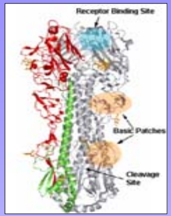


Protection of BALB/c Mice Challenged with H1NI Influenza Virus Following Vaccination with Liposomal H1N1 Hemagglutinin (L-HA/H1N1) or Liposomal H1N1 M2e (L-M2e/H1N1)

Meilen Chang Muñoz[1], Hana Kim[1], Jill P. Adler-Moore[1] and William A. Ernst[2]

[1]Department of Biological Sciences, California State Polytechnic University Pomona

[2]Molecular Express Inc., Rancho Dominguez, CA

ABSTRACT

Introduction: The HA and M2 viral proteins are present on the surface of the influenza virus in a ratio of 20:1. By incorporating each of the viral proteins as fusion proteins into separate formulations of small unilamellar liposomes (<100nm), both proteins can be expressed at high concentrations on the liposomes i.e. about 100:1 protein to liposome ratio. Our previous studies have shown that L-M2e/H1N1 was effective in protecting mice from homologous intranasal challenge with 10X LD_{50} H1N1 when a boost was given on day 56. The present study was done to investigate the following: 1) differences in protection using a day 28 boost rather than a day 56 boost; 2) comparison between the protection elicited by vaccination with L-HA/H1N1 or L-M2e/H1N1; and 3) effect of combining L-M2e/H1N1 vaccination with L-HA/H1N1 or L-HA/H5N1.

Methods: Female, 6 week old, BALB/c mice (n=12/group) were vaccinated subcutaneously day 0 and boosted intranasally day 28 with 15mg HA as L-HA/H1N1, 15ug M2 epitope as L-M2e/H1N1 or control (non-protein) liposomes; other groups of mice were boosted intranasally day 56 with , 15ug HA as L-HA/H1N1, 15ug HA as L-HA/H1N1 + 15ug M2 epitope as L-M2e/H1N1, 15ug HA as L-HA/H5N1 + 15ug M2 epitope as L-M2e/H1N1 or control liposomes. All the liposomes contained the adjuvant Monophosphoryl Lipid A (MPL). One week post-boost (day 35 or day 63) mice were challenged intranasally with 10X LD_{50} H1N1 virus (PR8 strain). Five days post-challenge, lungs were harvested from 5 mice/group, weighed and frozen for subsequent foci analysis of lung viral burden. The remaining 7 mice/group were monitored for clinical signs, weight loss, and survival for one month. For the foci assay, infected lungs were homogenized and incubated with $6x10^6$ MDCK cells/ml for 48h. To visualize the foci of infection, cells were incubated with mouse anti-NP antibody, enzyme labeled secondary antibody, and a substrate which appeared brown at the sites of infection when viewed microscopically.

Results: Mice immunized with L-HA/H1N1 provided some protection against H1N1 challenge (43% survival) following a boost at day 56, but there was no protection for mice given a 28 day boost, or for those given the control liposomes. Mice immunized with L-M2e/H1N1 showed an 86% survival with a boost at day 28, but there was no additional protection observed when the mice were co-immunized with L-HA/H1N1 (71% survival). In addition, vaccination with L-HA/H5N1 did not interfere with the protection provided by the L-M2e/H1N1 when the mice were immunized with a combination of L-HA/H5N1 and L-M2e/H1N1 and boosted at day 56 (86% survival). Disease signs in each group paralleled the survival, although the viral lung burden (foci/g lungs) at day 5 post-challenge did not correlate with survival. The lungs at day 5 were collected from mice that had minimal to mild signs of disease.

Conclusion: Both the HA protein and the M2e peptide corresponding to the H1N1 strain PR/8/34 incorporated into small unilamellar liposomes generated protection against an H1N1 challenge, although the M2e provided more protection. An interval of 8 weeks between the prime and boost immunization was optimal for the HA protein, but protection could be generated by the M2e with either a 4 week or 8 week boost.

INTRODUCTION

Hemagglutinin (HA) is one of the major surface envelope proteins of Influenza virus. HA is responsible for host-cell binding to sialic acid residues on host cell surfaces, and is also involved in the subsequent fusion of viral and host membranes in the endosome after the virus has been taken up by the cell via endocytosis (1). HA is highly immunogenic, but is susceptible to high levels of mutations and/or recombination (2).

The M2 transmembrane ion channel protein is another surface envelope protein of Influenza virus although it is present in the virus envelope in much lower concentrations relative to HA (1:20). The primary role of the M2 protein is to uncoat the virus in the endosome after it is engulfed by the host cell. Due to the M2 protein's highly conserved sequence, especially in the first 23 AAs, and its high concentration on the surface of infected epithelial cells (1 M2:1.5 HA), it is being investigated as a likely candidate for a universal antigen to induce protection against different influenza viral subtypes, such as H1N1, H2N2, H3N2 and H5N1 (3).

Our previous studies have shown that an M2 epitope (AAs 1-23) combined with a hydrophobic protein to form a fusion protein can be incorporated into small unilamellar liposomes (L-M2e/H1N1). These liposomes were effective in protecting mice from intranasal challenge with 10X LD_{50} H1N1 when the mice were vaccinated on day 0 and boosted on day 56 (4). The present study was done to investigate: 1) if boosting on day 28 rather than on day 56 would alter vaccine protection; 2) if the protection elicited by a vaccine targeting HA from H1N1 (L-HA/H1N1) would be equivalent to the protection elicited by the L-M2e/H1N1 vaccine; and 3) if combining L-M2e/H1N1 vaccination with L-HA/H1N1 or L-HA/H5N1 vaccination would alter the protection associated with L-M2e/H1N1 vaccination.

RESULTS – DAY 28 BOOST

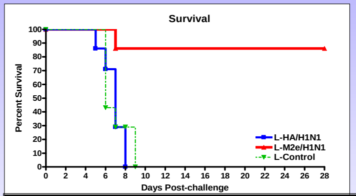
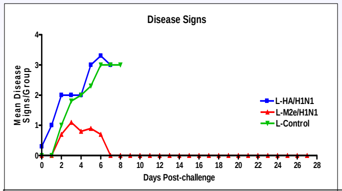


Figure 1. Mice boosted with L-M2e/H1N1 one month after the priming dose were significantly protected (p=0.0103) against challenge with 10X LD_{50} H1N1 (86% survival), whereas mice boosted with L-HA/H1N1, or L-Control were moribund by day 8 or day 9 post-challenge, respectively.



1 – decreased grooming
2 – decreased grooming, decreased activity
3 – decreased grooming, decreased activity, cachexic
4 - moribund

Figure 3. Mice boosted with L-M2e/H1N1 one month after the priming dose had mild disease signs up to 6 days post-challenge, which completely resolved by day 7. Mice vaccinated with L-HA/H1N1 or L-Control had severe disease signs until all animals became moribund on day 8 or 9, respectively.

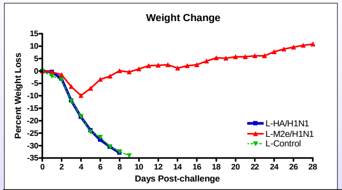


Figure 5. Mice boosted with L-M2e/H1N1 one month after the priming dose lost approximately 10% of their original body weight during the first 4 days post-challenge, then continuously gained weight throughout the remainder of the study. Mice vaccinated with L-HA/H1N1 or L-Control lost over 30% of their original body weight until all animals became moribund on day 8 or day 9 post-challenge, respectively.

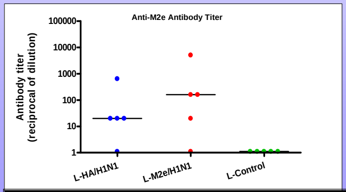


Figure 7. Mice boosted with L-M2e/H1N1 one month after the priming dose had the highest viral precipitation serum antibody titer compared to the other treatment groups. However, the titers of both the L-M2e/H1N1 and the L-HA/H1N1 groups were significantly higher than the L-Control group (p=0.03).

RESULTS – DAY 56 BOOST

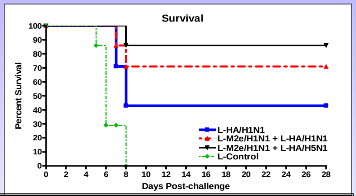


Figure 2. Mice boosted with L-M2e/H1N1 + L-HA/H5N1 or L-M2e/H1N1 + L-HA/H1N1 two months after the priming dose were significantly protected against challenge with 10X LD_{50} H1N1 (86% survival and 71% survival; p=0.0007 and p=0.0047 vs. control, respectively). Mice vaccinated with L-HA/H1N1 showed less, but still significant protection against challenge with the virus (43% survival, p=0.04 vs. control). All mice administered L-Control were moribund by day 8 post-challenge.

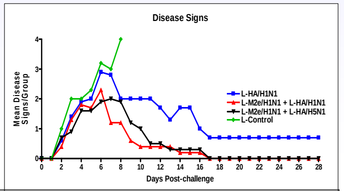


Figure 4. Mice boosted with L-M2e/H1N1 + L-HA/H1N1 or L-M2e/H1N1 + L-HA/H5N1 two months after the priming dose had moderate disease signs until day 8 post-challenge, which completely resolved by day 17. Mice vaccinated with L-HA/H1N1 had moderate to severe disease signs until day 17, with mild disease signs in the survivors for the duration of the study. Mice administered L-Control had severe disease signs until all animals became moribund on day 8.

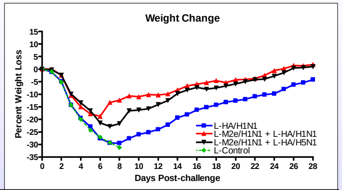


Figure 6. Mice boosted with L-M2e/H1N1 + L-HA/H1N1 or L-M2e/H1N1 + L-HA/H5N1 two months after the priming dose lost approximately 20% of their original body weight during the first 6 or 7 days post-challenge, respectively, then continuously gained weight throughout the remainder of the study. Mice vaccinated with L-HA/H1N1 lost approximately 30% of their original body weight during the first 8 days, and the survivors continuously gained weight throughout the remainder of the study. Mice administered L-Control lost over 30% of their original body weight until all animals became moribund on day 8.

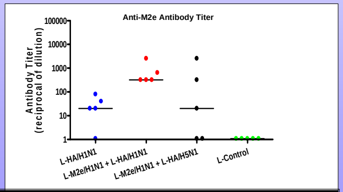


Figure 8. Mice boosted with L-M2e/H1N1 + L-HA/H1N1 two months after the priming dose had the highest viral precipitation serum antibody titer compared to the other treatment groups. The antibody titer for group L-M2e/H1N1 + L-HA/H1N1 was significantly higher than the L-HA/H1N1 (p=0.008) and the L-Control group (p=0.008). The titer of the L-HA/H1N1 was significantly higher then the L-control (p=0.03).

MATERIALS AND METHODS

Day 0: Subcutaneous liposome vaccine prime * (L-HA/H1N1; L-M2e/H1N1; L-M2e/H1N1 and L-HA/H1N1; L-M2e/H1N1 and L-HA/H5N1; or control liposomes; and 25ug MPL)

Day 28: Intranasal liposome vaccine boost * (L-HA/H1N1; L-M2e/H1N1; L-M2e/H1N1 and L-HA/H1N1; L-M2e/H1N1 and L-HA/H5N1; or control liposomes; and 25ug MPL)

Day 34: Blood collected by cardiac puncture**

Day 35: Intranasal 10X LD_{50} H1N1 (strain PR/8/34) challenge***

Day 56: Intranasal liposome vaccine boost * (L-HA/H1N1; L-M2e/H1N1; L-M2e/H1N1 and L-HA/H1N1; L-M2e/H1N1 and L-HA/H5N1; or control liposomes; and 25ug MPL)

Day 62: Blood collected by cardiac puncture**

Day 63: Intranasal 10X LD_{50} H1N1 (strain PR/8/34) challenge***

Day 63: End point

Day 91: End point

*** 15ug antigen/dose**
**** n = 5 mice/group**
***** n = 12 mice/group**

PRECIPITATION ASSAY FOR DETERMINING ANTIBODY TITER

-100ul of two-fold dilutions from 1:20 up to 1:40,960 of the serum in PBS in one 96-well agglutination plate was made.
- 100ul of 1:5 viral dilution added to one row of serum, and 100ul of 1:10 viral dilution to the next row of serum.
- Plate placed in a humidity chamber to prevent evaporation, and incubated at 37ºC for 24h to optimize the reaction.
- Incubated the plate at 4ºC for 24h to further enhance the visibility of the precipitation reaction.
- Spun the plate at 1500 rpm for 5 min, and placed it at room temperature for 30 min before reading the plates to allow any condensation on the bottom of the plate to evaporate.

CONCLUSIONS

•The M2e peptide corresponding to the H1N1 strain PR/8/34 incorporated into small unilamellar liposomes generated significantly more protection against the PR/8/34 challenge than the modified HA protein incorporated into liposomes, indicating that a small peptide of only 23aa (M2e) was more immunogenic than the tenfold larger modified protein (HA).
•An interval of 8 weeks between the prime and boost vaccinations was optimal for the HA liposomes, but protection could be generated by the M2e liposomes with either a 4 week or 8 week boosting interval further underscoring the M2e liposome's better immune stimulating properties.
• The high titer of viral precipitating antibodies generated by the M2e liposomes suggests that these antibodies may play a critical role in clearing the virus from the host either by phagocytic cell uptake of the antigen/antibody complexes, clearance of these complexes by the kidneys and/or neutralization of virus infectivity. This will be investigated in future studies.

REFERENCES

1 – Fan, J. et al. Vaccine, 2004. p.2993-3003.
2 – Seo, S.H. et al. Nat Med, 2002. p.950-4.
3 – Neirynck, S. et al. Nat.Med, 1999. p.1157-63.
4 – Ernst, W. A. et al. Vaccine, 2006. p.5158-68.

ACKNOWLEDGEMENTS

This study was funded by a Cal Poly Pomona ARI Research grant, with matching funds from Molecular Express, Inc., Rancho Dominguez, CA.