Immunization with pneumococcal neuraminidases NanA, NanB and NanC to generate neutralizing antibodies and to increase survival in mice

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Abstract

Purpose. Pneumococcal virulence protein-based vaccines can provide serotype-independent protection against pneumococcal infections. Many studies, including clinical observational studies on Thomsen–Friedenrich antigen exposure and haemolytic uremic syndrome, defined the role of neuraminidases NanA, NanB and NanC in host-pneumococcus interaction. Since neuraminidases are major virulence proteins, they are potential targets for both vaccines and small molecule inhibitors. Here we explored the utility of three neuraminidases as protein vaccine antigens to generate neutralizing antibodies and to increase survival following pneumococcal infections.

Methodology. Rabbits and mice were immunized subcutaneously with enzymatically active recombinant NanA, NanB and NanC as individual or a combination of the three neuraminidases. Antisera titres were determined by ELISA. Neuraminidase activity inhibition by antiserum was tested by peanut lectin and flow cytometry. Clinical isolates with serotype 3, 6B, 14, 15B, 19A and 23F were used to infect immunized mice by tail vein injection.

Results/Key findings. Presence of high levels of IgG antibodies in antisera against NanA, NanB and NanC indicates that all of the three neuraminidases are immunogenic vaccine antigens. To generate potent NanA neutralizing antibodies, both lectin and catalytic domains are essential, whereas for NanB and NanC a single lectin domain is sufficient. Immunization with triple neuraminidases increased the survival of mice when intravenously challenged with clinical isolates of serotype 3 (40 %), 6B (60 %), 15B (60 %), 19A (40 %) and 23F (30 %).

Conclusion. We recommend the inclusion of three pneumococcal neuraminidases in future protein vaccine formulations to prevent invasive pneumococcal infection caused by various serotypes.

INTRODUCTION

Streptococcus pneumoniae is an important human pathogen. It can colonize the upper respiratory tract and subsequently cause otitis media and community-acquired pneumonia (CAP) and invasive pneumococcal diseases (IPDs) such as necrotizing pneumonia, bacteremia and meningitis [1–3]. Pneumococcal pneumonia following influenza infection increases the disease severity and in a few cases causes death [4, 5]. One of the most severe complications of invasive pneumococcal infection is haemolytic uremic syndrome (HUS) that mainly occurs in children and is associated with haemolytic anemia, thrombocytopenia and acute renal failure [6, 7]. Although S. pneumoniae encodes many virulence factors, neuraminidase A (NanA) and pneumococcal surface protein C (PspC) are involved in the pathogenesis of HUS [7–10]. Neuraminidase cleaves N-acetylneuraminic acid (sialic acid) residues on red blood cells (RBCs), platelets and endothelial cells leading to the exposure of the Thomsen–Friedenrich antigen (TA) [8, 11–13]. It is widely accepted that normally circulating anti-T antigen antibodies react with the exposed TA on cells and can cause haemolysis and endothelial injury [8, 11–13]. But, Burin et al., showed that anti-TA are low-titre antibodies with extremely weak haemolyzing capacities compared with ABO antibodies [14]. Grewal et al. confirmed that thrombocytopenia is the result of the hepatic Ashwell receptor-dependent clearance of platelets that are first desialylated by NanA [15].
Desialylation also triggers an alternative pathway of complement (APC) dysfunction on RBCs [11, 16, 17]. Recent investigations found that transient complement dysregulation and consumption during HUS and genetic variations of complement genes in some patients may play a role in the pathogenesis of HUS [11, 16, 17].

Routine immunization with the pneumococcal conjugate vaccines (PCV7/PCV10/PCV13) significantly decreased the burden of CAP and IPDs worldwide [18, 19]. However, in the post-PCV era an increase of non-vaccine serotypes in both nasopharyngeal carriage and clinical isolates was observed [20–25]. These observations stress the need to develop pneumococcal protein vaccines that have broader serotype coverage. Pneumococcal proteins that have been shown from animal models to have vaccine potential include autolysins (LytC and LytA), CbpE, GlpO, IgA1, NanA, PavA/B, PcpA, PcsB, PhtA/B/D/E, Pili (RrgA, RrgB, RrgC), Ply, PspA, PspC, PotD, StkP, ZmpC etc. [26–28]. These proteins have divergent biological activities at different stages of infection [1, 3, 28]. Previously, several research groups used different NanA protein purification methods and animal models to determine the vaccine potential of NanA. In a murine intranasal challenge model, Lock et al. observed a small but significant increase in survival in mice when the neuraminidase antigen was purified from cultures of S. pneumoniae and treated with formaldehyde [29]. Long et al. demonstrated that NanA could significantly reduce nasopharyngeal colonization and the incidence of otitis media with effusion [30]. Tong et al. demonstrated that rNanA affords protection against S. pneumoniae 6A nasopharyngeal colonization in a chinchilla model [31]. Recently, Anderson et al. reported that in an intranasal challenge model, rNanA immunization reduced the pulmonary burden of the serotype 3 pneumococci and increased survival of CBA/N mice [19]. However, the pulmonary burden of the serotype 8 pneumococci was not reduced in BALB/c mice after intranasal challenge [27]. In children, S. pneumoniae colonization can induce antibodies against specific pneumococcal virulence proteins and decrease the number of respiratory tract infections [32]. Sera from patients convalescing from IPD and from healthy individuals can also react with multiple pneumococcal protein antigens [33, 34]. Data from the Finnish otitis media cohort study showed that culture-confirmed pneumococcal colonizers could induce serum anti-NanA IgG antibodies in early childhood [35]. However, the concentrations of serum anti-NanA were not significantly associated with subsequent pneumococcal carriage or acute otitis media (AOM). Simell et al. hypothesized that this might be due to low concentrations of anti-NanA antibodies, while higher concentrations could be reached by vaccination [35].

In addition to NanA, NanB and NanC that are encoded in the S. pneumoniae genomes could significantly enhance pathogenicity with separately defined roles in pathogenesis [1, 36–38]. The three pneumococcal neuraminidases, NanA, NanB and NanC, have a molecular mass of 115, 78 and 82 kDa, respectively, NanB and NanC share 50 % sequence identity, whereas they both share only 25 % identity with NanA [39–41]. NanA showed activity toward both α2-3 and α2-6 sialyl linkages, while both NanB and NanC cleaved only α2-3 sialyl linkages [39–42]. NanA has been shown to contribute to nasopharyngeal colonization, biofilm formation, spread of pneumococci to the lungs, inflammation, sepsis, development of otitis media and invasion of the blood-brain barrier [15, 37, 43–45]. Studies also showed that the nanA mutant in the ply deletion background did not result in an additional reduction of virulence and the encapsulated strain D39 nanA mutant had no effect on the ability to colonize mice [46, 47]. In addition, no difference in adhesion of the D39 nanA mutant to cells of human upper airway epithelial lines was found [47]. However, the nanA mutant of the R6 strain (non-encapsulated D39) was impaired in adherence to epithelial cells when compared with parental strain R6 [47]. In contrast, Brittan et al. showed that adhesion to cultured epithelial cells is reduced in nanA and nanB mutants of D39 [48]. In a mouse model, NanB was linked to the development of upper and lower respiratory tract infection and sepsis [36]. Neuraminidase gene nanA is present in both invasive and non-invasive isolates of S. pneumoniae and nanB in more than 96 % of the isolates. However, nanC is present in only 41–47 % of the non-invasive isolates, but in a higher percentage (58–89 %) of the invasive isolates [37, 49]. Pettigrew et al. reported that the prevalence of nanC was 1.41 times higher among cerebrospinal fluid isolates than among carriage isolates [37]. In a recent study, we observed that nanC was present in 89 % of HUS isolates when compared with 41 % of patients with culture-confirmed IPD without HUS [49]. However, two other investigations found no correlation between nanC distribution and HUS [50, 51]. Additionally, in in vitro studies no significant difference in the overall neuraminidase activity between HUS isolates and controls was found [50, 51]. Our laboratory investigations also showed that all of the three pneumococcal neuraminidases expose TA on cell surface proteins and on serum sialoglycoproteins [49, 52]. In this study, we explored the possibility of combining the three neuraminidases together as vaccine antigens to devise a universal protein vaccine to prevent systemic pneumococcal infection.

**METHODS**

**Bacterial strains and cell lines**

S. pneumoniae serotypes 3, 6B, 14, 15B, 19A and 23F used in this study were clinical isolates collected from patients with severe infections, including pneumonia, necrotizing pneumonia and HUS [21, 49, 52]. Serotypes 3, 6B, 14 and 23F are among the most predominant serotypes prevalent in communities in the last decade [13, 21, 22, 53]. Serotypes 19A and 15B included in this study are the non-vaccine serotypes that have become common in PCV7 and PCV13 vaccinated populations [21–25]. S. pneumoniae, Escherichia coli DH5α and E. coli BL21 (DE3) were grown as described previously [9, 40].

**Protein expression and purification**

Neuraminidase genes nanA, nanB and nanC were detected in clinical isolates using primers as described earlier [37]. PCR fragments containing the protein-coding sequences were
amplified using genomic DNA from a serotype 14 S. pneumoniae clinical isolate CGSP14 (Refseq: NC_010582, Table S1, available in the online version of this article) [54]. Amplified fragments were digested with KpnI and XhoI and ligated to pET29b+ (Novagen, Madison, WI, USA), with the resulting plasmids transformed into E. coli DH5α (Fig. 1 and Table S2). NanA, NanB and NanC recombinant proteins were expressed in E. coli BL21 (DE3) (RBC Bioscience, Taipei, Taiwan) and purified according to the manufacturer’s instructions for Ni²⁺ affinity chromatography using Nickel-Chelating Resin (Ni Sepharose 6 Fast Flow, GE Healthcare, Uppsala, Sweden) [40].

Protein preparations were evaluated by SDS-PAGE and coomassie blue staining. Purity of >95 % was observed for all of the proteins after coomassie-staining of the gels.

**Immunization of mice and antisera production**

To generate antisera against NanA, NanB and NanC proteins, individual or combination of neuraminidases; NanA, NanB and NanC (10 µg each) were injected subcutaneously. Four-week-old BALB/C mice were immunized with proteins four times at a 2-week interval. Only PBS was used as a negative control. Complete Freund’s adjuvant (CFA) was used

![Schematic diagram displaying recombinant neuraminidase clones and sub-clones.](image-url)

**Fig. 1.** Schematic diagram displaying recombinant neuraminidase clones and sub-clones. PCR fragments were amplified by using genomic DNA of serotype 14 S. pneumoniae strain CGSP14; PCR-amplified fragments of full-length neuraminidase genes, including nanA, nanB and nanC, and partial neuraminidase genes were cloned into the expression vector pET29b+, using restriction enzymes KpnI and XhoI. Lectin domain or CBM40 refers to type 40 carbohydrate-binding module (CBM) that recognizes sialic acid. Sialidase superfamily refers to catalytic domain. (a) Full-length protein NanA (NanA), NanA catalytic domain (NanACat), conserved catalytic domain (NanAConCat), lectin domain (NanALec), lectin+catalytic domain (NanALecCat) are shown. (b) Full-length protein NanB (NanB), full-length protein NanC (NanC), NanB and NanC central conserved domain (NanB/CCenCon), catalytic conserved domain (NanB/CCat-Con), NanB lectin domain (NanBlec), and NanC lectin domain (NanClec) are illustrated.
for first time immunization and incomplete Freund’s adjuvant (IFA) was used for the last three immunizations with the ratio of 1:1 to the antigen(s). To confirm the presence of anti-neuraminidase antibodies in the antisera, we performed ELISA. NanA, NanB and NanC proteins were first coated on an ELISA plate (Corning, New York, USA). The anti-neuraminidase antiserum was serially diluted twofold and added to the ELISA plate, and the antigen–antibody interaction was quantified by using goat HRP-conjugated anti-mouse/anti rabbit immunoglobulin G (IgG) as a secondary antibody (Millipore, Billerica, MA, USA) and TMB/peroxide (R&D Systems, Minneapolis, MN, USA) as a colour-developing substrate. Control sera from the pre-immune and the negative control with only PBS plus CFA and IFA were also examined. Initially, rabbits were injected subcutaneously with 0.50 mg of single neuraminidase in CFA. Later three booster doses of 0.25 mg of single neuraminidase in IFA were given at 2-week intervals. The purification of IgG fraction from the pooled antiserum was carried out by using protein A sepharose beads. To confirm the presence of anti-neuraminidase antibodies in the antiserum, we performed ELISA.

**Vaccination and intravenous challenge**

To evaluate the degree of protection of NanA, NanB and NanC protein antigens against *S. pneumoniae* intravenous challenge, individual or a combination of neuraminidases, NanA, NanB and NanC (10 µg each), were injected subcutaneously. Four-week-old BALB/C mice were immunized with proteins four times at a 2-week interval prior to *S. pneumoniae* (3 × 10⁵ c.f.u.) intravenous challenge [55]. The 23-valent Pneumovax (PPV23) polysaccharide vaccine (Merck Sharp and Dohme, NJ, USA) and PBS were used as positive and negative controls, respectively. Serotypes used in the intravenous infection in mice are included in PPV23. Freund’s complete adjuvant was used for first time immunization and Freund’s incomplete adjuvant used for the last three immunizations with the ratio of 1:1 to the antigen(s). *S. pneumoniae* cells were grown to mid-logarithmic phase were harvested, washed and resuspended in PBS. The inoculum used was determined by colony counts of serial dilutions plated on tryptic soy agar (TSA). *S. pneumoniae* cell suspension (0.1 ml) was used to infect anesthetized mice by tail vein injection. The challenge c.f.u. are given in figure legends. Survival studies were performed at least twice, and similar results were observed in all replicate experiments. Animals were monitored for 14 days post-challenge to determine the survival rate (%). The Chang Gung University institutional animal care and use committee approved all animal studies.

**Detection of T-antigen exposure on RBC**

The antisera against individual neuraminidase (NanA, NanB or NanC) were also tested for their inhibitory effect on the activity of neuraminidases. Different amounts of neuraminidases in 100 µl PBS were pre-incubated with 10 µl-immunized serum (30 µg) for 5 min. The antiserum-treated neuraminidase was mixed with RBCs (10⁶–10⁷ cells ml⁻¹) for 2 h of incubation at 37 °C. Inhibition of neuraminidases (NanA, NanB and NanC) activity by mice antiserum was quantified by TA exposure on RBCs using flow cytometry [52]. If a specific anti-neuraminidase serum neutralizes neuraminidase activity, the exposure of TA antigen on RBC cells, and the value of fluorescence intensity will be reduced. Flow cytometric analysis was conducted using a flow cytometer, FACScan instrument (Becton-Dickinson, FACSCanto II, USA). The data are representative of three or more independent experiments performed in duplicates. Values are expressed in mean±SD.

**Complement C3 deposition on the pneumococcal surface by flow cytometry**

Complement deposition assay was performed as described previously [56, 57]. Briefly, a volume of 100 µl of bacterial culture (OD₆₀₀ 0.45) was washed twice with PBS and resuspended in the 10 µl preimmune, anti-NanA, B, C, AB and ABC sera at 37 °C for 1 h. After incubation cells were washed with PBS twice and resuspended in 200 µl of FITC conjugated goat anti-mouse C3 IgG (1:100 dilution in PBS) (Mybiosource, San Diego, CA, USA) at 37 °C for 30 min. The washed bacteria were resuspended in 300 µl of 2% paraformaldehyde. One control consisted of replacing both the serum and labelled antibodies with PBS. Another control was bacteria incubated with FITC conjugated antibodies. Flow cytometric analysis was conducted using a flow cytometer, FACScan instrument (Becton-Dickinson, FACSCanto II, USA).

**Statistical analysis**

Statistical analysis was performed by using software SPSS 20.0 (SPSS, Chicago, IL, USA), GraphPad version 3.01 (GraphPad Software, San Diego, CA, USA) and SigmaPlot 11 (Systat Software, San Jose, CA, USA). The significance was determined using one-way ANOVA, the Kaplan–Meier method and log-rank test, and by the paired t-test. *P*<0.05 was considered statistically significant.

**RESULTS**

**Production of anti-neuraminidase antibodies in mice**

To confirm the production of antibodies against neuraminidases in the mouse and evaluate the effectiveness of immunization, we performed ELISA with serum collected 2 weeks after the fourth dose. As shown in Fig. 2, we observed high (4–6 logarithm fold) IgG class antibody titres against NanA, NanB, and NanC; this indicates that all of the three neuraminidases are immunogenic vaccine antigens. Sera from mice immunized with trivalent neuraminidases (NanA+NanB+NanC) also recognized individual NanA, NanB and NanC proteins, indicating no reduction in immunogenicity. The data from Western blot results also showed that the serum of NanA or NanB or NanC immunized mice identified NanA, NanB and NanC proteins, respectively (data not shown).
Inhibition of neuraminidase activity by immunized antisera

Detection of TA exposure on RBCs using PNA is considered to be the most appropriate test to support the diagnosis of pneumococcal HUS [8, 17]. Coats et al. demonstrated that neuraminidase A, but not neuraminidase B, was necessary for exposure of T-antigen on RBCs in vivo [9]. Our laboratory ex vivo assays showed that all of the three neuraminidases, NanA, NanB and NanC, are functional and capable of exposing TA on RBCs [49, 52]. Initially, we tested antineuraminidase antisera from rabbits and its capacity to inhibit the neuraminidase activity by preventing the exposure of T-antigen on RBCs. Inhibition of neuraminidase (NanA, NanB and NanC) activity by rabbit antiserum (r) was quantified by TA exposure on RBCs using flow cytometry. If neuraminidase activity is neutralized by anti-neuraminidase serum, the exposure of TA antigen on RBCs and the value of fluorescence intensity will be reduced. As shown in Fig. 3, NanA activity was higher than NanB and NanC. NanA activity was completely inhibited by anti-NanA antiserum when both 0.1 and 0.01 µg NanA was used, while only 20 % activity was inhibited when 1 µg NanA was used. The NanB activity was completely inhibited by anti-NanB antiserum (Fig. 3b). However, only 40 % NanC activity was inhibited by anti-NanC antiserum, but 90 % NanC activity was inhibited by anti-NanC antiserum IgG fraction purified by protein A sepharose beads (Fig. 3c).

We also tested the cross-reactivity of anti-neuraminidase (including NanA, NanB and NanC) antisera. Anti-NanA antiserum did not show an inhibitory effect on the activity of NanB and NanC. Anti-NanB antiserum did not show an inhibitory effect on the activity of NanA and NanC. However, anti-NanB antiserum could inhibit NanC activity by 74 %. Non-purified and purified anti-NanC antisera inhibited NanB activity by 40 and 76 %, respectively. In addition, neutralizing antibody titres (NAbs) were assessed by pre-incubating a twofold dilution series of the sera with a constant amount of neuraminidase in PBS for 30 min at 37 °C before adding RBCs (Fig. 4). The inhibitory capacity of the antisera was expressed as the serum dilution that inhibited 50 % of the neuraminidase activity. We found that IC50 NAbs titres for 1, 0.1 and 0.01 µg ml Nana were 1, 18 and 32 respectively. For NanB (1 µg), the NAbs titre was 16, while for NanC (1 µg) it was only 1. To increase the NAbs titre, the IgG fraction from anti-NanC antiserum was purified by protein A sepharose beads, we found an eightfold increase in the NAbs titre with purified NanC antisera. Anti-neuraminidase antisera raised from the mouse also inhibited neuraminidase activity (Fig. 5). The mouse anti-NanA and anti-NanB sera could completely inhibit the activities of NanA and NanB, respectively. However, anti-NanC serum was only able to neutralize 50 % activity of NanC. The results indicated that the antisera generated from mice and rabbits can inhibit neuraminidase-mediated TA exposure on RBCs.

To identify the neuraminidase domains that are necessary to generate antisera for efficient inhibition of neuraminidase activity, a series of protein domains of neuraminidases were used to immunize mice (Fig. 6a–c). Lectin or carbohydrate-binding module (CBM) of NanA, NanB and NanC binds to sialic acid containing glycoconjugates, CBMs increase the catalytic efficiency of the neuraminidases, mainly towards polysaccharide substrates [40]. In NanA lectin domain 1–325 amino acids (325 aa, 36 kDa), conserved sialic acid-binding residues Glu195 and Arg197 are present; while in NanA catalytic domain 501–780 aa (280aa, 31 kDa), amino acids Glu647, Arg663 and Tyr752 important residues for the catalytic activity are present [58, 59]. We found that the
mouse antisera (m\text{a}) against full-length NanA (m\text{aNanA}) and NanA lectin+catalytic domain (m\text{aNanALecCat}), efficiently inhibited (>90\%; P<0.001) neuraminidase activity (P<0.001). However, antisera against NanA lectin domain (m\text{aNanALec}), catalytic domain (m\text{aNanACat}), conserved catalytic domain (m\text{aNanAConCat}) could inhibit the enzyme activity partially but significantly (P<0.001). Overall, NanB and NanC proteins share 51\% sequence identity and 68\% similarity; NanB and NanC lectin domains, showed lower (41\%) sequence identity and 61\% similarity [40, 41]. But, catalytic conserved domains (NanB/CCatCon) 578–663 aa showed higher (63\%) sequence identity and

**Fig. 3.** Serum from rabbit immunized with neuraminidase inhibits neuraminidase activity. Neuraminidase-mediated TA exposure on RBCs was quantified by FITC-labelled PNA lectin and flow cytometry. Enzymatically active (a) NanA (1, 0.1 and 0.01 \mu g), (b) NanB (1 \mu g) and (c) NanC (1 \mu g), were mixed with 10 \mu l (30 \mu g ml\textsuperscript{-1}) sera from rabbits (r\text{a}) immunized with NanA (r\text{aNanA}), NanB (r\text{aNanB}), NanC (r\text{aNanC}) and purified anti-NanC antiserum IgG fraction (Purified r\text{aNanC}). Buffer only and pre-immune serum were used as controls. Data are averages of three or more independent experiments. Error bars represent SD. Statistical significance was determined using one-way ANOVA by SPSS software version 20.0, followed by Tukey's post hoc test. Asterisks represent statistical significance (*, P<0.05; **, P<0.01; ***, P<0.001) when compared to the pre-immune serum.
75% similarity, this region also contains three key catalytic residues. We predicted that higher sequence identity and similarity might increase antisera cross-reactivity. Comparison of NanB and NanC central conserved domains (NanB/CCenCon) 382–473 aa, showed 53% sequence identity and 75% similarity, NanB/CCenCon represents the inserted domain of NanB which is formed predominantly from β-strands and the role of this inserted domain remains unknown [40]. Antisera against the NanB lectin domain (mA NanBLec), NanC lectin domain (mA NanCLec), were sufficient to neutralize their enzyme activity (P<0.001). However, antisera against the high homology region of both NanB and NanC could inhibit enzyme activity partially but significantly (P<0.001), including antisera against the NanB and NanC central conserved domain (mA NanB/CCenCon) and the catalytic conserved domain (mA NanB/CCatCon).

Complement C3 deposition on the pneumococcal surface
Deposition of large C3 cleavage products on pneumococcal surfaces greatly enhances bacterial uptake by phagocytic cells [56, 57, 60, 61]. In addition, complement can directly kill bacteria via formation of a membrane attack complex (MAC) [61]. Therefore, S. pneumoniae was incubated with immunized sera and C3 deposition was tested by flow cytometry. We found that C3 deposition was greater (P<0.001) in the presence of NanA antisera alone when compared to pre-immune sera or sera from divalent or trivalent neuraminidase formulations (Fig. 6d). When compared to NanB and NanC, NanA has an additional C-terminal domain containing an LPETG motif that anchors the enzyme to the bacterial surface [40]. Therefore, C3 deposition on the pneumococcal surface was less when NanB and NanC antisera were used.

Vaccination and intravenous challenge model
We evaluated the vaccine potential of individual or a combination of three neuraminidases, NanA, NanB and NanC, in an intravenous challenge model using serotype 3. Serotype 3 is a virulent serotype that can cause complicated pneumonia in adults as well as in children [49, 62–64]. In addition, PCV13 vaccine efficacy is very low against serotype 3 [65, 66]. As shown in Fig. 7, in the PBS control group, mice did not survive past 5 days post-infection. Although, the survival rates of mice varied among immunized groups, >40% of the mice survived 14 days post-infection. The survival rate was 60% (P<0.001) in mice immunized with a combination of three neuraminidases (NanA+NanB+NanC),
Fig. 5. Sera from BALB/C mice (mα) immunized with neuraminidase inhibit neuraminidase activity. Neuraminidase-mediated TA exposure on RBCs was quantified by FITC-labelled PNA lectin and flow cytometry. Enzymatically active (a) NanA (1, 0.1 and 0.01 µg), (b) NanB (1 µg) and (c) NanC (1 µg) were mixed with 10 µl (30 µg ml⁻¹) mouse antisera, including mαNanA, mαNanB and mαNanC. Buffer only and pre-immune serum were used as controls. *: P<0.05 (when compared to the controls). Data are averages of three or more independent experiments. Error bars represent SD. Statistical significance was determined using one-way ANOVA by SPSS software version 20.0, followed by Tukey’s post hoc test. Asterisks represent statistical significance (*, P<0.05; **, P<0.01; ***, P<0.001) when compared to the pre-immune serum.
Fig. 6. Neuraminidase inhibitory activity of serum from BALB/C mice (m) immunized with NanA, NanB or NanC protein domains. Neuraminidase-mediated TA exposure on RBCs was quantified by FITC-labelled PNA lectin and flow cytometry. Enzymatically active (a) NanA (0.0005 µg), (b) NanB (0.05 µg) and (c) NanC (0.05 µg) were mixed with 10 µl (30 µg ml<sup>-1</sup>) antisera. Buffer only, pre-immune serum and sera from rabbits (r) immunized with neuraminidases were used as controls. (d) Complement C3 deposition on the pneumococcal surface by flow cytometry. Bacterial strain (serotype 3) was incubated with different anti-neuraminidase sera from BALB/C mice followed by incubation with 10% baby rabbit complement. Bacterial strain only (C1), C3 antibody only (C2) and pre-immune serum (mPre) were used as controls. Data are averages of three or more independent experiments. Error bars represent SD. Statistical significance was determined using one-way ANOVA by SPSS software version 20.0, followed by Tukey's post hoc test. Asterisks represent statistical significance (*, P<0.05; **, P<0.01; *** P<0.001) when compared to the pre-immune serum.
which was similar to the PPV23 control group. The survival rate at the end of 14 days for mice immunized with only NanA, or NanB, or NanC, and NanA+NanB was 56, 45, 45 and 56% respectively. The data suggest that the level of protection provided by the combination of three neuraminidases is marginally higher when compared to individual neuraminidases.

To assess the protein vaccine potential of the combination of three neuraminidases on different serotypes several clinical isolates were used: serotypes 6B, 14 and 23F are among the most predominant serotypes prevalent in communities in the last decade [53]. Serotypes 19A and 15B included in this study are the non-vaccine serotypes that have become common in PCV7 and PCV13 vaccinated populations respectively [21, 25]. BALB/c mice were immunized with a combination of three neuraminidases, NanA, NanB and NanC. Later, mice were intravenously challenged with different serotypes: *S. pneumoniae* serotype 3 (5 × 10⁸ c.f.u.), serotype 6B (7 × 10⁹ c.f.u.), serotype 14 (1 × 10⁹ c.f.u.), serotype 15B (7 × 10⁸ c.f.u.), serotype 19A (7 × 10⁹ c.f.u.) and serotype 23F (1 × 10⁸ c.f.u.). As shown in Fig. 6, immunization with a combination of NanA, NanB and NanC caused a significant increase in survival rates relative to the negative control; in serotype 3, 6B, 14, 15B, 19A and 23F survival was 40% (P<0.05), 60% (P<0.001), 40%, 60% (P<0.001), 40% (P<0.05) and 30%, respectively (Fig. 8). The results indicate that the immunization with a combination of three neuraminidases could protect mice against infection caused by different pneumococcal serotypes.

**DISCUSSION**

Neuraminidases are potential targets for both vaccines and small molecule inhibitors. Since three neuraminidases vary in substrate specificities, mechanism and kinetic parameters, developing a single potent inhibitor that can target all three neuraminidases is challenging [4, 38–42, 67]. NanA, NanB and NanC are large proteins (78–109 kDa), determining minimal antigenic domains or epitopes will help us in developing vaccination formulations and analysing the immune responses. In addition, none of the previous studies analysed the potential of immunized sera to neutralize all of the pneumococcal neuraminidases in an *ex vivo* assay. Antisera against full-length neuraminidases from both rabbits and mice could inhibit the neuraminidase activity significantly (P<0.001). Since NanB and NanC share 51% amino acid sequence identity, anti-NanB and anti-NanC antiserum could cross react and inhibit activity of both NanB and NanC. To increase the NanC NAb titre present in rabbit serum by eightfold, IgG fraction from antisera was purified by protein A sepharose beads. To further increase the NanC immunogenicity, highly immunogenic fragments need to be identified and different adjuvants need to be tested. When antisera was raised against neuraminidase protein fragments involved in sialic acid recognition, we found that to generate potent NanA NAb, both lectin and catalytic domains were essential, whereas for NanB and NanC only lectin domains were sufficient. Although in NanA protein domains important amino acids residues of the lectin and catalytic activity were present; we observed that antisera against the lectin and catalytic domain could reduce NanA activity significantly, but not completely [58, 59].

![Fig. 7. Increased survival of NanA, NanB and NanC-immunized mice after intravenous challenge with *S. pneumoniae* serotype 3. BALB/C mice (n=9) were immunized with individual or a combination of three neuraminidases (NanA, NanB and NanC; 10 µg each). Two weeks after the fourth immunization, mice were intravenously injected with *S. pneumoniae* serotype 3 (3 × 10⁹ c.f.u.) and the survival rate (%) of mice was monitored for 15 days. Mice immunized with PBS plus Freund’s adjuvant served as a control. Data are averages of three or more independent experiments. Kaplan–Meier survival curves were performed by SPSS software version 20.0. PPV23: pneumococcal polysaccharide vaccine; n: number of mice. Asterisks represent statistical significance (*, P<0.05; **, P<0.01; ***, P<0.001) when compared to the control.](image-url)
Immunogenicity of the purified domains and protein folding may also play a role in the development of antibodies. Therefore, anti-NanA antibodies derived from full-length NanA protein appear necessary to provide complete inhibitory activity. 

Chen et al. reported that neuraminidases increase *S. pneumoniae* pathogenicity by targeting the CD24-SiglecG interaction and suggested the use of neuraminidase inhibitors to protect mice against sepsis [38]. Therefore, we believe that immunization with trivalent neuraminidase protein vaccine might help in increasing anti-neuraminidase antibody concentration and reduce the severity of pneumococcal infections, including HUS by neutralizing the neuraminidase activity [38]. We recommend the use of flow cytometer-based ex vivo assays to screen and analyse the panel of polyclonal and monoclonal antibodies to identify neuraminidase activity neutralizing antibodies.

Although, PCV13 provides significant protection for most of the vaccine serotypes, Choi et al. showed that the poor efficacy of serotype 3 PCV was linked to capsular polysaccharide released during growth and suggested the use of alternative immunization approaches for serotype 3 [65]. Andrews et al. predicted that in humans, a higher serum IgG concentration (2.83 µg ml⁻¹) for serotype 3 would be needed for protection [66]. In this study survival rate after challenge with serotypes 3, 6B, 15B, 19A and 23F was between 30–60% in mice immunized with a combination of three neuraminidases. We predict that different levels of

Fig. 8. Combinational neuraminidase vaccine increased survival of mice after intravenous challenge with different *S. pneumoniae* serotypes. BALB/C mice (n=5) were immunized with all three neuraminidases (NanA, NanB and NanC; 10 µg each). Two weeks after the fourth immunization, mice were intravenously injected with (a) *S. pneumoniae* serotype 3 (5 × 10⁶ c.f.u.), (b) serotype 6B (7 × 10⁸ c.f.u.), (c) serotype 14 (1 × 10⁶ c.f.u.), (d) serotype 15B (7 × 10⁸ c.f.u.), (e) serotype 19A (7 × 10⁸ c.f.u.), (f) serotype 23F (1 × 10⁸ c.f.u.). Mice survival rate (%) was monitored for 15 days. Mice immunized with PBS plus Freund’s adjuvant served as a control. Data are averages of three or more independent experiments. Kaplan-Meier survival curves were performed by GraphPad version 3.01. PPV23: pneumococcal polysaccharide vaccine; n: number of mice. Asterisks represent statistical significance (*, P<0.05; **, P<0.01; ***, P<0.001) when compared to the PBS control.
protection observed in mice might be due to neuraminidase gene diversity and expression [41, 67, 68]. Previously, sequence analysis of pneumococcal strains showed that the nanA gene is highly diverse and amino acid substitutions in the lectin domain and the inserted domain of NanA were found to be associated with the biochemical properties of the enzyme [41, 68]. However, Smith et al. showed by in vitro assays that no particular nanA allele was associated with HUS isolates and that there was no significant difference in overall neuraminidase activity between HUS isolates and controls [51]. Although the protection levels are low and different for strains used in this study, real benefits of immunization with neuraminidases should be linked to their ability to neutralize the neuraminidase activity, thereby enhancing protection with different combinations of proteins and adjuvants [69]. When serotype 14 was used in an intravenous infection model, LD50 was difficult to establish and reproduce. At low c.f.u. (≤1 × 10^6), bacteremia could not be established, but at high c.f.u. (≥1 × 10^8) it was lethal. Silva et al. showed that three pneumococcal strains of the same ST and serotype (ST124, serotype 14) behave differently in an infection model [70]. The virulence factors and complement system interactions determine the outcome of many microbial infections including pneumococcal infections. Dalia et al. showed that on the surface of nanA, mgaA and strH mutants complement C3 deposition is more when compared to the wild-type strain. This indicates that NanA, MgaA and StrH, is important for resistance to complement deposition and subsequent phagocytic killing of S. pneumoniae [57]. Although complement component C3 was found to be the major opsonin in normal human serum responsible for killing by human neutrophils, it was predicted that resistance could require deglycosylation of multiple complement system proteins or have an indirect role in promoting resistance to opsonophagocytic killing [57]. Recently we confirmed that certainly NanA and NanC can cleave sialic acid residues from complement system protein components including C1r, C1s, C3, complement factor B, C4b-binding protein alpha chain and C9 [52]. Therefore, we hypothesize that increased survival in neuraminidase-immunized mice is due to the presence of neuraminidase activity inhibiting antibodies in serum and subsequent increased C3 deposition on the surface of S. pneumoniae, which might enhance bacterial uptake by phagocytic cells and formation of a membrane attack complex (MAC). Although this study enhances our understanding of neuraminidase-based protein vaccine candidates that can increase survival. Further studies will be needed to evaluate the potential of triple neuraminidase-based vaccines on the prevention of nasopharyngeal carriage, compatibility with other protein antigens, optimal dose required and proper adjuvants. We recommend that secreted virulence proteins (NanB, NanC, pneumolysin, CbpA, etc.) that can significantly alter the host cellular and molecular functions should be investigated further and included in the strategies to reduce the pathogenicity of the pneumococcal isolates [28, 71]. Since virulence factor expression varies at different stages of infection and virulence proteins exhibit differences in strain-specific sequences, a combination of different proteins can provide better non-serotype-dependent protection [1–3, 71, 72]. Ogguniyi et al. showed that the median survival times for mice immunized with double or triple combinations of PdB, PspA and PspC were significantly longer than those for mice immunized with any of the single antigens [72]. A quadrivalent protein (PcsB, StkP, PsaA and PspA) formulation was found to protect neonatally immunized mice from both bacteremia and lung infections [69]. In a human clinical trial, investigational formulations containing proteins PhID and pneumolysoid were found to be immunogenic when administered to healthy adults as a standalone protein vaccine or combined with PHID-CV conjugates [73, 74]. Although pneumolysin remains the most widely investigated vaccine [75, 76], isolation of non-haemolytic pneumolysin in S. pneumoniae clinical isolates showed that targeting a single virulence factor might not be highly effective to prevent pneumococcal infections [77, 78]. Antigenic and structural diversity in protein vaccine candidates can be observed in different geographical regions; it is therefore necessary to include multiple conserved protein antigens in vaccine formulations in the future [79]. Even after several clinical observational studies (TA exposure vs HUS outcome) and abundant experimental evidence defining the role of neuraminidases in host-pneumococcal interactions, none of the human clinical trials included neuraminidases as vaccine candidates.

In conclusion, neuraminidases, one of the major virulence proteins, are potential targets for both vaccines and small molecule inhibitors. Our study showed that to generate effective neutralizing antibodies for NanA, both lectin and catalytic domains are essential, whereas for NanB and NanC lectin domains are sufficient. We also found a significant increase in mouse survival in neuraminidase-immunized groups in the bacteremia model. We recommend the inclusion of three pneumococcal neuraminidases in future protein vaccine formulations to prevent IPD.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Ethical statement**

Animal and pathogen protocols were approved by the institutional animal care and use committee of Chang Gung University (numbers: CGU10-046, CGU11-069, CGU14-159). This study was supported in part by grants from Chang Gung Memorial Hospital (CMRP3E0623, CMRP3B0853, CMRP3F1142 and CMRP3G1971) and the Ministry of Science and Technology (104–2320-B-182A-008 and 105–3011-F-182A-001) in Taiwan.

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