

ANNUAL REPORT PROJECT NC-229

PERIOD COVERED: June 2008 to November 2009

INSTITUTION OR STATION: Centro Nacional de Biotecnología (CNB), CSIC. Spain

A. NC-229 REPRESENTATIVE:

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B. PROGRESS OF WORK AND PRINCIPAL ACCOMPLISHMENTS:

*Per station for ALL Accomplishment = Maximum 3,000 characters including spaces;
Full NC229 report for ALL Accomplishment = Maximum 30,000 characters):*

This section focuses on intended activities, outputs, and short-term outcomes. The report should also reflect on the items that stakeholders want to know, or want to see. The accomplishments should cover only the current year of the project.

Objective 1. Elucidate the mechanisms of host-pathogen(s) interactions.

Indicate progress in the following areas.

1. Research related to pathogenesis/persistence
2. Research related to virus evolution
3. Research related to mechanisms of transmission
4. Research related to viral Immunity and cross-protection
5. Research related to epidemiology

Objective 2. Understand the ecology and epidemiology of PRRSV and emerging viral diseases of swine.

Indicate progress in the following areas.

1. Research related to pathogenesis/persistence
2. Research related to virus evolution
3. Research related to mechanisms of transmission
4. Research related to viral Immunity and cross-protection
5. Research related to epidemiology

Objective 3. Develop effective and efficient approaches for detection, prevention and control of PRRSV and emerging viral diseases of swine.

Indicate progress in the following areas.

1. Research related to pathogenesis/persistence
2. Research related to virus evolution
3. Research related to mechanisms of transmission
4. Research related to viral Immunity and cross-protection
5. Research related to epidemiology

4. Progress in Enjuanes group research is summarize as follows:

Concerning the animal experiments using the TGEV vector expressing PRRSV GP5 and M proteins, it was found that all animals present a high antibody response against TGEV, therefore, the vector infected target tissues as expected. Also, vaccinated animals showed a clear antibody response against the PRRSV antigens (i.e., GP5 and M proteins). After a challenge with a virulent PRRSV isolate, a fast recall response was observed, as vaccinated animals induced higher antibody titers against PRRSV antigens and earlier than control animals. Nevertheless, the immune response was not strong enough to provide full protection against PRRSV. That was likely due to the low levels of neutralizing antibodies produced before challenge.

rTGEV expressing PRRSV M protein and GP5 mutants with altered glycosylation. Low levels of neutralizing antibodies were produced after rTGEV inoculation, similarly to what occurs with PRRSV infection. This could be due to a steric hindrance caused by the glycosylation sites present close to the neutralizing epitope in GP5. Therefore, a set of rTGEV vectors expressing M protein and GP5 mutants with a modified glycosylation pattern were generated. Just one of them was stable, a rTGEV expressing PRRSV M protein and a GP5 N46S mutant, lacking the glycosylation site overlapping neutralizing epitope. This rTGEV vector expressed high levels of GP5 and M PRRSV proteins in 74 % and 85% of the infected cells, respectively. A short in vivo immunization protocol was performed. One-week old piglets were inoculated with 1×10^8 pfu of the rTGEV by three routes: oral, nasal and intragastric. Blood samples were collected at different times post inoculation, and humoral immune responses were evaluated by ELISA. All animals present a high antibody response against TGEV, therefore, the vector infected target tissues as expected. Vaccinated animals showed a clear humoral response against PRRSV. Nevertheless, low levels of neutralizing antibodies were produced, similar to those obtained by vaccination with rTGEVs expressing wild-type GP5.

Formulation of a killed vaccine expressing GP5 mutants with alterations in the glycosylation pattern. A killed vaccine was also developed based on rTGEV expressing GP5-N46S mutant and M proteins. The protection conferred by this vaccine was tested. Vaccinated animals induced higher and faster antibody titers against PRRSV antigens than control animals. Neutralizing antibodies titers were also higher in the vaccinated animals when compared with non-vaccinated ones, suggesting that the elimination of glycosylation site close to the neutralizing epitope improves protective immune response against PRRSV. Therefore, results using rTGEV as a platform were promising, as antibodies against PRRSV antigens was elicited and a certain degree of protection was observed.

Expression of GP5 mutants lacking the decoy epitope. The presence of an immunodominant (decoy) epitope close to the neutralizing epitope in GP5 could be deleterious for a strong neutralizing immune response. Therefore, an rTGEV vector was constructed, expressing a GP5 protein lacking the non-neutralizing (decoy) epitope and the N46 glycosylation site. The virus was recovered with high titer and GP5 and M protein expression was stable in 65% and 90% of the infected cells, respectively.

C. IMPACT AND VALUE OF RESEARCH TO STAKEHOLDERS:

Impact statements (500 characters per statement)

This section focuses on actual or intended potential long-term outcomes and impacts, covering only the current year of the project. The report should also reflect on the items that stakeholders want to know, or want to see. List any grants, contracts, and/or other resources obtained by one or more project members as a result of the project's activities. Include the recipients, funding source, amount awarded and term if applicable.

D. PRRS PUBLICATIONS ISSUED OR “IN PRESS”

1) Refereed publications

* Cruz, J.L.G., Zuñiga, S., Sanchez, C.M., Ceriani, J.E., Urniza, A., Plana-Duran, J. and Enjuanes L. Immunogenicity of a TGEV-based vector expressing porcine reproductive and respiratory syndrome virus GP5 and M proteins. Vaccine. *Submitted*

2) Abstracts or Proceedings

* Cruz, J. L. G., Zúñiga, S., Sánchez, C. M., Ceriani, J. E., Plana, J., and Enjuanes, L. 2008. Design of a recombinant TGEV vector to protect against porcine reproductive and respiratory syndrome. EuroPRRSnet Workshop. Combating PRRS in Europe

* Cruz, J. L. G., Zúñiga, S., Sánchez, C. M., Urniza, A., Bru, T., Ceriani, J. E., Plana, J., and Enjuanes, L. 2008. Construction of a TGEV vector to protect against porcine reproductive and respiratory syndrome. 2008 PRRS Symposium

* Cruz, J. L. G., Zúñiga, S., Sánchez, C. M., Ros, S., Juanola, S., Plana, J., and Enjuanes, L. 2009. Design of a TGEV vector to protect against porcine reproductive and respiratory syndrome. 2009 PRRS Symposium

3) Book chapters or monographs

Give full citation

E. FUNDING SOURCES FOR PRRSV RESEARCH

1) Current

* Induction of cross-protective immunity without exposure to live PRRSV (NPB #08-197). National Pork Board. 2008-2009

* Mechanisms inducing protection against coronaviruses and arteriviruses. Fort Dodge, S.A. 2008-2009

* Plant Production of Vaccines (PLAPROVA, EU 227056). European Communities. 2009-2011

F. WORK PLANNED FOR NEXT YEAR

Evaluation of the protection conferred by the rTGEV expressing PRRSV M protein and GP5 mutants with altered glycosylation. In vivo protection experiments have been already initiated. One-week old piglets were inoculated with wild-type rTGEV or rTGEV viruses expressing PRRSV antigens, and with a plasmid encoding porcine GM-CSF. A boost was performed two weeks post-inoculation. Animals were infected with a virulent EU-type PRRSV strain six weeks after the first vaccination. Samples have been collected to evaluate both humoral and cellular immune response. Viremia, lung damage, and clinical signs will also be evaluated.

Expression of other PRRSV envelope proteins. To analyze other correlates of protection, an rTGEV vector was constructed, expressing PRRSV GP2a, GP3 and GP4. These minor structural proteins are exposed on virus surface assembled as a heterotrimer, and may play a role on protection against PRRSV. A tricistronic rTGEV vector has been constructed, expressing each protein from a separate transcription regulating sequence (TRS). Previous data from our group shown that GP3 expressed from rTGEVs, alone or in combination with GP5, is highly unstable. Therefore, to minimize toxicity effects of GP3, its expression is driven by a weak TRS and the rest of the components of the heterotrimer (GP2a and GP4) are expressed from the same recombinant vector.

Modification of the type of immune response to rTGEVs. The presence of immunostimulatory molecules in coronavirus-derived vectors improves the protection conferred by these vectors. Granulocyte-macrophage colony-stimulating factor (GM-CSF) has been used as an effective adjuvant to induce mucosal immunity (Toka, D., and T., 2004). It has been described that intranasal inoculation of vectors expressing GM-CSF stimulates the production of IFN- γ and IL12p40 in lung (Bukreyev et al., 2001). It has also been recently described that co-administration of GM-CSF improves the efficacy of a vectored vaccine expressing PRRSV antigens (Wang et al., 2009). Mouse hepatitis coronavirus (MHV)-derived vectors co-expressing heterologous antigens and GM-CSF improved vector immunogenicity. Therefore, recombinant TGEV vector expressing porcine GM-CSF is being engineered for its co-administration with rTGEVs expressing the selected PRRSV antigens. Stability and expression levels of GM-CSF will be analyzed previously to its use *in vivo*. The combination of the *in situ* expression of both PRRSV antigenic structures (i.e., GP5 and M) and GM-CSF, by using a modified live attenuated vectors based on TGEV, is a novel approach that will be explored to determine its efficacy in the protection against PRRSV.

Elimination of negative signals in GP5. The objective of this approach is the elimination of epitopes providing T cell negative regulatory signals that may reduce the strength of the immune response to PRRSV antigenic domains involved in protection. Although GP5 is the main inducer of NAbs, previous vaccination approaches based on GP5 expression have shown a delay in the immune response, similar to that obtained after PRRSV infection. One possible explanation is that some domains in GP5 stimulate regulatory T cell negative signals, preventing the development of a strong and rapid immune response against GP5 protein epitopes relevant in protection. In fact, it has been recently described that PRRSV infected dendritic cells induced Treg cells (Silva-Campa et al., 2009). Consequently, a collection of GP5 protein deletion mutants, with deletions distributed throughout the whole protein will be constructed. The humoral and cellular immune responses elicited by these rTGEVs expressing GP5-M heterodimers, containing truncated GP5 proteins, will be analyzed. The protection induced by these vectors will also be evaluated. Using this approach, domains of PRRSV GP5 representing negative antigenic signals will be most likely identified. The final objective is to express from rTGEV vectors the GP5 mutants lacking those domains, to improve the immune response against GP5.

Improvement of TGEV-derived vector stability. rTGEV vectors expressing PRRSV antigens were not fully stable, mainly due to GP5 protein toxicity resulting in a significant loss of GP5 expression in 8 to 10 passages. In contrast, M protein expression is fully stable, with at least 95% of infected cells expressing M protein for more than 10 passages in tissue culture. Loss of PRRSV antigens expression could be the cause for the modest results obtained in protection experiments using live rTGEV vectors, specially compared to those obtained when using killed vaccines formulated with antigens expressed from rTGEV. Therefore, two different strategies will be developed:

1. As PRRSV M protein is fully stable when cloned in rTGEV vectors, it will be the base for vectors co-expressing this protein and different small GP5 protein domains containing the neutralizing epitope. Stability of GP5 domain expression, and GP5 fragment-M heterodimer formation will be monitored in each case. This strategy is complementary to the one described above for the elimination of GP5 protein negative signals.
2. It has been described that coronavirus genomes encoding a mutated nsp14 exonuclease protein (ExoN) displayed a mutator phenotype (Eckerle et al., 2007). TGEV vectors encoding a mutated ExoN and expressing PRRSV antigens will be constructed. It is expected that these rTGEVs accumulate mutations throughout the viral genome, including PRRSV genes. With serial tissue culture passages, only viable rTGEV viruses will be recovered and, from those, the ones stably expressing GP5 protein will be selected. PRRSV ORF5 gene will then be sequenced to analyze the mutations introduced leading to efficient GP5 protein expression. As it is very likely that rTGEV viruses obtained will be highly attenuated for their growth in vivo, the mutated GP5 genes will be introduced in a wild-type rTGEV vector for their use as vaccine vectors.

References

Bukreyev, A., Belyakov, I. M., Berzofsky, J. A., Murphy, B. R., and Collins, P. L. (2001). Granulocyte-macrophage colony-stimulating factor expressed by recombinant respiratory syncytial virus attenuated viral replication and increases the level of pulmonary antigen-presenting cells. *J. Virol.* **75**, 12128-12140.

- Eckerle, L. D., Lu, X., Sperry, S. M., Choi, L., and Denison, M. R. (2007). High fidelity of murine hepatitis virus replication is decreased in nsp14 exoribonuclease mutants. *J. Virol.* **81**, 12135-12144.
- Silva-Campa, E., Flores-Mendoza, L., Resendiz, M., Pinelli-Saavedra, A., Mata-Haro, V., Mwangi, W., and Hernandez, J. (2009). Induction of T helper 3 regulatory cells by dendritic cells infected with porcine reproductive and respiratory syndrome virus. *Virology* **387**, 373-379.
- Toka, F. N., D., P. C., and T., R. B. (2004). Molecular adjuvants for mucosal immunity. *Immunol Rev.* **199**, 100-112.
- Wang, X., Li, J., Jiang, P., Li, Y., Zeshan, B., Cao, J., and Wang, X. (2009). GM-CSF fused with GP3 and GP5 of porcine reproductive and respiratory syndrome virus increased the immune responses and protective efficacy against virulent PRRSV challenge. *Virus Res.* **143**, 24-32.