2009 Harry M. Zweig Memorial Fund for Equine Research Summary Report

2009 celebrated the 30th anniversary of the collaboration between the Harry M. Zweig Memorial Fund for Equine Research and Cornell University. We are pleased to enclose the 2009 Annual Report covering the period of January 1, 2009 through December 31, 2009.

For this reporting period, The Harry M. Zweig Committee granted approval of 12 of 18 submitted projects. Five were new studies, one was a renewal, and six were continuation awards. The total amount allocated for 2009 awards was $594,339. Copies of the investigators' reports are provided in Appendix A.

2009 Harry M. Zweig Memorial Fund for Equine Research Awards

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<th>CONTINUATION</th>
<th>AWARD</th>
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<tr>
<td>Norm Ducharme</td>
<td>Factors Affecting Airway Stability at Exercise: A Combined Neuroanatomical, Clinical and Engineering Methodology (Year 2)</td>
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<td>Julia Flaminio</td>
<td>The Phagocyte Response Against R. equi in Foals (Year 2)</td>
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<td>Susan Fubini</td>
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<tr>
<td>Name</td>
<td>Project Description</td>
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<tr>
<td>Dorothy Ainsworth</td>
<td>Deciphering the Mechanism of Equine Inflammatory Airway Disease in Vitro (1 Year)</td>
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<tr>
<td>Douglas Antczak</td>
<td>Expression Microarrays and Equine Placental Development (2 Year)</td>
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<td>Lisa Fortier</td>
<td>The Role of Telomerase and Small G-Proteins in Senescence of Articular Chondrocytes (1 Year)</td>
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<td>Gillian Perkins</td>
<td>Immunization Against Strangles Using a Vectored Equine Herpesvirus Vaccine (1 Year)</td>
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<td>Bettina Wagner</td>
<td>Analysis of the Innate Immune Response to EHV-1 Infection (2 Year)</td>
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<td>Sylvia Bedford-Guaus</td>
<td>Further Characterization of the Specific Activity and Ultrastructural Localization of Phospholipase C Zeta in Fertile and Subfertile Stallions (1 year)</td>
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<td><strong>TOTAL:</strong></td>
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Completed 2008 Awards
Dr. Dorothy Ainsworth’s project entitled “Modeling Equine Pulmonary Disorders in vitro: Epithelial-Derived Proteins and Inflammatory Airway Diseases” received a no cost extension through July 31, 2009. A Final report is included in this report (Appendix B).

Dr. Fortier’s project entitled “Characteristics of Stem Cells Derived from Bone Marrow Aspirate, Adipose Tissue and Muscle” received a no-cost extension through June 30, 2009. A final report is included in this report (Appendix B).

Dr. Gillian Perkins’ project entitled “Therapy and Prevention of Equine Herpesvirus-1 (EHV-1)-Induced Disease” was awarded a no cost extension through December 31, 2009. A final report is included in this report (Appendix B).

FURTHER SECURED FUNDING FROM RESEARCH AWARDS IN 2009
In November 2009, the Zweig Committee voted to increase the amount of support provided by the Incentive Program from $10,000 to $20,000. The Incentive Program enables the Fund to leverage its investment in Zweig-sponsored research by encouraging Veterinary College faculty to seek either additional or supplementary monies from external sponsors that base their award decisions on a process that involves informed scientific review. The external grant must be for a closely related project. Eligible sponsors include, but are not limited to, the Grayson Foundation, the NIH, the NSF, and the USDA’s National Research Initiative. Recipients provide an annual report on the use of these funds.

The following external grant awards resulted from Zweig funding:

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<th>Principal Investigator</th>
<th>External Award</th>
<th>Sponsor</th>
<th>Project Period</th>
<th>Awarded Amount</th>
<th>Incentive Award</th>
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<tr>
<td>Dr. Lisa Fortier</td>
<td>Genetic Background and Efficient Generation of Induced Pluripotent Stem (iPS) Cells</td>
<td>NYS Department of Health-NYSYSTEM</td>
<td>01/01-09-12/31/10</td>
<td>$190,700</td>
<td>$10,000</td>
</tr>
<tr>
<td>Dr. Douglas Antczak &amp; Dr. Leela Noronha</td>
<td>Characterization of Lymphocyte Modulation in Pregnancy</td>
<td>NIH-National Research Service Award</td>
<td>09/16/08-09/15/11</td>
<td>$209,635</td>
<td>$5,000</td>
</tr>
<tr>
<td>Dr. Alan Nixon &amp; Dr. Ashlee Watts</td>
<td>Gene Induction of Stem Cell Chondrogenesis</td>
<td>NIH-National Research Service Award</td>
<td>04/01/09-03/31/12</td>
<td>$189,498</td>
<td>$5,000</td>
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Bettina Wagner, DVM, PhD  
Harry M. Zweig Assistant Professor in Equine Health

At the November 20, 2009 Annual meeting, Dr. Bettina Wagner was appointed to her 2\textsuperscript{nd} year as the Harry M. Zweig Assistant Professor in Equine Health. Her three-year appointment is for the period January 1, 2009 through December 1, 2011. A progress report is included in Appendix-A of this report.

CORNELL CLINICAL FELLOW IN EQUINE HEALTH

At the 2007 Annual meeting, the Harry M. Zweig Committee approved the allocation of funds to help support a Cornell Clinical Fellow in Equine Health. Dr. Sophy Jesty was selected as Cornell’s 1\textsuperscript{st} Clinical Fellow, supported in part by Zweig funds. Cornell’s College of Veterinary Medicine’s new two-year Clinical Fellows Program is the first in the country to address a growing shortage of academic veterinarians who conduct research on animal diseases and basic biology. The program is designed to help students meet the financial and time demands of qualifying for a position in veterinary academic medicine, which has traditionally required students to complete an M.S. or Ph.D. after they finish their doctorate in veterinary medicine (DVM). The two-year program, available to veterinarians who have completed a three-year residency, offers an annual salary of $50,000 plus benefits and an additional $15,000 per year to fund a research project.
PUBLICATIONS

Publications resulting from awards from the Harry M. Zweig Memorial Fund for Equine Research during 2009 were:

1. Foal monocyte-derived dendritic cells become activated upon rhodococcus equi infection" [Link to Pubmed]

2. Expression of essential B cell genes and immunoglobulin isotypes suggests active development and gene recombination during equine gestation. [Link to Pubmed]

3. Ex vivo generation of mature equine monocyte-derived dendritic cells" [Link to Pubmed]

4. Equine bronchial epithelial cells differentiate into ciliated and mucus producing cells in vitro [Link to Pubmed]

5. Leptospira immunoglobulin-like protein A variable region (LigAvar) incorporated in liposomes and PLGA microspheres produces a robust immune response correlating to protective immunity. [Link to Pubmed]

6. Immune response and prophylactic efficacy of smegmosomes in a hamster model of leptospirosis [Link to Pubmed]

7. Leptosome-entrapped leptospiral antigens conferred significant higher levels of protection than those entrapped with PC-liposomes in a hamster model [Link to Pubmed]

Syed M. Faisal, WeiWei Yan, Sean McDonough, Chao-Fu Chang, Ming-Jeng Pan, Yung-Fu Chang
8. Image-guided tissue engineering of anatomically shaped implants via MRI and micro-CT using injection molding

Ballyns JJ, Gleghorn JP, Niebrzydowski V, Rawlinson JJ, Potter HG, Maher SA, Wright TM, Bonassar LJ.

9. Temporal growth factor release from platelet-rich plasma, trehalose lyophilized platelets, and bone marrow aspirate and their effect on tendon and ligament gene expression

McCarrel T, Fortier L.

http://www.novoseek.com/DocumentDetailAction.action?numdocs=0&filters=&corpus=MEDLINE&criterion=1&showType=2&docId=19615831

Perkins, Gillian A ; Goodman, Laura B ; Tsujimura, Koji ; Van de Walle, Gerlinde R ; Kim, Sung G ; Dubovi, Edward J ; Osterrieder, Nikolaus;

11. A single-nucleotide polymorphism in a herpesvirus DNA polymerase is sufficient to cause lethal neurological disease.


12. Effective treatment of respiratory alphaherpesvirus infection using RNA interference

Fulton A, Peters ST, Perkins GA, Jarosinski KW, Damiani A, Brosnahan M, Buckles EL, Osterrieder N, Van de Walle GR.

13. A point mutation in a herpesvirus polymerase determines neuropathogenicity

Patent updates for 2009

As previously reported, Patent No. 7,036,460 entitled “Throat Support Device and Methods of Using Same” was issued to Dr. Norm Ducharme, etc. al. During 2007 he has been concentrating on obtaining the foreign applications for approval of this device. At the present time this application is still pending.

Dr. Yung-Fu Chang’s 2006 patent application No. 11,102,476 entitled “Novel Immunologenic Proteins of Leptospira” has been approved.

During 2007, Dr. Chang applied for another provisional patent entitled “Immunologenic Proteins for Genome-Derived Outer Membrant of Leptospira and Compositions and Methods Based Thereon.” At the present time this application is still pending.

Zweig News Capsules

There were two issues of the Zweig News Capsule published in 2009. Copies of these issues can be found in Appendix (G).

All Zweig News Capsules (#48 & #49) and can be found at: http://web.vet.cornell.edu/public/research/zweig/Newsletter/index.html.

SUMMARY OF EXPENDITURES

The 2009 Summary of Expenditures was presented and approved at the Zweig Committee Annual Meeting in November 2008. (Appendix C).

PUBLIC RELATIONS AND ADMINISTRATIVE BUDGET

The 2010 Public Relations and Administrative Budget was presented and approved by the Zweig Committee at their November 2009 annual meeting (Appendix D).

2010 ZWEIG PROGRAM

Nine projects were approved for funding, from a roster of 17 applications, at the Harry M. Zweig Memorial Fund annual November (2009) meeting. Of these studies, five were new studies, one was a supplemental, and three were continuations. The list for projects funded for 2010 are shown in (Appendix E).
APPENDIX A

Progress & Final Reports Resulting from 2009 Funding
2009 Harry M. Zweig Memorial Fund for Equine Research
Progress Reports

Final & Annual Reports Resulting from 2009 Funding

Dr. Ainsworth          Deciphering the Mechanism of Equine Inflammatory Airway Disease in Vitro
Dr. Antczak            Expression Microarrays and Equine Placental Development
Dr. Bedford-Guaus      Further Characterization of the Specific Activity and Ultrastructural Localization of Phospholipase C zeta in fertile and Subfertile Stallions
Dr. Ducharme           Factors Affecting Airway Stability of Horses at Exercise: A Combined Neuroanatomical, Clinical and Engineering Methodology
Dr. Flaminio           The Phagocyte Response Against R. Equine in Foals
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Dr. Perkins            Immunization Against Strangles Using a Vectored Equine Herpesvirus Vaccine
Dr. Wagner             Analysis of the Innate Immune Response to EHV-1 Infection
Harry M. Zweig Memorial Fund
for Equine Research

2009 Annual Report

P.I.: Dr. Dorothy Ainsworth

Title: Deciphering the Mechanism of Equine Inflammatory Airway Disease in vitro

Project Period: 1/1/09-12/31/09
Reporting Period: 1/1/09-12/31/09
Dr. Ainsworth was granted a no cost extension through December 31, 2010. An annual report is provided.
Title: Deciphering the mechanism of equine inflammatory airway disease in vitro

Principal Investigators: Dorothy M. Ainsworth, Hollis N. Erb (statistical consultant)

Hypothesis: Inhalation of hay dust components activates the NF-κB intracellular signaling pathway via specific cell surface receptors to induce epithelial chemokine production.

Goals: To determine which cell surface receptors are involved in hay dust induced up-regulation of pro-inflammatory cytokine gene expression in bronchial epithelial cells. The specific receptors that would be examined in this study include the toll-like receptor 4 (TLR4); the tumor necrosis factor alpha receptor (TNF-α R) and the interleukin-1 receptor (IL-1R). All of these receptors eventually signal through the NF-κB pathway.

Rationale: Inflammatory airway disease is a common, performance limiting condition of the equine athlete. However, the driving force or stimulus for influx of inflammatory cells (neutrophils) into the lower airways is currently unknown. We hypothesize that inhaled hay dust interacts with the airway (bronchial) lining cells to promote inflammatory mediator production and neutrophil emigration into the lung. The specific receptors on the bronchial epithelial cells that are involved in this inflammatory pathway are unknown and are the subject of this investigation. By identifying the cellular receptors involved, appropriate intervening therapies may be designed to prevent inflammatory airway disease in racehorses.

Identification of the cell surface receptors activated by hay dust components would be conducted in vitro utilizing established equine bronchial epithelial cell cultures.

Progress to date: Both primary and differentiated epithelial cell cultures (air-liquid interface) have been established from 3 different horses. However, we have not been able to maintain the differentiated epithelial cell cultures for sufficiently long enough time periods that would allow us to activate and/or block the cell receptors and intracellular signaling pathways of interest. One of the biggest determinants to our success has been fungal (Aspergillus) contamination of the bronchial epithelial cell cultures at the differentiation stage. We are attempting to circumvent this problem by using different anti-fungals in our isolation medium and by collecting lung samples from horses that have been pastured outside (reducing inhalation of Aspergillus spores that are commonly found in the hay). We have also been in contact with several other individuals from 2 different universities (Cleveland, Fort Collins) who have successfully established differentiated epithelial cell cultures in other species with the hope of adapting some aspects of their protocols to improve our success rates.
Harry M. Zweig Memorial Fund
for Equine Research

2009 Annual Report

P.I.: Dr. Douglas Antczak

Title: Expression Microarrays and Equine Placental Development

Project Period: 1/1/09-12/31/10
Reporting Period: 1/1/09-12/31/09
PROJECT TITLE: Expression Microarrays and Equine Placental Development

PRINCIPAL INVESTIGATOR(S): Douglas F. Antczak

In the coming two years (2009 and 2010) we propose to use the exciting genome-level technology of expression microarrays to explore how the cells of the placenta interact with the mother’s immune system cells and with other cells in the uterus during the period of implantation. We will determine the genes expressed by invasive placental cells that may lead to maternal immunological tolerance during pregnancy and to successful implantation. We will also test the hypothesis that pregnancy induces a state of partial immune tolerance in the pregnant mare that reduces her ability to respond to pathogenic viruses such as equine herpesvirus type 1. Lymphocytes from pregnant and non-pregnant mares, and lymphocytes from the uterus and peripheral blood will be compared in tightly controlled experiments.

Background and Significance: Over the past decade the Zweig Memorial Fund has provided critical support for Cornell’s participation in the international collaboration of the Horse Genome Project. This support has enabled the College of Veterinary Medicine to play a leading role in the development of the resources in equine genomics that are now available to veterinary scientists worldwide. Now that the complete sequence of the horse genome has been determined, many new avenues of equine research have been opened. It is important to consider how Cornell can continue to play a leadership role in equine research and remain at the forefront of genetic-based medicine for the horse.

There are two principal areas of application of the equine genome. First is the study of genetic variation in horses; it is widely accepted that the use of Single Nucleotide Polymorphisms (SNPs) is the method of choice at this time. The so-called “SNP chips” allow variation at thousands of sites across the genome to be compared between horses in a single experiment. Second is the use of gene expression assays with microarrays to determine the relative activity of most or all of the estimated 20,000+ genes in the genome, again in single experiments that compare normal and disease states, or responses to different therapeutic treatments.

The enormous scale of these two whole-genome approaches will allow scientists to evaluate not just the activity of a few genes in an experiment, but many thousands of genes. The computer support (bioinformatics) required for analysis of such experiments can be daunting, but fortunately Cornell has invested well in this developing area of research over the past several years, and opportunities for collaboration with leading bioinformatics scientists abound in Ithaca.

Specific Aims for 2009-2010:
Aim 1 (year 1, 2009): we would compare global gene expression in invasive chorionic girdle trophoblast cells with expression in the non-invasive trophoblast of the allantochorion and other conceptus tissues. The main hypothesis for this aim is that the invasive trophoblast cells of the placenta will express genes associated with tumor invasion and immuno-modulatory molecules that are not expressed by the non-invasive trophoblast, which does not come into intimate contact with immune cells of the mother.

Aim 2 (year 2, 2010): we would contrast gene expression patterns in normal and failing early equine pregnancies and also compare gene expression in the endometrium under these two different conditions. These Antczak, DF Zweig Continuation for 2010 2 experiments may be able to distinguish between pregnancy loss from primary fetal / placental demise versus inadequate uterine environment for placental development.
Aim 3 (year 2, 2010): we would compare global gene expression between lymphocytes isolated from around the endometrial cups with control lymphocytes from other sites, including peripheral blood from pregnant and non-pregnant mares. In these experiments we would test the hypothesis that the T cells surrounding the invasive endometrial cups express a phenotype dominated by regulatory T cells compared to T cells from other sites.

**Recent Laboratory Progress (prior to 2009):**

**Horse Genome Project Workshop:**
The past two years, in which the entire genome sequence of the horse has been determined, have had a transforming effect on equine research. In the table below are listed several new web sites that contain information on the genome of the horse. All of these web sites were developed in the past two years as a result of the whole genome sequencing of the horse. The sequence data and related bioinformatic information in these sites is a tremendous resource for equine researchers worldwide.

**Websites relevant to horse genetics: Web Site Contents**
- MIT Broad Institute Horse Genome Project
- NIH Horse Genome Sequence News Release
- NIH NCBI Horse Genome Resources
- NCBI BLAST Horse Sequences
- NCBI Entrez Horse Genome Project
- UCSC Horse Genome Browser Gateway
- Horse Genome Sequence Assembly News Release
- Ensembl Database Horse Genome Sequence information

**Web Address**
- http://www.broad.mit.edu/mammals/horse/
- http://www.genome.gov/20519480
- http://genome.ucsc.edu/cgi-bin/hgGateway
Harry M. Zweig Memorial Fund
for Equine Research

2009 Annual Report

P.I.: Dr. Sylvia Bedford-Guaus

Title: Further Characterization of the Specific Activity and Ultrastructural Localization of Phospholipase C zeta in Fertile and Subfertile Stallions

Project Period: 1/1/09-12/31/10
Reporting Period: 1/1/09-12/31/09
CONTINUATION APPLICATION

PROJECT TITLE: Further Characterization of the specific activity and ultrastructural localization of phospholipase C zeta in fertile and subfertile stallions.

PRINCIPAL INVESTIGATOR(S): Sylvia Bedford-Guaus, DVM, PhD and Mark Roberson, PhD

Our standard methods for breeding soundness evaluation and routine sperm laboratory tests do not provide a direct assessment of stallion fertility. Measures of sperm motility or sperm morphology are only marginally correlated with pregnancy rates (Jasko et al., 1990, 1992). Fertile sperm must be able to reach the site of fertilization, fertilize the oocyte and trigger the initiation of embryonic development. Only tests that directly evaluate these sperm functions can be directly correlated with actual fertility. In this regard, we have begun the characterization of a sperm-specific enzyme, phospholipase C zeta (PLCζ), which is responsible for inducing embryonic development at fertilization in all mammalian species studied thus far. Because species-specific differences in PLCζ activity have been reported, it is essential to characterize PLCζ in the stallion prior to its use as a tool for fertility evaluation.

Notably, a subfertile stallion was recently presented for evaluation at our equine breeding facility at Cornell University. Although this stallion had borderline semen quality, he was able to produce an average of 700 million progressively motile, morphologically normal sperm per day, which should be sufficient to get mares in foal. However, this stallion was subfertile with pregnancy rates ≤ 20%. Semiquantitation of PLCζ showed that this stallion had relatively less amounts of PLCζ when compared to one of our proven fertile stallions, and this may explain the reported low fertility rates of this stallion. This example underscores the importance of characterizing PLCζ in fertile and subfertile/infertile stallions with the long term goal of using this as part of a battery of tests to evaluate sperm function and thus stallion fertility.

What is phospholipase C zeta (PLCζ)?

In all mammalian species studied thus far, including the horse (Bedford et al., 2003, 2004), the sperm’s contribution at fertilization is not limited to providing the male’s DNA. Importantly, the sperm is also responsible for initiating and maintaining embryonic development. The way in which the sperm triggers embryonic development is by introducing a protein within the oocyte (egg) at the time of fertilization which induces intracellular calcium ([Ca²⁺]) rises that are termed ‘oscillations’. These [Ca²⁺] oscillations occur with a frequency that is species-specific and last for several hours (reviewed by Fissore et al., 1998).

Recent research has shown that the protein introduced by the sperm (referred to as the ‘sperm factor’) responsible for these [Ca²⁺] oscillations is the enzyme PLCζ (Saunders et al., 2002). This is a testis-specific protein that has been shown to be the ‘sperm factor’ in all mammalian species studied thus far (Cox et al., 2002; Saunders et al., 2002; Kurokawa et al., 2005; Bedford et al., 2006, 2008). PLCζ catalyzes the hydrolysis of phosphoinositides to liberate an important second messenger, inositol trisphosphate or IP3. In turn, IP3 facilitates the release of intracellular calcium within stores via a specific IP3 receptor present on the endoplasmic reticulum. The gene sequence for PLCζ has been fully characterized in humans, cynomolgus monkeys, mice, rats (reviewed by Swann et al., 2006), cattle, pigs and dogs. Although this protein is highly conserved, differences in sequence appear to be critical for species-specific differences in PLCζ activity. We have cloned the sequence for equine PLCζ. Understanding how this sequence relates to its specific activity is the next step in the characterization of PLCζ for evaluation of stallion fertility.
PLCζ in the Context of Male Fertility

In human couples seeking in vitro technology to have a baby, it is estimated that about 3% of cases of unexplained infertility in men are characterized by phenotypically ‘normal’ sperm with an inability of the sperm to initiate embryonic development of the egg (Eldar Geva et al., 2003; Mahutte and Arici, 2003). In couples where the men have abnormally shaped sperm, this percentage is much higher (~70%; Battaglia et al., 1997; Rybouchkin et al., 1997; Mahutte and Arici, 2003), supporting the possibility that dysfunction in the PLCζ protein may be causal in defining important mechanisms of male infertility.

Proposed Research

Our preliminary conclusions in the characterization of equine PLCζ thus far can be summarized as: i) the predicted protein sequence for equine PLCζ shows highest homology with that of porcine PLCζ, with lesser homology observed in the region known to be important for its specific activity; ii) equine PLCζ is expressed in later stages of the spermatogenic sperm cell lineage, namely starting at the round spermatid stage; and, iii) in all stages of sperm maturation, namely epididymal, ejaculated and capacitated sperm, PLCζ is localized to both head and tail regions of the sperm. In order to further characterize equine PLCζ we propose three Specific Aims:

1. To characterize the specific [Ca2+]-releasing activity of immature testicular sperm cells, of different regions of mature sperm, and of equine PLCζ in oocytes. Our hypothesis is that round spermatids obtained from equine testicular tissue and both the head and tail of equine sperm have the ability to induce [Ca2+]i oscillations in oocytes and this is correlated to the specific activity of equine PLCζ. Our hypothesis is based on preliminary results showing that PLCζ expression is observed in round sperm cells in testicular tissue and that PLCζ localizes to both head and tail regions of stallion sperm. To test our hypothesis, round spermatids, sperm heads or tails will be injected into oocytes to monitor their [Ca2+]-releasing activity. These studies might reveal fundamental differences in the physiology of PLCζ expression from what has been reported in other species. For instance, mice round spermatids do not possess PLCζ activity, and thus cannot initiate embryonic development when injected into oocytes (Kimura and Yanagimachi, 1995). Moreover, PLCζ activity has not been identified in the tail of any other mammalian species studied thus far (Yoon and Fissore, 2007; Young et al., 2008). In order to test the specific activity of equine PLCζ, we will produce and purify equine PLCζ RNA in vitro and different concentrations of this product will be injected into oocytes. In these studies, oocytes will be monitored for [Ca2+], oscillations as previously described (Bedford et al., 2006, 2008). These studies are critical to determine the bioactivity of PLCζ expressed in different stages of sperm maturation and/or different regions of the equine sperm, and of the novel equine PLCζ clone.

2. To investigate the ultra structural localization of PLCζ in equine sperm. Our hypothesis is that PLCζ with high enzymatic activity is localized to specific membrane fractions known as membrane rafts. Previous research suggests that PLCζ may be localized in specific membrane regions, in particular that underlying the acrosome (cap over the sperm head; Kurokawa et al., 2005). Immunofluorescence studies in our lab have localized PLCζ over areas of the sperm head and tail. However, these findings do not give us detailed information as to the ultrastructural localization of PLCζ and whether it is associated with specific membrane regions. We are interested in investigating whether PLCζ is stored in specific regions of enriched enzymatic activity (membrane rafts). For this purpose, sperm domains (membrane rafts, organelles) will be fractionated by differential centrifugation and the presence of PLCζ in the different membrane domains identified with the use of PLCζ-specific antibodies. Additionally, these fractions will be injected into oocytes to ascertain their ability to trigger [Ca2+]-oscillations.

3. To determine PLCζ levels and regional expression in equine sperm from stallions of variable fertility. Our hypothesis is that the innate level of PLCζ protein and its localization in equine sperm are functionally correlated with the fertility potential. The relative amount of PLCζ in sperm from a give
species is correlated with the specific [Ca²⁺]-releasing activity of their sperm (Kurokawa et al., 2005). Thus, we predict that some stallions of low relative fertility will have markedly reduced levels of PLCζ/sperm compared to stallions of high relative fertility, as already observed for one stallion that presented to our clinic with adequate sperm numbers yet low fertility rates. To test this we will examine equine PLCζ protein levels in sperm samples collected from stallions of low and high fertility based upon breeding records and traditional methods of breeding soundness evaluation. In stallions displaying decreased PLCζ expression we will further investigate its regional localization in sperm. This may yield information not only regarding relative quantity of PLCζ but also its pattern of expression and bioavailability as correlated to its ability to induce oocyte activation and thus as it relates to stallion fertility.

In summary, we propose to fully characterize equine PLCζ in regards to bioavailability, specific activity, and as a tool to evaluate stallion fertility. This research will greatly benefit the race-horse breeding industry as it relates to using PLCζ as a molecular tool to predict and/or evaluate stallion fertility. Moreover, we have already identified some relevant species-specific characteristics of equine PLCζ; further in depth study of these differences will greatly enhance our overall knowledge in basic mammalian reproductive physiology using the horse as a model.

References


Bedford SJ, Kurokawa M, Hinrichs K, Fissore RA. Patterns of Intracellular Calcium Oscillations in Horse Oocytes Fertilized by Intracytoplasmic Sperm Injection: Possible Explanations for the Low Success of This Assisted Reproduction Technique in the Horse. Biol Reprod 2004:7;936-944.


Kimura Y, Yanagimachi R. Mouse oocytes injected with testicular spermatozoa or round spermatids can develop into normal offspring. Dev 1995;121;2397-2405.


**PROGRESS REPORT:**

In order to further characterize equine PLC\(\zeta\) in fertile and subfertile stallions, we proposed three **Specific Aims:**

1. **To characterize the specific [Ca\(^{2+}\)]\(_i\)-releasing activity of immature testicular sperm cells, of different regions of mature sperm, and of equine PLC\(\zeta\) in oocytes.** Our *hypothesis* is that round spermatids obtained from equine testicular tissue and both the head and tail of equine sperm have the ability to induce intracellular calcium ([Ca\(^{2+}\)]\(_i\)) oscillations in oocytes and this is correlated to the specific activity of equine PLC\(\zeta\).

2. **To investigate the ultrastructural localization of PLC\(\zeta\) in equine sperm.** Our *hypothesis* is that PLC\(\zeta\) with high enzymatic activity is localized to specific membrane fractions known as membrane rafts.

3. **To determine PLC\(\zeta\) levels and regional expression in equine sperm from stallions of variable fertility.** Our *hypothesis* is that the innate level of PLC\(\zeta\) protein and its localization in equine sperm are functionally correlated with the fertility potential.
Our specific aims have not changed and below we summarize the results obtained to date and immediate future plans.

B) Describe the studies direction, positive and/or negative, toward the specific aims during the current budget year and the results obtained.

SA-1: To characterize the specific \([\text{Ca}^{2+}]\)-releasing activity of immature testicular sperm cells, of different regions of mature sperm, and of equine PLC\(\zeta\) in oocytes.

Our preliminary data demonstrated positive immunoblotting for PLC\(\zeta\) both in separated head and tail stallion sperm fractions. In order to investigate whether this result translated into catalytically active PLC\(\zeta\) for both sperm fractions, we injected heads and tails that had been separated by sonication into matured mouse oocytes. As anticipated, 7/8 oocytes injected with a single sperm head and 12/16 oocytes injected with a single sperm tail displayed \([\text{Ca}^{2+}]\) oscillations (\(P>0.05\); Fig. 1); conversely, none (0/10) of the oocytes injected with the sperm-containing medium or sham injected displayed \([\text{Ca}^{2+}]\) responses. For both sperm heads and tails, \([\text{Ca}^{2+}]\) oscillations lasted for as long as they were monitored (80-140 min). This is an intriguing result that reflects inherent differences in the expression of enzymatically active PLC\(\zeta\) to what has been reported in all other species studied thus far.

Our equine PLC\(\zeta\) clone presented some problems that precluded our ability to synthesize a protein of the appropriate size, suggesting errors in nucleotide sequence. This has now been addressed, and our PLC\(\zeta\) clone yields a protein of the appropriate size (~73kD) when expressed in a cell-free system. The cDNA sequence of this clone was tagged with an HA epitope to facilitate protein detection. As shown in Fig. 2, the resulting protein is recognized both by antibodies against the HA epitope and an antibody against the N-terminal epitope of PLC\(\zeta\). This is an important finding since it relates to our ability to synthesize equine PLC\(\zeta\) cRNA that can be injected into oocytes to analyze its specific catalytic activity.
SA-2: To investigate the ultrastructural localization of PLCζ in equine sperm.

We have presently followed a detergent-based method to separate raft fractions in equine sperm lysates followed by immunoblotting for PLCζ. Preliminary experiments suggest that PLCζ protein may partition, at least partially, into non-soluble fractions. This suggests that at least some of the PLCζ expressed in equine sperm may be localized to membrane raft fractions.

A3. To determine PLCζ levels and regional expression in equine sperm from stallions of variable fertility.

We have analyzed sperm from 6 additional stallions, four of which were subfertile as assessed by either ≤30% pregnancy rates/season and/or only achieving adequate pregnancy rates (~60%) when bred to a small book of mares (~15 season). Sperm from the two fertile stallions, as expected, displayed normal levels of PLCζ expression. One of the four subfertile stallions also expressed normal levels and localization of PLCζ; as expected, not all cases of subfertility can be attributed to problems with PLCζ expression. Interestingly, the other three subfertile stallions displayed normal levels of PLCζ as assessed by semiquantitative immunoblotting; however, indirect immunofluorescence showed a pattern of PLCζ expression that we have not previously observed. In sperm from all fertile stallions analyzed thus far, PLCζ localizes over the acrosomal region, subacrosomal ring, midpiece-to-head junction, and principal piece of the flagellum. Moreover, we have now also ascertained that the induction of acrosomal exocytosis, and thus loss of the acrosome, results in concomitant loss of PLCζ over the acrosomal region (data not shown). In the three subfertile stallions 14, 10 and 1% of sperm cells, respectively, showed no PLCζ immunostaining over the acrosomal region, despite the presence of an intact acrosome (Fig. 3). Therefore, our results to date suggest that PLCζ deficiencies in subfertile stallions may manifest as decreased levels or abnormal localization.

C) Summarize your plans to address the Specific Aims during the next year of support.
SA-1: To characterize the specific [Ca^{2+}]-releasing activity of immature testicular sperm cells, of different regions of mature sperm, and of equine PLCζ in oocytes. We are presently synthesizing equine PLCζ cRNA and microinjections into mouse oocytes to begin the characterization of its [Ca^{2+}]-releasing activity should begin by mid-October 2009. The separation of immature testis cells for the characterization of PLCζ catalytic activity will be performed during the second year of support beginning in January 2010.
Harry M. Zweig Memorial Fund
for Equine Research

2009 Annual Report

P.I.: Dr. Norm Ducharme

Title: Factors Affecting Airway Stability of Horses At Exercise: A Combined Neuroanatomical Clinical and Engineering Methodology

Project Period: 1/1/08-12/31/09
Reporting Period: 1/1/09-12/31/09
Dr. Ducharme was granted a no cost extension through June 30, 2010. An annual report is provided.

Investigators: Norm G Ducharme and Jeremy Rawlinson

The overall goal of this proposal was to couple bioengineering methods with clinical research to improve our understanding and develop better treatments to correct instability of the upper airway produced by dorsal displacement of the soft palate (DDSP) and recurrent laryngeal neuropathy (RLN). The experiments we performed determined the effect of laryngohyoid position, soft palate stiffness, and laryngeal biomechanics on upper airway stability. We used a combined approach of in vivo, in vitro, and in silico (computational) methods to tackle the complexity of these problems. Specifically, through in vivo and in vitro experiments, we identified the intrinsic and extrinsic tongue muscles controlling laryngohyoid conformation and nasopharyngeal stability. Using in vitro mechanical testing and with computational constitutive analysis, we calculated the biomechanical properties of the larynx and soft palate. Coupled with medical imaging reconstructions, these data allowed us to generate computer models of these soft tissue structures and investigate implant properties and implantation technique to improve airway stability. These models were also compared with in vitro experiments of palate and laryngeal function as a validation step.

Specific Aims and Findings:

Aim 1. Determine the effects of selected muscles (hyoglossus, genioglossus, styloglossus, and geniohyoideus) activity on laryngohyoid conformation and nasopharyngeal stability by in-vivo studies using imaging (radiography and CT) and a treadmill stress test to collect airway pressures EMG, and video endoscopic images.

(Abstract from publication)

The equine upper airway is highly adapted to provide the extremely high oxygen demand associated with strenuous aerobic exercise in this species. The tongue musculature, innervated by the hypoglossal nerve, plays an important role in airway stability in humans who also have a highly adapted upper airway to allow speech. The role of the hypoglossal nerve in stabilizing the equine upper airway has not been established. Isolated tongues from eight mature horses were dissected to determine the distal anatomy and branching of the equine hypoglossal nerve. Using this information, a peripheral nerve location technique was used to perform bilateral block of the common trunk of the hypoglossal nerve in 10 horses. Each horse was subjected to two trials with bilateral hypoglossal nerve block and two control trials (unblocked). Upper airway stability at exercise was determined using videoendoscopy and measurement of tracheal and pharyngeal pressure. Three main nerve branches were identified, medial and lateral branches and a discrete branch that innervated the geniohyoid muscle alone. Bilateral hypoglossal block induced nasopharyngeal instability in 10/19 trials, and none of the control trials (0/18) resulted in instability (P<0.001). Mean treadmill speed (+/-SD) at the onset of instability was 10.8+/-2.5 m/s. Following its onset, nasopharyngeal instability persisted until the end of the treadmill test. This instability, induced by hypoglossal nerve block, produced an expiratory obstruction similar to that seen in a naturally occurring equine disease (dorsal displacement of the soft palate, DDSP) with reduced inspiratory and expiratory pharyngeal pressure and increased expiratory tracheal pressure. These data suggest that stability of the equine upper airway at exercise may be mediated through the hypoglossal nerve. Naturally occurring DDSP in the horse shares a number of anatomic similarities with
obstructive sleep apnea. Study of species with extreme respiratory adaptation, such as the horse, may provide insight into respiratory functioning in humans.

Aim 2. Determine cricoarytenoid joint movement using MRI studies and determining the biomechanical properties of the laryngeal cartilages and soft palate by testing fresh laryngeal cadavers.

In the first part of this aim, we used reconstruction software to segment the cartilage tissue in medical images from a magnetic resonance imaging (MRI) dataset. The MRI data were obtained in an in vitro experiment using prosthetic laryngoplasty where the arytenoid was sequentially placed in greater abduction between imaging sessions. Thus, we produced several models to relate the full 3D position of the arytenoid with respect to the cricoid during abduction under increasing suture forces.

(Abstract from conference presentation)

With a prosthetic laryngoplasty for recurrent laryngeal neuropathy, the suture anchor site at the cricoid or continuing loss of arytenoid abduction limit clinical success. The purpose of this study was to measure the compressive mechanical properties of the hyaline cartilage to better understand the anchoring and deformation characteristics. Eight larynges were harvested from Thoroughbreds and Standardbred at necropsy. Biopsy specimens were obtained from three sites within the dorsal cricoid (caudal, middle, and rostral) and two central sites in the arytenoids (inner, outer). In a mechanical testing frame, the specimens were loaded to calculate the compressive stiffness according to bioengineering protocols; the data were analyzed using nested ANOVA. There were significant observations of higher modulus with increasing age (0.13 MPa per year; p=0.012) and stiffer cricoid cartilage (2.27 MPa) than the arytenoids (0.43 MPa; p<0.001), but no difference was observed between the left and right sides. Linear contrasts showed that the rostral aspect (2.51 MPa) of the cricoid was 24% stiffer than the caudal aspect (2.02 MPa; p=0.007), with no difference between the arytenoid sites. These results indicate that the suture, therefore, would pass through the least stiff region of the cricoid. The arytenoid is also a structure of relatively lower stiffness in the central region, for reference, equine articular cartilage is ~0.21 MPa. These characterizations are important to understand the laryngeal function and will continue with tissue failure properties.

Aim 3. Measure palatal stiffness and the maximal physiological increase possible by implants to test how changes in soft palate stiffness and laryngohyoid position affects upper airway wall pressures using a computer fluid model.

Using both in vitro and in silico methods, we investigated the use of implants to reduce tissue deformation (Pillar procedure) and improve airway stability. In this study custom made materials used for treatment of palate vibration in humans are investigated.

*In vitro modeling:* We first developed an ex-vitro model of exhalation at 2Hz producing leading to oscillatory expiratory flow at physiological range of horses exercising at maximal exercise intensity. Adult equine soft palates (n=7) were collected at necropsy from various breeds and immediately frozen to -20ºC. Twelve hours prior to testing frozen soft palates were thawed in a refrigerator and then placed in a physiologic saline bath once thawed. The caudal aspect of the soft palates (mean length ± SD = 7.9 ± 1.9 cm) were placed in the model and allowed to vibrated under high speed video recording (i.e. 1000 fps). The treatment group consisted of 3 implants placed on the caudal edge of the soft palate parallel to the long axis of the soft palate and each other. We found no effect of treatment on palate vibration as measured through video capture or through fundamental frequency of the sound produced. The amplitude of the oscillation of the palate was unaffected by the treatment and therefore the degree of obstruction was unaffected by treatment as
assessed by calculation of impedance of the system. This led to the conclusions that either the principle of treatment is wrong or the optimal configuration of the implants was not obtained.

*In silico* testing: To obtain the biomechanical properties of the palate we used a confined compression test similar to the laryngeal cartilage. A large variability with a coefficient of variation greater than 100% was observed in these tissue properties (HA=0.15±0.20 MPa) due to sections being friable. This required an adaptation in the models to incorporate such variation. The model was first ‘tuned’ so that for the baseline condition, the amount of palate displacement corresponded to the baseline condition for the in vitro experiment. In essence this calibrates the model within the range observed in the mechanical testing. The same implant tests performed in vitro were conducted in the model and the relative changes between the two were calculated. By assessing the relative effects, we validated the model within the range of tissue properties. Further variations to the implant technique were incorporated in the model to rapidly assess new positions and implant dimensions. We determined that while rostral-caudal stiffeners implanted at the midline do reduce palate displacement, the relative change is modest and may not be clinically significant. The models indicated that rotating the implants in more lateral positions (30-45-90 degrees to the midline) had a greater reduction in palate displacement due to the bowed shape of the palate during DDSP.

**Aim 4.** Determine the ideal implant for stabilizing the larynx using biomechanical modeling to quantify the direction and magnitude of the forces acting on the muscular process.

In this aim, we developed a 3D model of arytenoid and cricoid deformation based upon the tissue properties calculated and the images reconstructed in Aim 2. One limitation that arose in developing these model concerns the cricoarytenoid joint – the saddle shaped surface that guides the motion of the arytenoid with respect to the cricoid. This obstacle arose due to the resolution of the MR images. From our biomechanical assessment, the elliptical contours of the joint surface were approximately 15 x 8 mm and oblique to the plane of the MR images. This geometry means that with the MRI spacing at 3mm, only 1-2 slices of MR data were obtained in the region of the joint. This was not adequate for properly reconstructing the joint surface and, therefore, not adequate for modeling the relative motion of the arytenoid with respect to the cricoid. Mechanically, the load path we were analyzing includes the pressure acting to deform the arytenoid. This generates a force on the muscular process, induces motion at the cricoarytenoid joint, stretches the prosthetic suture, and compresses the caudal cricoid. In this load path, the deformation at the muscular process was a limiting step; its tissue structure varies from both the arytenoid and cricoid with possibly higher ratio of elastic and fibrocartilage compared to the hyaline cartilage of the other two. Thus, the deformation characteristics are potentially very different. We are addressing this with additional mechanical testing to identify the stiffness of the tissue in the region of the muscular process. Thus, the model requires some ongoing additions to ensure physiological validity before we can assess the relative deformations along this chain of support.

**Significance:**
There were several significant findings as well as areas of improvement in our experimental approaches. Understanding the neuroanatomical basis has generated new hypotheses for treatment - stability of the equine upper airway at exercise may be mediated through the hypoglossal nerve. This led to a new model of the disease and we are now investigating whether a new treatment targeting the action of the hypoglossal nerve can be designed using neuroprosthesis. Quantifying the mechanical properties of the laryngeal tissue has identified the regions of concern in the cartilaginous support and
provided new thoughts for research in tissue engineering. These elements have led to the first reports of properties for these tissues in horses; this is a sine qua non basis of this year’s new grant proposal to Zweig. Lastly, we have quantified the effects of different surgical techniques on airway stability to improve surgical correction of the soft palate. Although the technique used in humans does not appear valuable to horses, the modeling suggests the length and exact position of similar devices might be useful in horses to reduce airway obstruction or noise.

**Conference Presentations:**


Ducharme NG. Treatment of DDSP. World Equine Airway symposium, Bern Switzerland August 2009.

Ducharme NG. Update on the treatment of DDSP. World Equine Veterinary Congress, Guarujá, Brazil September 2009.

**Publications:**


Passman SN, Cheetham J, Bonassar LJ, Ducharme NG, Rawlinson JJ. Biomechanical Stiffness of Equine Laryngeal Hyaline Cartilage. Equine Veterinary Journal (to be submitted Dec 2009)

Priest D, Passman SN, Cheetham J, Ducharme NG, Rawlinson JJ. A Vibrational Experiment and Computational Analysis of Palate Implants in the Equine Airway. Equine Veterinary Journal (to be submitted Jan 2010)

Cheetham J, Rawlinson JJ, Burnett N, Ducharme NG. Magnetic Resonance Imaging and Three Dimensional Analysis of In Vitro Arytenoid Motion in the Equine Larynx. (In Preparation)
Listing of grant applications and their status resulting from Zweig funding:

Pending:

Qatar Foundation 2010: “Integrating medical imaging, neurodiagnostic, and engineering approaches to diagnose respiratory dysfunction in the equine athlete” Norm Ducharme and Jeremy Rawlinson. Co-Investigators: Jon Cheetham, Robin Gleed, Tom Divers, Hussni Mohammed and Heidi Reesink

Approved:
Harry M. Zweig Memorial Fund
for Equine Research

2009 Final Report

P.I.: Dr. Julia Flaminio

Title: The Phagocyte Response Against R. equi in Foals

Project Period: 1/1/08-12/31/09
Reporting Period: 1/1/09-12/31/09
Summary:
Susceptibility to R. equi pneumonia in foals is exclusive to the first few months of life. This bacterium causes severe pneumonia, enteritis and occasionally joint infection, with significant economic losses to the horse industry, and critical concerns about equine health and well-being. R. equi is prevalent in the horse environment (soil), and foals are exposed to it immediately after birth. Importantly, R. equi survives and replicates inside immune cells (macrophages). Therefore, R. equi evolved a mechanism to escape bactericidal activity in macrophages, which, paradoxically, are important cells of the immune system that perform surveillance, removal and killing of microorganisms. Phagocytes participate in the early stages of immune response by removing and killing pathogens. Foal neutrophils are bactericidal to R. equi. In contrast, R. equi readily invades alveolar macrophages, multiplies and causes their destruction, favoring its replication in the respiratory environment. Nevertheless, efficient killing of R. equi by macrophages is dependent on their activation by cytokines, for the generation of bactericidal products. In the respiratory tract, these cytokines and other mediators are produced by both immune cells and respiratory epithelial cells.

Our hypothesis is that phagocytes of the susceptible foal have impaired killing activity against R. equi because of inadequate signaling from the airway epithelial cells.

Specific Aims and Findings:
In this proposed investigation, we have:
1) tested the capacity of foal phagocytes to become activated in the presence or absence of cytokines or R. equi in culture. We measured a) the expression of cell surface molecules in macrophages that support acquired immune system (T cell) activation (MHC class II molecule) (Figure 1A); b) the expression of pro-inflammatory cytokine TNFα (Figure 1B); c) and the generation of pathogen killing products (oxidative burst activity) (Figure 1C) in foal macrophages or neutrophils, and compared those to adult horse cells.

Figure 1A - Foal macrophages (MO, n = 8) had comparable expression of MHC class II to adult horse macrophages (n = 8). Lipopolysaccharide (LPS) and interferon gamma (IFNγ) treatment for 48 hours, with the presence of R. equi for the last 12-14 hours of culture did not change significantly the expression of this molecule. Nevertheless, overall, foal macrophages presented greater variability in the expression of MHC class II than adult horse macrophages, supporting our previous observation of age-dependent expression of this molecule on antigen presenting cells of foals. For these experiments, macrophages were cultured in the absence of bronchial epithelial cells.
Figure 1B - In the absence of R. equi, foal macrophages (n = 8) had lower expression (* p < 0.03) of TNF • than adult horse macrophages (n = 8), despite LPS or IFN • stimulation for 48hrs of culture. In contrast, in the presence of R. equi for the last 12 hrs of culture, the difference between foal and adult macrophages was eliminated (p > 0.05), suggesting that R. equi stimulated foal macrophages. Indeed, foal macrophages had lower TNF • expression (**) p < 0.05) in the absence of R. equi than in its presence. Curiously, LPS treatment, but not IFN •, inhibited foal macrophage expression of TNF • (** p < 0.02); in addition, LPS treatment cancelled the IFN • effect. For these experiments, macrophages were cultured in the absence of bronchial epithelial cells.

Figure 1C - Foal neutrophils (Np, n = 6) had increased oxidative burst activity in comparison with adult neutrophils (* p = 0.009) in the absence of bacterial stimulus (Np alone), or in the presence of non-opsonized R. equi, suggesting a greater activation status of foal cells when incubated with opsonized R. equi for 15 minutes. Indeed, the activation status of these cells impaired a significantly statistical difference between non-opsonized and 40% opsonized R. equi (green ∆ trend p = 0.06) treatments. In contrast, adult horse neutrophils revealed greater (**) p = 0.008) oxidative burst activity when stimulated with 40% opsonized bacteria than non-opsonized bacteria. For these experiments, neutrophils were cultured in the absence of bronchial epithelial cells.
2) examined how the function of phagocytes is affected by contact with the airway epithelium and, conversely, the modulation of epithelial cells by the presence of phagocytes and R. equi.

With the support from the Harry M. Zweig Memorial Fund, we have developed a three dimensional culture system of equine bronchial epithelium that fully differentiates into ciliary beating and mucus producing cells. The advantage of this system is the access to functional bronchial epithelium for systematic co-culture with phagocytes and R. equi, in an attempt to mimic the lower airway environment; importantly, we were able to use different combinations of co-culture, and stop the interaction at different time-points. In this controlled in vitro system, we asked specific questions about activation and signaling mediated by soluble or cell surface molecules from bronchial epithelial cells that would increase the capacity of phagocytes to remove and kill bacteria in the respiratory tract. This is a very unique model that utilizes excess tissues from horses in necropsy, therefore with no need to euthanize animals for this purpose. The Figure 2 illustrates the steps for equine bronchial epithelial cell harvesting, digestion and culturing.

![Figure 2](image)

Figure 2 - Examined how the function of phagocytes is affected by contact with the airway epithelium and, conversely, the modulation of epithelial cells by the presence of phagocytes and R. equi.

Figure 3A demonstrates equine bronchial epithelium with ciliated and goblet (mucin producing) cells after growing the isolated respiratory cells on porous supports placed at air-liquid interface. We also studied the topology of the airway surface liquid interface, using a nonaqueous fixative (OsO₄ solution dissolved in a perfluorocarbon FC-72) that preserves airways mucins in situ. After fixation, samples were processed for epoxy resin embedding using conventional procedures, and 0.5 µm thick sections prepared for light microscopy. This technique allowed us to demonstrate a thick mucus layer covering the epithelium as seen in Figures 3B and 3C.

![Figure 3A](image)

Figure 3A - Demonstrates equine bronchial epithelium with ciliated and goblet (mucin producing) cells after growing the isolated respiratory cells on porous supports placed at air-liquid interface. We also studied the topology of the airway surface liquid interface, using a nonaqueous fixative (OsO₄ solution dissolved in a perfluorocarbon FC-72) that preserves airways mucins in situ. After fixation, samples were processed for epoxy resin embedding using conventional procedures, and 0.5 µm thick sections prepared for light microscopy. This technique allowed us to demonstrate a thick mucus layer covering the epithelium as seen in Figures 3B and 3C.

![Figure 3B](image)

Figure 3B - Equine bronchial epithelial cells cultured on membranes under ALI conditions developed into mucin producing and ciliated cells. (A) Paraffin-embedded sections were stained with Alcian blue/PAS stain; secretory (goblet) cells containing granules of acidic glycoproteins (mucin) appear purple; (B and C) Epoxy embedded sections were stained with Richardson’s stain. Note thick mucus layer upon cilia.

![Figure 3C](image)

Figure 3C - Mucin forms a barrier to protect underlying cells from microorganisms and irritants that are constantly inhaled. However, when mucus secretion becomes excessive, it impairs lung function and the airways become obstructed. Such quantitative changes in mucus have pathophysiological importance because they could alter the local mucosal host defenses. Mucins are the key structural components of mucus. Recently, Muc5b and Muc5ac have been reported to be the major oligomeric mucins in equine airway mucus, and these glycoproteins have a similar macromolecular organization to their counterparts found in human airways. We therefore analyzed the expression of the two gel-forming mucin species using reverse transcriptase (RT)-PCR. For this purpose, we isolated total RNA from differentiated and undifferentiated equine bronchial epithelial cell cultures grown on membranes (4.7 cm²) for 18 days or 3 days, respectively. As demonstrated in Figure 4, both mucin species Muc5b and Muc5ac mRNA levels were
detected in samples of differentiated cells obtained from tissues from 3 different horses (Figure 4A) but not in the sample of undifferentiated cells when grown for 3 days (Figure 4B). PCR of RT-negative samples verified the absence of DNA contamination (data not shown). Our data indicate that Muc5b seems to be equally or more expressed than Muc5ac, which was faintly detected in all three tested cell cultures obtained from different horses.

These data demonstrate that our developed respiratory epithelial cell culture system retains functional characteristics similar to those found in vivo. Our goal was then to study whether macrophages could phagocytose R. equi in the presence of mucus and ciliary beating cells. Mucus has been shown to interfere with bacterial survival, phagocytic function, and pathogenicity of Mycobacterium spp. and Pseudomonas aeruginosa in the airways. Using confocal microscopy, we examined phagocytic capture of live R. equi in mucus. The adult horse monocyte-derived macrophages were labeled with the CellTracker™ Green CMFDA and the bacteria with CellTracker™ Red CMTPX. When we added the phagocytes and bacteria (MOI 1:10) to the apical cell surface of the epithelium (established from bronchial epithelial cells from an adult horse), we were able to monitor the movement of phagocytes and bacteria across the epithelium due to the ciliary beating of the epithelial cells. Still, after 3h of incubation, phagocyte-adhered and ingested bacteria were seen as demonstrated by the co-localization of live bacteria within live macrophages resulting in yellow stained areas (Figure 5).

We also succeeded in showing live phagocytes transmigrating across the airway epithelium in the more physiologic basolateral-to-apical direction. We inverted a transwell with bronchial epithelial cells while maintained in medium. Phagocytes were then placed on the bottom side of the membrane and allowed to attach for 60 min. After the inserts were inverted back to their original orientation, the airway epithelium was infected with R. equi and incubated for 3 h. Figure 6 upper panel shows a sequence of optical sections (Z-stack) collected at different levels from the epithelium using computer controlled stepping in the Z (vertical) direction by preset distances. These images confirm that phagocytes (green) are able to migrate from...
underneath the membrane through the pores of the membrane to the apical surface of the epithelium to capture R. equi. Indeed, we found single macrophages, which also migrated through the epithelium, with intracellular bacteria (Figure 6 lower panel).

Next, we designed an experiment to quantitate bacteria-associated phagocytes and the number of intracellular bacteria in the presence of bronchial epithelial cells to determine whether the phagocytic activity of macrophages was inhibited in mucus. We incubated macrophages either in the mucus layer of in vitro generated adult horse airway epithelium or on collagen coated membranes (which are the same to grow the airway epithelial cells). After incubating macrophages with R. equi for 4 hours, the macrophages were harvested from the epithelial surface and fixed in 1% glutaraldehyde (Figure 7A). The cells were embedded in epoxy and 0.5 µm thick sections stained with Richardson stain. Indeed, we could see that the number of macrophages with intracellular bacteria and the number of intracellular bacteria per macrophage seemed lower when macrophages were incubated with R. equi in the presence of mucus (Figures 7B and 7C). Since the incubation time was only 4h, we know that the difference in the number of intracellular bacteria is not due to replication of R. equi for the bacterial growth rate is about 6 hours. Thus, the mucus produced by the airway epithelial cells obviously reduced the phagocytic activity of macrophages. Most interestingly, when we added foal macrophages to foal bronchial epithelial cells, we made the opposite observation (Figures 8A and B), i.e. many macrophages were loaded with R. equi. We therefore wanted to explore whether the in vitro equine bronchial epithelial system would allow the study of molecular signaling between bronchial epithelial cells and phagocytes, and the production of mucus, during R. equi infection.

Although, R. equi has never been seen within bronchial epithelial cells, studies of similar pathogens (e.g. mycobacteria) have shown that cells elaborate immune signals in response to exogenous stimuli, likely via their TLR-2 binding. Importantly, equine bronchial epithelial cells and our in vitro bronchial epithelial cells express TLR-2. When we cultured the equine bronchial epithelial cells with R. equi, we measured a decrease in TLR-2 expression in comparison to non-infected cells using real-time RT-PCR. In addition, we learned that epithelial growth factor receptor (EGFR) expression was concomitantly up-regulated when equine bronchial epithelial cells were infected with R. equi (Figure 9). Although originally described as a receptor for epidermal growth factors, recent studies have demonstrated that EGFR also acts as a receptor for viruses, and as a negative regulator for Haemophilus...
The presence of *R. equi* for 4h induces the expression of EGF-R, and down-regulates the expression of TLR-2 in adult horse equine bronchial epithelial cells (epithelial preparations from 2 different horses; STDEV ±0.4.) in comparison to non-infected cells using real-time RT-PCR.

**Figure 9** - The presence of *R. equi* for 4h induces the expression of EGF-R, and down-regulates the expression of TLR-2 in adult horse equine bronchial epithelial cells (epithelial preparations from 2 different horses; STDEV ±0.4.) in comparison to non-infected cells using real-time RT-PCR.

The results from Objective 1 of our study suggest that, in the absence of bronchial epithelial cells, foal and adult horse macrophages do not respond to IFN cytokine stimulus for increased expression of the MHC class II molecule. The same occurs when LPS is used for stimulus. The MHC class II molecule is essential for macrophage activation of the acquired immune system (T cell) after encounter with pathogen. IFN and LPS, individually or in combination, have been shown to promote upregulation of MHC class II expression. The presence of *R. equi* in the treatment conditions showed a mild trend in the upregulation of this molecule in foal macrophages. Altogether, these data indicated that increased expression of MHC class II molecule on macrophages would require additional stimulus than the ones tested herein, and we would like to further evaluate the expression of MHC class II in macrophages cultured with bronchial epithelial cells. It is possible that the latter may provide the necessary stimulus for macrophage expression of MHC class II, and this response may be also dependent on the presence of *R. equi*.

Interestingly, the response with TNF production, a pro-inflammatory cytokine, under the same conditions was different. Foal macrophages revealed to be more refractory to LPS and IFN stimulation than adult macrophages. Nevertheless, the presence of *R. equi* changed the TNF response of foal macrophages to IFN, in which these cells expressed higher levels of TNF. Curiously, LPS treatment inhibited foal macrophage expression of TNF, and cancelled the IFN effect. This is a striking finding because LPS is recognized by macrophages via toll-like receptor 4 (TLR-4), whereas *R. equi* is recognized by TLR-2. We expected that LPS + IFN stimulus would synergistically activate macrophages for detection and activation of *R. equi*; yet, LPS downregulates that response specifically in the foal, but not in adult horse cells. We would like to further explore the mechanisms by which LPS turned off foal macrophage response, which could involve regulation of the expression of TLR-2 (to detect *R. equi*), or other primary anergic effect.

Our studies and others have shown that foal neutrophils are very active at this age, and these cells, in contrast to macrophages, are bactericidal to *R. equi*. In addition, opsonization of *R. equi* with hyperimmune plasma containing *R. equi*-specific antibodies enhances the oxidative burst activity (production of killing chemicals) of neutrophils (Figure 1C) and macrophages (not shown). Although opsonization of *R. equi* increases activation of phagocytes, the mucus produced by our adult horse airway epithelial cell culture system decreased the number of macrophages with intracellular bacteria, and the number of intracellular bacteria per...
macrophage; surprisingly, the same was not true for foal macrophages exposed to foal epithelial cells. Greater number of intracellular bacteria could indicate phagocytosis efficiency, but it could also suggest virulence efficiency and infection permissiveness (i.e., the pathogen penetrates macrophages more easily, as they use this cell for its proliferation, and foal cells are more tolerant to this infection). Therefore, further studies are necessary to understand such difference between foals and adult horses, in order to evaluate foal macrophage function (activation) when it encounters *R. equi* in the respiratory environment. Furthermore, TLR2 is down-regulated in adult horse epithelial cells upon *R. equi* infection, which could contribute to the prevention of host tissue damage during bacterial infections, thus favoring the host considering that overactive inflammatory and immune response is detrimental to the host. We have isolated RNA from epithelial cells from additional adult horses, which are being tested for the expression of EGFR, TLR2, and the two mucin species Muc5b and Muc5ac using real-time RT-PCR. At this time, we do not know the effect of *R. equi* on foal bronchial epithelial cell expression of TLR2 and the other signaling molecules; we have isolated RNA from epithelial cells of one foal, and are hopeful to add at least two more foals in the near future. Also, we would like to further test how *R. equi* infection affects mucin production and, conversely, how respiratory mucus affects *R. equi* opsonization and uptake, and examine the effect of mucus on intracellular bacterial growth and phagocytic activation using our adult horse and foal bronchial epithelial cell culture systems.

This question has clinical relevance to the management of foals on farms with endemic *R. equi* pneumonia. In the neonate, the passive transfer of immunoglobulins via colostrum or plasma transfusion could be helpful in providing the initial protection until its own cellular and humoral immunity are developed. Nevertheless, the effect of *R. equi*-specific antibodies in preventing disease is not clear. In normal lung defense, the presence of bacteria directly induces mucin production by the airway epithelial cells. Such quantitative changes in mucus have pathophysiological importance because they could alter the local mucosal host defenses. Perhaps mucus production by airway epithelial cells affect *R. equi* opsonization, clearance and, consequently, replication in the airways during infection. There are only a few studies that examined the function of phagocytes in a mucus environment, and its role in *R. equi* infection is unknown. Foal susceptibility to *R. equi* disease can be associated with phagocyte bactericidal capacity during initial infection.

With a better understanding of the mechanisms for effective phagocytic activation and pathogen killing, we hope to identify elements of distinct susceptibility to disease in foals, and elaborate means to protect them more effectively during the period of vulnerability. The earlier the foal can assemble its protective immune system to fight infection, the more resistant it will be to disease.

**Publications:**


Dawson DR, Flaminio MJBF, Nydam D, Graham JE, Cynamon M, Divers TJ. Opsonization of Rhodococcus equi with hyperimmune plasma decreases bacterial viability and promotes phagocyte activation. Manuscript under submission.

Harry M. Zweig Memorial Fund
for Equine Research

2009 Final Report

P.I.: Dr. Lisa Fortier

Title: The Role of Telomerase and Small G-Proteins in Senescence of Articular Chondrocytes

Project Period: 1/1/09-12/31/09
Reporting Period: 1/1/09-12/31/09
PROJECT TITLE: The Role of Telomerase and Small G-Proteins in Senescence of Articular Chondrocytes

PRINCIPAL INVESTIGATOR: Lisa A. Fortier

Summary: The broad objectives of this proposal are to define how telomerase activity changes with age in articular chondrocytes and to evaluate the potential of Rho-subfamily GTPases to increase telomerase in senescent chondrocytes. The hypothesis of this proposal is that telomerase activity will be lost in chondrocytes after puberty and that it can be restored by over-expression of Rho-subfamily GTPases. Further, we hypothesize that restoration of telomerase activity in chondrocytes will diminish age-associated changes in cellular replication and extracellular matrix protein synthesis. The long-term expectation of this study is that avoiding senescence will diminish age-associated changes in the synthesis and assembly of cartilage matrix, thereby decreasing the susceptibility to OA in age.

Specific Aims and Findings: Two specific aims were designed to test our hypothesis.

Specific Aim 1. To measure telomerase activity in articular chondrocytes harvested from horses ages newborn – aged.

Findings: Telomerase activity was present in pre-pubescent and pubescent horses and absent in post-pubescent and mature horses. Pre-pubsecent animals were not significantly different from pubescent animals, and similarly, post-pubescent samples were not significantly different than mature.

Specific Aim 2. To determine if expression of Rho-subfamily GTPases can restore telomerase activity in senescent chondrocytes, chondrocytes were transfected with wild type, constitutively active, fast cycling, and dominant negative Rho-subfamily GTPases Cdc42, Rac, and Rho.

Findings: Our findings suggest that active Cdc42 increases telomerase and active Rho decreases telomerase activity. Knock down studies using chemical inhibitors to the small GTPases are presently being performed and will provide further information to confirm the initial findings.

Significance: Our results indicate that telomerase activity is present in articular chondrocytes until the horse enters the post-pubescent stage at approximately 15 months of age, as defined by serum insulin-like growth factor-I and insulin-like growth factor-I binding protein concentrations and full formation of the tidemark in articular cartilage. These findings are consistent with studies that demonstrate diminished responsiveness of aged chondrocytes to anabolic stimulation with insulin-like growth factor-I and implicate that these changes might begin as early as puberty. These data suggest that aging of cartilage begins as early as post-puberty and that investigations into therapeutics to enhance cartilage repair should carefully consider the age of target populations in laboratory and clinical studies. The loss of telomerase and the termination of replication due to an absence of telomerase upon entering post-pubescence may play a role in the limited capacity for articular cartilage repair in mature individuals.

Publications:
Novakofski KD, Donocoff RS, Fortier LA. Telomerase activity is lost in articular chondrocytes after puberty. Trans 56th Annual Meeting ORS 2010; CDROM.
Donocoff RS, Novakofski KD, Fortier LA. Telomerase activity is lost after puberty in articular chondrocytes. Annual ACVS Veterinary Symposium 2009; CDROM.
Harry M. Zweig Memorial Fund for Equine Research

2009 Annual Report

P.I.: Dr. Susan Fubini

Title: Indices of Intra-Abdominal Fibrinolysis in Colic Foals: Pathogenic and Prognostic Markers

Project Period: 1/1/08-12/31/09
Reporting Period: 1/1/09-12/31/09
Dr. Fubini was awarded a supplemental award through December 31, 2010. An annual report is provided.
Title: Indices of Intra-abdominal Fibrinolysis in Colic Foals: Pathogenic and Prognostic Markers

PI: Dr. Susan L. Fubini

Specific Aims or Objectives:

To measure indices of fibrinolysis (fibrinogen, plasminogen, D-dimer, antiplasmin) in paired plasma and peritoneal fluid samples from adult horses and foals, from both normal animals and those presented for colic. We aim to recruit 20 healthy horses (10 adults, 10 foals) to establish reference values, and 20 horses (10 adults, 10 foals) with colic in a 2-year study period. We plan to determine if there is any difference in fibrin deposition and clearance between the different age groups and disease states.

1) Summary of Scientific Findings:

The broad objective of this project is to test the hypothesis that colic induces dysregulation of fibrinolysis in horses and that the resultant hemostatic imbalance is more pronounced in foals than adult horses.

We have been collecting plasma and abdominal fluid from horses less than 6 months of age. This includes “normal” foals that are being euthanized for life threatening problems (fractures, neurologic disease, severe limb deformities) and foals presenting with abdominal pain that are subjected to a ventral midline celiotomy.

In this study period we have made one change in laboratory assay procedures: a new, quantitative D-dimer assay has been substituted for the previously utilized semi-quantitative D-dimer test. The new assay is an automated turbidometric assay using commercially available monoclonal antibodies bound to latex beads (Hemosil D-dimer, Instrumentation Laboratories). This assay method yields a specific value, rather than concentration range. In human medicine, quantitative immunoturbidometric D-dimer assays are accurate indicators of systemic thrombosis and are used in clinical diagnostic algorithms. The increased sensitivity and accuracy of this new D-dimer test will improve our ability to assess abdominal and systemic fibrinolysis, and our data will provide new information on clinical utility of quantitative D-dimer monitoring in colic foals.

We have encountered problems in obtaining samples from all eligible cases, especially when Dr. Watts and Fubini are not directly involved with admission and case management. To help increase clinician awareness of the study and increase case enrollment, we have posted study contact information, sent reminder emails, and reminder announcements in rounds and staff meetings. We decided recently to start a raffle for a nice dinner as an incentive program for samples collected or brought to our attention.

Nevertheless, we are close to our targeted recruitment goals. As of September 2008 we have collected a total of 10 foals with abdominal disorders (6 of which had strangulating obstructions) and 8 true “normal” foals. We have 5 “normal” adults and 1 colic foal sample being processed. We anticipate being able to secure more adult samples easily.
Results to date show that when compared with healthy animals, foals with colic had a significant increase in their peritoneal fluid fibrinogen (6 fold increase) and plasminogen (2.5 fold increase) and a trend of increased peritoneal D-dimer and antiplasmin.

Based on the lack of significant increases in D-dimer in the plasma of foals with colic in the face of significantly elevated peritoneal plasminogen and fibrinogen, it appears that fibrinolysis may be down-regulated in the foal with colic which is in contrast to published data in adult horses with colic. These differences may help to explain an apparent increased risk of intra-abdominal adhesion formation following abdominal surgery in foals.

We intend to finish data collection by June 2009 and be ready to present the data at the American College of Veterinary Surgeons meeting in October of 2009 in Washington, DC.

Future work in the pathophysiology of foal and adult horse colic will focus on the effects of intra-abdominal inflammation on systemic hemostatic balance. The Coagulation laboratory has recently acquired a global hemostasis analyzer (thromboelastograph) and the instrumentation to measure thrombin generating potential. These sophisticated tools and techniques hold promise for better defining hypercoagulable syndromes and may ultimately have clinical applications in identifying horses at risk for adhesion formation, thromboembolism, laminitis, and disseminated intravascular coagulation (DIC). This would be of tremendous benefit when determining appropriate therapy for critical clinical cases.

**Significance to the Equine Industry**

In the current study, we aim to identify characteristics of foal fibrinolysis that favor intra-abdominal adhesion formation following abdominal surgery. This may ultimately improve clinical decision making in treating the foal with colic; providing owners and breeders more clear cut data regarding treatment and prognosis. Like every good study, other questions surface along the way. Our data from this study will generate new hypotheses regarding the interplay of inflammation and hemostasis in horses. Further investigation and new approaches are urgently needed to improve clinical outcomes for horses with inflammatory syndromes that lead to the catastrophic complications of laminitis and DIC.
Harry M. Zweig Memorial Fund
for Equine Research

2009 Annual Report

P.I.: Dr. Alan Nixon

Title: Genomic Profiling of Osteochondritis Dissecans Using an Equine Whole Transcript Exon Array

Project Period: 1/1/08-12/31/09
Reporting Period: 1/1/09-12/31/09

Dr. Nixon was awarded a no cost extension through June 30, 2010. An annual report is provided.
PROJECT TITLE:
Genomic Profiling of Osteochondritis Dissecans Using an Equine Whole Transcript Exon Array

PRINCIPAL INVESTIGATOR(S):  Alan J. Nixon

LAY SUMMARY:  Provide the original lay summary from last year’s award. If it has changed, an updated summary should be submitted.

Significance:
Osteochondritis dissecans (OCD) remains a prominent and debilitating musculoskeletal syndrome among growing horses. In concert with other forms of developmental orthopedic disease (DOD), including physitis, angular limb deformity, cuboidal bone collapse, and wobbler syndrome, this complex continues to affect 35 to 40% of the annual weanling and yearling crop. For individual animals, the impact has largely been through joint and growth plate pain, deformity and mechanical dysfunction, and subsequent osteoarthritis (Fig 1)

Figure 1. Radiographic (A) and morphologic appearance (B) of early OCD (arrows) in the stifle of the horse, compared to normal appearance (C,D). These early affected tissues may yield important causative gene expression information that is at the center of this grant. Defining gene family changes will allow a more focused study of specific gene dysfunction that may flesh out the genetic basis or otherwise of OCD. At the very least, this study should elucidate the series of early gene dysfunctions that set OCD in motion deep within the joint surface.
The economic costs of lameness and cosmetic disfigurement, add to the morbidity associated with OCD, spinal ataxia, and angular limb diseases. Further, osteochondrosis (OC) seems to affect the more rapidly growing animals, making this group of related diseases a frustrating syndrome for owners and farm managers. Despite adoption of environmental controls such as trace mineral supplementation to mares and weanlings, overall growth rate restrictions, and consistent exercise regimens, the incidence of osteochondrosis (OC) and OCD remains unacceptably high. The predominant sites for occurrence vary between horse breeds, but epiphysitis (physitis) and OCD are particularly common in Thoroughbreds, Standardbreds, Warmbloods, Arabians and Quarter Horses. In addition to the pain and debility of physitis and joint disease, there are serious mechanical consequences to OCD induced joint surface disruption. Fetlock and shoulder OCD frequently degenerate to osteoarthritis with permanent lameness. Other common OCD sites such as the hock and stifle can also be debilitating, and frequently require surgery or intraarticular medication for adequate resolution. Better longer-term control will only come through an understanding of the cause and cascade of events in OCD formation.

We consider OCD and other forms of DOD remain as unresolved key targets in equine health research. Defining the cause of OC at a molecular and genetic level is fundamental to reducing the incidence and consequences of DOD.

**Progress:**

Despite corrections to pasture and water quality for mares, and improvements in the composition and volume of supplements for weanlings, DOD and particularly OCD remain at a high incidence plateau. Our pilot studies using global gene scans of cartilage specimens from OCD and normal animals identified several candidate causative genes which were subsequently analyzed in more detail. These studies determined that OCD cartilage has a prominent footprint of gene expression derangements, spanning from increases in growth factors and bioactive peptides important in the control of growth plate development and maturation, to changes in gene modulators and proteins controlling the cell cycle and cell developmental states. The prioritization and interaction of these developmentally important proteins in OCD remains essentially uncharted. This largely stems from the lack of global gene array tools. Although the equine genome project completed the horse genome sequence detection earlier in 2007, it is not clear that a full genome equine gene chip will ever be commercialized due to market limitations. Given these constraints our group developed a custom gene chip capable of interrogating the equine genome.

**Research Plan:**

We hypothesize that OCD is the result of aberrations in the signaling processes of key regulatory proteins controlling cartilage maturation and formation into bone.

Objectives of this study include a complete gene expression profiling in OCD tissues using the Cornell equine gene chip, developed by our lab for this project, and providing interrogation of the full equine genome. Commercial versions will not be available for several years, if at all. This proposal expands previous work on 4 animals with early OCD lesions. Microarray techniques will be used for global gene expression mapping, and selected microarray and targeted gene data will be verified with precise gene quantitative assays, in situ definition of gene expression dysfunction on actual microscopic tissue sections, and laser microdissection to punch out microscopic islands of specific regions of the OCD flap to establish gene profiles of discrete zones within the diseased cartilage. Functional verification of the role of key molecules in OCD will use gene overexpression and gene knockdown techniques to study the impact of differential gene regulation on cartilage matrix structure and competency. In so doing we will characterize whether the suspect genes actually perturb cartilage formation in the way we see in the natural disease.
PROGRESS REPORT: (Not to exceed 2 pages) The progress report should include the following:

A) State the Specific Aims as actually funded. If they have been modified, provide the revised aims and the reason for the modification. Substantial changes must be reviewed by the Associate Dean for Research & Graduate Education as directed by the Harry M. Zweig Memorial Fund post-award guidelines.

1) Determine differentially expressed genes in OCD cartilage using a custom exon array equine GeneChip.
2) Verify predominantly dysregulated genes by quantitative real time PCR measurement of gene expression in OCD and normal cartilage.
3) Assess the spatial distribution of gene dysfunction in focal OCD lesions by laser-capture microdissection of specific cell groups and study of the differentially expressed genes within precise zones or clusters by quantitative PCR.
4) Verify the direct impact of overexpressed genes identified on microarray by transient transfection of chondrocytes and quantification of cartilage matrix response genes.
5) Use RNA interference to examine the effects of candidate gene knockdown on chondrocyte gene profiles and matrix assembly

B) Describe the studies direction, positive and/or negative, toward the specific aims during the current budget year and the results obtained.

TIMETABLE: (Provide a tentative sequence or timetable for the investigation for the two-year funding period).

<table>
<thead>
<tr>
<th>Date Range</th>
<th>Activity Description</th>
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<tbody>
<tr>
<td>January 1, 2008 - Sept 1, 2008</td>
<td>Collect OCD and normal cartilage samples</td>
</tr>
<tr>
<td>March 1, 2008 - Dec 31, 2008</td>
<td>Isolate RNA and run microarray, decal &amp; section osteochondral blocks</td>
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<tr>
<td>Sept 1, 2008 – March 31, 2009</td>
<td>Analyze chip data; Analyze candidate gene expression by taqman</td>
</tr>
<tr>
<td>April 1, 2009- Dec 31, 2009</td>
<td>Transient overexpression and gene silencing experiments</td>
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Osteochondral blocks and cartilage for RNA isolation have been collected from a total of 17 OCD cases and 14 age and site matched controls. All have been decalcified and sectioned. Histology verified the classical clefting and separation of OCD cartilage from underlying bone (Fig 1). Microarray data using version 1 of the CU Equine gene chip showed over 800 genes were differentially regulated in OCD compared to normal cartilage (p<0.05, fold-change ≥1.2, ≥50% present call in ≥1 group). The new version 2 chip was delayed in production but has now been secured and is being used in new expression profiling. A wide range of differentially expressed mRNAs were identified in OCD cartilage, which encoded key players of various functional pathways, biological processes, and functionally related gene families (Fig 2). Validation of 4 predominantly dysregulated genes on microarray using real-time PCR (Taqman) showed the same differential expression pattern in OCD cartilage (Table 1). Major gene expression abnormalities in OCD affected cartilage included (i) VEGF ligand and receptor pathway overexpression, (ii) increased signaling through Ephrin ligand over-abundance, (iii) increased apoptotic activity by overexpression of caspase 3 & 7 and downregulation of bcl-2, (iv) increased protein ubiquitination, (v) altered wnt/beta catenin pathways, and (vi) structural derangement by reduced aggrecan, collagen type II, and PRG-4 (lubricin) expression, overexpression of enhanced GAG degradation through elevated MMP-13 levels.
Harry M. Zweig Memorial Fund
for Equine Research

2009 Annual Report

P.I.: Dr. Alan Nixon

Title: Pro-Inflammatory Cytokine Targets in Joint Disease as Check-Points for Gene Inhibition

Project Period: 1/1/08-12/31/09
Reporting Period: 1/1/09-12/31/09

Dr. Nixon was awarded a no cost extension through December 31, 2010. An annual report is provided.
PROJECT TITLE:
Pro-inflammatory Cytokine Targets in Joint Disease as Check-Points for Gene Inhibition

PRINCIPAL INVESTIGATOR(S):  Alan J. Nixon

LAY SUMMARY:  Provide the original lay summary from last year’s award. If it has changed, an updated summary should be submitted.

Athletic horses often start to develop insidious concussive joint erosions as the intensity of training and competition increases. The early warning signs in 2-year-old racehorses include joint fluid build-up and pain on manipulation of the affected joints. Alert trainers treat these signs by changes in exercise patterns and intensity, cold therapy, oral anti-inflammatory agents, and occasionally systemic joint medication. Rarely, flaking and chip fragmentation of the joint surface requiring surgery develops. As 3- to 4-year-olds, actual cartilage loss limits the response to most therapies. Steroids are introduced to more potently quell the symptoms, but often accelerate structural damage to the joint and can lead to insidious arthritic degradation with increasing pain and dysfunction. Estimates of the annual cost of joint injury and arthritis to the equine industry approach $100 million. It has become increasingly clear that control of arthritis will not progress until we understand the key degradatory cytokines in equine joint disease. Prevention of arthritis is the major focus of our research in cell-based cartilage repair using growth factor gene or protein methods to drive healing of traumatized joints. Research in the investigator’s laboratory has focused on both cartilage and adult stem cell transplants, bolstered by growth factor composites added at surgery. Application of mixtures of cartilage cells and IGF-1 has been used in over 120 equine cases suffering from stifle, shoulder, fetlock, and knee injuries and developmental syndromes such as OCD. However, these systems add cells and growth factors to stimulate cartilage cell function, but do not quell the enzymes and degradation pathways that continue to erode the joint surface.

Osteoarthritis has a complex of precipitating traumatic causes. However, it follows a common pathway after perturbation of cartilage homeostasis, with proliferation of degradatory enzymes and other inflammatory proteins that erode the joint.

Our research program aims to better understand the fundamental interaction of these inflammatory mediators in acute joint injury, chronic surface erosion and chip fracture, and subsequent arthritis. Targeted treatment with cytokine gene silencing may halt the progression from joint surface injury and fragmentation to

Figure 1. Acute stifle injury often leads to chronic degeneration and arthritis, with meniscal tearing and joint surface erosions (left). The number and type of degradatory enzymes and other destructive agents in equine joint disease that weaken and fibrillate the cartilage (right), are largely unknown. This grant profiles these agents, develops mechanisms to control their flux, and tests them separately and in combination.
arthritis. Two cytokines, interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), are thought to be the principal messengers of destruction in arthritis. Our previous work suggests that controlling IL-1 through over-expression of its own natural inhibitor or gene silencing through IL-1 RNA interference restores cartilage function for several weeks. Despite the benefit of local knockdown of the IL-1 gene product (before it can be synthesized into the active cytokine), our studies indicate continued expression of other degradatory players such as TNF-α may partly account for the poor recovery of suppressed cartilage structural components. The current proposal will examine the fundamental interactions of cytokines in natural joint disease, develop a tier of inflammatory targets for treatment, examine the repercussions of single gene knockdown on other cytokine profiles, and finally develop multimodal gene silencing constructs that may provide a comprehensive therapeutic product.

We hypothesize that numerous inflammatory cytokines and enzymes are elevated in arthritis and that IL-1 and TNF are the master regulators, both of which need to be silenced before cartilage structural genes can function to rebuild cartilage matrix.

Our previous studies show catabolic IL-1 gene fluxes can be controlled in articular tissues by gene knockdown. Despite reduced IL-1 formation, our experiments show a recalcitrant suppression of cartilage component formation, essentially failing to rebuild the damage to the joint surface. This grant will examine the fundamental interactions of degradatory cytokines in natural joint disease, develop a tier of inflammatory targets, examine the repercussions of single gene knockdown on other cytokine profiles, and finally develop multicomponent gene silencing constructs that may provide a therapeutic modality. Previous Zweig Funded grants during 2005-2006 screened IL-1 silencing motifs and in 2007 allowed development of stable IL-1 gene induced silencing suitable for clinical application. This proposal uses the Cornell equine genome project gene chip, developed in our lab, to deepen our understanding of the inflammatory agent profile in equine traumatic joint disease, and assesses the interplay of inflammatory cytokines/enzymes and growth factors. Until we understand these molecular events, we can not hope to appropriately target the most destructive cytokines, nor which cytokine group more profoundly suppresses active cartilage reformation.

This 2-year proposal aims to:

1) Use gene chip and other molecular expression assays to profile damaging gene expression in equine joint disease, specifically horses with knee chips.

2) Test IL-1 and TNF gene silencing motifs alone and in combination to determine how completely they can control joint reaction.

3) Evaluate expression of other cytokines and pro-inflammatory mediators suppressing cartilage rebound after silencing IL-1 or TNF.

Previous work has identified one highly efficient IL-1 gene knockdown agent from dozens tested in cartilage and joint lining cells. This coding segment has been assembled into a DNA fragment capable of self-sustained production of new silencing agents from the individual target cells. This “silencing microfactory” is unique in medicine and has cross-over to arthritis in man. Moreover, we have established a collaboration with Dr John Rossi in California, a leader in the field of gene silencing to control HIV infection, who has guided our work in developing multiple coding regions in the one construct. Additionally, through another collaboration with Dr Perry Hackett of the University of Minnesota, we have further formed this coding region into a gene package (coined Sleeping Beauty™) capable of permanent integration into target cells so the gene is not lost during cell division. Both plasmids effectively knockdown IL-1, but do little to help rebuild cartilage. We have developed two strategies to overcome this. 1) Supplement stimulatory growth factors through gene therapy which has been a focus of our lab for 6 years. (now the major part of a pending NIH grant). 2) Verify the target hierarchy in naturally occurring joint disease and widen the search by assessing expression of all major degradatory agents.

The experiments in this two-year grant will use gene expression arrays and histologic techniques to profile pro-inflammatory cytokine expression in the joint lining from a range of Thoroughbred and Standardbred knee chip and arthritis cases, employ laboratory culture techniques to examine the efficiency of
IL-1 and TNF cytokine RNA interference products, and use gene and protein assays to measure cross-talk between these cytokines and other agents in the joint lining and adjacent cartilage. The project long-term goal is to assemble multiple coding regions targeting the key elements in joint destruction onto the one DNA backbone, and eventually to test this gene based anti-inflammatory motif in animals with cartilage injury and finally in horses with arthritic joints. Ultimately, we hope to return horses with possible career-ending joint injury back to being competitive athletes.

PROGRESS REPORT: (Not to exceed 2 pages) The progress report should include the following:

A) State the Specific Aims as actually funded. If they have been modified, provide the revised aims and the reason for the modification. Substantial changes must be reviewed by the Associate Dean for Research & Graduate Education as directed by the Harry M. Zweig Memorial Fund post-award guidelines.

  **Aim 1)** Profile cytokine gene expression in equine joint disease using gene chip and other molecular expression assays to develop a hierarchy of targets for molecular control.

  **Aim 2)** Develop and test IL-1 and TNF gene silencing motifs alone and in combination to determine how completely they control joint reaction.

  **Aim 3)** Evaluate expression of other cytokines and pro-inflammatory mediators suppressing cartilage rebound after silencing IL-1, TNF, or both IL+TNF.

B) Describe the studies direction, positive and/or negative, toward the specific aims during the current budget year and the results obtained.

**TIMETABLE:** (This was the timetable for the two-year funding period).

- Jan 1, 2008 – Sept 30, 2008  
  Harvest synovial membrane and fluid from chip cases.

- May 1, 2008 – Sept 30, 2008  
  Prep RNA, assay IL-1 and TNF in membrane and fluids. Develop pSIREN-TNF.

- Oct 1, 2008 – March 31, 2009  
  Microarray data assessment. qPCR of candidate genes.

- Jan 1, 2009 – April 1, 2009  
  Evaluate pSIREN and SB for IL-1 shRNA. Evaluate pS-TNF & SB-TNF.

- Apr 1, 2009 – Dec 31, 2009  

Eleven Thoroughbred and 1 Standardbred have had chip fractures removed from the carpi since the grant commenced January 2008. Synovial fluid was saved and synovial tissue harvested and stored. Similar fluids and tissues were harvested from carpi of 15 normal horses in this same time period. Size and location of the fracture fragment, extent of cartilage damage beyond the fragment (scored using published scale 1-4), and concurrent radiographic OA score (1=normal; 2=perimeter osteophytes; 3=osteophytes and joint space narrowing; 4=osteophytes/exostoses/joint collapse) were recorded for each horse. The synovial tissue has been homogenized and RNA extracted. Sample collection continues to achieve the goal of 20 samples from affected and 20 from normal horses. Anticipated candidate gene qPCR assay of gene expression is expected to start within the next 30 days. Candidate genes will include IL-1α & β, IL-6, IL-10, IL-17, TNF-α, MMP-1, MMP-3, MMP-9, MMP-13, ADAMTS-4 (aggrecanase 1), ADAMTS-5 (aggrecanase 2), nitric oxide synthase, interferon (IFN), NFκB, collagen type I, and decorin. A poorly performing TNF-α qPCR has been replaced with new primer probe sets after comparison of 3 new primer pairs using Primer Express. Microarray gene chips have been designed and manufactured, and were delivered September 18, 2008. RNA purification is complete and representative samples from carpi with similar OA stages have been pooled. Microarray is expected to commence on schedule.

Small interfering RNA’s (siRNA) targeting TNF-α were synthesized and screened by electroporation into chondrocyte monolayers exposed to LPS to stimulate an inflammatory state. Gene expression for catabolic cytokines and cartilage matrix gene expression was determined by real-time PCR. The most efficient siRNA ribo-oligo’s were used to design a short-hairpin coding region to code for sense and antisense RNA interference motifs in a pSIREN plasmid, to prolong TNF-α knockdown (pSH-TNF2). TNF-α expression was substantially reduced by 2 of seven siRNA targeting TNF (Fig 1). LPS treated chondrocyte cultures showed a small rebound
in matrix collagen type II expression when transduced with TNF-α siRNA #2 alone and in combination with IL1b siRNA (Fig 2.)

Chondrocytes transduced with pSH-TNF2 demonstrated a >50% knockdown of TNF-α gene expression. (Fig 3.) and had a small rebound in aggrecan expression (Fig 4.)

This project has allowed us gather the majority of the projected samples required to complete the subsequent screening assays of cytokine expression. The remaining samples should be collected in the remaining months of 2008, given the current case load. In vitro work has screened TNF-α silencing motifs and resulted in development of a plasmid based shRNA TNF silencing motif (pSH-TNF2). These data show novel TNF knockdown using a plasmid coding a short hairpin loop (shRNA) form of a TNF interference motif. Moreover, development of this plasmid by recombination into the Sleeping Beauty (SB) transposon has been completed and provides the potential for chromosomal integration and selection of chondrocytes resistant to TNF effects. These culture experiments have been finished and await qPCR data. Target gene knockdown provides a non-competitive path to control of TNF action, compared to previous receptor decoy and soluble antibody approaches. Similarly, post-transcriptional gene silencing has the advantage of persistence, particularly when combined with simple plasmid or integrating vectors such as SB. Combinatorial therapy, targeting both IL-1 and TNF-α, has been developed to achieve global knockdown of the two major catabolic agents and improve anabolic recovery. Preliminary data on longer term combination silencing of IL-1b and TNF-α in chondrocyte cultures indicate that aggrecan and collagen type II expression can be returned to levels closer to normal.

C) Summarize your plans to address the Specific Aims during the next year of support.

Much of the work in aims 2 and 3 has already commenced ahead of the projected timeline. This should allow concentrated focus on the microarray studies, which will consume much of the 08-09 winter period.
Harry M. Zweig Memorial Fund
for Equine Research

2009 Final Report

P.I.: Dr. Gillian Perkins

Title: Immunization Against Strangles Using a Vectored Equine Herpesvirus Vaccine

Project Period: 1/1/09-12/31/09
Reporting Period: 1/1/09-12/31/09

Dr. Perkins was awarded a no cost extension through December 31, 2009. An annual report is provided.
Title: Immunization Against Strangles Using a Vectored Equine Herpesvirus Vaccine

Principal Investigators: Gillian Perkins and Nikolaus Osterrieder

SUMMARY/DESCRIPTION OF THE PROJECT

The overall goal of this work is to 1) gain an understanding of what is required for an efficacious, long-lasting immune response to S. equi and to 2) establish equine herpesvirus type 1 (EHV-1) as a platform to deliver immunogens from S. equi to horses to avoid the many complications of S. equi-induced disease. Our hypothesis is that a modified live EHV-1 vaccine derived from a non-pathogenic EHV-1 strain (RacH) can safely and efficiently deliver S. equi antigens and induce a protective immune response against both S. equi and EHV-1 infection.

Specific aim 1: To engineer EHV-1 strain RacH recombinants expressing three S. equi antigens (SeM, Se18.9 and IdeE).

Specific aim 2: To test the generated EHV-1 virus expressing the S. equi proteins in mice and horses.

PROGRESS OF THE PROJECT – 1st YEAR

Specific aim 1:

Construction of EHV-1 strain RacH recombinants expressing S. equi antigens SeM, Se18.9 and IdeE

1. Description of the work

The nucleotide sequences of SeM and Se18.9 separated by an internal ribosomal binding site (named SeM-IRES-Se18.9) were synthesized de novo by a commercial supplier (GenScript). IdeE sequence was separately synthesized by the same company. The sequences were codon-optimized versions of the genes to enable efficient expression in mammalian cells.

Two shuttle plasmids were constructed by cloning separately the SeM-IRES-Se18.9 and IdeE sequences into pEP_CMV plasmid, resulting in the control of the genes by the human cytomegalovirus immediate early promoter and the bovine growth hormone as a transcriptional terminator sequence. The pEP_CMV plasmid also contains a unique I-SceI site and an aphA1 gene conferring kanamycin resistance. These elements allow the cloning strategy into the RacH genome. Transfer constructs were generated by PCR and two-step Red recombination was used to insert the SeM-IRES-Se18.9 and IdeE genes into the infectious bacterial artificial chromosome (BAC) clone of the EHV-1 RacH (pRacH). The recombinants pRacH_SeM-IRES-Se18.9 and pRacH_Ide DNAs were isolated and the structure of the BAC recombinants was verified by restriction enzyme and PCR analysis.
2. Future actions
EHV-1 viruses will be reconstituted from the two BAC clones. Expression of *S. equi* antigens by the two recombinant viruses will be assessed by immunofluorescence and western blotting using SeM, Se18.9 and IdeE antibodies.

**Expression of *S. equi* SeM, Se18.9 and IdeE proteins in *Escherichia coli***

1. Description of the work

The SeM, Se18.9 and IdeE genes were PCR amplified and cloned separately into an arabinose-inducible expression vector pBAD24. In order to facilitate purification from *E. coli*, a carboxy-terminal tag of six histidine residues (6-His) was added to each recombinant protein. BL21-Rosetta 2 *E. coli* cells were transformed with the expression plasmids. Protein expression was monitored by SDS-PAGE and Western Blotting using Penta-His antibodies. Bands of approximately 60 kDa, 40kDa and 19 kDa corresponding to SeM, Se18.9 and IdeE proteins respectively, were detected.

Protein expression and further purification has been optimized testing different growth temperatures, induction time and inducer (arabinose) concentration.

2. Future plans

Antiserum against the purified SeM, Se18.9 and IdeE proteins will be produced in rabbits and used to check expression of the *S. equi* proteins by the recombinant EHV-1 viruses.

Purified proteins will be used in an ELISA system designed to recognize the anti-SeM, anti-Se18.9 and anti-IdeE antibodies in mice and horses experimentally infected with the recombinant viruses.

*Specific aim 2:*

**Testing the generated EHV-1 virus expressing the *S. equi* proteins in mice and horses**

Once the recombinant RacH-based *S. equi* vaccines are completed and optimized, they will be tested initially in the mouse model of infection. This is planned for December 2009 and January 2010.
Harry M. Zweig Memorial Fund
for Equine Research

2009 Annual Report

P.I.: Dr. Bettina Wagner

Title: Analysis of the Innate Immune Response to EHV-I Infection

Project Period: 1/1/09-12/31/10
Reporting Period: 1/1/09-12/31/09
Project Title: Analysis of the Innate Immune Response to EHV-1 Infection

Principal Investigator(s): Dr. Bettina Wagner  
Dr. Cynthia Leifer  
Dr. Klaus Osterrieder

Lay Summary: Equine herpesvirus type 1 (EHV-1) negatively impacts the equine industry, both economically and medically. Existing vaccines are of low efficacy or offer only partial protection from infection. Therefore, improved strategies to protect horses from EHV-1 are needed. A protective vaccine of high efficacy would induce an immune response that resembles the natural host response required to cure infection and develop immunity. Studies on the protective immune response to EHV-1 infection will provide this information and allow the development of more effective vaccines. In the proposed study we will identify key protective responses to EHV-1 infection.

Innate immune response mechanisms provide a first line of host defense after infection with viral pathogens. The innate immune system is composed of cells expressing receptors that are at the ready to respond to the first sign of infection. The response is mainly through direct killing mechanisms to eliminate the invader and through the production of inflammatory mediators that both control the growth of the invader and call other cells of the immune system to the site of infection where they are recruited into the fight to eliminate the invader. The response is initiated by specific receptors expressed on cells of the innate immune system.

The most important of these receptors is the family of Toll-like receptors (TLRs). TLRs are transmembrane proteins that directly bind to pathogens and induce the activation of innate immune cells that express them. Stimulation of TLRs induces the production of interferons (IFNs) and chemokines that direct the ensuing immune response. Two TLR family members have been demonstrated to play an important role in host defense against other herpesviruses, TLR2 and TLR9 (Figure 1). In Aim 1 of this proposal we will define the role of equine TLR2 and TLR9 in the immune response to EHV-1.

Figure 1. TLRs important in host response to herpesvirus. TLR 2, together with TLRs 1 and 6, primarily induce cytokines and chemokines, while TLR9 induces IFN-α. The information is based on studies on herpes simplex virus, a virus closely related to EHV-1, in mouse models.
Immune modulators that produced during viral infection and control the immune response include IFN-\(\alpha\) and chemokines. IFN-\(\alpha\) inhibits viral replication, activates cells of the immune system and regulates the immune response. Chemokines are critical for the recruitment of immune cells to the site of infection and for their activation. Activation of cells through TLRs induces the production of both IFN-\(\alpha\) and chemokines. In Aim 2 of this proposal we will identify and characterize the IFNs and chemokines that are produced in response to EHV-1 infection, and provide details about the cells critical for this production.

In order to improve research of the immune response in veterinary species including the horse, a network of investigators has been assembled whose main goal is the development of reagents for immunological studies. Dr. Wagner’s group at Cornell is one of the three core facilities of this US Veterinary Immune Reagent Network. This ongoing US wide collaborative effort started in 2006. For the horse, our network plans to develop 12 monoclonal antibodies, four of which are to cytokines and eight to cell surface markers. Several reagents, including novel antibodies to equine cytokines and interferon have already been produced by our group (Wagner et al. 2006, 2008 and in press). In Aim 3 of this proposal we seek complementary funding for the development of additional key reagents to investigate the immune response to EHV-1 in horses. We propose to generate two antibodies to the most interesting equine chemokine candidates in EHV-1 infection. The potential candidates are not yet part of the reagent initiative mentioned above. Our main focus will be on molecules that have recently become of high interest and are at the cutting edge of immunological research in herpesvirus pathogenesis. The approach will provide new tools for the identification of the early immunological events that lead to protection against EHV-1 infections and neurological disease.

Overall the goal of this project is the identification of innate immune response cascades and their consequences for disease susceptibility in EHV-1 infection in horses. This foundational approach is essential for the rational design and development of more efficient vaccines and novel treatment strategies to improve equine health and well-being.

It is important to note that due to the ability of TLRs to activate innate immune responses, ligands for TLRs have been used to improve experimental vaccines in horses. For example, the TLR9 ligand CpG DNA mixed with Emulsigen improved the serological response of horses vaccinated with killed equine influenza virus vaccine (Lopez et al 2006). Therefore, understanding the role that TLRs play in the induction of IFN-\(\alpha\) and chemokines may provide useful targets for the improvement of vaccines and for development of novel therapies designed to enhance and complement the normal immune response to EHV-1.

**PROGRESS REPORT:**

**Aim 1 as actually funded:** We will determine whether EHV-1 induces IFN and chemokines by directly activating TLR2 and TLR9. Equine TLR2 and TLR9 will cloned and transfected into an equine fibroblast cell line. Stimulation by EHV-1 will be tested using NF-\(\kappa\)B and IFN reporter assays established in Dr. Leifer’s lab. We will also determine whether neuropathogenic EHV-1 strains differ from non-neuropathogenic EHV-1 strains in their potential to stimulate the innate immune response through TLRs.

**Results:** We have performed polymerase chain reactions to obtain the cDNA of equine TLR2 and TLR9. The genes were sequenced and compared to the available equine, human and/or murine TLR2 or TLR9 sequences for confirmation. Afterwards, both genes were cloned in a mammalian expression vector and used to transfect equine fibroblast cells (NBL-6) and CHO cells. While NBL-6 cells expressed the His\textsubscript{6}/myc tagged TLR proteins very weakly, both proteins could be expressed successfully in CHO cells.
Plans to address aim 1 during the next year of support: CHO cells transfected with the TLR constructs will be infected with different EHV-1 strains (Ab4, RacL11 and NY03) and NF-κB and IFN reporter assays will be performed in Dr. Leifer’s group to determine the TLR-dependent signaling and stimulation by the different EHV-1 strains.

**Aim 2** as actually funded: We will identify the major chemokines that are produced in response to EHV-1 infection after TLR stimulation. Blood cells lacking expression of T, B and monocyte cells will be enriched from equine peripheral blood. These cells typically represent dendritic cells that are high producers of IFN and play an important role in host-defense against viral infection. The cells will be infected with different EHV-1 strains and assayed by PCR for chemokine gene expression. Supernatants will be tested for cytokine and chemokine production in Aim 3.

**Results:** Initial experiments were performed with equine PBMC. These cells were infected with different EHV-1 strains (Ab4, RacL11 and NY03). The cells were collected for RNA isolation to perform PCR for different chemokines. Supernatants were obtained for cytokine detection (IFN-α and IL-10).

The qualitative PCR analysis showed a clear suppression of CCL3 expression after infection of equine PBMC with the EHV-1 strain RacL11 (Fig. 1, #2, thin arrows). The PCR also suggested an increase in CXCL9 expression after EHV-1 infection (Fig. 1, #5, bold arrows).

To confirm these changes real-time PCR primers were subsequently designed. The real-time PCR analysis of the expression of all chemokine genes after infection of PBMC with all three EHV-1 strains is currently ongoing. The preliminary data suggest that EHV-1 actively influences chemokine expression and modulates the innate immune response early after infection.

The induction of IFN-α and IL-10 was measured after infection of PBMC with the three different EHV-1 strains (Fig. 2). While the production of IFN-α increased with the viral dose for RacL11 and NY03, IFN-α secretion was clearly diminished after Ab4 infection with high viral doses (Fig. 2, left panel). In addition, the production of the anti-inflammatory cytokine IL-10 was decreased after Ab4 infection compared to RacL11 and NY03 strains at medium and high viral infection doses (Fig. 2, right panel).
These results illustrate the differences in the ability to induce anti-viral IFN-\(\alpha\) and anti-inflammatory IL-10 by these EHV-1 strains, and indicate that the neuropathogenic Ab4 strain inhibits IFN-\(\alpha\) production in high doses and IL-10 in high and medium doses. This suggests to mechanisms that down-regulate type I interferon production and fail to regulate inflammation in the host after infection with neuropathogenic strains of EHV-1. The variations in innate IFN-\(\alpha\) and IL-10 secretion might influence the development of a protective immune response in the host, the pathogenicity of the virus and finally the outcome of disease.

Plans to address aim 2 during the next year of support: The real-time PCR experiments will be completed and analyzed. The IFN-\(\alpha\) expression experiments will be repeated with different cell populations to identify the source of IFN-\(\alpha\). Data analysis and publication of the data is also planned for next year.

**Aim 3 as actually funded:** We will generate monoclonal antibodies to two of the most important chemokines in the TLR-mediated innate immune response to EHV-1 infection. Dr. Wagner’s lab will use an established expression system to produce these monoclonal antibodies.

**Results:** We were able to obtain yeast expressed equine CCL3 and CXCL9 from Kingfisher Bioscience and performed fusions with both chemokines. The yeast expressed CXCL9 did not result in any clones that recognize the native equine chemokine. The fusion for CCL3 is still ongoing.

**Plans to address aim 3 during the next year of support:** The CXCL9 fusion will be repeated with mammalian expressed CXCL9 using our established Ig fusion protein system. The CCL3 fusion will be further characterized and if not successful, also repeated with mammalian expressed protein. If antibodies are developed, they will be used to test the supernatants of EHV-1 infected equine PBMC to confirm the PCR results obtained in aim 2.
APPENDIX B

Final Reports Resulting from 2008 Funding
Harry M. Zweig Memorial Fund for Equine Research

2008 Final Report

P.I.: Dr. Dorothy Ainsworth

Title: Modeling Equine Pulmonary Disorders in vitro: Epithelial-Derived Proteins and Inflammatory Airway Disease

Project Period: 1/1/08-7/31/09
Reporting Period: 1/1/09-7/31/09

Dr. Ainsworth was granted a no cost extension through July 31, 2009. A final report is provided.
Title: “Modeling Equine Pulmonary Disorders in vitro: Epithelial-Derived Proteins and Inflammatory Airway Disease.”

Principal Investigator: Dorothy M. Ainsworth DVM PhD

Summary: Our overall goal is to better understand the pathophysiological mechanisms underlying the development of equine inflammatory airway disease (IAD). This etiopathogenesis of this disease, which occurs in racehorses and in young performance horses, is poorly understood and has been hypothesized to result from exposure to viral agents, endotoxin, cold air or blood. Our data demonstrate for the first time that the bronchial epithelium, upon exposure to hay dust or autologous blood (but not to autologous plasma), up-regulates inflammatory cytokine expression. Our findings suggest a major role of the bronchial epithelium in inducing inflammatory airway disease in athletic horses.

Specific Aims and Findings: We hypothesized that the airway epithelium is a major player in inciting the pulmonary inflammatory response of IAD following its exposure to organic dusts (stabling) or to autologous blood (EIPH). Using an in vitro model of the bronchial epithelium that we developed as part of this study, we sought to determine:

Aim one: if chemokine and mucus gene expression in airway epithelial cells was up-regulated in response to exposure to autologous blood or plasma and how this response compared to organic dust exposure (hay dust).

Aim two: if antimicrobial peptide gene expression is up-regulated in airway epithelial cells exposed to autologous blood, endotoxin or isoleucine exposure.

Aim one: Bronchial epithelial cell cultures were established from 6 young healthy horses that had previously been pastured for 2 months. Horses were confirmed free of IAD by endoscopic examination of the lower respiratory tract and cytological examination of bronchoalveolar lavage fluid. Bronchial epithelial cell cultures were incubated for 24 h with solutions of saline, hay dust, endotoxin, autologous serum and autologous blood. Gene expression of interleukin1-beta (IL-1β), tumor necrosis factor alpha (TNF-α), IL-8 and CXCL2 (all cytokines that induce inflammatory cell influx into the airway lumen) was measured by real-time PCR. The gene expression of MUC5AC and MUC5b, two genes that control mucus expression in the airway epithelium, were also measured by real-time PCR.

We found that with the exception of TNF-α, MUC5AC and MUC5b, all target genes were expressed in the airway cells following treatment with saline. Treatment of the airway epithelial cell cultures with autologous blood significantly up-regulated IL-1β, IL-8 and CXCL2 expression relative to that induced with saline treatment. Indeed, the fold-increase in these cytokines following autologous blood treatment was comparable to that induced with a hay dust treatment. Interestingly, autologous plasma exposure failed to up-regulate chemokine expression in the airway epithelial cells.

Aim two: This portion of the study is currently being undertaken by a Cornell University undergraduate (Pre-Med) student who has previous experience working with cellular based anti-microbial peptides. We anticipate completion of this portion of the study by spring semester, 2010.
Significance: The data from aim one directly support our hypothesis that the airway epithelium plays an important role in inducing IAD in young healthy horses. The implication of our findings is that either organic (hay) dust exposure (a consequence of prolonged stabling) or pulmonary hemorrhage (a result of strenuous exercise—EIPH) have the potential to induce IAD through epithelial-based mechanisms—increased production of inflammatory cytokines. The finding that blood but not plasma induced chemokine up-regulation also suggested that erythrocytes or their intracellular components (hemoglobin) are pro-inflammatory in nature.

Our studies have additional significance: Having validated this in vitro model of the equine bronchial epithelium system has enhanced international collaborations investigating equine IAD. For example, Dr. Andres Diaz, from the Ontario Veterinary College-University of Guelph—recently spent four weeks in our laboratory learning methodology to examine the role of equine rhinovirus B in inducing IAD. A second collaboration has recently been initiated with Dr. Eliane Marti at the University of Berne, Switzerland. Her laboratory will be using our airway epithelial model to investigate the role of TSLP in equine IAD. TSLP or thymic stromal lymphopoietin is an epithelial-derived cytokine that recently has been found to play a significant role in orchestrating human airway disease.

Publications:


Reyner C, Matychak MB Ainsworth DM. The airway epithelium as a source of chemokines: a potential link between inflammatory airway disease and exercise-induced pulmonary hemorrhage. (In preparation, target date for submission to the Am J Vet Res is February 2010).*

* Ms. Reyner, an undergraduate student who conducted much of the research on this project, is currently enrolled in the College of Veterinary Medicine, University of Georgia. We hope to complete the manuscript over the semester holiday break.

**At least one publication and one abstract is expected following completion of aim two.

Listing of grant applications and their status resulting from Zweig funding:

None submitted at this time.
Harry M. Zweig Memorial Fund
for Equine Research

2008 Final Report

P.I.: Dr. Lisa Fortier

Title: Characteristics of Stem Cells Derived from Bone Marrow Aspirate, Adipose Tissue and Muscle

Project Period: 1/1/08-6/30/09
Reporting Period: 1/1/09-6/30/09
Dr. Fortier was awarded a no cost extension through June 30, 2009. A final report is provided.
Title: Characteristics of Stem Cells Derived from Bone Marrow Aspirate, Adipose Tissue and Muscle

Principal Investigator: Lisa A. Fortier

Summary: The broad objectives of this proposal were to define the subpopulations of mesenchymal progenitor cells (MPCs) in adult equine tissues using a combination of molecular and biochemical techniques. The expectation was that these studies would provide important insight into the identification and isolation of MPC subpopulations that form more differentiated cell types, with the long term goal of improved quality in cell-based grafting procedures.

Specific Aims and Findings: The experiments in this proposal were focused upon answering two primary questions. First, what are the differences in molecular characteristics and quantity of mesenchymal progenitor cells (MPCs) harvested from bone marrow aspirate or other tissue sources? Second, what are the unique molecular characteristics of subpopulations of MPCs within a tissue source and how do the subpopulations vary in their ability to terminally differentiate into cells of bone and cartilage phenotypes? During the funding period, we focused on defining MPCs from adult bone marrow aspirates.

As detailed in the publications listed below, we successfully developed a cell surface marker profile to identify MPCs in equine bone marrow-derived MPCs. Further, we determined the temporal changes in this marker profile as bone marrow-derived MPCs were terminally differentiated into bone, cartilage, and adipose tissue lines. A second manuscript is in preparation and further details the differences between the subpopulations of MPCs in bone marrow aspirate in their ability to differentiate into cartilage, bone, and adipose tissue.

Significance: These studies were the first to generate and validate a molecular marker profile to identify equine MPC from isolation through differentiation into cartilage, bone, and fat. The results led to more fundamental questions regarding the epigenetic status of MPCs and in compliment, clinically applicable questions regarding how to enrich or purify MPCs from bone marrow and what the ideal biological vehicle would be for transplantation of MPCs into cartilage or tendon defects.

Publications:

Listing of grant applications and their status resulting from Zweig funding:
Fortier (Mentor) Radcliffe (fellow)
Sponsor: NIH-NRSA
Title: Defining mesenchymal progenitor cell subpopulation
This award in intended to support Dr. Radcliffe’s salary during her post-doctoral period.
Status: funded

Fortier PI
Sponsor: New York State Department of Health
Title: NYSTEM Grant for Institutional Development of Stem Cell Research Capability.
The primary objective of this award is to determine the histone acetylation status of bone
marrow-derived mesenchymal stem cells.
Status: funded

Fortier (Mentor) Radcliffe (fellow)
Sponsor: Grayson- Jockey Club Research Foundation, Inc.
Title: Temporal analysis of mesenchymal progenitor cells
This award in intended to provide supplemental salary support for Dr. Radcliffe during her
post-doctoral period.
Status: submitted, pending review
Harry M. Zweig Memorial Fund for Equine Research

2008 Final Report

P.I.: Dr. Gillian Perkins

Title: Therapy and Prevention of Equine Herpesvirus-1 (EHV-1)-Induced Disease

Project Period: 1/1/08-12/31/09
Reporting Period: 1/1/09-12/31/09
Specific Aim 1: To test the efficacy of siRNA directed against the gB and UL9 genes and combinations thereof in vitro and in mice.

Specific Aim 2: To test the efficacy of siRNA directed against the gB and UL9 genes in horses.

No modifications were made to these broad aims.

Specific Aim 1: Small inhibitory RNAs (siRNAs) were developed against two essential and highly conserved EHV-1 genes. The siRNAs targeted (1) open reading frame (ORF) 33 encoding the envelope glycoprotein B (gB), which is essential for viral entry and cell-to-cell spread (named sigB3), and (2) ORF53 (UL9 homologue) encoding the origin-binding protein (ORI) helicase (named siOri2). ORI also is essential for virus growth, because this protein is required for the initiation of herpesviral genome replication. Initially, the siRNAs were tested in vitro by treating rabbit kidney (RK-13) cells with the siRNAs and then determining the effect on growth of equine herpesvirus (EHV-1) as measured by viral titers (PFU/mL) and plaque sizes. A clear reduction (up to 80-fold) in EHV-1 growth was seen in the cell culture system using sigB3 and siOri2 separately, and when the two siRNAs were used in combination, a much lower dose of each was required suggesting a synergistic inhibition of EHV-1 replication. With this promising data, we tested sigB3 and siOri2 in a mouse model of EHV-1 infection. The mice were treated intranasally before and after infection with EHV-1 with various doses of the sigB3 and siOri2, alone and in combination, and using different carriers [phosphate buffered saline (PBS) and lipofectamine]. These were compared to negative controls (siRNA against firefly luciferase – siLuc, and no siRNA). Mice treated with the EHV-1 specific siRNAs were protected against clinical signs, such as weight loss and the viral loads in the lungs of mice were significantly lower as assessed by viral titration of lung tissue, quantitative real-time PCR and histological scoring. Another promising result was that there was no significant difference between the effectiveness of the EHV-1 siRNAs complexed with the transfection reagent lipofectamine or with buffer (PBS).

Specific Aim 2: Two equine EHV-1 challenge experiments were performed to test the efficacy of the sigB3 and siOri2 in the target species. In the first experiment, ten horses were given 750 pmol of each siRNA intranasally using a nasal atomizer, 12 hours before and 12 hours after infection with EHV-1 strain Ab4, a neuropathogenic virus strain. Four horses were used as controls and given siLuc at the same time the EHV-1 specific siRNAs were applied. There was no difference in the duration or severity of fever and viral load in the nasal secretions or peripheral blood mononuclear cells (PBMCs) as measured by viral culture and quantitative real-time PCR for the three weeks after infection. However, neurologic signs developed in 3 of the 4 control horses and required humane euthanasia due to the severity of the ataxia and inability to urinate and defecate. Only 2 of the 10 horses treated with sigB3 and siOri2 developed mild neurologic symptoms and were mildly affected (grade 1 ataxia) at the end of the study. The discrepancy between the percentage of horses developing severe neurologic symptoms necessitating euthanasia and the fact that there was no difference in the viral load in the PBMCs was curious because it has been proposed that equine herpes myeloencephalopathy (EHM), the neurologic form of EHV-1-induced disease, generally arises in horses with a higher level of EHV-1 circulating within blood lymphocytes and for a longer period of time. Thus a second horse experiment was performed using higher and
more frequent doses of the siRNAs (24 h before and 12, 24, 36 and 48 h after EHV-1 infection). Seven horses were treated with the sigB3 and siOri2 (5 nmoles each) and six were given siLuc as controls. Once again there was no difference in the viral loads present in the nasal secretions or PBMC. In addition, in the second experiment there was no difference between groups with respect to the development of the neurologic symptoms. EHM was seen in 5 horses (2 siLuc and 3 sigB3/siOri2) with the most severely affected horse being in the sigB3/siOri2 treatment group. Our conclusions were that sigB3 and siOri2 using these dosing protocols did not protect or reduce the severity of clinical signs of EHV-1 infection. Further work is necessary to determine if higher doses, or newer more efficient siRNAs and carriers to assist in nasal absorption of the agent would be effective.

From the two EHV-1 experiments using Ab4 (neurologic strain of the virus at 1 x 10^7 PFU per horse) administered intranasally using a fine mist (nasal atomizer) we have been able to produce EHM in 10 out of 27 horses or 37%. Defining our EHV-1 experimental model has been a valuable exercise and should aid in further investigation of treatment, prevention and pathogenesis of EHV-1 infection in general and EHM in particular.

*Other work partially funded by the Zweig grant.

Our laboratory has had continued interest in the single point mutation of the equine herpes virus-1 (N752 to a D752) that is linked with the development of EHM (Goodman et al., 2007; Van de Walle et al., 2009). During the course of this proposal we started a side-project investigating the prevalence of the neurologic equine herpes virus type 1 from isolates sent to the New York State Animal Health and Diagnostic Laboratory from 1984-2007. Allelic discrimination quantitative real-time PCR (Allen, GP. 2007) and sequencing of the ORF30 (Pol gene) region where the single point mutation occurs was performed on 176 EHV-1 isolates and the genotype of the virus was compared to the clinical history. We found that the neurologic strain of the virus (ORF30 G2254 or D752) was highly associated with outbreaks of EHM. However, the “non-neurologic” form of the virus (ORF30 A 2254 or N752) was isolated from nearly 25% of the EHM cases suggesting that other factors may also contribute to the onset of EHM.

Scientific Findings:
Failure of conventional drugs and vaccines to produce the desired level of protection against EHV-1 has led to our investigation of an alternative novel approach for prophylaxis and treatment using RNA interference. To the author’s knowledge, we are the first to investigate RNA interference in a domestic species. In vitro and in a mouse model of EHV-1 infection our synthetic siRNAs targeted against essential genes of the EHV-1 (sigB3 and siOri2) were effective in reducing clinical signs of EHV-1 infection, lung pathology and viral shedding. Application of this RNA interference technology in the target species, the horse, showed less favorable results. sigB3 and siOr2 given around the time of EHV-1 infection in two separate experiments using a total of 27 horses did not alter the clinical signs including neurological symptoms, EHV-1 nasal shedding and viremia compared to control horses. RNA interference has been rapidly evolving and there have been modifications to the original siRNAs that we developed making them more efficient at knocking down the target RNA. With further development of siRNAs and optimizing dosage and improving transfection agents that would increase uptake by the equine nasal mucosa, RNA interference as a metaphylactic and preventative treatment for EHV-1 may be worth further investigation in the future.
Publications:


Presentations at national meetings in an abstract form:

2006 ACVIM Forum, San Antonio, TX.
2007 Dorothy Havemeyer Equine Herpes 1 Workshop, Steamboat Springs, CO


2009 AAEP, Las Vegas, Nevada

Perkins GA, Nicola Pusterla, Nikolaus Osterrieder. RNA interference does not protect against experimental equid herpesvirus type 1 (EHV-1) infection.

2010 ACVIM Forum, Anaheim, CA.

References


APPENDIX C

SUMMARY OF 2009 EXPENDITURES

2009 Research Awards $594,339
2009 Public Relations and Administrative Budget $27,500
Incentive Awards $20,000
30th Anniversary Celebrations $3,000

Total Expenditures: $644,839
APPENDIX D

2009 PUBLIC RELATIONS AND ADMINISTRATIVE BUDGET
Harry M. Zweig Memorial Fund for Equine Research  
Public Relations and Administrative Budget

**BUDGET**

**Fiscal Year 2010**  
(07/01/09 - 6/30/10)

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**Administration:**  
- Administrative Services: $ 14,000
  - .20 FTE for Administrative Assistant
  - Xeroxing, Mailing, Communications, Web Maintenance, $ 1,800
  - Zweig News Capsule Coordination
  - 30th Year Celebration $ 3,000
  - Contingency $ 400

**Total Administrative Costs**  
$ 19,200

**TOTAL BUDGET**  
$ 27,500
APPENDIX E

POSTER SESSION PRESENTATIONS FOR 30TH ZWEIG ANNIVERSARY
Cornell University & Harry M. Zweig Memorial Fund for Equine Research
Poster Session & Seminar – 30th Zweig Anniversary
November 19, 2009
Dr. Harry M. Zweig Memorial
CELEBRITY JULIET

July 10, 2009
One Mile Trot
Owner: Celebrity Farms
Trainer: Staffan Lind
Driver: Mike LaChance
Race: 7
Time: 1:56:1

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Nichols, New York
Fred J Brown Studios
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Welcome to the Poster Session in celebration of the 30th Anniversary of the Harry M. Zweig Memorial Fund for Equine Research.

The posters have been created by faculty, graduate students and residents who have been a recipient of an award from the Fund.

We would like you to enjoy looking at these posters and feel free to ask questions. We would also like to thank the presenters for taking the time to join us in celebration of the Zweig Memorial Fund.

Thank you for your attendance.

Dr. Robert Gilmour, Associate Dean for Research and Graduate Education
Laura Mathews, Zweig Secretary
#1 - Title: A D752N Mutation in the Equine Herpesvirus-1 (EHV-1) Polymerase Abolishes Neurovirulence in the Natural Host

Authors: Gillian Perkins, Klaus Osterrieder, Laura Goodman, Arianna Loregian, Josie Nugent, Elizabeth Buckles, Beatrice Mercorelli, Julia Kydd, Giorgio Palu, Ken Smith, Nicholas Davis-Poynter

Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853

Abstract: Equine herpesvirus type 1 (EHV-1) is spread by aerosol and is the causative agent of the most common neurologic disease of horses. Outbreaks of the neurologic form of EHV-1 can be devastating to individual animals and entire herds, and approximately one-third of the affected horses generally are at risk of death or suffer so extensively that euthanasia becomes necessary. Our report provides evidence for a direct causal link between the genotype of EHV-1 strains and their neurovirulence, and thereby gives a long-awaited explanation for the conundrum of the different clinical outcomes following EHV-1 infection. We proved that alteration of one amino acid in the key viral enzyme, DNA polymerase, which is conserved in all herpesviruses, renders the virus unable to cause neurologic disease. The improved clinical outcome is likely due to the reduction in virus levels in the bloodstream, ultimately resulting in less virus reaching the central nervous system. In summary, our study shows that herpesvirus virulence and tissue tropism in the natural host are linked with the function of a key virus-encoded enzyme involved in DNA replication.

#2 - Development of a Bead-Based Multiplex Assay for Simultaneous Quantification of Cytokines in Horses

Authors: Bettina Wagner, Heather Freer, Department of Population Medicine and Diagnostic Sciences, Animal Health Diagnostic Center, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853

This work was supported by the USDA and the Method Development funds of the Animal Health Diagnostic Center at Cornell University.

Abstract: The detection and quantification of equine cytokines has been hampered by the lack of antibodies for many years. With the development of antibody pairs for equine cytokines during the past years, the quantification of these essential regulators of the immune response became possible. After being successfully tested by enzyme-linked immunosorbent assays (ELISA), three of these anti-cytokine reagents were used here to establish the first cytokine multiplex assay for equine IL-4, IL-10 and IFN-a. A fluorescent bead-based system was used as matrix for this assay that allows the simultaneous detection of the cytokines in a single sample by a Luminex analyzer. Equine recombinant cytokine/IgG fusion proteins were validated as standards for quantification of the individual cytokines. The analytical sensitivities of the multiplex assay were found to be 40 pg/ml for IL-4 and 15 pg/ml for IL-10 and IFN-a. The sensitivity of cytokine detection by the multiplex assay was increased by 13 to 150-fold compared to the corresponding ELISA. The specificity of the multiplex assay was validated using cell culture supernatants from equine peripheral blood mononuclear cells (PBMC) stimulated with different mitogens or infected with equine herpesvirus type 1 (EHV-
1). As predicted, supernatants from PBMC stimulated with different mitogens contained IL-4 and IL-10, but no IFN-a. EHV-1 infection of PBMC resulted in a dose-dependent secretion of IFN-a. Low concentrations of IL-10 were also measured. IL-4 was not detectable in these samples. The resulting detection pattern found for the multiplex analysis and assays performed with individual standard cytokines indicated that individual bead assays did not interfere or cross-react during simultaneous detection of equine IL-4, IL-10 and IFN-a. The equine cytokine multiplex assay is a valuable and cost-effective tool for quantification of IL-4, IL-10 and IFN-a and can be used for manifold immunological applications. In the future, the assay can also be expanded by adding bead assays for other equine cytokines and chemokines to the existing platform.

#3 - Occurrence and IgE Binding of Mucosal Mast Cells in Neonates

Authors: Bettina Wagner, Julie Hillegas, M Julia Flaminio, Mary Beth Matychak, Bronwen A Childs, Seana M Thrasher, Douglas F Antczak.
Department of Population Medicine and Diagnostic Sciences, Department of Clinical Sciences, Baker Institute for Animal Health, Cornell University, Ithaca, NY

Abstract: Maternal IgE is exclusively transferred to the neonatal foal via colostrum after birth. The primary contact site for maternal IgE is the foal’s gut tissue. Our goal was to investigate neonatal tissues for the presence of mucosal mast cells (MCs), and to test whether intestinal neonatal MCs can bind maternal IgE. Tissues from the small intestine, mesenteric and peripheral lymph nodes were harvested immediately after birth (before colostrum uptake), and on days 5, 9 and 36 from healthy neonates. Mucosal MCs were detected using Alcian blue staining. At birth, most MCs were found in the crypt areas of the small intestine. On days 5 and 9, only a few mucosal MCs could be detected. On day 36, many MCs were found in the submucosa. Mucosal MCs were observed in the T-cell areas of lymph nodes at all time points. To investigate whether intestinal MCs can bind maternal IgE directly after birth, MCs were isolated from the mucosa of the jejunum. The cells were cultured in the presence of fluorochrome conjugated IgE and measured by flow cytometry. Two populations of IgE+ cells were detected. First, an IgE+/MHC class II- population; and second, cells of a IgE+/MHC class II+ phenotype. We concluded that intestinal mucosal MCs express IgE receptors to rapidly bind maternal IgE from ingested colostrum.
#4 - Sensitization of Skin Mast Cells with IgE Antibodies to Culicoides Allergens Occurs Frequently in Clinically Healthy Horses

Authors: Bettina Wagner, William H. Miller Jr., Hollis N. Erb, D. Paul Lunn, Douglas F. Antczak - Department of Population Medicine and Diagnostic Sciences, Department of Clinical Sciences, and Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Ithaca, NY, Department of Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO 80523

Abstract: IgE antibodies are mediators of mast cell degranulation during allergic diseases. The binding of IgE to its high-affinity IgE receptor on mast cell surfaces is called "sensitization" and precedes the development of clinical allergy. Previously, intradermal injection of anti-IgE or the anti-IgG(T) antibody CVS40 induced immediate skin reactions in horses. This suggested that both IgE and IgG(T) sensitize equine skin mast cells. Here, we investigated sensitization to allergen and with IgE or IgG(T) in clinically healthy horses of different age groups. In addition, immediate skin reactions to Culicoides were determined by intradermal testing in non-allergic horses. A total of 14% of the young horses 1-3 years old and 38% of the adult animals showed skin reaction to Culicoides allergen extract. Sensitization with IgE and IgG(T) was evaluated in skin mast cells and peripheral blood basophils to determine whether sensitization with IgG(T) preceded that with IgE in young horses. Anti-IgE stimulated immediate skin reactions in 18 of 21 young horses, but only 7 of them reacted to the anti-IgG(T) antibody CVS40. The equine IgG(T) fraction is composed of IgG3 and IgG5. We used several newly developed monoclonal antibodies to IgG3 and IgG5 for intradermal testing to improve our understanding about the mast cell reaction induced by the anti-IgG(T) antibody CVS40. None of these antibodies induced a skin reaction in young or adult horses. To determine sensitization with IgE in neonates and foals at 6 and 12 weeks of age an in vitro histamine release assay was performed using peripheral blood cells. The histamine concentration released by anti-IgE stimulation from foal basophils increased between birth and 12 weeks of age, while almost no histamine release was observed after anti-IgG(T) treatment of the cells. In summary, IgE was the major immunoglobulin involved in the sensitization of mast cells and basophils in horses at various ages. IgG(T) antibodies did not play a major role in the activation of mast cells or basophils in young horses and their role in the sensitization of adult horses remains unclear. Sensitization to Culicoides allergen in the absence of clinical disease was frequently found in horses of all age groups. Because many clinically healthy horses developed skin reactions to this allergen, sensitization results are useful to diagnose Culicoides-induced allergy only in horses with allergic conditions.
#5 - Basophils are Producers of Initial Interleukin-4 in Neonates

**Authors:** Bettina Wagner, Julie M Hillegas, Bronwen Childs, Alexandra Burton, Hollis Erb, Dorothy M Ainsworth - Department of Population Medicine and Diagnostic Sciences, and Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA

**Abstract:** Interleukin-4 (IL-4) is a key cytokine of the T helper 2 cell response. IL-4 has been found to be a major regulator of immunoglobulin class switching to IgE and has important functions in the regulation of allergic diseases. Here, the onset of the IL-4 production after birth was investigated in equine neonates. The form of equine placentation does not support the transfer of cytokines or immunoglobulins in utero and maternal immunity is exclusively transferred to the neonate with the colostrum after birth. IL-4 producing cells were measured in peripheral blood mononuclear cells (PBMC) of neonates and foals by flow cytometric analysis. At day 3-6 after birth, a small population of IL-4 producing cells was observed in the absence of any stimuli. The IL-4+ population was not detectable at 6 or 12 weeks of age. Other cytokine producing cells (IFN-g, IL-10) were not detected using these conditions. The stimulation of neonatal PBMC with PMA and ionomycin did not alter the IL-4+ cell population. Phenotyping of the neonatal IL-4+ cells showed that they were IgE+/MHCII-/CD4- cells. The occurrence of CD4+ IL-4 producing cells after PMA stimulation increased slowly with age and did not reach adult levels by 12 weeks after birth. Magnetic cell sorting of the IgE+/MHCII+ cells identified them as basophils. Previous work has shown that foals do not produce endogenous IgE for at least six months of life. IgE bound to the surface of neonatal basophils was found to be of maternal origin and transferred with the colostrum after birth. Here, the stimulation of neonatal PBMC with anti-IgE induced the secretion of IL-4 at day 5 after birth. Neonatal PBMC collected before colostrum uptake did not produce IL-4 in response to anti-IgE. In summary, equine neonates provide a model to investigate IgE mediated IL-4 responses after birth. The transfer of maternal IgE from allergic individuals could potentially provide a direct mechanism for the early induction of an allergen-specific neonatal IL-4 response mediated by the mare’s accumulated acquired immunity to allergens.

#6 - Putative Mesenchymal Progenitor Cells from Equine Bone Marrow are Enriched Using the Hematopoietic Marker CD14

**Authors:** Catherine Radcliffe, Julia Flaminio, Lisa Fortier, Department of Clinical Sciences, Cornell University, Ithaca, NY 14853

**Abstract:** Bone marrow contains a heterogeneous mixture of hematopoietic and nonhematopoietic cells. There are also cells with reported capacity to differentiate into bone, cartilage, and other tissues. The terminology used to describe these cells varies and includes mesenchymal stem cell (MSC), stromal stem cell (SSC), and mesenchymal progenitor cell (MPC), used herein. One of the defining features of MPCs in humans and other species is the lack of expression of the cluster of differentiation (CD) marker CD14, also known as the lipopolysaccharide receptor (LPS-R) on their cell surface (Dominici, M et al, 2006). Only certain lineages of hematopoietic cells (e.g. monocytes, macrophages, dendritic cells, and to a lesser extent neutrophils), are known to express CD14 on their surface. Therefore, in this study we proposed to enrich for MPC colony formation by removal of the CD14 positive population from equine bone marrow cells in a cell sorting experiment utilizing a CD14 antibody.
#7 - Biomechanical Stiffness of Hyaline Cartilage In The Equine Larynx

**Authors:** Jeremy Rawlinson, Jonathan Cheetham, Lawrence Bonassar, Norm Ducharme, Samantha Passman - College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, and Civil and Environmental Engineering, Cornell University, Ithaca, NY, Biomedical Engineering, Cornell University, Ithaca, NY

**Abstract:** Airway obstruction is a common problem in the equine athlete as the soft tissues of the larynx collapse into the airway. With a prosthetic laryngoplasty for recurrent laryngeal neuropathy, the suture anchor site at the cricoid or continuing loss of arytenoid abduction limit clinical success. The purpose of this study was to measure the compressive mechanical properties of the hyaline cartilage to better understand the anchoring and deformation characteristics. Eight larynges were harvested from Thoroughbreds and Standardbred at necropsy. Biopsy specimens were obtained from three sites within the dorsal cricoid (caudal, middle, and rostral) and two central sites in the arytenoids (inner, outer). In a mechanical testing frame, the specimens were loaded to calculate the compressive stiffness according to bioengineering protocols; the data were analyzed using nested ANOVA. There were significant observations of higher modulus with increasing age (0.13 MPa per year; p=0.012) and stiffer cricoid cartilage (2.27 MPa) than the arytenoids (0.43 MPa; p<0.001), but no difference was observed between the left and right sides. Linear contrasts showed that the rostral aspect (2.51 MPa) of the cricoid was 24% stiffer than the caudal aspect (2.02 MPa; p=0.007), with no difference between the arytenoid sites. These results indicate that the suture, therefore, would pass through the least stiff region of the cricoid. The arytenoid is also a structure of relatively lower stiffness in the central region, for reference, equine articular cartilage is ~0.21 MPa. These characterizations are important to understand the laryngeal function and will continue with tissue failure properties.

#8 - Using The Equine Bronchial Epithelial Cell Culture System to Study Immune Response to Rhodococcus equi Infection

**Authors:** U Schwab, ML Fulcher, SH Randell, S Caldwell, DV Nydam, MB Matychak, DH Schlafer, DG Russell, MJBF Flaminio - Dept. of Microbiology and Immunology, Clinical Sciences, Dept. of Population Medicine and Diagnostic Sciences, and Biomedical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA; and the Cystic Fibrosis/Pulmonary Research and Treatment Center, The University of North Carolina at Chapel Hill, NC

**Abstract:** *Rhodococcus equi* causes pyogranulomatous pneumonia and enteritis in foals less than 5 months of life, but not in older horses. During the establishment of natural infection in foals, the lower respiratory tract seems to be the major site of inflammatory response. It is unknown how *R. equi* establishes infection in the respiratory tract of foals. We have developed a 3-D in vitro equine bronchial epithelial cell culture system that fully differentiates into ciliary beating and mucus producing cells. Using this system, we evaluated phagocytic activity of macrophages, and molecular signaling between bronchial epithelial cells and macrophages upon *R. equi* infection. Our hypothesis is that, in contrast to the adult horse, bronchial epithelial cells of young foals cannot signal phagocytes for effective bacterial removal and killing upon *R. equi* infection. Our goal is to identify key immune response signals modulated by *R. equi* infection that are distinct between foals and adult horses. Using live confocal microscopy, we observed...
macrophages as they interact with mucus in their natural environment, while patrolling the airway surface to eradicate the bacteria. We were able to monitor the movement of phagocytes and bacteria in the midst of ciliary beating of the epithelial cells. After 3h of incubation, phagocyte-adhered and ingested bacteria were seen as demonstrated by the co-localization of bacteria within live macrophages. To evaluate if mucus affected the phagocytic activity of macrophages, we incubated adult horse monocyte-derived macrophages with \( R.\ equi \) for 4 h either in the mucus layer of in vitro generated adult horse airway epithelium or on collagen coated membranes. The macrophages were harvested, pelleted and fixed in glutaraldehyde. Next, the cells were embedded in epoxy and Richardson stained sections examined by light and electron microscopy. The number of macrophages with intracellular bacteria, and the number of intracellular bacteria per macrophage seemed lower when macrophages were incubated with \( R.\ equi \) in the presence of mucus. Most interestingly, when we added foal macrophages to foal bronchial epithelial cells, we made the opposite observation, i.e. many macrophages were loaded with \( R.\ equi \). To determine whether the in vitro equine bronchial epithelial system would allow the study of molecular signaling between bronchial epithelium and phagocytes during \( R.\ equi \) infection, we cultured the equine bronchial epithelial cells with \( R.\ equi \), and measured the expression of TLR-2 (involved in \( R.\ equi \) recognition) and EGF- R (involved in mucin production). Using real-time RT-PCR, we observed a decrease in TLR-2 expression and a concomitant increase in EGF-R expression when equine bronchial epithelial cells were infected with \( R.\ equi \), in comparison to non-infected cells. Currently, we are studying the expression of these genes in foal epithelial cells. These data demonstrate that our differentiated respiratory epithelial cell culture system retains functional characteristics similar to those found in vivo.

Further studies using this system will investigate whether the respiratory environment in the foal responds differently to \( R.\ equi \) infection, and the effect of mucus on opsonization and phagocytosis.

#9 - Equine-1 Influenza Virus as a Model for Emergence of Pandemic Influenza in Mammalian Species

**Authors:** Xiangjie Sun, Gary Whittaker,
Department of Microbiology & Immunology, Cornell University, Ithaca NY 14853

**Abstract:** Influenza virus is currently of major biomedical interest due to the emergence of new pandemic viruses in humans (e.g. novel H1N1, or swine flu). There is also great concern regarding the ability of highly pathogenic avian influenza viruses to undergo direct transmission to humans. This has occurred with fatal consequences for several viruses i.e. H5N1, H7N7, and can also occur for other subtypes (e.g. H9N2). However, humans are not the only mammalian species into which highly pathogenic avian influenza viruses can undergo direct transmission. The equine-1 influenza viruses are examples of avian viruses that have emerged from birds and become established mammalian viruses. As such they serve as excellent models for the emergence of pandemic influenza. A key determinant of influenza pathogenesis is mutation in the proteolytic cleavage site of the viral hemagglutinin (HA) gene, which allows cleavage of HA
by cellular proteases, activation of viral fusion and entry, and spread within the host. Low pathogenicity forms of influenza have a single basic amino acid at the cleavage site (a monobasic site) and are cleaved by trypsin-like proteases. However, highly pathogenic avian influenza typically has a mutation in the cleavage site, which now contains multiple basic amino acids (a polybasic cleavage site). These highly pathogenie viruses can be cleaved by different proteases (e.g. furin). The equine-1 (H7N7) viruses are unusual in that they are a rare example of an influenza virus containing a polybasic cleavage site (RKKR) that has established itself in the mammalian population. Our studies are designed to characterize the function and activation properties of A/equine-1/Cornell/74 (H7N7) as a model of influenza emergence in human and equine populations.

#10 - Concentrations of Cardiac Troponin 1 Measured with an I-STAT®1 Analyzer in Normal Horses Undergoing A Standard Treadmill Performance Examination
Authors: Mark Kraus, Thomas Divers, Daryl Nydam, Sophy Jesty, Anna Gelzer, Norm Ducharme - Department of Clinical Sciences, Department of Population Medicine & Diagnostic Sciences, Cornell University, Ithaca, NY 14853

Abstract: Elevated serum concentration of cardiac troponin I (cTnI) is a biomarker for myocardial damage in horses. Treadmill exercise is a common method for evaluation of horses with poor performance, but detection of occult heart disease is difficult. Treadmill exercise may induce increased cTnI concentrations, thereby unmasking myocardial disease. We hypothesize that in healthy horses, treadmill exercise does not induce cTnI concentrations above the normal range. Our objective was to determine cTnI concentrations in normal horses undergoing a standardized treadmill performance examination. Eleven healthy horses (8 Thoroughbreds, 2 Standardbreds and 1 Warmblood) were exercised using a stepwise incremental treadmill protocol. Blood samples for cTnI were taken prior to exercise and at 5, 60, 180 minutes, and 24 post exercise and then daily for 5 days post exercise. Heparinized plasma samples were frozen at -20º C and analyzed for cTnI concentration utilizing the (i-STAT®1, Heska Corporation). Within each horse the change in cTnI over time was assessed using linear regression. The regression coefficients were then compared to baseline levels of cTnI using a 1 sample Wilcoxon rank test. The pvalue was 0.61, indicating that there was no significant change in cTnI concentration over time in these 11 horses. Clinical significance of this study is that cTnI concentrations are not increased in clinically healthy horses undergoing a standardized treadmill exam. Thus, cTnI concentrations above the reference range measured in a horse at any time point following similar treadmill exercise should be considered abnormal.
**#11 - Telomerase Activity is Lost After Puberty in Articular Chondrocytes**

**Authors:** Donocoff RS, Novakofski KD, Fortier LA  
Department of Clinical Sciences, Cornell University, Ithaca, NY 14853

**Abstract:** Increasing age is the most predominant risk factor for developing osteoarthritis. With increased age, somatic cells reach replicative senescence, the maximum number of cellular divisions. Previous studies suggest that replicative senescence is initiated by telomere erosion, which has been linked to a loss in telomerase activity. The Rho family of GTPases, including Cdc42, Rac, and Rho, participate in cellular responses including replication. In this study, our aim was to measure telomerase activity in chondrocytes as a function of age and GTPase activity. Chondrocytes were isolated from equine articular cartilage and grown in monolayer for 24 hrs in Ham’s F-12 + 10% FBS media, or transfected with wild type, dominant negative, or constitutively active Cdc42, Rac, or Rho for 48 hrs. Presence or absence of telomerase activity was determined with TeloTAGGG Telomerase PCR ELISA kit (Roche Applied Science). Telomerase activity was examined in four age groups: pre-pubescent, pubescent, post-pubescent, and mature (n=4 in each age group). Samples were considered positive for telomerase activity if the blank-corrected absorbance was greater than 0.2.

Pre-pubescent and pubescent chondrocytes both reached the 0.2 threshold for telomerase activity. Chondrocytes from post-pubescent and mature horses did not have telomerase activity, with neither group achieving the 0.2 threshold. Transfection with wild type Cdc42 and Rho increased telomerase in pubescent cells. This study examined the age at which telomerase activity declines in primary equine chondrocytes. According to this study, telomerase remains active in chondrocytes until the horse enters the post-pubescent stage at 15 months of age. Further investigations are required to determine if decreased telomerase activity is related to the limited intrinsic capacity for repair in mature cartilage.

**#12 - Polymorphism and Genomic Organization of the Equine MHC Class II Region**

**Authors:** Donald Miller and Douglas F. Antczak

**Abstract:** Studies of the organization and gene content of the Equine Major Histocompatibility Complex (MHC), also known as the Equine Leukocyte Antigen (ELA) region, have benefited in recent years from the construction of the equine Bacterial Artificial Chromosome (BAC) library, and the whole genome sequence (WGS) of the horse. These tools were derived from DNA of two closely related Thoroughbreds, each homozygous for the ELA-A3 haplotype, and members of the research herd at Cornell University. The BAC library provided a framework for the genomic organization of ELA, and provided sequence data that has enabled us to study gene content and polymorphism in other ELA haplotypes. The WGS has advanced these studies by confirming earlier sequence data and identifying gene loci that were previously undetected. In this study we examine nine DQ and DR genes located in the ELA Class II region, and compare the gene alleles of the ELA-A3 haplotype with those of the ELA-A2, -A5, -A9, and –A10 haplotypes. Our findings reveal a higher level of polymorphism in the beta genes compared with the alpha genes, and three of the five beta gene loci studied have unique alleles for each of the five ELA haplotypes.
#13 - GCM1 RNA is induced in Differentiating Equine Chorionic Girdle Trophoblast Cells

Authors: Amanda M. de Mestre, Donald Miller, Jenny Liford, Lisay Chizmar, and Douglas F. Antczak

Abstract: The aim of this study was to identify key molecules that regulate equine chorionic girdle (CG) differentiation. Equine conceptus tissue was obtained at days 15, 21, 25, 28, 30, 31, 32, 33, 34, 43 and 46 of pregnancy. Expression of Glial cell missing-1 (GCM1), equine chorionic gonadotrophin (eCG)/luteinizing hormone (LH) β subunit and Major Histocompatibility Complex Class I (MHC Class I) mRNA in conceptus tissue and in cultured CG cells was determined using quantitative RT-PCR. Initially, twenty-four conceptus tissues were screened by qualitative RT-PCR using molecular probes for fifteen genes. Expression of GCM1 was found primarily in CG and endometrial cups. Using real time RT-PCR, we found GCM1 mRNA was expressed greater than 10-fold higher in day 34 CG, when compared with other day 34 extraembryonic tissues. GCM1 expression in CG tissue (day 28 to 34) increased over time, with maximal expression observed at day 34. Furthermore, GCM1 expression in CG correlated with expression of eCG/LHβ mRNA. To investigate the cell type that expressed GCM1, we harvested mixed binucleate/uninucleate cultured CG cells using trypsin (enriches for binucleate cell fraction) followed by scraping (enriches for uninucleate cell fraction). We found that GCM1 and eCG/LHβ mRNA was expressed primarily in the binucleate enriched fraction, not the uninucleate enriched fraction. Consistent with previous findings, MHC Class I was expressed at mildly elevated levels in the uninucleate enriched fraction. GCM1 mRNA is highly expressed by equine CG. Expression of GCM1 correlates with CG maturity, binucleatism and eCG/LHβ mRNA expression. Based on these expression patterns it is likely GCM1 is an important molecule in CG differentiation.

#14 - Evidence of an Antigen-Independent Reduction in Cell-Mediated Immunity During Equine Pregnancy

Authors: L.E. Noronha and D.F. Antczak
Baker Institute for Animal Health, Cornell University College of Veterinary Medicine, Ithaca, NY.

Abstract: Mammalian pregnancy has been likened to a semi-allograft existing within the pregnant mother. Consequently, immunological tolerance of the fetus is critical for a successful outcome. During pregnancy, horses demonstrate a “split” immunological tolerance whereby anti-paternal cell-mediated immunity decreases while the antibody mediated compartment remains active. We have previously observed that pregnant female horses (mares) demonstrate a decrease in their ability to generate effective cytotoxic T lymphocyte (CTL) responses against target cells from the breeding stallion. The aim of this study was to determine: a) if this decrease in CTL activity is observed against non-paternal antigens, and b) if this decrease in activity requires fetal expression of foreign MHC antigens. Mares of known MHC haplotypes were inseminated with semen from one of two stallions homozygous at the MHC region to establish 6 MHC mismatched and 4 MHC matched pregnancies. PBMC were isolated and CTL were generated in vitro by stimulation with irradiated cells from individuals with an MHC haplotype distinct from both the mare and stallion (so-called “3rd party”).
The CTL were then assayed for the ability to lyse the 3rd party target cells in a chromium release assay. Cells from the tested mares demonstrated a reduction in CTL activity against 3rd party cell targets while carrying a MHC mismatched pregnancy. Cells from the mares carrying a MHC matched pregnancy likewise showed reduced CTL activity. These data provide evidence that the reduction of CTL activity observed during pregnancy is not specific for paternal alloantigen and that there may be a broader antigen-independent immunological tolerance during pregnancy. They also suggest that the presence of foreign paternal MHC alloantigens may not be required for the generation of the impaired CTL activity.

#15 - Leptosome-entrapped antigens conferred significantly higher levels of protection against leptospirosis in hamsters compared to those associated with conventional liposomes

Authors: Syed M. Faisal, WeiWei Yan, Sean P. McDonough, Ming-Jeng Pan, Chao-F Chang and Yung-Fu Chang - College of Veterinary Medicine, Cornell University, Ithaca, New York, USA

Abstract: We prepared novel liposomes from total polar lipids of non-pathogenic Leptospira biflexa serovar Potac (designated leptosomes) and evaluated their vaccine delivery/adjuvant potential with novel protective antigens (Lp0607, Lp1118 and Lp1454) against leptospirosis in hamsters. The immune response induced against multiple antigens and protective efficacy was evaluated and compared to those induced by conventional liposomes and with liposomes of similar lipid profile viz. E. coli lipid liposomes (escheriosomes). Four-week-old hamsters were immunized subcutaneously twice at a three-week interval, bled at various time points to evaluate antibody response and sacrificed to isolate splenocytes for lymphocyte proliferation and cytokine profiles in response to recall antigen. For the challenge test, 10 MLD50 (modified lethal dose 50%) of virulent L. interrogans serovar Pomona were administered intraperitoneally. Our results demonstrate that leptosomes associated with antigens are better adjuvants than conventional liposomes as revealed by enhanced long term antibody response, lymphocyte proliferation and significant enhancement of both Th1 (IFN-γ) and Th2 (IL-4 and IL-10) cytokines. Additionally, leptosome and escheriosomes induced significantly higher level of memory responses than conventional liposomes did. Moreover, these novel liposomal vaccines induced significantly higher levels of protection than those achieved with conventional liposomes as revealed by enhanced survival, reduced histopathological lesions in vital organs and reduced leptospiral load in kidneys. Taken together, the results of the present study clearly reveal that both leptosomes and escheriosomes have emerged as promising delivery vehicles/adjuvants that can be widely exploited with newly discovered antigens in future leptospira vaccines.
#16 - Immune response and prophylactic efficacy of Smegmosomes in a hamster model of leptospirosis.

Authors: Syed M. Faisal, WeiWei Yan, Sean P. McDonough, Hussni Mohamed, Thomas Divers, and Yung-Fu Chang - College of Veterinary Medicine, Cornell University, Ithaca, New York, 14853

Abstract: Leptospirosis is an important zoonotic disease worldwide. Subunit vaccines are an attractive intervention strategy against this disease, but potent, nontoxic adjuvants are necessary components to any effective vaccine. Among various adjuvant candidates, liposomes have garnered recent attention for their capacity as carriers of vaccines. In the present study we prepared novel liposomes using total polar lipids from the nonpathogenic bacterium, *M. smegmatis* (designated smegmosomes). The potential for smegmosomes as a vaccine delivery/adjuvant system was evaluated with novel leptospira protective antigens (Lp0607, Lp1118, Lp1454) and compared with conventional aluminum hydroxide adjuvant (alum) in a hamster model of leptospirosis. Four-week-old hamsters were immunized subcutaneously twice at three weeks intervals and either bled at various time points to evaluate antibody responses, sacrificed to isolate splenocytes for lymphocyte proliferation and cytokine profiles in response to recall antigen, or challenged intraperitoneally with a modified lethal dose (10 x MLD50) of virulent *L. interrogans* serovar Pomona. Our results demonstrate that smegmosomes carrying antigens are better adjuvants than alum as revealed by enhanced and long term antibody response, lymphocyte proliferation and significant enhancement in both Th1 (IFN-γ) and Th2 (IL-4, IL-10) cytokine production. Additionally, smegmosomes were found to induce memory responses that are significantly higher than those of alum. Above all, smegmosomes were observed to impart a significantly higher level of protection than alum as revealed by enhanced survival, reduced histopathological lesions and bacterial load in vital organs. Taken together, the data of the present study suggests that smegmosomes will serve well as a promising delivery vehicle/adjuvant system that can induce both Th1 and Th2 type immune responses and provide a novel tool in development of improved vaccines for leptospirosis and other infectious diseases.
#17 - Repeated domains of *Leptospira* Immunoglobulin-like proteins interact with elastin and tropoelastin

**Authors:** Yi-Pin Lin, Dae-Won Lee, Sean P. McDonough, Linda Nicholson, Yogendra Sharma, and Yung-Fu Chang - Department of Population Medicine and Diagnostic Sciences, Department of Biomedical Science, College of Veterinary Medicine, and Department of Molecular Biology and Genetics, College of Agriculture and Life Science, Cornell University, Ithaca, New York, USA, and Center for cellular and molecular Biology, Uppal Road, Hyderabad 500 007, India.

**Abstract:** *Leptospira* spp., the causative agents of leptospirosis, adhere to components of the extracellular matrix, a pivotal role for colonization of host tissues during infection. Previously, we and others have shown that *Leptospira* Immunoglobulin-like proteins (Lig) of *Leptospira* spp. bind to Fn, laminin, collagen and fibrinogen. In this study, we report that *Leptospira* can be immobilized by human tropoelastin (HTE) or elastin from different tissues including lung, skin, and blood vessels, and that Lig proteins can bind to HTE or elastin. Moreover, both elastin and HTE bind to the same LigB immunoglobulin-like domains including LigBCon4, LigBCen7'-8, LigBCen9, and LigBCen12 as demonstrated by ELISA and competition ELISA assays. The LigB immunoglobulin-like domain binds to the 17th to 27th exons of HTE (17-27HTE) as determined by ELISA (LigBCon4, KD = 0.50 μM; LigBCen7'-8, KD = 0.82μM; LigBCen9, KD = 1.54μM; LigBCen12, KD = 0.73μM). The interaction of LigBCon4 and 17-27HTE was further confirmed by steady state fluorescence spectroscopy (KD = 0.49μM) and ITC (KD = 0.54μM). Furthermore, the binding was enthalpy-driven and affected by environmental pH, indicating it is a charge-charge interaction. The binding affinity of LigBCon4D341N to 17-27HTE was 4.6-fold less than that of wild type LigBCon4. In summary, we show that Lig proteins of *Leptospira* spp. interact with elastin and THE and conclude this interaction may contribute to *Leptospira* adhesion to host tissues during infection.

#18 - *Leptospira* Immunoglobulin-like proteins bind to the C-terminal fibrinogen αC domain inhibiting fibrin clot formation, platelet adhesion and aggregation

**Authors:** Yi-Pin Lin, Sean P. McDonough, Yogendra Sharma, and Yung-Fu Chang - Department of Population Medicine and Diagnostic Sciences, Department of Biomedical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York and Center for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India

**Abstract:** *Leptospira* Immunoglobulin-like (Lig) proteins belong to Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM; LigB and C) or Secretable Expanded Repertoire Adhesive Molecules (SERAM; LigA) that contribute to leptospiral adhesion. In this study, the Fg and fibrin binding sites of LigBCen2 (amino acids 1014-1165 of LigB) are localized to LigBCen2R, the partial 11th and 12th Ig-like domain (amino acids 1014-1119). Fg and fibrin also bind to 10th, 12th, and 13th Ig-like domains of LigA (LigA10, LigA12, LigA13), and 7'-8th, 9th, and 10th Ig-like domains of LigB. In addition, LigBCen2R binds to the C-terminal αC domain of Fg (FgαCC; amino acids 392-644 in Fg α chain; ITC, KD = 0.382 μM; Fluorescence spectrometry, KD =0.364 μM).
The quenching and blue shift observed from the intensities of maximum wavelength on the tryptophan fluorescence spectra of FgaCCY570W upon LigBCen2RW1073C binding suggests a RGD motif close to the sole tryptophan on FgaCCY570W is buried in LigBCen2R saturated with FgaCC. Conformational change of the LigBCen2R bound FgaCC RGD motif blocks further binding to integrin αIIbβ3 on platelets and their aggregation. LigBCen2R binding to FgaCC reduces clot formation but does not affect plasminogen (PLG) and tissue-type plasminogen activator (tPA) interactions with FgaCC. This study is the first to report that a spirochetal protein binds to the C-terminal αC domain of Fg that regulates thrombosis and fibrinolysis and may be related to the pulmonary hemorrhage and thrombocytopenia seen in the clinical cases of leptospirosis.

#19 - Coagulation and Fibrinolysis in the Abdomen of Foals and Adult Horses

Authors: Ashlee E. Watts, Marjory Brooks, Hollis Erb, Susie L. Fubini
Department of Clinical Sciences, Population Medicine & Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York

Abstract: Fibrous bands of scar tissue (intra-abdominal adhesions) can form following colic or surgical trauma as a generalized peritoneal response to intra-abdominal trauma and inflammation. Anecdotal evidence and long-standing veterinary dogma suggests that adhesion formation is much more common in foals versus adults. We hypothesized that foals have decreased fibrinolytic capacity within the abdomen, predisposing them to intra-abdominal adhesion formation following peritoneal trauma. Abdominal fluid and blood were collected from normal foals (group A; n=22), foals with colic (group B; n=16), normal adult horses (group C; n=20), and adult horse with colic (group D; n=19). Samples were analyzed for Fibrinogen, Plasminogen, D-dimer, and Antiplasmin. When comparing normal to colic within each age group, normal values were significantly different (lower) for abdominal fluid and not significantly different for plasma, supporting the conclusion that the coagulation and fibrinolytic systems are upregulated in the abdomen of colics. In foals with colic, there was an increased procoagulant environment in the abdomen. There was also an increase in the profibrinolytic system. In foal colics, higher fold change values in all 4 parameters indicate that both the coagulation and the fibrinolytic system are generally more active in the abdomen of foals compared to adults with a greater increase in activity of the profibrinolytic system. As we complete case collection (n=20) we may reject our hypothesis. If foals do have increased risk for adhesion formation, it may be due to their increased rate of healing exceeding their fibrinolytic capacity, despite the fact that it is up-regulated.
APPENDIX F

AWARDS FOR 2010
## 2010 Harry M. Zweig Memorial Fund for Equine Research Awards

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<thead>
<tr>
<th>CONTINUATION</th>
<th>AWARD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Douglas Antczak</td>
<td>Expression Microarrays and Equine Placental Development</td>
</tr>
<tr>
<td>Sylvia Bedford-Guaus</td>
<td>Further Characterization of the Specific Activity and Ultrastructural Localization of Phospholipase C Zeta in Fertile and Subfertile Stallions</td>
</tr>
<tr>
<td>Bettina Wagner</td>
<td>Analysis of the Innate Immune Response to EHV-1 Infection</td>
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</tbody>
</table>

| Sub-Total: | $121,661 |

<table>
<thead>
<tr>
<th>NEW</th>
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<tbody>
<tr>
<td>Norm Ducharme</td>
<td>Tissue Engineered Cartilage in the Equine Airway (1 Year)</td>
</tr>
<tr>
<td>Lisa Fortier</td>
<td>Optimization of Platelet Rich Plasma Components for the Treatment of Tendonitis (2 Year)</td>
</tr>
<tr>
<td>Robert Gilbert</td>
<td>Controlled Postponement of Ovulation by Progestagen Treatment (1 Year)</td>
</tr>
<tr>
<td>Vicki Meyers-Wallen</td>
<td>Generation of a Molecular Resource to Identify Gene Mutations Causing Inherited Equine Sterility and Infertility (1 Year)</td>
</tr>
<tr>
<td>Alan Nixon</td>
<td>Targeted Delivery of Stem Cells for Pro-Inflammatory Cytokine Suppression in Arthritic Joints (2 Year)</td>
</tr>
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<tr>
<td>Susan Fubini</td>
<td>Indices of Intra-Abdominal Fibrinolysis in Colic Foals: Pathogenic and Prognostic Markers</td>
</tr>
</tbody>
</table>

| Sub-Total: | $322,044 |
| TOTAL:     | $443,705 |