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Vaccine 26 (2008) 3842–3852

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

A recombinant subunit vaccine formulation protects against lethal Nipah virus challenge in cats

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ARTICLE INFO

Article history:
Received 9 February 2008
Received in revised form 25 April 2008
Accepted 7 May 2008
Available online 2 June 2008

Keywords:
Henipavirus
Subunit vaccine
CpG
Mucosal immunity

ABSTRACT

Nipah virus (NiV) and Hendra virus (HeV) are closely related deadly zoonotic paramyxoviruses that have emerged and re-emerged over the last 10 years. In this study, a subunit vaccine formulation containing only recombinant, soluble, attachment glycoprotein from HeV (sG_{HeV}) and CpG adjuvant was evaluated as a potential NiV vaccine in the cat model. Different amounts of sG_{HeV} were employed and sG-induced immunity was examined. Vaccinated animals demonstrated varying levels of NiV-specific Ig systemically and importantly, all vaccinated cats possessed antigen-specific IgA on the mucosa. Upon oronasal challenge with NiV (50,000 $TCID_{50}$), all vaccinated animals were protected from disease although virus was detected on day 21 post-challenge in one animal. The ability to elicit protective systemic and mucosal immunity in this animal model provides significant progress towards the development of a human subunit vaccine against henipaviruses.

1. Introduction

Hendra virus (HeV) and Nipah virus (NiV) are closely related highly pathogenic paramyxoviruses that continue to cause morbidity and mortality in animals and humans. Flying foxes in the genus *Pteropus* are considered to be the natural reservoir for both viruses and their geographic distribution encompasses all locations where HeV and NiV outbreaks have occurred [1,2]. HeV has appeared sporadically in Australia since 1994 where infection has been predominantly in horses, although human infection has also occurred (reviewed in Ref. [3]). Of the two reported fatal human cases, one presented with severe respiratory disease while the other succumbed to encephalitis. Recent outbreaks where horse fatalities were documented include 1999, 2004, 2006 and 2007 and one human case was reported in 2004 [4–7]. The first NiV outbreak occurred in peninsular Malaysia and Singapore in 1998–1999 where the majority of infections were in pigs with subsequent transmission to humans (reviewed in Refs. [8,9]). Humans developed severe febrile encephalitis with high case fatality and 25% of cases also exhibited respiratory signs. NiV has re-emerged repeatedly since 1998: twice in Bangladesh and India in 2001, four times in Bangladesh in 2003, 2004 and 2005, and most recently in 2007 in India and Bangladesh [10–14]. These more recent outbreaks have included a higher incidence of acute respiratory distress syndrome in conjunction with encephalitis, epidemiological findings consistent with person-to-person transmission [15], and apparent higher case fatality rates (~75%). Furthermore, direct transmission of NiV from flying foxes to humans has been suggested [11,16].

NiV and HeV are distinguished from all other paramyxoviruses because of their broad species tropism and highly pathogenic nature; and they have been classified into the new genus *Henipavirus* within the family *Paramyxoviridae* [17]. Classified as zoonotic biosafety level 4 (BSL-4) agents, both viruses have also been included among the pathogenic agents of biodefense concern by the Centers for Disease Control and Prevention (CDC) and the National Institute of Allergy and Infectious Diseases (NIAID) mainly due to their accessibility in the environment and their relative ease of propagation and dissemination. Currently there are no vaccines available for preventing NiV or HeV infection. As BSL-4 agents, human efficacy studies for testing potential henipavirus therapeutic products are not easily achievable; however, henipavirus animal disease models have been developed (reviewed in Ref. [18]). As HeV and NiV are closely related, the development of one cross-reactive vaccine would be ideal for protection against infection by either virus.

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doi:10.1016/j.vaccine.2008.05.016

Paramyxoviruses are large, enveloped, negative-sense ssRNA viruses [19]. Entry of paramyxoviruses into host cells is a two-step process mediated by the attachment glycoprotein which binds the host receptor, and the fusion (F) glycoprotein which drives virus-host cell membrane merger [19]. For HeV and NiV, the attachment glycoprotein (G) lacks haemagglutinin and neuraminidase activities and the F glycoprotein is a typical class I fusion glycoprotein (reviewed in Ref. [20]). For paramyxoviruses, the majority of neutralising antibody is elicited against the envelope glycoproteins in an infected host [19]. Furthermore, neutralising antibodies are the key vaccine-induced protective mechanisms for the human paramyxoviruses mumps and MeV [21,22]. Three approaches have emerged for henipavirus vaccine development and all incorporate one or both of the henipavirus envelope glycoproteins (reviewed in Ref. [18]). Recombinant vaccinia and canarypox viruses encoding either NiV F or G were shown to protect against NiV challenge in golden hamsters and pigs, respectively [23,24]. Recombinant HeV and NiV G (sG_{HeV} and sG_{NiV}, respectively) were used as subunit vaccines in a cat NiV challenge model and all animals were protected from disease [25]. Although either sG_{HeV} or sG_{NiV} was able to protect against NiV, sG_{HeV} elicited a higher-titre cross-reactive henipavirus immune response. Recent serological studies have also suggested that a more cross-reactive henipavirus immune response is generated upon infection by HeV as compared to NiV [26]. Together, both data sets suggest that a HeV-derived vaccine would protect against challenge with either virus. Here we describe a vaccination and challenge trial in cats using a new sG subunit vaccine formulation where varying doses of sG_{HeV} were used as the vaccine immunogen and small CpG DNA molecules were used as the adjuvant. All vaccinated animals were challenged oronasally with a high dose of a low passage NiV isolate. Pre- and post-challenge immune responses were examined and our data indicated that this new subunit vaccine formulation induced systemic as well as mucosal immunity. Viral loads were significantly reduced in vaccinated animals as compared to adjuvant alone control animals and all vaccinated animals were protected from NiV-mediated disease.

2. Materials and methods

2.1. Animals, accommodation, handling, and biosafety

Eight adult (12–24 month old) domestic shorthair cats (four castrated males and four entire female) were used for the vaccine efficacy study. The animal husbandry methods and experimental design were endorsed by the CSIRO Australian Animal Health Laboratory's Animal Ethics Committee. Animals were allowed to free roam in a single room during the vaccination phase of the study (day 1–day 39) and were subsequently relocated to individual cages in a single room at BSL-4 for the remainder of the experiment. Individual cage size and environmental conditions were as previously described [25]. All animals were within visual and auditory contact of each other and were fed twice daily with a complete premium dry food and provided with water *ad lib*. The BSL-4 room was fitted with a surveillance camera which allowed regular monitoring of the animals. For collection of all specimens, cats were anaesthetised as previously described [25]. Tracheobronchial washes were taken from the cats by the endotracheal tube method [27] with the cat in sternal recumbency and the head held up. Following laryngeal de-sensitisation with anaesthetic spray (Cophenylcaine Forte, ENT Technologies, Melbourne, VIC, Australia), the cat was intubated and a sterile dog urinary catheter (size 6 French) was gently inserted through the endotracheal tube as far as it would go. Via attached syringe, 5 ml of sterile phosphate buffered saline (PBS) was instilled into the catheter and immediately aspirated. Usually the aspirate contained floccules of mucus and the procedure was deemed successful. If no mucus was present in the aspirate the procedure was repeated once. Anesthetic reversal was done as previously described [25]. Staff wore fully encapsulated suits with breathing apparatus while in the BSL-4 animal room or BSL-4 laboratory. Serum neutralisation tests, virus isolations and the initial stages of RNA extraction and post-challenge ELISA (Ig and cytokine) were carried out at BSL-4.

2.2. Vaccine preparation

Production and purification of sG_{HeV} was done as previously described [28]. CpG oligodeoxynucleotide (ODN) 2007 (TCGTCGTTGTCGTTTTGTCGTT) containing a fully phosphorothioate backbone was purchased from Coley Pharmaceutical Group (Wellesley, MA, USA) and Allhydrogel™ was purchased from Accurate Chemical & Scientific Corporation (Westbury, NY, USA). Vaccine doses containing fixed amount of ODN 2007, varying amounts of sG_{HeV} and aluminum ion (at a weight ratio of 1:25) were formulated as follows: 50 μg dose: 50 μg sG_{HeV}, 1.25 mg aluminum ion and 150 μg of ODN 2007; 25 μg dose: 25 μg sG_{HeV}, 625 μg aluminum ion and 150 μg of ODN 2007; and 5 μg dose: 5 μg sG_{HeV}, 125 μg aluminum ion and 150 μg of ODN 2007. For all doses, Allhydrogel™ and sG_{HeV} were mixed first before ODN 2007 was added. Each vaccine dose was adjusted to 1 ml with PBS and mixtures were incubated on a rotating wheel at room temperature for at least 2–3 h prior to injection.

2.3. Immunisation and pre-challenge sampling

Eight adult cats were immunised intramuscularly with vaccine preparations on day 0 and on day 21. Each cat received the same 1 ml dose for both prime and boost injections. All vaccine doses were given via intramuscular injection. Two animals received 50 μg doses (cat 29-50 and cat 30-50), two animals received 25 μg doses (cat 31-25 and cat 32-25), two animals received 5 μg doses (cat 33-5 and cat 34-5) and two animals received adjuvant-alone (cat 27-0 and cat 28-0). Each dose group contained one male and one female cat. Animals were bled on day 0, day 21 and day 35 and respiratory washes were collected on day 0 and day 35 as described above.

2.4. Radio-telemetry measurements of body temperature

On day 39, under anesthesia, as described above, single stage transmitters fitted with an internal loop antenna and coated with an inert two-pot epoxy resin (Sirtrack, Havelock North, New Zealand) were implanted subcutaneously in the flank (final dimensions, 25 mm × 15 mm × 6 mm). Real-time monitoring of body temperature for all animals using single stage transmitters was done as previously described [25].

2.5. Nipah virus infections

On day 42, all animals were inoculated oronasally with 50,000 $TCID_{50}$ of a low passage NiV isolate (EUKK 19817; stock virus titre 4.3×10^6 $TCID_{50}$/ml) prepared as described previously [29]. Cats were assessed daily and scored out of 10 for a range of clinical observations, including alertness, grooming behavior, curiosity, depression, food consumption, faeces production and respiration rate. Clinical scores for each day were combined to provide a cumulative daily score. Rectal temperatures and body weights were recorded during sampling periods which occurred every second day. Once cats were clinically assessed to be exhibiting signs of disease, animals were then immobilised as described

above. Blood was collected via intracardiac puncture, and animals were immediately euthanised by intravenous injection of sodium pentabarbitone.

2.6. Sample collection post-challenge

Heparinised blood, oral swabs and urine were collected 6 days post-infection (dpi), 8 dpi, 10 dpi, 13 dpi and 21 dpi. Mucosal washes were collected 8 dpi, 13 dpi and 21 dpi as described above. Immediately following sampling, two 0.5 ml aliquots of whole blood were removed from each blood sample and plasma was collected and aliquoted. Duplicate swabs were immediately placed in 0.5 ml of PBS or 0.5 ml RNA*later* (Ambion Inc., Austin, USA). Urine was collected from cats by manual expression. All specimens were placed at −80 °C for further analysis. After euthanasia, tissue samples were collected aseptically from lung (apical and diaphragmatic lobes), brain (olfactory and occipital lobes), heart, bronchial lymph nodes, spleen, liver, kidney, bladder, and adrenal gland. Additionally, in female cats, the uterine horn and ovaries were collected and in male cats the testes were collected. Tissues were either fixed in 10% neutral buffered formalin for 48 h prior to histological processing or submerged in 0.5 ml RNA*later* or PBS, and stored at −80 °C until further analysis.

2.7. Serum neutralisation test

Virus neutralisation antibody titres were determined as previously described [28]. Briefly, plasma samples were diluted in Eagle's minimal essential medium (EMEM) containing 10% fetal calf serum (FCS) (EMEM-10) and subject to doubling dilution starting at 1:2 (pre-challenge) or 1:16 (post-challenge). Fifty microlitres of each plasma dilution was added to wells in quadruplicate in a 96-well plate followed by 50 μl virus containing 200 $TCID_{50}$ of either HeV or NiV and incubated at 37 °C for 30 min. Twenty thousand Vero cells were added to all wells in 150 μl EMEM-10 and incubated at 37 °C for 4 days in a humidified 5% CO_2 incubator. Neutralisation titres were determined as the dilution where no cytopathic effect (CPE) was observed.

2.8. Ig ELISA

The 96-well ELISA plates were coated with 75 ng of either sG_{HeV} or sG_{NiV} [25] and incubated overnight at 4 °C. For all assays, the sG_{HeV} and sG_{NiV} antigens were from the same batch preparations. Plates were washed three times with PBS/0.05% Tween-20 (PBST). All wells were blocked with 5% skim milk/PBST for 1 h at 37 °C and transferred to the BSL-4 laboratory. All plasma samples and mucosal washes were diluted in Ca^{2+}/Mg^{2+} free PBS (PBSA) and assayed in duplicate. Plasma samples and washes were added and incubated for 1 h at 37 °C. Plates were washed three times with PBST and subsequently fixed in ice-cold absolute methanol for at least 20 min prior to air-drying outside the BSL-4 laboratory. Plates were washed three times with PBST. All horseradish peroxidase (HRP) conjugates were purchased from Bethyl Inc. (Montgomery, TX, USA) and optimal working dilutions were determined. HRP-goat anti-feline IgG (1:5000), HRP-goat anti-feline IgM (1:10,000) or HRP-goat anti-feline-IgA (1:5000) were added and incubated for 1 h at 37 °C. Plates were washed three times with PBST and developed with a 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Sigma–Aldrich, St. Louis, MO, USA). The absorbance of each well was measured at 450 nm. Pre-challenge plasma and mucosal washes were not tested in the BSL-4 laboratory; therefore, for these samples, a methanol fixation step was not done.

2.9. Feline cytokine ELISA

All feline cytokine kits and relevant cytokine ELISA reagents were purchased from R&D Systems Inc. (Minneapolis, MN, USA) and protocols were followed as per the manufacturer's instructions with several variations. Briefly, 96-well ELISA plates were coated with kit supplied capture antibodies: goat anti-feline IL-2 (400 ng/well); mouse anti-feline IL-4 (400 ng/well); mouse anti-feline IL-10 (400 ng/well) or goat anti-feline IFN-γ (400 ng/well) and incubated for 1 h at 37 °C. Plates were washed three times with PBST. All wells were blocked with 1% BSA/PBSA for 1 h at 37 °C and transferred to the BSL-4 laboratory. All feline cytokine standards, plasma samples, mucosal washes and tissue homogenates, including lung (apical and diaphragmatic lobes), bronchial lymph nodes and spleen (prepared as described below) were diluted in PBSA and assayed in duplicate. IL-2, IL-4 and IFN-γ standard concentrations ranged from 16 ng/ml to 0.125 ng/ml; IL-10 standard concentrations ranged from 8 ng/well to 0.0625 ng/ml. Cytokine standards, plasma samples, mucosal washes and tissue homogenates were added and incubated for 1 h at 37 °C. Plates were washed three times with PBST. Biotinylated goat anti-canine IL-2 (1:500), biotinylated goat anti-feline IL-4 (1:500), biotinylated goat anti-feline IL-10 (1:500), or biotinylated goat anti-feline IFN-γ (1:500) were added and incubated for 1 h at 37 °C. Plates were washed three times with PBST and subsequently fixed in ice-cold absolute methanol for at least 20 min prior to air-drying outside the BSL-4 laboratory. Plates were washed three times with PBST. Streptavidin–HRP (1:200) was added to each well and incubated for 1 h at 37 °C. Plates were washed three times with PBST and developed with a TMB substrate (Sigma). The absorbance of each well was measured at 450 nm. Pre-challenge plasma samples and mucosal washes were not tested in the BSL-4 laboratory; therefore, for these samples and the corresponding standard curves, a methanol fixation step was not done.

2.10. RNA isolation

RNA was isolated from blood cells using the RiboPure-Blood kit (Ambion Inc.), from swabs and urine using the QIAamp viral RNA kit (Qiagen Pty Ltd., Clifton Hill, Australia) and from tissues using the RNeasy Mini kit (Qiagen Pty Ltd.). Briefly, under BSL-4 conditions, 300 μl blood was added to 800 μl RiboPure lysis buffer and 50 μl sodium acetate. One hundred and forty microlitres of swab solution or urine was added to 560 μl AVL viral lysis buffer (QIAamp). Tissues were homogenised by removing the tissue from RNAlater and adding it to tubes containing 600 μl RLT lysis (RNeasy) containing 6 μl β-mercaptethanol and 250 μl volume of 1 mm zirconia/silica beads (BioSpec Products Inc., Bartlesville, USA). Samples were then homogenised for 2 cycles of 30 s using a Mini-Bead Beater (BioSpec Products Inc.), centrifuged to pellet cell debris and the supernatant transferred to a new tube. All samples were then removed from the BSL-4 lab and processed according to manufacturer's instructions supplied with each kit.

2.11. NiV Taqman PCR assay

All Taqman PCR assays were preformed as previously described [25]. The samples were amplified in a GeneAmp 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Standard curves were obtained for NiV specific cDNA in triplicate for each assay. Threshold cycle (Ct) values obtained from the standard dilutions were plotted against the input cDNA concentration to create a standard curve (data not shown). Linear regression analysis was used to quantify NiV cDNA. Ct values representing NiV genome in samples were further analysed relative to the standard

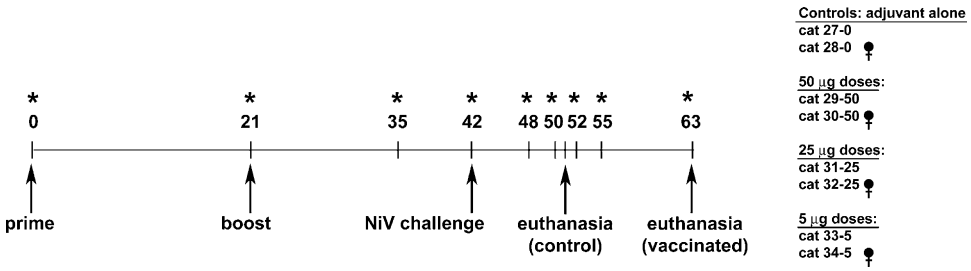

Fig. 1. Schematic diagram of vaccination and challenge schedule. Dates of vaccination, challenge and euthanasia are indicated by arrows below. Blood and swab samples were collected on all days indicated immediately above the timeline (*). Swabs were only collected post-challenge. Cat number and vaccine dose groups are also shown. ♀ indicates female cat.

NiV cDNA dilution (3.6 pg NiV cDNA) and data are shown as relative NiV genome levels.

2.12. Virus isolation

Virus isolation was done as previously described [25]. Virus isolation was only attempted from specimens positive for NiV genome.

2.13. Histopathology and immunohistochemistry

Tissues were processed by routine histological methods and sections of tissue were stained with hematoxylin and eosin to examine for histopathological changes. Separate sections were stained by immunohistochemical techniques as previously described [30] using a rabbit polyclonal antiserum against recombinant NiV nucleoprotein.

3. Results

3.1. Immunogenicity of sG_{HeV}

In our previous NiV vaccine trial, three high dose sG_{HeV} or sG_{NiV} immunisations were administered to cats along with a potent adjuvant yielding exceptionally high neutralising antibody titres, and challenge was carried out with a 500 $TCID_{50}$ dose of NiV injected subcutaneously [25]. All animals survived and virus was not recovered at any time point post-challenge suggesting sterilising immunity. Here we sought to better assess sG-specific immune responses thus a new vaccine trial was devised that employed varying doses of sG_{HeV}. Additionally, as HeV and NiV replicate and cause severe pathology in the lung, CpG adjuvant was included in an attempt to elicit mucosal immunity. Eight adult cats were immunised as described in the methods and a schematic diagram outlining vaccination, specimen collection and NiV challenge is shown in Fig. 1. Each animal received one prime and one boost intramuscularly over a 21-day period. Animals were bled and plasma antibody responses, specific for either sG_{HeV} or sG_{NiV}, were measured and results are shown in Fig. 2. As expected, for all cats, regardless of Ig subclass, sG_{HeV}-specific antibody levels were slightly higher than those specific for sG_{NiV}. All cats had significant levels of IgG on day 21 which further increased upon boost (day 35) and there did not appear to be notable differences among different vaccine doses. Plasma IgM levels varied, and although all cats demonstrated sG_{HeV}-specific IgM, only cat 29 (50 μg dose) (cat 29-50) and cat 32 (25 μg dose) (cat 32-25) demonstrated increased levels of sG_{NiV}-specific IgM. Unlike IgG responses, IgM levels appeared to decrease upon boost. Significant levels of plasma IgA were detected in cat 29-50 and levels decreased upon boost; whereas cat 33 (5 μg dose) (cat 33-5) appeared to have a slight increase in IgA upon boost. All plasma samples were further evaluated in NiV serum neutralisation tests (SNT) and results are shown in Table 1. Most of the animals had low levels of neutralising antibody 21 days after prime and cat 34 (5 μg dose) (cat 34-5), like the two adjuvant-only controls (cat 27-0 and cat 28-0), was negative. Upon boosting, SNT titre increased for all animals. No large differences in titre were observed between the cats vaccinated with 50 or 25 μg; although the male cats had a slightly higher titre than the females. SNT titres were lower in the animals that received 5 μg of sG_{HeV}. All cats demonstrated higher SNT titres to HeV, and those animals that received 5 μg sG_{HeV} again exhibited the lowest SNT titres (data not shown). To determine if vaccination targeted mucosal surfaces, tracheobronchial washes were collected and sG_{HeV}-specific secretory IgA was measured and results are shown in Fig. 3. All cats had increased levels of mucosal IgA compared with day 0. Cat 29-50, cat 30 (50 μg dose) (cat 30-50) and cat 32-25 had lower secretory IgA levels demonstrating that higher vaccine dose did not generate the highest mucosal antibody response. Additionally of these three animals, the two females, cats 30-50 and 32-25, had the lowest amount of mucosal IgA. IgM was not detected in mucosal washes (Fig. 3).

3.2. Oronasal challenge with 50,000 $TCID_{50}$ NiV

To examine the extent of sG_{HeV}-induced immunity and its ability to protect against a lethal NiV challenge, a high dose of virus (50,000 $TCID_{50}$) was employed. Oronasal challenge was used to mimic a more natural route of infection. By 7 days post-infection (dpi), the two control animals, cats 27-0 and 28-0, developed an acute febrile disease (>39 °C) comparable to that seen previously in experimental infection of cats given 50,000 $TCID_{50}$ NiV orally and intranasally [30]. The rise in temperature observed in both animals preceded an increase in respiratory rate by approximately 24 h (data not shown), as expected based on prior studies [25]. A rise in cumulative daily clinical scores was observed in parallel to the increase in respiratory rate (data not shown). For welfare reasons, cats were not allowed to progress to serious clinical disease and the

Table 1
Pre-challenge NiV serum neutralisation test (SNT) titres

Dose	Animal # (sex)	NiV SNT titre		
		Day 0	Day 21	Day 35
Controls	27 (M)	<1:2	<1:2	<1:2
	28 (F)	<1:2	<1:2	<1:2
50 μg	29 (M)	<1:2	1:16	1:512
	30 (F)	<1:2	1:16	1:256
25 μg	31 (M)	<1:2	1:16	1:512
	32 (F)	<1:2	1:32	1:256
5 μg	33 (M)	<1:2	1:4	1:128
	34 (F)	<1:2	<1:2	1:32

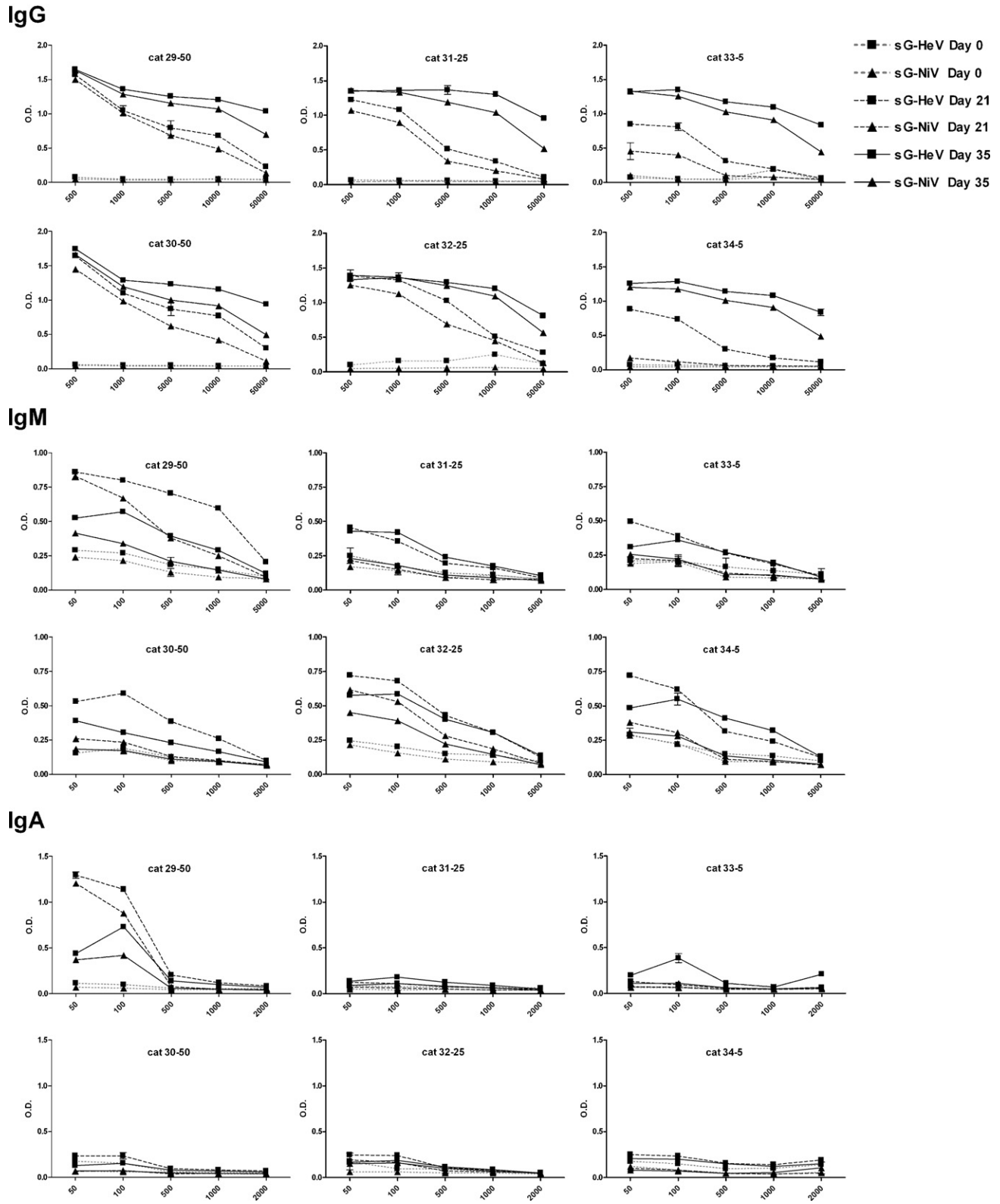

Fig. 2. Pre-challenge sG_{HeV}- and sG_{NiV}-specific immunoglobulin (Ig) in plasma. Cats were vaccinated on day 0 and day 21 and were bled on days 0, 21 and 35 as described in the methods. Antigen-specific IgG, IgM and IgA were measured by ELISA. High dose cats are shown on the left, 25 μg dose cats are shown in the middle, and low dose cats are shown on the right. Plasma samples from adjuvant-alone cats were not assayed. Reciprocal plasma dilution is shown on the *X*-axis for all panels. All assays were performed in duplicate; the average optical density (O.D.) is shown for each sample and error bars represent the range of O.D.

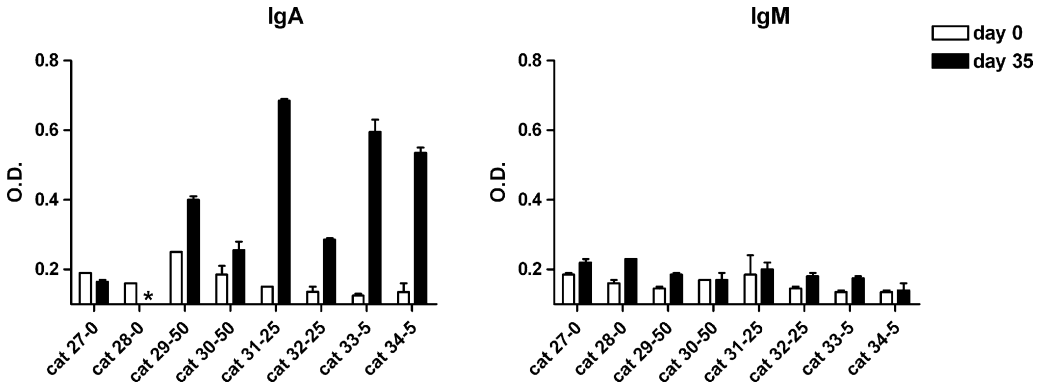

Fig. 3. Pre-challenge sG_{HeV}-specific mucosal IgA. Tracheobronchial washes were collected from vaccinated cats on day 0 and day 35 as described in the methods. sG_{HeV}-specific IgA and IgM were measured by ELISA. Cat number and sG_{HeV} dose are indicated on the *X*-axis. All washes were assayed at a dilution of 1:5 for IgA or 1:10 for IgM and * indicates that the sample was not tested. Assays were performed in duplicate; the average O.D. is shown for each sample and error bars represent the range of O.D. For IgA, four day 0 samples were not assayed in duplicate due to poor sample recovery and are indicated by the absence of error bars.

control animals were euthanised 9 dpi. On necropsy examination the only grossly visible change was a discrete reddened lesion at the margin of the lung tissue of one control animal (cat 28-0). On microscopic examination both control animals had multiple foci of necrotising alveolitis in the lung parenchyma. Cat 28-0 had an advanced lesion at the margin of the lung, which consisted of alveolar necrosis, interstitial inflammatory infiltration predominantly with neutrophils, and necrotising bronchiolitis. Affected bronchiole and alveolar epithelium consisted of swollen cells or fused multinucleate cells (syncytia). The affected epithelial cells had frequent eosinophilic intracytoplasmic inclusion bodies (Fig. 4A). The bronchial lymph node of cat 28-0 had sub-capsular focal necrosis, with small numbers of syncytia. Both spleens had multiple foci of necrosis affecting the red pulp and splenic nodules. Intranuclear and intracytoplasmic antigen staining was present in affected bronchiole and alveolar epithelium of both control cats (Fig. 4B), and within the lymph node lesion. Conversely, all vaccinated cats did not demonstrate any clinical signs of NiV-mediated disease and survived until euthanasia on 21 dpi. Body temperature rose gradually only in cat 33-5 over the course of infection, however, no other clinical signs of disease were present. Upon post-mortem examination, significant lesions and viral antigen were not detected in any of the six vaccinated cats (data not shown).

3.3. Post-challenge immunoglobulin profile

Plasma Ig levels were measured using sG-specific ELISA in all animals post-challenge and no changes in titre were noted (data not shown), with the exception of plasma IgA and IgM in the two low dose animals (Fig. 5). By 8 dpi, cat 33-5 had increased IgM but by 10 dpi, IgM levels had returned to pre-challenge levels (Fig. 5A). Cat 33-5 IgA levels decreased following NiV challenge; however by 10 dpi, both sG_{HeV}- and sG_{NiV}-specific IgA increased and levels remained elevated until euthanasia (Fig. 5B). For cat 34-5, IgM levels had increased by 10 dpi were further elevated on 13 dpi but returned to pre-challenge levels by 21 dpi (Fig. 5C). Cat 34-5 had increased levels of IgA by 8 dpi which decreased to pre-challenge levels by 10 dpi. A slight increase was also noted on 13 dpi, particularly for sG_{NiV}-specific IgA (Fig. 5D). Plasma samples from all animals were also evaluated using SNT and results are shown in Table 2. Control cat 27-0 had a low SNT titre by 8 dpi whereas control cat 28-0 remained SNT negative. For most vaccinated cats SNT titre had decreased on day 42 as compared to day 35. For cats 29-50, 30-50 and cat 31 (25 μg dose) (cat 31-25), SNT titre changed slightly over the course of infection. Cat 32-25 had a fourfold increase in SNT titre by 21 dpi and cat 34-5 had an eightfold increase in titre by 8 dpi, together these data demonstrate an ele-

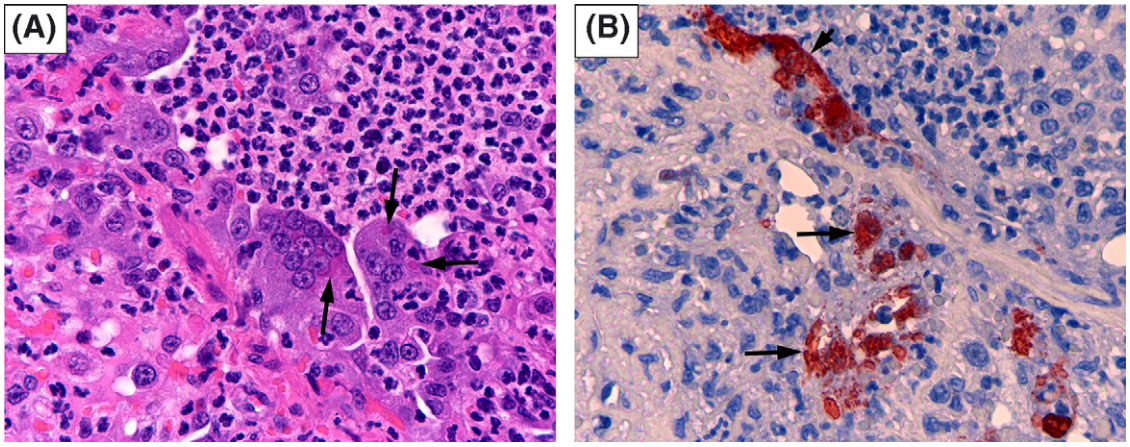

Fig. 4. Lung section from adjuvant-alone control cat infected with 50,000 $TCID_{50}$ NiV. Lung tissue was collected from cat 28-0 upon euthanasia and prepared as described in the methods. (Panel A) Fused bronchiolar epithelial cells (syncytia) containing intracytoplasmic inclusion bodies (arrows). A suppurative inflammatory exudate is present in the bronchial lumen (top right), with a necrotising alveolitis (bottom left). Haematoxylin and eosin stain. (Panel B) Immunohistochemical stain showing viral antigen staining in syncytia of affected bronchiolar epithelium (short arrow) and lung parenchyma (long arrows).

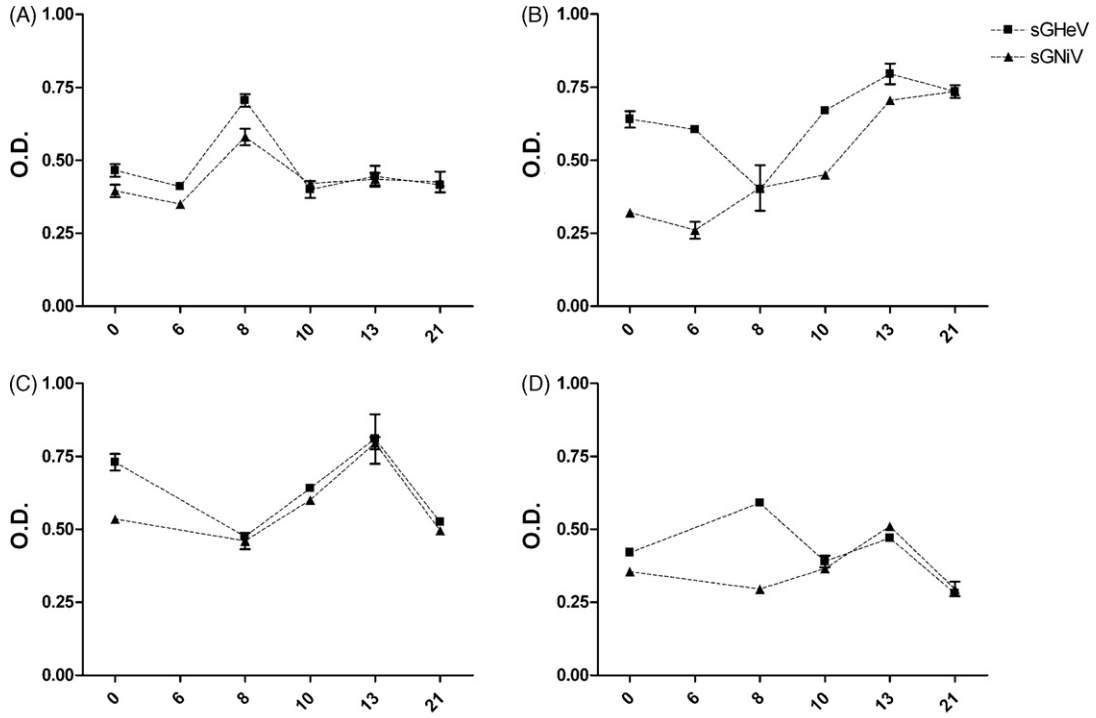

Fig. 5. Post-challenge sG_{HeV}- and sG_{NiV}-specific plasma IgM and IgA in low vaccine dose cats. Cats were challenged with NiV on day 42 post-vaccination (day 0). Plasma was collected on days 0, 6, 8, 10, 13 and 21 post-infection and antigen-specific IgM and IgA were measured by ELISA. All plasma samples were tested at a 1:50 dilution. All assays were performed in duplicate; the average O.D. is shown for each sample and error bars represent the range of O.D. For all panels, days post-infection are shown on the *X*-axis. (Panel A) Cat 33-5 IgM; (Panel B) cat 33-5 IgA; (Panel C) cat 34-5 IgM; (Panel D) cat 34-5 IgA.

vated immune response following NiV challenge. Cat 33-5 SNT titre remained relatively stable but then increased significantly by 13 dpi and 21 dpi. Such a large increase in titre most likely represented an increased immune response to replicating NiV. To determine if mucosal Ig levels changed post-challenge, tracheobronchial washes collected 8 dpi, 13 dpi and 21 dpi were evaluated for sG-specific IgA and IgM. As was seen pre-challenge, IgM was not detected in mucosal washes (data not shown). Conversely, measurable IgA was detected in most animals post-challenge and results are shown in Fig. 6. Cats 27-0 and 28-0 had significant amounts of IgA 8 dpi whereas IgA levels were low in vaccinated animals by comparison. By 13 dpi, mucosal IgA levels had increased in all vaccinated animals except cats 31-25 and 34-5 which had high levels of pre-challenge mucosal IgA. The two female animals, cats 30-50 and 32-25, which had the least amount of mucosal IgA pre-challenge had the most significant increase post-challenge demonstrating an increased immune response to NiV challenge. By 21 dpi, mucosal IgA was almost undetectable.

3.4. Post-challenge cytokine levels

Plasma, mucosal and tissue-specific IL-2, IL-4, IL-10 and IFN-γ levels were measured using feline-specific cytokine ELISA as described in the methods. Cytokines were not detected in pre- or post-challenge samples for seven of eight animals, including the two control cats. In contrast, cytokines were detected in plasma from cat 33-5 (data not shown). Cat 33-5 had significant levels of IL-2 and IFN-γ and detectable levels of IL-10 and IL-4 from day 0 through day 35. On day 42, all cytokines were still detectable but had decreased significantly. Upon NiV challenge, all cytokines increased, with the highest elevations in IL-2 and IFN-γ.

Table 2
Post-challenge NiV SNT titres

Dose	Cat #	35 dpv[a]	42 dpv[b]	6 dpi[c]	8 dpi	10 dpi	13 dpi	21 dpi
Controls	27	<1:16	<1:16	<1:16	1:16			
	28	<1:16	<1:16	<1:16	<1:16			
50 μg	29	1:512	1:128	1:128	1:128	1:64	1:512	1:256
	30	1:256	****	****	1:256	1:128	1:128	1:64
25 μg	31	1:512	1:256	1:256	1:256	1:256	1:128	1:256
	32	1:256	1:256	****	1:64	1:256	1:256	1:1024
5 μg	33	1:128	1:64	1:64	1:128	1:128	1:8192	1:16384
	34	1:64	1:32	****	1:256	1:128	1:64	1:128

****Sample not collected.
[a] Days post-vaccination = dpv.
[b] NiV challenge.
[c] Days post-infection = dpi.

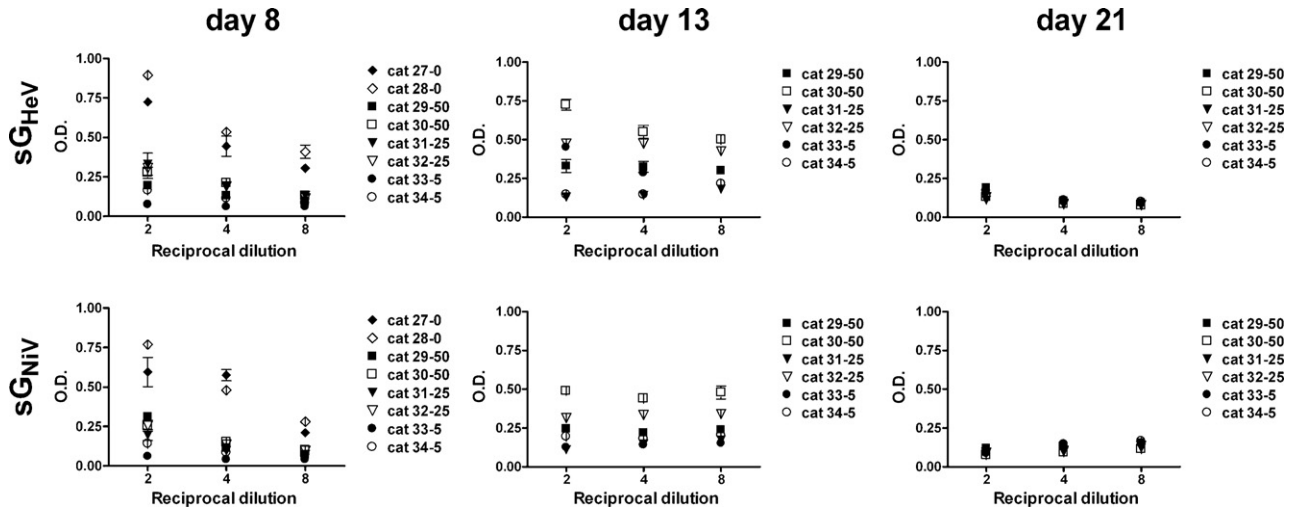

Fig. 6. Post-challenge sG_{HeV}- and sG_{NiV}-specific mucosal IgA. Tracheobronchial washes were collected from challenged cats on days 8, 13, and 21 post-infection as described in the methods. Antigen-specific IgA was measured by ELISA. Reciprocal wash dilution is shown on the *X*-axis for all panels. All assays were performed in duplicate; the average O.D. is shown for each and error bars represent the range of O.D. Top panels represent sG_{HeV}-specific IgA and bottom panels represent sG_{NiV}-specific IgA.

3.5. Virus shedding and tissue tropism

To ascertain NiV tropism and shedding post-challenge, blood, oral swabs, urine and tissues were collected from the cats. Samples were initially screened using a quantitative Taqman PCR assay that targets NiV RNA in the nucleoprotein gene coding region. Previous studies in cats have demonstrated that detection of NiV genome by Taqman PCR, particularly in blood, oral swabs and urine, was a more sensitive assay than virus isolation [25]. In the present study, NiV genome was not detected in blood samples from any animal at any time point (data not shown). NiV genome was detected in oral swabs from all cats post-challenge and results are shown in Fig. 7A. The highest level of apparent shedding occurred on 6 dpi and 8 dpi. Cats 32-25, 33-5 and 34-5 appeared to shed virus at low levels until 21 dpi consistent with post-challenge SNT data, which suggested an increased immune response to replicating virus in these animals. Virus isolation was attempted on all NiV genome positive oral swabs; unfortunately cell toxicity in all samples precluded any successful isolation. Urine samples were collected opportunistically from cats post-challenge, assayed using Taqman PCR and results are shown in Fig. 7B. Similar to the swab data, the highest level of apparent shedding in urine occurred at 6 dpi and 8 dpi. When virus isolation was attempted, all urine samples were negative but again, variable successful isolation of NiV from urine samples of infected cats has been reported [25,30]. Although based on Taqman PCR, not virus isolation, these data suggest that all cats shed significant amounts of NiV following challenge. The data from 6 dpi and 8 dpi may have represented clearance of the initial inoculum, not replicating virus; however, the detection of NiV genome in urine and in swabs from low dose cats on 21 dpi was perhaps a stronger indication of replicating virus in these animals, despite the failure of virus isolation. Tissues collected upon euthanasia were also assayed for viral RNA and results are shown in Fig. 8. Similar to previous studies of NiV infection in cats, both control cats (27-0 and 28-0) had significant amounts of NiV genome in a wide range of tissues, with the highest levels present in the lung and spleen (Fig. 8A). Virus isolation was conducted on all tissues that were positive for the presence of NiV genome; however, virus was only isolated from lung and spleen. The majority of tissues assayed from vaccinated cats at day 21 were negative for NiV genome; however significant levels of genome were detected in the brain (Fig. 8B). Unexpectedly, cats 29-50, 30-50 and 31-25 demonstrated the highest level of genome in brain tissues, and levels were significantly higher than those seen in the control cats. Virus isolation confirmed the presence of NiV in cat 31-25 brain-olfactory whereas for cats 29-50 and 30-50, no virus was isolated. No viral antigen was detected in any of the brains by immunohistochemistry. Clearly, for all vaccinated animals, systemic NiV-mediated disease and multiple organ system pathology was reduced almost entirely. As an exception, NiV genome was detected in the brain of several animals and the significance of these data remains to be elucidated.

4. Discussion

There are presently no active or passive therapeutic procedures for preventing or treating NiV or HeV infection resulting from a natural outbreak, laboratory accident, or deliberate misuse. Here, we have conducted a second sG-henipavirus vaccine trial in cats to further progress a human henipavirus vaccine candidate. As person-to-person transmission of NiV has occurred in recent outbreaks, a vaccine may prove useful in future outbreaks to decrease transmission. Additionally, a henipavirus vaccine would be beneficial to BSL-4 laboratory staff and first responders who may encounter a deliberate misuse of either virus. As HeV and NiV are BSL-4 pathogens, human efficacy studies are not permitted during vaccine development; however, the U.S. Food and Drug Administration (FDA) has implemented the Animal Efficacy Rule for the evaluation of therapeutic products under these circumstances. Specifically, FDA can rely on evidence derived from animal studies in the evaluation of product effectiveness. However, a well-understood mechanism for the pathogenicity of the agent in conjunction with the underlying mechanism of action of the product must be documented. In previous studies, we documented the mechanism for the pathogenicity of the agent and demonstrated protection from NiV challenge in cats using recombinant sG subunit vaccines. Our studies suggested that vaccinated animals developed sterilising immunity [25]. Here we have completed a second sG vaccine trial aimed at influencing and understanding, in a more detailed manner, the mechanism of sG-induced immunity. A CpG motif adjuvant which had been employed previously in humans [31–33] and was known to stimulate mucosal and Th1 immunity [34–36] was utilized. Varying doses of sG and a minimum immunisation regimen of one prime and one boost were employed with the goal of eliciting a range of humoral immune responses. Finally,

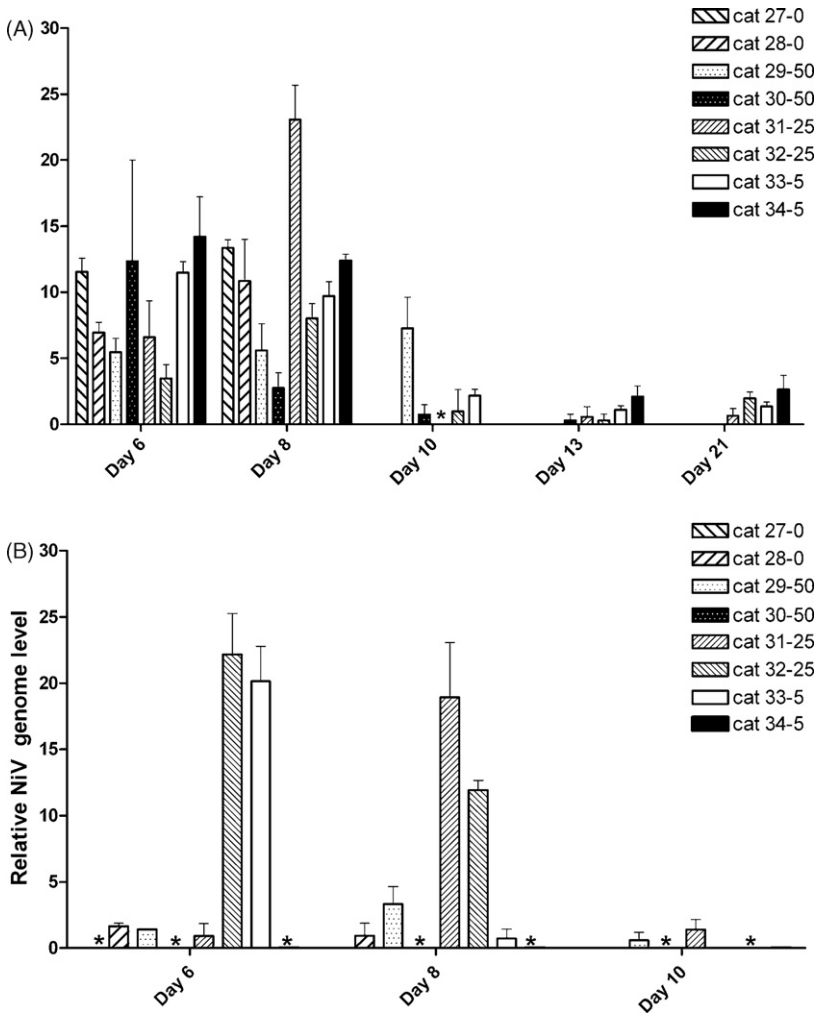

Fig. 7. NiV genome in oral swabs and urine. Oral swabs and urine were collected from all cats post-challenge. * Indicates that no sample was collected. RNA was extracted as described in the methods and further assayed using a Taqman PCR assay that detects the NiV nucleoprotein gene. Threshold cycle (Ct) values were analysed against a known NiV cDNA control and results are shown as relative NiV genome level. For both panels, days post-infection are shown on the *X*-axis. (Panel A) Oral swabs; (Panel B) urine.

unlike the previous sG vaccine trial, where only 500 $TCID_{50}$ was employed subcutaneously, in this study a high NiV challenge dose was employed and administered oronasally, a route of challenge that more closely mimicked a natural infection.

Upon vaccination, antigen-specific plasma Ig was generated in all animals with IgG being the most abundant subclass. IgM and IgA levels varied according to vaccine dose which suggested subtle differences in the potency of the immune response in vaccinated animals. A significant percentage of antigen-specific Ig had neutralising capability, as demonstrated by SNT titre. Mucosal IgA was detected in all vaccinated animals suggesting that CpG adjuvant helped target the immune response to the mucosa; however, IgA levels did not correlate with vaccine dose. The absolute level of plasma Ig did not vary greatly post-challenge. SNT titres did increase in three of the lowest dose cats suggesting virus replication did occur. The observed differences between increased SNT titre post-challenge and relatively unchanged levels of sG-specific plasma Ig might represent antibody responses directed at other viral proteins besides the attachment glycoprotein. Equally possible, although sG-specific antibodies did not increase, an increase in sG-specific antibody affinity could have led to increased potencies in SNT. The combination of sG_{HeV} and CpG induced sufficient levels of antigen-specific plasma Ig, neutralising antibodies and antigen-specific mucosal IgA to protect all cats from lethal NiV challenge. In four of six vaccinated animals some evidence of viral replication was evident whether indicated by increases in SNT titre or mucosal IgA. The immune response elicited by vaccination was sufficient to protect animals from systemic NiV-mediated disease.

An unexpected finding was that the cytokines examined in this study were undetectable in most animals, including the adjuvant-alone controls. To be thorough, plasma, mucosal washes and tissue homogenates from the lung, spleen and bronchial lymph nodes were tested. Unlike other highly pathogenic human viral respiratory pathogens, such as severe acute respiratory syndrome (SARS), RSV, and highly pathogenic influenza virus, a cytokine storm does not appear to play a role in the early stages of NiV-mediated disease. Future studies aimed at understanding the function of T-cells will be critical in determining the role of Th1 cytokines in protection from disease.

To indirectly measure the effects of vaccine-induced immunity, we assessed viral shedding, viral loads and NiV-associated pathology in cats post-challenge. Prolonged apparent viral shedding was demonstrated in oral swabs from three of the vaccinated cats. In all vaccinated animals, systemic NiV-mediated disease was practically negated as evidenced by the lack of clinical disease and gross or microscopic pathology, the absence of immunohistochemical antigen staining and minimal detection of NiV genome in various tissues. Surprisingly, although no viral antigen was detected

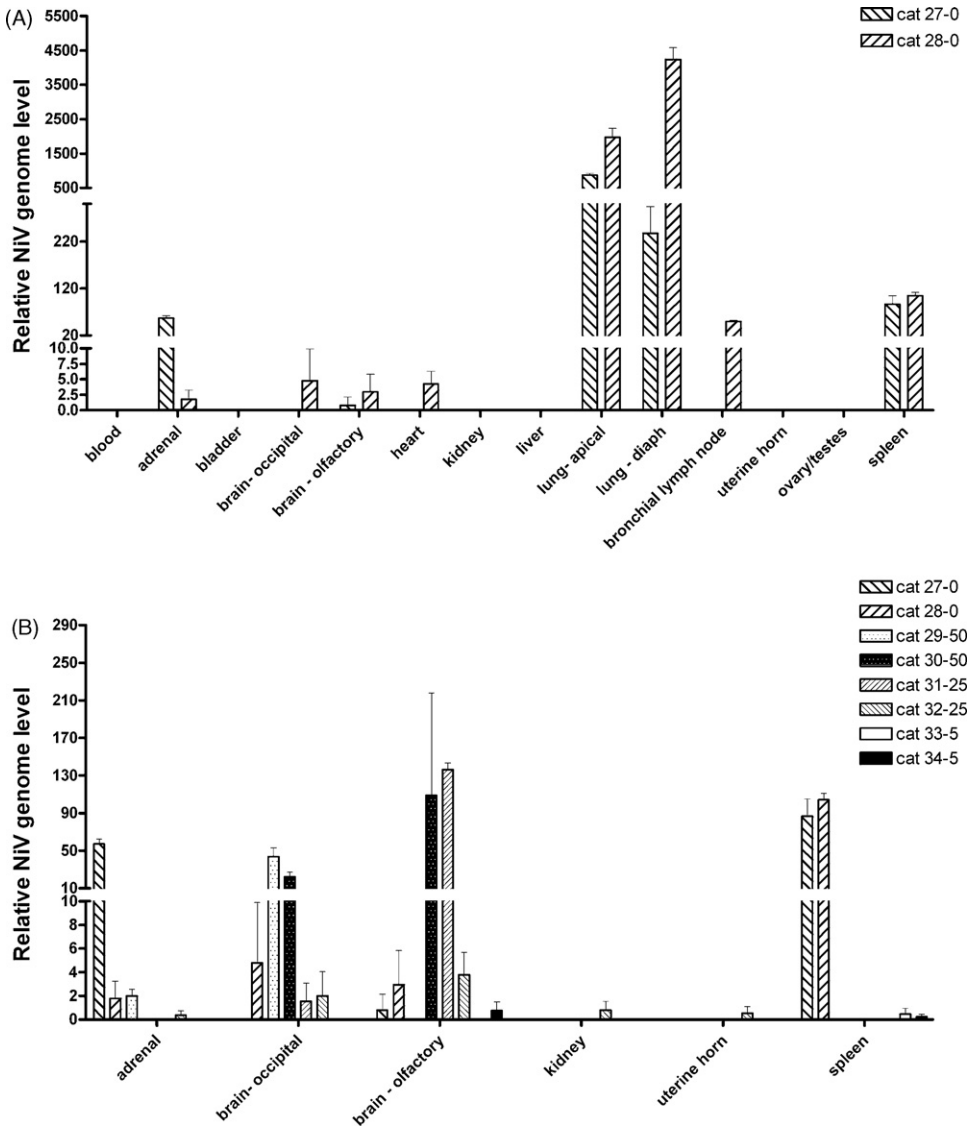

Fig. 8. NiV genome in tissues collected upon necropsy. Various tissues, as indicated by the *X*-axis, were collected from cats upon euthanasia. RNA was extracted as described in the methods Section 2 and further assayed using the NiV Taqman PCR assay. Ct values were analysed against a known NiV cDNA control and results are shown as relative NiV genome level. For control cats (27-0 and 28-0) (Panel A) results from all tissues are shown. For vaccinated animals (Panel B), only positive tissues are shown and control cats are included for comparison.

in any of the brain tissues from vaccinated animals, relatively high amounts of NiV genome were detected in the brain of four of the higher vaccine dose cats. NiV was isolated from one brain tissue confirming the presence of infectious virus. In the two low vaccine dose animals, which had the lowest pre-challenge SNT titres, the level of NiV genome in the brain was either undetectable or just barely above the threshold of the assay. Notably, all the vaccinated cats that demonstrated NiV genome in the brain had significant antibody titres prior to challenge. These observations indicate that persistent infection of NiV in cats may occur despite the presence of pre-existing immunity and that persistent infection may even be enhanced by particular levels of immunity. The mechanisms of this effect are unclear and require further investigation. Due to the constraints with working at BSL4 only small numbers of cats were used. It would be useful to strengthen the statistical significance of these findings by repeating similar trials, and thereby increasing the numbers of infected animals. The data suggest that although protection from disease was achieved in the current vaccine study, the antigen dose used was not sufficient to prevent virus from infecting the brain. What remains unknown is whether the NiV detected within the brain of some animals would eventually be cleared by the host or if neurological disease would manifest at a later date. However, considering the significantly longer periods that such recurrences of disease have been documented to occur in humans (ranging from several months to nearly 4 years) [37], such long-term studies are nearly impossible to conduct under BSL-4 containment.

In summary, we have achieved protection in cats from a lethal NiV challenge using a recombinant sG_{HeV} subunit vaccine in conjunction with a CpG adjuvant. Importantly, our data suggests that one vaccine could be developed for both henipaviruses. Furthermore, a better understanding of the mechanism of sG-induced immunity to NiV infection has been elucidated, which provides important information required by the FDA for any human vaccine candidate. The vaccine doses employed here have revealed subtle differences in immune responses and protection from disease and have highlighted important considerations for future sG vaccine trials.

Acknowledgments

The authors wish to thank Stuart Isaacs from the University of Pennsylvania for helpful advice pertaining to CpG adjuvants and antigen dose. We thank Dayna Johnson and Jessica Klippel from the Australian Animal Health laboratory (AAHL) for implanting all radio telemetry devices and help with sampling and necropsy of all animals. Additional thanks to Don Carlson, John Muschialli and Bruce Mungall from AAHL for their help in monitoring of cats at BSL-4. Final thanks to Glenn Marsh and Mary Tachedjian for critical review of this manuscript prior to its submission. This study was supported by a Jack Brockhoff Foundation grant to KNB, in part by AB-CRC grant 1.013RE to LW and NIH, NIAID Middle Atlantic Regional Center of Excellence grant AI057168 to CCB.

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