Preclinical efficacy studies of influenza A haemagglutinin precursor cleavage loop peptides as a potential vaccine

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A universal influenza vaccine that does not require annual reformulation would have clear advantages over the currently approved seasonal vaccine. In this study, we combined the mucosal adjuvant alpha-galactosylceramide (αGalCer) and peptides designed across the highly conserved influenza precursor haemagglutinin (HA₀) cleavage loop as a vaccine. Peptides designed across the HA₀ of influenza A/H3N2 viruses, delivered to mice via the intranasal route with αGalCer as an adjuvant, provided 100% protection following H3N2 virus challenge. Similarly, intranasal inoculation of peptides across the HA₀ of influenza A/H5N1 with αGalCer completely protected mice against heterotypic challenge with H3N2 virus. Our data suggest that these peptide vaccines effectively inhibited subsequent influenza A/H3N2 virus replication. In contrast, only 20% of mice vaccinated with αGalCer-adjuvanted peptides spanning the HA₀ of H5N1 survived homologous viral challenge, possibly because the HA₀ of this virus subtype is cleaved by intracellular furin-like enzymes. Results of these studies demonstrated that HA₀ peptides adjuvanted with αGalCer have the potential to form the basis of a synthetic, intranasal influenza vaccine.

INTRODUCTION

Influenza causes significant morbidity (3–5 million cases) and mortality (500,000) annually worldwide, particularly amongst the young, elderly and immunocompromised individuals. The current inactivated influenza vaccine relies on prediction of the forthcoming viral strain and requires annual reformulation. This vaccine predominantly induces a humoral immune response with production of antibodies that bind to the HA₁ subunit (Wilson & Cox, 1990). These antibodies are thought to opsonize the input virus, enhancing immune-mediated removal or inhibition of virus attachment to the cellular receptors (Treanor, 2004; Wilson & Cox, 1990). Due to antigenic shift and drift, the current vaccine would be unlikely to provide significant protection should a novel strain emerge in the community. This was indeed the case with the 2009 influenza pandemic caused by swine origin influenza A/H1N1 virus (www.who.int). Accordingly, development of novel vaccines that provide heterotypic protection, without the requirement for annual reformulation based on known circulating strains, would facilitate influenza control and prevention. The influenza virus precursor haemagglutinin (HA₀) cleavage loop, which is essential in the replication cycle of the virus, provides a promising target for a peptide-based vaccine. The connecting peptide across the HA₀ cleavage site is a prominent surface loop (Chen et al., 1998) containing amino acid residues that are highly conserved across all influenza A and B viruses, including the highly pathogenic avian influenza (HPAI) strains. Peptide vaccines designed around the HA₀ cleavage loop have previously been shown to provide protective immunity. One of the first reports used a haemagglutinin multi-peptide construct (Horváth et al., 1998) where mice vaccinated with either linear peptide sequences across HA₀ or multiple copies of HA₁- or HA₂-peptides induced specific antibodies and provided significant protection.
against 2 LD_{50} influenza A/H1N1 (A/PR8/35). Recently, a peptide vaccine based on the conserved HA0 cleavage site of influenza B haemagglutinin, conjugated to the outer-membrane protein complex of Neisseria meningitidis and adjuvanted with alum or QS21, gave variable protection to mice when challenged with different influenza B lineages (Bianchi et al., 2005). In the above studies, the peptide vaccines were administered via the intraperitoneal route.

Intranasal delivery of an influenza vaccine would be attractive as this is non-invasive and, importantly, stimulates local immune responses at the site of virus entry and induction of secretory IgA (Renegar & Small, 1991; Renegar et al., 2004). The glycolipid, α-galactosylceramide (αGalCer), has been shown to be a potent mucosal adjuvant. When combined with subunit influenza vaccine and administered to mice via the intranasal route; this protected against lethal influenza A virus (Ko et al., 2005; Youn et al., 2007), presumably by modulating the acquired immune response. Natural killer T-cells (NKT), a distinct subset of lymphocytes, can be activated by sponge-derived or synthetic forms of αGalCer and other bacterial glycolipids presented on CD1d (Borg et al., 2007). This MHC I-like molecule, expressed on dendritic, B- and T-cells, macrophages and hepatocytes (Bendelac et al., 1997; Brossay et al., 1997), interacts with NKT cells resulting in rapid secretion of both T-helper 1 (TH-1) and T-helper 2 (TH-2) cytokines, such as gamma interferon (IFN-γ) and interleukin 4 (IL-4) (Au-Yeung & Fowell, 2007). αGalCer has been approved for use in humans (Nieda et al., 2004; Veldt et al., 2007), is inexpensive and provides an ideal mucosal adjuvant for testing with our HA0 peptide vaccine.

In this study, we designed and synthesized peptides, conjugated to diphtheria toxoid (DT), across the influenza HA0 cleavage loops of H3N2 and H5N1 influenza viruses. In addition to HA0 peptides, two T-helper peptides were synthesized to aid induction of antibody responses. Comprehensive testing was performed with the H3 HA0 peptides, culminating in a vaccine regimen that provided 100% protection against H3N2 influenza virus. Following heterologous or homologous challenge of mice vaccinated with H5 HA0 cleavage loop peptides, our results showed that HA0 peptides with αGalCer have the potential to form the basis of a synthetic, intranasal influenza vaccine.

RESULTS

Complete protection against H3N2 influenza challenge following H3 HA0 peptide vaccination

We established the optimal vaccination protocol in BALB/c mice (Supplementary Fig. S1, available in JGV Online). In a coded experiment, groups of 20 mice received either three intranasal vaccinations containing saline or H3 HA0 peptides (see Methods) adjuvanted with 0.3 µg of αGalCer. The selection of the latter concentration of αGalCer was based on the highest protection level obtained in BALB/c mice (see Supplementary Fig. S1). Mice cages were coded to prevent bias during virus challenge. All mice were challenged with 6 mouse infectious dose (MID_{50}) influenza A/X31 virus 7 days after the final vaccination. Three days post-infection (p.i.), lung samples were collected from eight of each group of 20 BALB/c mice for viral RNA and histological analysis. The remaining 12 control mice lost 20% of their body weight by day 5 p.i. and were sacrificed (Fig. 1a). Prior to virus challenge, two vaccinated mice died from non-vaccine related events. The remaining 10 vaccinated mice lost <5% of their initial body weight by days 2–3 p.i. but fully recovered by day 5 p.i. (Fig. 1a).

Similar results were obtained in a different genetic strain of mice – C57/B6. Although no interim sampling was performed,
the body weight loss observed in the control or vaccinated C57/B6 mice following virus challenge was similar within days 1 and 2 p.i. An increase in body weight and improvement in clinical condition was evident by day 3 p.i. in the vaccinated mice (Fig. 1b). Statistical analysis using the log-rank test indicated that the period to reach 20% body weight loss was significantly longer in the peptide-αGalCer-vaccinated groups in both BALB/c and C57/B6 mice, compared with saline and αGalCer + saline, respectively (P<0.001).

**Quantification of H3N2 viral RNA in lung**

Real-time RT-PCR showed that the quantity of influenza virus RNA in lung tissues of vaccinated BALB/c mice was threefold lower than in control mice at day 3 p.i., and approximately 10-fold lower at day 5 p.i. when the experiment was terminated (Fig. 2a). An independent samples t-test showed that the differences in the mean RNA levels were significantly lower in the peptide-αGalCer-vaccinated mice at days 3 (P=0.0133) and 5 p.i. (P=0.0021).

**Influenza A/H3N2 viral histopathology and immunohistochemistry**

Coded microscopic examination of lung sections from control mice sacrificed at days 3 (Fig. 3a) and 5 p.i. (Fig. 3c) revealed a severe necrotizing bronchiolitis characterized by marked loss of respiratory epithelium and resulting intraluminal accumulation of desquamated cellular debris (day 5 p.i., Fig. 3e). In lesions of longer duration, there was substantial reparative hyperplasia of the lining epithelium, with some polypoid causing luminal obstruction and occasionally dysplastic. Type II pneumocyte metaplasia was prominent, with occasional syncytia formation and predominantly lymphoplasmacytic with macrophage infiltration of the alveolar interstitium. Pulmonary congestion, high-protein oedema and multifocal haemorrhage were present. In contrast to control mice, the most striking histological feature in vaccinated mice was marked perivascular lymphocytic or lymphoplasmacytic cuffing and hyperplasia of bronchial-associated lymphoid tissue. Cells of the perivascular lymphocytic cuffs (Fig. 3f, day 5 p.i., H&E staining) showed strong staining with anti-CD3 antibodies, indicating the presence of a high density of T lymphocytes (Fig. 3g, day 5 p.i.). Fig. 3(h) shows NK cells – in lungs of vaccinated mice – stained with rabbit anti-Asialo GM1 antisera (day 5 p.i.).

Lungs were stained for influenza virus antigen by immunoperoxidase. In all saline control mice, intense virus staining was observed in the cytoplasm of bronchial and bronchiolar lining epithelial cells (Fig. 3a, c, see also Supplementary Figs S3a, a–h and 3b, a–l, available in JGV Online). The extensive viral pathology observed in control mouse lung is consistent with severe acute necrotizing bronchilitis (Fig. 3e). There was significant viral antigen staining observed in degenerate epithelium desquamated into bronchiolar lumina. By contrast (and in concordance with H3N2 viral RNA levels, Fig. 2a), viral antigen was substantially reduced in lungs, collected at days 3 and 5 p.i., from H3 HA0-vaccinated mice (Fig. 3b, d respectively, see also Supplementary Figs S3a, i–n and 3b, m–x). The histopathology and viral RNA results suggest a successful first round of virus infection.

**Complete protection against H3N2 influenza virus challenge following H5 HA0 peptide vaccination**

Since our data clearly demonstrated that the H3 vaccine protected against homologous H3N2 influenza virus challenge, we tested heterotypic protection using two overlapping linear peptides across the H5 influenza HA0 cleavage site (Table 1). Initial testing demonstrated that the H5 HA0 peptides with αGalCer were less immunogenic, inducing antibody titres two logs lower than the H3 HA0 peptide-vaccinated mice (Table 2). However, all 12 H5 HA0 peptide-vaccinated mice survived challenge with 6 MID50 H3N2 virus, indicating that this peptide vaccine...
gave cross protection against H3N2 virus. All control mice succumbed to the infection by day 5 p.i. (Fig. 4).

**Anti-HA₀ peptide antibody characterization post-H3N2 virus challenge**

Using ELISA to measure antibody responses, we detected HA₀ peptide-specific total immunoglobulins, IgA, IgG1 and IgG2a antibodies in sera and mucosal IgA in nasal washes of mice following vaccination and a serum-transfer experiment (Table 2). The H3 HA₀ peptide-specific antisera showed no reactivity to the egg-produced, mature H3N2 virus (data not shown) in which the HA₀ is expected to have been cleaved by extracellular enzymes (Klenk et al., 1975; Lazarowitz & Choppin, 1975). Viral antibody was not detected in the antisera as judged by haemagglutination inhibition activity (titre <1/4) with the egg-grown X31 virus and 0.25 % guinea pig erythrocytes as indicator cells (Lennette & Schmidt, 1979). The antisera failed to neutralize egg-grown H3N2 virus infection of Madin–Darby canine kidney (MDCK) monolayers.

To examine the anti-peptide antibodies further, a passive-antibody-transfer experiment was performed. Groups of eight 10-week-old BALB/c mice were inoculated (intraperitoneum) with 100 µl of pooled sera from 12 normal mice or pooled HA₀ antisera from 10 vaccinated mice, 24 h prior to and on the day of H3N2 influenza virus challenge. The ELISA HA₀ end-point antibody titre in the recipient mice was $9.3 \pm 65.1 \times 10^{3}$ (Table 2). No significant protection was observed, indicating an absence of sterilizing antibodies in the anti-peptide sera (see Supplementary Fig. S2, available in JGV Online).

Although specific antibodies from H3N2 HA₀ peptide-vaccinated mice were detected by ELISA, other tests with the antisera were negative viz. (i) no reaction with or neutralization of egg-grown H3N2 virus (HA₀ cleaved), (ii) no detectable influenza A virus antibody by haemagglutination inhibition and (iii) passive-antibody-transfer experiment did not confer protection. We then studied the reaction of the anti-HA₀ peptide antibodies with uncleaved HA₀ in progeny virus. MDCK cells were infected with egg-grown X31 virus (HA₀ cleaved) and cultured without trypsin for 24 and 72 h. Following fixation, the X31 virus-infected cell monolayers were reacted with normal mouse serum, pooled HA₀ peptide antisera or polyclonal rabbit anti-influenza A by immunofluorescence test to localize newly synthesized influenza A/H3N2 virus on MDCK cells (HA₀ uncleaved). No specific staining was observed in the cells reacted with normal mouse serum (Fig. 5a, d). As expected, strong virus-specific staining was observed with the pooled HA₀ peptide antisera (Fig. 5b, e) or rabbit antisera (Fig. 5c, f) at 24 h p.i., with more intense staining at 72 h p.i. These results confirm that HA₀ anti-peptide antibodies are reactive to the newly made influenza virus prior to HA maturation and this is probably linked to the virus challenge protection results in mice.

**Partial protection against H5N1 influenza virus challenge following H5 HA₀ peptide vaccination**

Mice were vaccinated three times intranasally with H5 HA₀ peptides and 0.3 µg zGalCer, while control mice received saline and 0.3 µg zGalCer. Low-levels of H5 HA₀ peptide
antibodies were detected in the sera of these mice prior to challenge and nasal wash-specific IgA titre was <10 (Table 2). All mice were challenged intranasally with the H5N1 virus 7 days after the final vaccination. Control mice developed greasy fur from days 4 to 5 p.i., which progressively became more ruffled until they were sacrificed between days 8 and 10 p.i., having lost ~20 % body weight with varying degrees of inactivity and dehydration, while two mice recovered, having lost 10 and 16 % body weight by days 8 and 11 p.i., respectively (Fig. 6). The clinical protection data from this experiment indicated that H5 HA₀ peptide vaccination prolonged the lives of vaccinated mice by about 2 days compared with the controls and, notably, 20 % of the mice recovered from H5N1 infection. The peptide-αGalCer-vaccinated group took a longer period to show 20 % weight loss compared with the controls (P<0.0015).

**Quantification of H5N1 viral RNA**

Real-time RT-PCR analysis of lung tissue collected from three mice autopsied at days 3 (P=0.0178) and 6 (P<0.0001) p.i. showed significant decreases in the levels of viral RNA in the peptide-αGalCer-vaccinated mice.
Table 2. Antibody titres in three groups of BALB/c mice post-
H3 HA0, -H5 HA0 peptide vaccination or passive-antibody transfer. Mice received intraperitoneal inoculation with pooled
antisera from H3 HA0 peptide-immunized (intranasal) mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Antibody titre</th>
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<tbody>
<tr>
<td>Anti-H3 peptide serum</td>
<td></td>
</tr>
<tr>
<td>Total immunoglobulins</td>
<td>$50.2 \pm 10.2 \times 10^3$</td>
</tr>
<tr>
<td>IgA</td>
<td>$5.8 \pm 1.7 \times 10^6$</td>
</tr>
<tr>
<td>IgG1</td>
<td>$24.2 \pm 8.2 \times 10^9$</td>
</tr>
<tr>
<td>IgG2a</td>
<td>$0.9 \pm 0.5 \times 10^7$</td>
</tr>
<tr>
<td>Nasal wash IgA</td>
<td>$0.1 \pm 0.25 \times 10^9$</td>
</tr>
<tr>
<td>Anti-H5 peptide serum</td>
<td></td>
</tr>
<tr>
<td>Total immunoglobulins</td>
<td>$2.3 \pm 1.3 \times 10^7$</td>
</tr>
<tr>
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<tr>
<td>Passive-antibody transfer</td>
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<tr>
<td>Total immunoglobulins</td>
<td>$9.3 \pm 65.1 \times 10^3$</td>
</tr>
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compared with the controls, indicating some degree of
protection against H5N1 challenge (Fig. 2b).

**DISCUSSION**

This study investigated the vaccine potential of intranasal
inoculation of synthetic peptides – with αGalCer adjuvant –
to the conserved viral HA0 cleavage site to induce heterotypic immune responses against influenza A infection. Using four overlapping peptides designed around the conserved influenza A HA0 cleavage loops, intranasal vaccination of mice with the H3 HA0 peptides with αGalCer provided 100% protection (Fig. 1). Mice vaccinated with the H3 HA0 peptides with αGalCer, induced high titres of peptide-specific anti-H3 HA0 IgG1, IgG2A and IgA antibody subclasses in serum and IgA in nasal washes. These antibodies reacted strongly to newly made virus prior to HA maturation. In the vaccination/challenge experiments, there was concordance between protection against H3N2 influenza A virus in mice and the anti-peptide antibody levels. These observations suggested that the anti-peptide antibodies were probably involved in protection. This is in contrast to the passive-antibody-transfer experiment in which no protection was found. This may be explained by insufficient peptide antibodies in the passive-antibody-transfer experiment, as the dilution of antisera was approximately 1:15, taking the average volume of blood in a BALB/c mouse as 1.5 ml. The total immunoglobulin level in mice with passive-antibody transfer was more than fivefold lower compared with H3 HA0 peptide-immunized mice (see Table 2). NKT cells (Gazit et al., 2006) may be activated and contribute to protection in the peptide-vaccinated mice, but not in those which received passive transfer antibodies.

Vaccination of mice with H5 HA0 loop peptides induced 250-fold lower anti-peptide antibody levels than vaccination with the H3 HA0 peptides (Table 2). Several attempts to increase the antibody response to the H5 HA0 peptides were unsuccessful. The reason for the lower antibody response is unclear. It is possible that the multiple basic amino acid insertions (Table 1) may have enabled degradation of the H5 HA0 peptides more readily by tryptase-like enzymes in the respiratory tract of mice, resulting in possible lower immunogenicity. The 3’ half of H5N1 and H3N2 HA0 cleavage loops are similar, offering the possibility of inducing cross-reacting antibodies with homologous as well as one-way cross protection. This was clearly observed – H5 HA0 peptide antibodies protected against H3N2 virus challenge (see Fig. 4 and Table 1). However, the 20% protection data obtained in the homologous H5 virus challenge indicates that lower level (compared with H3 HA0 peptides, Table 2) of antibodies produced were not fully effective against the biologically more virulent influenza A/H5N1 virus. The latter viral HA0 is cleaved by intracellular furin-like enzymes which results in production of infectious virus within the cells, thus causing systemic infection in the animals (Nishimura et al., 2000), which may not be neutralized by extracellular antibodies. In addition, the challenge dose that we used in this experiment was not optimized and may have been too high. Further experimental studies in mice and ferrets would add to the understanding of the protective roles played by the HA0 peptide antibodies with heterologous and homologous viral strain challenges.

In this study, the anti-peptide antisera did not show viral neutralization in vitro or in vivo (Supplementary Fig. S2), suggesting that the observed protection was not from sterilizing serum antibodies. This is consistent with the presence of viral antigen and RNA in the lungs of vaccinated/challenged mice at day 5 p.i. (Fig. 3, see also Supplementary Fig. S3a, b). In addition, the peptide-
specific antibodies did not bind significantly to egg-produced, mature virus (where the HA₀ has been cleaved), when used as a coating antigen in ELISA, but recognized influenza A virus-infected MDCK cells, suggesting that the anti-HA₀ antibodies specifically recognized newly formed, uncleaved HA₀ molecules in the infected cells (Fig. 5). These HA₀-specific antibodies could inhibit virus matura-

tion and therefore play a role in protection by preventing multiple rounds of virus replication.

Influenza-specific cytotoxic T-lymphocytes have been shown to be important for recovery from influenza and protection against subsequent infection (Thomas et al., 2006). Fc-receptor-mediated cell killing plays a pivotal role in the clearance of respiratory viral infections as the result of antibody dependent cellular cytotoxicity by NK cells (Huber et al., 2001). The accumulation of T-lymphocytes in perivascular cuffing, observed only in the vaccinated mice, could be a result of protective cytokines released in response to the HA₀ peptide vaccine (Fig. 3f and g). The presence of serum IgG1 and IgG2a (Table 2) suggests that TH-2 or TH-1 mechanisms may be operating. A significant number of NK cells within the lungs of these mice were also evident (Fig. 3h). Therefore, the observed protection is unlikely due only to the action of serum neutralization of the virus, but may involve NK cell-mediated elimination of the anti-peptide antibody-coated influenza-infected cells in the airways. This has the potential to restrict the spread of virus by enhancing immune-mediated clearance of the infected cells. Further studies on this mechanism are warranted to understand more fully the protection afforded by these HA₀ peptide vaccines.

In conclusion, we have shown 100 % homologous protection with H3N2 HA₀ peptides against H3N2 influenza infection and cross protection with the H5N1 HA₀ peptide vaccine. The protection against homologous challenge with HPAI H5N1 was modest. This was equivalent to that shown with the antiviral drug Oseltamavir (Yen et al., 2005). Finally, this peptide-based
vaccine regimen, with further development, has the potential to form the basis of an intranasal, universal influenza virus vaccine that may not require annual reformulation.

**METHODS**

**Peptides and αGalCer synthesis.** All peptides were synthesized by Mimotopes Pty. Ltd, Australia. Overlapping R/G peptides, designed around the HA0 cleavage site of H3N2 (GenBank accession no. AAA43178) and H5N1 (GenBank accession no. AFIQ2672) influenza A viruses were synthesized unconjugated or conjugated to DT via an additional cysteine. The latter conjugation was used to increase peptide immunogenicity (Tam, 1994). Two additional CD4 T-helper peptides were also synthesized to enhance the T-helper cell function needed for the generation of strong and lasting antibody responses (Table 1). The first-helper peptide was made to a human CD4 T-helper cell epitope (Danke & Kwok, 2003; Gebe et al., 2001). The second was a tetanus toxoid peptide (Hlp 2) that has been tested in a phase 1 trial of a melanoma vaccine (Singluff et al., 2001). αGalCer (KRN7000) was synthesized from phytosphingosine and 4-tetra-O-benzyl-D-galactopyranoside (Du & Gervay-Hague, 2005).

**Viruses and virus titration.** A/H3N2 strain X31 influenza A virus was obtained from Assoc. Professor Lorena Brown, University of Melbourne, Australia. Stocks of the virus were grown in the allantoic cavity of 10-day-old embryonated chicken eggs at 37 °C for 48 h, clarified by centrifugation and stored at −70 °C until use. Influenza A/Vietnam/1203/2004 (H5N1) was provided by the WHO Collaborating Centre for Reference and Research on Influenza (Melbourne, Australia) and passage twice in 10-day-old embryonated chicken eggs at 37 °C for 48 h. Virus stocks were prepared as clarified allantoic fluid and stored at −70 °C before use.

Stock virus titrations in 13-week-old female BALB/c mice and H5N1 virus in Vero cells were performed as described previously (Alsharif et al., 2009). The TCID50 and MLD50 were calculated using the method of Reed & Muench (see Lennette & Schmidt, 1979). The stock X31 virus titre was 9 × 106 TCID50 ml−1 as assessed by plaque-titration on MDCK cells or 10−3 MLD50 ml−1. The stock influenza A/ H5N1 virus titre was 10−6 TCID50 ml−1 or 10−6 MLD50 ml−1.

**Mice, vaccination and influenza A virus challenge**

**Vaccination.** Six-week-old female BALB/c or C57/B6 mice were obtained from the Animal Care Facility, IMVS, Adelaide or Animal Resource Centre (Perth, Australia). An optimal vaccination regimen was determined in preliminary experiments. For H3 vaccination, mice were inoculated with 8.3 μg of each DT-conjugated H3 HA0 peptide (Table 1: 3A1, 3A2, 3B1 and 3B2) and 8.3 μg of each T-helper peptide (Table 1: Hlp1 and Hlp2) combined with 0.3 μg αGalCer. For H5 vaccination, mice were inoculated with 16.7 μg of each DT-conjugated H5 HA0 peptide (Table 1: 5A1 and 5B1) and 8.3 μg of T-helper peptides (Table 1: Hlp1 and Hlp2) combined with 0.3 μg αGalCer. In H3 HA0 peptide vaccine studies, control mice received saline whilst in all C57/B6 mice and H5N1 challenge studies, control mice received 0.5 μg αGalCer in saline. Unless otherwise stated, 40 μl (20 μl per nostril) of vaccine containing the pooled peptides, with 0.3 μg αGalCer in normal saline, was administered intranasally following anaesthesia induced by inotraperine administration of 100 mg ketamine HCl kg−1 and 10 mg xylazine HCl kg−1 (Illum Veterinary Products, Australia). In all studies, mice received doses of vaccine on days 0, 14 and 21. Mice were challenged with H3N2 or H5N1 virus on day 7 following the final vaccination. Serum samples were collected for determination of anti-peptide antibodies one day prior to virus challenge. Nasal lavages were collected following ligation of the trachea with a suture and subsequent washing of the nasal cavity with 400 μl of saline.

**Passive-antibody transfer.** In passive-antibody-transfer experiments, serum samples were pooled from either 10 mice vaccinated with H3 HA0 peptides or 12 non-vaccinated mice. Then 100 μl of the pooled sera were administered twice to naive mice via the intraperitoneal route at 24 h and immediately prior to challenge with 6 MID50 H3N2 (X31) virus.

**Virus challenge.** Serum HA0 antibody levels in recipient mice were measured by ELISA prior to challenge with 35 μl of virus (6 MID50 of H3N2 or 5 MID50 of H5N1) administered either into the left nostril or both nostrils, respectively. Mice were weighed and examined once daily for development and progression of clinical signs consistent with influenza A/H3N2 infection. Following H5N1 challenge, mice were examined twice daily for clinical signs of infection and weighed. Mice were sacrificed when there was loss of 20 % of the pre-challenge body weight, development of any neurological sign or inability to eat or drink. For interim sampling, three mice were sacrificed on days 3 and 5 (H3N2) or days 3 and 6 (H5N1) p.i. and the right lung was collected for viral RNA test. To prevent cross contamination between samples, tissues were removed using separate sterile forceps and scissors, diced with single-use sterile disposable scalps and stored on ice and transferred to −80 °C for longer term storage. The lung was immersed in >10 volumes of 10 % PBS-formalin for subsequent histopathology examination (see below). All animal handling procedures and protocols were carried out in accordance with the IMVS and Australian Animal Health Laboratory (AAHL, Geelong, Australia) Animal Ethics committees. Procedures with influenza A/ H5N1 virus were performed under Biosecurity Level 3 enhanced containment at AAHL.

**Immunoperoxidase staining of formalin fixed lung tissue.** Lung tissues were fixed in 10 % PBS-formalin for 24 h prior to paraffin embedding. Serial 8 μm sections were cut onto polylysine (Menzel-Glaser) coated microscope slides. For the influenza and NK-specific staining, antigen was retrieved with citrate buffer while target retrieval solution (cat # S-1699; Dako) was used for CD3, followed by trypsin type II digestion (cat # T-8128; Sigma). To minimize background staining, the tissue sections were treated with a 1 : 30 dilution of normal goat serum after quenching possible endogenous peroxidase with 0.05 % H2O2 for 15 min. The primary antibodies used were rabbit anti-H3N2 produced in-house (Kok et al., 1994), diluted 1:1000 in 10 % FCS/PBS; rabbit anti-CD3 (cat # A0452; Dako), diluted 1:6000 in 3 % normal horse serum (NHS) and rabbit anti-Asialo GM1 (cat # 986-10001; www.wako-chem.co.jp), diluted 1:2000 in 10 % FCS/PBS. Slides were incubated with the appropriate primary antibodies at 37 °C for 1 h or overnight at 4 °C followed by HRP-conjugated goat anti-rabbit (KPL Cat # 04-1506) antibody, diluted 1:200 in 10 % FCS/PBS, or sheep anti-mouse HRP, diluted 1:200 in 3 % NHS/PBS, and incubated for 1 h at 37 °C. In between these incubations the slides were washed before the addition of substrate 3, 3′ di-aminobenzidine (cat # D6815; Sigma) for 15 min in the dark, counterstained with haematoxylin and mounted in Depex.

**Peptide-specific total immunoglobulins, IgA, IgG1 and IgG2A ELISA.** Antibody tests by ELISA were performed with U16 Maxisorp strips (Nunc). Antisera were diluted in 5 % skimmed milk in PBS containing 0.05 % Tween 20 (SMPBST) in a volume of 100 μl and incubated for 1 h at 37 °C unless stated otherwise.

Plates were coated with either egg-grown X31 influenza A virus (9 × 106 TCID50 per well, in a volume of 100 μl) or 100 ng of pooled non-conjugated peptides (Table 1: 3A3, 3A4, 3B3 and 3B4 for H3 studies or 3A2 and 3B2 for H5 peptide studies) in 100 μl PBS and
incubated at 37 °C overnight. Prior to use, the peptide-coated plates were washed and blocked with 200 μl per well with SMPBST. Nasal lavage or serum samples, diluted 1 : 10 in SMPBST, were added, then followed by four 10-fold falling dilutions and incubated for 1 h at 37 °C. The immunoglobulin isotype was detected with 50 μl per well of peroxidase-conjugated sheep anti-mouse total Igs (1 : 5000) (NXA-931; Amersham), rabbit anti-mouse IgG1 (1 : 200) (610-4340; Rockland) or rabbit anti-mouse IgG2A (1 : 200) (610-4341; Rockland). For the IgA-specific ELISA, mouse IgA was detected with goat anti-mouse IgA (1 : 200) (01-18-01; KPL) followed by peroxidase-conjugated donkey anti-goat Ig (1 : 2500) (605-703-125; Rockland). Wells were washed three times with SMPBST following antibody incubations, except prior to the addition of substrate where the plates were washed three times with PBS. O-Phenylenediamine dihydrochloride (cat # P9187; Sigma) was used as a substrate, incubated for 15 min at room temperature in the dark and the reaction was stopped with an equal volume of 0.5 M H2SO4. Absorbance was read at 490 nm and results are shown as the log10 end-point titre to achieve an optical density of 0.2 (cut-off).

**Real-time RT-PCR analysis (H3N2).** Lung tissues were excised at the time of autopsy, immerged in RNAlater (Qiagen), incubated overnight at 4 °C and stored at −20 °C until used. Viral RNA was extracted from lung tissues with an RNeasy Mini kit (cat # 74106; Qiagen). Briefly, 10 mg of frozen lung tissue was homogenized in lysis buffer, eluted in the supplied columns with RNase free water in a 30 μl volume and stored at −80 °C until used. The influenza A matrix gene, selected as the target for amplification, is highly conserved (Stone et al., 2004). PCR primers were used: MAfw1: 5’-GACCAA-TTCTGTCCACCTCTGA-3’ and MArev1: 5’-GTATATGAGGGCCA- TRCAACT-3’. RT-PCR was performed using a Quantitect SYBR Green RT-PCR kit (Qiagen) with 0.5 μM of each primer in a final volume of 25 μl. Reactions were performed using a Rotorgene PCR machine with the following cycling conditions: 50 °C for 30 min (RT), 95 °C for 15 min (PCR activation), 45 cycles at 94 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s. Uniform RNA loading was controlled using a standard curve constructed from normal lung tissue spiked with 10 μl of stock virus where normal lung RNA was added to normalize total RNA in each reaction. Normalization of RNA added was performed by a separate real-time RT-PCR under the same conditions as the influenza-specific reaction using primers spanning the intron/exon junctions of the mouse β-actin gene. The β-actin primers used were βACTfsw: 5’-CTCTTCTGGGTATGAATC- 3’ and βACTrev: 5’-GGAGCAATGATCTTGATCT-3’. Single melt curves and agarose gel electrophoresis revealed a 200 bp product in both DNase-treated and -untreated samples confirming the specificity of the primers (data not shown).

For H5N1-infected mice, RNA was extracted from the lungs and tested by real-time RT-PCR as described previously (Alsharifi et al., 2009). All samples were analysed in triplicate and included test for 18S rRNA to exclude possible PCR inhibition. For relative quantification of viral RNA, a standard curve was generated using 10-fold serial dilutions of a cDNA template generated from virus stock. For data analysis, 1 U of viral RNA was arbitrarily defined as the number of RNA molecules which, when reverse transcribed and subjected to real-time PCR, produced a Ct value of 36.

**Immunofluorescence of MDCK cells.** Confluent monolayers of MDCK cells were infected with 9 × 107 TCID50 X31, without trypsin for 1 h, then incubated for 24, 48 and 72 h prior to fixation with 95 % (v/v) methanol in water. The m.o.i. was estimated as 0.18. All antiserums were used at 1 : 100 dilution and cell monolayers were stained either pooled normal mouse serum or pooled mouse anti-H3 HA peptide sera. Rabbit anti-influenza A was used as a positive control (Kok et al., 1994). Bound antibodies were detected with either anti-mouse or anti-rabbit Alexa 488-conjugated antibodies.

**Statistical analysis.** To compare the period taken for 20 % weight loss with different treatments, Kaplan–Meier survival curves and log rank tests were used. For comparison of PCR results, independent sample t-tests were performed using SAS Version 9.1 (SAS Institute Inc., Cary, NC, USA).

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