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A COMPARATIVE STUDY OF THE EFFECTS OF LIPOPOLYSACCHARIDE AND POLY I:C IN RATS

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A dissertation submitted to the Faculty of Science, University of the Witwatersrand, in fulfillment of the requirements for the degree of Master of Science

Johannesburg, 2006

DECLARATION

I declare that this dissertation is the product of my own, unaided work. It is being submitted for the degree of Master in Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Candidate signature _____

Shaheen Fajandar

_____ Day of _____ 2006

ABSTRACT

The acute phase response induced by bacterial pyrogens, but not by viral pyrogens, has been thoroughly investigated. Polyinosinic:polycytidylic acid (poly I:C) is a synthetic viral pyrogen that is used to simulate viral infection. This dissertation describes how I determined an effective peripheral route and dose of administration of poly I:C to rats. Thereafter I investigated whether poly I:C induced sickness behaviour, and whether repeated administration of poly I:C resulted in the development of tolerance. Intraperitoneal administration of at least 1000µg/kg poly I:C induced fevers in rats, but not sickness behaviour. Unlike repeated administration of LPS, repeated administration of poly I:C in rats did not result in the development of tolerance.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

ANOVA - analysis of variance

APR - acute phase response

CNS - central nervous system

COX - cyclooxygenase

dsRNA - double stranded ribonucleic acid

HPA - hypothalamic-pituitary-adrenal

IFNs - interferons

IL-1 - interleukin 1

IL-1ra - interleukin 1 receptor antagonist

IL-6 - interleukin 6

LPS - lipopolysaccharide

MDP - muramyl dipeptide

NSAIDs - non-steroidal anti-inflammatory drugs

Poly I:C - polyinosinic:polycytidylic acid

PG - prostaglandin

TRI - thermal response index

TLR - toll-like receptors

TNF-α - tumour necrosis factor-alpha

1. INTRODUCTION

Fever is one of the most common symptoms observed in an animal which has been infected with a pathogenic micro-organism, whether bacterial, viral or protozoal in origin (Chuang *et al.*: 1990, Mitchell & Laburn: 1997, Luker *et al.*: 2000, Cartmell *et al.*: 2002, Engeland *et al.*: 2003). In addition to an increase in body temperature, there are a variety of other brain-mediated, behavioural changes, collectively referred to as sickness behaviour (Bluthe *et al.*: 1992, Mitchell & Laburn: 1997, Engeland *et al.*: 2003, Dantzer: 2004), which accompany fever. Sickness behaviour includes malaise, hypoactivity, fatigue, hypophagia, an increase in slow wave sleep, a decrease in sexual behaviour, anhedonia, hyperalgesia and activation of the hypothalamic-pituitary-adrenal (HPA) axis (Bluthe *et al.*: 2000, Bluthe *et al.*: 2001, Dantzer: 2004, Vollmer-Conna *et al.*: 2004). Fever combined with these behavioural changes is known as the acute phase response (APR) (Mitchell & Laburn: 1997). The components of the APR are not specific to any particular sex (Engeland *et al.*: 2003) or animal species, but are seen in humans and a variety of animals (Hart: 1988a) including invertebrates (LeGrand: 2000).

1.1 Mediators of the Acute Phase Response

The administration of an exogenous pyrogen results in a cascade of events, beginning with the activation of the host's peripheral phagocytic cells, which include blood monocytes, tissue macrophages and a variety of other cells. These immune-competent cells produce and secrete a family of polypeptides called cytokines, which themselves

are potent mediators of the acute phase response and are also referred to as endogenous pyrogens (Kluger *et al.*: 1995, Mitchell & Laburn: 1997, Engeland *et al.*: 2003).

The important cytokines mediating the acute phase response induced by a bacterial product are interleukin-1 (IL-1α and β), interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF- α) (Kluger *et al.*: 1995, Luheshi: 1998, Swiergiel & Dunn: 1999, Bluthe *et al.*: 2000, Engeland *et al.*: 2003, Conti *et al.*: 2004, Dantzer: 2004). Cytokines regulate the release of other cytokines causing a hierarchical response (Swiergiel & Dunn: 1999). For example, *in vitro* studies have shown that administration of the bacterial pyrogen, lipopolysaccharide (LPS) in experimental animals results in the release of TNF-α and IL-1β which induce the secretion of each other and both induce the secretion of IL-6. IL-6 in turn, however, down-regulates the secretion of TNF-α and IL-1β (Swiergiel & Dunn: 1999, Blatteis: 2004). The APR is centrally mediated; specific cytokine receptors exist throughout the brain (Engeland *et al.*: 2003) and central administration of cytokine antagonists, such as the IL-1 receptor antagonist (IL-1ra) in rats, attenuate many of the components of the APR (Bluthe *et al.*: 1992, Dantzer: 2004).

The endogenous pyrogens elicit their effects by catalyzing the formation of prostaglandins (PGs) in the central nervous system (CNS) (Roth & de Souza: 2001). The cyclooxygenase (COX) enzyme system is critically important in the formation of

PGs (Roth *et al.*: 2002, Botting: 2006), as these enzymes initiate the first step in the transformation of arachidonic acid to prostanoids (Lefkowith: 1999, Botting: 2006). The principal action of antipyretic and non-steroidal anti-inflammatory drugs (NSAIDs) therefore rests in their ability to inhibit the enzyme COX and interrupt the synthesis of inflammatory PGs, thereby attenuating the APR (Ziel & Krupp: 1975, Aronoff & Neilson: 2001, Dantzer: 2004, Botting: 2006). PGs of the E_2 series are believed to be the central and probably the key mediator of fever and sickness behaviour (Roth & de Souza: 2001, Dantzer: 2004). The theory that PGE_2 is the central and key mediator of the APR stems from studies reporting that microinjections of PGE_2 administered centrally in experimental animals, almost immediately results in a monophasic fever (Ziel & Krupp: 1975, Abul *et al.*: 1997, Blatteis: 2004) which can be inhibited with the administration of NSAIDs (Ziel & Krupp: 1975, Lefkowith: 1999, Blatteis: 2004).

1.2 Fever and Sickness Behaviour

Fever is a component of the APR and is defined as a rise in core body temperature induced by a controlled increase in the so-called temperature "set-point" due to changes in the neuronal activity in the preoptic area of the hypothalamus (Conti *et al.*: 2004). The magnitude and duration of the fever is dependent on the nature of the pyrogen, the route of administration and the dose of the pyrogen (Cartmell *et al.*: 2002), as well as the species and strain of the experimental animals used (Kimura *et*

al.: 1994, Romanovsky *et al.*: 1996, Plata-Salaman *et al.*: 1998, Kamerman & Fuller: 2000, Grinevich *et al.*: 2001).

The febrile response is one of the best examples of the communication between the immune system and the brain (Kluger *et al.*: 1995, Luheshi: 1998, Dantzer: 2004). The immune system functions as a sensory organ relaying information to the brain and other parts of the body via signals such as the pro-inflammatory cytokines (Kent *et al.*: 1992, Dantzer: 2004). The raised body temperature reached during fever in turn stimulates immune responses (Dinarello & Wolff: 1982, Kent *et al.*: 1992) and decreases the proliferation of pathogens which are thermo-sensitive (Kent *et al.*: 1992).

Previously, sickness behaviour was thought to be the result of the incapacitating process that occurs during infection (Kent *et al.*: 1992, Konsman *et al.*: 2002). Evidence now indicates that the behavioural changes observed during infection are not maladaptive but are a crucial part of the natural homeostatic reaction that the body uses to fight infection (Hart: 1988b, Kent *et al.*: 1992, Konsman *et al.*: 2002).

The most extensively used model to study the APR and to induce reproducible febrile responses has been to inject healthy laboratory animals with exogenous pyrogens, particularly bacterial pyrogens.

1.3 Bacterial Pyrogens

The most commonly used bacterial product is lipopolysaccharide (LPS), the pyrogenic moiety of Gram-negative bacteria (Plata-Salaman *et al.*: 1998, Cartmell *et al.*: 2002, Engeland *et al.*: 2003). LPS forms the major part of the outer cell wall of Gram-negative bacteria that is released after bacteriolysis. Another bacterial product is muramyl dipeptide (MDP), the pyrogenic moiety of Gram-positive bacteria (Engeland *et al.*: 2003). During phagocytosis other bacterial cell wall constituents which are abundant in Gram-positive bacteria are released after bacteriolysis. MDP is the minimal structure from Gram-positive bacteria having immune-stimulating properties (Engeland *et al.*: 2003).

Although Gram-positive and Gram-negative bacteria share some common mechanisms of immuno-competent cell activation, as CD-14 receptors on circulating phagocytes are capable of recognizing both LPS and MDP (Engeland *et al.*: 2003), there are quite distinct differences in the effects induced by these two pyrogens. It is well documented that there are differences in the pattern of fevers induced by Gram-negative and Gram-positive bacteria, for example, administration of Gram-negative bacteria can result either in a biphasic fever or monophasic fever under different conditions in laboratory animals, whereas administration Gram-positive bacteria results in a monophasic fever response (Romanovsky *et al.*: 1996, Condrad *et al.*: 1997, Cartmell *et al.*: 2002, Engeland *et al.*: 2003). Gram-positive and Gram–negative bacteria also induce different molecular and behavioural effects in the brain

(Plata-Salaman *et al.*: 1998). For example, LPS is more potent than MDP in inducing hypophagia and in up-regulating TNF-α and IL–1β mRNAs in certain regions of the brain (Plata-Salaman *et al.*: 1998). MDP on the other hand, is more potent in up-regulating the expression of the anti-inflammatory cytokine, IL-1ra, mRNAs centrally (Plata-Salaman *et al.*: 1998).

A further characteristic of LPS which is not attributed to MDP, is that repeated administration of LPS in animals results in an attenuation of the febrile effects after as little as one injection (O'Reilly *et al.*: 1988, Roth *et al.*: 1997), a phenomenon referred to as endotoxin tolerance. In contrast to repeated administration of LPS, repeated administration of the Gram-positive pyrogen, MDP, does not readily result in the development of tolerance to the febrile effects of this pyrogen (Soszynski *et al.*: 1991, Roth *et al.*: 1997, Zeisberger & Roth: 1998). Thus there appear to be differences in the characteristics of the acute phase response induced by Gram-negative and Gram-positive bacterial products.

Another class of exogenous pyrogens, whose effects have received limited research, are those of viral origin.

1.4 Viral Pyrogens

While the mechanisms mediating fever and sickness behaviour in response to a bacterial challenge, particularly in response to LPS administration, have been thoroughly investigated and reported on (Hart: 1988b, Kluger *et al.*: 1995, Mitchell & Laburn: 1997, Engeland *et al.*: 2003, Conti *et al.*: 2004), the mechanisms mediating virally-induced infection have yet to be fully elucidated. If the pyrogenic effects of different bacterial pyrogens differ, it is reasonable to speculate that fevers induced by a viral pyrogen would differ from fevers induced by a bacterial pyrogen.

Much of our knowledge of the interaction between viruses and cells has been obtained through studies with various models for viral infection. A candidate substance for inducing the pyrogenic and behavioural effects to many viral infections is double-stranded ribonucleic acid (dsRNA), which is generated by most viruses as a result of viral replication (Jacobs & Langland: 1996). The polyinosinic:polycytidylic acid copolymer (poly I:C) is a synthetic viral dsRNA and has been administered to laboratory animals, primarily rabbits, as a model to simulate virally-induced infections (Lindsay *et al.*: 1969, Homan *et al.*: 1972, Won & Lin: 1988, Kimura-Takeuchi *et al.*: 1992, Katafuchi *et al.*: 2003). Poly I or poly C, administered as individual polymers to rabbits does not result in a febrile response, indicating a synergy between these two polymers (Lindsay *et al.*: 1969).

As with bacterially-induced infection, the induction of a viral infection results in a strong host-response (Mogensen & Paludan: 2001). The interaction between viral surface proteins and specific cellular receptors initiates a cellular reaction causing the production and release of cytokines (Mogensen & Paludan: 2001). The effect can be simulated by the administration of poly I:C which induces the production of cytokines by macrophages and the activation of macrophages, via the toll-like receptor TLR-3 (Alexopoulou *et al.*: 2001, Pruett *et al.*: 2003, Fortier *et al.*: 2004).

Exposure to viruses induces a similar cytokine cascade as bacterially-induced infection (Majde: 2000), but the primary cytokines involved in viral infections are the interferons (IFNs). Thus poly I:C is referred to as an interferon-inducer (Won & Lin: 1988, Davidson *et al.*: 2001). IFNs mediate immune reactions and cause central nervous system-mediated effects, such as fever, by activating PGs (Won & Lin: 1988). Therefore, similar to a bacterial infection, the effects of a viral infection, such as fever, are also dependent on the crucial role played by cytokines and prostaglandins.

There remains a lot of controversy in the exact mechanisms by which poly I:C mediates its effects. A study performed on rabbits showed that the APR induced after administration of viral pyrogens, comparable to that after administration of bacterial pyrogens, causes PGE_2 to enter the brain from the peripheral circulation resulting in the fever response (Davidson *et al.*: 2001). Poly I:C-induced production of PGE_2 was

confirmed by studies using anti-inflammatory drugs, such as ketoprofen, which resulted in an attenuation in the febrile response induced by poly I:C (Ziel & Krupp: 1975, Rotondo *et al.*: 1987, Won *et al.*: 1991, Aronoff & Neilson: 2001). Chuang *et al.* (1990) showed with the use of a somatostatin antagonist (SS-14) that a somatostatinergic pathway in the rat hypothalamus may mediate the fever induced by interferon or its inducer poly I:C. Another study reported that poly I:C may act to induce fever through the endogenous release of noradrenalin from the rat hypothalamus (Liu *et al.*: 1989).

Poly I:C- and LPS-induced infections differ somewhat in the APRs they induce, for example, although the sensitivity of rabbits to these two stimuli is similar in terms of pyrogencity when administered intravenously, the profile of the febrile response differs, with LPS-induced fevers peaking after three hours and diminishing by six hours, while administration of poly I:C results in a more extended fever response lasting longer than six hours (Kimura *et al.*: 1994). A possible reason for the differences in the APRs induced by LPS and poly I:C in laboratory animals is the different cytokine profiles that are involved, such as the fact that administration of LPS does not cause an increase in IFN-α, whereas administration of poly I:C does (Kimura *et al.*: 1994). Another difference in the APR induced by LPS and poly I:C is that LPS induced anorexia has been shown to be IL-1 and PG dependent (Swiergiel & Dunn: 1999) whereas poly I:C-induced anorexia, is not IL-1 dependent (Fortier *et al.*: 2004).

1.5 Study Objectives

Our knowledge of the molecular mechanisms mediating viral infection has grown considerably over the years, but there are still many important unanswered questions. Differences in APRs accompanying these two classes of infection, specifically regarding the pyrogenic and behavioural effects, have received limited study. The high prevalence of viral infection in humans underlines the importance of better understanding the mechanisms mediating virally-induced infection.

The objectives of this study were to compare different aspects of bacterially-induced infection to a virally-induced infection. Before I could proceed with my objectives however, I firstly had to determine an effective dose and route of administration of poly I:C to induce fevers in rats, a species seldom used to study the effects of poly I:C. My first objective was to compare the body temperature responses induced by a viral (poly I:C) and bacterial (LPS) pyrogen as well as an aspect of sickness behaviour, namely the suppression in cage activity. The next objective was to determine whether, similar to repeated administration of LPS, repeated night-time administration of poly I:C results in the development of tolerance to the respective pyrogen's thermal and behavioural effects.

2. GENERAL METHODS

2.1 Animals

Adult male Sprague-Dawley rats with an initial body mass of 180-220g were used in the experiments. The animals were individually housed in perspex cages in a temperature controlled environment (22°C ± 1°C). The rats were kept on a 12:12h light:dark cycle (lights on at 07:00). Standard rat food pellets (Epol, Johannesburg, South Africa) and water were available *ad libitum* throughout the experimental period. Cages were cleaned every four days and at the same time the animals were weighed. All procedures and protocols were approved by the Animal Ethics and Screening Committee of the University of the Witwatersrand (AESC 2004/35/4, 2004/78/4, 2004/79/4).

2.2 Measurement of Body Temperature

Seven days before the start of each experiment, all rats had sterile, wax-coated temperature-sensitive radiotelemeters (DataScience, St Paul, USA) implanted into their peritoneal cavities for the continuous measurement of body temperature. The surgical procedures were performed under anaesthesia using ketamine hydrochloride (Anaket-V, Bayer, SA, 80mg/kg) and xylazine (Chanazine, Bayer, SA, 4mg/kg). Before surgery, telemeters were calibrated in a water bath against a precision quartz-crystal thermometer (Quat 100, Heraeus, Germany), such that abdominal temperature could be measured to an accuracy of 0.1^{o}C. After implantation, the frequency emitted by each telemeter was monitored by a receiver plate (RTA 500 Mini Mitter, USA)

which was placed under each rat's cage and fed into a peripheral processor (Datacol-3 Automated Data Acquisition System, Mini Mitter) connected to a personal computer. The output was expressed in degrees centigrade and body temperature recordings were made at 5 min intervals.

2.3 Measurement of Cage Activity

The Data Acquisition System also allowed us to continuously monitor cage activity of the rats based on the detection of the movement of the telemeter over the receiver plate, which was the size of the cage. Activity counts were accumulated over 5 min intervals and were consolidated to match the temperature data.

2.4 Pyrogenic Agents

Poly I:C (Lot No 033K4011 and 074K4051, Sigma, USA) was prepared by dissolving the pyrogen in sterile, pyrogen-free saline (0.9%NaCl). Three doses of poly I:C were prepared (500µg/kg, 1000µg/kg and 2000µg/kg) and administered at a final volume of approximately 0.3ml. For any particular study the same batch of poly I:C was used throughout that experimental period.

LPS (from *E. coli,* serotype 0111:B4, Sigma, USA) was prepared by dissolving the pyrogen in sterile pyrogen-free saline. One dose of LPS was prepared (100µg/kg), and administered intramuscularly at a final volume of approximately 0.3ml. The

intramuscular route of injection and dose chosen of LPS have previously been used to induce fevers in rats (Kamerman & Fuller: 2000, Kamerman *et al.*: 2002). A study performed in rabbits showed that the sensitivity to intraperitoneal administration of LPS is much lower compared to intramuscular administration (Cartmell *et al.*: 2002). All animals which served as controls were injected with sterile saline.

2.5 Data Analysis

Data are expressed as mean (±SD). The body temperature for each group of rats was averaged at 15 min intervals and consolidated to represent a 24h period from 07:00 to 07:00. One-way analysis of variance (ANOVA) followed by the Tukèy *post hoc* test was used to detect significant differences between the experimental groups (receiving pyrogen) and the control groups (receiving saline). Mean body temperatures of each group were calculated and compared. Differences in mean body temperature compared to saline treated groups were also calculated and compared. To obtain thermal response indices (TRIs), I calculated differences in the responses of each rat by calculating the area under the temperature curve vs. time. The TRI is a useful measure of temperature changes as it takes into account not only the magnitude of the temperature rise but also the duration.

The cage activity of each group of rats was averaged for 12 hours from lights on (07:00) to lights off (19:00) and expressed as a mean 12h day-time activity. Mean 12h night-time activity was calculated from lights off (19:00) to lights on (07:00).

One-way ANOVA followed by the Tukèy *post hoc* tests was used to detect significant differences between the experimental groups and the control groups for the day-time and night-time activity. P values of < 0.05 were considered significant.

3. AN EFFECTIVE DOSE AND ROUTE OF POLY I:C ADMINISTRATION IN RATS

3.1 Introduction

Administration of exogenous pyrogens in laboratory animals at different doses via different routes of administration results in fevers with differing profiles (Cartmell *et al.*: 2002). Often an effective pyrogen is rendered ineffective when administered at an inappropriate dose or route (Cartmell *et al.*: 2002). The most commonly used exogenous pyrogen to induce symptoms of infection is LPS, the pyrogenic moiety from Gram-negative bacteria. The mechanisms mediating the effects induced by Gram-negative bacteria have been thoroughly investigated by administering LPS at various doses and routes of administration (Cartmell *et al.*: 2002) and in a variety of animal species and strains, with rats being the most extensively used (Roth *et al.*: 1994, Tripp *et al.*: 1998, Kamerman & Fuller: 2000, Luker *et al.*: 2000, Cartmell *et al.*: 2002). The same, however, is not true for viral pyrogens such as poly I:C. Studies investigating the effects of poly I:C and the mechanisms mediating virally induced infection have been primarily performed on rabbits (Lindsay *et al.*: 1969, Lin: 1981, Won *et al.*: 1991, Kimura *et al.*: 1994), or via a central route of delivery in rats (Liu *et al.*: 1989, Chuang *et al.*: 1990).

There exists a species variation in the pyrogenicity of poly I:C when administered via different routes. Lindsay *et al.* (1969) reported that poly I:C is highly pyrogenic in rabbits, resulting a 0.8^{o}C increase in body temperature, after intravenous

administration at a dose of only 0.5µg/kg. Whereas, another study reported that poly I:C administered intravenously at a dose of 0.25µg/kg results in an increase in body temperature of 1.2^{o}C (Kimura *et al.*: 1994). Intranasal administration of poly I:C at a dose of 2000µg/kg to rabbits also resulted in a fever with a 1.2^{o}C increase in body temperature (Lindsay *et al.*: 1969). Microinjections of poly I:C into the anterior hypothalamic area produces fevers in rabbits (Liu *et al.*: 1989). Intracerebroventricular injections of poly I:C at a dose of 1ng does not elicit an increase in core body temperature whereas a dose of 10ng and 100ng result in a 0.7^{o}C and 1^{o}C increase in temperature respectively (Kimura *et al.*: 1994).

In guinea pigs, intravenous administration of poly I:C at a dose of 30µg/kg results in a 0.6^{o}C increase in body temperature whereas injections given intraperitoneally required a dose of 50µg/kg to evoke a similar response (Li *et al.*: 2000). A dose of 800µg/kg is required to result in a 1.2^{o}C increase in body temperature when administered intramuscularly (Lindsay *et al.*: 1969).

In rats intrahypothalamic injections of poly I:C also cause fever (Liu *et al.*: 1989, Chuang *et al.*: 1990), as does intravenous administration but with intravenous administration there is a longer latency period compared to intrahypothalamic administration (Liu *et al.*: 1989).

The aim of this study was to determine an effective peripheral route and dose of poly I:C administration to consistently induce fevers in rats. Poly I:C was administered to rats at three different doses via three routes of delivery i.e. the subcutaneous-, intramuscular- and intraperitoneal routes. Another objective was to determine the effects of peripheral administration of poly I:C on cage activity; the suppression in voluntary activity is a sign of the presence of sickness behaviour (Engeland *et al.*: 2003). The effects of injection of poly I:C on aspects of sickness behaviour have been investigated but to my knowledge, not fully understood.

3.2 Experimental Protocol

After recovery from telemeter surgery, rats were randomly assigned to three experimental groups (n=18/group) and received a single injection of either sterile saline, 500µg/kg poly I:C or 1000µg/kg poly I:C via one of the following routes: intraperitoneal, intramuscular or subcutaneous administration. Intraperitoneal injections were given in the abdominal cavity avoiding the bladder, intramuscular injections were given in the muscle mass of the upper thigh of the hind leg and the subcutaneous injections were given dorsally between the scapulae into the nape of the neck. Injections were given on one or other side of the body. All injections were given at 09:00 and body temperature and cage activity were monitored for four days before- and three days after the poly I:C or saline injections.

Poly I:C administered at a dose of 1000µg/kg and by the intraperitoneal route was the only dose/route combination which proved successful in causing a significant increase in body temperature (see Figure 1), but with inconsistent results. Therefore, in a separate group of animals a dose of 2000µg/kg was administered intraperitoneally in an attempt to obtain more consistent fevers. The additional group consisted of seven rats which received a single injection of 2000µg/kg poly I:C intraperitoneally. Poly I:C administered subcutaneously and intramuscularly at doses of 500µg/kg and 1000µg/kg was ineffective in causing as increase in body temperature, therefore higher doses were not pursued via these routes.

3.2.1 Data analysis

The mean body temperatures of rats receiving poly I:C at a dose of either 500µg/kg, 1000µg/kg, via subcutaneous and intramuscular routes and at doses of 500µg/kg, 1000µg/kg, and 2000µg/kg via the intraperitoneal route of administration, were compared to rats receiving sterile saline via the particular route of administration. Thermal response indices (TRIs, ^{o}C.h) were calculated for the six-hour time period after the start of the fever, for all rats receiving poly I:C via one of the three routes of administration and compared to the respective rats receiving saline. Mean 12h day-time and 12h night-time activity counts of rats receiving poly I:C via one of the three routes of administration were calculated and compared to the rats receiving sterile saline via the particular route of administration. Statistically significant differences in body temperature and cage activity between the groups were determined using one-

way ANOVA followed by the Tukèy *post hoc* test for multiple comparisons. P values of < 0.05 were considered statistically significant.

3.3 Results

Body temperature

There was no increase in the mean body temperatures of the rats receiving poly I:C subcutaneously or intramuscularly at either dose of 500μg/kg and 1000μg/kg, when compared to the rats receiving sterile saline (Figure 1 A and B). There was a significant increase in the mean body temperatures of rats receiving poly I:C intraperitoneally at a dose of 1000μg/kg and 2000μg/kg when compared to the rats receiving sterile saline ($P<0.05$) (Figure 1C). The rats' body temperatures started to rise approximately 2h after poly I:C administration and the fevers peaked approximately 4h after the injections. The average maximum increase in body temperature after poly I:C administration at a dose of 1000μg/kg and 2000μg/kg was $1^{o}C$ and $1.5^{o}C$ respectively, with the fevers lasting ± 6 and 7 h respectively.

All the rats receiving either pyrogen or sterile saline injections showed a transient rise in body temperature which lasted for approximately 30min immediately after the injection (Figure 1). This short-lived increase in body temperature was ascribed to the stress hyperthermia associated with handling during injections which is well documented (Long & Satinoff: 1990, Cabanac & Briese: 1992).

The body temperatures of all the rats returned to pre-treatment levels during the dark-phase after the poly I:C or sterile saline administration except for the rats receiving 2000µg/kg which showed a second rise in body temperature during the dark-phase lasting approximately 12h (Figure 1C).

Cage Activity

Figure 2 shows that there was no effect, either for day-time- or night-time activity in rats receiving poly I:C at either 500µg/kg or 1000µg/kg via subcutaneous and intramuscular routes or at either 500µg/kg, 1000µg/kg or 2000µg/kg via the intraperitoneal route, when compared to rats receiving sterile saline.

Figure 1

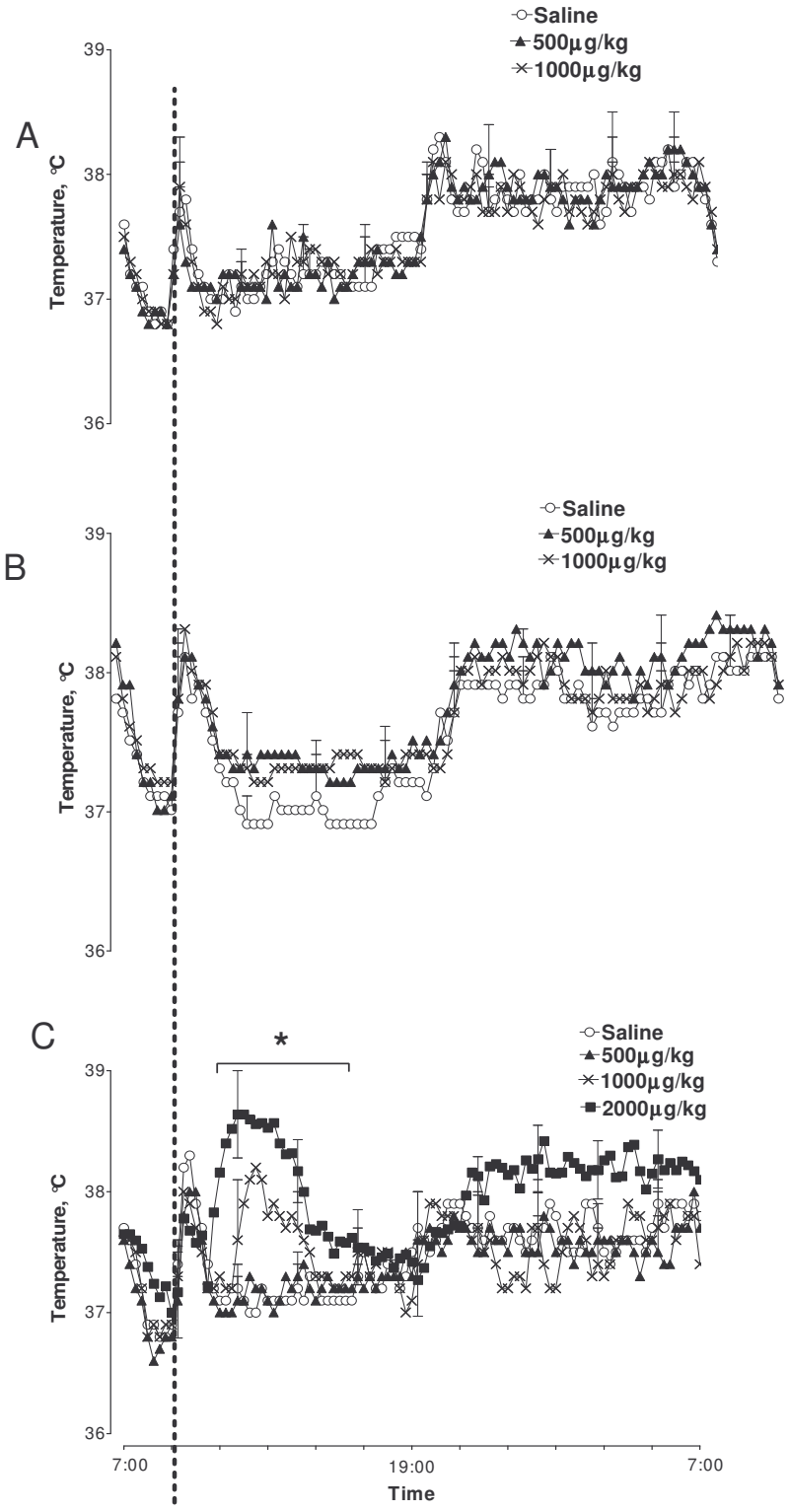

A
Saline
500µg/kg
1000µg/kg
Temperature, ℃
39
38
37
36
B
Saline
500µg/kg
1000µg/kg
Temperature, ℃
39
38
37
36
C
*
Saline
500µg/kg
1000µg/kg
2000µg/kg
Temperature, ℃
39
38
37
36
7:00
19:00
7:00
Time

Fig 1. Mean body temperature changes in rats after administration of either 500μg/kg (▲) 1000μg/kg (×) and 2000μg/kg (■) poly I:C or sterile saline (○) via subcutaneous (A), intramuscular (B) or intraperitoneal (C) route. * Indicates a significant increase in the mean body temperature of rats receiving poly I:C intraperitoneally at a dose of 1000μg/kg and 2000μg/kg when compared to the rats receiving sterile saline ($P<0.05$) for the period form 11:00-17:00. The dashed line indicates the time of injection. Ordinate: body temperature in ^{o}C. Abscissa: 24h clock time.

Figure 2

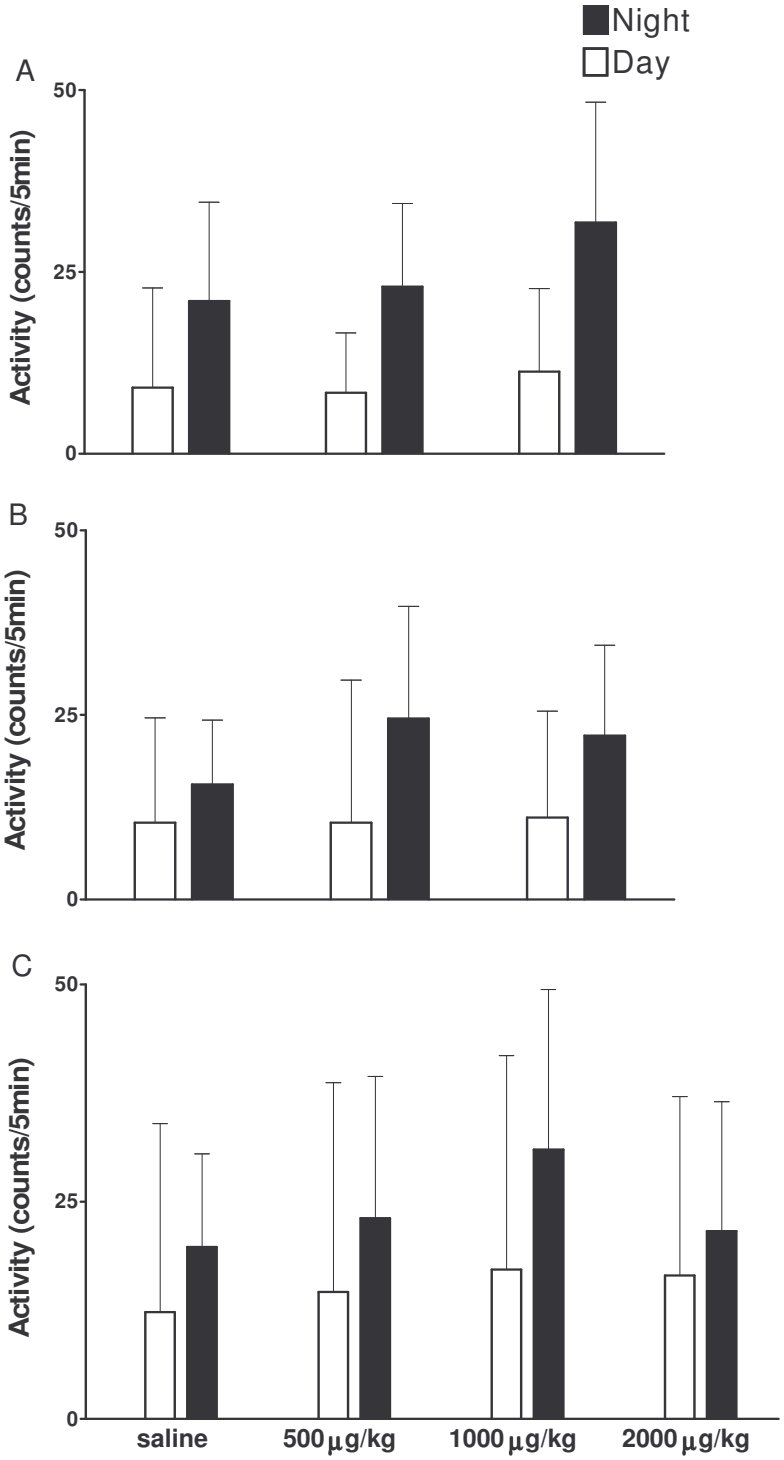

Night
Day
A
B
C
Activity (counts/5min)
50
25
0
saline
500 µg/kg
1000 µg/kg
2000 µg/kg

Fig 2. Changes in mean 12h day-time (open bars) and mean 12h night-time (filled bars) cage activity counts for rats receiving either 500µg/kg, 1000µg/kg and 2000µg/kg poly I:C or sterile saline via subcutaneous (A), intramuscular (B) or intraperitoneal (C) route. Ordinate: cage activity in counts/5min. Abscissa: different experimental groups receiving poly I:C at respective doses.

3.4 Discussion

Intraperitoneal administration of poly I:C at a dose of 1000µg/kg or 2000µg/kg in rats resulted in fevers significantly greater than the change in body temperature occurring as a result of sterile saline injection ($P<0.05$) by that same route. The 1000µg/kg dose produced less consistent fevers compared to the larger dose (2000µg/kg) but both doses resulted in significant rises in body temperature.

Poly I:C administered intraperitoneally at a dose of 2000µg/kg resulted in fevers of longer duration when compared to the 1000µg/kg dose. Fevers induced by both high doses of poly I:C were monophasic, starting about two hours after injection and peaking about four hours after administration and lasting for six (1000µg/kg) to seven (2000µg/kg) hours, although the larger dose of poly I:C also resulted in a second rise in body temperature during the dark-phase (Figure 1C).

Poly I:C administered either subcutaneously or intramuscularly, at a dose of 500µg/kg and 1000µg/kg were ineffective in causing a rise in body temperature (Figure 1A and B).

The fact that poly I:C administered either subcutaneously or intramuscularly fails to cause a fever at a dose which is effective via the intraperitoneal route may be the result of a lack of macrophages in the surrounding area to trigger the release of

cytokine mediators. Another reason could be that the doses used via the subcutaneous and intramuscular route were not adequate to cause an immune response. Another possibility is that subcutaneous administration of poly I:C results in tissue macrophages being primarily stimulated, decreasing the capacity to excite central receptors (Cartmell *et al.*: 2002).

The doses used in this study are dissimilar to studies using other animals species such as rabbits, but appear to be consistent with other studies (Cooper *et al.*: 1988, Katafuchi *et al.*: 2003, Fortier *et al.*: 2004). Although intramuscular injections administered in this study did not result in fevers, intramuscular injections of poly I:C, administered to guinea pigs does result in an increase in body temperature (Cooper *et al.*: 1988), confirming that there is a species variation in the effectiveness of poly I:C. The results of this study are supported by Fortier *et al.* (2004) who reported that 500µg/kg of poly I:C administered intraperitoneally does not result in a significant increase in body temperature (Fortier *et al.*: 2004).

Another important and unexpected finding of this study was that although intraperitoneal administration of poly I:C at doses of 1000µg/kg and 2000µg/kg resulted in significant fevers there was no accompanying suppression in cage activity (Figure 2). The suppression of activity is a common observation in rats made febrile by LPS injection (Hart: 1988b, Dantzer: 2004). Fortier *et al.* (2004) demonstrated that fever but not anorexia, which is another common characteristic which accompanies

fever (LeGrand: 2000), induced by poly I:C, unlike that induced by LPS, is IL-1 and prostaglandin-dependent (Fortier *et al.*: 2004). Therefore, a possible explanation for not observing a decrease in cage activity is that the mediators which induce an increase in body temperature may differ from those mediating activity during a virally-induced infection. Interferons (IFNs) are the primary mediators thought to mediate virally-induced infection, but not much is known about the effects of this cytokine on inducing sickness behaviour.

The lack of suppression in cage activity induced by poly I:C is contradictory to Katafuchi *et al.* (2003) who demonstrated that the administration of poly I:C results in an increase in body temperature of about 1^{o}C as well as a suppression of physical activity in rats but at a much higher dose of poly I:C (3000µg/kg) (Katafuchi *et al.*: 2003). Thus another possible reason for the findings of my study was that the dose was inadequate to elicit sufficient cytokine mediators to result in the suppression in cage activity.

In conclusion, I have shown that the efficacy of poly I:C to induce fever in rats is dependent on the dose and the route of administration. This study has shown that intraperitoneal administration of poly I:C at a minimum dose of 1000µg/kg is effective in inducing a fever response in rats but does not result in a suppression of cage activity in rats.

For all subsequent studies I describe in this dissertation, poly I:C was administered at a dose of 2000μg/kg via the intraperitoneal route.

4. THE EFFECTS OF REPEATED ADMINISTRATION OF LPS AND POLY I:C IN RATS

4.1 Introduction

Repeated administration of the Gram-negative bacterial product, LPS, to laboratory animals results in the development of tolerance to its pyrogenic effects (Beeson: 1947, Roth *et al.*: 1994, Chemo *et al.*: 1997, Tripp *et al.*: 1998, Almeida *et al.*: 1999), which is the progressive diminution in the febrile response. The tolerance phenomenon results not only in the attenuation of the fever response induced by LPS, but also an attenuation to aspects of the accompanying sickness behaviour (Chemo *et al.*: 1997, Tripp *et al.*: 1998, Almeida *et al.*: 1999). Not much, however, is known about whether tolerance to the pyrogenic effects of a viral pyrogen develops after repeated administration in rats.

The development of tolerance associated with repeated administration of LPS has been shown in a variety of animals including rabbits, guinea pigs and different strains of rats (He *et al.*: 1992, Chemo *et al.*: 1997, Tripp *et al.*: 1998). There exists a species variation in the rate of the development of tolerance, with the tolerance effect being more pronounced in rats, compared to rabbits and guinea pigs, which have a more gradual attenuation of the fever response (Tripp *et al.*: 1998).

Previous studies have also shown that tolerance to both the physiological and behavioural effects of Gram-negative LPS forms rapidly, often after a single exposure (O'Reilly *et al.*: 1988, Roth *et al.*: 1997, Tripp *et al.*: 1998) whereas the development of tolerance in response to repeated administration of the Gram-positive bacterial muramyl dipeptide (MDP) results more slowly or not at all (Soszynski *et al.*: 1991). If there are differences in the development of tolerance between bacterial products, it seems reasonable to speculate that there would be differences between the development of tolerance induced by bacterial and viral pyrogens in rats.

Rats exhibit nycthermal body temperature variations, with body temperature being ~1^{o}C lower during the light-phase (day) than at night. Therefore fever responses induced during the dark-phase may be masked by the natural nocturnal rise in body temperature. To maximize the ability to discern changes in body temperature responses as a result of repeated injections, therefore, tolerance studies in rats usually are performed during the day, and no studies, I believe, to date have investigated the effects of repeated night-time administration of LPS on body temperature and cage activity in rats, and it is not known if the tolerance to LPS would be as evident as it is after repeated day-time injections.

Many different experimental protocols have been used to study the mechanisms mediating the development of tolerance. One such protocol is the use of osmotic pumps or pellets which are implanted subcutaneously in laboratory animals and allow

for the slow, continuous release of pyrogens over a period of days. Another protocol used is consecutive injections of a pyrogen, in which animals receive a single injection daily for a number of days. The interval between injections, however, has differed among the various studies (Soszynski *et al.*: 1991, Roth *et al.*: 1994, Tripp *et al.*: 1998).

The objective of this study was therefore to determine whether repeated administration night-time administration of LPS would result in an attenuation of its pyrogenic and behavioural effects. I also wanted to compare the effects of repeated administration of LPS and poly I:C on body temperature and cage activity.

4.2 Experimental Protocol

After recovery from the surgery necessary for implantation of radiotelemeters (see chapter 2.2), rats were randomly assigned to three experimental groups and received a daily injection of either i) sterile saline intramuscularly (n=5), ii) poly I:C 2000µg/kg intraperitoneally (n=7), or iii) LPS 100µg/kg intramuscularly (n=5), for five consecutive days.

LPS is known for its reliability in producing tolerance when injected repeatedly intramuscularly (Rosenthal *et al.*: 1996, Roth *et al.*: 1997, Soszynski & Krajewska: 2001). I have shown that poly I:C does not produce fevers when administered intramuscularly, but does produce fevers when administered intraperitoneally.

Because repeated injection of LPS, both intramuscularly and intraperitoneally, produce tolerance (Roth *et al.*: 1994, Rosenthal *et al.*: 1996, Tripp *et al.*: 1998, Soszynski & Krajewska: 2001), it was not deemed necessary for a separate comparative study.

Injections were given at 17:00, two hours before the start of the dark period in which the rats are most active.

4.2.1 Data analysis

The body temperatures of rats receiving either repeated LPS or poly I:C were compared to rats receiving repeated sterile saline. The mean body temperatures for all the groups of rats were calculated for the six hour period from 19:00-01:00. The mean body temperatures were calculated from 19:00 and not 17:00 to omit the stress hyperthermia from the fever response. Mean temperatures of rats receiving either LPS or poly I:C were compared to the mean temperature of rats receiving sterile saline.

Thermal response indices (TRIs, ^{o}C.h) were calculated over a six hour period from 19:00-01:00, following all five injections, for rats receiving either LPS, poly I:C or sterile saline.

Mean 12h day-time and 12h night-time activity counts of rats receiving either LPS or poly I:C was calculated and compared to the rats receiving sterile saline. Statistical significance was determined using one-way ANOVA followed by the Tukèy *post hoc* test for multiple comparisons. $P<0.05$ was considered statistically significant.

4.2 Results

Body Temperature

There was a significant rise in the body temperatures of rats after receiving the first injection of 100µg/kg LPS intramuscularly (n=5) when compared to the rats receiving saline (n=5) ($P<0.01$) (Figure 3). The rats' body temperatures started to rise approximately 2h after LPS administration and the fevers peaked approximately 4h after injection. The maximum increase in body temperature after LPS administration was approximately $2^{o}C$. Not only was rat body temperature significantly elevated for approximately 6h after injection but the rats receiving LPS had a significantly elevated body temperature the morning after the first injection compared to the rats receiving saline ($P<0.05$) (Figure 4). The increase in body temperature the morning after LPS administration was approximately $0.5^{o}C$, with the fever lasting approximately 10h. There was no significant increase in body temperature in rats after receiving the second, third, fourth and fifth injection of 100µg/kg LPS intramuscularly when compared to the rats receiving saline (Figure 4 and Figure 5). Figure 6 shows results of comparison of mean body temperature for the period after injection (19:00-01:00), between LPS, poly I:C and saline groups over the five

consecutive injections. The figure shows that there was a significant increase in mean body temperature, over that of saline-injected rats, only after the first LPS injection. It would have been preferable to have a group of rats injected intraperitoneally with saline for control purposes. However, I expected either no, or a negligible effect on body temperature of rats injected intraperitoneally or intramuscularly with saline. Therefore, it was not considered ethical to do a separate group of rats receiving saline intraperitoneally.

Figure 7 shows the six hour thermal response indices (^{o}C.h), calculated for all three groups of rats. The figure shows that the rats receiving LPS responded with a significant increase in body temperature compared to the saline-treated controls only after the first injection and not following subsequent injections.

There was a significant increase in the body temperatures of rats after receiving the first injection of 2000µg/kg poly I:C when compared to the rats receiving sterile saline ($P<0.01$) (Figure 3). The rats' body temperatures started to rise approximately two hours after poly I:C administration and the fevers peaked approximately three hours after injection. The maximum increase in body temperature after poly I:C administration was approximately 1^{o}C, with the fevers lasting about six hours. The rats receiving poly I:C responded with fevers with a similar profile after the second, third, fourth and fifth injections when compared to the rats receiving saline ($P<0.05$) (Figure 4 and 5). The six hour thermal response indices (^{o}C.h), and mean differences

in body temperature for rats receiving repeated poly I:C were significantly elevated on all five nights following each injection when compared to rats receiving sterile saline ($P<0.01$) (Figure 7 and 6 respectively).

Once again all the rats receiving either pyrogen or saline injections showed a transient rise in body temperature of approximately $0.5^{o}C$, except for the fourth injection when animals were weighed in which there was an approximate $1^{o}C$ increase, which lasted for approximately 30min after the injection (Figure 3, 4 and 5). Notably the rats did not show a tolerance to this stress-induced rise in body temperature. This could be indicative of a different pathway being used compared to that induced during infection.

Cage Activity

There was a significant suppression in the night-time cage activity of rats after receiving the first three injections of LPS when compared to the rats receiving sterile saline (Figure 8, Day 1, 2 and 3). There was no change in the night-time cage activity after the 4^{th} and 5^{th} injection of LPS (Figure 8, Day 4 and 5).

Poly I:C administration had no significant effect on the cage activity of rats, either night- or day-time, after any of the five injections when compared to the rats receiving sterile saline (Figure 8).

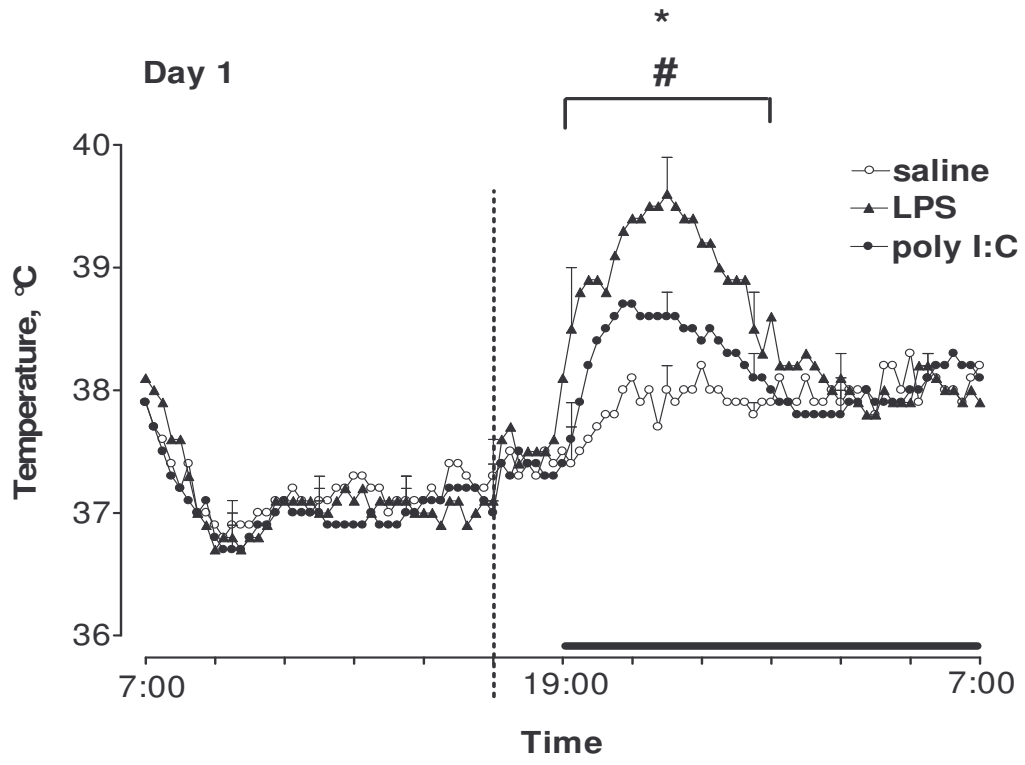


Fig 3. Mean body temperature changes (±SD) in rats receiving either 100µg/kg LPS intramuscularly (▲, n=5), 2000µg/kg poly I:C intraperitoneally (●, n=7) or sterile saline intramuscularly (○, n=5). * Indicates a significant increase in the body temperatures of rats receiving LPS, and #, a significant increase in the mean body temperatures of rats receiving poly I:C, when compared to the mean body temperatures of rats receiving sterile saline ($P<0.01$) for the period 19:00-01:00. Dashed line indicates time of injection, and solid bar indicates the night-time period. Ordinate: body temperature of rats in ^{o}C. Abscissa : 24-h clock time.

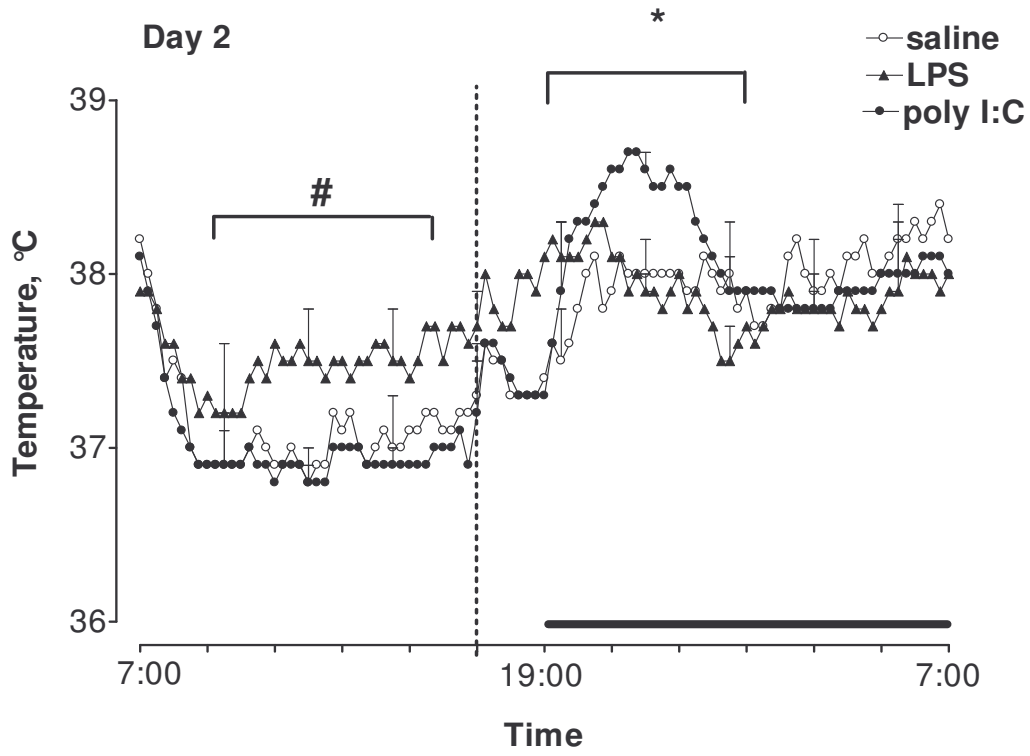


Fig 4. Mean body temperature changes (±SD) in rats receiving either 100µg/kg of LPS intramuscularly (▲, n=5), 2000µg/kg poly I:C intraperitoneally (●, n=7) or sterile saline intramuscularly (○, n=5) the day after the first injection (Day 2) and the night of the second injection. # Indicates a significant increase in the body temperature of rats receiving LPS the morning following the first injection when compared to rats receiving sterile saline ($P<0.05$) for the period 09:00-17:00. * Indicates a significant increase in the body temperature of rats receiving the second injection of poly I:C when compared to rats receiving sterile saline ($P<0.05$) for the period 19:00-01:00. There was no significant difference ($P>0.05$) in the mean body temperatures of rats receiving the second injection of LPS compared to the rats receiving sterile saline injection. Dashed line indicates time of second injection, and solid bar indicates the night-time period. Ordinate: body temperature of rats in ^{o}C. Abscissa: 24-h clock time.

Figure 5

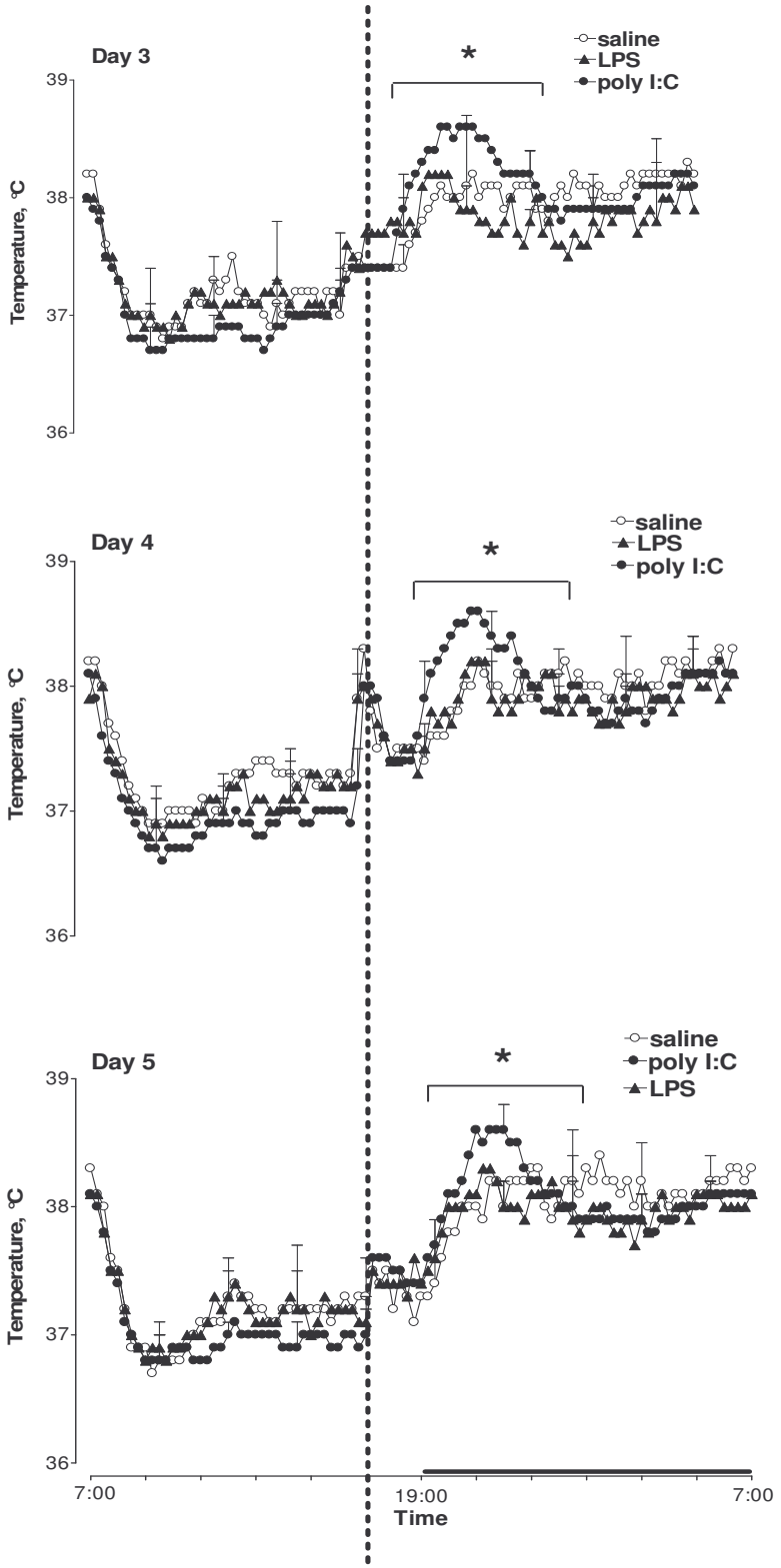

Day 3
Day 4
Day 5
saline
LPS
poly I:C
*
Temperature, ℃
39
38
37
36
7:00
19:00
7:00
Time

Fig 5. Mean body temperature changes (±SD) in rats after receiving the third (Day 3), fourth (Day 4) and fifth (Day 5) injections of either 100µg/kg of LPS intramuscularly (▲, n=5), 2000µg/kg poly I:C intraperitoneally (●, n=7) or sterile saline intramuscularly (○, n=5). There was no significant increase in the mean body temperature of rats receiving LPS on either days 3, 4 and 5 compared to rats receiving sterile saline. * Indicates a significant increase in the body temperature of rats receiving the third, fourth and fifth injection of poly I:C when compared to rats receiving sterile saline ($P<0.05$) for the period 19:00-01:00. Dashed line indicates the time of injection, and solid bar indicates the night-time period. Large spike in body temperatures at the time of injection on day 4 was due to weighing of animals. Ordinate: body temperature of rats in ^{o}C. Abscissa: 24-h clock time.

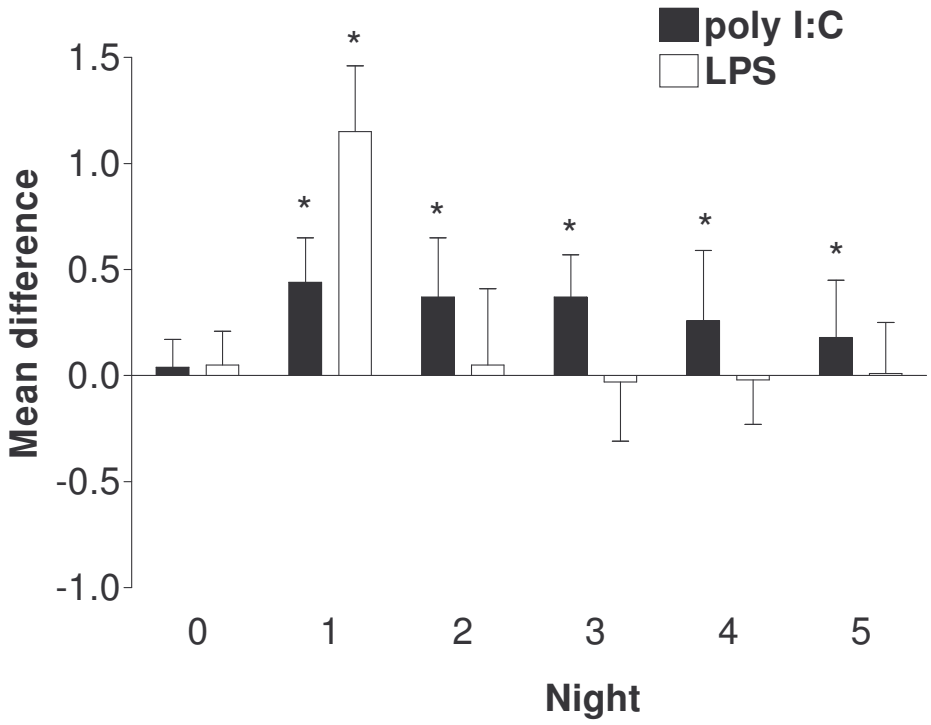


Fig 6. Mean difference in body temperature (±SD) between rats receiving either 100µg/kg of LPS, and sterile saline intramuscularly (n=5, filled bars) or between rats receiving 2000µg/kg poly I:C intraperitoneally, and sterile saline intramuscularly (n=7, open bars). No difference between pyrogen and saline responses represented by the zero line. Differences were calculated for the period 19:00-01:00. * Indicates a significant differences in the effect of the pyrogen ($P<0.05$). Ordinate: mean difference in body temperature in ^{o}C. Abscissa: successive nights on which injections were given. Night 0 = the night before the first injection.

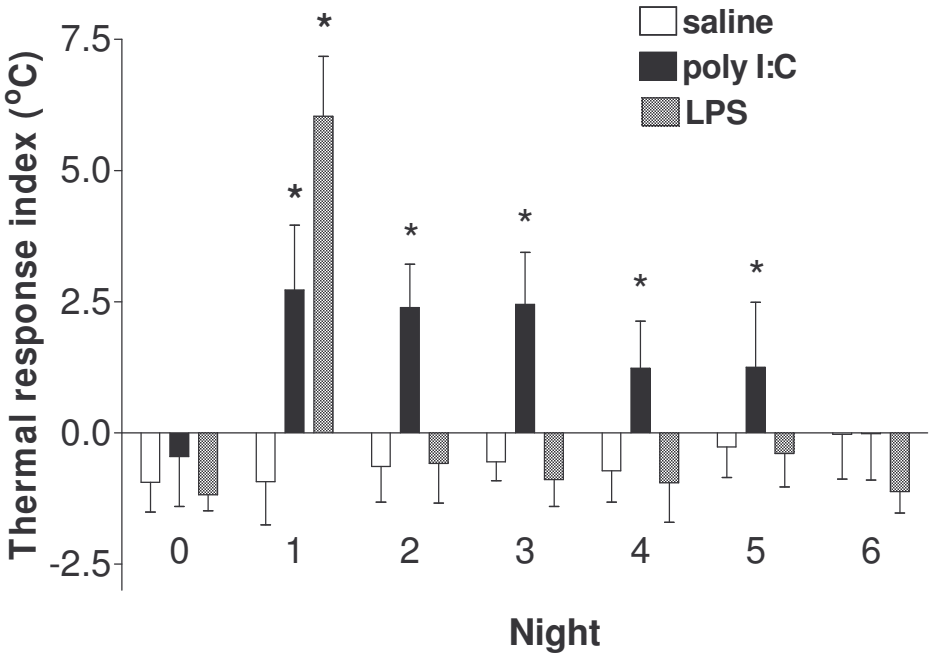


Fig 7. Mean thermal response index (°C.h, ±SD) for the six hour period between 19:00 and 01:00 calculated for rats receiving injections (at 17:00) for five consecutive nights, of either 100µg/kg LPS intramuscularly (n=5) (checked bars), 2000µg/kg poly I:C intraperitoneally (n=7, filled bars) or sterile saline intramuscularly (n=5, open bars). * Indicates a significant increase in the thermal response index of rats receiving LPS and poly I:C when compared to rats receiving sterile saline ($P<0.05$). Ordinate: thermal response index (°C.h). Abscissa: successive nights on which injections were given**.** Night 0 = the night before the first injection.

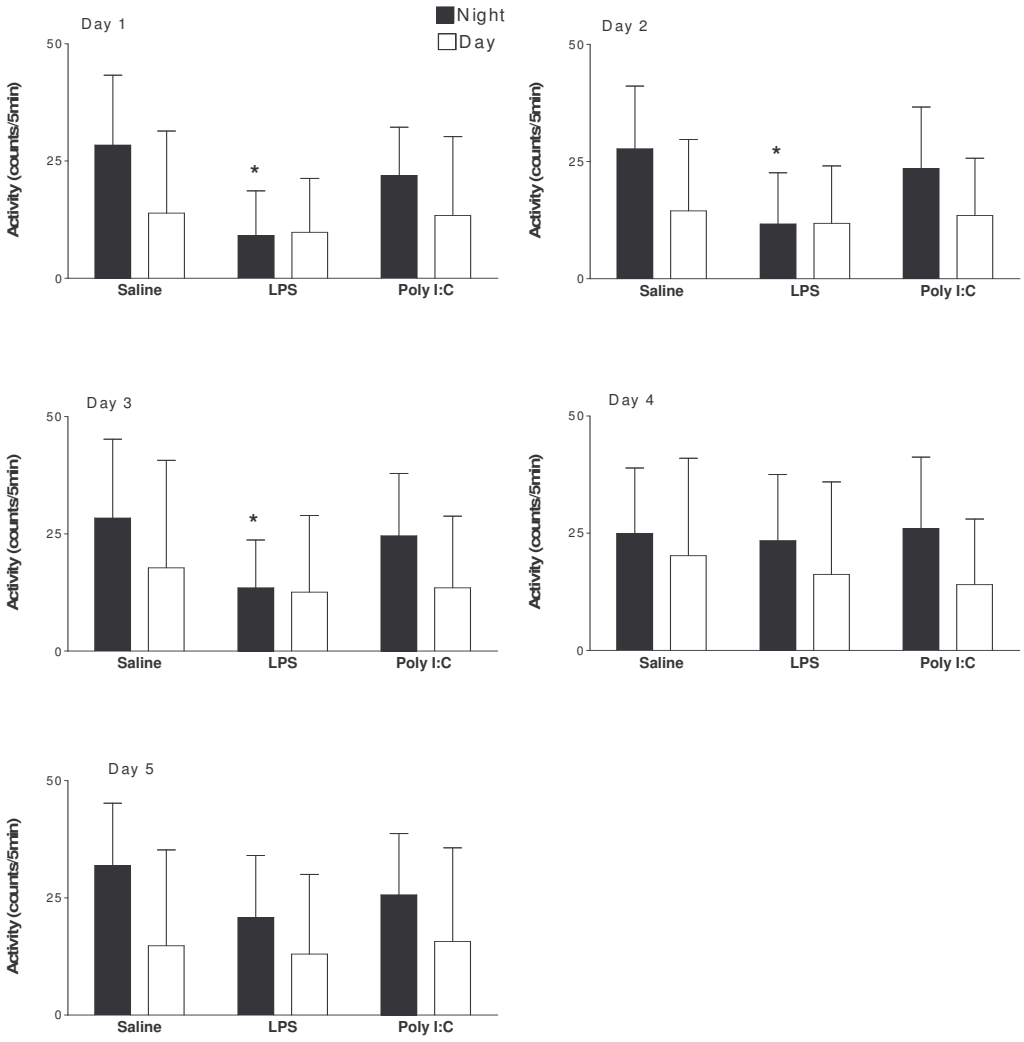


Fig 8. Changes in mean cage activity counts of rats receiving either 100µg/kg LPS intramuscularly (n=5), 2000µg/kg poly I:C intraperitoneally (n=7) or sterile saline intramuscularly (n=5) daily for five consecutive nights (Day1-5). Open bars represent the mean 12 h day-time cage activity counts for the day before the night of injection and the filled bars represents the mean 12 h night-time cage activity counts for the night of the injection. * Indicates a significant suppression in cage the activity of rats receiving 100µg/kg LPS intramuscularly when compared to rats receiving sterile saline ($P<0.05$). Ordinate: cage activity in counts/5min. Abscissa: different experimental groups.

4.3 Discussion

I have shown that LPS and poly I:C injected into rats two hours prior to the night-time active period, causes fever with increases in body temperature being quite distinct from the normal night-time rise in rat body temperature (Figure 1). The magnitude and duration of fevers induced by LPS were distinctly larger when compared to those induced by poly I:C-induced fevers (Figure 1). Furthermore, I have shown that repeated daily administration of LPS to rats results in an attenuation of the febrile response, as well as to the suppression in cage activity. LPS-treated rats became tolerant to the thermal effects of the pyrogen after a single injection, while the attenuation of the cage activity occurred after the third injection. In contrast, however, rats receiving poly I:C continued to respond with a significant rise in body temperature after each of the five consecutive injections. Poly I:C administration did not affect cage activity.

The development of tolerance to the thermal effects of LPS after a single intramuscular night-time injection found in this study, is consistent with previous studies which have been performed during the day and with studies in which LPS was administered intraperitoneally (Soszynski *et al.*: 1991, Roth *et al.*: 1994, Chemo *et al.*: 1997, Tripp *et al.*: 1998). The time of LPS administration, whether during the active- or inactive phase, therefore has no effect on the development of tolerance to repeated LPS administration. Although there is a variation in the rate of development of thermal tolerance across different species thermal tolerance is a consistent feature

of repeated LPS administration (Soszynski *et al.*: 1991, Roth *et al.*: 1994, Tripp *et al.*: 1998).

In my study, tolerance did not develop to the thermal effects of poly I:C administration. Poly I:C consistently induced fevers of similar magnitude after each of the five injections (Figure 1-3). My data support previous findings, described for other animal species such as rabbits and guinea pigs (Cooper *et al.*: 1988, Soszynski *et al.*: 1991). Soszynski *et al.* (1991) reported that repeated intravenous administration of poly I:C in rabbits also did not result in thermal tolerance (Soszynski *et al.*: 1991). However, Cooper *et al.* (1988) reported that repeated administration of poly I:C in guinea pigs showed a transient tolerance to the febrile effect after three to four injections but continued administration of poly I:C resulted in fevers with increasing magnitude, a finding which they could not explain (Cooper *et al.*: 1988).

The development of tolerance to repeated administration of LPS is thought to be mediated partly through a down-regulation of the gene transcription for- and the secretion of pro-inflammatory cytokines (Zeisberger & Roth: 1998) and an increase in the secretion of anti-inflammatory cytokines (Tripp *et al.*: 1998). Thus development of tolerance to repeated administration of LPS appears at least to be related to an altered function of macrophages, to *inter alia,* secrete cytokines involved in the fever response. Roth *et al*. (1994) showed that repeated administration of LPS

was accompanied by a strongly correlated reduction in circulating concentrations of tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6), which corroborated evidence from *in vitro* studies showing that repeated administration of LPS resulted in reduced macrophage transcription and translation for cytokine mRNAs and cytokine proteins (Roth *et al.*: 1994). It also was postulated that the hypothalamus might be unable to respond with an increase in prostaglandin E_2 (PGE_2) production to repeated administration of LPS. This latter theory, however, was not supported by Chemo *et al.* (1997) who showed that tolerance to LPS was more likely related to the altered responsiveness of neuronal receptors to PGE_2 or due to alterations in intracellular signal transduction events (Chemo *et al.*: 1997).

Why tolerance does not occur after repeated administration of poly I:C in rats remains unclear. I did not measure circulating cytokine concentrations of the rats in my study, but it is likely that repeated poly I:C administration may not result in a similar down-regulation of the cytokine mechanisms such as occurs after repeated LPS administration (Fortier *et al.*: 2004). This study provides evidence that different mechanisms may mediate fevers induced by poly I:C, compared to fevers induced by LPS.

While the mechanisms of pyrogen-induced fever and the cytokines involved in those mechanisms have been well documented (Kluger *et al.*: 1995, Luheshi: 1998, Conti *et al.*: 2004), much less is understood about the cytokine mediators of sickness

behavior. In my study, cage activity was attenuated after the first injection of LPS, consistent with data from previous reports (Yirmiya *et al.*: 1994, Engeland *et al.*: 2003) including that cage activity also is reduced by injection of Gram-positive agents (Luker *et al.*: 2000). In contrast to the thermal response, which was tolerant after the first injection, cage activity remained suppressed until after the third injection of LPS (Figure 6).

The suppression of activity is a common observation in rats made febrile by LPS injection (Hart: 1988b). Unexpectedly a dose of poly I:C which caused a robust fever consistently, had no effect on cage activity. In contrast, Katafuchi *et al.* (2003) demonstrated that the administration of poly I:C results in an increase in body temperature as well as a suppression of physical activity in rats but at a much higher dose of poly I:C (3000µg/kg) (Katafuchi *et al.*: 2003).

My data point to the fact that different cytokine networks may underlie the responses to LPS and poly I:C. Fortier *et al.* (2004) demonstrated that the fever induced by poly I:C similar to LPS, is IL-1- and prostaglandin-dependent (Fortier *et al.*: 2004). However, the anorexia which accompanies the poly I:C-induced fever, appeared not to be effected by the same mediators (Fortier *et al.*: 2004). This finding is further supported by Swiergiel and Dunn (1999), who reported that IL-1β, IL-6 and TNF-α contribute to the hypophagia induced by LPS, but antagonism of all three of these cytokines did not attenuate the decrease in food intake and weight loss induced by

influenza virus infection (Swiergiel & Dunn: 1999). Thus, a possible explanation for not observing a decrease in cage activity after poly I:C injection is that the mediators which induce the sickness behaviour after poly I:C injection may differ from those induced by LPS to produce fever and suppression in cage activity.

Interferons (IFNs) are the primary mediators thought to be involved in virally-induced infection (Majde: 2000, Mogensen & Paludan: 2001), with little or no involvement in bacterial infections (Kimura *et al.*: 1994). Poly I:C and indeed other viral simulants may activate a cascade of cytokine release which exclude those, like IL-1α and/or TNF-α, which appear to mediate a suppression of physical activity. Alternatively viral simulants may activate primarily pathways leading to IFN synthesis. It has been reported that intraperitoneal administration of poly I:C in rats induces the expression of IFN-α mRNA in the brain (Katafuchi *et al.*: 2003). Kimura *et al.* (1994) reported that LPS, unlike poly I:C, fails to induce IFN-α in the mouse macrophage cell line RAW 264.7, whereas administration of poly I:C minimally induces IL-6 mRNA (Kimura *et al.*: 1994). Interferon-alpha has been shown to result in hypoactivity and hypophagia in mice (Crnic & Segall: 1992) as well as in poly I:C-induced hypoactivity in rats (Katafuchi *et al.*: 2003). Thus, those cytokines responsible for activity suppression may be released by viral simulants, but only at very high doses of them.

An intriguing question arises from my observations; why does tolerance to LPS-induced hypoactivity occur only after three injections, when the thermal effects are tolerant after a single injection of LPS? I believe an explanation may lie in different cytokines mediating LPS-induced fever, versus LPS-induced suppression of activity. IL-6 is widely thought to be the most likely, if not the most essential, mediator of the rise in body temperature induced by LPS (Swiergiel & Dunn: 1999, Bluthe *et al.*: 2000, Conti *et al.*: 2004). It is the expression of mRNA for- or synthesis of the protein of IL-6 which appears to be attenuated in the process of tolerance development, leading to decreased circulating concentrations (Roth *et al.*: 1994). My findings would suggest that IL-6 plays a lesser role in the regulation of cage activity, as it does in the generation of fever. Alternatively, cytokines other that IL-6, may mediate the suppression of sickness behaviour, and candidate substances may include the interleukins 1-α and -β. Mechanisms involving these cytokines may be more resistant to down-regulation during the process of tolerance development.

A further question is raised by my observation of a disassociation in the time-course of tolerance to fever and activity suppression, that is why the infected organism would develop tolerance more readily to the thermal, compared to the non-thermal effects of Gram-negative infection. A prolonged period of fever would be metabolically expensive. A prolonged state of rest (inactivity) additionally conserves energy. Hart (1988) also has suggested that inactivity also would decrease the risk of predation, in some species.

In conclusion, I have shown that unlike during simulated bacterial infection, simulated viral infection appears not to induce one of the behaviours characteristic of infection, which is inactivity. Moreover, in contrast to the LPS-induced response, poly I:C in rats does not induced thermal tolerance, at least after repeated night-time administration. I have also shown a more rapid development of tolerance to the febrile effect of LPS compared to the development of tolerance to the inactivity which accompanies LPS fever. My results raise interesting questions concerning the mechanisms of induction of the acute phase response during bacterial as distinct to viral infections.

5. SUMMARY DISCUSSION AND CONCLUSIONS

The overall purpose of this dissertation was to compare aspects of the acute phase response induced by LPS to those aspects which would be induced by a viral pyrogen in rats. In order to achieve the objective of this study, I firstly had to establish an effective peripheral route of- and dose of administration of poly I:C, the chosen synthetic viral pyrogen, that consistently induced fevers in rats, a species seldom used for the study of poly I:C-induced fevers. The first of the differences I exposed between Gram-negative pyrogen and a viral pyrogen was that whereas LPS fever can be induced by injection via subcutaneous, intramuscular and intraperitoneal routes (O'Reilly *et al.*: 1988, Kamerman & Fuller: 2000, Cartmell *et al.*: 2002, Engeland *et al.*: 2003), poly I:C is effective only via the intraperitoneal route, at least in rats (see Chapter 3). I have shown that the synthetic viral pyrogen, poly I:C, is effective in causing an increase in body temperature in rats when administered intraperitoneally at a dose of at least 1000µg/kg, and 500µg/kg is ineffective in causing an increase in body temperature. Neither subcutaneous nor intramuscular administration of poly I:C were effective in causing an increase in body temperature, at least at doses below 1000µg/kg.

A second difference in the characteristics between a Gram-negative and virally-induced acute phase response, is that unlike for LPS and Gram-positive pyrogens (Luker *et al.*: 2000, Engeland *et al.*: 2003), poly I:C fails to affect physical activity. The different responses observed with exposure to LPS and poly I:C and the lack of

an effect of poly I:C administration on cage activity, could be indicative of different cytokine networks induced by the two chemically and molecularly different pyrogens. This theory is supported by Fortier et al. (2004) and Kimura *et al.* (1994) who demonstrated that there are some differences in the cytokine networks induced during LPS- and poly I:C induced APRs.

Another well known characteristic of LPS is that repeated administration of this pyrogen in laboratory animals results in the development of tolerance to its pyrogenic effects. I was keen to observe whether repeated poly I:C administration also resulted in the development of tolerance. Repeated night-time administration of LPS resulted in tolerance to its pyrogenic effects after only one injection; the second consecutive injection of LPS had no effect on the rats' body temperature. Interestingly, tolerance to the behavioural effects of LPS, specifically the attenuation in cage activity, only developed after the third consecutive injection. Repeated administration of poly I:C, unlike LPS, did not result in the development of tolerance to its pyrogenic effects. Again I believe the answer may lie with the cytokine mediators induced by LPS and poly I:C.

In conclusion, although both LPS and poly I:C induce fevers in rats, there are fundamental differences in the characteristics of the thermal and behavioural effects induced by these two classes of pyrogens. These findings may indicate that the effects of these two molecularly and chemically different pyrogens are mediated via different

mechanisms. Future research should focus on the different cytokine networks involved in mediating the acute phase response in response to a bacterial versus a viral challenge.

REFERENCES

Abul, H. T., Davidson, J., Milton, A. S. & Rotondo, D. (1997). Prostaglandin E2 enters the brain following stimulation of the acute phase immune response. *Ann N Y Acad Sci* **813,** 287-295.

Alexopoulou, L., Holt, A. C., Medzhitoz, R. & Flavell, R. A. (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* **413,** 732-738.

Almeida, M. C., Trevisan, F. N., Barros, R. C., Carnio, E. C. & Branco, L. G. (1999). Tolerance to lipopolysaccharide is related to the nitric oxide pathway. *Neuroreport* **10,** 3061-3065.

Aronoff, D. M. & Neilson, E. G. (2001). Antipyretics: mechanisms of action and clinical use in fever suppression. *Am J Med* **111,** 304-315.

Beeson, P. B. (1947). Tolerance to bacterial pyrogens. Factors influencing its development. *J Exp Med* **86,** 29-38.

Blatteis, C. M. (2004). The cytokine-prostaglandin cascade in fever production: fact or fancy? *J Therm Biol* **29,** 359-368.

Bluthe, R. M., Bristow, A., Lestage, J., Imbs, C. & Dantzer, R. (2001). Central injection of interleukin-13 potentiates LPS-induced sickness behavior in rats. *Neuroreport* **12,** 3979-3983.

Bluthe, R. M., Dantzer, R. & Kelley, K. W. (1992). The effects of interleukin-1 receptor antagonist on the behavioral effects of lipopolysaccharide in rat. *Brain Res* **573,** 318-320.

Bluthe, R. M., Michaud, B., Poli, V. & Dantzer, R. (2000). Role of IL-6 in cytokine-induced sickness behavior: A study with IL-6 deficient mice. *Physiol Behav* **70,** 367-373.

Botting, R. M. (2006). Cyclooxygenase: Past, present and future. A tribute to John R. Vane (1927-2004). *J Therm Biol* **31,** 208-219.

Cabanac, A. & Briese, E. (1992). Handling elevates the colonic temperature of mice. *Physiol Behav* **51,** 95-98.

Cartmell, T., Mitchell, D., Lamond, F. J. D. & Laburn, H. P. (2002). Route of administration differentially affects fevers induced by Gram-negative and Gram-positive pyrogens in rabbits. *Exp Physiol* **87,** 391-399.

Chemo, A., Fraifeld, V., Adramovich, L., Sod-Moriah, U. A. & Kaplanski, J. (1997). Tolerance to lipopolysaccharide is not related to the ability of the hypothalamus to produce prostaglandin E2. *Life Sci* **61,** 813-818.

Chuang, J., Lin, M. T., Chan, S. A. & Won, S. J. (1990). Febrile effects of polyriboinosinic acid: polyribocytidylic acid and interferon: relationship to somatostatin in rat hypothalamus. *Pflugers Arch* **415,** 606-610.

Condrad, A., Bull, D. E., King, M. G. & Husband, A. J. (1997). The effects of Lipopolysaccharide (LPS) on the fever response in rats at different ambient temperatures. *Physiol Beh* **62,** 1197-1201.

Conti, B., Tabarean, I., Andrei, C. & Bartfai, T. (2004). Cytokines and fever. *Front Biosci* **9,** 1433-1449.

Cooper, K. E., Blahser, S., Malkinson, T. J., Merker, G., Roth, J. & Zeisberger, E. (1988). Changes in body temperature and vasopressin content of brain neurons, in pregnant and non-pregnant guinea pigs, during fevers produced by Poly I:Poly C. *Pflugers Arch* **412,** 292-296.

Crnic, L. S. & Segall, M. A. (1992). Behavioural effects of mouse interferons-alpha and -gamma and human interferon-alpha in mice. *Brain Res* **590,** 277-284.

Dantzer, R. (2004). Cytokine-induced sickness behaviour: a neuroimmune response to activation of innate immunity. *Eur J Pharmacol* **500,** 399-411.

Davidson, J., Abul, H. T., Milton, A. S. & Rotondo, D. (2001). Cytokines and cytokine inducers stimulate prostaglandin E2 entry into the brain. *Pflugers Arch* **442,** 526-533.

Dinarello, C. A. & Wolff, S. M. (1982). Molecular basis of fever in humans. *Am J Med* **72,** 799-819.

Engeland, C. G., Kavaliers, M. & Ossenkopp, K.-P. (2003). Sex differences in the effect of muramyl dipeptide and lipopolysaccharide on locomotor activity and the development of behavioral tolerance in rats. *Pharmacol Biochem Behav* **74,** 433-447.

Fortier, M.-E., Kent, S., Ashdown, H., Poole, S., Boksa, P. & Luheshi, G. N. (2004). The viral mimic, polyinosinic:polycytidylic acid, induces fever in rats via an interleukin-1 dependent mechanism. *Am J Physiol Regul Integr Comp Physiol* **287,** R759-766.

Grinevich, V., Ma, X. M., Herman, J. P., Jezova, D., Akmayev, I. & Agguilera, G. (2001). Effect of repeated lipopolysaccharide administration on tissue cytokine expression and hypothalamic-pituitary-adrenal axis activity in rats. *J Neuroendocinol* **13,** 711-723.

Hart, B. L. (1988). Biological basis of the behavior of sick animals. *Neurosci Biobehav Rev* **12,** 123-137.

He, W., Fong, Y., Marano, M. A., Gershenwald, J. E., Yurt, R. W., Moldawer, L. L. & Lowry, S. F. (1992). Tolerance to endotoxin prevents mortality in infected thermal injury: association with attenuated cytokine responses. *J Infect Dis* **165,** 859-864.

Homan, E. R., Zendzian, R. P., Schott, L. D., Levy, H. B. & Adamson, R. H. (1972). Studies on poly I:C toxicity in experimental animals. *Toxicol Appl Pharmacol* **23,** 579-588.

Jacobs, B. L. & Langland, J. O. (1996). When two strands are better than one: the mediators and modulators of the cellular responses to double-stranded RNA. *Virology* **219,** 339-349.

Kamerman, P. & Fuller, A. (2000). Effects of nitric oxide synthase inhibitors on the febrile response to lipopolysaccharide and muramyl dipeptide in guinea pigs. *Life Sci* **67,** 2639-2645.

Kamerman, P. R., Mitchell, D. & Laburn, H. P. (2002). Effects of nitric oxide synthase inhibitors on the febrile response to muramyl dipeptide and lipopolysaccharide in rats. *J Comp Physiol [B]* **172,** 441-446.

Katafuchi, T., Kondo, T., Yasaka, T., Kubo, K., Take, S. & Yoshimura, M. (2003). Prolonged effects of polyriboinosinic:polyribocytidylic acid on spontaneous running wheel activity and brain interferon-[alpha] mRNA in rats: a model for immunologically induced fatigue. *Neuroscience* **120,** 837-845.

Kent, S., Bluthe, R. M., Kelley, K. W. & Dantzer, R. (1992). Sickness behavior as a new target for drug development. *Trends Pharmacol Sci* **13,** 24-28.

Kimura, M., Toth, L. A., Agostini, H., Cady, A. B., Majde, J. A. & Krueger, J. M. (1994). Comparison of acute phase responses induced in rabbits by lipopolysaccharide and double-stranded RNA. *Am J Physiol Regul Integr Comp Physiol* **267,** R1596-1605.

Kimura-Takeuchi, M., Majde, J. A., Toth, L. A. & Krueger, J. M. (1992). The role of double-stranded RNA in induction of the acute-phase response in an abortive influenza virus infection model. *J Infect Dis* **166,** 1266-1275.

Kluger, M. J., Kozak, W., Leon, L. R., Soszynski, D. & Conn, C. A. (1995). Cytokines and fever. *Neuroimmunomodulation* **2,** 216-223.

Konsman, J. P., Parent, P. & Dantzer, R. (2002). Cytokine-induced sickness behavior: Mechanisms and implications. *Trends Neurosci* **25,** 154-159.

Lefkowith, J. B. (1999). Cyclooxygenase-2 specificity and its clinical implications. *Am J Med* **106,** 43S-50S.

LeGrand, E. K. (2000). Why infection-induced anorexia? The case for enhanced apoptosis of infected cells. *Med Hypothesis* **54,** 597-602.

Li, S., Sehic, E., Ungar, A. L. & Blatteis, C. M. (2000). Complement does not mediate the febrile responses of guinea pigs to muramyl dipeptide and polyriboinosinic-polyribocytidylic acid. *J Therm Biol* **25,** 51-58.

Lin, M. T. (1981). Polyriboinosinic-polyribocytidylic acid complex induces fever reaction in the rabbit. *Neuropharmacology* **20,** 1105-1109.

Lindsay, H. L., Trown, P. W., Brandt, J. & Forbes, M. (1969). Pyrogenicity of Poly I:C in rabbits. *Nature* **223,** 717-718.

Liu, H. J., Young, C. M. & Lin, M. T. (1989). Depletion of hypothalamic norepinephrine reduces the fever induced by polyriboinosinic acid: polyribocytidylic acid (Poly I:Poly C) in rats. *Experientia* **45,** 720-722.

Long, N. C. & Satinoff, E. (1990). Stress-induced rise of body temperature in rats is the same in warm and cold environments. *Physiol Behav* **47,** 773-775.

Luheshi, G. N. (1998). Cytokines and fever. *Ann N Y Acad Sci* **856,** 83-89.

Luker, F. I., Mitchell, D. & Laburn, H. P. (2000). Fever and motor activity in rats following day and night injections of *Staphyloccocus aureus* cell walls. *Am J Physiol Regul Integr Comp Physiol* **279,** R610-R616.

Majde, J. A. (2000). Viral double-stranded RNA, cytokines, and the flu. *J Interferon Cytokine Res* **20,** 259-272.

Mitchell, D. & Laburn, H. P. (1997). *Macrophysiology of fever.* August Krogh Institute, Copenhagen, 349-363.

Mogensen, T. H. & Paludan, S. R. (2001). Molecular pathways in virus-induced cytokine production. *Microbiol Mol Biol Rev* **65,** 131-150.

O'Reilly, B., Vandeer, A. J. & Kluger, M. J. (1988). Effects of chronic infusion of lipopolysaccharide on food intake and body temperature of the rat. *Physiol Behav* **42,** 287-291.

Plata-Salaman, C. R., Ilyin, S. E., Gayle, D. & Flynn, M. C. (1998). Gram-negative and gram-positive bacterial products induce differential cytokine profiles in the brain: analysis using an integrative molecular-behavioral in vivo model. *Int J Mol Med* **1,** 387-397.

Pruett, S. B., Fan, R. & Zheng, Q. (2003). Acute ethanol administration profoundly alters poly I:C-induced cytokine expression in mice by a mechanism that is not dependent on corticosterone. *Life Sci* **72,** 1825-1839.

Romanovsky, A. A., Kulchitsky, V. A., Akulich, N. V., Koulchitsky, S. V., Simons, C. T., Sessler, D. I. & Gourine, V. N. (1996). First and second phases of biphasic fever: two sequential stages of the sickness syndrome? *Am J Physiol* **271,** R244-R253.

Rosenthal, M., Roth, J., Storr, B. & Zeisberger, E. (1996). Fever responses in lean (Fa /-) and obese(fa/fa) Zucker rats and its lack to repeated injections of LPS. *Physiol Behav* **59,** 787-793.

Roth, J. & de Souza, G. E. P. (2001). Fever induction pathways: Evidence from responses to systemic or local cytokine formation. *Braz J Med Biol Res* **34,** 301-314.

Roth, J., Hubschle, T., Pehl, U., Ross, G. & Gerstberger, R. (1997). Fever and production of cytokines in response to repeated injections of muramyl dipeptide in guinea pigs. *Pflugers Arch* **434,** 525-533.

Roth, J., Hubschle, T., Pehl, U., Ross, G. & Gerstberger, R. (2002). Influence of systemic treatment with cyclooxygenase inhibitors on lipopolysaccharide-induced fever and circulating levels of cytokines and cortisol in guinea pigs. *Pflugers Arch* **443,** 411-417.

Roth, J., McClellan, J. L., Kluger, M. J. & Zeisberger, E. (1994). Attenuation of fever and release of cytokines after repeated injections of lipopolysaccharide in guinea pigs. *J Physiol* **477,** 177-185.

Rotondo, D., Abul, H. T., Milton, A. S. & Davidson, J. (1987). The pyrogenic actions of the interferon-inducer, polyinosinic: polycytidylic acid are antagonised by ketoprofen. *Eur J Pharmacol* **137,** 257-260.

Soszynski, D., Kozak, W. & Szewczenko, M. (1991). Course of fever response to repeated administration of sublethal doses of lipopolysaccharides, polyinosinic:polycytidylic acid and muramyl dipeptide to rabbits. *Experientia* **47,** 43-47.

Soszynski, D. & Krajewska, M. (2001). Lack of cross tolerance between pyrogenic effects of LPS and turpentine in rats. *J Therm Biol* **27,** 229-237.

Swiergiel, A. H. & Dunn, A. J. (1999). The role of IL-1, IL-6 and TNF in the feeding responses to endotoxin and influenza virus infection in mice. *Brain Behav Immun* **13,** 252-265.

Tripp, A., Roth, J., Storr, B., Voigt, K. & Zeisberger, E. (1998). Febrile tolerance develops in response to repeated administration of bacterial lipopolysaccharide but not of interleukin-1[beta] in rats. *J Therm Biol* **23,** 387-394.

Vollmer-Conna, U., Fazou, C., Cameron, B., Li, H., Brennan, C., Luck, L., Davenport, T., Wakefield, D., Hickie, I. & Lloyd, A. (2004). Production of pro-inflammatory cytokines correlates with the symptoms of acute sickness behaviour in humans. *Psychol Med* **34,** 1289-1297.

Won, S. J. & Lin, M. T. (1988). Pyrogenicity of interferon and its inducer in rabbits. *Am J Physiol Regul Integr Comp Physiol* **254,** R499-507.

Won, S. J., Lin, M. T., Ko, Y. H. & Chuang, J. (1991). The fever induced by polyriboinosinic:polyribocytidylic acid is not related to interferon synthesis in the rabbit's hypothalamus. *J Interferon Res* **11,** 165-169.

Yirmiya, R., Rosen, H., Donchin, O. & Ovadia, H. (1994). Behavioral effects of lipopolysaccharide in rats: involvement of endogenous opioids. *Brain Res* **648,** 80-86.

Zeisberger, E. & Roth, J. (1998). Tolerance to pyrogens. *Ann N Y Acad Sci* **856,** 116-131.

Ziel, R. & Krupp, P. (1975). The significance of inhibition of prostaglandin synthesis in the selection of non-steroidal anti-inflammatory agents. *Int J Clin Pharmacol* **186,** 186-191.