Humane Endpoints
The current state of affairs of animal use in infectious disease research

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Summary

Laboratory animals are currently used as models for studying the pathogenesis, treatment and prevention of infectious diseases. Historically many of the animal models have been lethality studies in which death is the scientific endpoint of the study. Measures of survival, such as the number of remaining animals or the mean time to death following infection are the criteria used to determine the success or failure of a preventative or therapeutic intervention. The aim of this study is to determine the extent to which death as an endpoint, rather than alternative indicators of morbidity and mortality, is used in infectious disease models. We conducted a review of recently published animal studies in two prominent peer-reviewed infectious disease journals, The Journal of Infectious Diseases and Infection and Immunity. Studies were evaluated based on the use of death as the study endpoint. Other factors such as animal species, class of infectious agent and study type were also examined. One fourth (24%) of the research reports reviewed involve the use of animal subjects, of these 31% are lethality studies that report death as the endpoint. The species of animal used is related to the prevalence of lethality studies. Of the 175 lethality studies 157 or 89.7% were conducted in rats and mice. Also, 37% of studies conducted with rats and mice use death as the study endpoint while only 14% report death as an endpoint in all other species. Differences were also observed with respect to the type of pathogen used as well as the type of study.

This study describes the current use humane endpoints and lethality studies and may help to inform scientists regarding the scope and professional acceptance of data collected with more humane endpoints. IACUCs, regulatory agencies, animal welfare organizations, and scientific publishers may also find this type of information to be of use in the evaluation and development

of policies that emphasize the adoption of humane endpoints not only in infectious disease studies but also in other disciplines where death is often the outcome.

Introduction

Biological investigation and the development of therapeutics and prevention strategies for human and animal diseases are in many ways dependent on the use of laboratory animals. Justification of and ethical considerations raised by the use of laboratory animals are often discussed in the political and public arenas. The American public continues to support the use of animals as research subjects as evidenced by public opinion surveys conducted between 1989 and 1999 that indicate 60 to 65 percent approval (1). These surveys also show that the public approval rating is dependent upon the species of animals used, the amount of pain and suffering involved and the perceived benefits of the research. The US Congress supports animal research and "finds that the uses of animals is instrumental in certain research and education for advancing knowledge of cures and treatment for diseases and injuries which afflict both humans and animals." This statement of policy in the Animal Welfare Act (AWA) also supports development of non-animal testing, minimization of unnecessary duplication of animal experiments, and measures that reflect public concern for animal treatment to the extent that research will progress (2). The acceptance of the necessity and validity of animal models by the general public, congress, regulatory agencies and the scientific community ensures that animal experimentation will continue as long as there are no acceptable alternatives. It is not likely that animal experimentation will be completely replaced by non-animal models, but there are many opportunities to reduce or ameliorate the pain and distress that some animals will experience. To this end, practical considerations dictate that the use of animal models for the generation of sound and relevant scientific data should be accompanied by a continual search for methods that result in the least possible pain and distress to the animals.

Animal models of disease are intended to simulate the mechanism and clinical course of the corresponding disease in humans. The outcome of animal studies can in some instances be predictive of the human conditions though not necessarily on an individual animal basis. Some of the features of animal models that allow for correlation to human disease include standardization of the animal subjects, conditions for the initiation of disease, and administration of potential therapeutics. The ability to control as many variables as possible in a disease model is desirable in order to produce reproducible data.

There are many instances where disease models produce progressive and severe disease resulting in high rates of mortality. Research in oncology, autoimmune disease, metabolic and infectious diseases all have the potential to cause the death of subjects, and in some instances, death is the traditional and desired endpoint.

In infectious disease and other fields there is a cumulative body of knowledge that is rooted in traditional types of investigations. Laboratories all over the world are engaged in various types of research utilizing animals, humans, plants and other biological systems to study disease transmission, prevention, and possible therapeutics. The inclination by scientists is to continue to use traditional and standardized experimental techniques in order to produce data that is 1) compatible with that of other laboratories and 2) acquired with methodology acceptable to other scientists. The survival study is one of the fundamental experiments of infectious disease research that has as its key feature the death of the animals as the scientific data point. Survival studies were instrumental in determining the efficacy of therapeutics and vaccinations and have been adapted for use in basic research studies.

However, advances in understanding of the immune system, host responses, and mechanisms of infection on organismal, cellular and molecular levels raise questions regarding

the necessity of lethality studies from both scientific and ethical perspectives. A major question that can therefore be asked is - should animals be subjected to unrelieved pain and distress if there are other scientific methodologies available that could either reduce the suffering or replace it altogether?

Advances in scientific techniques and technologies have been paralleled by changes in the oversight and regulations of the use of animals in research studies, the details of which will be discussed later. In brief, with respect to lethality studies investigators submitting proposals for the use of animals must demonstrate that alternative methods for all potentially painful procedures were considered and must provide scientific justification for experiments causing unrelieved pain and distress.

There is little published evidence but ample anecdotal evidence among IACUC professionals that researchers commonly justify the use of death as the scientific endpoint by asserting that it is standard practice and necessary to maintain scientific credibility and data integrity. The motivation for this study is partially based on verifying the claims that survival studies are standard practice and that no other alternatives are acceptable to the scientific community.

The use of the humane endpoint is one method of ensuring that the pain and suffering of the research animals is minimized and that the scientific objectives can also be met. Despite considerable literature describing humane endpoints and how they can be defined there is no evidence describing the extent to which humane endpoints have been put into practice. This study will examine the status of humane endpoints and lethality studies published in infectious disease literature for the purposes of describing the prevalence of lethality studies and identifying successful applications of humane endpoints and areas where more work is needed.

Background

The three R's

The alternatives approach to the use of laboratory animals as codified by the Three R's has become well known in the research community. The seminal work most often cited is titled The *Principles of Humane Experimental Technique* published in 1959 by Russell and Burch (3). This work eloquently defines the problem as being that of "determining what is and what is not humane, and how humanity can be promoted without prejudice to scientific and medical aims." Briefly, the three R's refers to the Replacement of animals by either non-animal studies or replacement with studies that involve animals that experience minimal or no distress, such as ex vivo studies where animal tissues are harvested for use in laboratory experiments. The second R, Reduction, is described as the development of experimental design strategies that control variance resulting in the reduction of the numbers of animals needed to obtain a defined set of data. Refinement, the third R, refers to the modification of experimental procedures to minimize the pain and distress experienced by the animals. Refinements can be as simple as choosing the appropriate size needle to induce the least amount of pain during an injection or as complex as selecting an appropriate analgesia following a surgical procedure. The three R's sometimes overlap but the desired cumulative goal is to conduct animal studies only when there is no suitable replacement, using the smallest numbers of animals with the minimum amount of pain and distress.

Since their introduction, the three R's have evolved somewhat but maintained their basic characteristics and have become the mantra of Institutional Animal Use and Care Committees (IACUC), laboratory animal organizations, animal welfare groups and federal regulatory agencies charged with overseeing animal welfare. The application of three R's is also offered

by the research community as evidence of their cooperation in improving the welfare of laboratory animals. Progress has certainly been made since the Russell and Burch publication, most notably in the areas of replacement and reduction. Animals have also benefited from refinement of living conditions, improved husbandry practices and more humane experimental conditions such as increased use of anesthesia and analgesia. Replacement and reduction alternatives in toxicology testing and risk assessment have become widely used and have reduced the numbers of animals used for routine quality control and toxicity testing (4). In addition the development of *in vitro* high-throughput target-based drug discovery has also replaced some animal models previously used for drug screening (5). This type of *in vitro* screening can test tens of thousands of potential drug candidates against a huge array of possible diseases, prior to initiating any animal testing.

Advances in refinement, the "neglected R," is more difficult to assess (5). Refinement requires that animal health and well being be incorporated into the experimental design. One of the successful areas of refinement is the evaluation and alleviation of pain and distress in laboratory animals. The recognition of and alleviation of pain and distress in laboratory animals was not always standard practice. The first milestone was the recognition that pain and distress produces physiologic changes in the animal that may have an effect on the study and introduce additional confounding data. A long held belief was that any introduction of pain management strategy could negatively impact the quality of the data collected. Pain and distress are now recognized to have biological impacts that can and do have an effect on the experimental outcome as does any experimental procedure (6) and the same is true of analgesics, anesthetics any other compound administered to the animals (7).

Investigators may have had and still have concerns over the possibility that anesthetics and analgesics and even supportive care may interfere with experimental objectives, but the use of anesthesia and analgesia in research is now commonplace. From a regulatory perspective, both the USDA and the PHS require that analgesia and / or anesthesia be considered for potentially painful procedures and that unrelieved pain and distress must be scientifically justified (8).

Refinements can apply to the animal model itself or to the specific circumstances of the individual animals. For example, refinements to the disease models can include modifications such as using inbred or genetically modified animals, which might lead to reduction and possibly replacement of certain procedures. Refinements to animal conditions in the context of the disease model can include providing supportive care such as frequent monitoring, supplemental food or fluids, warming pads, wound care, antibiotics or even softer bedding.

Humane endpoints as a form of refinement

The goal of refinement is the minimization of pain and distress experienced by the animals. In cases where palliative measures are not scientifically feasible and the animals experience unrelieved pain, the establishment of humane endpoints may be a suitable refinement technique. The humane endpoint defines the finite endpoints of a study that provide a scientifically valid answer to the experimental question with the least amount of pain and distress to the animals.

The scientific endpoint must be a measurable outcome of the study, and may be a point in time, a physical characteristic of a disease or in some instances the survival of an animal. When mortality is the endpoint, the animals may experience considerable pain and suffering prior to

death as a consequence of the experimental procedures often without the potential for supportive care or analgesia. In some cases, however, the recorded death endpoint may not be a direct result of the experimental procedure but from factors related to the lack of supportive care such as dehydration or hypothermia. For example, an experimentally induced infection may result in the inability of an animal to access its drinking water and subsequently lead to severe dehydration and death. The animal might be erroneously recorded as an experimental death due to infection but it might have recovered if given supplemental fluids. To attribute of cause of death to the experimentally induced infection it is essential to have a clear understanding of the animals, the disease model and predictive criteria for evaluating the progression of the disease. Survival may be the desired discriminating factor between a treatment modality that works and one that fails but confounding factors such as dehydration or even side effects of the experimental treatments may diminish the value of death as a scientifically valid endpoint.

To satisfy the objectives of a study and to simultaneously consider the best interests of the animals, our best efforts must be made to find that point where both are reasonably achieved. If a study is terminated before crucial data is collected the animals may have been used for nothing but if the experiment continues past the point where useful data are collected then the animals may have suffered needlessly (9). Once the humane endpoint is identified the result can be removal of an animal from the study, or administration of a therapeutic agent to reverse the disease or of an analgesic or anesthetic to reduce the pain and distress. Euthanasia is a commonly used intervention once the humane endpoint is reached. In most cases the animals are euthanized at the point where it has been determined that they will not recover and before they become moribund or comatose. The supposition is that euthanasia is less painful and distressing than natural death by disease.

In some cases euthanasia of moribund animals is described as a humane endpoint. The moribund condition is described as a severely debilitated state that precedes imminent death (10). Animals that are unable to ambulate, unresponsive and possibly comatose, often characterize this state. As an animal in the moribund state has likely progressed through the point where it has suffered the most, killing an animal that is moribund may not in fact realistically be considered a humane endpoint. Preemptive euthanasia can also provide some benefits to research, such as an opportunity to collect tissues or other samples for analysis. Once the animal has become moribund some of the physiological changes could be interpreted as either the effect of the disease process or agonal in origin and not related to the disease.

Establishing humane endpoints

The determination of humane endpoints does not fit into a universal set of guidelines and every type of study may require a different solution and endpoint. There are multiple sources to consult for practical help in defining and establishing humane endpoints in different fields. (11,12,13,14,15). In all cases it is essential for the establishment and implementation of humane endpoints to be fully integrated into the design and execution of the experiments. The investigator must have a clear research question in mind, select the appropriate model, understand the disease process under study, and design the experiments with these factors in mind. In basic investigations, it is more challenging since many of the characteristics of the disease model may still be unknown. Pilot experiments utilizing smaller numbers of animals can provide some basic yet valuable information that can lead to developing humane endpoints for subsequent studies.

Clearly outlined plans for monitoring and treatment of animals, especially the untreated control animals or those receiving the highest infectious or therapeutic doses, are essential. Those animals receiving the highest infectious dose with no treatment are likely to suffer the most extreme symptoms of the disease. Likewise, it is possible that the animals that are receiving a high dose of anti-infective therapy may have a lesser pathogenic burden but have an adverse response to the chemotherapeutic. In both of these situations, it behooves the investigator to closely monitor the animals to better interpret the data.

Almost all of the published guides to selecting humane endpoints include some type of monitoring scheme. Monitoring and documentation is essential to defining the humane endpoints and may be useful in the final analysis of data by identifying subtle differences between groups. The monitoring could take different forms based on the individual study but may have some universal features such as physiologic parameters, body condition scoring and behavioral or clinical signs (16). Monitoring serves several purposes; it increases the basic knowledge about the disease model, provides data regarding humane endpoints, and may also provide data that could be useful in ultimately leading to a cure or prevention. Once collected the data must be scrutinized to identify any variables that may be signals of imminent death that could trigger sample collection or preemptive euthanasia.

When subjective measures are used, such as clinical observations or body condition scoring, there may be some bias or inconsistencies depending upon the observer. Individuals differ in judging severity of clinical signs even when efforts are made to synchronize those observations. One of the concerns raised is that monitoring is often conducted by observers who are aware of the treatment group identities, which may introduce a bias into the determination of when to euthanize. It has been suggested that these observations should be done without

knowledge of treatment and decisions to euthanize should be done regardless of the treatment (17). Unfortunately, blinding the observers to the treatment groups may interfere with the observations that may lead to a more thorough understanding the subtleties of the model.

The establishment of objective and measurable criteria such as weight change, fluctuations in body temperature, or analysis of biochemical markers that may be present in blood or serum might require additional handling and manipulation of the animals, which can add pain, anxiety and distress to an animal that is ill. Use of less invasive monitoring such as the testing of urine or feces or monitoring temperature remotely by telemetry or thermography should also be considered. Biochemical markers such as acute phase proteins or cytokines can provide experimental data as well as predict imminent death; however this also requires handling the animal to withdraw a blood sample. There may also be a time lag between when the sample is collected and the analysis is conducted, during which time the animal may continue to decline.

All of the steps described above require a significant investment in time and resources on the part of the investigators and research technicians. This investment can be quite costly and the development of humane endpoints may seem to be less of a priority than the other demands in the laboratory. It may be that once the initial groundwork has been done, however, the monitoring and scoring could become routine, conserve resources by shortening studies, and provide valuable data.

Obstacles to the acceptance of humane endpoints

In addition to the practical challenges of establishing humane endpoints, there are other reasons why they might not be accepted or adopted. These reasons may stem from lack of resources, information or commitment. One of the greatest obstacles may be the reluctance of

the research community to alter a “tried and true” experimental technique. Related to this is the perception that the use of alternate models may prevent the acceptance of data and reports in peer review journals.

There is the concern over the comparability of data between and within laboratories, of experiments conducted over long periods of time. For example, if previous experiments reported actual death, reporting preemptive euthanasia in subsequent studies may raise questions regarding the continuity and comparability of the data. There is the suspicion of a possibility that some of the animals might have recovered spontaneously and, under the new paradigm, are now to be reported as dead because they showed a symptom that seemed predictive of death. The issues relating to the reporting and analysis of data can often be resolved by the selection of the appropriate endpoint and the proper training and synchronization of observations by the study monitors.

Although there is a large body of literature on the subject of humane endpoints the bulk of it is not published in the widely read scientific journals, and therefore may not be well disseminated among investigators. A search of PubMed for humane endpoints yielded 33 articles, only 2 of which were published in basic science journals, namely Shock and Archives of Virology. A third was published in a toxicology trade publication. The remaining 30 were published in journals directed toward animal research professionals such as The ILAR Journal, Contemporary Topics in Lab Animal Medicine, Lab Animal Science and Altex (18). The information published in these journals may slowly filter into the research community through animal facility management, attending veterinarians, IACUCs and interested scientists, yet is likely that these journals are not widely read by research scientists. Mainstream scientific

journals occasionally publish commentaries or some papers on the ethics of animal research and humane endpoints but may lack scientific credibility if they are not supported by data.

Another obstacle may be the limitations in knowledge and influence of the reviewing IACUCs. The basic premise behind IACUC review is that the institution can self-regulate the animal studies of an institution by a review panel. Although the regulations governing the mandate of an IACUC are universal, the actual deliberations and philosophy of individual committees differ. The review committee must also assess pain and distress and determine if the benefits of the research justify the pain and suffering of the animals. In addition the IACUC must also approve the scientific justification of any unrelieved pain. In these situations the IACUC is in the difficult position of addressing scientific merit or ethical questions for which it has no formal training or mandate to undertake.

In addition, committee reviewers are often colleagues of the investigators submitting proposals and may be hesitant to question the justification for lethality studies. There may also be institutional pressure to approve proposals especially in situations where the receipt of grant funding is contingent upon approval.

The nature of infectious disease research

Animals were central to early investigations into many infectious diseases. Infections that plagued domesticated animals and threatened the lives and livelihoods of humans presented some of the early epidemiological data that hinted that transmissible agents were the cause of disease. Animals were also instrumental in advancing the validity of the germ theory of disease, the concept and practice of vaccination, the development of anti-infective agents and to some extent the adoption of sterile technique. The early innovators in microbiology used animals in

their research; Robert Koch used many different types of animals in his studies of anthrax, tuberculosis, typhoid fever and cholera to positively identify those microbes as the agents of disease. Emile Roux and Emil Behring, the discoverers of diphtheria antitoxin used scores of guinea pigs to understand the diphtheria bacillus and develop a treatment for this lethal disease (19). Louis Pasteur's basic studies of fermentation, microbial propagation and sterile technique preceded his work with anthrax in sheep and chickens, fowl cholera in domestic birds and led to the development of rabies vaccination. There are many other examples of the reliance on animals for developments in the fields of microbiology and immunology is evidenced in many sources chronicling the birth of these fields (20). The development of vaccines and antibiotic therapies has increased the life span of people in the developed and developing world.

Animal diseases of economic or public health significance are also studied in animal models. Agricultural practices which concentrate animals in close proximity to each other have produced conditions whereby animals are exposed to more pathogens and are often more susceptible to infection. Control of infection in livestock presents both economic and public health concerns which drive research into these diseases. Infections that can be transmitted between wildlife, domestic animals and humans have always been a concern and increasingly so due to the globalization of pathogens. International agencies such as the World Bank and World Health Organization and US agencies such as the Center for Disease Control have recognized that zoonoses have the potential to infect millions of people and animals causing very serious public health and economic effects. New research collaboratives, research centers, and grant opportunities have all been established to support efforts to study newly emerging and re-emerging infectious diseases and the possibilities of interspecific disease transmission.

The study of infectious diseases is not limited to the study of the infectious agents themselves but also the evolving interactions between the organisms and the hosts. The field of immunology and other studies of host response have become an integral part of the study of infection. The ability of the host to stop or slow an invader is as important as the ability of the infectious agent to evade the immune system.

Mice have been the species of choice in immunology research and with developments in genetic modification new models are continually being developed. Animals engineered to be missing one or multiple components of the immune system are used to study the factors responsible for controlling certain infections. Although highly useful tools for gaining insight into the mechanisms of immune system function, genetically modified animals present unique considerations with reference to humane endpoints. Such animals may suffer from unknown conditions as a result of the genetic manipulation that could affect the disease model or result in pain and distress in ways that are difficult to anticipate (21).

Current uses of animals

A typical infection study involves the administration of a putative or known infectious agent into a living animal after which the animal is monitored during the course of infection. In the case where death is the endpoint of the study, the animals are monitored periodically and counted as dead or alive. The data collected is represented as a survival or mortality curve, which shows the number of survivors over the time course of the study. Other measures such as mean time to death and LD_{50} are also reported. The LD_{50} refers to the infectious dose at which 50% of the animals die. The binary determination of dead or alive provides an unambiguous data point for analysis, this approach however, is very crude in that the opportunities to collect other

points or types of data are missed. There are many biochemical, histological and clinical parameters that could be studied in the interval between infection and death that could provide important insights into the disease process that could potentially lead to treatments, vaccines or prevention strategies.

Many studies do in fact capitalize on those opportunities. In these instances, the animals are infected and at predetermined time points during the course of the disease they are either monitored for clinical signs, euthanized for study or blood and tissue samples taken. The samples taken throughout the course of infection can be analyzed for immune response, and for colonization, migration, and replication of the pathogen in the animal, and produce an array of data that can provide a broad description of the infection that improves upon the crude estimations of survival. New and more sophisticated techniques are being developed to monitor clinical signs and progression of infection in real time in an infected individual. Telemeters can be implanted to monitor temperature, heart rate and other parameters that not only provide data but also can be useful in determining imminent death and subsequent assignment of humane endpoints. It must be noted however that telemeter implantation involves stress and pain and could have an impact on the animal and the experimental procedures. Moreover, the use of alternate endpoints or monitoring strategies does not eliminate the pain and distress experienced during the course of the experiment.

The scope of infectious disease research goes far beyond the basic in vivo infection model. The investigations delve into the molecular basis of virulence, pathogenesis, host – pathogen interactions and many other areas. For example, vaccine development is no longer limited to the crude injection of microbial extracts followed by challenge but is often a combination of in vitro and in vivo studies. Preliminary, systematic studies of the pathogen

including sequencing of the genome, analysis of virulence factors, and possible antibiotic resistance are routinely conducted. For example, cell surface molecules present on infectious pathogens can be studied to determine which ones are likely to produce neutralizing antibody response once injected into an animal. The techniques developed for in vitro studies can be applied to tissues collected from animals in vivo. In many cases animal studies are a confirmatory component of a larger project.

Oversight of animal experiments

Animal studies are regulated by federal and local statute as well as by governmental regulation and institutional policies. On the federal level, the Animal Welfare Act (AWA) and the Health Research Extension Act both regulate the use of research animals. The Animal and Plant Health Inspection Service (APHIS) of the Department of Agriculture (USDA) enforces the former, and the Public Health Service, a department of Health and Human Services, oversees the latter.

The AWA applies to all animals used in research with the exception of birds, rats of the genus *Rattus* and mice of the genus *Mus*. The USDA is responsible for writing and enforcing regulations that require institutions to register and submit to inspections of animal holding areas, laboratories, and animal use protocols. The USDA is not authorized to "promulgate rules, regulations or orders with regard to design, outlines, or experimentation…. or the performance of actual research or experimentation by a research facility…." Every registered institution must establish an internal review panel to establish and oversee the care and use of experimental animals and ensure that "procedures involving animals will avoid or minimize discomfort, distress, and pain to the animals." Investigators are required to consider alternatives to painful

procedures and appropriate anesthesia and analgesia. Any “animals that otherwise experience severe or chronic pain or distress that cannot be relieved will be painlessly euthanized at the end of the procedure or if appropriate during the procedure” (22). Also, “ discomfort and pain to animals will be limited to that which is unavoidable for the conduct of scientifically valuable research, including provision for the use of analgesic, anesthetic and tranquilizing drugs where indicated.”

The Health Research Extension act applies to all vertebrate animals used in research studies supported by federal funds. The Public Health Service relies on individual institutions to review protocols, conduct internal investigations and report any deficiencies to OLAW. In cases of noncompliance federal funds may be withdrawn. In 1993, the Health Research Extension Act was amended to require the National Institutes of Health to conduct and develop research methods that do not require animals, that reduce the number of animals used and that produce less pain and distress in animals (23). In addition to the PHS policy, an interagency commission published a set of guidelines to align the principles guiding the use of animals by government agencies and research supported by federal funds. The US Government Principles for the Utilization and Care of Vertebrate Animals used in Testing, Research and Training was published in 1985 and sets forth universal principles with the intent that all governmental agencies follow the same general principles (24).

Three R’s and the establishment of humane endpoints are not specifically mentioned in the laws or regulations but both the USDA and the PHS support the concepts embodied in the Three R’s. They have posted published papers and information on their websites supporting humane endpoints and discourage death as an experimental endpoint. The USDA regulations, PHS policy, and the government principles specify that an internal review committee, often

known as an Institutional Animal Care and Use Committee, review all proposed research. This effectively places the implementation of humane endpoints in the purview of the IACUCs and the institutions that they serve.

Methods

Research articles containing original data generated in United States laboratories and published in the last 18 months in two prominent infectious disease journals was reviewed. The research studies were evaluated with a focus on the design of the animal model, the types of data collected, data presentation, and the collection of additional markers that may indicate imminent death. The scope of the journals selected is similar and of interest to physicians, immunologists, microbiologists, epidemiologists and other infectious disease professionals. The first, The Journal of Infectious Diseases (JID), is sponsored by the Infectious Diseases Society of America and published by the University of Chicago Press. It is by its own description " the premier publication in the Western Hemisphere for original research on the pathogenesis, diagnosis and treatment of infectious diseases; on the microbes that cause them and on disorders of host immune mechanisms" (25). Laboratory, clinical, and epidemiologic studies in the fields of microbiology, infection and host response are all considered for publication. The instructions for authors regarding the use of laboratory animals in JID does not require a statement indicating that the animals use has been reviewed. The only reference to animal studies refers to the use of appropriate controls in experiments utilizing genetically modified mice. The second journal selected, Infection and Immunity (I&I) is published by the American Society for Microbiology. The range of topics is similar to that of JID and includes mechanisms of infection, host resistance, molecular genomics of the pathogens and evaluation of therapeutics, vaccines, and

prevention strategies. Manuscripts submitted to I & I must include a statement indicating that research involving animals complies with "relevant federal guidelines and institutional policies" and documentation of compliance must be available upon request (26).

Inclusion and exclusion criteria

To further define the study, the research reports surveyed included only studies conducted in the United States. In collaborative situations involving non-US laboratories, the inclusion of a study was based on where the animal portion of the studies took place. Articles published between January 2005 and July 2006 were reviewed. These dates reflect a snapshot of research conducted in the last few years. Most articles were submitted for review from the first quarter of 2004 to the end of 2005. Reviews, invited articles, and commentary were not included in this study.

The animal studies were separated into those conducted using animals covered by the Animal Welfare Act (AWA) and those that are not. According to the AWA, " animal means any live or dead dog, cat, nonhuman primate, guinea pig, hamster, rabbit or any other warm-blooded animal, which is being used, or is intended for research, testing, experimentation, or exhibition purposes, or as a pet. This term excludes: birds, rats of the genus *Rattus* and mice of the genus *Mus* bred for use in research. Facilities housing and using animals covered under the AWA are subject to unannounced facility inspections, review of protocols and administrative procedures. All vertebrate animals including rats and mice used in studies supported by funding provided by PHS and other government granting agencies are covered by the Office of Laboratory Animal Welfare of the Public Health Service.

Rats and mice have continued to be the species of choice for many infectious disease studies, but mice are by far the largest single species used. Mice are small, easily contained, easy to handle, and can be used in groups large enough to overcome individual animal variations. There are also many genetically modified animals that either lack certain immune functions or contain human genes that make them attractive animal models. Due to their small size, lesser amounts of precious laboratory-generated experimental agents are needed to conduct experiments. There are also many more reagents available for testing mouse tissues. Finally, the concept of replacement also includes a provision that animals be replaced with less sentient animals where possible. On the current continuum of sentience, mice are considered to occupy the low end of the scale and have become the replacements for experiments once conducted in many other species such as dogs or primates.

This review excluded studies that used animals exclusively for reagent generation such as reagent antibody production or tissue harvests for *ex vivo* studies such as normal bone marrow, splenocytes or other tissues.

Study Types

The type of study may be a factor in the choice of scientific endpoints. For purposes of evaluation each study was classified into one of four categories: virulence, animal model development and pathogenesis, host and immune response, and vaccine and therapeutic development.

Virulence is defined as the ability of an agent to produce disease (27). The ability to produce disease is not exclusively a function of the pathogen but also reflects the immune and health status of the host. There are however, unique characteristics of pathogens that may confer

increased ability to evade the immune system. In this sense, the virulence studies in this survey are those focused on fitness and survival of the pathogen and its ability to produce disease in animals. In these studies different strains, serovars or genetically modified pathogens are administered to the animals to identify the determinants present in the infectious agent that confers the ability to cause disease.

The assessment of pathogenesis, disease progression and animal model development were grouped together. Pathogenesis is defined as "the pathologic, physiologic, or biochemical mechanism resulting in the development of a disease or morbid process" (27). In our classification, pathogenesis includes the study of the mechanisms of infection, the progression of the disease and in some cases the development and description of an animal model, which approximates human infection.

Studies focused mainly on the host immune response to microbial challenge were grouped together. The host response category includes all host responses including barriers to infection, non-inducible anti-infective strategies such as nitric oxide (NO), co-infection by competing microbes as well as the classical immune responses. The sophisticated techniques developed for studying the many facets of the immune response may provide greater opportunities for humane refinements.

The last category, that of vaccine development and therapeutic development, includes those experiments specifically designed to test a therapeutic agent, or vaccine. These studies typically involve the induction of disease followed by therapeutic intervention. Animals in vaccine studies are immunized with the test vaccine and are subsequently infected to determine the protective effect of the vaccine. Unvaccinated controls and untreated animals will often suffer the full effect of the infection.

Classification by pathogen type

The studies were also classified by type of pathogen. The term pathogen is defined as any virus, microorganism, or other substance causing disease. (27) The pathogens were grouped into bacterial, fungal, parasitic, viral and toxins classification. In the cases where toxin-producing bacteria were directly injected into the animals they were classified as bacterial. The toxin classification only applies where toxin components were directly administered.

Classification of humane endpoints

The use of endpoints other than death proved difficult to classify. The entire text of each published report was reviewed in order to make the assessments. The components examined were the aim of the study as described in the abstract and introduction, and the animal study design as described in the materials and methods. The presentation of data was important in determining the endpoints in many cases; the inclusion of terms or indicators such as survival plots, mean time to death and LD_{50} indicated that death was the endpoint. The discussion section was also reviewed for evidence that criteria other than mortality and moribundity were considered.

The studies were classified into those using death as an endpoint and those that did not. A study was classified as having used death as an endpoint if data were consistent with mortality studies, or if it was specifically stated that the data points collected represented the natural infection-induced death of the animals. Also included in this tally are studies where moribund animals were euthanized, since, as discussed previously, euthanasia at this stage does not necessarily preempt suffering of the animal and we did not consider it to be a humane endpoint.

Unless alternative criteria for euthanasia were defined and used, the study was classified as a lethality study.

Studies that did not report or use death as an endpoint, indicated other criteria for defining the scientific endpoints including periodic pre-determined observations or collection of samples for the purposes of measuring severity of disease, pathogen burden, immunological parameters and other markers of disease. Studies that were terminated based on clinical signs such as body condition scoring, or other measures were considered to have used humane endpoints. The subset of the studies that use alternative humane endpoints often report detailed clinical information regarding the course of the disease, the clinical signs and other markers that they use to predict the imminent death of the animal and criteria for euthanasia.

In many studies, the endpoints are defined in the experimental design as a point in time following infection or intervention and without knowing the clinical course and actual fate of the experimental animals it is not possible to determine if the endpoint is humane or not. In some cases, a cohort of animals is euthanized at each timepoint for the collection of tissues or other assays while the remaining animals in the study continue to experience the clinical course of the disease. Without a description of the clinical course of the infection or the condition of the animals, it is not possible to eliminate the possibility that some of the later time points, sampling is actually conducted on moribund or possibly even dead animals

The data were compiled to reflect the studies that can be determined to have used death as an endpoint and those that have not. In some studies where death is not the reported endpoint, the animals may still have suffered from unrelieved pain and serious morbidity that led to death.

Funding sources and IACUC Statement and Review

In order to get an idea of oversight of the animal studies several pieces of data were collected. The source of funding of a research project and the presence of an IACUC Statement in the published report both contribute to determining whether an institutional review committee has reviewed a study. As mentioned previously, not all research studies are subject to IACUC review, specifically studies conducted using rats and mice and not supported by government funds.

Information on the identity of the funders of the research was gathered in an effort to ascertain whether the use of humane endpoints was aligned with the guidelines stated by their respective sources of funding. The studies surveyed were assigned to broad categories. The first category, government support, includes all research funded by the Departments of Agriculture (USDA), Health and Human Services (HHS), Defense and the Veterans Administration. The other two funding categories are private research foundations and pharmaceutical companies. The second piece of information gathered was the inclusion of a statement indicating that the animal research was approved and by inference any unrelieved pain and distress was scientifically justified and that the relief of pain and distress was considered. If either of these two conditions were met it was assumed that there was a review of the proposed studies and that adequate scientific justification was provided to conduct the research studies.

Results

A Total of 2279 peer review articles published between January 2005 to July 2006 in the *Journal of Infectious Diseases* (JID) and *Infection and Immunity* (I&I) were surveyed for content that would reveal the current status of the use of humane endpoints. Of these articles 556 were

research reports of animal studies conducted in United States laboratories. The other 1723 articles were studies conducted throughout the world, nonanimal studies, and human clinical and epidemiology studies.

The animal studies were subsequently divided into two groups. The first group contains all studies conducted with rats and mice and the second includes studies using all other species. Table 1 shows the total number of journal articles surveyed and the breakdown of studies by types of animals used. Overall 72% of the studies used were rats and mice; the remaining 28% used mostly other mammals. Two studies used chickens, two used non-laboratory fish (bass and trout) and one study used amphibians. Rabbits and pigs made up the largest groups within the all other species group. Both JID and I&I contained more rodent experiments than all other species, but they differed in relative percentages (62% in JID and 80% in I & I).

Table 1. Journal issues published between January 2005 and July 2006. Percentages of studies using Rats and Mice and All Other Species is shown for each journal.

Journal	Total # Articles	# Animal Studies (US Labs)	Mice and Rats (non-AWA covered)	All other Species (AWA covered)
Journal of Infectious Diseases	758	78	48/78 (62%)	30/78 (38%)
Infection & Immunity	1521	478	382/478 (80%)	96/478 (20%)
Total	**2279**	**556**	**430/ 556 (72%)**	**126/556 (28%)**

To get an overview of the field, the data from both journals was combined for further analysis. For all animal species studied, bacterial studies represented the largest class of

infectious agent studied, 65% of all studies were conducted with bacteria, followed by fungal and parasitic studies at 11% and 12% and lastly toxin and viral studies at 6% each (Table 2). When rat and mouse only were grouped by pathogen, a similar relative pattern is seen. Among all other species, there were no studies conducted with fungi and only 1 with microbial toxins.

Table 2. Number of studies reviewed classified by pathogen type. Numbers of studies and percentages within each group are shown for All Species, Rats and Mice and All other Species.

Pathogen type	All Species # Studies (% total)	Rats and Mice # Studies (% group)	All other Species # Studies (% group)
Bacterial	363/556 (65%)	279/430 (65%)	85/126 (67%)
Fungal	63/ 556 (11%)	63/430 (15%)	0/ 126 (0%)
Parasitic	66/556 (12%)	46/430 (11%)	20/126 (16%)
Toxin	31/556 (6%)	30/430 (7%)	1/126 (< 1%)
Viral	32/556 (6%)	12/430 (3%)	20/126 (16%)
TOTAL	**556**	**430**	**126**

The study type and experimental design also can determine the endpoints of interest. Among the studies surveyed 41% are focused in the immune and host response to the pathogen in question followed by 28% examining virulence factors. Vaccine and therapeutic development and pathogenesis studies make up the remainder of the studies at 17% and 14% respectively. Studies conducted in rats and mice have roughly the same distribution pattern. Among all other species the type of study is more evenly distributed.

Table 3. Number of studies reviewed and classified by type of study. Numbers of studies and percentages within each group are shown for All Species, Rats and Mice and All other Species

Study type	# Studies (% total)	Rats and Mice # Studies (% group)	All other Species # Studies (% group)
Virulence	156/556 (28%)	124/430 (29%)	32/126 (25%)
Model Development / Pathogenesis	79/556 (14%)	55/430 (13%)	24/126 (19%)
Immune / Host Response	226/556 (41%)	192/430 (45%)	34/126 (27%)
Vaccine / Therapeutic Development	93/556 (17%)	58/430 (14%)	35/126 (28%)
Other (diagnostic)	1/556 (<1%)	---	1/556 (<1%)
TOTALS	**556**	**430**	**126**

Death as an Endpoint

Of the 556 studies reviewed 175 or 31% reported death as an endpoint. There was a slightly higher percentage of lethality studies in The Journal of Infectious Diseases compared to Infection and Immunity. A similar relative difference was observed between studies conducted in the rats and mice compared with studies conducted with all other species (Table 4).

Table 4. Summary of numbers of studies where death is the endpoint in all animals, rats and mice and all other species.

Journal	All Animals # Lethal studies/Total #	Rats and Mice # Lethal studies/# Rat &Mouse	All Other Species # Lethal studies/# other species
Journal of Infectious Diseases	31/78 (40%)	22/ 48 (46%)	9/30 (30%)
Infection and Immunity	144/478 (30%)	135/382 (35%)	9/96 (9%)
Totals	**175/556 (31%)**	**157/ 430 (37%)**	**18/126 (14%)**

We sought to determine if death as an endpoint is related to the type of pathogen, study type or species of animal used. The species of animal used appears to be related to the use of lethality studies. Of the 175 lethality studies 157 or 89.7% were conducted in rats and mice. As shown above in table 4, 37% of studies conducted with rats and mice use death as the study endpoint while only 14% report death as an endpoint in all other species.

Table 5 illustrates the relationship of pathogen type to use of death as the study endpoint. Rats and mice are tabulated separately from all other species. In experiments conducted with rats and mice, 50% or more of authors studying fungi, microbial toxins or viruses use death as the study endpoint. Among all other species, there were no experiments conducted with fungi and only one with microbial toxin and 30% of viral studies used death as the endpoint. For bacterial studies 32% of studies conducted with rats and mice report death as an endpoint while lethality studies only make up 13% among all other species. A similar finding is true among parasite studies, 24% among for the rats and mice and 5% for all other species.

Table 5. Death as an endpoint by pathogen type in Rats and Mice (Panel A) and All other species (Panel B). Percentages of studies using death as an endpoint were determined for each study type.

A. Rats and Mice

Pathogen type n = # studies	Death as Study Endpoint YES	Death as Study Endpoint NO
Bacteria (n= 279)	88/ 279 (32 %)	191/ 279 (68 %)
Fungi (n= 63)	34/63 (54 %)	29/63 (46 %)
Parasites (n= 46)	11/46 (24 %)	35/46 (76 %)
Toxins (n=30)	18/ 30 (60 %)	12/30 (40 %)
Viruses (n=12)	6/ 12 (50 %)	6/ 12 (50 %)
Overall (n=430)	**157/430 (37%)**	**273/430 (63%)**

B. All other Species

Pathogen type n = # studies	Death as Study Endpoint YES	Death as Study Endpoint NO
Bacteria (n= 84)	11/ 84 (13%)	73/ 84 (87%)
Fungi (n= 0)	----	----
Parasites (n= 20)	1/20 (5%)	19/20 (95%)
Toxins (n=1)	1/ 1 (100%)	----
Viruses (n= 20)	6/ 20 (30%)	14/ 20 (70%)
Overall (n=126)	**18/126 (14%)**	**108/126 (86%)**

Table 6. Summary table of death as an endpoint in Mice and Rats vs. All other Species as grouped by pathogen type. Percentages are calculated within each pathogen type for each species class.

Pathogen type	Rats and Mice # lethal studies/ # rat & mouse	All other Species # lethal studies/ # all others
Bacterial	88/ 279 (32 %)	11/84 (13 %)
Fungal	34/63 (54 %)	-----
Parasitic	11/46 (24 %)	1/20 (5 %)
Toxin	18/ 30 (60 %)	1/ 1 (100%)
Viral	6/ 12 (50 %)	6/20 (30 %)
Overall	**157/ 430 (37%)**	**18/126 (14%)**

Table 7. Death as an endpoint by study type in Rats and Mice (Panel A) and All other species (Panel B). Percentages of studies using death as an endpoint were determined for each study type.

A. Rats and Mice

Study type n = # studies	Death as Study Endpoint YES	Death as Study Endpoint NO
Virulence (n=124)	45/124 (36%)	79/124 (64%)
Model Development/ Pathogenesis (n=55)	20/55 (36%)	35/55 (64%)
Immune /Host Response (n= 192)	61/192 (32%)	131/192 (68%)
Vaccine/ Therapeutic Development (n=58)	30/58 (52%)	28/58 (48%)
Overall (n =430)	**157/430 (37%)**	**273/430 (63%)**

B. All other Species

Study type n = # studies	Death as Study Endpoint YES	Death as Study Endpoint NO
Virulence (n=32)	4/32 (13%)	28/32 (87%)
Model Development/ Pathogenesis (n=24)	2/24 (8%)	22/24 (92%)
Immune /Host Response (n= 34)	2/34 (6%)	32/34 (94%)
Vaccine/ Therapeutic Development (n=35)	10/35 (29%)	25/35 (71%)
Overall (n =126)*	**18/126 (14%)**	**108/126 (86%)**

*The total number of studies is 126, one of which was the development of a diagnostic test and is not included in this table.

Table 8. Summary table of death as an endpoint in Mice and Rats vs. All other Species as grouped by pathogen type. Percentages are calculated within each pathogen type for each species group.

Study Type	Rats and Mice (non-AWA covered species)	All other Species (AWA covered species)
Virulence	44/124 (36%)	18/32 (13%)
Model development / Pathogenesis	20/55 (36%)	4/32 (8%)
Immune / Host response	61/192 (32%)	2/34 (6%)
Vaccine / Therapeutic Development	30/58 (52%)	10/35 (29%)
Overall	**157/ 430 (37 %)**	**18/126 (14 %)**

IACUC Statement

Of the 175 lethality studies 94% contained statements indicating IACUC approval. 93% of all lethality studies conducted using rats and mice were approved while 100% of all lethality studies conducted in all other species were approved (Table 9). The main source of funding for research projects was difficult to assess as many of the projects are supported by several different groups. No inferences could be made regarding the influence of the supporting institutions positions on the use of humane endpoints.

Table 9. IACUC statements in lethality study reports in rats and mice compared with all other species. IACUC review was confirmed by direct statement of IACUC review or statement of government funding.

	Lethality Studies		
	# Studies	# Reviewed	(%)
Rats and Mice	157	146/157	93%
All other Species	18	18/18	100%
Total	**175**	**164/175**	**94%**

Additional Observations

Many of the findings in this review do not fit neatly into a table nor are they amenable to calculation or statistical analysis. These findings fall into the category of impressions and or statements that may have significant implications.

The group of studies designated as not using death as an endpoint contains several different types of studies. Some studies are conducted with non-lethal pathogens where the animals may suffer from a localized or limited infection from which they recover spontaneously. They may be euthanized and tissues or other samples harvested for analysis. Another subset of studies features pre-selected timepoints for euthanasia and tissue collection during the course of

infection. These studies may require many animals since a group of animals must be killed at each timepoint and animals harvested at the later time points may be quite ill and even die prior to their designated timepoint. A newer technology that utilizes real time imaging was used in a few studies. The disease progression in each individual animal can be followed on a periodic basis with the possibility that data can be collected prior to the animals suffering severe morbidity. A few studies of this type were published and show promise for reducing the numbers of animals needed and, if the image data can be effectively correlated with clinical signs of disease, this can be a powerful tool to help define humane endpoints in some situations.

Studies using and defining humane endpoints:

The studies that used humane endpoints described the clinical signs of morbidity and defined their criteria for euthanasia. There does not seem to be a universal pattern for these descriptions and they sometimes appear in unexpected sections of the research reports. The following are some examples of the how humane endpoints were reported.

The first two examples clearly describe the humane endpoints and clinical parameters used to define those endpoints.

> "A study of the pathogenesis of enterohemorraghic *E.coli* measured hematologic parameters, clinical signs and renal disease by histopathological means. Animals experiencing acute disease characterized by hemorrhagic diarrhea and weight loss were euthanized" (28).

> "The mice were euthanized 48 h after instillation of bacteria. Mice were euthanized earlier if they exhibited loss of >15% of original body weight or 3 of the following: dehydration (evaluated by skin tenting test), lethargy and decreased movement, abnormal posture (such as hunching), ruffled fur, pale eyes, or loose feces." (29).

Several of the studies state the IACUC required the adoption of the humane endpoints and are quoted below.

> "Institutional Animal Care and Use Committee guidelines do not permit the use of death as an experimental endpoint in animal studies, so morbid animals that were judged to be incapable of surviving infection were humanely sacrificed, and the data have been reported with respect to the percentage of animals that were "nonmorbid." Mice identified as morbid typically exhibit hunched posture, ruffled fur, weight loss, and decrease responses to stimuli and have been judged to be incapable of surviving infection." (30).

> Tsenova et al (31) developed a clinical scoring system for the evaluation of meningitis in rabbits as follows: "stage 0 (normal), stage 1 (hyperesthesia, head tilt, and lethargy), stage 2 (monoparesis), stage 3 (hemiparesis and recumbency), stage 4 (quadriplegia), and stage 5 (anorexia, and CNS depression progressing to moribund state and death). Rabbits at stages 4 and 5 were killed as required by our IACUC."

Discussion

Animal studies in infectious disease research have been integral to the development of antibiotics, sterile technique and public health disease prevention strategies. There are some who argue that the use of animals was not necessary and that some of the same advances would have been made without them. However, it might be safe to say that countless human and animal lives have been saved by the uses of animals in the efforts of the pioneers in bacteriology. In the early 1800's much was unknown about the infectious agents responsible for diseases that ravaged human and animal populations such as anthrax, cholera, rabies, diphtheria, smallpox, and plague, including their means of transmission and the processes of infection. The search for answers was almost desperate, and in the early 1900's scientists were just beginning to develop the tools necessary to study and identify the causative agents and prove that they were indeed the cause of the sickness. Animal models became one of those tools; the animals were used to identify one microbe as the causative agent of a specific disease, a significant breakthrough that

became one of the standard ways to confirm an agent as pathogenic. Animals also became the test subjects for possible treatments and the new field of vaccination. At that time, the animal models may well have been among the most sophisticated experimental techniques.

In 2006, the variety and specificity of tools used to study infectious disease have increased considerably. Microscopy has advanced to include high-powered electron microscopy and computer-assisted confocal microscopy that can provide fine structural detail about the organisms. Within the last 20 years, the advent of molecular biology has made it possible to study the genetic characteristics of known pathogens and also to rapidly characterize previously unknown agents as well as study the molecular interactions of pathogen and host. Similar advances in immunology and pharmaceutical development have expanded the tool kit of infectious disease researchers. The animal models have also advanced in reliability and specificity, and from a welfare perspective the condition of the animals has also improved. The importance of animal health and uniform husbandry to the outcome of experiments has been recognized, and nutrition, housing, and experimental conditions have much improved since the time when animals were stacked in cages in the corners of laboratories. Although the conditions in which the animals live have changed, basic design of the studies has not, and the animals continue to suffer from the sequelae of the diseases with which they are infected.

Opposition to the use of animals for these studies also continues. Scientists of 2006 face criticism regarding the pain and suffering that are inflicted upon the animals as a result of the infections that they endure. The main question is that now, with a huge array of precise tools available to researchers, is the use of animals as necessary? If the answer to this is yes, then the next question is, is it necessary for the animals to progress to death or are there other, possibly

more precise, endpoints that can be obtained through a creative use of the new methods available, and are there more humane options available?

Humane refinement of research studies is not a new concept. As previously mentioned the Three R's were described in the late 50's and are now firmly entrenched in federal and local regulations, institutional policy of many universities, and even in the lexicon of practicing scientists and their professional organizations. There are many websites, books, journal articles, conferences, workshops and other sources of information to provide guidance on the development of humane alternatives to some types of studies. Federal granting agencies require that grant submissions proposing to use vertebrate animals include approval of the study by an IACUC as well as a PHS Assurance, which describes how animals will be housed, cared for and treated in accordance with federal regulations. The USDA, OLAW and other groups have posted extensive bibliographies on their websites. The prospect and possibility of alternatives to animals in research has also penetrated the popular press. Although many would prefer that no animals be used for experimentation, some animal welfare organizations such as The Humane Society of the United States publish literature that refers to the three R's as a path toward more humane studies.

With this in mind, one of the objectives of this study was to determine if the available information on humane endpoints and more sophisticated methodologies has been internalized and reduced to practice, in a specialized field of research, namely infectious diseases. The second objective of this study was to find evidence as to whether the use of lethality studies can be scientifically and ethically justified by the assertion that they are standard practice within the field and are necessary for acceptance and publication. To address both of these issues it was

first necessary to determine the current status and prevalence of lethality studies and humane endpoints in the field of infectious diseases.

The infectious diseases field was selected because it has historically used animal models of disease that often lead to significant morbidity and even death. Most people have personal experiences with infectious diseases and can relate to the potential benefits of research aimed at treatment and prevention. One of the studies reviewed in this study referred to the pathogen in question as being particularly "loathsome" (31). The severity and of the diseases studied in this field may lead the reader and IACUCs to make the case for justification of lethality studies if indeed the result is to rid the world of a loathsome disease.

We were limited by the information described in the published reports, and the absolute numbers and percentages differ between the journals reviewed, possibly due to the types of laboratories and institutions submitting reports. The objective in reviewing and combining the data from two journals was to try to obtain a broader view of the field and to lessen the effect of editorial bias. The articles published in Journal of Infectious Diseases and Infection and Immunity were largely funded by government grants and the studies were mostly conducted at universities, medical centers and government research facilities. The animal studies used in infectious disease research at private pharmaceutical and biotechnology companies and contract research laboratories are not equally represented here. The magnitude of research at these other institutions is not known but it may be safe to say that in light of the increase of drug-resistant infectious diseases and newly emerging infectious diseases the hunt for new anti-microbials and vaccines are progressing at a rapid rate and involve many studies involve the use of laboratory animals in potentially lethal experiments.

Within those limitations, this study confirms that lethality studies continue to be a prominent feature in the infectious disease literature. Almost one third (31%) of the published studies sampled describe death as the desired study endpoint. Of the 69 percent that do not, even though it is not the study endpoint, it is possible that in the course of these experiments some of the animals may have progressed to death without intervention. However, the finding that the majority of studies do not use death as an endpoint would suggest that lethality studies are not the standard methodology and that other more humane options are being successfully published.

The combined data reveals that regardless of journal, more lethality studies were conducted with rats and mice than with all other species combined. This finding may be important in light of the fact that rats and mice are not regulated under the Animal Welfare Act although they are covered under the PHS Policy.

Most of the studies in this review received government funding and were likely to have been submitted for institutional review. The greater use of rats and mice in lethality studies may not only reflect the lack of USDA oversight but also may reflect differences in perceived benefits vs. the harms when mice and rats are the proposed subjects as opposed to "more sentient" species such as dogs, or non-human primates. The finding that lethality studies are more commonly conducted in rats and mice is consistent across different types of studies and pathogen type used.

The type of pathogen under investigation may underlie the choice of death as the desired endpoint. The largest percent of lethal experiments (greater than 50% overall) were in the fungal and toxin studies. All fungal studies and all but one toxin study were conducted in rats and mice. Viral studies were equally represented between rats and mice and all other species; 50% of rat and mouse viral studies were lethal while only 30% were lethal in all other species. The possible

reasons for this may be due to the nature of fungal, viral and toxin studies. Fungi and toxins may have a greater propensity to cause mortality and in the case of toxins the effects may be very rapidly fatal with few opportunities for intervention. Fungi in particular are often opportunistic but once established they could be rapidly lethal. The rarity of fungal or toxin studies in animals other than rats and mice may be due to experimental concerns, or possibly the concern that these studies may have the possibility of causing a great deal of unrelieved pain and suffering and were conducted in rats and mice as a refinement.

Viral diseases can be more species specific, which may explain why similar numbers of studies were conducted in rats and mice and in all other groups. The experimental difficulties presented by the species barrier may provide added justification for their use in higher species. For example, the use of simian immunodeficiency virus (SIV) in primates may be seen to be a better model for human immunodeficiency virus (HIV) infections than models in other species.

It is possible that within the fields of mycology or virology there is a greater tendency toward lethality studies. To further explore this it would be useful to select a few of these studies and search for similar reports and determine if death as an endpoint is commonly used. For example, for a single pathogen it is possible that similar studies in different laboratories all use the same disease model including the selection of death as the endpoint. This may be due to the nature of the pathogen but also the desire of the different laboratories to produce comparable interlaboratory data.

In summary the data suggests that death as an endpoint is still in use, but that humane endpoints and non-lethal experiments are also being considered and used. This is encouraging in light of the assertions that lethality studies are standard and necessary for acceptance into peer-reviewed publications. The finding that studies conducted with fungi and toxins have a greater

percentage of lethality studies might imply that within these areas of investigation the use of the lethality study may be considered to be an acceptable standard. With regard to fungal studies, further study should include a search within mycology journals to ascertain whether lethality studies are more prevalent in the field as a whole or if it is more pathogen specific.

The continued use of death as an endpoint presents a challenge to researchers, IACUCs and laboratory animal professionals. The data show that in all categories lethality studies are more prevalent in studies conducted with rats and mice. Aside from the possible scientific and practical reasons, it is possible that mice and rats are considered to be less sentient than other species and the cost to the animals is determined to outweigh the potential benefits. The reviewing committees may view the use of mice rather than another species as a refinement in and of itself. Mice and rats present unique difficulties with regard to monitoring and determining humane endpoints but it has been demonstrated that pain and distress can be identified and humane endpoints put into place.

Almost all research articles included in this study were reviewed and presumably approved by an IACUC or similar panel. One of the most difficult and least well-defined functions of the IACUC is the review of protocols, especially with regard to lethality studies. This requires evaluation of the scientific reasons for withholding anesthesia, analgesia supportive care, and the possibility of preemptive euthanasia. Even if there are good reasons for conducting a study, the potential benefits may not be adequate to justify the harms. The benefits resulting from basic research on normal biological processes and the natural history of disease are often less measurable than that of toxicity or potency tests of a therapeutic agent. Due to the cumulative nature of research many studies serve to increase the base of knowledge but may not produce practical benefits in the near term. For studies whose aim is to increase basic

knowledge of a natural process, it is very difficult to justify a study that entails significant harms to the subjects, in contrast to a study that will cause significant harm but determine the efficacy of a life saving drug.

Over 94% of all lethality studies included in this study received IACUC review. Since IACUC review requires scientific justification for lethal studies it can be concluded that the reviewers were able to determine that these studies were necessary and that presumably the best efforts were made to minimize pain and distress.

Humane endpoints in the literature

This study was aimed at the use of humane endpoints and lethality studies in infectious diseases as reported in the scientific literature. An additional and encouraging finding is that a few studies that we reviewed actually describe and define the humane endpoints.

Humane endpoint literature is focused on why and how to define and implement humane endpoints and is directed toward animal research professionals, while the scientific literature aimed toward scientists is focused on how to comply with IACUC mandates. The missing piece is the lack of compelling published evidence that the establishment of humane endpoints benefits the animals but also produces more precise and reproducible data. This information is not readily available or even searchable by Medical Subject Headings (MeSH).

In addition, to really prevent the needless replication of animal experiments it is important to support the publication of negative data. For example, there is no database of animal models; so multiple labs could be simultaneously working on a model for disease X in species Y. Several researchers may have failed to do so in the past and may have even discerned a reason why it is not feasible. Yet such failures are rarely published and it is likely that others

will needlessly repeat the experiments. It is hard to imagine that there would be much incentive for scientists to take the time and effort to submit their animal model refinements.

Publications such as I & I and JID are in a position to encourage the publication of alternatives. Editorial policies should encourage publication of methodologies as supplemental information as part of the electronic journals. Most journals allow for and sometimes request supplemental information to be provided for publication on their websites. This simple measure could disseminate useful information, buried in laboratory notebooks that could significantly enhance the use of humane endpoints. This could also increase the interlaboratory comparability of data and possibly reduce unnecessary duplication of studies. As mentioned above it would also be useful for journals to invite articles, commentary and supplemental information specifically directed at animal models and efforts by researchers to develop humane endpoints. Such information could help to standardize animal models and the use of humane endpoints and possibly prevent the replication of studies and in doing so free up scarce resources for innovative work.

Going Forward

Animals will continue to be used for research and testing in infectious disease research until there are suitable replacement models. The need for new anti-infectives and prevention strategies to combat the increasing numbers of multi-drug resistant bacteria and newly emerging infectious diseases will drive the search for new animal models and in vitro methods as well. The current regulations governing the approval of new drugs and therapies include requirements for testing in animal models and are not likely to change. It is in this regulatory climate that we are faced with the challenge to ensure that animal studies are necessary and are as humane as possible. One small part of this is to continue to advocate for and find ways to make humane

endpoints the standard rather than the exception in research that involves disease models that create severe morbidity, unrelieved pain and distress, and ultimately death of the animal. Several people have suggested that there should be a fourth R, Responsibility, which should be shared by the research community, IACUCs and policy makers among others. Based on our findings a few strategies are proposed below.

There needs to be a public commitment within the infectious disease research community that humane endpoints should be the gold standard for disease models. This commitment should be one of practice not only of discourse. All research proposals should include a pilot study that is designed to provide evidence for the feasibility of humane endpoints as an alternative to a lethality study. As an adjunct to this commitment, there must also be the realization that there may be some cases where some studies cannot be scientifically or ethically justified and should be abandoned. Conversely there should be an open mind toward the possibility that some studies that produce unrelieved pain and distress will also produce a significant benefit and should be allowed to proceed. The infectious disease research community is not a monolith that can be easily persuaded to commit to such controversial ideas. The activist wing of the animal rights movement has served to solidify the positions of the pro-animal research propagandists and perhaps made them resistant to approaches that they perceive to threaten their autonomy with respect to their own research. The various professional societies and the journals that they sponsor are a likely place to encourage dialog on the benefits of humane endpoints both for the animal subjects and the research quality itself. From that perspective the professional societies should strive to bridge the gaps between the humane endpoint literature and the mainstream scientific literature. Evidence that supports measurable and positive effects of the use of humane or alternative endpoints on the precision and quality of research should be encouraged. There

may be such evidence buried in lab notebooks, or anecdotal evidence communicated at cocktail parties, but very little has been published. As in all things encouragement should be supported by incentive, supplemental grant support or financial awards should be established to fund development of alternatives and humane endpoints.

The animal use review panels should have full institutional support and autonomy. The IACUCs do not currently have the mandate to deny studies that are not conducted in a humane manner. There are several ways to accomplish this through individual institutional policy and through USDA or PHS policy revisions. The IACUCs could also benefit from better guidelines or training aimed at evaluating the value and justification of proposed studies.

Policy makers, funding agencies and the public also share the responsibility for the fate of research animals. As primary beneficiaries of animal experimentation in the form of new drugs and vaccines, the public should take the responsibility of being informed as to the types of studies that are required to approve new drugs. The public supports research in a general and abstract way but faced with the reality of the experiments they may feel differently. The finding that mice and rats bear the brunt of the lethal studies in infection research points to the possibility that this may be due to their omission from the definition of animal by the Animal Welfare Act. AWA protection for mice and rats has been proposed and rejected several times. There are millions of mice and rats used for research and their addition would represent many challenges to the regulators and the regulated. Their inclusion as animals worthy of protection under the Animal Welfare Act may be necessary to enhance the scrutiny that they receive when lethal studies are planned, reviewed and published.

The findings of this study and the suggestions described above can also be extrapolated to other fields where lethality studies are used. The modeling of disease in animals in fields such as

oncology and metabolic diseases often results in serious morbidity leading to death. Creative uses of new technologies, coupled with knowledge of the natural history and clinical manifestations of disease can be powerful tools to produce valuable scientific data without causing unnecessary pain, distress and death to the experimental animals. As suggested above, the shared responsibility and committed actions of the research community, the regulatory community, policy makers and the public at large can improve the welfare of experimental animals, including rats and mice.

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