Introduction

The obligation to minimize or eliminate unnecessary pain and distress animals may experience when they are used in biomedical research, teaching, and testing is clearly stated in the Canadian Council on Animal Care guidelines on choosing an appropriate endpoint in experiments using animals for research, teaching, and testing:

“In experiments involving animals, any actual or potential pain, distress, or discomfort should be minimized or alleviated by choosing the earliest endpoint that is compatible with the scientific objectives of the research. Selection of this endpoint should involve consultation with the laboratory animal veterinarian and the animal care committee” (CCAC 1998, p. 2).

Additional guidance is provided for animals used in infectious disease studies, as follows:

“For all infectious disease research, including virulence tests in animal models, endpoints should be established that minimize the potential for pain and/or distress in the animals” (CCAC 1998, p. 15).

Since the work of pioneers such as Jenner and Pasteur (Fenner et al. 1997) more than a century ago, animal models have provided essential information in the study of infectious diseases. Although much experimentation can now be accomplished with in vitro systems, animals continue to be necessary for this field for studying the process of inflammation, specific infectious diseases, and pharmacologic treatment of infectious diseases; vaccine development and efficacy and safety testing; virulence testing; and, for diagnostic purposes, in both human and veterinary medicine. The use of whole animal models, both natural and induced (including transgenic models), is considered important to the understanding of the very complex temporal relationships that occur in infectious disease involving the body, its neuroendocrine and immune systems, and the infectious organism.

Animals used in infectious disease research may experience significant pain and/or distress as part of the disease process. Seeking earlier, more humane endpoints can result in significant reductions in the potential pain and distress experienced by these animals. However, the use of earlier endpoints give rise to a valid scientific concern—An earlier endpoint should accurately predict the outcome of the experiment. The earlier endpoints should be based on scientifically valid values of variables, not just honest judgments based on an appreciation of the distress and pain the animal may be experiencing. For example, animals becoming sick in an infection challenge/antibiotic efficacy trial might be euthanized for humane reasons when in fact they could have survived the challenge, proving that a (new) antibiotic was an effective treatment. One earlier endpoint that has been validated for several infectious agents involves the use of decreased body temperatures as a predictor of impending death, which is discussed further below.

Biochemical changes that occur in response to infection are being investigated for their potential to serve as earlier, more humane endpoints in infectious disease studies. Research during the past decade has revealed a great deal about how the body responds to infection. Infectious organisms invading the body stimulate the immune system into rapid action, initiating (up-regulating) a complex array or cascade of activities that include biochemical, endocrinologic, physiologic, behavioral, and pathologic changes. This activation of immune system-mediated defense mechanisms is called the acute phase response. An overview of the acute phase response is presented in Figure 1. Such work is now providing clues to measurements that can be used to set earlier, more humane endpoints, before the animal’s welfare is seriously compromised by the infectious or inflammatory process being studied.

Many of the biochemical and endocrinologic changes that occur early in the process of the response to infection have been measured. Increases in serum levels of cytokines and acute phase proteins (APPs\(^1\)) occur early, before severe behavioral and physiologic changes. These biochemical changes can serve as indicators of the presence of infectious disease and as predictors of both the severity and the outcome of infectious disease. This approach has been used for other reasons in human medicine and veterinary medicine, and it appears to have application in selecting earlier, more humane endpoints in infectious disease animal models. We propose that measuring some of the species-typical major APPs can provide objective scientific information on the

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\(^1\)Abbreviations used in this paper: APP, acute phase proteins; Hp, haptoglobin.

Volume 41, Number 2 2000 99
Cytokines produce a number of rapidly occurring systemic effects if the infection becomes generalized or prolonged (Gregory 1998; Schijns and Horzinek 1997; van Deuren et al. 1992). In liver hepatocytes, the cytokines up-regulate production and release of a group of proteins known as acute phase proteins (Figure 1). In the brain and nervous system, the cytokines act to produce fever (probably through the prostaglandin system, changing the body temperature set point), lethargy/sleep, inappetence (Gregory 1998; Schijns and Horzinek 1997; van Deuren et al. 1992), and hyperalgesia (increased sensitivity to pain) (Dray 1995).

Sickness Behavior Associated with the Acute Phase Response

The behavioral effects produced by the cytokines acting in the brain (lethargy, sleep, inappetence/anorexia) have been termed “sickness behaviour” (Gregory 1998). As the infectious disease state progresses, there is increasing deviation from normal physiologic and behavioral states that may involve increasing pain and distress. These deviations from normal are evaluated through observations/checklists in an effort to establish endpoints that are predictive of the eventual experimental outcome. The experiment may then be terminated when the deviation from normal (behavior and physiology) exceeds a preset point (the endpoint).

The use of immunologically deficient or genetically modified rodents in infectious disease research presents additional problems. First, the signs of disease may be altered from the “normal” rodent. Preliminary or pilot studies will help define which observations need to be made to determine the endpoint. Second, the isolator housing necessary to maintain immunocompromised rodents makes it more difficult to observe them thoroughly and frequently. In these circumstances, implanted telemetry technology may enhance the ability to perform frequent observations without stressing the animals or subjecting them to increased risk of contamination.

Acute Phase Response—The Animal’s Bodily Response to Inflammation

The initial bodily response to foreign agents (e.g., bacteria, fungi, parasites, viruses) is usually local—at the site of infection—and involves neutrophils, macrophages, and other immune cells. The response includes the release of small proteins known as cytokines from macrophages and other neutrophils. Cytokines form a group of secreted regulatory proteins that orchestrate the development and regulation of the immune response. They act as intercellular messengers to coordinate cellular responses throughout the body as the immune system responds to infection (Godson et al. 1995, 1997; Gregory 1998; Schijns and Horzinek 1997; van Deuren et al. 1992). The cytokines interleukin-1, interleukin-6, and tumor necrosis factor have been studied the most extensively.
known that animals in a septic state lose the ability to maintain body temperature. A decrease in body temperature beyond a certain point (e.g., more than 4 to 6°C) has been correlated with death as an outcome in several infectious disease models. Studies of mice infected with bacteria (Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus epidermidis) indicated that a hypothermia of 34°C (about 4.5°C lower than normal) was predictive of eventual mortality (Soothill et al. 1992). The recommended endpoint for chronic infection of mice with systemic Candida albicans was the point when the body temperature decreased more than 4°C (Siems and Allen 1989). Body temperature less than 32°C (about 6.5°C less than normal) in mice infected with an influenza virus was predictive of mortality (Wong et al. 1997). Kort et al. (1998) reported that for mice infected with Klebsiella pneumoniae, a decrease in body temperature to less than 36°C was predictive of mortality and could be used as an endpoint that would not skew the results obtained.

Monitoring body temperature should therefore be part of monitoring any infectious disease animal model. The frequency of monitoring will depend on the progression of the infection and the time frame to expected severe signs. Monitoring body temperature in small laboratory animals can be accomplished without undue disturbance using laser directed infrared temperature scanners (Love et al. 1996), tympanic infrared thermometers (Love et al. 1996), or implanted thermistor microchips (Kort et al. 1998).

Weight Loss

As noted above, one effect of cytokines on animals is the production of inappetence or anorexia. Thus, the resulting weight loss is a cardinal indicator of the severity of infectious disease in animal models. Body weight can be monitored by a number of scoring systems, and we recommend using a predetermined amount of weight loss (e.g., 10 to 20% or 20%) as an endpoint (CCAC 1998; Morton and Townsend 1995; Workman et al. 1998). Prolonged inappetence can lead to cachexia. The total amount of the weight lost, as well as its duration and consistency, should be used to determine the endpoint for infectious disease animal models. Animals that are recumbent or have lost the ability to reach food and water will rapidly lose weight, and this observation should influence the chosen endpoint.

Other Behavioral and Physiologic Changes

Decreased activity (lethargy or sleep) is also an effect of the cytokines. This change in activity level and alertness should be monitored in infectious disease animal models. In addition to primary signs of infectious disease, there are many secondary signs that can be used to track deteriorating condition. For the common laboratory animal species, a number of publications are available that detail signs of pain and distress (Acred et al. 1994; Baumans et al. 1994; CCAC 1998; Flecknell 1994; Morton 1990, 1997a; Morton and Townsend 1995; NRC 1992; Wallace et al. 1990; Wolfensohn and Lloyd 1994). For common domestic species, the veterinary literature will be a valuable resource for information on the signs and symptoms of infectious diseases.

Detailed observational checklists for the purposes of selecting an earlier endpoint have contributed in a significant way to moving away from death or moribundity as the endpoint for animals (Morton 1990; Morton and Townsend 1995; Workman et al. 1998). The condition scoring checklist should be developed specifically for each infectious disease animal model because models can vary considerably in the number of observations required to determine the endpoint. Veterinary consultation will assist in establishing the list of observations that should be used to assess the animal’s condition. Examples of observational checklists for infectious disease animal models (Acred et al. 1994; CCAC 1998) demonstrate the extent to which detailed observations can be made and scored to find a more humane endpoint.

APPs as Diagnostic and Prognostic Indicators in Infectious Disease in Animals

Elevated serum cytokine levels are associated with infectious disease and its severity. However, the transient nature of elevated cytokine levels and the rapid production of binding or regulatory factors limit the value of using elevated levels to determine earlier endpoints in infectious disease animal models. Measuring the biologic consequences of cytokine production, such as increased levels of APPs, however, holds promise as a more practical method for setting earlier endpoints in infectious disease research.

In the normal healthy animal, APPs are either not detectable or occur at very low levels in the plasma. Plasma levels rise rapidly (within hours) after cytokine up-regulation of the hepatocytes in response to infection, inflammatory process, certain cancers, and trauma. Based on the magnitude of the rise in plasma APPs during the acute phase response, they are grouped as “major,” “moderate,” or “minor” (Table 1). Efforts have been directed primarily at measuring the major APP specific to the species of animal being studied.

Depending on the animal species, the serum profile of the APP varies and is related to the type of inflammatory response (Conner et al. 1989; Eckersall and Conner 1988). This variation occurs even in closely related species like the mouse and rat; the major APP in the mouse is serum amyloid A, whereas in the rat it is α2-macroglobulin. The specific APP to be measured depends on the species and the type of infection/inflammatory response being studied.

High levels of the major APP correlate well with the presence and severity of infectious disease. Thus, they can be used for diagnosis (the presence and extent of inflammatory lesions), for prognosis in infectious disease cases, for measuring the response to treatment, and as predictors of outcome in experimental situations. Research is needed in
Table 1 Some acute phase proteins (APPs) in various species

<table>
<thead>
<tr>
<th>Species</th>
<th>Major APP (10- to 1000-fold increase)</th>
<th>Moderate APP (2- to 4-fold increase)</th>
<th>Minor APP (about 50% increase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>SAA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Fb,&lt;sup&gt;b&lt;/sup&gt; AGP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CRP&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rat</td>
<td>AMG&lt;sup&gt;b&lt;/sup&gt;</td>
<td>AGP</td>
<td>CRP</td>
</tr>
<tr>
<td>Dog</td>
<td>SAA, CRP</td>
<td>Hp, AGP</td>
<td></td>
</tr>
<tr>
<td>Pig</td>
<td>CRP</td>
<td>Hp</td>
<td></td>
</tr>
<tr>
<td>Cow</td>
<td>Hp, SAA</td>
<td>AGP, AP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Fb, AMG</td>
</tr>
<tr>
<td>Horse</td>
<td>SAA</td>
<td>Hp, AGP</td>
<td>CRP</td>
</tr>
<tr>
<td>Human</td>
<td>SAA, CRP</td>
<td></td>
<td>Fb, Cp</td>
</tr>
</tbody>
</table>


<sup>b</sup>AGP, α, acid glycoprotein; AMG, α<sub>2</sub>-macroglobulin; AP, α, antiproteinase; Cp, ceruloplasmin; CRP, C-reactive protein; Fb, Fibrinogen; Hp, haptoglobin; SAA, serum amyloid A.

several areas for the use of APP measurements to become a practical means of setting more humane endpoints in the rodent models of infectious disease. In other words, a specific level of APP beyond which the animal never recovers is needed. Such research is necessary to clarify the nature and extent of the acute phase response in rodent models for specific infectious agents. Standardized, rapid tests for APPs using small volumes of blood or serum will also be necessary.

Haptoglobin in Cattle Infectious Diseases

The study of haptoglobin (Hp<sup>1</sup>) levels in cattle serves as an excellent example of how an acute phase protein useful for diagnostic, prognostic, and treatment evaluation purposes can also serve as an early indicator suitable for determining an earlier, more humane endpoint in an infectious disease model. Hp has been associated with experimental inflammation in cattle (endotoxin administration, Conner et al. 1989; turpentine injection, Conner et al. 1988) and many clinical diseases (e.g., Conner et al. 1989; Godson et al. 1996; Hirvonen et al. 1996; Hofner et al. 1994; Horadagoda et al. 1994; Salonen et al. 1996). Hp levels also increase after surgery (Makimura and Suzuki, 1982, Morimatsu et al. 1992) and after certain stressful conditions such as transport (Murata and Miyamoto 1993). Hp has very low values in normal healthy cattle (McNair et al. 1995). The increase in Hp after infection can be on the order of 1000-fold and is associated with the severity of the insult; however, there is a range of response in individual animals given a specific challenge.

Studies on a bovine respiratory disease model in weaned beef calves indicated that elevations in Hp levels, which correlated with the subjective clinical sickness score, elevated body temperature, and weight change could be used to discriminate between clinical outcomes (Table 2; Godson et al. 1996). The clinical sickness score was determined using a clinical observational check list developed specifically for this model. A version of the clinical observational check list was published in the appendices to the CCAC endpoints guidelines (CCAC 1998). Hp levels began increasing 24 to 48 hr after the calves were exposed to the bacterial agent Pasteurella haemolytica and were significantly higher by 72 hr in calves that were euthanized (or died) (Figure 2; Godson et al. 1996). Thus Hp levels served as an early objective indicator of disease severity and could be included in the criteria for making a decision to euthanize the animal. Confidence in the decision may be improved by including measurement of additional acute phase reactants. For instance, levels of blood trace minerals such as copper, zinc, and iron also change during the acute phase response. In the experiment described above, zinc levels, which the authors measured, decreased during the same 24- to 48-hr period as Hp levels began increasing. Survivors and nonsurvivors were more clearly differentiated by this combination of Hp and zinc measurements (Figure 2).

Methods for measuring serum levels of Hp and other APPs have been developed and include the hemoglobin binding assay (for Hp) (Makimura and Suzuki, 1982), radial immunodiffusion assay (Morimatsu et al. 1992), fluorometric competitive immunoassay (McNair et al. 1995), and enzyme-linked immunosorbent assay (Godson et al. 1996; Wagner et al. 1996). Depending on the assay, the volume of serum needed will be small (perhaps less than 50 μl) and the results may be obtained within hours: 4 to 6 hr for enzyme-linked immunosorbent assay; 24 hr for radial immunodiffusion. We are aware of one company (Cardiotech Services Inc., Louisville, Kentucky; http://www.equineinfo.com/cardiotech/) that sells an APP test (for α, acid glycoprotein) for several species including rats and mice. Each radial immunodiffusion test costs about $5 to $7 and requires 24 hr to...
### Table 2 Association of haptoglobin concentration with other measures of disease severity

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Alive</th>
<th></th>
<th>Dead</th>
<th>Correlation with haptoglobin&lt;sup&gt;c&lt;/sup&gt; r²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>95% CI&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Mean</td>
<td>95% CI&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Haptoglobin&lt;sup&gt;e&lt;/sup&gt; (µg ml&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>661</td>
<td>(414-908)</td>
<td>2812</td>
<td>(1579-4045)</td>
</tr>
<tr>
<td>Sick score</td>
<td>1.17</td>
<td>(0.9-1.4)</td>
<td>3.1</td>
<td>(2.8-3.4)</td>
</tr>
<tr>
<td>Temperature&lt;sup&gt;f&lt;/sup&gt; (°C)</td>
<td>40.7</td>
<td>(40.5-41.0)</td>
<td>41.5</td>
<td>(41.4-41.6)</td>
</tr>
<tr>
<td>Weight change&lt;sup&gt;g&lt;/sup&gt; (kg)</td>
<td>3.4</td>
<td>(0.7-6.2)</td>
<td>12.8</td>
<td>(16.3-9.2)</td>
</tr>
<tr>
<td>Zinc&lt;sup&gt;h&lt;/sup&gt; (ppm&lt;sup&gt;i&lt;/sup&gt;)</td>
<td>0.57</td>
<td>(0.50-0.63)</td>
<td>0.19</td>
<td>(0.16-0.22)</td>
</tr>
</tbody>
</table>


<sup>b</sup> Forty-nine calves were challenged with BHV-1 on day 0 and with *Pasteurella haemolytica* on day 4. The data were grouped according to whether the animal survived the challenge. Ten animals died in the trial. Results are expressed as the mean and 95% confidence interval for each parameter. All measures of disease severity were significantly different between the groups (P< 0.001).

<sup>c</sup> All correlations were significant (P< 0.001).

<sup>d</sup> CI, confidence interval; ppm, parts per million.

<sup>e</sup> Day 8 serum haptoglobin concentration determined by enzyme-linked immunosorbent assay.

<sup>f</sup> Maximum sick score after *P. haemolytica* challenge: 0, healthy; 4, severe disease.

<sup>g</sup> Maximum rectal temperature after *P. haemolytica* challenge.

<sup>h</sup> Difference in weight from the start of the trial to its completion on day 11.

<sup>i</sup> Day 8 plasma zinc concentration determined by atomic absorption spectroscopy.

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The process of establishing more humane endpoints in infectious disease research must involve the scientist, laboratory animal veterinarian, institutional animal care and use committee, and technical staff (CCAC 1998; Olfert 1995). In any research program in which an earlier endpoint is desirable for welfare reasons, agreeing on the point that minimizes the laboratory animal’s pain and/or distress while meeting the scientific needs for objective evaluation will often require much consultation between the researcher, veterinary staff, and animal care committee. The recently published CCAC endpoints guidelines (CCAC 1998), developed to assist animal care committees and investigators with that process, will be useful to all involved with establishing endpoints in infectious disease animal models.

### Figure 2


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Develop the volume of serum needed is small (e.g., 50 µl). Development of rapid tests for APPs, similar in format to the home pregnancy test, has been initiated.

### Responsibility for Establishing Humane Endpoints

The process of establishing more humane endpoints in infectious disease research must involve the scientist, laboratory animal veterinarian, institutional animal care and use committee, and technical staff (CCAC 1998; Olfert 1995). In any research program in which an earlier endpoint is desirable for welfare reasons, agreeing on the point that minimizes the laboratory animal’s pain and/or distress while meeting the scientific needs for objective evaluation will often require much consultation between the researcher, veterinary staff, and animal care committee. The recently published CCAC endpoints guidelines (CCAC 1998), developed to assist animal care committees and investigators with that process, will be useful to all involved with establishing endpoints in infectious disease animal models.
References


CCAC [Canadian Council on Animal Care]. 1998. CCAC guidelines on: Choosing an appropriate endpoint in experiments using animals for research, teaching, and testing. Canadian Council on Animal Care, Ottawa, Canada.


