Protocol: 1
Species (common name): Ferret
Number: 2
Explanation of procedure producing pain and/or distress:
Infection of ferrets with highly pathogenic H5N1 or H7N7 virus may cause severe morbidity, including neurological symptoms and death. Therefore, we believe that a Category E pain class is warranted in cases where animals exhibit severe respiratory symptoms as well as multiple organ dysfunction.

Justification why pain and/or distress could not be relieved:
Two kinds of drugs were considered: 1) Non-steroidal anti-inflammatory drugs (NSAIDs, COX inhibitors) and related analgesics. This class of drugs cannot be used because they may alter viral replication levels, body temperature and body weight loss; all of which are important parameters in our studies. NSAID therapy is not considered because of its potentially detrimental impact on the interpretation of the effects of the vaccine. 2) Opioid drugs. These agents cause depression of respiratory control centers in the CNS. Highly pathogenic influenza virus infection in ferrets can cause an acute respiratory distress syndrome which may be aggravated by opioid drugs. Consequently, opioid therapy is not considered because of its potentially detrimental impact on the interpretation of the effects of the vaccine. Pain will be minimized by observation of the animals as frequently as 4-hour intervals to evaluate the severity of the symptoms and make the earliest possible decision (according to predetermined criteria) on humane termination by euthanasia.

Protocol: 2
Species (common name): Hamster
Number: 29
Explanation of procedure producing pain and/or distress:
The focus of this study is two-fold; first, to establish a Syrian hamster model of Andes virus infection; second, to use this robust model to evaluate the efficacy of promising therapeutic drugs. The nature of the study therefore requires the animals to be infected with lethal Andes virus.

Justification why pain and/or distress could not be relieved:
Pain and distress will be minimized as much as practically possible by close adherence to the post-infection monitoring and euthanasia algorithms described in the protocol description section. All efforts will be taken to ensure that the hamsters experience the least amount of pain and distress as is absolutely necessary to accomplish the goals of the experiment. Analgesics have been proven to interfere with immunologic responses and can exacerbate liver damage and cause catabolism of plasma. It is also known that inflammation and inflammatory mediators play a major role in the pathogenesis of flaviviral diseases. Based on these factors, analgesics should not be used since they could affect the clinical outcome of the disease and alter the theoretical basis of the experiment.

References include:

Protocol: 3
Species (common name): Prairie Dog
Number: 8
Explanation of procedure producing pain and/or distress:
Animals will be anesthetized during sampling to avoid pain and discomfort. However, pain associated with infection will not be treated with anesthetics or analgesics such as metacam because metacam is shown to interfere with the Nuclear Factor Kappa B (inflammatory pathway) which is believed to be an important process for pox virus infections.

Justification why pain and/or distress could not be relieved:
The nuclear factor kB (NF-kB) transcription factor is involved in the transcription of many proinflammatory as well as antiapoptotic genes and therefore is an important component in the progression of inflammatory diseases, including poxviruses such as monkeypox (Barnes et al 1997). Orthopoxviruses appear to have acquired mechanisms to inhibit antiviral effects of NF-kB activation; encoding multiple proteins that act in various ways to prevent NF-kB activation (Reviewed by Moss and Shisler 2001; Seet et al 2003). The ability of orthopoxviruses to modulate NF-kB activation likely plays an important role in the ability of these viruses to cause disease, and therefore the utilization of substances that inhibit NF-kB activation would greatly change the normal virus lifecycle. Numerous drugs have been shown to inhibit NF-kB and therefore have anti-inflammatory results in experimental models (Reviewed by D'Acquisto, May and Ghosh 2002). These include salicylates, NSAIDs, and glucocorticoids to name a few. Metacam is commonly prescribed by veterinarians and falls into the NSAID category. Because this study is aimed at better understanding the pathogenesis of the two monkeypox strains, including through the use of recombinant viruses, it is important that the life cycle of the two virus strains as well as wild-type and recombinant viruses not be altered by the use of NSAIDs or other anti-inflammatory agents.

References:

Protocol: 4
Species (common name): Ferret
Number: 9
Explanation of procedure producing pain and/or distress:
Ferrets are used to model influenza virus infection because they respond in similar ways as humans when infected and they have a similar distribution of receptors in their respiratory tracts as humans. Infection of ferrets with highly pathogenic H5N1 viruses may cause severe morbidity and even death in some cases. Every attempt will be made to euthanize the animal prior to it reaching a moribund state. Animals will be monitored daily and will be euthanatized if they reach 10 points on the 10-point euthanasia scale detailed in the Protocol.
Justification why pain and/or distress could not be relieved:
The crux of our study is to understand the mechanisms of disease caused by influenza virus infection. Disease is assessed based on clinical signs observed in infected animals such as lethargy, fever, weight loss, etc… that would be obscured by pain relieving drugs. After influenza virus infection, the initial inflammatory response, as well as the subsequent adaptive T and B cell responses, are crucial to the progression of disease and directly affects the clinical signs observed in the animals (1, 2). Any manipulation of the inflammatory response with analgesics will affect the overall immune response and will prevent an accurate assessment of disease. For example, Type I and II interferon and NK cell responses are important components of the immune response to influenza. Manipulation of these responses with anti-inflammatory drugs such as NSAIDS or opioids directly affect virus shedding, incidence of fever and additional disease parameters (3-6). Our preliminary studies suggest that a majority of viruses to be evaluated in this study will actually be attenuated for ferrets. Therefore, we estimate that only 25% of animals at most may experience symptoms that require euthanasia.


Protocol: 5
Species (common name): Ferret
Number: 17

Explanation of procedure producing pain and/or distress:
Ferrets are used to model influenza infection because they are susceptible to virus infection and have a course of disease that is similar to humans. It is generally believed that some pain and/or distress will occur when ferrets are infected with influenza strains that are considered highly pathogenic. During FY 2011, under this protocol, some ferrets were infected with highly pathogenic influenza strains which caused severe morbidity. Death was not an endpoint and every attempt was made to euthanize the animal prior to the development of severe disease. Animals were monitored twice daily and euthanatized when they reached 10 points on the 10-point euthanasia scale detailed in the Protocol.

Justification why pain and/or distress could not be relieved:
Infection of ferrets with highly pathogenic H5N1 viruses may cause severe morbidity and even death in some cases. Every attempt will be made to euthanize the animal prior to it reaching a moribund state. Animals will be monitored daily and will be euthanatized if they reach 10 points on a 10-point euthanasia scale.

The focus of our study is to understand the mechanisms of pathogenesis and transmission of influenza viruses delivered via aerosols. After animals are infected with influenza virus, an initial inflammatory response occurs that is important to disease progression and virus shedding which directly affects the ability of a virus to transmit from one animal to another (1,2). Any
manipulation of the inflammatory response with analgesics will affect the overall immune response and will prevent an accurate assessment of disease and transmissibility. For example, Type I and II interferon and NK cell responses are important components of the immune response to influenza. Manipulation of these responses with anti-inflammatory drugs such as NSAIDS or opioids directly affect virus shedding, fever, activity levels and additional disease parameters that play a role in the frequency of virus transmission and severity of disease (3-6). Our preliminary studies suggest that a majority of viruses to be evaluated in this study will actually be attenuated for ferrets. Therefore, we estimate that only 25% of animals at most may experience symptoms that require euthanasia.


Protocol: 6
Species (common name): Ferret
Number: 40
Explanation of procedure producing pain and/or distress:
Ferrets are used to model influenza virus infection because they respond in similar ways as humans when infected and they have a similar distribution of receptors in their respiratory tracts as humans.

Part of the study outlined in this protocol is to evaluate influenza disease caused by H5N1 viruses. Infection of ferrets with some strains of highly pathogenic H5N1 viruses causes significant morbidity. These observations (lethargy, anorexia, etc) would be obscured by the use of pain relieving drugs (supported by references below). The majority of influenza viruses studied in under this protocol were low pathogenic for ferrets, however, approximately 25% of animals experienced significant morbidity, sometimes requiring euthanasia. Every attempt was made to euthanize the animal prior to it reaching severe morbidity. Any animal that lost greater than 25% body weight and/or accrued a total score of 10 on the scale detailed in the protocol, indicating severe illness, were humanely euthanized. The observation of animals by protocol associates were monitored no less than twice daily.

Justification why pain and/or distress could not be relieved:
It has been demonstrated that analgesic drugs or anti-inflammatory agents affect the respiratory inflammation during influenza infections (references on form). We have chosen not to use pain relieving drugs because they can have serious consequences towards the outcome of virus infection. The main purpose of this animal protocol is to better understand the mechanisms of disease cause by highly pathogenic influenza virus infection. Disease is assessed based on clinical signs observed among infected animals such as lethargy, fever, weight loss, leucopenia and cytokine production, many of which would be obscured by pain relieving drugs. After influenza virus infection, the initial acute inflammatory response, as well as subsequent adaptive T and B cell responses are crucial to the progression of disease and directly affects the clinical signs observed in the animals. Any manipulation of the inflammatory responses with analgesics.
will affect the acute assessment of the disease. Moreover, manipulation of these responses with anti-inflammatory drugs such as NSAIDS or opioids directly affect virus shedding, incidence of fever, and additional disease parameters as indicated in references. It is also important to note that this animal model is being used to model human influenza virus infection where in most cases; infected individuals will not be taking NSAIDS or opioids. Thus, the data obtained from treated animals would not model the real-life situation. Our ultimate goal is to understand the unmanipulated disease process so we can more precisely target strategies of treatment.


Protocol: 7
Species (common name): Ferret
Number: 24

Explanation of procedure producing pain and/or distress:
The ferrets are inoculated by intranasal instillation with live influenza virus. Infection of ferrets with highly virulent influenza viruses may cause severe morbidity and even death in some cases. Most ferrets exhibit a modest rise in temperature, minimal weight loss and little to no decrease in activity when infected with seasonal influenza virus. Many viruses that are highly pathogenic in domestic poultry will also cause only moderate disease in ferrets. Disease is assessed based on clinical signs observed among infected animals such as lethargy, fever, weight loss, leucopenia, and cytokine production, many of which would be obscured by pain relieving drugs. After influenza virus infection, the initial acute inflammatory response, as well as the subsequent adaptive T and B cell responses, are crucial to the progression of disease and directly affects the clinical signs observed in the animals.

Justification why pain and/or distress could not be relieved:
Most ferrets exhibit a modest rise in temperature, minimal weight loss and little to no decrease in activity when infected with seasonal influenza virus. Many viruses that are highly pathogenic in domestic poultry will also cause only moderate disease in ferrets. Disease is assessed based on clinical signs observed among infected animals such as lethargy, fever, weight loss, leucopenia, and cytokine production, many of which would be obscured by pain relieving drugs (1, 2). After influenza virus infection, the initial acute inflammatory response, as well as the subsequent adaptive T and B cell responses, are crucial to the progression of disease and directly affects the clinical signs observed in the animals (1, 2). In a few animals, progression of the disease may cause more severe respiratory and systemic symptoms. When retained through the second week of infection, these animals may also develop neurological symptoms. Onset of neurological symptoms may occur as early as day 7 or as late as 13 days post-infection. Neurological signs include torticollis, ataxia and hind-limb paresis. When these neurological signs are observed, the animal is immediately euthanized. Since animals are observed twice a day, ferrets would exhibit signs for less than 1 day before euthanasia. The onset of these symptoms and progression of the disease is rapid, often measured in less than 24 hours, leaving little time to effectively treat the
ferrets. The stress of receiving the treatment can also hasten the death of the ferret. Any manipulation of the inflammatory response with analgesics will affect the acute and adaptive immune response and will preclude an accurate assessment of disease. For example, Type I and II interferon and NK cell responses are important components of the immune response to influenza. Manipulation of these responses with anti-inflammatory drugs such as NSAIDS or opioids directly affect virus shedding, incidence of fever and additional disease parameters as indicated in the following references (3-6). In addition, the use of analgesics could alter the pathogenic and immunologic response to infection, thus making it impossible to interpret the data obtained in this study. Narcotic analgesics were shown to interfere with the mechanism(s) responsible for interferon production (7-8). Moreover opioids can suppress Natural Killer (NK) cell activity (9). Also, analgesics including buprenorphine can cause histamine release (10, 11) and respiratory depression (12). Histamine is a well-known inflammatory mediator and plays a central role in the pathogenesis of allergic and inflammatory diseases by modulating vascular and airway responses. Histamine has been shown to induce activation of human macrophages (13), inhibit interferon alpha release from dendritic cells (10) and increase the synthesis and release of IL-10 from human macrophages (14). Clearly, the analgesic-induced release of histamine would directly interfere with the inflammatory process. Investigators, using an established murine model of endotoxemia, showed that the opioids fentanyl and buprenorphine directly altered the outcome of their experiments by modulating the immune response (15). In this case, both opioids caused significant decreases in circulating levels of tumor necrosis factor alpha following administration of lipopolysaccharide (LPS). For these reasons, at the first sign of any neurological symptom, the animal is euthanized. In very rare cases, animals may die without exhibiting respiratory or neurological symptoms and with disease apparently no more severe than those that survive. However, every effort is made to prevent this from happening.


Protocol: 8
Species (common name): Prairie Dogs
Number: 60
Explanation of procedure producing pain and/or distress:
Infection with monkeypox virus that was not treated with analgesics.

Justification why pain and/or distress could not be relieved:
The nuclear factor kB (NF-kB) transcription factor is involved in the transcription of many proinflammatory as well as antiapoptotic genes and therefore is an important component in the progression of inflammatory diseases, including poxviruses such as monkeypox (Barnes et al. 1997). Orthopoxviruses appear to have acquired mechanisms to inhibit antiviral effects of NF-kB activation; encoding multiple proteins that act in various ways to prevent NF-kB activation (Reviewed by Moss and Shisler 2001; Seet et al. 2003). The ability of orthopoxviruses to modulate NF-kB activation likely plays an important role in the ability of these viruses to cause disease, and therefore the utilization of substances that inhibit NF-kB activation would greatly change the normal virus lifecycle. Numerous drugs have been shown to inhibit NF-kB and therefore have anti-inflammatory results in experimental models (Reviewed by D’Acquisto, May and Ghosh 2002). These include salicylates, NSAIDs, and glucocorticoids to name a few. Metacam is commonly prescribed by veterinarians and falls into the NSAID category. Because the goal of this study is to determine the efficacy of therapeutic treatment of monkeypoxvirus infection with an antiviral, it is important that the life cycle of monkeypox virus not be altered by the use of NSAID’s or other anti-inflammatory agents.

References:

Protocol: 9
Species (common name): Ferret
Number: 13
Explanation of procedure producing pain and/or distress:
Ferrets are used to model influenza virus infection because they respond in similar ways as humans when infected and they have a similar distribution of receptors in their respiratory tracts as humans. Infection of ferrets with highly pathogenic H5N1 viruses may cause severe morbidity and even death in some cases. Every attempt will be made to euthanize the animal prior to it reaching a moribund state. Animals will be monitored daily and will be euthanatized if they reach 10 points on the 10-point euthanasia scale detailed in the Protocol.

Justification why pain and/or distress could not be relieved:
The focus of our study is to understand the mechanisms of transmission of influenza virus. After animals are infected with influenza virus, an initial inflammatory response occurs that is important to disease progression and virus shedding which directly affects the ability of a virus to transmit from one animal to another (1,2). Any manipulation of the inflammatory response with analgesics will affect the overall immune response and will preclude an accurate assessment of disease and transmissibility. For example, Type I and II interferon and NK cell responses are important components of the immune response to influenza. Manipulation of these responses with anti-inflammatory drugs such as NSAIDS or opioids directly affect virus shedding, fever, activity levels and additional disease parameters that can play a role in the frequency of virus transmission (3-6). Our preliminary studies suggest that a majority of viruses to be evaluated in this study will actually be attenuated for ferrets. Therefore, we estimate that only 25% of animals at most may experience symptoms that require euthanasia.


Protocol: 10
Species (common name): Skunk
Number: 5
Explanation of procedure producing pain and/or distress:
The development of clinical rabies infection is expected to occur in a subset of the infected animals. There are occasionally animals that progress to a terminal state before we are able to humanely euthanize them.

Justification why pain and/or distress could not be relieved:
All animals infected with rabies virus will be euthanized at the onset of clinical signs of rabies or if neurological illness is evident (according to pain scores). Beginning approximately 7 days post infection animals will be examined at least daily by rabies staff (beginning at approximately day 7 post-infection through approximately day 21, or after the first animal displays clinical signs through day 21 by rabies staff) and ad hoc by ARB staff during routine husbandry so that euthanasia can be promptly administered. However, occasionally animals progress rapidly from an apparently healthy to a terminal state. This can occasionally occur overnight or between routine check periods. The majority of animals will be euthanized at the first onset and immediately euthanized if neurological signs are evident. We do not expect a significant number of animals to rapidly progress without prior signs, as described above.

Protocol: 11
Species (common name): Hamster
Number: 79
Explanation of procedure producing pain and/or distress:
The development of clinical rabies infection is expected to occur in a subset of the infected animals. There are occasionally animals that progress to a terminal state before we are able to humanely euthanize them.

**Justification why pain and/or distress could not be relieved:**
All animals infected with rabies virus will be euthanized at the onset of clinical signs of rabies. Approximately 7 days following inoculation of rabies virus, control animals may start to show signs of rabies. After day 7 post infection (7 days post through day 21), all experimentally infected animals will be checked twice daily by members of the rabies program in addition to routine husbandry checks performed by ARB. Additionally, PRB associates are on call at all times during an infection. This information is posted outside of all animal treatment rooms. However, occasionally animals progress rapidly from apparently healthy to a terminal state. This can occasionally occur overnight or between routine check periods. The majority of animals will be euthanized at first onset and we do not expect a significant number of animals to rapidly progress as described above. Based on prior experience with hamsters under similar experimental protocols we might expect 3-10% of animals that develop signs of rabies to progress to death before euthanasia can be administered. These animals will subsequently be categorized as pain category E.

**Protocol:** 12  
**Species (common name):** Prairie Dog  
**Number:** 27  

**Explanation of procedure producing pain and/or distress:**
All animals that a challenged with MPXV - whether vaccinated or not - have the potential to become ill with MPXV infection.

**Justification why pain and/or distress could not be relieved:**
Although all sampling procedures will be performed on anesthetized animals and any uninfected animal that becomes ill will be pulled from the study and treated as deemed appropriate by the Attending Vet; infected animals that exhibit monkeypox disease in these studies will not be treated unless the animal requires humane euthanasia based on the established pain score described later in this protocol and attached here as an image.

We are currently planning a study to test the effects of analgesics in MPXV studies in these animals in hopes of determining if they can be used in future studies. Until then, we have initiated non-pharmacological support measure (i.e. fluids) for administration to those animals that need them to help alleviate some discomfort. These measures can be found on the attached pain scale. Our reasoning behind not using NSAIDs or opioids as analgesics are found below with references:

**NSAIDs**

The nuclear factor kB (NF-kB) transcription factor is involved in the transcription of many proinflammatory as well as antiapoptotic genes and therefore is an important component in the progression of inflammatory diseases, including poxviruses such as monkeypox (Barnes et al 1997). Orthopoxviruses appear to have acquired mechanisms to inhibit antiviral effects of NF-kB activation; encoding multiple proteins that act in various ways to prevent NF-kB activation (Reviewed by Moss and Shisler 2001; Seet et al 2003). The ability of orthopoxviruses to modulate NF-κB activation likely plays an important role in the ability of these viruses to cause disease, and therefore the utilization of substances that inhibit NF-κB activation would greatly change the normal virus lifecycle. Numerous drugs have been shown to inhibit NF-kB and
therefore have anti-inflammatory results in experimental models (Reviewed by D’Acquist, May and Ghosh 2002). These include salicylates, NSAIDs, and glucocorticoids to name a few. Metacam is commonly prescribed by veterinarians and falls into the NSAID category. The administration of an anti-inflammatory medication to ease distress would alter viral disease progression in unknown ways and could potentially interfere with or alter the therapies being tested in these studies. Either of these would severely confound our results making them impossible to analyze.

References:


Opioids

Our reasoning behind not using opioid based analgesics for infected animals is that opioid-based analgesics have multiple adverse effects that would interfere with both our assessment of MPXV disease and the immune response to MPXV infection in the prairie dog. MPXV infection can cause bloating, which could be confused with postoperative ileus brought on by the drugs and MPXV infection can cause respiratory distress which could be confused with the depression of respiratory function which can occur with opioid analgesics. In addition, opioid analgesics in rats have been shown to cause temperature deregulation and respiratory depression which could affect the safety of the animals during anesthesia and would also affect the behavior and eating habits of the animals. Since part of our experimental aims are to understand the ability of post-exposure vaccines to protect the animals from MPXV disease, any masking of the MPXV disease will make our mortality and morbidity results difficult to interpret, thus compromising our data. In addition, this experiment also seeks to understand the immune response to MPXV in this animal model and opioid based analgesics are known to increase TNF-alpha and IFN-gamma which are two major cytokines implicated in the immune response against MPXV infection, so use of these drugs would compromise our analysis of the immune response also. Lastly, MPXV can affect liver function and would this make dosing of the animals difficult to ascertain as these drugs are metabolized in the liver.

References:


Animals that become severely ill due to monkeypox disease will be humanely euthanized and those that are not severely ill typically start showing signs of disease at Day 7-9 post-infection and have recovered from all symptoms of the disease by Day 17-21 post infection. All animals infected with monkeypox are classified as category E. Animals that are not challenged with monkeypox, including those that are vaccinated, are listed as category D as we have previously shown that vaccination does not cause illness.
Protocol: 13
Species (common name): Ferret
Number: 4

Explanation of procedure producing pain and/or distress:
Ferrets are used to model influenza virus infection because they respond in similar ways as humans when infected and they have a similar distribution of receptors in their respiratory tracts as humans. Infection of ferrets with highly pathogenic H5N1 viruses may cause severe morbidity and even death in some cases. Every attempt will be made to euthanize the animal prior to it reaching a moribund state. Animals will be monitored daily and will be euthanatized if they reach 10 points on the 10-point euthanasia scale detailed in the Protocol.

Justification why pain and/or distress could not be relieved:
The focus of our study is to understand the mechanisms of transmission of influenza virus. After animals are infected with influenza virus, an initial inflammatory response occurs that is important to disease progression and virus shedding which directly affects the ability of a virus to transmit from one animal to another (1,2). Any manipulation of the inflammatory response with analgesics will affect the overall immune response and will preclude an accurate assessment of disease and transmissibility. For example, Type I and II interferon and NK cell responses are important components of the immune response to influenza. Manipulation of these responses with anti-inflammatory drugs such as NSAIDS or opioids directly affect virus shedding, fever, activity levels and additional disease parameters that can play a role in the frequency of virus transmission (3-6). Our preliminary studies suggest that a majority of viruses to be evaluated in this study will actually be attenuated for ferrets. Therefore, we estimate that only 25% of animals at most may experience symptoms that require euthanasia.


Protocol: 14
Species (common name): Guinea Pig
Number: 2

Explanation of procedure producing pain and/or distress:
Two animals died suddenly overnight after infection with virulent virus, before euthanasia was indicated by the clinical-illness and euthanasia algorithm.

Justification why pain and/or distress could not be relieved:
Even with enhanced monitoring according to the predetermined clinical-illness and pain algorithm it is possible that an animal may succumb to lethal arenavirus infection. These types of deaths are unanticipated but could happen suddenly while the animal is not being directly
observed by animal care personnel. As such approximately 5% of the total numbers of animals have been allocated to Category E classification. All efforts will be made to ensure timely euthanasia prior to the onset of lethal disease to minimize the numbers of animals that die unexpectedly following infection.