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Kaleb A. Reese for the degree of Master of Science in Microbiology presented on September 9, 2011.
Title: Development of Mucosal Vaccines for Avian Influenza A Virus Composed of Recombinant *Lactococcus lactis* and *Streptococcus gordonii*

Abstract approved:

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Bruce L. Geller

Novel mucosal vaccines (LL-M2e, LL-HAe, SG-HAe) were constructed from live, non-pathogenic *Lactococcus lactis* or *Streptococcus gordonii* that express conserved regions of HA or M2 antigens from avian influenza virus (AIV) A. All three vaccines evoked antigen-specific serum immunoglobulin G (IgG) responses in vaccinated chickens. The addition of the adjuvant cholera toxin B (CTB) increased the antigen specific IgG response. Fecal IgA and cell mediated immune responses were not detectable in the chickens. LL-M2e provided significant protection against lethal challenge compared to mock-vaccinated chickens. None of the chickens vaccinated with the control strain (LL-PIP) or mock-vaccine survived lethal challenge past 5 days, whereas 2 of the 12 chickens vaccinated with LL-M2e survived at least 7 days post-infection. An M2e-specific serum IgG concentration of 1100 ng/ml was found to be the minimum required for increased protection against AIV infection. SG-HAe, but not LL-HAe, provided a significant increase in the number of chickens that survived lethal challenge compared to control-vaccinated birds. However, no correlation between HAe-specific serum IgG and survival was found.

Development of Mucosal Vaccines for Avian Influenza A Virus Composed of Recombinant *Lactococcus lactis* and *Streptococcus gordonii*

by

Kaleb A. Reese

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Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Kaleb A. Reese, Author

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TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

Development of mucosal vaccines for avian influenza A virus composed of recombinant *Lactococcus lactis* and *Streptococcus gordonii*

Chapter 1

INTRODUCTION

Avian influenza is a viral disease that affects all avian species. It is caused by the influenza A virus from the Orthomyxoviridae family, which can infect a range of hosts including horses, dogs, humans and birds. However, out of the three types of influenza virus (A, B, and C), birds are only infected by A (12, 97). The natural reservoir of influenza A virus is aquatic birds, particularly migratory ducks and geese (32, 33, 92). Poultry livestock can come into contact with these migratory birds and the virus can spread rapidly through an entire flock (42, 43).

US poultry is regarded worldwide as high quality meat and eggs. In 2010, the United States Department of Agriculture (USDA) reported the total value of United States poultry production was $34.7 billion, up 10% from 2009 (63). Poultry livestock includes turkeys and four types of chickens: fryers, roasters, broilers, and egg-layers. Most of the production comes from broilers and egg-layers ($23.7 billion and $6.52 billion, respectively) (63). Poultry are housed in one of several different methods including: free-range, yarding, and intensive chicken farming (28). Both the free-range and yarding methods allow the chickens to roam freely in the environment with and without other farm animals, respectively. On the other hand, intensive chicken farming restricts the chickens to an enclosed space with close proximity housing. This method is

the most economical and common approach to poultry housing. All three approaches are potentially at risk for outbreaks of disease (42, 43).

Numerous outbreaks of highly pathogenic diseases in poultry, such as Marek's disease, Newcastle disease, infectious bronchitis, and avian influenza virus (AIV), caused major losses of this economically valuable resource (10, 11, 38, 42, 43, 70, 71, 79). Large outbreaks do not occur frequently because of vaccination programs and industry regulations, but when they happen they are usually severe. For example, in 2003 a Newcastle disease outbreak, starting in California, spread to four states and caused an estimated economic impact of $5 billion (9). In another example, the specific highly pathogenic avian influenza virus (HPAIV) (A/chicken/PA/1370/83) H5N2, that was isolated from a 1983 outbreak in Pennsylvania, was responsible for the slaughter of 17 million chickens, and an estimated direct economic impact of $70 million (75). If an AIV outbreak were to occur presently, the overall estimated economic impact within just one major egg producing plant is $194.2 million (75). One reason for such high monetary losses is because the general method used to deal with an AIV outbreak is depopulation of an infected area. The article, entitled *Avian Influenza: What It Is and How to Protect Against Its Establishment*, from the West Virginia University, Extension Service, illustrates the impact of depopulation during an AIV outbreak:

> "Since affected farms have to be depopulated, cleaned, disinfected, and allowed to sit without birds until they test negative, farmers lose a lot of income. Consumers pay an increased price for eggs and meat because of reduced numbers of birds and reduced egg production. Trade embargoes from neighboring states and other countries result in a loss of jobs for individuals and revenue for farmers, companies, poultry-producing states, and the affected country. State and federal officials commit extra time, personnel, and money to help test flocks, depopulate farms, disinfect them, and institute quarantine procedures. All this costs money. The ultimate result is economic in terms of money spent to control and eradicate the disease, as well as actual loss of birds, eggs, and income for farmers, poultry companies, and allied industry". (22)

Currently there is no approved vaccine for avian influenza in the US. However, there are multiple vaccines for avian influenza in use in other countries where the disease has become endemic. For example, in Mexico, a recombinant live fowlpox virus-vectored vaccine, expressing the H5 antigen from AIV, is being used against H5N2 LPAIV (40, 48, 49, 70, 71). Vaccination programs in Egypt and Italy both use inactivated heterologous vaccines against H5N1 HPAIV and H7N1 LPAIV, respectively (70, 71). AIV vaccines in these countries can provide significant protection to the birds, but require injections that are labor-intensive and impractical on poultry farms containing millions of birds. Additionally, these AIV vaccines do not provide cross protection against multiple influenza subtypes. In the US, because the disease is not endemic and vaccination can cause difficulty in distinguishing infected birds from those that have been vaccinated, vaccination is not recommended. If there is an outbreak in the US, the logical strategy is to depopulate the affected birds in order to eliminate the disease.

Avian influenza is also a zoonotic disease, and therefore of concern for human health. Evidence suggests deadly outbreaks of influenza virus in humans over the last

century such as 1918, 1957, and 1968 have been caused by AIV jumping from avian species to humans, each outbreak caused by a different subtype H1N1, H2N2, and H3N2, respectively (84, 86, 88). Recently, outbreaks of H5N1 highly pathogenic avian influenza virus (HPAIV) have been reported in multiple countries causing 330 fatalities out of 564 total confirmed infections since 2003 (97). The cases were most prevalent in South East Asia due to direct contact people had with infected birds during food preparation and inside of their living areas. These conditions allow the virus ample opportunity to zoonotically transfer from birds to humans. Fortunately, H5N1 HPAIV has not been easily transmitted between humans (12). In contrast to South East Asia, direct contact with avian species in the United States is localized to poultry farms. Certain AIV subtypes, such as H5N2 HPAIV, are not able to infect humans through a cross-species infection. While H5N2 HPAIV only circulates within avian species, it is still a major concern for poultry farmers because it can spread rapidly, and cause high mortality rates in poultry stocks (32, 48, 49, 92).

This research effort is directed at developing a safe, cross-subtype protective, inexpensive, and easily administered recombinant bacterial vaccine against AIV for the poultry industry. Two Gram positive, non-pathogenic lactic acid bacteria (LAB), *Lactococcus lactis* and *Streptococcus gordonii*, have been engineered to express conserved antigens from AIV on their surface. Chickens were vaccinated with either an AIV antigen (HA or M2) expressing bacterial construct or a control vaccine, and lethally challenged with (A/chicken/PA/1370/83) H5N2 HPAIV. Humoral and cell mediated immunity (CMI) responses were analyzed and correlated with survival.

Chapter 2

LITERATURE REVIEW

Poultry Vaccines

Over the last century, vaccination of poultry has been a valuable preventative measure against bacterial and viral diseases that would otherwise cause major economic losses in the poultry industry (42, 43, 54, 76). One important vaccine is directed against Marek's disease, which is caused by a herpes virus that infects and replicates within lymphocytic vesicles, spreads to all tissues within the chicken, and causes tumors to develop (38, 78). The vaccine is made from attenuated strains of individual serotypes that protect poultry from highly pathogenic strains of herpes virus. The protective effect of the vaccine at the cellular and molecular level is still poorly understood, but it is an effective vaccine on a practical basis (38). The vaccine has proven to be economically important as well. Before a vaccine was found in 1970 for Marek's disease, this virus caused an estimated annual loss of $150 million in the United States alone (38). The cost of the vaccine is around $0.20 cents per bird (25). Therefore, it is much less expensive to vaccinate an entire flock than to lose the flock to the disease by depopulation and other containment measures.

Another poultry vaccine, is for Newcastle disease, which is a respiratory and neurological disease caused by an Avulavirus (1, 13, 79). Live attenuated and inactivated vaccines are effectively used to control the disease. In 2003, a Newcastle disease outbreak that started in California and spread to Nevada, Arizona, and Texas caused an estimated economic loss of $5 billion (9). Even with the cost of the vaccine around $1.00

per bird, the estimated cost-benefit ratio of disease outbreak to vaccination for a flock of 27,078 birds is 7.2 (62).

Another vaccine protects poultry from infectious bronchitis (IB), caused by a Coronavirus that targets the respiratory tract and causes viremia (10, 11, 46). Only after respiratory infection does it spread to the kidneys and uro-genital tracts. IB is most pathogenic in young chickens, and secondary infections from common bacteria, such as *Escherichia coli*, are usually the cause of mortality within infected birds. Live attenuated or inactivated vaccines can be used for 100% protection against specific subtypes (42, 54). However, this protective effect is often short-lived due to many different subtypes existing of IB strains, and chickens have to undergo multiple vaccinations 2-3 weeks apart. Additionally, because so many subtypes can exist, vaccines do not provide 100% protection against IB, and outbreaks continue to occur. Nonetheless, vaccines against IB have greatly minimized the incidence of infection and continue to be a potent preventative measure.

These vaccines have proven to be both protective and economic to maintain health of poultry flocks from frequently occurring pathogens. An AIV vaccine with similar protection and cost would be a great addition to the ones currently in use.

Avian Influenza

AIV is from the Orthomyxoviridae family, which is a group of viruses with negative-sense ssRNA genomes. AIV is part of the influenza A group, which is classified based on the amino acid composition of two specific surface proteins,

hemagglutinin (HA) and neuraminidase (NA). There are 16 HA subtypes and 9 NA subtypes. However, many variations of these surface proteins can exist and further classification includes the geographical location of the virus, the infected host from which it was isolated, its pathogenicity, and the date it was discovered. The AIV genome consists of 8 RNA segments that encode 11 proteins. Three of these segments encode HA, NA, and matrix ion channel (M2). These three proteins are found in the virus capsid, and all have domains that protrude from the capsid. Because the external domains are exposed to the immune system, they are thought to be the best candidates for a vaccine (19, 21, 27, 30, 31, 39, 52, 61). Each of the three segments plays an important role during AIV infection. HA and NA are responsible for either attachment to a specific receptor to gain entry into a cell or cleavage of the virus from the cell after replication, respectively (55, 85). Additionally, the M2 antigen acts as proton-specific ion channel that lowers the pH of the virion, so that initiation of virus uncoating and release of the viruses contents into the host cell can occur (8, 99).

The genetic sequence of HA and NA are in constant flux due to genetic drift and genetic shift, while M2 remains more conserved (7, 72, 82, 85). Genetic drift is the result of small point mutations occurring in specific genome segments. Alternatively, genetic shift occurs when different influenza subtypes co-infect a host and abruptly change the composition of their 8-segment genome by reassorting different segments from 2 different viruses. Genetic drift and shift both have the capability to make new subtypes of influenza virus, which can have dramatic effects on pathogenicity. An example of this occurs every year, when seasonal flu vaccines are altered to match the specific circulating

strains that changed over the course of a year (37, 85). In another example, the H1N1 influenza A virus that was responsible for the 1918 pandemic was an AIV that had genetically shifted after reassorting its 8-segments during co-infection of an avian species with 2 different subtypes (88). The reassortment shifted the host range to humans and provided the virulence for human to human transmission.

Our lab has chosen, for its vaccines, conserved regions within the external domains of the surface proteins HA (called HAe), and M2 (called M2e). The HA protein is cleaved into two peptides (HA1 and HA2) by cellular proteases located at infectious sites of AIV (14, 87). HA1 and HA2 come together by a disulphide bond to form a fusion peptide that is required for attachment and connection of the viral membrane to the host cell. A 12 amino acid (RGLFGAIAGFIE) region of HAe was selected that is 100% conserved in all subtypes (74). Moreover, the selected 12 amino acid sequence spans the HA1 and HA2 cleavage site, so that the HA2 sequence becomes the N-terminus external domain of AIV after proteolytic cleavage. For M2e, the sequence of the whole peptide was used. The 23 amino acid sequence (SLLTEVETPTRNGWECKCSDSSD) is conserved among all strains, with small differences in amino acid residues occurring in high pathogenic subtypes (Fig. 2.1). These sequences have been shown by others to provide protection from an infectious challenge (21, 27, 31, 52, 60, 61).

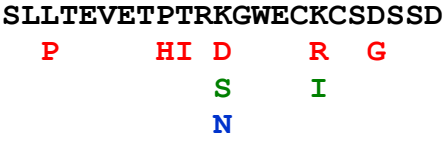

Fig. 2.1. M2e peptide sequence. H5N2 (black), H6N1 (red), H6N2 (green), H7N2 (blue) HPAIV subtype variations in amino acid residues are shown.

Avian Influenza Vaccines

An ideal vaccine must be safe, effective, shelf-stable, inexpensive, cross-subtype specific, easily administered, and protective with minimal inoculations (5, 51). However, these attributes appear elusive with current AIV vaccines, each having advantages and disadvantages associated with them. Some AIV vaccines are made of inactivated virus, while others contain live pathogenic viruses that have been attenuated and genetically engineered to express AIV-specific antigens. Each AIV vaccine is safe, effective, and cheap to make. However, all AIV vaccines are not shelf stable, easily administered, or effective with a single vaccination dose. Additionally, all of the AIV vaccines require subcutaneous injections, which can be both labor intensive and expensive on a poultry farm with millions of birds. The labor intensity and cost can be exacerbated even further in those AIV vaccines that require more than a single administration. The only current AIV vaccine that only requires a single dose for protection is a recombinant fowlpox vaccine expressing the H5 AIV antigen. As with many other vaccines, none of the current AIV vaccines are 100% effective and some animals may become sick even after vaccination. Therefore, vaccinated birds need to be monitored for viremia to effectively control AIV outbreaks (66, 70, 71).

Several countries have instituted vaccine programs for AIV, including Mexico (recombinant fowlpox virus vectored vaccine expressing H5 and a heterologous inactivated vaccine against H5N2 LPAIV) (40, 48, 49, 70, 71), Egypt (homologous inactivated vaccine against H5N1 HPAIV) (70, 71) and Italy (homologous inactivated vaccine against H7N1 HPAIV) (70). Each vaccine is specific to one influenza subtype.

The results have been mixed. Since initiating vaccinations in 1998, there have been no outbreaks of H5N2 LPAIV in Mexico (48). However, there have been outbreaks of AIV caused by other subtypes. Apparently, in Mexico, the vaccine is only protective against the one subtype, as predicted. In Egypt, the inactivated vaccine against H5N1 HPAIV seems to be effective at reducing outbreaks of this specific subtype (70, 71). However, there has been a breakdown in the management of Egypt's vaccination program. A small number of farms have never been given the government-funded vaccine, and some farms do not vaccinate their entire flock of birds. Therefore, the specific vaccine strain still persists along with other AIV subtypes.

A vaccine that is cross-protective against multiple influenza subtypes does not yet exist. Therefore, even if a country vaccinates their poultry stocks with the most prevalent influenza subtype, a new one can arise and cause a large economic loss. Other countries, including the United States, have yet to implement a poultry vaccination program because current AIV vaccines do not protect against multiple subtypes, and are difficult to administer.

The goal of this research program is to develop a cheap, shelf stable, easy to administer, and an effective cross-protective vaccine against multiple strains of AIV for the global poultry industry. A vaccine with these attributes would protect an economically valuable industry, as well as promote the health of both animals and humans.

Recombinant Bacterial Vaccine Systems

Lactococcus lactis and *Streptococcus gordonii* are Gram positive, non-spore forming, non-pathogenic, lactic acid bacteria (LAB) that are well studied for their ability to be used as recombinant bacteria (58, 73, 89, 95). The advantages to using these bacteria are that they are safe, effective at expressing high amounts of antigen, and they can induce mucosal immune responses (94). In addition to its use as a mucosal vaccine delivery vehicle, *L. lactis* has been used extensively in the food industry as a tool to produce fermentation products and a wide array of dairy products such as buttermilk and cheeses (35). *S. gordonii* is a human commensal bacterium that produces multiple antigenic proteins on its surface that help it adhere to and colonize the oral cavity, and in some cases reproductive sires. Neither bacterium can colonize the GI tract of endothermic animals, but they can remain metabolically active and survive passage through it (24, 45, 59).

Surface attachment of a foreign antigen to recombinant bacteria seems to evoke a better immune response than expressing the same foreign antigen in the cytoplasm or secreting it from the cell (50, 65, 95). Weak antigens, such as *Helicobacter pylori* urease subunit B, evoke a poor immune response when expressed in the cytoplasm of *L. lactis* and delivered by oral vaccination (50). Secreted proteins may lead to immune tolerance (15, 96). A direct comparison of surface attachment vs. cytoplasmic expression vs. secretion of tetanus toxin fragment C showed that the immune response (as measured by protective challenge) was 10-20 fold greater with surface-expressed antigen (65).

The addition of adjuvants to recombinant vaccines is known to significantly increase immune responses of vaccinated animals. Mucosally administered adjuvants are especially adept at significantly increasing IgA antibodies, compared to vaccines by themselves (2, 17, 90). One such mucosal adjuvant is the non-toxic B subunit of cholera toxin (CTB). Cholera toxin (CT) is a two-component toxin containing two peptide subunits called A and B. The B subunit binds to intestinal epithelial cells and opens up a channel for the A subunit to pass through. The A subunit acts as an ADP-ribosyltransferase enzyme that spikes cAMP levels and disrupts the ion balance of the intestinal cells, causing significant diarrhea. Therefore, CTB is a safe and effective adjuvant to use because it does not contain the A subunit and will not induce diarrhea, while still promoting an elevated mucosal humoral immune response. Furthermore, studies show CTB can induce an effective immune response. One study found that CTB-specific IgA antibodies could successfully protect mice from a lethal challenge of *Vibrio cholera* (90). CTB was not used as an adjuvant, but nevertheless was able to be recognized by the immune system and induce a mucosal immune response. Another study using an attenuated H5N1 intranasal vaccine co-administered with CTB was able to successfully protect mice from a lethal challenge of H5N1 HPAIV (2). When the attenuated vaccine was used by itself, no IgA antibodies were produced, and no protection was conferred upon the mice from lethal challenge. Therefore, it was deduced that CTB would be a good mucosal adjuvant for co-administration with the recombinant bacterial vaccines, used in this study, to increase the mucosal humoral immune responses.

L. lactis

There are many recent examples of *L. lactis* developed as an effective mucosal vaccine delivery vehicle (59, 77, 94, 95). In one study, the Pac antigen from *Streptococcus mutans* was expressed in *L. lactis,* and the molecularly cloned strains were administered intragastrically to mice (41). Significant Pac-specific peptide salivary IgA and serum IgG responses were induced, but no definable protection was seen. Another research group found that when *L. lactis,* expressing tetanus toxin fragment C was subcutaneously injected into mice, it evoked a protective systemic immune response against lethal challenge from tetanus toxin (94). However, mucosal delivery of *L. lactis* expressing the tetanus toxin fragment C produced a C-specific immune response in both the humoral and mucosal immune systems (65, 77). In addition, mucosal delivery of *L. lactis* gave 100% protection against a lethal challenge of tetanus toxin. Furthermore, mucosal delivery of the *L. lactis* vaccine produced a mucosal immune response at distal mucosal sites, as well as the site of vaccination. This confirms previously published reports that IgA-producing B cells stimulated by antigen in inductive sites, such as the Peyer's patches provide protection at all of the effector mucosal surfaces, such as the respiratory and intestinal mucosa (4).

Mucosally delivered *L. lactis* expressing viral antigens on its surface have been shown to evoke immune responses, and reduce viral load to provide protection (6, 98). For example, mice were vaccinated orally with *L. lactis* expressing an HIV envelope protein and this construct for this vaccine showed a 350-fold reduction of viral load after infectious challenge (98). In another study, mice were intranasally vaccinated with *L. lactis*

expressing E7 antigen of human papillomavirus, and this vaccine showed a significant reduction in tumor volume compared to mice vaccinated with *L. lactis* that did not express E7 antigen (6).

Many other examples exist of *L. lactis* vaccines carrying one or more different antigens including: glutathione S-transferase from *Schistosoma mansoni* (16), bovine rotavirus nonstructural protein 4 (26), VP2 and VP3 of infectious bursal disease virus (23), rotavirus vp7 antigen (69), and nucleocapsid protein of SARS-corona virus (68).

Our lab previously described an *L. lactis* mucosal vaccine that uses Pip, an endogenous plasma membrane anchored protein, as a carrier for the antigenic portion of the M6 protein from *Streptococcus pyrogenes* (53). The carboxy-terminus of Pip is amphipathic and anchors it to the plasma membrane, which leaves the amino terminus exposed on the surface of the bacteria. The external domain of the M6 protein was inserted into the amino terminus of the Pip protein allowing it to be exposed on the surface of *L. lactis*. The PipM6 gene fusion peptide is expressed from a high copy plasmid, pP16pip, using a very strong promoter (P16) originally isolated from *Lactobacillus acidophilus* (Fig 2.1) (36). The amount of the gene fusion peptide present within the cell, 7% of the total peptide concentration, was found to be comparable to expression levels found within other *L. lactis* vaccines necessary to produce effective mucosal immunity. Protective salivary IgA and serum IgG antibodies were produced in intranasally vaccinated mice, and promoted survival following an infectious challenge with the M14 *S. pyrogenes* strain. Additionally, it provided cross protection to a different serotype of the M protein.

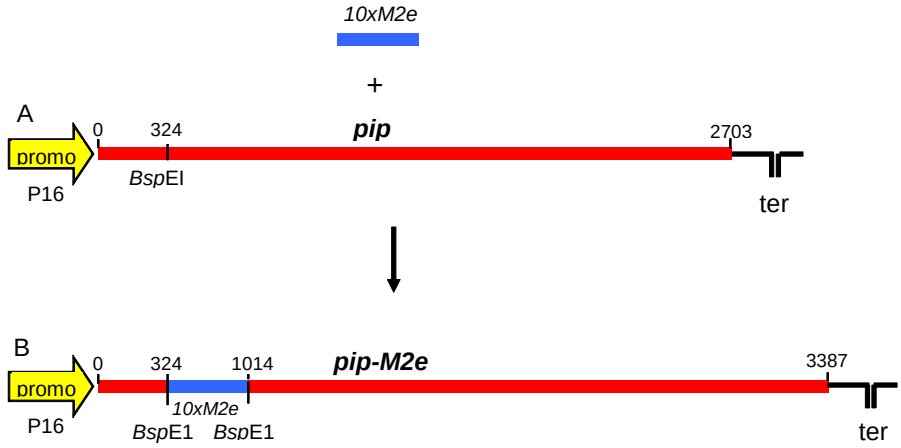

Fig. 2.2. Pip-M2e gene fusion peptide construction. A 10 tandem repeat of the coding region for M2e was cloned into the BspE1 restriction site of pip. Expression of *pip-M2e* from a strong promoter P16 forms the gene-fused peptide Pip-M2e.

S. gordonii

An *L. lactis* bacterial construct expressing a Pip-HAe gene fusion protein could not be made because of technical problems (consistently wrong orientation of the ten tandem repeat of HAe into Pip). Therefore, an alternate protein, M6 from *Streptococcus pyrogenes*, was used to carry HAe on the surface of our bacterial constructs. The M6 protein is native to the *Streptococcus* genus and so we chose to express an M6-HAe gene fused protein on the surface of *S. gordonii* in addition to *L. lactis.*

Like *L. lactis* there are many examples of *S. gordonii* being used as a mucosal vaccine delivery vehicle. In one study, *S. gordonii* was genetically engineered to express an antigen from simian immunodeficiency virus (SIV) called P27 on its surface (57). The peptide was found to induce $CD4^+$ T-cell response in the nasal associated lymphoid tissue (NALT) of mice. This showed that *S. gordonii* could induce both local and

systemic cellular and humoral immune responses. In another study, researchers expressed a surface adhesion protein from the pathogen *Porphyomonas gingivalis*, called FimA, on the surface of *S. gordonii* (80). Mice were intranasally vaccinated with the recombinant *S. gordonii*, and were found to have high levels of FimA antigen-specific protective salivary IgA and serum IgG antibodies. Furthermore, all vaccinated mice were 100% protected from alveolar tooth loss that is known to occur from *P. gingivales* infection. Another study, expressed a surface adhesion and virulence associated peptide from *Neisseria meningitides*, called NadA, on the surface of *S. gordonii* (20). NadA expressing *S. gordonii* vaccine was co-administered intranasally into mice with a mucosal adjuvant called LTR72, a partially inactivated mutant of *Escherichia coli* heat-labile enterotoxin. Both a local and systemic humoral immune response was induced. In addition, bactericidal activity was observed for the IgG antibodies produced.

S. gordonii has recently been used as the host for the pLEX expression system developed by SIGA technologies (Corvallis, OR) (91). The pLEX plasmid contains a number of features that make it a desirable expression vector (Fig. 2.1). pLEX is a high copy number plasmid that can be utilized in multiple Gram positive bacteria. pLEX also contains a selective marker for erythromycin resistance (*emm*). Furthermore, pLEX encodes for a strong promoter (*gtfG*), the promoters positive regulatory gene (*rgg*), and the *gtfG*-signal sequence just upstream of the M6 peptide. The pLEX specific M6 peptide is a surface protein native to *Streptococcus* species that enables surface adhesion to the oral cavity or reproductive sites. The M6 peptide encodes for a sortase enzyme allowing it to anchor to the cell wall. Additionally, the C-repeat region (CRR) is the

amino terminal domain of pLEX encoded M6, that has been shown to be highly immunogenic (29).

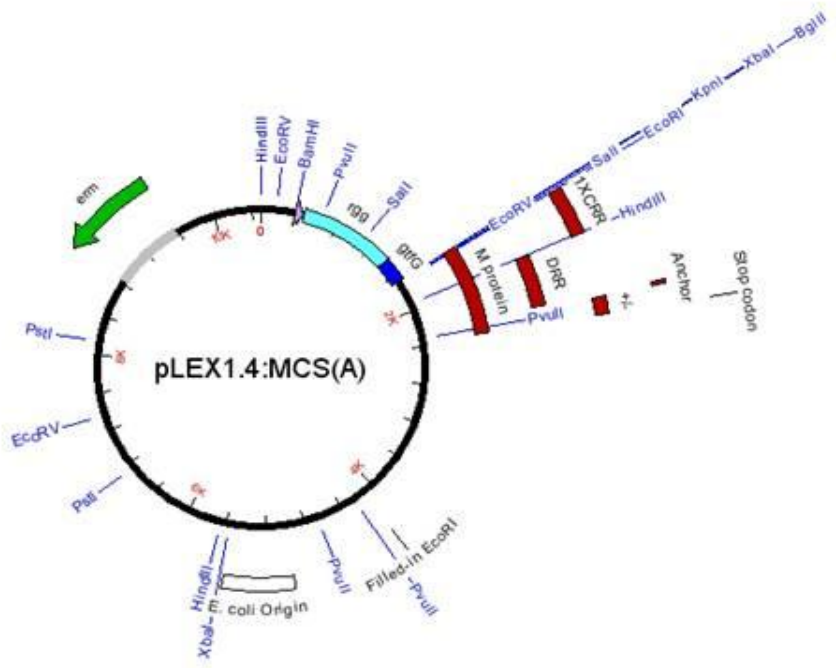

Fig. 2.3. pLEX plasmid map. Important features of the pLEX plasmid as an expression vector for making recombinant bacterial vaccines are: the erythromycin resistance gene, *emm* (in green), M6 protein (in red), the strong promoter for the M6 peptide, *gtfG* (in dark blue), and the positive regulator for the *gtfG* promoter, *rgg* (in light blue).

Chapter 3

MATERIALS AND METHODS

Construction of Recombinant Bacterial Strains

The DNA sequences coding for a 10 tandem repeat of the conserved peptide sequences for HAe and M2e (RGLFGAIAGFIE and SLLTEVETPTRNGWECKCSDSSD, respectively) were bought from Blue Heron Biotechnology (Bothell, WA). The coding region for M2e was molecularly cloned into the BspE1 restriction site of pP16pip as described previously (36). The coding region of HAe was amplified by PCR from the *Escherichia coli* strain EC100 containing pUC19-10xHAe using the following primers: 10xHAe forward (5'-CGAAGCGAATTCCGTGGATTATTTGGTGCAATC-3' containing a restriction site for EcoRI), and 10xHAe reverse (5'-GGATGCGAATTCTTCGATAAAACCTGCAATTGCA-3' containing a restriction site for EcoRI). The amplified fragment encoding 10xHAe was digested with EcoRI and cloned in frame into the M6 protein-coding gene (*1xCRR*) on pLEX 1.4a (pLEX) (SIGA, Corvallis, OR) using the EcoRI restriction site making the plasmid pHAeM6. The pLEX expression system was previously described by (91). The plasmids pHAeM6 and pLEX were each used to transform *S. gordonii* GP204 (SIGA, Corvallis, OR) and *L. lactis* LM2301 competent cells to obtain the recombinant *S. gordonii* pHAeM6 (SG-HAe), and *L. lactis* pHAeM6 (LL-HAe) strains that express M6-HAe fusion proteins at the bacterial surface, or *S. gordonii* pLEX (SG-M6), *L. lactis* pLEX (LL-M6) strains that just express the M6 protein at the bacterial surface. Procedures for cloning in *E. coli*, transformation

of *L. lactis* and *S. gordonii*, and genetic analysis of transformants were performed as previously described (67).

Recombinant Bacterial Strains and Media

L. lactis

LL-HAe, LL-M2e, LL-M6, LL-Pip were grown as previously reported at 30°C in M17G with 5 µg/ml of erythromycin to an optical density of 0.8 measured at 600 nm wavelength (36). The cells were harvested by centrifugation, washed twice with sterile phosphate buffered saline (PBS), and resuspended in sterile PBS with 15% glycerol to a final concentration between 1 x 10^{11} and 8 x 10^{11} CFU/ml, and stored at -80°C.

S. gordonii

SG-HAe and SG-M6 were grown at 37°C in Todd-Hewitt broth with 1% yeast extract and 5 µg/ml erythromycin to an optical density of 0.8 measured at 600 nm wavelength. The cells were harvested by centrifugation, washed twice with sterile phosphate buffered saline (PBS), and resuspended in sterile PBS with 15% glycerol to a final concentration between 1 x 10^{10} and 7 x 10^{10} CFU/ml, and stored at -80°C.

Analysis of Recombinant Bacteria

Polyclonal antisera were made by injecting 1 mg (subcutaneously) KLH-peptide conjugates into New Zealand white rabbits. Each of the conjugates was prepared by coupling pure, synthetic M2e or HAe to KLH using a commercial kit (Thermo Scientific,

Rockford, IL). Titermax Gold was included as adjuvant. Rabbits were boosted twice at 2 week intervals and sera were collected 2 weeks after the final boost.

Where indicated, antibodies either obtained from another laboratory or commercially were used to analyze HAe expression. A polyclonal rabbit antibody was obtained commercially and is specific to the HA2 region of HA (Invitrogen, Carlsbad, CA). Two other polyclonal antibodies used to test expression to HAe (HA-49, and HI) were generously donated by Dr. Siga Samal (Virginia-Maryland Regional College of Veterinary Medicine, University of Maryland, College Park, MD). Both HA-49 and HI are specific to the whole H5 HA antigen (64).

For the detection of the expressed protein in an ELISA assay, frozen vaccine stocks were thawed, adjusted to an OD_{600} of 1.0 with PBS, and then serially diluted (1:2) in coating buffer (carbonate buffer 0.05 M, pH 9.6) starting with an OD_{600} of 1×10^{-2}. One hundred µl of each dilution was then added to wells of a 96-well OptiPlate -96F HB plate (PerkinElmer, Waltham, MA). Antigen-negative controls contained only coating buffer in the wells. The plate was incubated overnight at 4°C to promote binding. Wells were washed three times with ELISA wash buffer (PBS containing 0.05% (w/v) Tween 20; PBST) and then blocked with 200 µl/well PBST containing 1% Bovine Serum Albumin (BSA) and 0.05% (w/v) Sodium Azide; PBSTBA or PBSTB (without azide). After incubating for 2 hours at room temperature, wells were washed again prior to addition of antigen specific antisera (diluted 1 in 200 in blocking solution, 100 µl per well) and incubated overnight at 4°C. Wells were washed as described above to remove unbound antibody and then incubated for 2 hrs with goat anti rabbit HRP conjugate

antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (diluted 1 in 5000 in PBSTB). Wells were washed 3x with PBST and then 3x with PBS. SuperSignal ELISA Pico Chemi-luminscent Substrate (Thermo Scientific, Rockford, IL) was then added at 50 µl per well. Plates were then analyzed for luminescence using a TECAN infinite F500 multifunctional plate reader (TECAN, Männedorf, Switzerland).

Immunization Protocol

Frozen stocks of vaccine were thawed and diluted with PBS to of either 2×10^9 CFU/ml for *Streptococcus gordonii* vaccines or 2×10^{10} CFU/ml for *Lactococcus lactis* vaccines. Chickens (groups of 8 per vaccine) were vaccinated intranasally by transfusing into both nostrils on 3 consecutive days with 200 µl of either a mock vaccine containing PBS or one of the recombinant bacterial strains. This schedule was repeated beginning 14 and 28 days later. Fourteen days after the last vaccination, blood samples were collected. In addition to blood samples, fecal samples from individual birds were also collected from chickens in Trials 3 and 4.

Serum Collection and Preparation

Peripheral blood samples were collected on days 14 and 53 from a wing vein, left at room temperature (RT) for 2h, and then centrifuged at 8,000 x *g* for 7 min. The serum was separated and stored at -20°C. Fecal samples were collected, diluted into 300 µl of fecal processing solution (0.5% bovine serum albumin, 0.02% NaN3, and 1x complete

protease inhibitor [Boehringer, Mannheim, Germany] in PBS), mixed, centrifuged (10,000 x *g*, 5 min), and stored at -20°C.

Fecal IgA and Serum IgG ELISA

M2e and HAe-specific IgA in fecal samples and IgG in serum were quantified by enzyme-linked immunosorbent assay (ELISA) as described previously (3, 56). Black, 96-well OptiPlate – 96F HB microplates (PerkinElmer, Waltham, MA) were coated with 2µg/ml purified, H5N2 M2e or conserved HAe protein. Fecal and peripheral serum was applied at 3 dilutions: 1:25, 1:50, and 1:100. The dilution that gave the highest specific signal that was different from background was used to calculate the concentration of antigen-specific antibodies. Standard curves were established on every plate with use of a twofold dilution series of Chicken IgA or Chicken IgG (Sigma Chemical Co., St. Louis, MO.). Goat anti-chicken IgA (Sigma) and IgG (Bethyl, Montgomery, TX.) conjugated to horseradish peroxidase and chemiluminescent substrate (SuperSignal ELISA; Pierce, Rockford, IL.) were used to develop the signal, which was read in a TECAN Infinite F500 Microplate reader (Männedorf, Switzerland). Concentrations of M2e and HAe-specific IgA and IgG were extrapolated from the standard curves.

Lymphocyte Proliferation Assay

The peripheral lymphocyte proliferation assay was performed as described previously (81) from peripheral blood samples taken 14 days after the final vaccination. Carboxyfluorescein diacetate succinimidyl ester (CFSE) was allowed to stain the isolated

and washed lymphocytes for 5 minutes at room temperature. CFSE staining reaction was quenched with 20% CS (w/v), and washed 3 times with RPMI-CS. Briefly, assays were established at 2 x 10^5 lymphocytes/well in V-bottomed 96-well microtiter tissue culture plates and co-cultured with RPMI 1640 supplemented with 10% Chicken serum (CS), 2mM L-Glutamine, 10,000 units of Penicillin, 10,000 units of Streptomycin, and 25 µg/ml Amphotericin B (RPMI-CS) (all supplements obtained from Invitrogen, Carlsbad, CA). Wells were then stimulated with either 20 µg/ml AIV specific peptide, 20µg/ml non-specific peptide, 10 µg/ml concanavalin A (ConA) (Sigma-Aldrich, St. Louis, MO), or RPMI-CS alone in a final volume of 150 µl/well. Microtiter plates were incubated at 39°C in an atmosphere of 5% CO_2 in air for 72Hrs. Proliferated lymphocytes were then isolated from RPMI-CS and resuspended in mouse-anti-chicken CD4-RPE (R-Phycoerythrin) clone CT-4 (Thermo Scientific, Rockford, IL). Samples were washed twice and resuspended in 300 µl PBS. Lymphocytes were then sent to Fred Schnell at AVI Biopharma inc. to be read on a Flow cytometer Cytomics FC 500 (Becton Dickinson, Franklin Lakes, NJ) and analyzed with FlowJo flow cytometry analysis software (Tree Star, Ashland, OR).

Challenge of Vaccinated Chickens (CL egg preps) (CDC Regulatory info)

Vaccinated chickens (groups of 6) were challenged with highly pathogenic (A/PA/1370/83) H5N2 influenza virus [1 x 10^4 Egg infective dose (EID)], by intranasal transfusion (100 µl) and intratracheal gavage (100 µl) in PBS. Birds were monitored daily for weight and body temperature. Food and water was available ad libitum. All

procedures involving animals were performed in compliance with federal and state laws and guidelines and approved by the Oregon State University Institutional Animal Care and Use Committee (approval no. 4063 and 4125).

Virus Titers in Tracheal swabs

Tracheal swabs were collected from AIV infected chickens on Days 3 or 5 post infection or day of death, and placed in 1ml of minimal essential medium (MEM) plus antibiotics and antimycotics and stored at -80°C. Madin-Darby canine kidney (MDCK) cells were bound 24 Hrs before infection to a 24 well cell culture plate to make a 90% confluent monolayer. The MDCK plates were washed twice with PBS and then inoculated with ten-fold serial dilutions of the tracheal swabs in duplicate and allowed to infect for 20 min at 37°C + 5% CO_2 with intermittent shaking. Negative and positive controls included medium only and a 10 fold serial dilutions of the H5N2 challenge strain. Infected cells were overlayed with 0.75 mls of 37°C fresh medium containing molten 1% low melting SeaPlaque agar (Lonza, Rockland, ME) and then continued to incubate for 72 Hrs. The plates were then fixed with 10% Formulin (PBS-Buffered) for 1 hr at room temperature. The formalin was aspirated and the agarose gel plugs removed. Plates were then stained with 0.05% Crystal Violet in 100% Methanol. After removing the stain, plaques were counted by visualizing plates in a variable light source, and PFU/ml was determined.

Statistical Analysis

Data were analyzed using GraphPad Prism software, version 4.0 (La Jolla, CA). The Mann-Whitney test was used to compare the mean serum IgG responses in the different treatment groups. Group means were calculated by including all individual values. Statistical significance was determined by Student's unpaired *t*-test. Simple linear regression and calculation of Pearson correlation coefficient were performed to assess the correlation between the antibody concentration and the survival time from the challenge. The significance level was set at $P < 0.05$ and $R^2 < 0.75$.

Chapter 4

RESULTS

Construction and Surface Expression Analysis of Recombinant Bacteria *L. lactis* expressing M2e and control strain

A highly conserved coding region for an externally protruding antigenic peptide of AIV, called M2e, was cloned into a plasmid expression system called pP16pip. This plasmid contains the coding region for a surface peptide of *L. lactis* called phage infection protein (Pip). M2e was inserted into a restriction site, BspEI, at the amino terminus of Pip, creating a gene fusion peptide known as Pip-M2e.

Expression of the Pip-M2e fusion protein on the surface of recombinant *L. lactis* was verified by whole-cell ELISA (Fig. 4.1). The amount of M2e detected on the surface of intact *L. lactis* that expresses M2e (LL-M2e) was proportional to the number of bacterial cells bound to the ELISA plate. ELISA analysis of the control strain that does not express M2e (LL-Pip) showed only non-specific, background signal. No M2e-specific expression was detected in either strain using pre-immune serum.

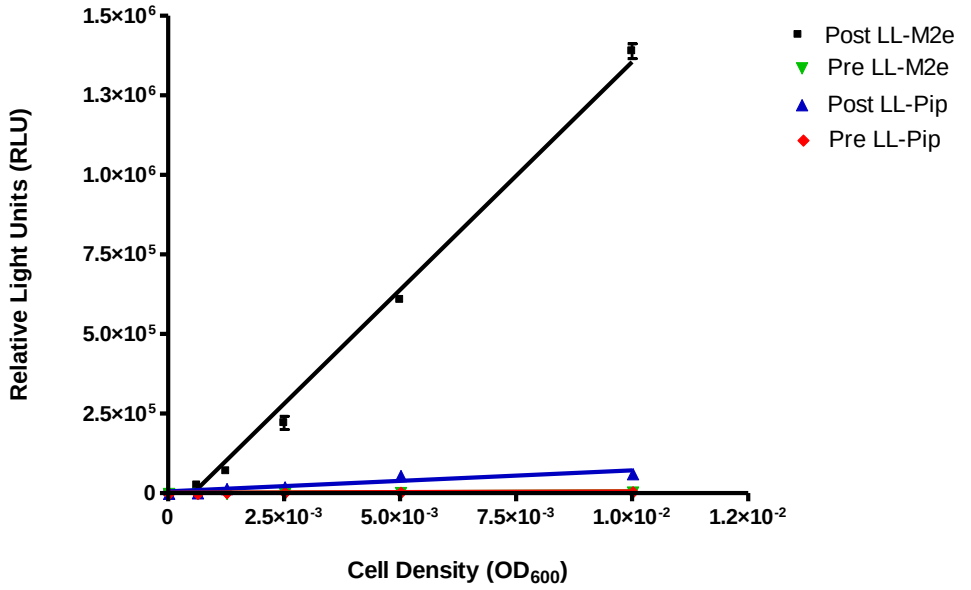

Fig. 4.1. Whole-cell ELISA analysis for M2e-specific surface expression. Pre- and Post-immune M2e-specific polyclonal antibodies were used to show surface expression of M2e in recombinant bacterial vaccine strains. LL-M2e (black squares and green inverted triangles) and LL-Pip (blue triangles and red diamonds) recombinant bacteria were measured by the relative light units given off by HRP conjugate enzymatic cleavage in commercial luminescent substrate. Standard deviation error bars were calculated for each data point, but in most cases were smaller than the size of the symbols used and may therefore, not be visible.

***L. lactis* and *S. gordonii* expressing HAe and control strains**

A recently developed plasmid expression system, called pLEX, was used to make recombinant *L. lactis* and *S. gordonii* vaccine strains. A highly conserved coding region for an externally protruding antigenic peptide of AIV, called HAe, was molecularly cloned into the coding region for the C-repeat region (CRR) of a truncated M6 surface peptide creating a gene fusion peptide known as M6-HAe.

The presence of the M6-HAe fusion protein on the surface of recombinant *L. lactis* and *S. gordonii* could not be verified by whole-cell ELISA (Fig. 4.2). *L. lactis* or *S.*

gordonii strains that expressed HAe (LL-HAe or SG-HAe, respectively) showed luminescent signals (relative light units [RLU]) that were proportional to the number of cells analyzed. Additionally Post-immune serum showed increased signal intensity compared to Pre-immune. However, the M6-HAe expressing vaccine constructs were not different from the control strains expressing only M6 (SG-M6 and LL-M6). An ELISA analysis of the antiserum used to detect HAe revealed that it did not recognize HAe peptide (Fig. 4.3). Three additional antibody and antiserum preparations specific for the whole H5 peptide protein or the HA2 domain were also tested, but none of them showed any HAe-specific binding to the vaccine strains.

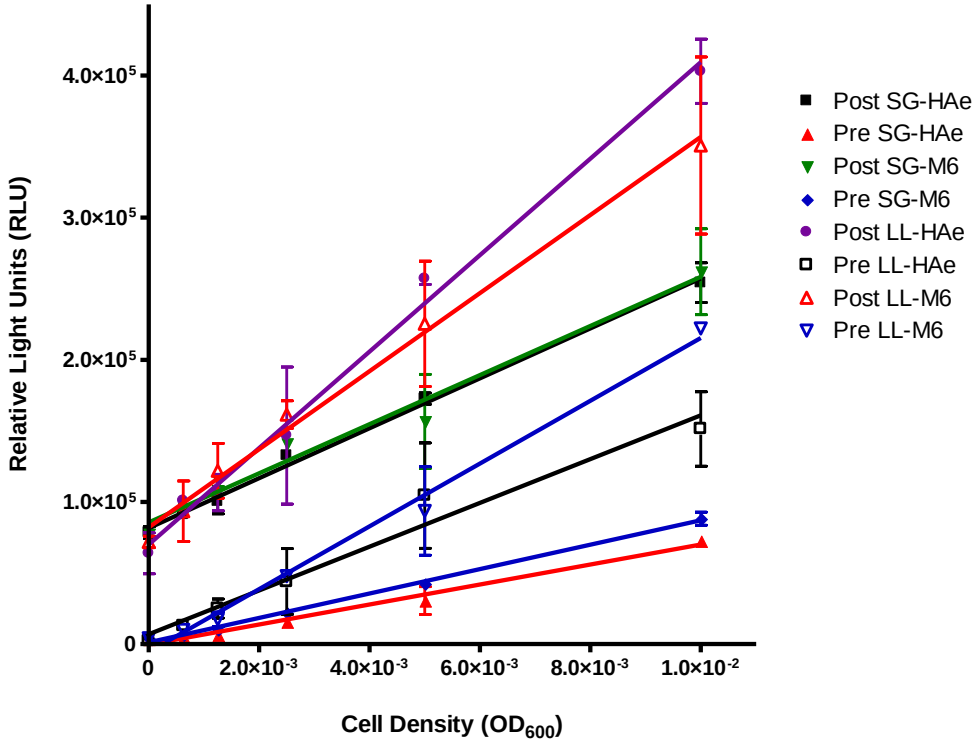

Fig. 4.2. Whole-cell ELISA analysis for HAe-specific surface expression. Pre- and Post-Immune HAe-specific polyclonal antibodies were used to show surface expression of HAe in recombinant bacterial vaccine strains. Recombinant bacteria were measured by the relative light units given off by HRP conjugate enzymatic cleavage in commercial luminescent substrate. Standard deviation error bars were calculated for each data point, but in most cases were smaller than the size of the symbols used and may therefore, not be visible.

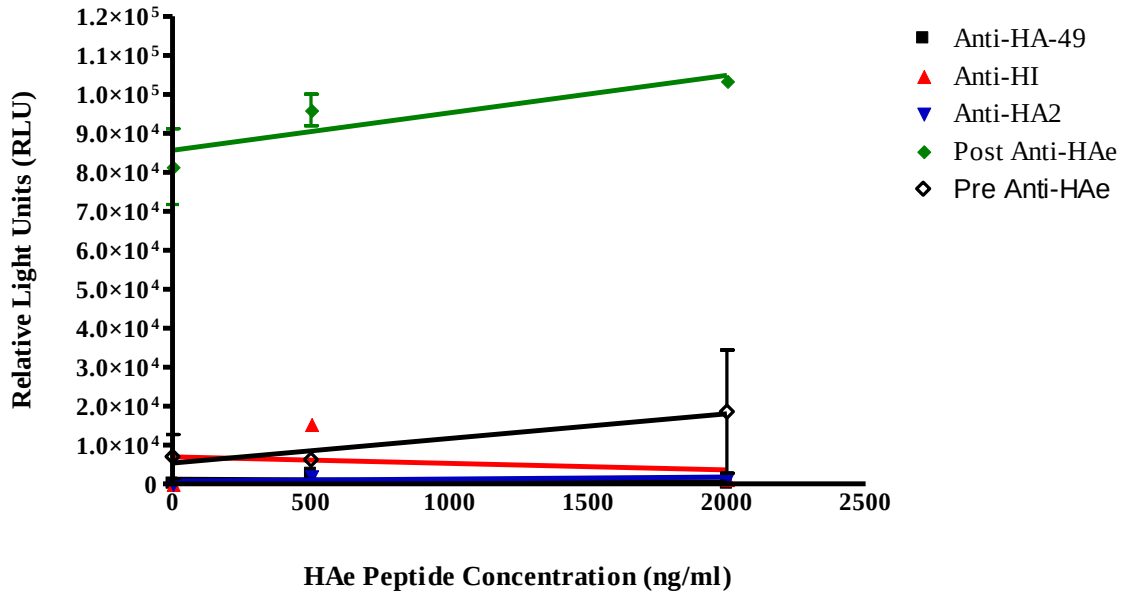

Fig. 4.3. ELISA analysis of various antibodies to HAe-specific peptide. Four different HA antibodies were tested for their ability to bind to our HAe peptide: Anti-HA-49, Anti-HI, Anti-HA2, and Anti-HAe. All antibodies are polyclonal and were either commercially obtained or made through specific peptide injection into rabbits. Specific antibody recognition of peptides was measured by relative light units. Standard deviation error bars were calculated for each data point, but in most cases were smaller than the size of the symbols used and may therefore, not be visible.

Trial 1: LL-M2e vs. Mock Vaccinated Chickens

Vaccination and Humoral Immune Response

Two groups of six, two-week old chickens were vaccinated intranasally with either LL-M2e or a control (mock) vaccine containing PBS. Vaccination was performed for a period of three days, and then repeated 14 and 28 days after initial vaccination. Two weeks after the final vaccination (about 60 days after initial vaccination), blood was collected and analyzed by ELISA for M2e-specific responses.

The results show that the group vaccinated with LL-M2e had a mean response of 160 ng/ml (Fig. 4.4). However, only one chicken had a considerable humoral response of 1104 ng/ml, and therefore the vaccine group had a significant standard deviation of 382 ng/ml. The mock-vaccinated treatment group had a mean response of 46 ng/ml. However, only three chickens in the mock vaccine group showed a response greater than 0 ng/ml. Therefore, the control group also had significant standard deviation of 71.2 ng/ml. The difference in response between these groups was not statistically significant ($P > 0.05$).

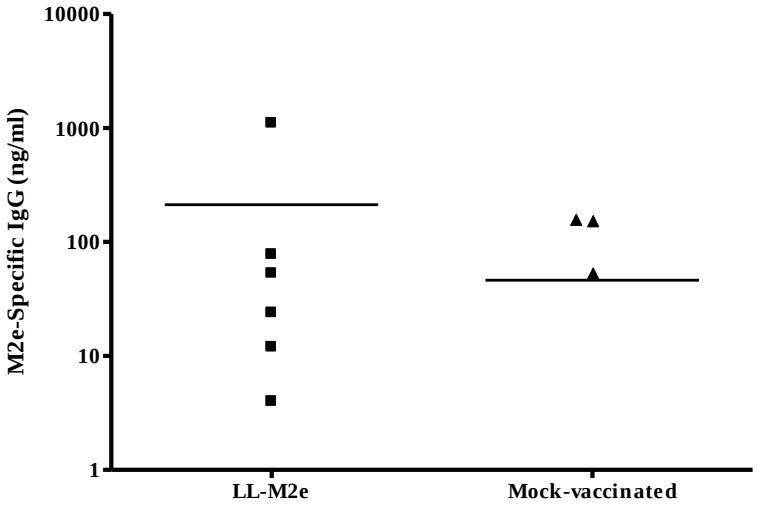

Fig. 4.4. Trial 1 serum IgG. M2e-specific IgG in peripheral blood serum was measured by ELISA. Vaccine groups were LL-M2e (black squares) or mock vaccinated (black triangles). No significant difference was seen ($P > 0.05$). * Chickens that had 0 ng/ml M2e-specific serum IgG, are not shown.

Lethal Challenge and Viral Titers

Two weeks after the last vaccination with either LL-M2e or the mock vaccine, all chickens were challenged nasally with a 10^4 egg infective dose (EID) of

(A/chicken/Pennsylvania/1370/1983) H5N2 HPAIV. Tracheal swabs were taken days 3 and 5 or day of deaths post-challenge. AIV infection was detected by performing a plaque assay. Viral titers (PFU/ml) were seen in all of the samples (Fig. 4.5). No significant difference was seen between LL-M2e and mock-vaccinated treatment groups from days 3 to 5. However, the last surviving bird in the trial, Yellow 22 (YL 22), had sequentially lower titers over the course of the challenge, and was almost undetectable on day 7.

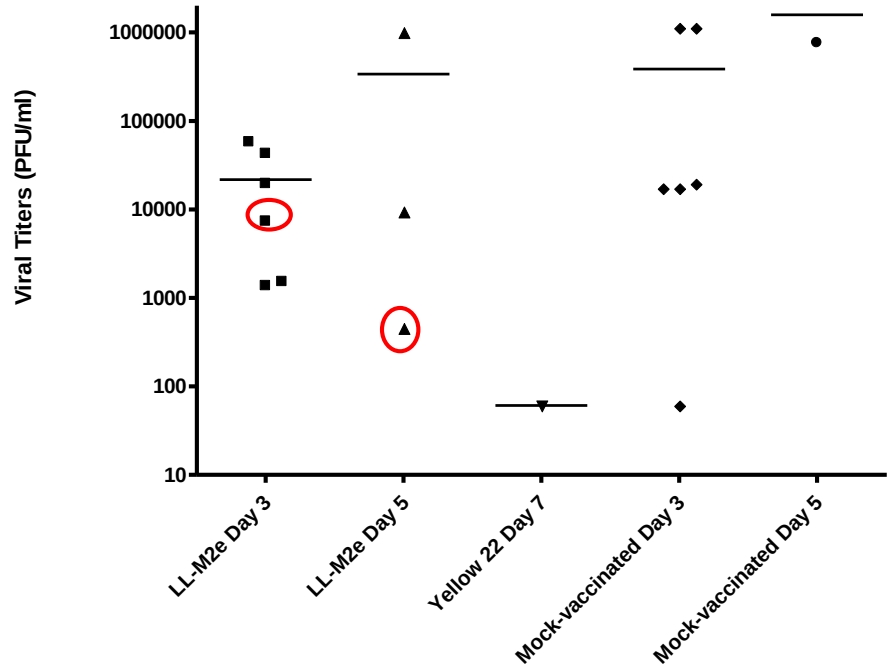

Fig. 4.5. Tracheal viral titer (PFU/ml) of Trial 1 treatment groups post-challenge. Tracheal swabs were taken days 3 and 5 or day of death. The chicken titers for YL 22 are circled in red. No significant difference was seen in LL-M2e vaccinated chickens between days 3 and 5 ($P > 0.05$). Additionally, no significant difference was observed between vaccine groups on the same day ($P > 0.05$).

Two chickens immunized with LL-M2e survived longer than the chickens treated with the mock vaccine that all died by day 6 of the challenge (Fig. 4.6). The LL-M2e vaccine conferred a longer median day of survival, compared to the mock vaccine group, by 1.5 days (5 days vs. 3.5 days, respectively). Additionally, group mean survival time of LL-M2e vaccinated birds was significantly greater ($P = 0.01$) than that of the mock vaccinated chickens. YL 22 was the longest surviving chicken, which was humanely euthanized on day 7 post infection. The weights and temperatures of all individual chickens were observed during lethal challenge. The weight of the chickens steadily decreased up until the day of death, including YL 22 (Fig. 4.7). The average temperature of all chickens initially rose 1.2°C from day of infection to 3 days post infection, and then sequentially lowered until day of death (Fig. 4.8).

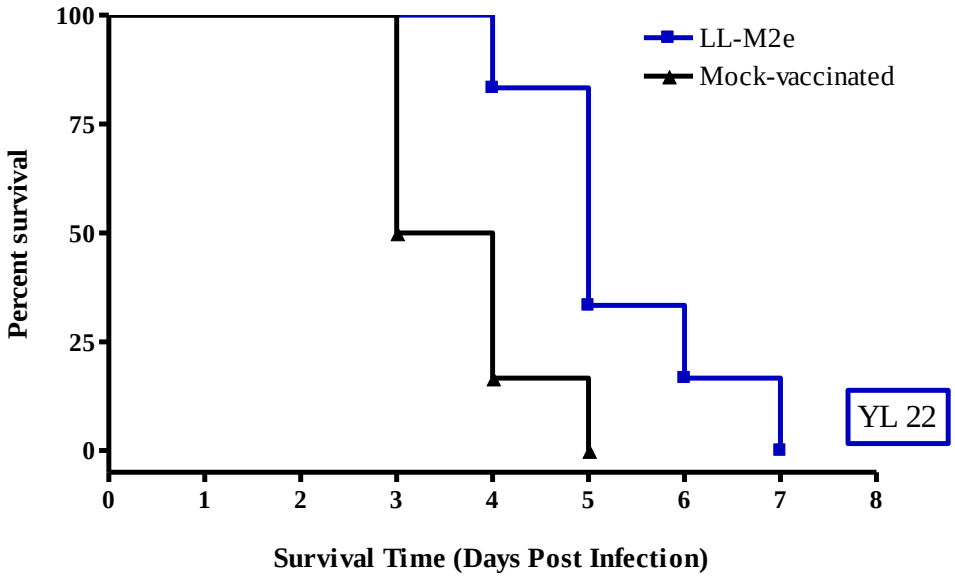

Fig. 4.6. Trial 1 survival curve. LL-M2e (blue) and mock vaccinated (black) vaccine groups were given a lethal challenge of (A/chicken/Pennsylvania/1370/1983) H5N2 HPAIV. 100% death in both vaccine groups occurred on day 7 of the trial.

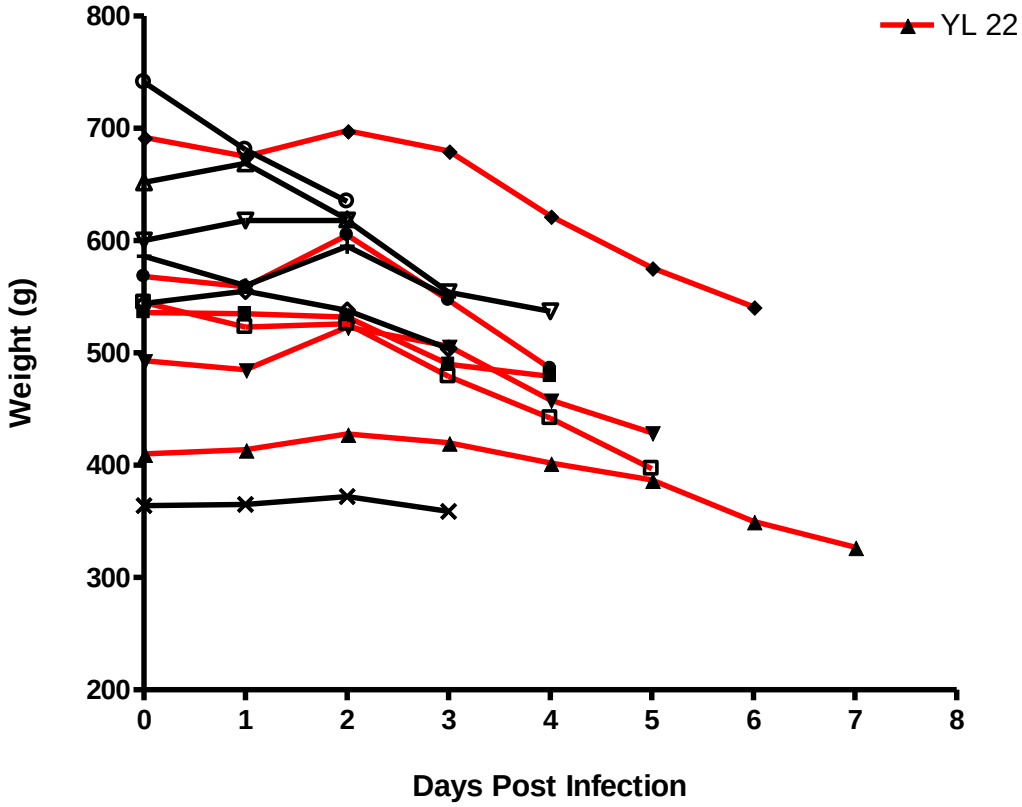

Fig. 4.7. Trial 1 distribution of weight (grams). Weights of all birds for each day post infection are shown for Trial 1. Each symbol denotes an individual chicken. LL-M2e symbols are connected by red lines and mock symbols by black lines. The surviving chicken, YL 22, is denoted by a black filled-in triangle.

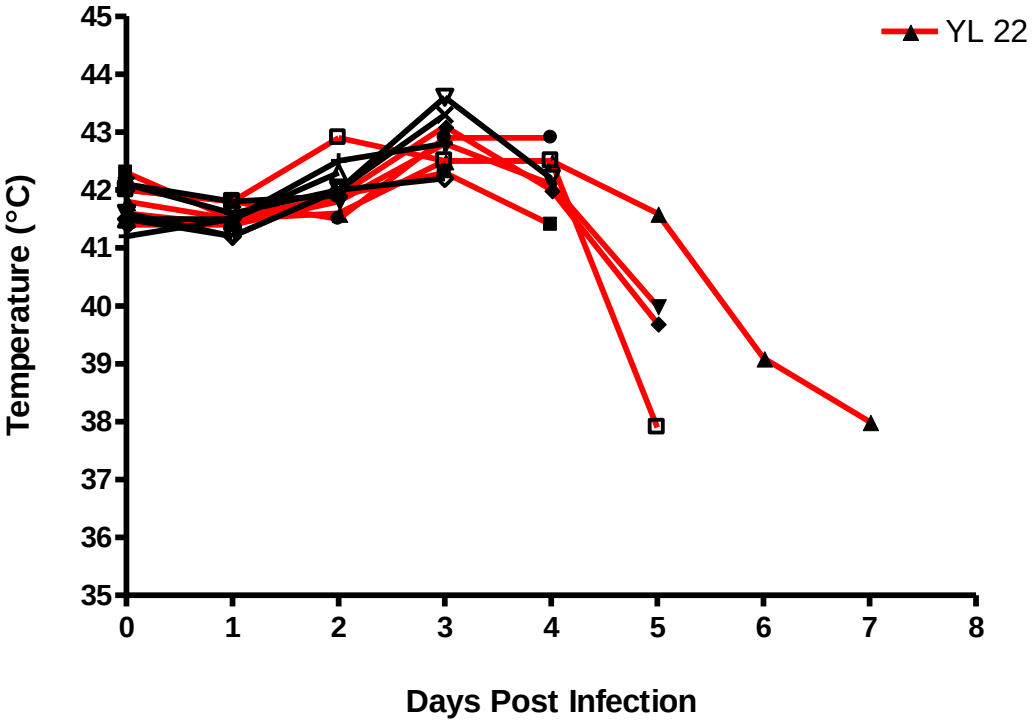

Fig. 4.8. Trial 1 distribution of body temperature (°C). Body temperatures of all birds for each day post infection are shown for Trial 1. Each symbol denotes an individual chicken. LL-M2e symbols are connected by red lines and mock symbols by black lines. The surviving chicken, YL 22, is denoted by a black filled-in triangle.

An analysis of all LL-M2e vaccinated chickens was calculated for a correlation between the M2e-specific serum IgG concentration and survival (Fig. 4.9). No significant correlation ($P = 0.056$, $R^2 = 0.64$) was found between the M2e-specific IgG concentration and survival. In fact, the second longest surviving chicken, YL 26, had 0 ng/ml M2e-specific IgG, while the longest surviving chicken, YL 22, had the highest M2e-specific serum IgG of both vaccine groups.

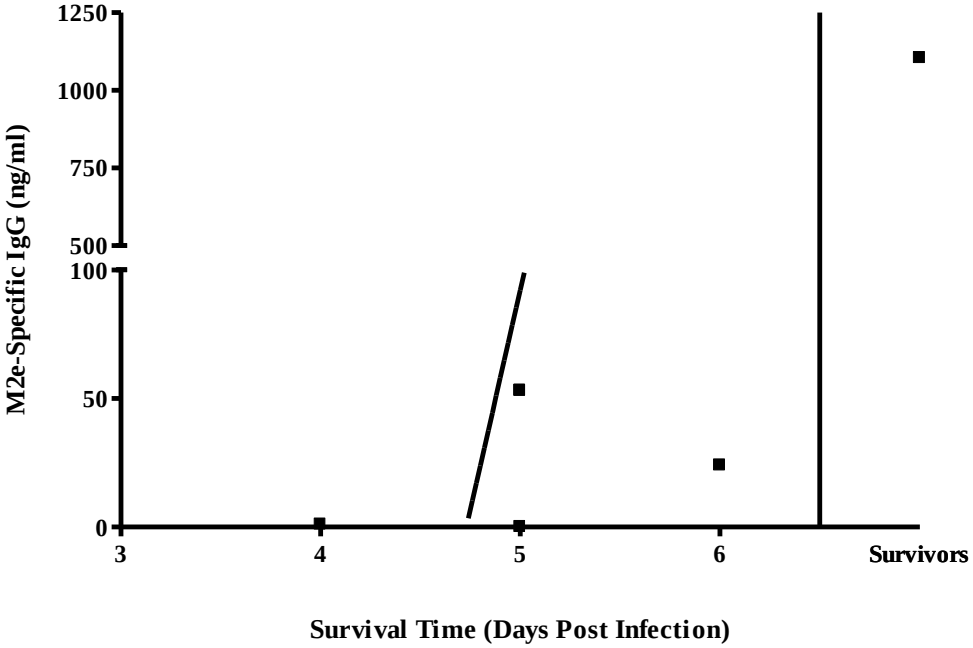

Fig. 4.9. Trial 1 correlation between LL-M2e elicited M2e-specific serum IgG and protection against lethal challenge. In the right panel the concentration of M2e-specific serum IgG in the chicken(s) that survived the challenge is reported; in the left, the direct correlation between the concentration of M2e-specific serum IgG and the survival time (days post infection) was found to have no significant difference ($P = 0.056$, $R^2 = 0.64$).

Trial 2: LL-HAe vs. SG-HAe

Vaccination and Humoral Response

The same vaccination strategy was used as Trial 1, except the treatment groups differed. One group (n = 7) was vaccinated with LL-HAe and the other group (n = 5) with SG-HAe. Additionally, the mock-vaccinated animals (n = 6) from Trial 1 were compared with LL-HAe and SG-HAe. Blood was collected and analyzed for HAe-specific IgG as before, but in addition, the cellular immune response was also measured.

HAe-specific serum IgG in birds vaccinated with LL-HAe had a mean response of 4.6 ng/ml (SD = 6.4), SG-HAe-vaccinated birds had a mean response of 1.8 ng/ml (SD = 3), and 0 ng/ml was found in mock-vaccinated chickens (Fig. 4.10). However, each HAe vaccine group had three chickens with 0 ng/ml humoral response. Furthermore, LL-HAe and SG-HAe did not show as much antigen specific titer response as Trial 1. The difference in response between the HAe vaccine groups was not statistically significant ($P > 0.05$). However, there was a significant difference between mock-vaccinated chickens and both LL-HAe and SG-HAe.

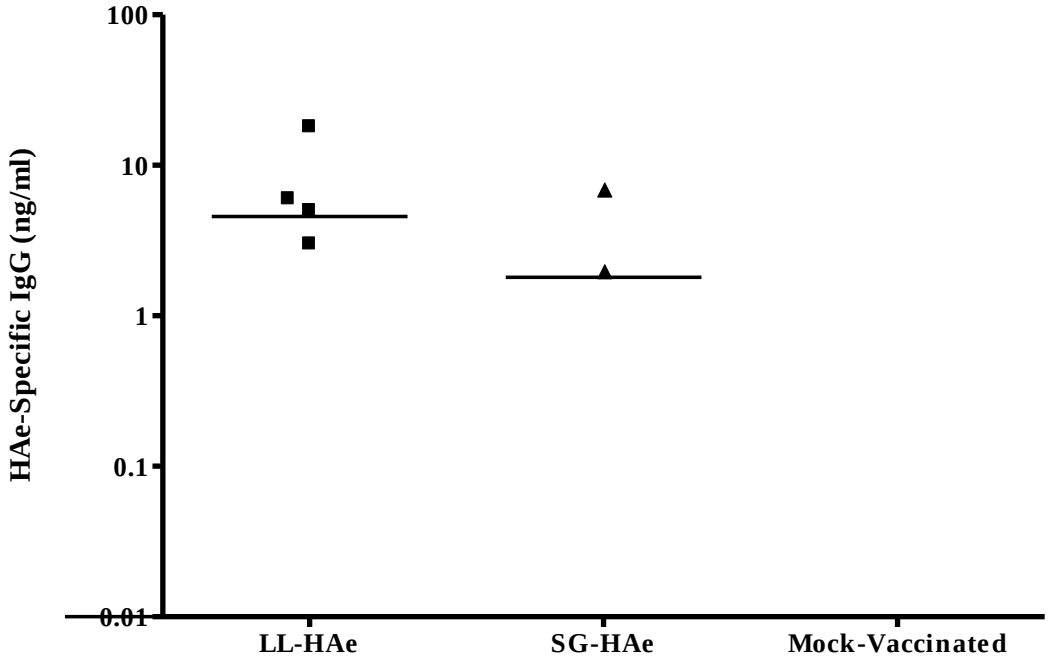

Fig. 4.10. Trial 2 serum IgG. HAe-specific IgG in peripheral blood serum was measured by ELISA. Treatment groups were LL-HAe (black squares), SG-HAe (black triangles), and Mock-vaccinated. A significant difference ($P < 0.05$) was seen between HAe vaccine groups and mock vaccinated. *Chickens that had 0 ng/ml HAe-specific serum IgG, are not shown.

Cell Mediated Immune Response

The mononuclear cell fraction from individual chickens of each treatment group was separated from red blood cells, platelets, antibodies, and other peripheral blood components. Peripheral lymphocytes were then stained with carboxyfluorescein diacetate succinimidyl ester (CFSE), a lipid bi-layer attaching stain, and incubated with either one of three different antigens, HAe-peptide, M2e-specific peptide, concanavlin A (Con A), or left un-stimulated. Additionally, lymphocytes were stained with RPE conjugated CD4 to isolate the T helper (TH) cell population during flow cytometric analysis. Con A stimulated TH cells underwent division as expected (Fig. 4.11). Fluorescent intensity was sequentially halved in each new generation due to dilution of CFSE stain with each division of the lipid bi-layer membrane. However, in each of the other stimulated samples, TH division was not observed. Furthermore, HAe peptide stimulated TH cells showed no difference in division compared to un-stimulated CD4 T-cells.

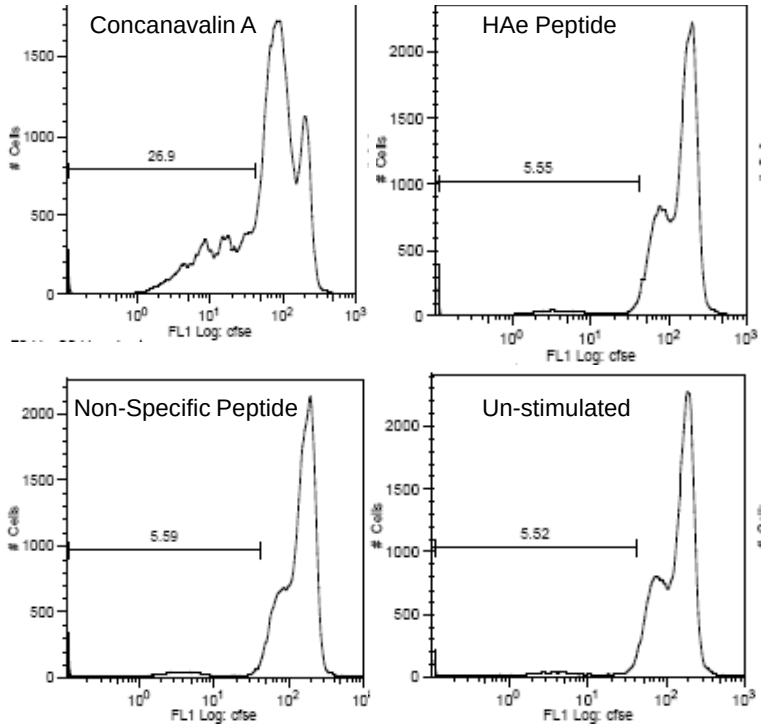

Fig. 4.11. Cell mediated immune response. Flow cytometric analysis of LL-HAe and SG-HAe treatment groups response to different antigens. Peripheral lymphocytes from each individual chicken were stained with CFSE and incubated separately with 3 different antigens, ConA, HAe peptide, Non-specific peptide, or left un-stimulated. Additionally, lymphocytes were stained with anti-chicken RPE conjugated CD4. No significant difference was seen between the HAe and non-specific stimulants or un-stimulated CD4 T-cell population.

Lethal Challenge and Viral Titers

Vaccinated birds were challenged as described above for trial 1, except the HPAIV was administered both intranasally and intratracheally. Viral load from tracheal swabs collected at days 3 and 5 post-infection indicated no significant difference ($P > 0.05$) between the LL-HAe and SG-HAe vaccinated chickens (Fig. 4.12). In addition, no significant difference ($P > 0.05$) was found between vaccinated and mock vaccinated birds. However, both LL-HAe and SG-HAe vaccinated chickens had lower

PFU/ml than mock vaccinated chickens on day 5. Furthermore, one of the birds that survived the trial had lower PFU/ml than 60% of the SG-HAe chickens, and had a significant reduction in viral titer from days 3 to 5 post infection.

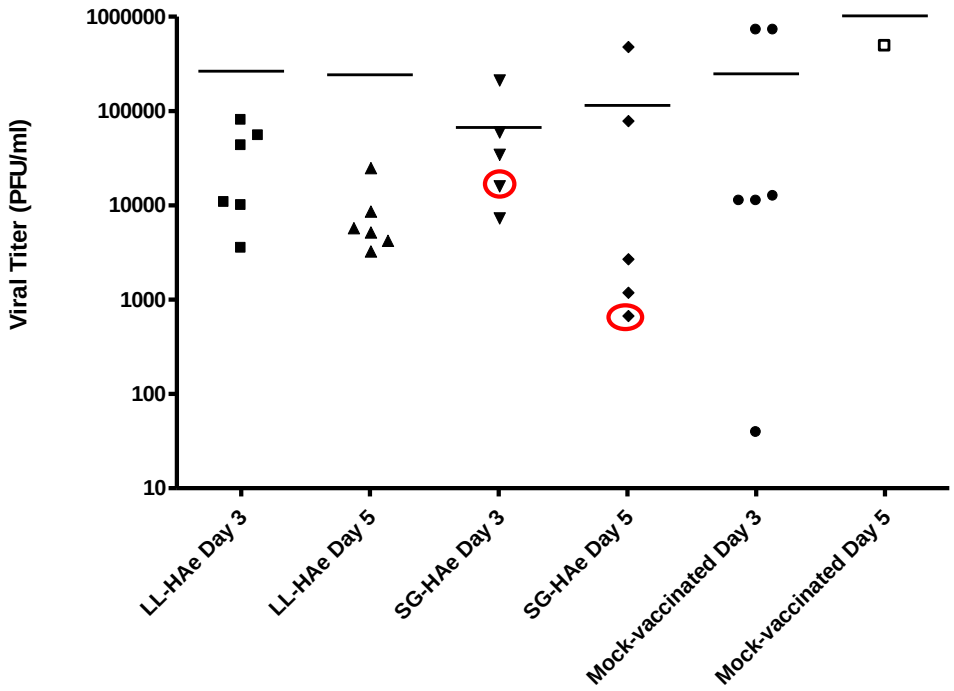

Fig. 4.12. Tracheal viral titer (PFU/ml) of Trial 2 vaccine groups post-challenge. Tracheal swabs were taken days 3 and 5 or day of death. The chicken titers for PU 20 are circled in red. No significant difference was found between vaccinated chickens in the same vaccine group between days 3 and 5 ($P > 0.05$). Additionally, no significant difference was found between the vaccine and mock vaccine groups on either days 3 or 5 ($P > 0.05$).

Survival showed that 100 % of both LL-HAe and mock-vaccinated birds died by day 6 post-infection, whereas 60 % of the SG-HAe-vaccinated birds died (Fig. 4.13). Both LL-HAe and SG-HAe similarly increased median days of survival, compared to the mock vaccine group, by 1.5 days (5 days vs. 3.5 days, respectively). In addition, the

group mean survival times of both LL-HAe and SG-HAe vaccinated chickens were significantly increased ($P = 0.01$ and $P = 0.02$, respectively) compared to mock vaccinated birds. The weights and body temperatures of each of the surviving birds, Purple 20 (PU 20) and Purple 23 (PU 23), decreased until 7 days post infection, and then either leveled off or increased until the end of the lethal challenge (Fig. 4.14, 4.15). All other individual chickens from both vaccine groups showed similar trends as observed in Trial 1.

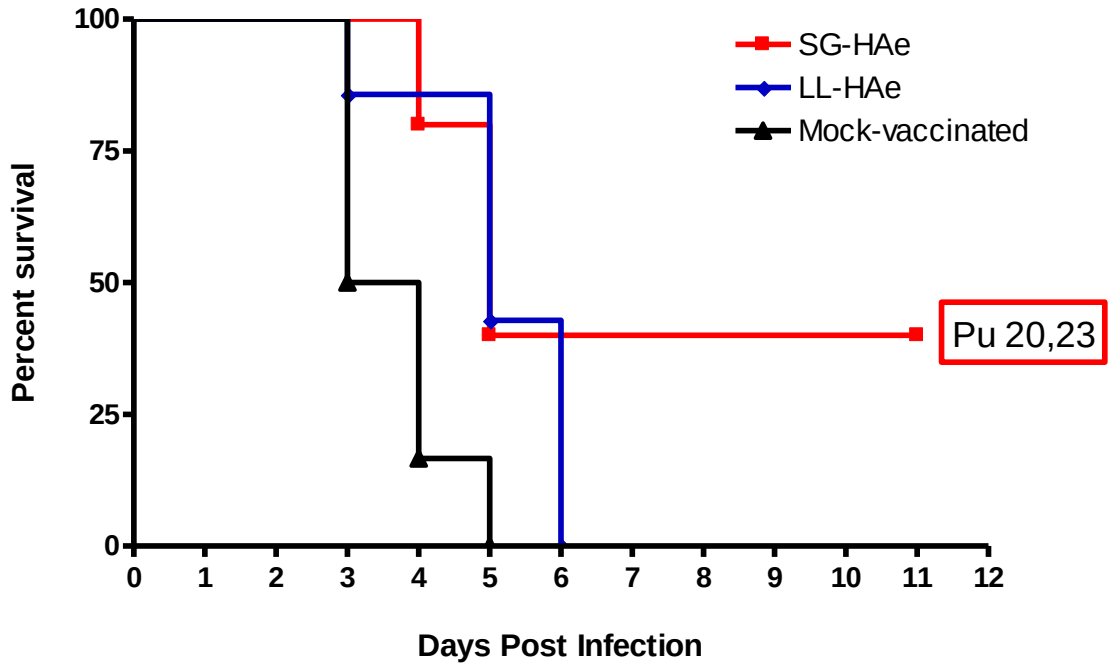

Fig. 4.13. Trial 2 survival curve. LL-HAe (blue), SG-HAe (red), and mock-vaccinated (black) vaccine groups were given a lethal challenge of (A/chicken/Pennsylvania/1370/1983) H5N2 HPAIV. 100% death of LL-HAe and mock vaccinated chickens occurred by day 6 of the trial, whereas 40% of the SG-HAe vaccine group survived.

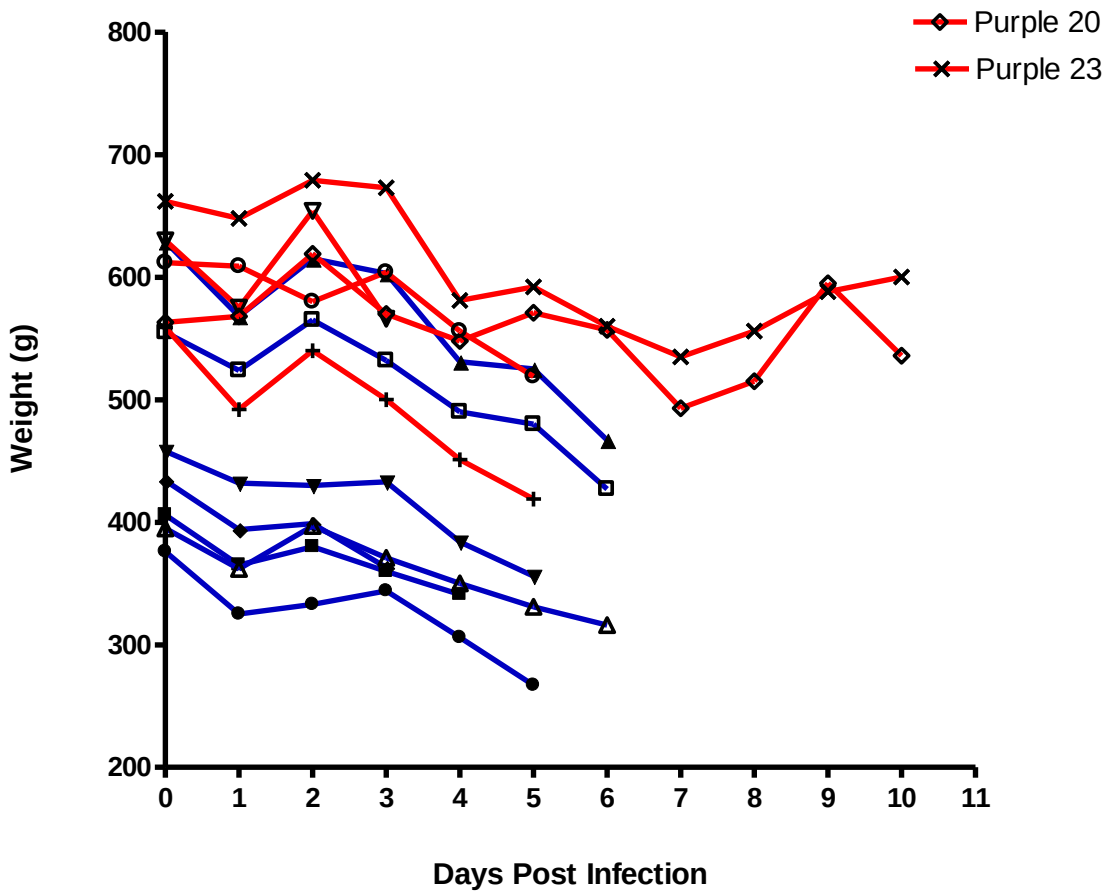

Fig. 4.14. Trial 2 distribution of weight (grams). Weights of all birds for each day post infection are shown for Trial 2. Each symbol denotes an individual chicken. LL-HAe symbols are connected by blue lines and SG-HAe symbols by red lines. The surviving chickens, PU 20 and PU 23, are denoted by a black diamond and a black X, respectively.

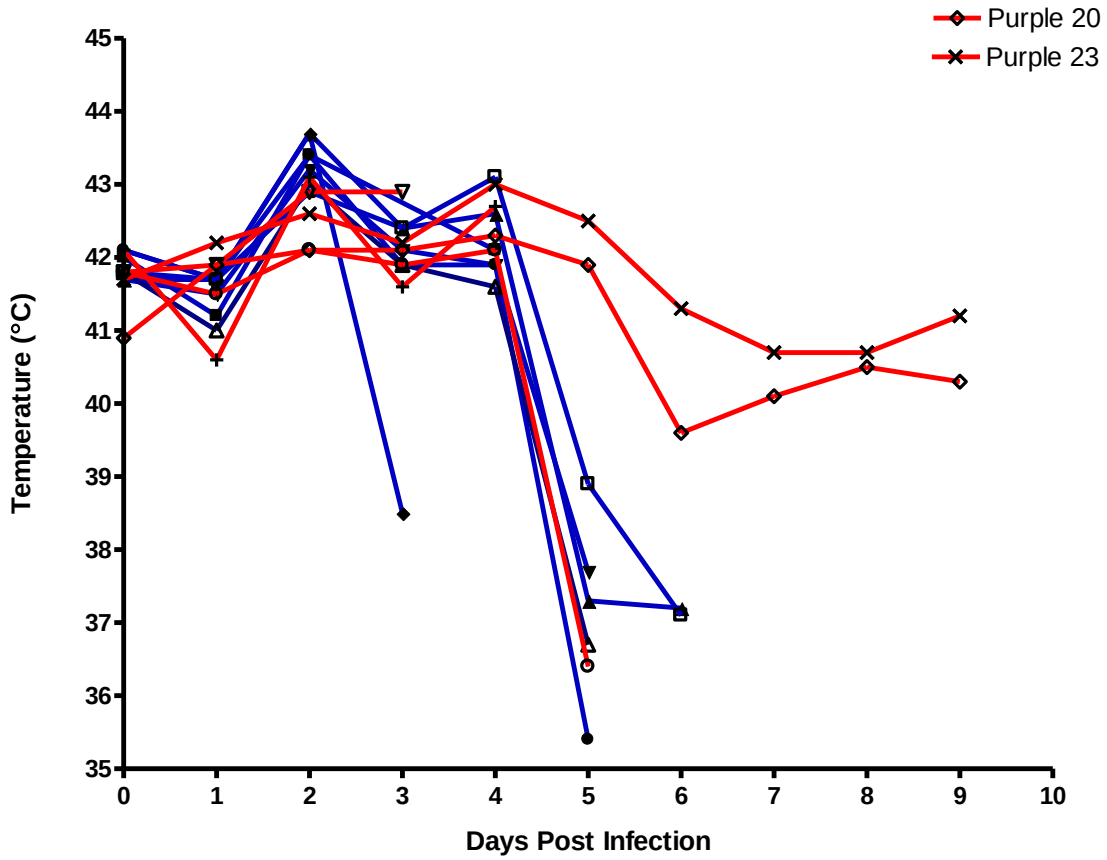

Fig. 4.15. Trial 2 distribution of body temperature (°C). Body temperatures of all birds for each day post infection are shown for Trial 2. Each symbol denotes an individual chicken. LL-HAe symbols are connected by blue lines and SG-HAe symbols by red lines. The surviving chickens, PU 20 and PU 23, are denoted by a black diamond and a black X, respectively.

No significant correlation ($P > 0.05$, $R^2 < 0.75$) was found between the HAe-specific serum IgG concentration and survival for either SG-HAe or LL-HAe (Figs. 4.16, 4.17). In fact, the bird with highest serum IgG from the LL-HAe vaccinated chickens was the first bird to die in the trial. Moreover, one of the birds that survived the challenge, PU 23, had 0 ng/ml IgG, while the other surviving bird, PU 20, had 7 ng/ml HAe-specific IgG.

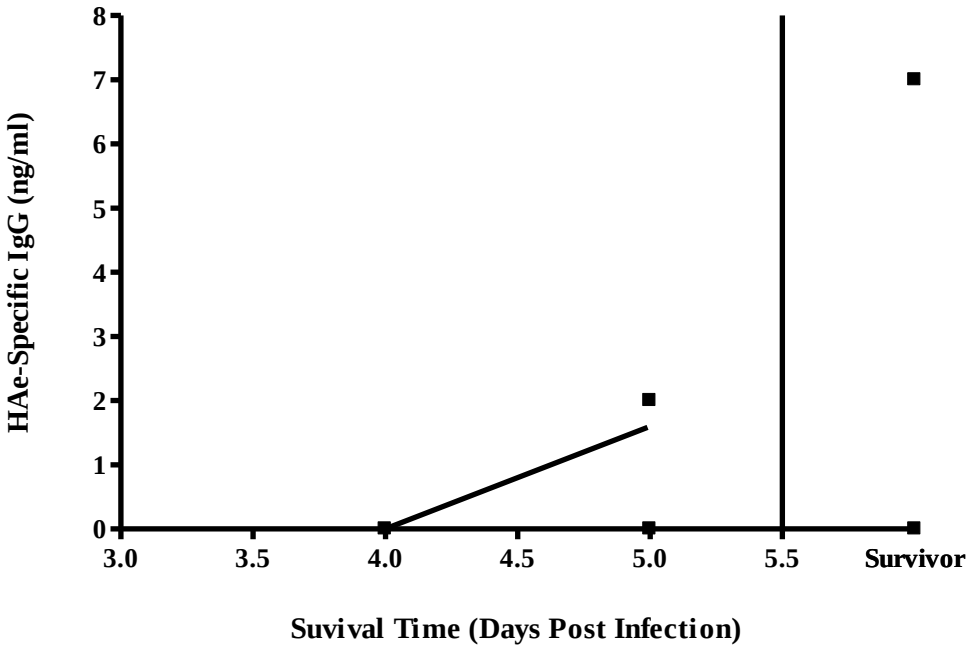

Fig. 4.16. Trial 2 correlation between SG-HAe elicited HAe-specific serum IgG and protection against lethal challenge. In the right panel the concentration of HAe-specific serum IgG in the chicken(s) that survived the challenge is reported; in the left, the direct correlation between the concentration of HAe-specific serum IgG and the survival time (days post infection) showed that no significant difference was found ($P > 0.05$, $R^2 < 0.75$).

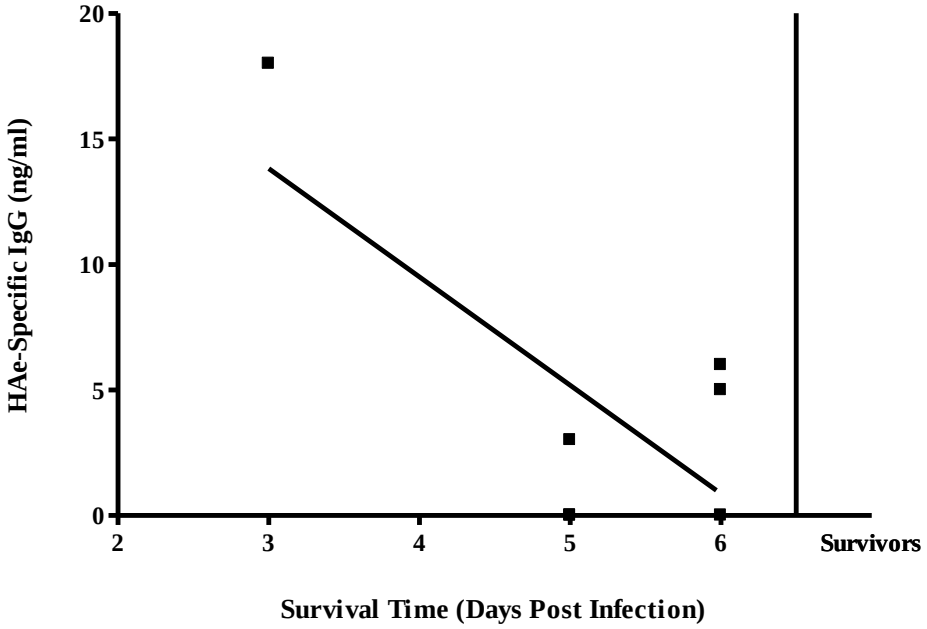

Fig. 4.17. Trial 2 correlation between LL-HAe elicited HAe-specific serum IgG and protection against lethal challenge. In the right panel the concentration of HAe-specific serum IgG in the chicken(s) that survived the challenge is reported; in the left, the direct correlation between the concentration of HAe-specific serum IgG and the survival time (days post infection) found that there was no significant difference ($P > 0.05$, $R^2 < 0.75$).

Trial 3: SG-HAe vs. SG-P

Vaccination and Humoral Immune Response

The same vaccination strategy was used as previous trials (n = 6 for both vaccine groups), except the vaccine groups differed, and an adjuvant, CTB, was co-administered with each vaccine. SG-HAe had evoked a consistently higher HAe-specific serum IgG than LL-HAe in Trial 2. Therefore, SG-HAe was tested against a control strain, SG-M6, in Trial 3. In addition, fecal matter was collected from each individual bird and analyzed for HAe-specific fecal IgA.

HAe-specific serum IgG was measured, and a greater humoral immune response was seen in both treatment groups compared to previous trials, possibly because of the adjuvant. However, SG-HAe had a mean response of 13.9 ng/ml (SD = 24.9), which was much lower than SG-M6 with a mean response of 102 ng/ml (SD = 172) (Fig. 4.18). Additionally, in each treatment group, 3 or more chickens had 0ng/ml HAe-specific antibodies. The difference in response between these groups was not statistically significant ($P > 0.05$). HAe-specific fecal IgA was undetectable in either vaccine group (data not shown).

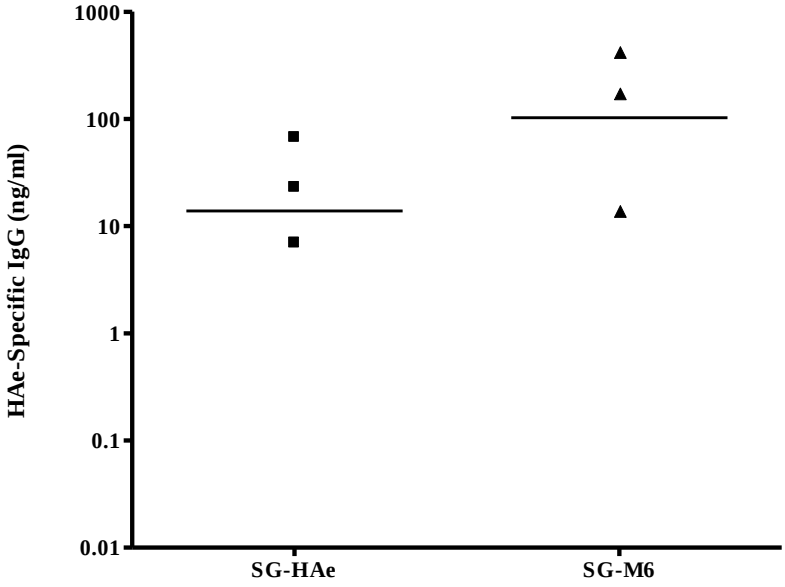

Fig. 4.18. Trial 3 serum IgG. HAe-specific IgG in peripheral blood serum was measured by ELISA. Treatment groups were SG-HAe (black squares) or SG-M6 (black triangles). No significant difference was seen ($P > 0.05$). *Chickens that had 0 ng/ml HAe-specific serum IgG, are not shown.

Cell Mediated Immune Responses

A cellular proliferation assay with the same antigen specific peptide stimulants and cellular stains was performed as in Trial 2. No significant difference in cell mediated immunity could be observed in either treatment group (data not shown).

Lethal Challenge and Viral Titers

Vaccinated birds were infected as described in trial 2. Viral load in tracheal swabs indicated titers (PFU/ml) in all of the samples, and no significant difference ($P > 0.05$) was seen between either tracheal swab days or vaccine groups (Fig. 4.19). The surviving birds, Blue 20 (BU 20) and Pink 40 (PI 40), from each treatment group had the lowest PFU/ml on day 5.

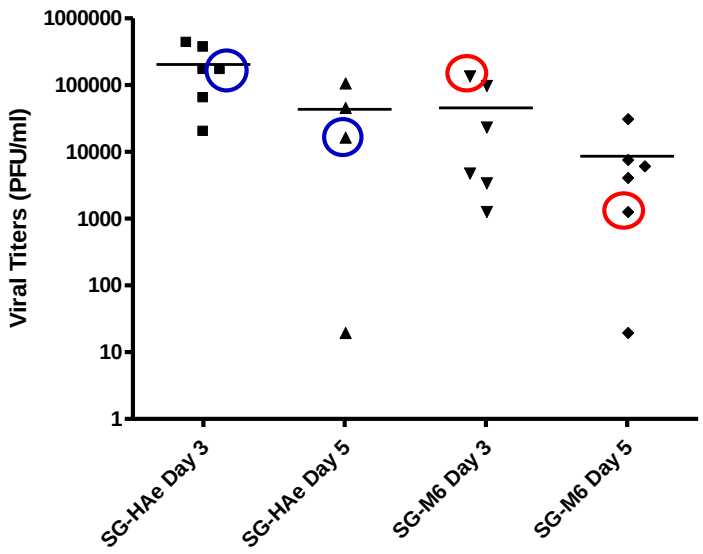

Fig. 4.19. Tracheal viral titer (PFU/ml) of Trial 3 treatment groups post-challenge. Tracheal swabs were taken days 3 and 5 or day of death. PFU/ml of BU 20 and PI 40 are circled in blue and red, respectively. No significant difference was seen in between vaccinated chickens in the same treatment group between days 3 and 5 ($P > 0.05$). Additionally, no significant difference was found between the treatment groups on either days 3 or 5 ($P > 0.05$).

One chicken from both SG-HAe and the control strain SG-M6 survived the infectious challenge, while the rest of each treatment group died on days 5 and 6, respectively (Fig. 4.20). The weights and body temperatures of each of the surviving birds, BU 20 and PI 40, decreased until 8 days post infection, and then either leveled off or increased until the end of the lethal challenge (Fig. 4.21, 4.22). All other individual chickens from both treatments groups showed similar trends as observed in previous Trials.

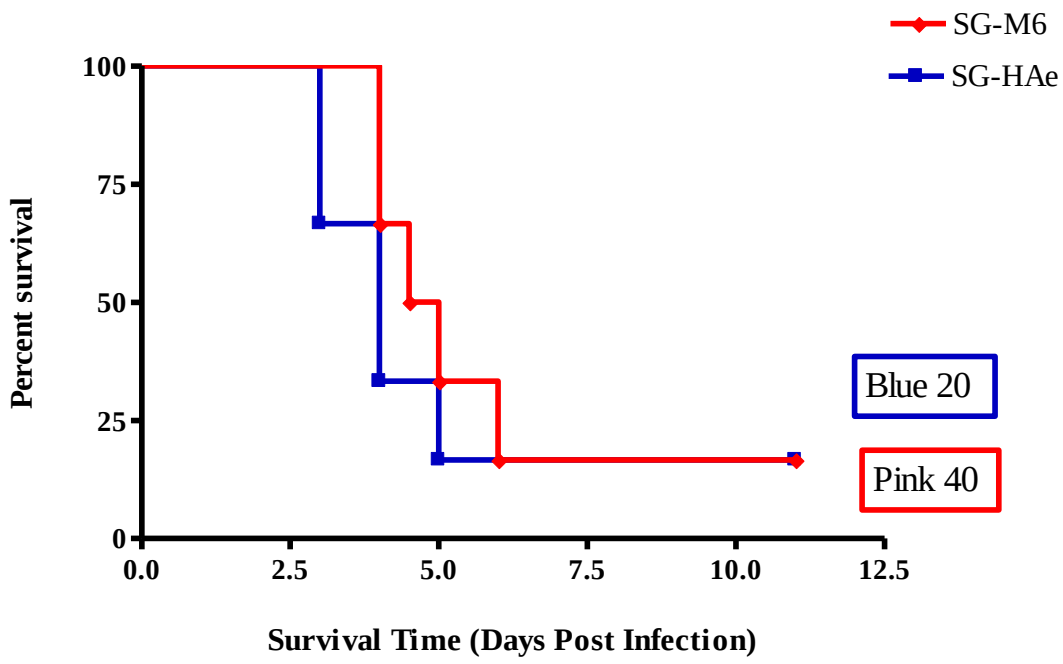

Fig. 4.20. Trial 3 survival curve. SG-HAe (blue) and SG-M6 (red) treatment groups were given a lethal challenge of (A/chicken/Pennsylvania/1370/1983) H5N2 HPAIV. One bird from each vaccine group survived the trial.

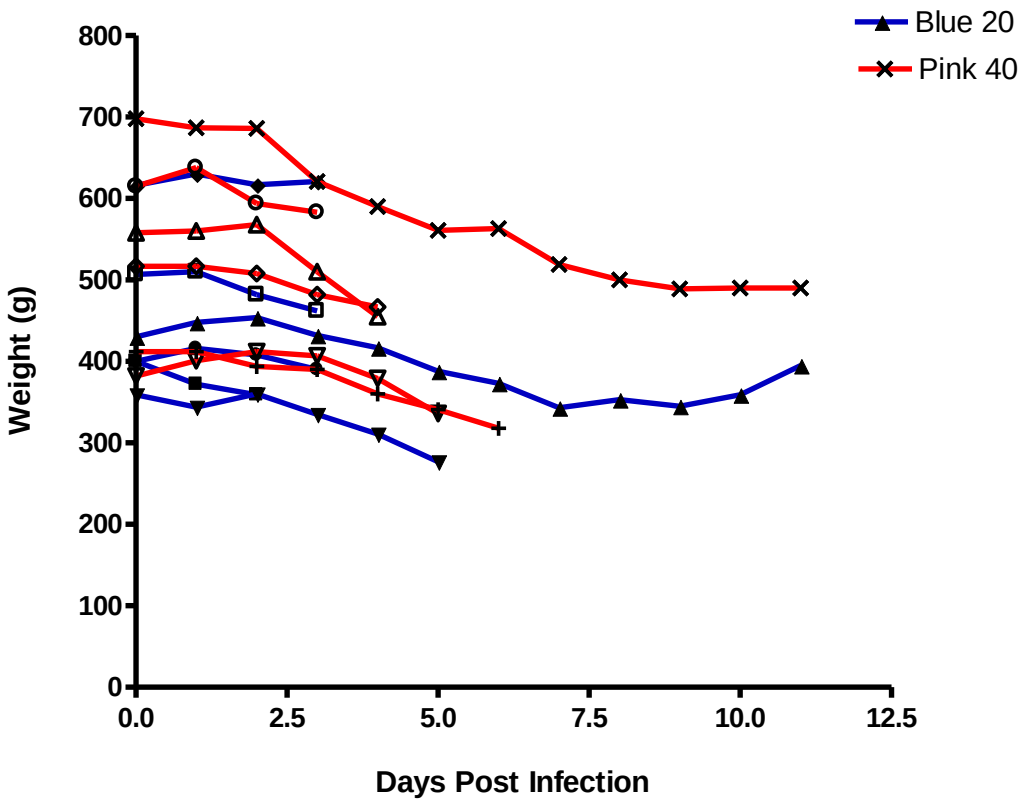

Fig. 4.21. Trial 3 distribution of weight (grams). Weights of all birds for each day post infection are shown for Trial 3. Each symbol denotes an individual chicken. SG-HAe symbols are connected by blue lines and SG-M6 symbols by red lines. The surviving chickens, BU 20 and PI 40, are denoted by a black filled-in triangle and a black X, respectively.

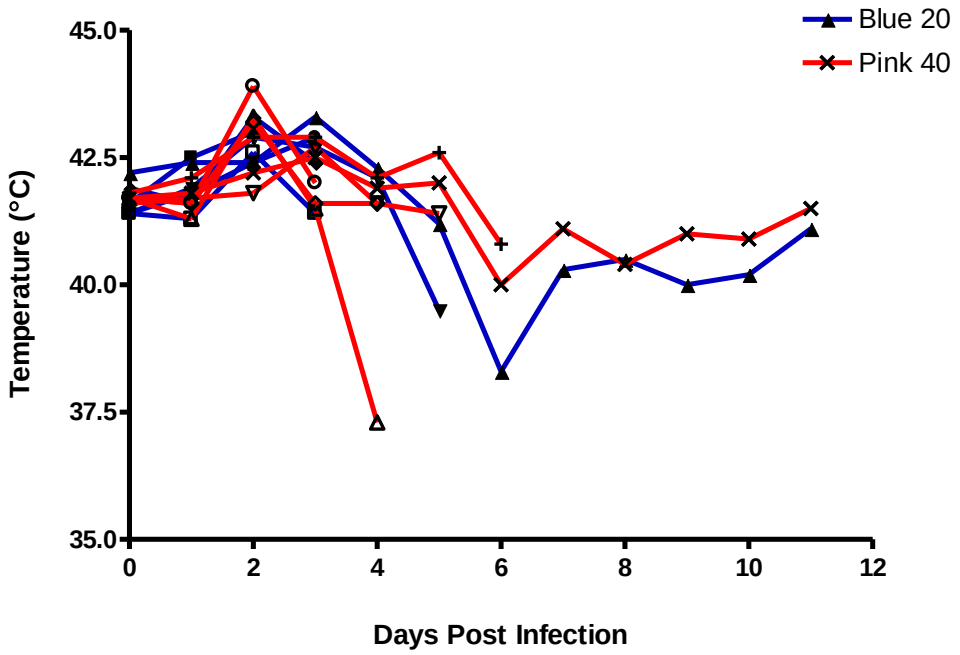

Fig. 4.22. Trial 3 distribution of body temperature (°C). Body temperatures of all birds for each day post infection are shown for Trial 2. Each symbol denotes an individual chicken. SG-HAe symbols are connected by blue lines and SG-M6 symbols by red lines. The surviving chickens, BU 20 and PI 40, are denoted by a black filled-in triangle and a black X, respectively.

There was no difference in survival between the treatment groups ($P > 0.05$). The HAe-specific serum IgG concentration, calculated for each chicken using a standard curve of pure chicken IgG, was correlated with the protection from lethal challenge for the SG-HAe vaccine (Figs. 4.23). No significant difference ($P > 0.05$, $R^2 < 0.75$) was observed between the HAe-specific IgG concentration of SG-HAe and survival. Moreover, the SG-HAe vaccinated birds that survived the challenge, BU 20, had 7 ng/ml IgG, while the other birds from the same treatment group had up to 67 ng/ml HAe-specific IgG.

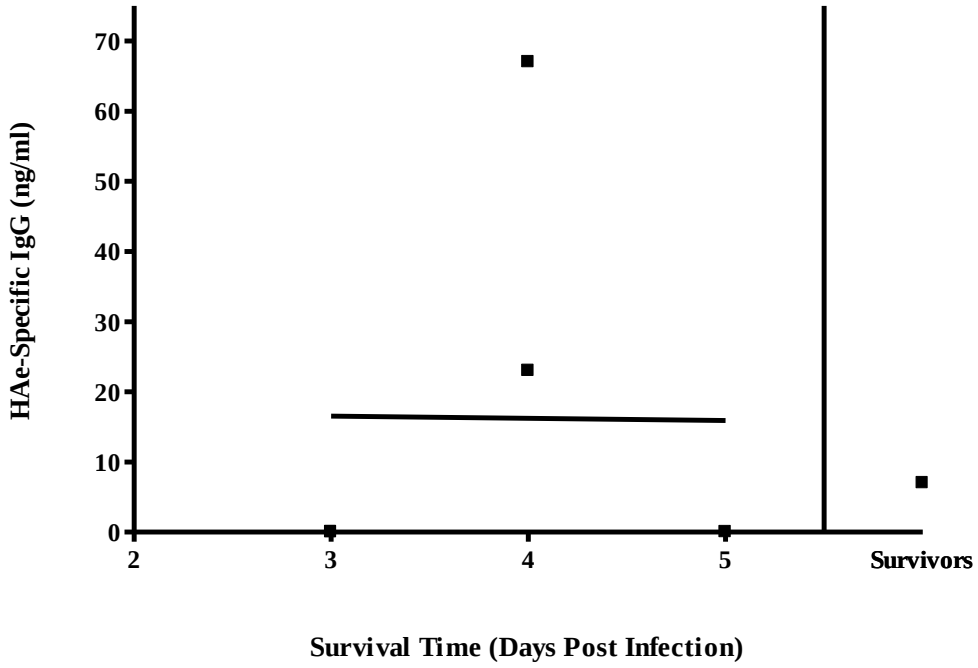

Fig. 4.23. Trial 3 correlation between SG-HAe elicited HAe-specific serum IgG and protection against lethal challenge. In the right panel the concentration of HAe-specific serum IgG in the chicken(s) that survived the challenge is reported; in the left, the direct correlation between the concentration of HAe-specific serum IgG and the survival time (days post infection) found that there was no significant difference ($P > 0.05$, $R^2 < 0.75$).

Trial 4: LL-M2e vs. LL-Pip

Vaccination and Humoral Immune Response

The same vaccination strategy was used as previously described in Trial 1 (n = 6 for both groups), except that the control strain, LL-Pip, was used instead of a mock vaccine control, and both vaccines were co-administered with CTB adjuvant. Peripheral blood was collected and analyzed for M2e-specific humoral response by ELISA or a cell mediated response by cellular proliferation assay.

M2e-specific serum in LL-M2e vaccinated chickens was significantly higher than in previous trials, possibly because of the co-administration of CTB. LL-M2e had a mean response of 2339 ng/ml (SD = 2782), which was much higher than LL-Pip with a mean response of 107 ng/ml (SD = 133) (Fig. 4.24). Additionally, all LL-M2e vaccinated chickens had a measurable serum titer, whereas 2 of 6 LL-Pip vaccinated chickens had an undetectable titer. When combining all individual chickens for each treatment group, the difference in response was highly significant ($P = 0.004$). There was no detectable M2e-specific fecal IgA in any of the samples from either vaccine group (data not shown).

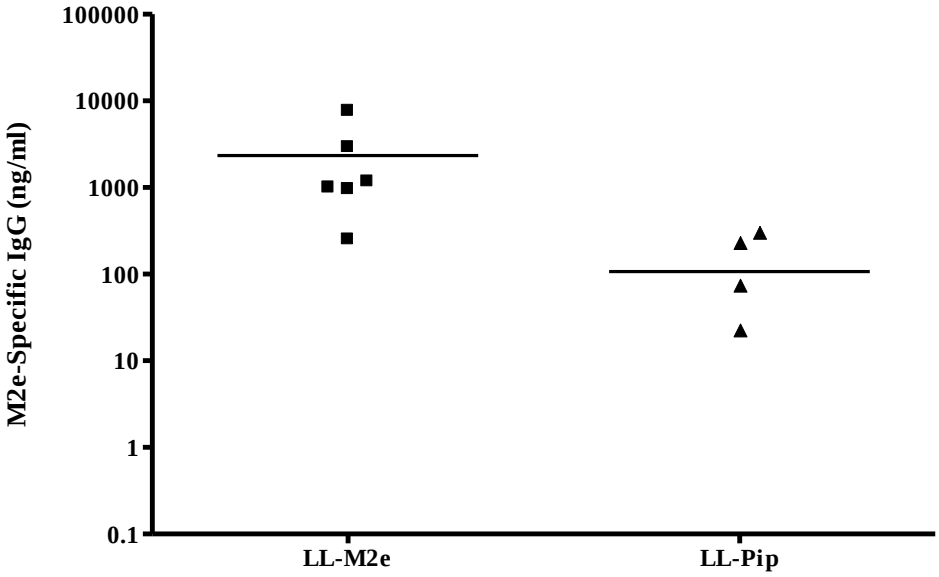

Fig. 4.24. Trial 4 serum IgG. M2e-specific IgG in peripheral blood serum was measured by ELISA. Treatment groups were LL-M2e (black squares) or LL-Pip (black triangles). A significant difference was observed between the M2e-specific IgG concentration of LL-M2e and LL-Pip vaccinated animals ($P = 0.004$). *Chickens that had 0 ng/ml M2e-specific serum IgG are not shown.

Cell Mediated Immune Response

A cellular proliferation assay with the same stimulants and cellular stains was performed as in Trial 2. The results indicate that there was no M2e-specific response in any of the samples from either group (data not shown).

Lethal Challenge and Viral Titers

The same infectious dose and challenge strategy was followed as described in Trial 2. Analysis of the viral load in tracheal swabs indicated no significant difference was seen between either tracheal swab days or treatment groups ($P > 0.05$) (Fig. 4.25). However, the chicken that survived the trial, Black 21 (BK 21), had lower PFU/ml than 90% of the total challenged birds for both days.

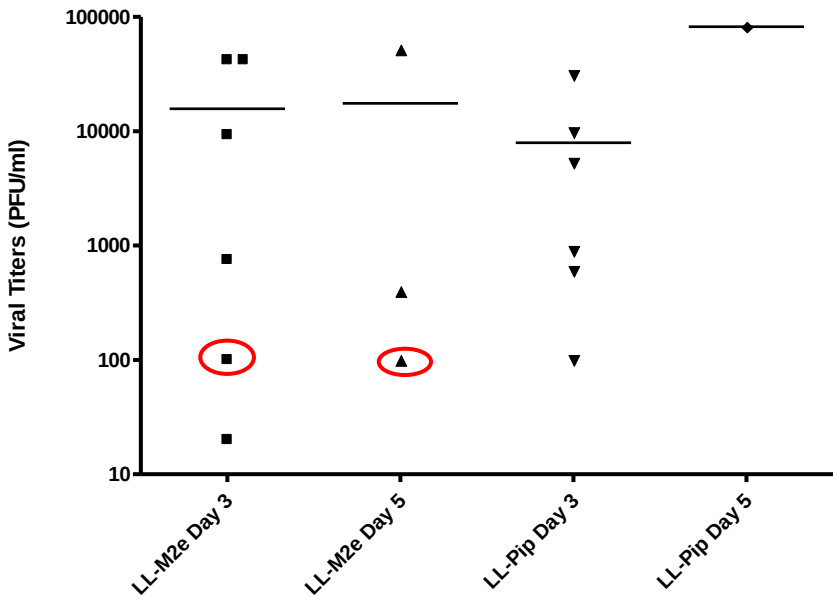

Fig. 4.25. Tracheal viral titer (PFU/ml) of Trial 4 treatment groups post-challenge. Tracheal swabs were taken days 3 and 5 or day of death. The viral titers for BK 21 are circled in red. No significant difference was seen in between vaccinated chickens in the same treatment group between days 3 and 5 ($P > 0.05$). Additionally, no significant difference was found between the treatment groups on either days 3 or 5 ($P > 0.05$).

Only one chicken from the LL-M2e-vaccinated group survived the challenge, and all other chickens died by day 6 (Fig. 4.26). The weight and body temperature of the surviving bird BK 21 decreased until 7 days post infection, and then increased until the end of the lethal challenge (Fig. 4.27, 4.28). All other individual chickens from both treatments groups showed similar trends as observed in previous Trials.

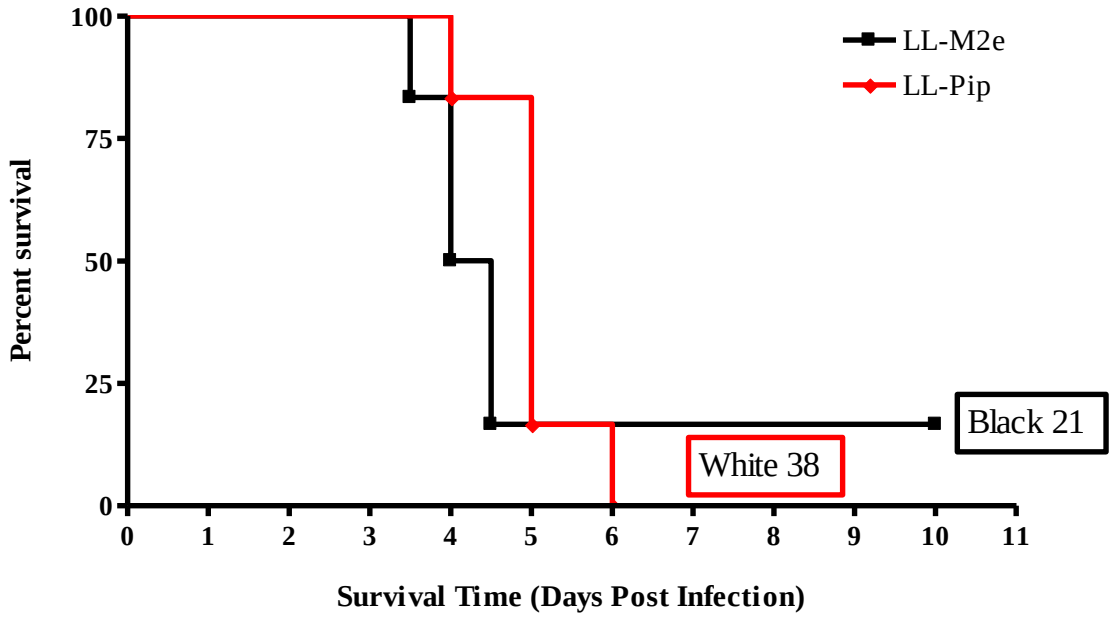

Fig. 4.26. Trial 4 survival curve. LL-M2e (black) and LL-Pip (red) vaccine groups were given a lethal challenge of (A/chicken/Pennsylvania/1370/1983) H5N2 HPAIV. 100% death of LL-Pip vaccinated chickens occurred on day 6 of the trial.

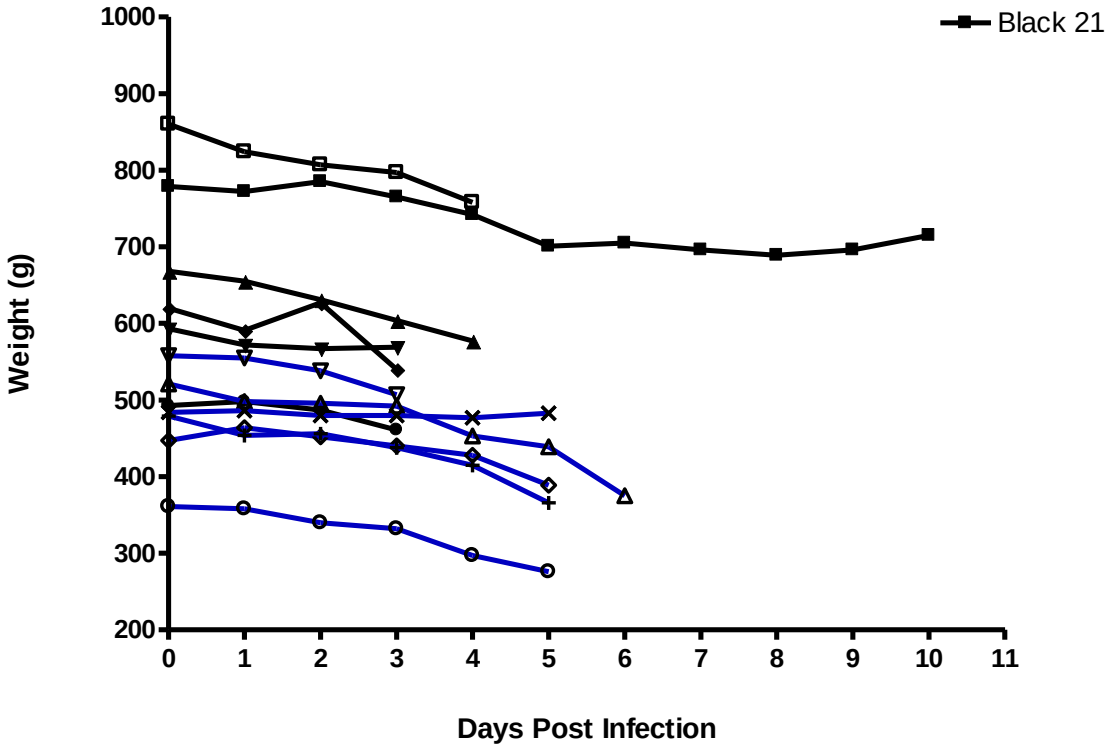

Fig. 4.27. Trial 4 distribution of weight (grams). Weights of all birds for each day post infection are shown for Trial 4. Each symbol denotes an individual chicken. LL-M2e symbols are connected by black lines and LL-Pip symbols by blue lines. The surviving chicken, BK 21, is denoted by a black square.

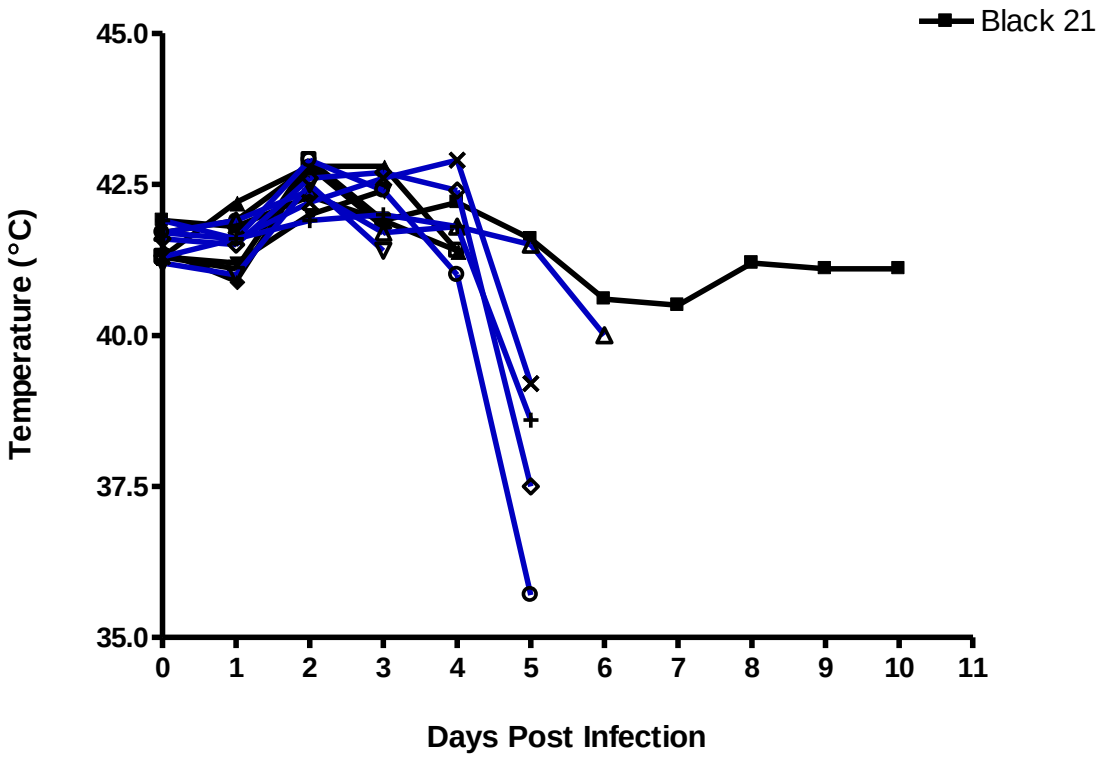

Fig. 4.28. Trial 4 distribution of body temperature (°C). Body temperatures of all birds for each day post infection are shown for Trial 2. Each symbol denotes an individual chicken. LL-M2e symbols are connected by black lines and LL-Pip symbols by blue lines. The surviving chicken, BK 21, is denoted by a black filled-in square.

The M2e-specific serum IgG did not correlate with the protection from lethal challenge (Fig. 4.29). No significant difference in M2e-specific serum IgG titers was observed between treatment groups ($P > 0.05$). Two of the LL-M2e vaccinated chickens that died on day 5 had higher M2e-specific serum IgG antibodies (7719 ng/ml and 2926 ng/ml) than the only bird to survive the trial (1171 ng/ml).

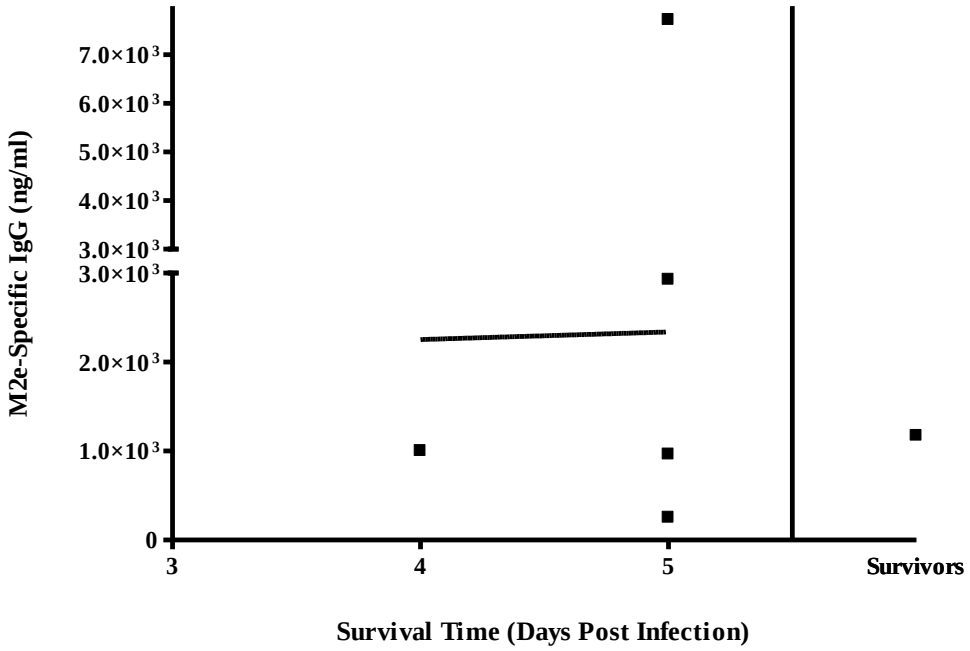

Fig. 4.29. Trial 4 correlation between LL-M2e elicited M2e-specific serum IgG and protection against lethal challenge. In the right panel the concentration of M2e-specific serum IgG in the chicken(s) that survived the challenge is reported; in the left, the direct correlation between the concentration of M2e-specific serum IgG and the survival time (days post infection) found that there was no significant difference ($P > 0.05$, $R^2 < 0.75$).

Chapter 5

DISCUSSION and CONCLUSION

Construction and Analysis of Surface Expression

L. lactis expressing M2e

We have constructed three vaccines for avian influenza. Each vaccine is composed of live lactic acid bacteria (LAB), which have been genetically engineered to express an AIV antigen on their cell surface. The first vaccine expresses the M2e antigen from *L. lactis* (LL-M2e). The M2e antigen is the conserved sequence from the external domain of the M2 capsule protein of AIV. Although the M2e region is conserved among subtypes, variations exist. We chose a variation that exactly matched all known low pathogenicity subtypes of AIV, and H5N2 HPAIV. The M2e peptide was expressed in 10 tandem repeats in the context of a plasma membrane protein named Pip. Whole-cell ELISA analysis of the LL-M2e vaccine was clearly recognized by M2e-specific Post-immune serum whereas the control strain LL-Pip was not. Additionally, no signal intensity was observed in the Pre-immune serum for either LL-M2e or LL-Pip. Expression was proportional to the number of cells analyzed. Therefore, M2e is expressed on the surface of LL-M2e.

L. lactis and S. gordonii expressing HAe

Two additional vaccines were constructed to express the HAe antigen from either *L. lactis* or *S. gordonii*. The HAe sequence that we chose is 100% conserved in every AIV subtype. We chose to express HAe in 10 tandem repeats in the context of the truncated plasma membrane protein named M6. Surface expression of HAe was

analyzed in SG-HAe, LL-HAe, and the control strains SG-M6 and LL-M6. The results showed that there was no difference between the HAe strains and the control strains. Additionally, the Post-HAe antiserum used to detect HAe was higher than Pre-HAe antiserum, but could not be differentiated from the background signal (non-specifically bound to the plate). Furthermore, the antiserum and antibody preparations used to detect HAe did not recognize the purified HAe peptide bound to the ELISA plate as standard. Four HA-specific antibodies were tested for recognition of HAe peptide: One commercial antibody made against whole HA protein, one made in our laboratory against a KLH-HAe conjugate, and two that were provided by a colleague who prepared them from recombinant NDV expressing whole H5 peptide (HA2, Pre and Post HAe, HI, HA-49, respectively). None of the antibodies tested showed any specific recognition of the HAe peptide. Therefore, detection of HAe on the surface of *L. lactis* and *S. gordonii* was inconclusive.

The HAe peptide was observed to be too hydrophobic, and insoluble to bind correctly to an ELISA plate, which may also explain the non-specific action of the Pre and Post HAe antibodies. In an effort to correct this problem of insolubility, we purchased a modified HAe peptide with several hydrophilic and charged amino acid residues added to the amino terminus. The modified HAe peptide was soluble and readily applied to the surface of the ELISA plate. Despite this improvement, none of the four antibodies directed against HAe recognized the modified peptide. In addition to ELISA analysis, a western blot was used to confirm expression, but a lack of specificity

for HAe was found. The results indicate, expression of HAe from our vaccine strains could not be verified, and therefore expression is inconclusive.

Other factors may have played a role in the non-recognition of HAe on the surface of the bacteria. One possibility is that HAe may have taken an abnormal conformation in the context of the M6 fusion. Specific recognition may have also been disrupted by the conformation of the gene-fusion protein, M6-HAe, which may have altered the secondary or tertiary structure of HAe protein. Additionally, assuming M6-HAe was expressed on the surface of *S. gordonii*, the tertiary structure of M6-HAe may have caused the HAe peptide to be fully or partially hidden from antibody recognition. Another possibility is that the antibodies raised against HA recognize the cleaved form of HA, which is normally cleaved into HA1 and HA2 and held together by a disulphide bond. Our HAe peptide starts with the last arginine residue from the cleavage site and continues 11 amino acids into the nascent N-terminus of the HA2 peptide after cleavage. The lack of HA1 amino acid residues and the full cleavage site within our HAe peptide may have contributed to a lack of HA-specific antibody recognition. Yet another possibility was that the genetic construct was mutated or incorrect in some unanticipated way. However, the pLEX-HAe construct was sequenced and found to be accurate with the coding region for 10 tandem repeats of HAe cloned in-frame with the coding regions for the C-terminal signal sequence and the N-terminal M6 CRR.

M2e Vaccine Trials

Peripheral blood from each treatment group in both M2e trials was analyzed by ELISA for M2e-specific serum IgG. In Trials 1 and 4, analysis of LL-M2e and control vaccinated chickens found M2e-specific serum IgG in all treatment groups. In Trial 1, the induced immune response in LL-M2e vaccinated birds was not significantly different ($P > 0.05$) from mock-vaccinated birds. However, Trial 4 LL-M2e vaccinated chickens co-administered with CTB had definitively improved M2e-specific serum IgG levels as a group compared to Trial 1, and were significantly different ($P = 0.004$) from the control, LL-Pip vaccinated birds. M2e-specific IgG immune responses in the control vaccinated chickens could have occurred during the vaccination period when the birds from both vaccination groups were housed together in the same pen. Chickens are coprophagic and perhaps the control birds consumed some of feces from LL-M2e-vaccinated birds. Studies have shown that LAB can survive passage through the gut and can be found in the fecal matter (24, 45, 59). We considered the mucosal IgA response to our vaccines. Studies performed by other labs have shown that protection from a number of pathogens by antigen presenting LAB recombinant vaccination, have been attributed to a mucosal IgA humoral immune response (4, 41, 53, 65, 77). Our results showed no detectable M2e-specific fecal IgA in any of the samples that we analyzed. We were unable to analyze salivary IgA response due to technical problems with collecting chicken saliva (it is too viscous to pipet and handle).

In Trials 1 and 4, mixed results were found in the mean survival time of LL-M2e-vaccinated chickens. A significant increase ($P = 0.01$) was found in the mean survival

time of Trial 1 LL-M2e vaccinated birds compared to mock vaccinated birds, whereas Trial 4 LL-M2e vaccinated chickens showed no significant difference ($P > 0.05$) compared to LL-Pip. This data contrasts with the M2e-specific serum IgG immune response that had found a significant difference between LL-M2e and LL-Pip vaccinated chickens. Although, in both Trials 1 and 4, no control vaccinated birds were able to survive lethal challenge. Moreover, the LL-M2e vaccinated birds that survived longest (YL 22 and BK 21) had some of the highest M2e-specific IgG titers and had the greatest reduction of tracheal PFU/ml. However, when all of the LL-M2e vaccinated birds were analyzed no significant correlation ($P > 0.05$, $R^2 < 0.75$) was found in either Trials 1 or 4 between the M2e-specific serum IgG titer and survival. In Trial 4, two chickens with a higher amount of IgG died 4 days before the surviving chicken (BK 21). Chickens that survived the longest had the same approximate M2e-specific serum IgG immune response of 1100 ng/ml. This suggests 1100 ng/ml may approximate the minimum IgG response required for survival, but that other factors are required for full protection. Cell mediated immunity was considered in Trial 4, but none of the individual chickens had a detectable response. In other studies, antibodies and CMI response against M2e has been shown to protect the infected animal as opposed to preventing infection (21, 27, 47). The fact that M2e vaccinated birds survived 1.5 days longer than mock-vaccinated birds in our vaccine trials is quite significant, because M2e vaccinated birds had less severe disease and had more time for the host immune response to fight back the infection.

A possible reason for lack of protection in the other 10 LL-M2e vaccinated chickens during Trials 1 and 4 is that the antigen-specific antibodies may not have

disrupted the function of M2. A recent study has shown that M2e, in conjunction with an activated T-cell ligand (CD154), expressed on the surface of *Salmonella* serotype Enteritidis can increase protection against LPAIV (47). However, M2e has never been shown to induce a fully protective response in recombinant bacteria, and has not been tested in *L. lactis* until now.

HAe Vaccine Trials

ELISA analysis of the treatment groups from the HAe vaccine trials showed mixed results. The results of trial 2 showed that LL-HAe and SG-HAe were able to evoke similar HAe-specific serum IgG responses ($P > 0.05$), that were significantly different from mock-vaccinated birds. Although, the HAe-specific IgG responses found were much lower than the M2e vaccinated chickens, studies have shown that HAe antibodies are more potent at neutralizing AIV in animals than M2e antibodies (83, 93). SG-HAe was further investigated in Trial 3 with the addition of CTB adjuvant. CTB was able to elicit a significantly higher HAe-specific serum IgG titer in SG-HAe-vaccinated birds compared to Trial 2. However, HAe-specific IgG induction in the SG-HAe vaccinated chickens was not significantly different ($P > 0.05$) from the control strain, SG-M6. The reason for this is unknown, but may have been a result of the housing conditions described in the M2e Vaccine Trial section. As in the M2e vaccine trials, cell mediated immunity and HAe-specific fecal IgA antibodies were tested, but no immune response was observed.

A mixed response in survival time was observed in both Trials 2 and 3. In Trial 2, analysis of both SG-HAe and LL-HAe vaccines showed a significant increase (P = 0.02 and P = 0.01, respectively) in the mean survival time from lethal challenge compared to the mock-vaccinated chickens. However, SG-HAe had two birds that survived the full length of the trial, whereas all LL-HAe vaccinated birds were all dead by day six. Therefore, SG-HAe was further investigated in Trial 3, this time with the addition of CTB. No significant difference ($P > 0.05$) in mean survival time was observed between SG-HAe and SG-M6 vaccinated birds, which was predicted by the HAe-specific IgG immune response. Additionally, one chicken from each treatment group was able to survive lethal challenge. In Trials 2 and 3, analysis of all the birds from each vaccine group showed no significant correlation ($P > 0.05$, $R^2 < 0.75$) between HAe-specific IgG and survival.

Analysis of the surviving chickens from both trials reveals inconclusive results. Each of the surviving birds fully recovered from lethal challenge, as evidenced by both the decrease in PFU/ml and the stabilization of body temperatures and weights. However, in Trial 2, one of the two surviving SG-HAe vaccinated birds had 7 ng/ml HAe-specific serum IgG and the other had 0 ng/ml. Additionally, CTB did not seem to improve the protection of the Trial 3 chickens. The SG-HAe vaccinated chicken had 7 ng/ml HAe-specific IgG, and the SG-M6 vaccinated chicken had 175 ng/ml. Therefore, the HAe-specific serum IgG humoral immune response seems to be insufficient for protection. Thus survival must be due to some protective effect outside of the adaptive

immune response tested for in this study. Other studies suggest host-cell factors involved in the innate immune response may have contributed to the unknown protection (34, 44).

Future Investigations

Certain areas of investigation were either not performed or were left inconclusive during the M2e and HAe vaccine Trials and should be considered for future investigation. HAe surface expression was not conclusively determined by ELISA, because of the lack of a HAe-specific antibody. However, the SG-HAe expressing bacterial vaccine increased survival of chickens from lethal challenge, which suggests that HAe is being expressed on the surface of S. gordonii. Therefore, a HAe-specific antibody should be made to clarify surface expression of HAe.

No correlation could be detected between either antigen-specific serum IgG or CMI and survival, even though LL-M2e, LL-HAe, and SG-HAe all increased survival of chickens from lethal challenge. Salivary and fecal IgA immune responses were considered, but antigen-specific fecal IgA could not be detected in any of the birds, and salivary IgA could not be measured, due to the thick and viscous nature of chicken saliva. Therefore, in future studies tracheal and lung washes should be performed on the chickens, after infectious challenge, in an attempt to find antigen-specific IgA by ELISA. Any antigen-specific tracheal or lung IgA could then be correlated with survival.

Another potential future investigation, could involve the administration of a single vaccine composed of multiple bacterial constructs expressing both M2e and HAe. This

would expose chicken immune cells to a variety of AIV antigens instead of just one, and may lead to an increased immune response.

An additional bacterial vaccine construct could be investigated. A conserved region from the other externally protruding protein in AIV, NA, could be cloned into M6, Pip, or another carrier protein, and tested for any antigen-specific immune responses in chickens. All other experimental procedures would be kept the same. Furthermore, after testing an NA expressing *L. lactis* or *S. gordonii* bacterial vaccine construct by itself in chickens, the NA vaccine could be used in conjunction with M2e and/or HAe vaccines.

Conclusion

Both LL-M2e and SG-HAe showed potential as future candidates for the vaccination of poultry without the use of an adjuvant. Each vaccine was able to induce antigen specific IgG in chickens and was able to increase mean survival times compared to control vaccinated groups. Furthermore, one or more chickens from each of these vaccines were able to fully recover and survive from a lethal challenge with H5N2 HPAIV, whereas the controls did not. Although the individual vaccine groups antigen specific IgG did not correlate with survival, significant trends were seen. This suggests that a few minor changes to the recombinant bacterial constructs may increase the protective response of antigen specific IgG.

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