

FOUNDATION: 1998 Grant Reports Title: Testing and Comparison of Analgesic Drug Action in Amphibians

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Practicing veterinarians are increasingly called upon to provide analgesia for amphibian and reptile species following trauma or surgical procedures. At present, there is little information on the effectiveness and safety of opioid and non-opioid analgesics in non-mammalian species. This project tested several types of analgesic agents used in mammals in a unique amphibian model to test analgesics. The agents were administered subcutaneously into the dorsal lymph sac of Northern leopard frogs, *Rana pipiens*, (for methods, see 1,2) and analgesic effectiveness was assessed for the ability of test agents to reduce the behavioral response to a dilute, weak acid noxious stimulus. For the first time, amphibians have been tested for analgesia following the injection of xylazine, ketamine, buprenorphine, butorphanol, diphenhydramine, histamine, chlorpromazine, haloperidol, chlordiazepoxide, flurazepam, phenobarbital, pentobarbital, ketorolac, and indomethacin.

None of the agents produced greater than an 80% analgesic effect at doses that were not lethal within 24 hours (morphine data from ref. 3 included for comparison). Select doses of antipsychotics, benzodiazepines, and partial opioid agonists produced greater than 45% analgesic effect (MPE, see Table 1). The same classes of agents, plus non-steroidal anti-inflammatory drugs (NSAIDs), antihistaminergics, barbiturates, and ketamine had efficacy of 20-45% analgesia. Although the safety index of tested agents was not determined, doses that produced lethality within 24h of injection are noted in Table 1. Agents that produced results less than 20% analgesia or demonstrated high lethality were not reported in Table 1.

These results suggest that careful clinical use of non-opioid analgesics and partial opioid analgesics may be warranted in amphibians and reptile species. Previous work demonstrated that opioids such as morphine, meperidine, and fentanyl are potent and safe analgesics in amphibians.^{3,4} However, as potent opioids are controlled substances (Schedule II) that require special licensing and reporting requirements, the tested non-opioid agents may be more accessible when mild to moderate analgesia is needed. In this regard, the partial opioid agonists may be judiciously used as butorphanol is a non-scheduled agent, and buprenorphine is a schedule V controlled substance. Finally, the actual doses used in the amphibian model may not be appropriate in other amphibian or reptile species. However, this project provides novel data on the use of non-opioid analgesics in amphibians in an experimental pain model, which gives initial guidance for selection and use of potential analgesic agents in other amphibian and reptile species. Further studies are needed to establish effective dose-response curves in clinical pain situations with these species.

[see February ACLAM newsletter for table of agent, class, and analgesic effects of drugs tested in the amphibian acetic acid test, or you may contact Dr. Marty Morin at morinasc@hpiug.org and request a copy.]

References

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Title: Development of PCR and RT-PCR Assays for the Detection of Rodent Adventitious Viral and Mycoplasmal Infectious Agents in Cell Lines and Biological Materials

Principal Investigator: Sanford H. Feldman, DVM, PhD, ACLAM Diplomate, University of Virginia

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The research objective of this project was to develop a method for rapid, cost-effective, sensitive, and pathogen specific screening of cell lines, transplantable tumors and biological materials for the presence of adventitious murine pathogens through polymerase chain reaction (PCR) and reverse transcription PCR (RT-PCR) in multiplex formats. This capability could replace expensive and time consuming mouse antibody production (MAP) tests and similar testing.

RT-PCR assays were successfully developed for Sendai virus, lactate dehydrogenase elevating virus (LDHV), Reovirus-3 (conserved sequence includes Reo-1 and Reo-2), coronaviruses (SDAV/RCV and MHV), Theiler's meningoencephalomyelitis virus (TMEV) and encephalomyocarditis virus (EMC) (picornaviruses) and SV-5 (parainfluenza virus). PCR assays were successfully developed for Mycoplasmas (16S rRNA sequence includes acholeplasmas and ureaplasmas), papovaviruses (K-virus and polyomavirus), parvoviruses (KRV, MVM, H-1 but should detect MPVs and RRVs) and cytomegaloviruses (MCMV, RCMV, GPCMV and rhesus CMV).

The ability to multiplex assays was attempted for several RT-PCRs without success in detecting the presence of all the viruses. It should be noted that in any given multiplex of a cell line infected with multiple RNA viruses at least one virus was detected alerting that the cell line was contaminated (although the extent was not disclosed). This information alone may be sufficient to reject inoculating this cell line into a rodent housed outside of a quarantine area. Rather than multiplex the PCR assays, genera specific tests were developed (e.g., for all parvoviruses).

This research was somewhat hampered by the inability to obtain positive control materials. Charles River Laboratories supplied positive control material - coronaviruses. HAI materials were purchased from CRL to obtain KRV, MVM and H-1.

PCR and RT-PCR assays are currently being run for PVM, EDIM and adenoviruses (FL and K87). This laboratory will continue to work on these assays until the study has been completed. At that time, a comparison of molecular assays to antibody production tests is planned for a

limited subset of the agents (parvoviruses and coronaviruses as representing DNA and RNA viruses, respectively).

The author wishes to express gratitude to the Charles River Laboratories and the ACLAM Foundation for their generous support of this study.

Title: Detection of Rodent Pathogens by Environmental Monitoring

Principal Investigator: Lela Riley, Ph.D., Associate Professor, Research Animal Diagnostic Laboratory, University of Missouri-Columbia, Columbia, Missouri, 65211, U.S.A.

In this project, environmental monitoring using polymerase chain reaction (PCR) assays for detection of specific murine pathogens was examined as an alternative method for health monitoring of rodents. Three types of environmental samples (bioaerosols, cage swipes, and bedding samples) and two microorganisms (*Corynebacterium bovis* and Minute Virus of Mice [MVM]) were evaluated. Mice were experimentally infected with each pathogen and environmental samples were collected at 4, 7, 14, and 28 days postinoculation. For *C. bovis*, cages containing infected mice were correctly identified 10 out of 11 times with cage swipe samples, and 3 out of 11 times with bioaerosol and bedding sampling. For MVM, cages containing infected mice were correctly identified 30 out of 32 times with bedding samples, 20 out of 32 times with cage swipes and 1 out of 32 times using bioaerosol sampling. Cages with sham-inoculated mice were uniformly negative at all time points. These findings suggest that PCR-based environmental monitoring may represent an alternative method of health monitoring and pathogen detection in laboratory rodents, although environmental samples needed for accurate detection may vary depending on the pathogen. A manuscript describing the results of this study is currently being prepared for submission and publication.

Title: Assessment of Distress in Mice Used for Monoclonal Antibody Production by the Ascites Method

Principal Investigator: Norman C. Peterson, DVM, PhD; Division of Comparative Medicine, Johns Hopkins University, Baltimore, MD 21205-2196

The effects of pristane inoculation, ascites accumulation, peritoneocentesis, and analgesics upon the well-being of mice used in monoclonal antibody (MAb) production protocols were investigated in this study. Four different experiments each containing 17-21, 6-8 week old male Balb/c mice were conducted. Each experiment involved a period in which baseline data was collected followed by pristane or phosphate buffered saline (PBS) intraperitoneal inoculations of each mouse. One week later mice received intraperitoneal inoculations of either hybridoma cells or PBS. Parameters used to assess well-being throughout each of these periods included: wheel-running activity, food and water consumption, open field box activity, clinical observation, and plasma corticosterone concentrations. When compared to controls, pristane inoculation had slight to no affect upon mice, and there was no evidence of distress in cell-inoculated mice prior to their gaining 25% of their baseline body weight. The number of times (up to three) that peritoneocentesis was performed did not have a significant impact upon the mice's well-being, but ascites yields were greater when multiple harvests were performed. Cell-inoculated mice that

gained weight slowly or developed high particulate ascites were at higher risk of being distressed. Buprenorphine (0.1 mg/kg) administered subcutaneously twice each day to ascitic mice of one experiment had no effect upon any of the parameters measured when compared to PBS inoculated ascitic mice. The effects of different dosages, treatment regimens, or analgesics were not investigated here, and further studies may lead to discovery of effective refinements.

Our observations indicate that the ascitic mice's well-being and productivity may have benefited from free access to the mouse wheels. Additional controlled studies are needed to define if this would be a useful adjunct to in vivo MAb production protocols. There is also some concern that non-terminal ascites harvesting may result in hypovolemic shock and that replacement of the removed ascites with warm saline may benefit the mouse's health. However, our observations seemed to indicate that mice benefited from the relieved abdominal pressure. Further analyses are needed to determine if replacement fluids are a practical refinement in these protocols. Anecdotal evidence also suggests that selection of nonaggressive, nonadherent hybridoma cell-lines may also positively influence the health and well-being of mice in these protocols and further work in this area may also benefit both the mice and productivity.

Based upon the findings presented in this report, the following is suggested: 1) Procedures should be performed by skilled individuals, who are familiar with the recognition of pain in mice, and, preferably, an institutional veterinarian should be involved, 2) A maximum of 0.2mls of pristane should be sufficient to prime mice, 3) Animals should be monitored and weighed at the day of cell inoculation and at least daily beginning no later than the fifth day after inoculation 4) Healthy robust mice can be harvested up to three times, as long as the initial harvest occurs between 20 and 25% of its baseline body weight, thereafter, body weight should not exceed 30%, and a 4 day period from the time of first to last harvest should be targeted, 5) Mice which are slow at gaining weight or have hemorrhagic/cloudy ascites should be monitored closely and considered for euthanasia. While we seek to improve the ascites method, emphasis should also be placed on the continued development of improved replacement/in vitro technology.

Title: Epidemiology of *Helicobacter hepaticus* Infection and Disease in Mouse Colonies

Principal Investigators: Mark T. Whary, DVM, PhD and James G. Fox, DVM, MS,
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A/JCr and C57BL/6 mice were compared for immune response, *Helicobacter hepaticus* colonization and development of hepatitis after experimental or natural infection. Eight week old mice were gavaged with *H. hepaticus* and after 30 days were cohoused for 5 months with helicobacter-free mice that were 3, 12 or 24 weeks old. Each group was then necropsied after a total period of 6 months. PCR confirmed fecal shedding of *H. hepaticus* in experimentally infected mice as well as natural acquisition of the organism by all cagemates. Serum IgG and Th2-associated IgG1 responses to *H. hepaticus* were similar, irrespective of mouse strain, gender, method of infection or age ($p=0.09$ and higher). Serum IgG reactivity to antigens of 50 to 75 kD on Western blots and mucosal IgA responses to outer membrane proteins by ELISA were higher in A/JCr mice ($p<0.00001$). A/JCr mice also developed more severe hepatitis ($p<0.0001$) and expressed higher gIFN mRNA in the liver compared to C57BL/6 mice ($p<0.05$) despite significantly lower *H. hepaticus* colonization in the cecum ($p<0.0001$). Compared to older mice,

both A/JCr and C57BL/6 mice first exposed to *H. hepaticus* as weanlings were colonized with higher CFUs of *H. hepaticus* after 6 months ($p < 0.01$). Young A/JCr mice developed higher Th1-associated IgG2a responses ($p < 0.02$) which were associated with more severe hepatitis ($p < 0.027$), particularly in weanling male A/JCr mice ($p < 0.04$). Th1-associated IgG2ab responses of C57BL/6 mice were similar across experimental groups ($p = 0.15$) and were not associated with significant hepatitis although female C57BL/6 had mild liver lesions compared to few or none in males. Thus, *H. hepaticus* colonization is lower in A/JCr than in C57BL/6 mice and is, therefore, inversely related to severity of hepatitis with young mice most susceptible.

As the result of ACLAM Foundation funding of this laboratory, a series of three papers have established or further supported several aspects of helicobacter pathogenesis in the mouse. All enterohepatic helicobacter species of the mouse (*H. hepaticus*, *H. bilis*, *H. rodentium*, others) are quickly spread by fecal-oral contact but simple husbandry guidelines (microisolator caging, change order of known clean to unknown status, forceps handling) can prevent cage-to-cage transmission. Because of fecal-oral transmission, the gold standard in developing a helicobacter-free mouse colony is embryo rederivation or caesarean section with cross fostering. This work has also established that positive serology is very reliable for detecting the presence of helicobacters at the genus level but species identification requires PCR or culture. Thus, use of serology from sentinel mice to monitor the helicobacter-status of established helicobacter-free colonies makes sense. To monitor the helicobacter status of conventional colonies, cecal scrapings of sentinels exposed via dirty bedding should be tested by PCR with either genus-specific or species-specific primers, dependent on the management objectives. From the latest work, weanling mice appear to be most susceptible to infection which is consistent with the epidemiology of *H. pylori* acquisition early in childhood. Mice exposed by fecal-oral contact at weaning will become colonized with the highest numbers of CFUs in the lower bowel and can be assumed to shed the greatest number of helicobacters in feces. Colonization of *H. hepaticus* in the hepatitis-prone A/JCr mice was significantly lower than in disease-resistant C57BL/6 mice. Although not directly tested, this may mean that clinically normal wild type mice may actually be shedding more organisms than clinically affected mice such as the IL-10 knockout which is very prone to colitis with rectal prolapse, particularly when infected with any of several enterohepatic helicobacters. This hypothesis is predicated on the assumption that inflammatory responses to helicobacter infections in mice are driven by Th1 immune responses and that this type of immune response will reduce, but not eliminate, colonization. Therefore, mice with impaired Th2 responses (i.e., IL10 KO), develop exaggerated Th1 driven inflammation secondary to helicobacter infection.

NOTE: Since these summaries can not capture the detail of the projects, we look forward to reading the full publications in the literature. We thank the authors and staffs for their efforts to expand the body of knowledge in our field.