resistance (7–9). Resistance to oseltamivir, the most frequently used NAI, has also been of concern, as shown during the 2008 to 2009 influenza season, during which >99% of the influenza A/Brisbane/59/07 (H1N1)-like strains isolated in North America and Europe carried the NAI-resistant H275Y NA change (10). Moreover, resistance to oseltamivir has also been reported in certain clinical settings of pediatric and immunocompromised patients infected with influenza A(H3N2) viruses in which the NA gene contained E119V and/or R292K substitutions (11, 12). Given this background, alternative therapeutic strategies are certainly needed to counterbalance the emergence of novel seasonal and pandemic influenza viruses carrying resistance mutations to the currently available anti-influenza drugs. Monoclonal antibodies (MAbs) recognizing broadly conserved influenza virus epitopes and harboring excellent neutralizing activity would fit as an immediate prophylactic and therapeutic tool. Strain-specific neutralizing epitopes are mainly located on the HA1 globular head, responsible for attachment to cell receptors (13). However, conserved broader-range epitopes shared among influenza virus isolates belonging to different subtypes are located on the fusion peptide within the HA2 subunit or in the stem region of the HA. The HA stem region epitopes can offer, therefore, an appropriate neutralization target for universal prophylactic/therapeutic purposes (14). The PN-SIA49 and PN-SIA28 human MAbs have been reported to recognize such epitopes (15, 16). In vitro studies showed successful neutralization of influenza A H1N1, H2N2, H5N1, and H9N2 and all H3N2 viruses from 1968 to 1975 by
TABLE 1 Impact of treatment with MAb PN-SIA28 on mortality rates of mice infected with influenza A (H1N1), A (H1N1)pdm09, and A (H3N2) viruses

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Group size (no.)</th>
<th>Mortality rate (%) on day 14 p.i.</th>
<th>A/WSN/33(^a) (H1N1)</th>
<th>A/Quebec/144147/09(^b) (A [H1N1]pdm09)</th>
<th>A/Victoria/3/75(^c) (H3N2)</th>
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</thead>
<tbody>
<tr>
<td>Uninfected/untreated</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Untreated/infected</td>
<td>8</td>
<td>80</td>
<td>75</td>
<td>37.5</td>
<td>37.5</td>
</tr>
<tr>
<td>MAB PN-SIA28 at 1 mg/kg</td>
<td>8</td>
<td>87.5</td>
<td>62.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MAB PN-SIA28 at 10 mg/kg</td>
<td>8</td>
<td>25(^d)</td>
<td>0(^d)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MAB e137 at 10 mg/kg</td>
<td>8</td>
<td>100</td>
<td>75</td>
<td>25</td>
<td>25</td>
</tr>
</tbody>
</table>

\(^a\) Viral inoculum of 3 × 10^5 PFU/mouse.
\(^b\) Viral inoculum of 1 × 10^3 PFU/mouse.
\(^c\) Viral inoculum of 5 × 10^3 PFU/mouse.
\(^d\) The P value is <0.01 compared to the untreated/infected group, using the Kaplan-Meier and Mantel-Cox tests.

PN-SIA28. However, the in vivo activity of this MAb has not been demonstrated yet.

In the present study, the protection conferred by PN-SIA28, a fully human MAB targeting a conserved region lying in the stem region of HA and encompassing amino acid residues on the HA2 and HA1 stem portion (16) was evaluated in mice challenged with a lethal dose of influenza A/WSN/33 (H1N1), A/Victoria/3/75 (H3N2), or A/Quebec/144147/09 (H1N1)pdm09-like viruses.

MATERIALS AND METHODS

Ethics statement. This study was carried out with the approval of the institutional animal care committee of the Centre Hospitalier Universitaire de Québec in accordance with the national guidelines for the use of animals in laboratory experiments (from the Canadian Council of Animal Care) (17).

Monoclonal antibodies. The MAB PN-SIA28 is a human IgG with heterosubtypic binding and neutralizing capabilities, and its preparation and development have been previously described (18, 19). Another MAB (e137), directed toward the hepatitis C virus E2 glycoprotein, served as a negative control (20, 21).

Viruses. The recombinant influenza A/WSN/33 (H1N1) virus was previously generated using a reverse genetics system that includes eight influenza virus RNA-coding transcription plasmids (pPOLI-PA, -PB1, -PB2, -NP, -HA, -NA, -M, and -NS) and polymerase and nucleoprotein expression plasmids (pCAGGS-PA, -PB1, -PB2, -NP) kindly provided by Peter Palese (Mount Sinai School of Medicine, New York, NY) (22). The mouse-adapted influenza A/Victoria/3/75 (H3N2) virus (a gift from Donald Smeec, Utah State University, Logan, UT) was passaged twice in Madin-Darby canine kidney (MDCK) cells prior to use (23). The recombinant influenza A/Quebec/144147/09 (GenBank accession numbers FN434457 to FN434464), which is an influenza A/H1N1)pdm09 virus, was rescued by reverse genetics, as previously described (24).

Virus titrations. Viral titer were determined by standard plaque assays using confluent ST6-Gal MDCK cells (25) for influenza A/Quebec/144147/09 and A/Victoria/3/75 strains, whereas Madin-Darby bovine kidney (MDBK) cells were used for the influenza A/WSN/33 strain.

Mouse infections and treatment regimens. Groups of 12 female, 6-week-old BALB/c mice (for A/WSN/33 experiments) or C57BL/6 mice (for A/[H1N1]pdm09 and A/Victoria/3/75 experiments) were purchased from Charles River (LaSalle, Quebec, Canada). Animals were randomized based on their body weight and housed four per cage in a biosafety level 2 environment that prevented cage-to-cage transmission. The animals were inoculated intranasally, under isoflurane anesthesia, with 3 × 10^5 PFU of influenza A/WSN/33 (H1N1), 5 × 10^3 PFU of influenza A/Victoria/3/75 (H3N2), or 1 × 10^3 PFU of influenza A/Quebec/144147/09 (H1N1)pdm09 virus. A group of 6 uninfected and untreated mice served as a naive control. At 24 h p.i., infected animals received a single intraperitoneal dose of PN-SIA28 (1 mg/kg or 10 mg/kg of body weight), e137 (10 mg/kg), or saline. Mice were monitored daily for body weight loss and mortality over a period of 14 days. Subgroups of 4 mice were sacrificed at day 5 postinfection (p.i.) for determination of lung viral titers (LVT). The lungs were sampled aseptically and homogenized in 1 ml of sterile phosphate-buffered saline. The homogenates were centrifuged at 600 × g for 10 min, and supernatants were titrated by using standard plaque assays.

Comparison of HA amino acids belonging to viruses tested against PN-SIA28 in vitro and in vivo, potentially involved in PN-SIA28 binding. Several amino acid parts of the stem portion of HA (HA1 and the HA2 subunits) were previously identified as major determinants of MAb PN-SIA28 affinity (16). Such residues were compared between the strains used in this study and the reference influenza A strains previously used to assess in vitro PN-SIA28 neutralizing capability after sequence alignments using the ClustalW algorithm.

Statistics and software. Mortality rates (MRs) were assessed with Kaplan-Meier survival curves and the Mantel-Cox test. Weight losses and LVT were compared with a one-way analysis of variance followed by Tukey’s comparison test. GraphPad version 5.0.288 software was used for all statistical analyses, whereas CLC Sequence Viewer version 7.5 (Qiagen-Aarhus A/S) was used for amino acid ClustalW alignment.

RESULTS

Activity of MAb PN-SIA28 against influenza A/WSN/33 (H1N1) infection. Intranasal inoculation of BALB/c mice with 3 × 10^5 PFU of the recombinant A/WSN/33 (H1N1) virus resulted in an MR of 100% (8/8 mice) in the untreated but infected animals (Table 1 and Fig. 1A). The same MR was recorded for mice that received 10 mg/kg of the nonspecific e137 MAb. An MR of 87.5% (7/8 mice) was observed in the animals that received 1 mg/kg of PN-SIA28, whereas the group that received 10 mg/kg of PN-SIA28 had an MR of 25% (2/8). Only the 10-mg/kg regimen of the MAB PN-SIA28 resulted in a significantly reduced MR compared to the untreated and infected group (P < 0.01).

Infection of mice with the influenza A/WSN/33 virus resulted in marked body weight loss in the untreated and infected group as well as in the animals that received PN-SIA28 (1 mg/kg and 10 mg/kg) and e137 (10 mg/kg) treatments during the first 4 days posttreatment (Fig. 2A). After this time point, a significant weight gain was only observed in the PN-SIA28 10-mg/kg group compared to the untreated and infected animals.

The mean ± standard error of the mean (SEM) LVT for the untreated and infected mice was 8.50 ± 4.13 × 10^5 PFU/ml (Fig. 3A). The PN-SIA28 treatment regimens of 1 mg/kg and 10 mg/kg were associated with an approximately 1-log reduction (4.10 ± 1.00 × 10^5 PFU/ml; P < 0.05) and a 4-log reduction (4.00 ± 2.67...
Infection of mice with influenza A/Quebec/144147/09 resulted in similar body weight losses for the untreated group and for animals that received PN-SIA28 (1 mg/kg or 10 mg/kg) during the first 4 days posttreatment (Fig. 2B). After this time point, a significant weight gain was observed in the PN-SIA28 10-mg/kg group, while the PN-SIA28 1-mg/kg and the e137 10-mg/kg treatment groups showed weight losses that were similar to those of the untreated and infected group.

The mean (± SEM) LVT for untreated and infected mice was $3.00 ± 0.47 × 10^6$ PFU/ml (Fig. 3B). Mice receiving the e137 (10 mg/kg) treatment regimen exhibited slightly higher LVT, i.e.,...
4.03 ± 0.29 × 10^6 PFU/ml. The PN-SIA28 1-mg/kg treatment group also had comparable LVT (2.34 ± 0.30 × 10^6 PFU/ml). In contrast, the LVT for the PN-SIA28 10-mg/kg treatment group was significantly lower than that of the untreated and infected group by approximately 1 log (5.93 ± 1.38 × 10^5 PFU/ml; P < 0.001).

Activity of MAb PN-SIA28 against influenza A/Victoria/3/75 (H3N2) infection. Intranasal inoculation of C57BL/6 mice with 5 × 10^3 PFU of the recombinant A/Victoria/3/75 (H3N2) virus resulted in an MR of 37.5% (3/8 mice) in untreated but infected animals (Table 1 and Fig. 1C). There were no mortalities in the groups that received the PN-SIA28 1-mg/kg and 10-mg/kg doses, whereas an MR of 25% was recorded for the group treated with 10 mg/kg of e137. Although there was a clear trend toward lower MRs in the PN-SIA28 treatment groups, this effect was not statistically significant compared to the untreated group.

Infection of mice with A/Victoria/3/75 (H3N2) resulted in a small body weight loss for the untreated group as well as for animals that received PN-SIA28 (1 mg/kg and 10 mg/kg) and e137 (10 mg/kg) during the first 4 days posttreatment (Fig. 2C). After this time point, a statistically significant weight gain, starting on day 6 p.i., was observed for the PN-SIA28 1-mg/kg and 10-mg/kg groups compared to the untreated and infected animals, while the e137 10-mg/kg treatment group did not show weight recovery. Although the weight difference was not significant between the 1-mg/kg and 10-mg/kg groups, the 1-mg/kg group showed delayed weight recovery between days 7 and 9.

The mean (± SEM) LVT for untreated and infected mice was 4.63 ± 1.88 × 10^6 PFU/ml (Fig. 3C). The e137 10-mg/kg group showed slightly higher LVT at 7.33 ± 0.56 × 10^6 PFU/ml. In contrast, the PN-SIA28 1-mg/kg and 10-mg/kg regimens showed significantly reduced the mean LVT (by at least 1 log) compared to that for the untreated and infected group (5.25 ± 3.25 × 10^5 PFU/ml and 1.55 ± 0.45 × 10^5 PFU/ml, respectively; P < 0.01).

Comparison of amino acids potentially involved in PN-SIA28 affinity. The three influenza strains used in this study share many potential affinity-related amino acid residues with other influenza A strains from different phylogenetic groups (Table 2). The H25, K57, T315, N336, M360, D362, G363, W364, and T384 residues are identical among the three strains evaluated. Moreover, many of these amino acids are also found in other group 1 and group 2 strains. On the other hand, residues at positions 45 (H/N), 358 (T/E), 361 (I/V), 392 (T/N), 395 (V/L), and 400 (E/I) differ among the three strains used, but most of the differences are related to the subtypic differences (i.e., group 1 or 2); however, positions 361 and 400 seem to exhibit subtype independent differences. Of interest, although the influenza A/Quebec/144147/09 strain was not previously characterized by in vitro neutralization assays, its HA protein shared most (13 of 15) residues involved in PN-SIA28 binding affinity with that of the influenza virus A/Milan/UHSRI/09 strain (Table 2).
In this *in vivo* study, we provide strong evidence of protection conferred by the PN-SIA28 IgG molecule after a single dose administered 24 h after a lethal challenge with influenza A/WSN/33 (H1N1), A/Quebec/144147/09 (A[H1N1]pdm09), or A/Victoria/3/75 (H3N2) strains. Indeed, we observed reduced mortality in mice infected with the three viral strains tested at both PN-SIA28 concentrations (1 mg/kg and 10 mg/kg), with a dose-dependent effect for the two influenza A(H1N1) viruses. The highest dose of PN-SIA28 significantly reduced body weight losses and lung viral titers compared to untreated animals or those receiving the e137 control MAb. The human MAb PN-SIA28 had never been tested against all strains tested in mice as a complete IgG (19). It thus is important and appropriate to mention that we did not observe any unexplained deaths or signs of toxicity when this compound was injected for the first time. All signs and symptoms observed in infected animals, rather, were related to the well-characterized influenza syndrome in susceptible mice (26, 27).

Phylogenetic studies segregate influenza HA subtypes into two major groups: group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13) and group 2 (H3, H4, H7, H10, H14, and H15) viruses (28). Previous studies, MABS PN-SIA28 and PN-SIA49 were tested for their neutralization against various strains, and PN-SIA28 was able to neutralize group 2 H3 influenza viruses from 1968 to 1975 in addition to highly divergent group 1 viruses. Although the epitope of PN-SIA28 involves Ile361 and Asp362 in the HA2 domain of influenza A(H1N1) viruses, a substitution at position 57 (Lys to another amino acid) in the HA1 domain resulted in decreased MAb affinity, and this seems to be responsible for the lack of activity against influenza A(H3N2) viruses that circulated in decreased MAb affinity, and this seems to be responsible for the lack of activity against influenza A(H3N2) viruses that circulated in 1934 influenza A virus reference isolate (18, 19). Few heterosubtypic neutralizing MAbs directed toward the influenza virus HA2 molecule have been reported, and most of them were found to neutralize either group 1 or group 2 HAs (30, 31). An HA2-based specific MAb recognizing influenza A(H3N2) viruses from 1947 to 2008 was also described; however, this MAb did not recognize group 1 HAs (32). Indeed, the origin of PN-SIA28 might explain its spectrum of activity. PN-SIA28 and PN-SIA49 were isolated as Fab fragments cloned from serum peripheral B cells able to recognize influenza virus reference isolates and collected from the donor before the 2009 H1N1 pandemic. Then, the full human IgG was produced, and the activity of the whole IgG was evaluated in vitro (15, 19).

We could not evaluate the effect of PN-SIA28 treatments against all strains tested in vitro to confirm its broad heterosubtypic neutralizing activity, as not all influenza A virus subtypes are pathogenic in mice (33). Nevertheless, mouse models of influenza A virus infections available at our laboratory and used in this study included the influenza A(H1N1) and A(H3N2) virus subtypes which have been responsible for important influenza epidemics and pandemics. Previous experimental infections of mice with influenza A viruses performed by our group demonstrated that the BALB/c mouse model was adequate for studying the influenza A/WSN/33(H1N1) strain, whereas higher titers were obtained with the C57BL/6 model for influenza A(H1N1)pdm09 and A/Victoria/3/75 (H3N2) strains (23, 34). Despite the fact that PN-SIA28 may not be sufficient to protect against all existing subtypes, it would be worthwhile to evaluate its activity as part of a cocktail of 2 or 3 heterosubtypic MAbs that could protect from influenza A virus strains of the entire phylogenetic HA diversity in the context of a new pandemic virus. Additionally, although we expect that PN-SIA28 could also exert a protective activity when administered prophylactically, delayed treatment (>24 h) should be evaluated to better mimic the clinical conditions. Finally, since mouse models of infection with more recent influenza A(H3N2) viruses are not available, evaluating PN-SIA28 against such strains in other animal models, such as guinea pigs or ferrets, would be warranted.

### TABLE 2 Comparison of HA amino acids potentially involved in PN-SIA28 affinity between influenza A strains of different subtypes

<table>
<thead>
<tr>
<th>Virus</th>
<th>Neutralization in vitro</th>
<th>Subtype</th>
<th>Phylogenetic cluster group</th>
<th>HA amino acid at position:</th>
<th>HA1</th>
<th>HA2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/PR/8/34</td>
<td>+</td>
<td>H1N1</td>
<td></td>
<td>H H K T N T M I D G W T T V E</td>
<td>25</td>
<td>45</td>
</tr>
<tr>
<td>A/WSN/33</td>
<td>+</td>
<td>H1N1</td>
<td></td>
<td>H H K T N T M I D G W T T V E</td>
<td>57</td>
<td>315</td>
</tr>
<tr>
<td>A/Milan/UHSR1/09</td>
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<td>H1N1</td>
<td></td>
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<td>361</td>
</tr>
<tr>
<td>A/Quebec/144147/09</td>
<td>NA</td>
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<td>362</td>
</tr>
<tr>
<td>A/Vietnam/1203/04</td>
<td>+</td>
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<td>Q H M A N S L V A G W T T V D</td>
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</tr>
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<td>A/chicken/Hong Kong/G9/97</td>
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<td>A/Aichi/2/68</td>
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<td>H N K T N E M I D G W T N L E</td>
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<tr>
<td>A/Fuku1/20/04</td>
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<td>H3N2</td>
<td></td>
<td>H N K T N E M I D G W T N L E</td>
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<tr>
<td>A/Washington/01/07</td>
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<td>H3N2</td>
<td></td>
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</tr>
<tr>
<td>A/NY/107/03</td>
<td>—</td>
<td>H7N2</td>
<td></td>
<td>H N K T N E M I D G W T N L E</td>
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</tr>
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</table>

Influenza strains toward which MAb PN-SIA28 showed a protective effect in the present study are presented in bold type. In vitro neutralization results based on a previous study (16). +, neutralization of the virus; NA, not available; —, no neutralization of the virus. H1 numbering using complete cDNA HA sequences.
In conclusion, the present study provides strong evidence that the MAb PN-S1A28 is an effective postinfection therapeutic agent with heterosubtypic potency in vivo. Our results, combined with previous in vitro data showing broad-spectrum activity against many HA from phylogenetic groups 1 and 2, position PN-S1A28 as a potential broad-range treatment for severe human influenza A virus infections.

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