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Variation in Plasma Prostaglandin E2 Level in Mouse During Infection with Trichinella spiralis and Trichinella pseudospiralis

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THESIS APPROVAL

The abstract and thesis of Zahra Mehdizadehkashi for the Master of Science in Biology were presented July 6, 1995, and accepted by the thesis committee and the department.

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ABSTRACT

An abstract of the thesis of Zahra Mehdizadehkashi for the Master of Science in Biology presented July 6, 1995.

Title: Variation in Plasma Prostaglandin E_2 Level in Mouse During Infection With *Trichinella spiralis* and *Trichinella pseudospiralis*.

Polymorphonuclear leukocytes infiltrate tissues in response to an inflammatory stimulus such as endotoxin or parasite products. Previous studies have shown an extensive cellular infilteration about the parasitized skeletal muscle of mouse infected with the nematode, *Trichinella spiralis*. Infection of the host with *Trichinella pseudospiralis*, on the other hand, is associated with a dramatic suppression of inflammatory cellular response.

Prostaglandin E_2 is a product of arachidonic acid metabolism and is synthesized by variety of cell types. Prostaglandins of the E series have been generally known to suppress inflammatory responses.

In the present study, I have investigated the possible relation between plasma prostaglandin E_2 levels and host cellular response in infected mice. Concentrations of prostaglandin E_2 in mice plasma were measured at 5, 11, and 21 days after infection with

larvae of either nematode species by enzyme immunoassay.

There were noticeable elevations in the concentrations of prostaglanding E_2 in samples of mice infected with *Trichinella pseudospiralis* compared to controls. Conversely, decreased levels of prostaglandin E_2 were observed in samples from the mice infected with *Trichinella spiralis*.

These results suggest that the differences observed in the host inflammatory response to infection with *Trichinella spiralis* versus *Trichinella pseudospiralis* might be associated with recognized properties of prostaglandin E_2. In this connection, I have suggested three possible mechanisms by which the differences of inflammatory response in relation to PGE_2 production may be explained.

VARIATION IN PLASMA PROSTAGLANDIN E_2 LEVEL IN MOUSE DURING INFECTION WITH *TRICHINELLA SPIRALIS* AND *TRICHINELLA PSEUDOSPIRALIS*

by

ZAHRA MEHDIZADEHKASHI

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Trichinella spiralis is a parasite nematode which occurs within the phylum Nematoda, class Amphasmidia, order Trichurata, family Trichinellidae (Schmidt and Roberts 1989). *Trichinella spiralis* was described by Owen in 1835 and it was considered the only species in the genus *Trichinella* (Kramar et al., 1981). However, within the last 20 years, researchers have employed a variety of methods for the study and description of the systematics of *Trichinella*. These include geographic distribution, nurse cell development, reproductive capacity index (RCI), resistance to freezing, alloenzyme markers and host pathogenicity (Pozio et al., 1992). Together, these studies demonstrate that the genus *Trichinella* is composed of eight distinct gene pools, termed T1 - T8 by La Rosa et al. in 1992. Five of these genetic groups were proposed as species: *Trichinella spiralis* sensus stricto (T1) described by Owen in 1835; *Trichinella pseudospiralis (T4)* described by Garkavi in 1972; *Trichinella nativa (T2), Trichinella nelsoni (T7), Trichinella britovi n. sp. (T3)* all described by Britov and Boev in 1972 (Pozio et al., 1992). Three other cluster groups, named T5, T6, and T8 have been recognized in the genus *Trichinella*, but at present their taxonomic level is uncertain.

Trichinella pseudospiralis is differentiated from *Trichinella spiralis* by differences in morphology, alloenzyme characteristics, host spectrum, the absence of a capsule or nurse cell surrounding the skeletal muscle larva (Kramar et al., 1981). Additionally, an interesting property of *Trichinella pseudospiralis* is the apparent ability of the parasite

to down-modulate inflammatory responses directed at larval forms in skeletal muscles (Neva and Brown, 1994). While there is an extensive cellular infiltration of polymorphonuclear neutrophils about the parasitized skeletal muscle of mouse infected by *Trichinella spiralis*, an infection of the mouse with *Trichinella pseudospiralis* is not accompanied by strong host inflammatory cellular response (Stewart and Larsen, 1989).

Among the supporting evidence is a study undertaken by Larsen et al. in 1989 on infection of Chinese hamster (*Cricetulus grigeus*) with *Trichinella pseudospiralis*. Chinese hamster has a strong innate resistance to the muscle phase of *Trichinella spiralis*. This is because the pre-encapsulated larvae in the skeletal muscles of the Chinese hamster are encountered by intense influx of inflammatory cells which surround and rapidly destroy the majority of worms (Ritterson, 1959). On the other hand, a much greater infectivity for the *Trichinella pseudospiralis* is seen in Chinese hamster. This is because the infection with *Trichinella pseudospiralis* is accompanied by markedly low level of myositis and cellular infiltration (Stewart et al., 1982). Larson et al. demonstrated that the concurrent infection of Chinese hamster with these two species of parasite led to an increase in infectivity of *Trichinella spiralis*. They suggested that during concurrent infection, suppression of myositis and cellular infiltration by *Trichinella pseudospiralis* lessens the destruction of *Trichinella spiralis* larvae by the host inflammatory response (Larsen et al., 1991).

Furthermore, Stewart and his co-workers in 1989 showed that modulation of host defenses by *Trichinella pseudospiralis* includes suppression of host inflammatory response and an increase in pulmonary natural killer cell activity. During the course of

this study, they attempted to eliminate natural killer cell activity in vivo by injection of antibody against a natural killer cell surface antigen (asialo GM_1). The outcome was a rapid and high mortality among mice infected with *Trichinella pseudospiralis*, but not among mice infected with *Trichinella spiralis* (Stewart et al., 1989).

Finally, Prulhiere in 1994 compared neutrophil migration in the mouse in response to individual infections with each of the two species of *Trichinella*. He showed that the muscle phase of infection in the mouse by *Trichinella spiralis* was accompanied by myositis and a pronounced neutrophil infiltration, whereas there were none to few neutrophils present in the *Trichinella pseudospiralis* infected muscle fibers (Prulhiere, 1994).

The purpose of my study was to determine whether the variation in inflammatory cell infiltration in the early stages of skeletal muscle infection induced by *Trichinella spiralis* and *Trichinella pseudospiralis* in the mouse was associated with the anti-inflammatory role of prostaglandin E_2. To investigate this we measured and compared the Prostaglandin E_2 level of mice plasma infected individually with each of the two species. This included the quantitation at three different time periods during the first 21 days postinfection. We concluded that the prostaglandin E_2 (PGE_2) plasma level was increased in hosts infected with *Trichinella pseudospiralis* compared with hosts infected with *Trichinella spiralis*.

REVIEW OF LITERATURE

The emigration of inflammatory cells from the blood into the tissues represents one of the most important components of the inflammatory response. Collectively, inflammation is a defensive response to invasion of the host by foreign material, often microbial in nature although mechanical trauma, toxins, and neoplasia also may lead to inflammatory responses. Inflammation involves vasodilation, increased vascular permeability, and cellular infiltration (neutrophils and mononuclear cells).

The local accumulation of leukocytes is commonly preceded by the binding of circulating leukocytes to the vascular endothelium, followed by diapedesis of the phagocytic cells through spaces between the endothelium (Snyderman and Uhing, 1992).

The category of phagocytic inflammatory cells includes neutrophils, eosinophils and mononuclear phagocytic series. Macrophages are central components of the mononuclear phagocytic system. The main role of tissue macrophages is to recruit more phagocytic cells to the site of infection by releasing a battery of cytokines.

The cytokines which are produced by monocytes and macrophages are called monokines. Monokines include interleukin-1, interleukin-6, interleukin-8, interleukin-12, and tumor necrosis factor-α and are synthesized upon recognition of microbial constituents by macrophages. Some of these monokines belong to a group of proteins termed chemokines. Chemokines are synthesized by macrophages, endothelial cells, the keratinocytes, the fibroblasts and smooth cells of connective tissue. The chemokines

serve as chemoattractants for phagocytic cells, recruiting macrophages and neutrophils from the circulation to the site of infection. Chemokines fall into two broad subclasses, α and β. The α chemokines encourage migration of neutrophils and the β chemokines promote migration of monocytes. While classical chemotactic agonists such as the cleavage product of the fifth component of complement (C5a), N-Formyl-methionyl-leucyl-phenylalanine (FMLP), and Leukotriene B_4 (LTB_4) attract different types of granulocytes, IL-8, an α chemokine, appears to be unusually selective for neutrophils (Janeway and Travers, 1994).

Additionally, leukocytes migrate from the circulation to accumulate at sites of inflammation in response to inflammatory mediators. These chemical substances modify blood flow, increase vascular permeability, add to adherence of circulating leukocytes to vascular endothelium, encourage migration of leukocytes into tissues, and stimulate leukocytes to destroy the invading agent (Goldyne and Stobo, 1981). Some of the well characterized mediators of inflammation include: histamine, a vasoactive amine stored performed in cytoplasmic granules of mast cells and basophils; serotonin, a vasoactive amine found in the blood platelets of all mammalians and in most cells of rodents and cattle; and prostaglandin, a group of fatty acids produced by most mammalian cells (Walker and Wilson, 1980).

The most obvious role for prostaglandins in the immune response is proinflammatory activity. The fact that inhibitors of prostaglandin synthesis are nonsteroid anti-inflammatory drugs, with aspirin being a prototype, is the best evidence for predominantly proinflammatory function of prostaglandins. The action of these anti-

inflammatory drugs is based on the inhibition of cyclo-oxygenase, the enzyme which catalyzes the initial conversion of arachidonic acid into the precursors of the various prostaglandins (Goodwin and Webb, 1980). A less well recognized function of prostaglandins is the negative regulation of humoral and cellular immunity, which would be considered an anti-inflammatory function. Due to their dual activity, prostaglandins are referred to as "regulators" or "modulators" of inflammation (Zurier, 1990).

Endogenous sources of arachidonic acid that give rise to prostaglandins and other eicosanoids (20 carbon fatty acids) are contained in the membrane phospholipids of a variety of resting mammalian cells. The synthesis of prostaglandins starts when cells are stimulated by antigenic stimuli (for example, endotoxins or parasite products) to release arachidonic acid. Table 1 summarizes the major sources of prostaglandins in acute inflammation (Davies and MacIntyre, 1992).

Prostaglandins (PGs), referred to as prostanoids, are oxygenation products of arachidonic acid, a long-chain derivative of linoleic acid. The term "prostaglandin" resulted from the erroneous assumption that the prostate gland was a primary source of this activity. It is only in the last 20 years that the chemical structure and the biological activity of many of the members of this family have been established (Higgs and Vane, 1983). Prostaglandins are a group of polyunsaturated, oxygenated 20 carbon atom fatty acids which contain a cyclopentane ring between carbon 8 and carbon 12.

Prostaglandins are named in alphabetical order and currently nine units (shown in Figure 1) are recognized (PGA through PGI). The first prostaglandins identified were named PGE and PGF because of their respective partitioning into ether and phosphate

Table 1. Synthesis of prostaglandins and thromboxanes by cells involved in inflammatory responses (adapted from Davies and MacIntyre, 1992)

	Cyclooxygenase products			
Cell Type	PGE_2	PFG_2	PGI_2	PGD_2
Platelets				
PMN leukocytes				
Mononuclear phagocytes	+			
Lymphocytes			+	
Endothelium	+			
Fibroblasts	+		+	
Mast cells				+

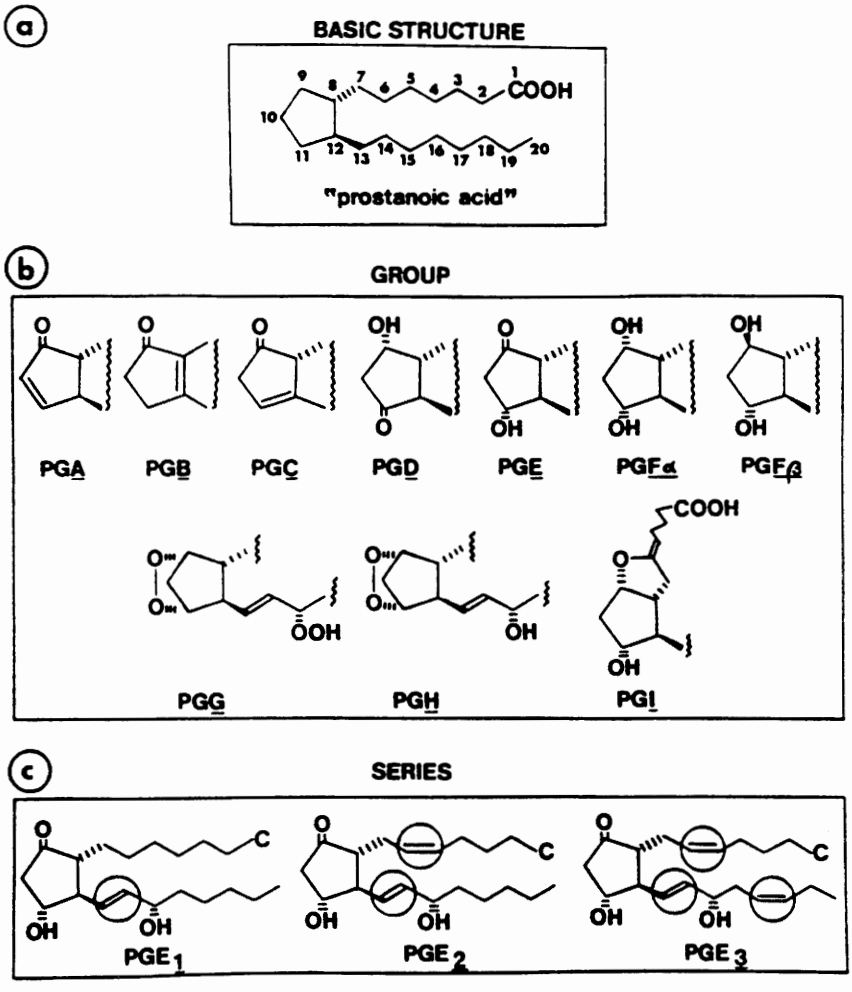

Figure 1. *Prostaglandin nomenclature: (a) basic structure common to all PGs, (b) groups are given letter designations according to the substitutions on the pentane ringe and in the case of PGG on C_{15}; PGFα and PFGβ differ in the orientation of the -OH group on C_9, (c) series are given numerical subscript following group name, which corresponds to number of double bonds in side chains (adapted from Goldyne and Stobo, 1981).*

buffer solvents, "E" representing ether and "F" representing phosphate (Fosfat in Swedish). Prostaglandins are further characterized by the number of double bonds in the molecule. The number of double bonds are indicated by a suffix; for example, PGE_2 has two double bonds (Goldyne and Stobo, 1981).

Prostaglandins are produced when arachidonic acid is metabolized by the enzyme cyclo-oxygenase, also known as prostaglandin G/H synthase. The enzyme catalyzes oxygenation of carbon 11 of arachidonic acid, with the formation of a cyclic endoperoxide in which an oxygen molecule attaches carbon 9 to carbon 11. Incorporation of a second oxygen molecule at carbon 15 produces PGG_2. Subsequently PGH_2 is formed as a result of peroxidase activity (Figure 2) (Davies and MacIntyre, 1992).

Prostaglandins of the E series (PGE) are known to inhibit a number of functions of the immune system (Chouaib et al., 1985). Kloeze in 1967 reported that PGE_1 inhibits platelet aggregation. This led to the notion that the arachidonic acid metabolites may have anti-inflammatory activity (Zurier, 1990). Other investigators then showed in various in vitro and in vivo assay systems that PGE_1 and PGE_2 could serve as inhibitors of inflammatory response.

Zurier et al. in 1973 reported that PGE_1 and PGE_2 suppressed acute carrageenan-induced inflammation and chronic joint inflammation characteristic of adjuvant disease in the rat (Zurier et al., 1973). Thompson and her co-workers in 1984 studied the role of PGE_2 in the regulation of human B cell proliferation in response to *Staphylococcus*

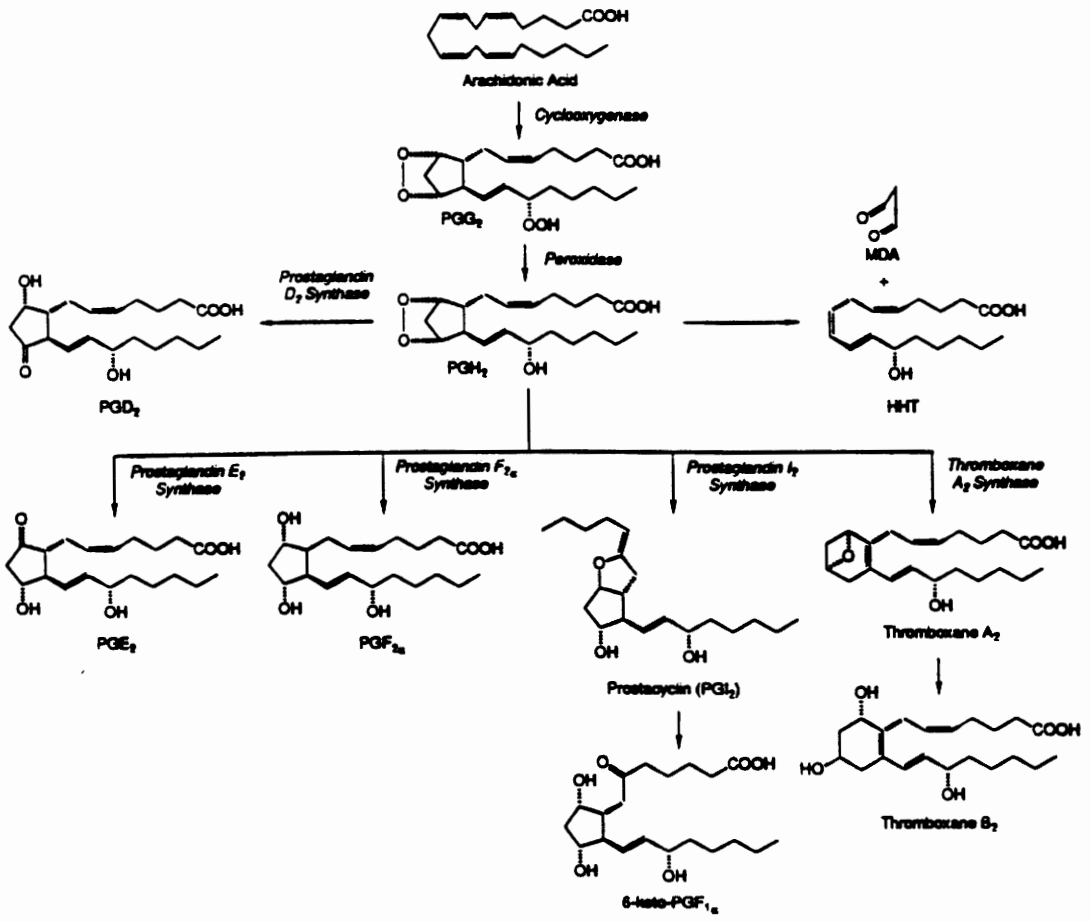

Figure 2. *The synthesis of prostaglandins and thromboxanes (adapted from Davies and MacIntyre, 1992)*

aureus. They found that PGE_2 suppressed B cell DNA synthesis and proliferation stimulated by *Staphylococcus aureus* (Thompson et al., 1984).

Rivkin et al. in 1975 showed that PGE_1 and PGA_1 inhibited neutrophil chemotaxis induced by *Escherichia coli* culture filtrate (Rivkin et al., 1975). Fantone and his co-workers in 1980 investigated the effect of PGE_1 on vasopermeability induced by vasoactive mediators such as histamine and serotonin which initiate inflammatory reactions. They concluded that PGE_1 or its stable derivative, 15-(S)-15-methyl-prostaglandin E_1, markedly reduce the increase in vasopermeability induced by these chemicals (Fantone et al., 1980).

Rossi and O'Flaherty in 1989 showed that PGE_2 and PGD_2 inhibited polymorphonuclear (PMN) neutrophil degranulation responses to infection-induced leukotriene B_4 (leukotriene B_4 is produced by cells of myelomonocytic lineage and is a potent proinflammatory mediator which induces chemotaxis, chemokinesis, aggregation, and degranulation of PMN leukocytes, as well as PMN adherence to endothelial cells. It is also a chemoattractant for monocytes and amplifies inflammatory reactions) (Rossi and O'Flaherty, 1989).

Raud and his associates in 1988 conducted an experiment in hamsters to characterize the influence of PGE_2 and indomethacin (a nonsteroidal anti-inflammatory drug which inhibits cyclooxygenase) on microcirculatory changes during inflammation induced by antigen challenge. They revealed that indomethacin reduced antigen-induced vasodilation while potentiating plasma leakage, leukocyte accumulation and histamine release of the mast cells in cheek pouches of immunized hamsters. Then they observed

that the topical application of PGE_2 reversed the indomethacin-induced inflammatory responses to antigen. Prostaglandin E_2 induced a marked vasodilation and effectively reversed the enhanced number of emigrated leukocytes after indomethacin treatment (Raud et al., 1988).

All of the above studies support the concept that several prostaglandins, mainly prostaglandins of the E series, predominantly inhibit acute inflammation. Further investigations are necessary to unravel the functions of eicosanoid mediators in the inflammatory response. I have taken a step toward these directions by measuring and comparing the plasma PGE_2 level in the mouse infected with *Trichinella pseudospiralis* or *Trichinella spiralis*.

MATERIALS AND METHODS

Animals and Parasites

Six-to-eight-week-old MRL^{++} mice were used as experimental hosts and controls. Animals were maintained on a 12-hour light-dark photoperiod and provided water and commercially prepared food ad libitum. The care of all animals was in accordance with the guidelines set forth by the National Institute of Health provided in the "Guide for the Care and Use of Laboratory Animals".

The Indiana bore strain of *Trichinella spiralis* isolated by Kazacoskr (Kazacoskr et al., 1986) was obtained from Dr. Green, University of Missouri, Columbia. *Trichinella pseudospiralis* was obtained from H. Ray Gambel, USDA, Beltsville, Md. Both species of parasites were maintained through passage in rats and mice at Portland State University.

Establishment of Infection

Infective larvae were isolated from the carcasses of older MRL^{++} mice infected with *Trichinella spiralis* or *Trichinella pseudospiralis*. Mice were killed by cervical dislocation and skeletal muscle containing the infective larvae were recovered. Small pieces of muscle tissue containing approximately 150-200 infective larvae were selected. The presence of larvae in each tissue portion was confirmed by examining it under a dissecting microscope.

To assure rapid consumption of the infected tissue, the mice were restricted from food for approximately 15-20 hours prior to infection. A panel of five to six mice, held individually in a cage without litter, were fed with *Trichinella spiralis* or *Trichinella pseudospiralis* infected tissue that was moistened with water. During the feeding period, the mice were periodically observed to ensure that the tissue pieces were moist and available to each animal. When the meat pieces were consumed, the mice would be returned to the diurnal rhythm of light with food and water ad libitum.

Additionally, five to six mice were left uninfected to serve as control for provision of in vivo base line concentrations of prostaglandin E_2.

Blood Collection and Processing

Animals were anesthetized with Metophane approximately 5-10 minutes before blood collection (Metophane was obtained from Oregon Health Sciences University, Animal Care Center). Fresh blood was collected by cardiac puncture from 5-6 mice of each infection on 5, 11, and 21 days postinfection (dpi). The control specimen was obtained by bleeding 5-6 normal, uninfected mice and pooling the collection into one control specimen. Once drawn into a sterile syringe, the blood was immediately injected into 3 mL vacutainer tubes containing the anticoagulant ethylenediaminetetraacetic acid (EDTA).

Following blood collection each mouse was evaluated for infection. A midline laparotomy was performed and several sections of the host intestine and diaphragm were removed for microscopic evaluation on the stage of infection. The infection of the host was assessed by visually observing the adult worms of each species in the host intestine

at day 5 postinfection. Additionally, I examined diaphragms from infected mice which appeared normal at this time, suggesting that the larvae had not yet reached the diaphragm in sufficient number to be easily seen.

Upon examination of the host diaphragm at day 11 postinfection, we observed the presence of a few larvae. Therefore, an early stage of muscle infection was confirmed in the host infected with each species.

Finally, I found a great number of larvae in the host diaphragm at day 21 postinfection. This was indicative of a heavy infection of each species in the host.

The blood samples were centrifuged at 1500 x g for 13 minutes at room temperature. Plasma was removed and delivered to sterile polypropylene tubes. Each sample and control tube was composed of a pool of 2 - 2.5 mL plasma from five to six mice. All plasma samples were stored at -80°C and assayed within five weeks.

Plasma Purification

Prior to assaying for PGE_2, all plasma samples were obtained from -80°C, thawed at room temperature and purified. The purpose of purification was to minimize the interferences resulting from many substances contained in the plasma, especially active proteins.

Plasma samples were also concentrated 10 fold by purifying a larger volume of the sample and reconstituting in a small amount of buffer. The purpose of concentration was to bring the expected level of PGE_2 content of samples within the readable range of the standard curve. A 2 mL aliquot of each plasma sample was placed into a clean test tube. Methanol at 8 mL was added to the samples. The samples were vortexed and

allowed to stand at 4°C for five minutes. The samples were then centrifuged at 3000 x g for 10 minutes at room temperature to remove the precipitated proteins.

The supernatant was transferred into a clean container and 32 mL of 0.1 M phosphate buffer, pH 4, was added and the container was vortexed briefly. Sep-pak C_{18} (C_{18}) cartridges (Water Associates; Milford, MA) were assembled by removing the plunger from a 10-milliliter sterile syringe and placing the long end of a C_{18} cartridge on the luer tip of the syringe barrel. The C_{18} cartridges were then activated one at a time by rinsing with 5 mL methanol (see Appendix A) and then with 5 mL ultrapure water (see Appendix B).

Each sample was passed through an activated C_{18} cartridge. The cartridge was rinsed with 5 mL ultrapure water followed by 5 mL of HPLC grade hexane. Both washes were discarded and expected PGE_2 was eluted to a clean test tube with 5 mL ethyl acetate containing 1% methanol. The ethyl acetate was evaporated to dryness under a stream of dry nitrogen. Enzyme Immunoassay (EIA) buffer at 200 mL was added and the tube was vortexed. The sample was then ready for quantitation assay.

Prostaglandin E_2 Kit

A prostaglandin E_2 - Monoclonal Enzyme Immunoassay Kit was purchased from Cayman Chemical Company; Ann Arbor, MI and stored at -20°C until used. The kit is validated for use with urine, culture media and plasma samples, and has an assay sensitivity of 7.8 pg/mL. Table 2 summarizes the specificity of the kit for PGE_2 compared with other prostaglandins (PG_s).

Table 2 Prostaglandin E_2 - Monoclonal Enzyme Immunoassay Kit Specificity

Prostaglandin	% Specificity
PGE_2	100%
PGE_3	43%
PGE_1	18.7%
6-Keto PG $F_{1\alpha}$	1%
PGA_1	< 0.01%
PGA_3	< 0.01%
PGB_1	< 0.01%
PGB_2	< 0.01%
PGD_2	< 0.01%
15-Keto PGE_2	< 0.01%
13, 14 - Dihydro -15-Keto $PGF_{2\alpha}$	< 0.01%
13, 14 - Dihydro -15-Keto PGE_2	< 0.01%
$PGF_{1\alpha}$	< 0.01%
$PFF_{2\alpha}$	< 0.01%
$PGF_{3\alpha}$	< 0.01%
Thromboxane B_2	< 0.01%

The kit contains a 96 well microtiter plate. The wells are pre-coated with goat anti-mouse polyclonal antibody. The polyclonal antibody is produced as follow: PGE_2 hapten which is attached to a strong immunogen is injected into a recipient mouse to produce an antibody responsive to PGE_2. This antibody serves as a haptenic antigen for injection into the goat. The antibodies produced by the goat are anti-mouse polyclonal antibody.

Among other contents of the Kit is PGE_2 - acetylcholinesterase conjugate. Acetylcholinesterase is isolated from the electric organ of the electric eel, *Electrophorus electricus*. This stable enzyme is covalently attached to a molecule of the analyte (PGE_2) and serves as a tracer in the test system. The enzyme is capable of high turnover for the hydrolysis of acetylthiocholine. Quantification of the tracer is accomplished by measuring its acetylcholinesterase activity with Ellman's Reagent provided in the kit.

Ellman's Reagent is made up of acetylthiocholine and 5, 5'-dithio-bis-(2-nitrobenzoic acid). Acetylcholinesterase hydrolizes the acetylthiocholine to produce thiocholine (Figure 3). The 5, 5'dithio-dis-(2-nitrobenzoic acid) will then nonenzymatically react with thiocholine to produce 5-thio-2-nitrobenzoic acid which has a strong absorbance at 412 nm.

The test is performed by adding all reactants (samples containing PGE_2, PGE_2 tracer, and PGE_2 monoclonal antibody) to the goat anti-mouse coated wells. The assay is based on the competition between sample PGE_2 and a PGE_2 tracer for a limited amount of PGE_2 monoclonal antibody. Since the concentration of the PGE_2 tracer is constant, yet the concentration of sample PGE_2 is variable, the amount of PGE_2 tracer

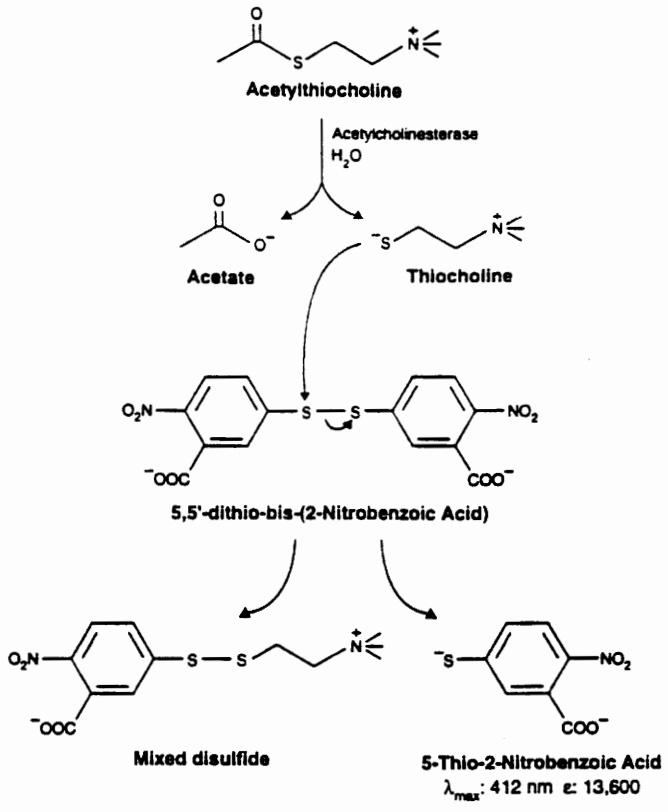

Figure 3. Reaction catalyzed by acetylcholinesterase (adapted from Cayman Chemical Company instruction handbook)

which binds to the PGE_2 monoclonal antibody is inversely proportional to the concentration of sample PGE_2.

The antibody-PGE_2 complex will attach to the attached goat anti-mouse antibody in the wells. The plate is washed to remove any unbound reagents. In the final step an appropriate substrate (Ellman's Reagent) is added to the wells. The enzyme hydrolyzes the substrate, forming a distinct yellow color whose intensity, determined spectrophotometrically, is proportional to the amount of PGE_2 tracer bound to the well. A schematic of this process is shown in Figure 4.

Preassay Preparation

The Enzyme Immunoassay Buffer and the wash buffer were made by dissolving the content of EIA and wash buffer packet (proprietary formulation) in 500 mL ultrapure water. Tween 20 at 0.25 mL was added to the wash buffer and mixed by swirling. Both buffers were stored at 4°C until needed. PGE_2 tracer and PGE_2 monoclonal antibody were reconstituted with 6 mL EIA buffer and stored at 4°C.

A vial of PGE_2 standards was reconstituted with 1 mL EIA buffer. The concentration of this solution (the bulk standard) was 10 ng/mL. To prepare the standards for establishing an eight point standard curve, 8 test tubes were obtained and labeled #1 through #8. 900 μL of EIA buffer was added to tube #1 and 500 μL EIA buffer was added to tubes #2-8. 100 μL of the bulk standard (10 ng/mL) was transferred to tube #1 and mixed thoroughly. The standard was serially diluted as follows: 500 μL of solution from tube #1 was transferred into tube #2. After sufficient mixing, 500 μL solution from tube #2 was transferred to tube #3 and was mixed well. This process was

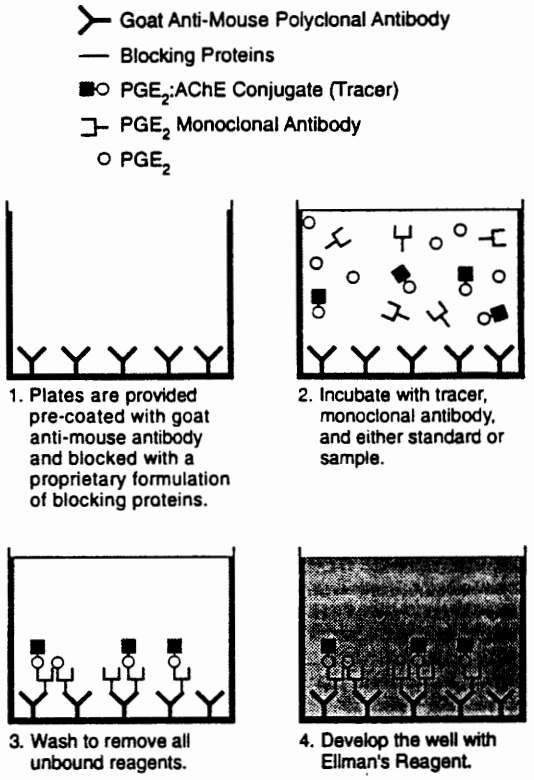

Figure 4. Schematic of Enzyme Immunoassay (adapted from Cayman Chemical Company instruction handbook)

repeated for tubes #4-8. The product of this dilution represented standards numbered 1 through 8; with concentration of 1000, 500, 250, 125, 62.5, 31.3, 15.6, and 7.8. Standards were stored at 4°C and used within one hour of preparation.

Plate Set Up

Plate was prepared to contain two blanks (B), two non-specific binding wells (NSB), two maximum binding wells (B_o), and an eight point standard curve, run in duplicate (Appendix C contains definition of key terms). Each sample was assayed in duplicate. We also included a 1:2 dilution of each sample, set in duplicate, in the assay to confirm the samples appropriate purification. This is because a significant disparity between the apparent concentration of two different dilutions of the same sample would indicate interference. Plate format, and the location and contents of the wells is shown in Table 3.

Performing the Assay

Plate packet was opened immediately prior to use and wells were rinsed once with wash buffer. The unused strips were put back in the plate packet without rinsing and stored at 2-4°C.

The EIA buffer at 100 μL was added to non-specific binding (NSB) wells. The EIA buffer at 50 μL was added to maximum binding (B_0) wells. The PGE_2 standards (S_1 -S_8) at 50 μL were added to the respective wells. Each sample and each 1:2 diluted sample at 50 μL was added to the appropriate wells (see Test Plate Format; Table 3). The PGE_2 tracer at 50 μL was added to each well, except the total activity (TA) and the

Table 3. Test Plate Format showing the location and contents of wells

	1	2	3	4	5	6	7
A	B	S_1	S_5	Ts (UND) 5 dpi	Ts (1:2) 5 dpi	Tp (UND) 5 dpi	Tp (1:2) 5 dpi
B	B	S_1	S_5	Ts (UND) 5 dpi	Ts (1:2) 5 dpi	Tp (UND) 5 dpi	Tp (1:2) 5 dpi
C	NSB	S_2	S_6	Ts (UND) 11 dpi	Ts (1:2) 11 dpi	Tp (UND) 11 dpi	Tp (1:2) 11 dpi
D	NSB	S_2	S_6	Ts (UND) 11 dpi	Ts (1:2) 11 dpi	Tp (UND) 11 dpi	Tp (1:2) 11 dpi
E	B_0	S_3	S_7	Ts (UND) 21 dpi	Ts (1:2) 21 dpi	Tp (UND) 21 dpi	Tp (1:2) 21 dpi
F	B_0	S_3	S_7	Ts (UND) 21 dpi	Ts (1:2) 21 dpi	Tp (UND) 21 dpi	Tp (1:2) 21 dpi
G	B_0	S_4	S_8	Control	Control 1:2		
H	TA	S_4	S_8	Control	Control 1:2		

Key: (B) Blank; (TA) Total Activity; (NSB) Non-Specific Binding; (B_0) Maximum Binding; (S1-S8) Standards 1-8; (Ts) *Trichinella spiralis* (Tp) *Trichinella pseudospiralis*; (UND) Undiluted sample; (1:2) 1:2 diluted sample.

blank (B) wells. The PGE_2 monoclonal antibody at 50 μL was added to each well except the total activity (TA), the non-specific binding (NSB), and the blank (B) wells.

The plate was covered with plastic film and incubated for 18 hours at room temperature. After incubation was completed, a vial of Ellman's Reagent was reconstituted with 20 mL ultrapure water. The wells were emptied and rinsed five times with wash buffer, followed by addition of 200 μL Ellman's Reagent to each well. In addition 5 μL of tracer was added to the TA well. The plate was covered with plastic film and rotated on an orbital shaker for 90 minutes. This process is referred to as development. Following development, the plate was read at 405 nm using a microplate autoreader model EL311 (Bio-Tek Instruments, Inc.; Winooski, VT).

RESULTS

The absorbance readings, raw data, from the wells, are shown in Table 4. The results were calculated manually by averaging the absorbance readings from the NSB and B_0 wells and then subtracting the NSB average from the B_0 average. This resulted in the corrected B_0 (corrected maximum binding). The % B/B_0 (percent sample or standard bound/maximum bound) was calculated for the remaining wells by subtracting the average NSB absorbance from each sample and standard absorbance and dividing the value by the corrected B_0. The result was then multiplied by 100 to obtain % B/B_0. The results of corrected B_0 and % B/B_0 for all standards and samples are presented in Table 5.

NOTE:	The plate reader had subtracted the absorbance readings of the blank wells from the absorbance readings of the rest of the plate.

Using Sigma Plot software, version 5.0, a standard curve was generated by plotting % B/B_0 for standards S1-S8 versus PGE_2 concentration in pg/mL (Figure 5). I determined the concentration of each sample by identifying the % B/B_0 on the standard curve and reading the corresponding values on the x-axis. Since samples were assayed in duplicate, an average of the two absorbance readings was used to determine the PGE_2 concentration.

Table 4. Presentation of raw data; the Location of each well corresponds to that of plate format presented in Table 3.

	1	2	3	4	5	6	7
A	0.000	0.034	0.217	0.118	0.181	0.072	0.148
B	0.000	0.033	0.209	0.121	0.175	0.065	0.152
C	0.005	0.057	0.246	0.125	0.194	0.062	0.146
D	0.003	0.055	0.251	0.131	0.189	0.071	0.148
E	0.309	0.101	0.294	0.168	0.209	0.051	0.114
F	0.308	0.096	0.275	0.175	0.215	0.058	0.109
G	0.309	0.156	0.306	0.118	0.245		
H	0.998	0.158	0.302	0.121	0.249		

Table 5. Corrected Maximum Binding, and % Sample and Standard Bound/Maximum Bound (% B/B_0)

	Sample	Corrected B_0	% B/B_0
Standards	S1	0.03 0.029	9.84 9.51
	S2	0.053 0.051	17.38 16.72
	S3	0.097 0.092	31.80 30.16
	S4	0.152 0.154	49.84 50.49
	S5	0.213 0.205	69.83 67.21
	S6	0.242 0.247	79.34 80.98
	S7	0.290 0.271	95.08 88.85
	S8	0.302 0.298	99.02 97.70
Trichinella spiralis	5 dpi	0.114 0.117	37.37 38.36
	11 dpi	0.121 0.127	39.67 41.16
	21 dpi	0.164 0.171	53.77 56.07
	5 dpi (1:2)	0.177 0.171	58.03 56.07
	11 dpi (1:2)	0.190 0.185	62.30 60.66
	21 dpi (1:2)	0.205 0.211	67.21 69.18
Trichinella pseudospiralis	5 dpi	0.068 0.061	22.30 20.00
	11 dpi	0.058 0.067	19.00 21.96
	21 dpi	0.047 0.054	15.41 17.70
	5 dpi (1:2)	0.144 0.148	47.21 48.52
	11 dpi (1:2)	0.142 0.144	46.56 47.21
	21 dpi (1:2)	0.110 0.105	36.06 34.43
Control	Undiluted	0.102 0.097	33.44 31.80
	Diluted (1:2)	0.241 0.244	79.02 80.00

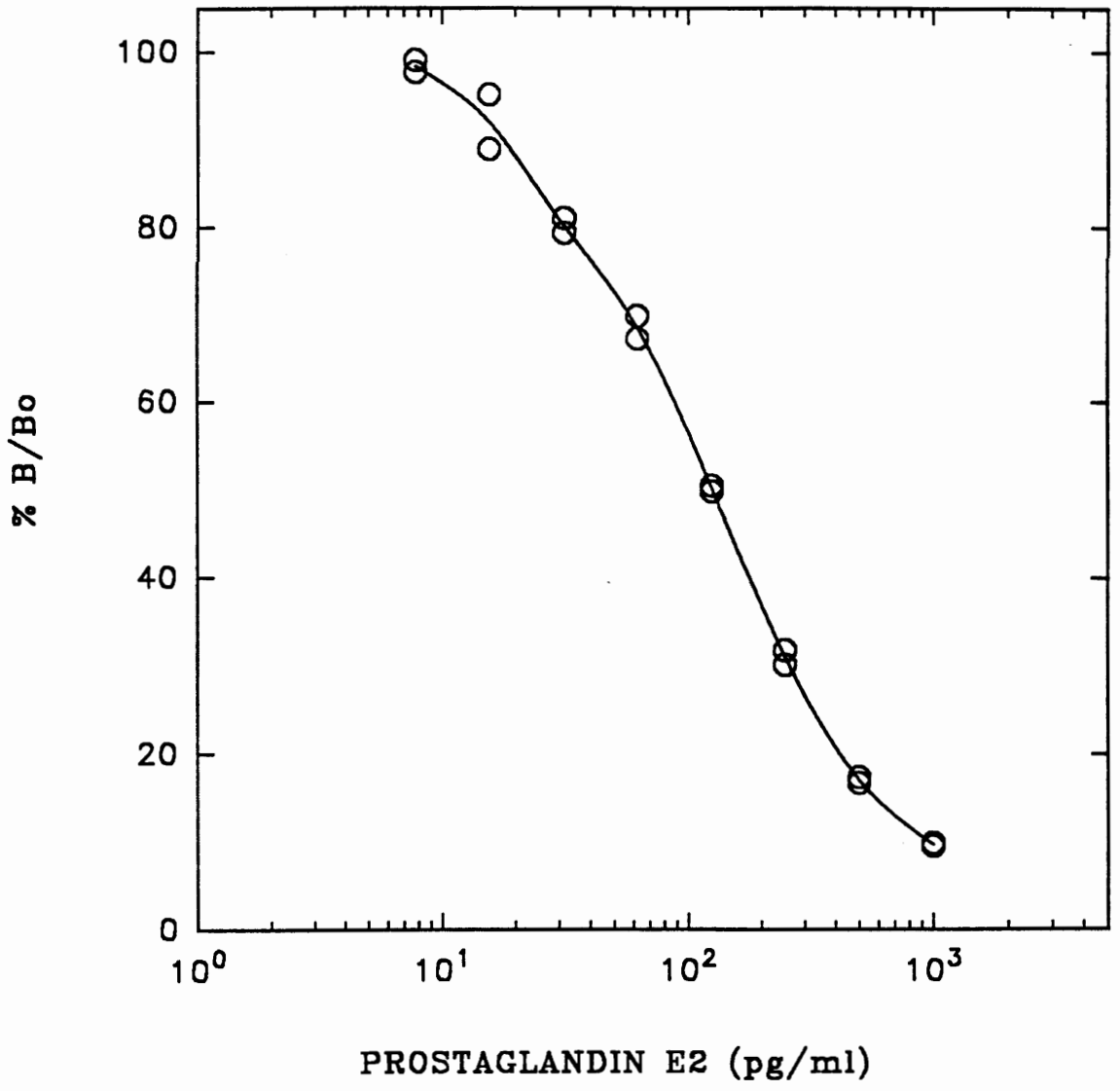

Figure 5. Standard curve for PGE_2 using the Prostaglandin E_2 monoclonal enzyme immunoassay kit.

The concentrations of PGE_2 in the plasma from the various groups of mice are summarized in Table 6. Because the samples were concentrated by a factor of 10 in purification process, the values obtained for PGE_2 concentration were divided by 10. As shown in Table 6, PGE_2 concentration was increased in mice infected with *Trichinella pseudospiralis* compared with uninfected mice. While there was a significant elevation of 21 dpi, samples collected 5 dpi and 11 dpi exhibited varying, but not significantly different levels of PGE_2. The PGE_2 levels, on the other hand, were decreased in mice infected with *Trichinella spiralis*. Samples obtained at various time intervals showed various levels of PGE_2. A comparison of PGE_2 levels in infected and uninfected animals is presented in Figure 6. The concentrations of samples diluted at 1:2 were not determined. This was because a number of the % B/B_0 values would fall out of the linear range of the standard curve due to the low PGE_2 concentration of the diluted samples. However, I estimated the concentration of some of the diluted samples whose % B/B_0 were determinable on the standard curve (data not shown). These values, considering their dilution factor, were within 10% of those of undiluted samples. This reassured that the purification process on the plasma had eliminated the possible interferences.

Table 6. PGE_2 concentration of known standards (shaded) and unknown various groups of mice (unshaded).

Sample	PGE_2 Concentration pg/mL
S1	1000
S2	500
S3	250
S4	125
S5	62.5
S6	31.3
S7	15.6
S8	7.8
TS; 5 dpi	19
TS; 11 dpi	17
TS; 21 dpi	11.5
TP; 5 dpi	29
TP; 11 dpi	29.5
TP; 21 dpi	40
Control (Uninfected)	24

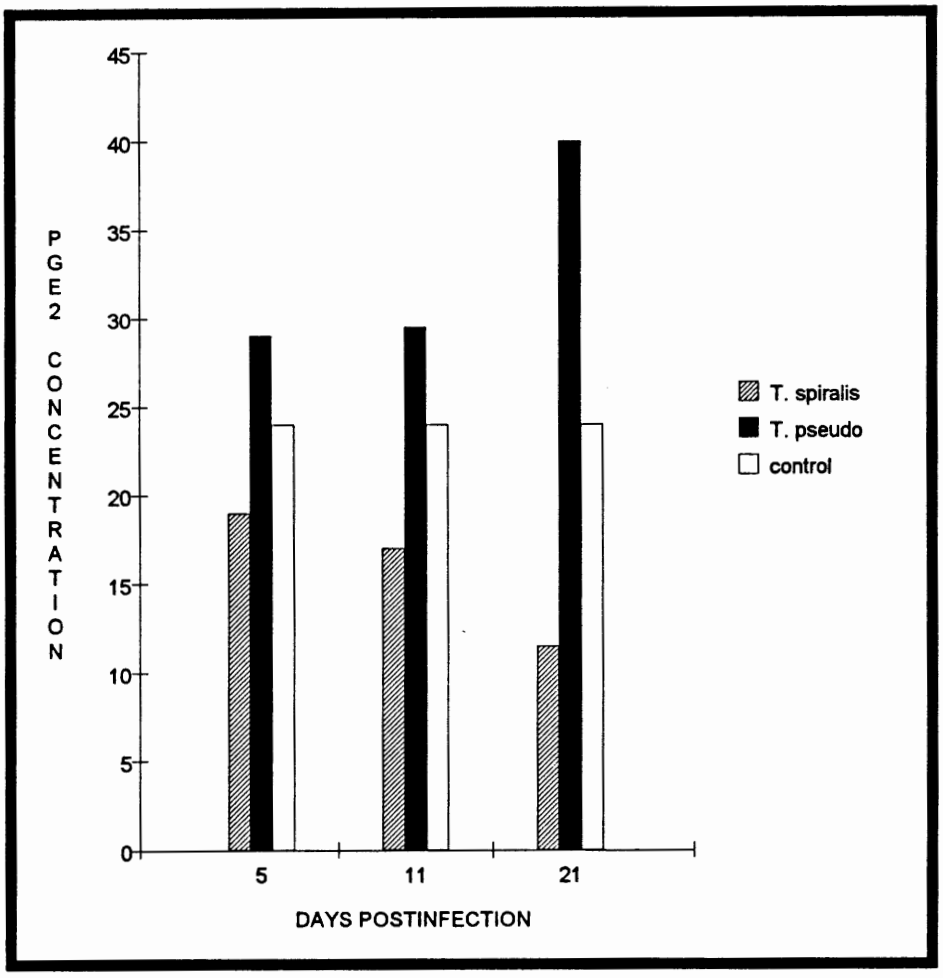

Figure 6. Comparison of PGE_2 variation in the mouse infected with *Trichinella spiralis* versus *Trichinella pseudospiralis* at various intervals postinfection. Normal control values are included for comparison. PGE_2 reported values are in pg/mL.

DISCUSSION

Host protective immunity to the skeletal muscle dwelling larvae of the nematode *Trichinella spiralis* is mediated by an extensive cellular infiltration. Opposingly, the muscle stage infection of *Trichinella pseudospiralis* does not elicit an immune response that is characterized by elevated levels of inflammatory phagocytic cells.

Inflammatory cells are recruited to the relevant site of inflammation via a number of mediators and enzymes. These cellular products are either released from granules and other cytoplasmic storages or are synthesized *de novo* from membrane phospholipids.

The purpose of this study was to determine a change in concentration of PGE_2 in the mouse plasma as a possible correlation between these differences in host inflammatory response to infection with *Trichinella spiralis* versus *Trichinella pseudospiralis*.

I approached my investigation by measuring circulatory PGE_2 levels in each one of the infections and then assumed a correlation between possible variations of these measurements and the host inflammatory response to each infection observed in previous studies.

The results obtained in this study showed elevated levels of PGE_2 in mice infected with *Trichinella pseudospiralis* compared with uninfected animals. Conversely, we observed a decreased PGE_2 level in the host infected with *Trichinella spiralis*.

Therefore, I suggest an anti-inflammatory action for PGE_2 based on the assumption that the inhibition of neutrophil migration in response to *Trichinella pseudospiralis* infection is associated with the increase of PGE_2 level. The observed decreased level of PGE_2 in this study and its possible relation to the enhancement of cellular infiltration in infection with *Trichinella spiralis* is in accordance with our suggestion for anti-inflammatory role of PGE_2.

Prostaglandin E_2 has been extensively studied in association with inflammation. This fatty acid has been regarded as proinflammatory mediator since it is a strong vasodilator and can act synergistically with other inflammatory mediators (Leal-Berumen et al., 1995). However, there are circumstances when PGE_2 may have an anti-inflammatory role. For example, it has been reported that PGE_2 blocks a variety of responses to chemotactic peptides (O'Flaherty et al., 1979).

Nonetheless, the literature in this field contains a number of conflicting observations. Several in vivo studies have shown that the neutrophil accumulation at local sites of inflammation is enhanced by PGE_2 (Issekutz, 1981; Issekutz and Movat, 1982; Buckley et al., 1991). Additionally, Dong et al. in 1993 demonstrated that PGE_2 concentration in burned-infected rats enhanced neutrophil migration (Dong et al., 1993).

On the other hand, Goetzl and Gorman in 1977 revealed that the PGE_2 exhibited negative chemokinetic activity and inhibited chemotactic migration of PMNs (Goetzl and Gorman, 1977). Furthermore, Raud and his co-workers in 1988 reported that PGE_2 reduced the number of emigrated leukocytes (Raud et al., 1988). The inhibitory action

of PGE_2 that I have theorized based on our observations is in accordance with the latter reports.

After an extensive search of literature in attempt to understand the mechanism(s) underlying the immunosuppressive action of PGE_2, I found three possible mechanisms by which the anti-inflammatory role of PGE_2 might be explained.

One possible mechanism may be that the PGE_2 mediates its effect on neutrophil migration through cyclic- 3', 5'-adenosine monophosphate (cAMP).

Bjornson et al. in 1992 demonstrated that intracellular cAMP is increased in PMNs following thermal injury in the guinea pig, and the elevation of intracellular cAMP had important effect on defective chemotaxis of the PMNs in response to chemoattractants (Bjornson et al., 1992). Furthermore, it was found that PGE_2 increased cAMP level in human mononuclear cells which in turn inhibited the mitogenesis of the cells (Goodwin and Webb, 1980). Also, Chouaib and his associates in 1985 demonstrated that PGE_2 inhibits T lymphocyte activation and proliferation by increasing the intracellular concentration of cAMP.

I propose that the following sequence of events may be responsible for the suppression of inflammatory response observed in mice infected with *Trichinella pseudospiralis*.

When macrophages encounter inflammatory stimuli (*Trichinella pseudospiralis* antigens), they respond by releasing a number of products including PGE_2 (Humes et al., 1977). This is accomplished by phagocytosis of the antigen which triggers the release of lysosomal phospholipases. The phospholipases hydrolyze phospholipids of cell

membranes and yield arachidonic acid. The production of PGE_2 is by enzyme activity of cyclooxygenase on produced arachidonic acid (Higgs and Vane, 1983). The PGE_2 synthesized and secreted by macrophages can then modulate the action of other immunocompetent cells, including neutrophils. Prostaglandin E_2 activates the cAMP-dependent intracellular signal transduction pathway in neutrophils by binding to the specific receptors on neutrophil plasma membrane (Alberts et al., 1989; Becker and Deamer, 1990).

The neutrophil receptors activate a group of membrane-bound guanosine triphosphate-binding proteins, called G-proteins. When active, the G-proteins bind guanosine triphosphate (GTP) and activate the enzyme adenylate cyclase which catalyzes the reaction that converts adenosine triphosphate (ATP) to cAMP (Tizard, 1995). Thus, the intracellular cAMP level increases and activates cAMP-dependent protein Kinase A. This enzyme phosphorylates myosin light-chain kinase which in turn greatly reduces the affinity of the kinase for the calcium calmodulin complex (Becker and Deamer, 1990). The light-chain kinase catalyzes the process of myosin molecules aggregation into small dipolar filaments. The kinase itself is activated by Ca^{2+} - calmodulin complex (Barrett, 1988). In this way PGE_2 inhibits migration of neutrophils because in these cells directed migration requires actin filaments (Alberts et al., 1989).

In addition to the proposed sequence of event, the elevated cAMP may exhibit its effect through the suppression of chemokines. Strieter et al. in 1988 reported that an increase in cAMP interfered with endotoxin-induced transcription of the Tumor Necrosis Factor (TNF-α) gene. Tumor Necrosis Factor is known to dramatically increase the

binding of neutrophils to endothelium (Bouma et al.,1994). In vivo migration of neutrophils to sites of acute inflammation requires the adhesion and binding of these cells to vascular endothelial cells (Hallman et al., 1991). Therefore, elevated cAMP may inhibit the neutrophil migration into tissues by suppressing the production of TNF-α, the chemokine which mediates the neutrophil attachment to vascular endothelium.

Regardless of its mode of action, increased cAMP appears to decrease neutrophil migration into tissues.

The second possible mechanism for the inhibitory role of PGE_2 on neutrophilic response is associated with corticosteroids. PGE_2 is known to increase steroidogenesis (Zurier et al., 1973). This effect of PGE_2 is well reviewed in literature (Flack et al., 1969; Saruta and Kaplan, 1971). Corticosteroids decrease neutrophil adherence to vascular endothelium (margination) and reduce neutrophil emigration into inflamed tissues. Corticosteroids also depress abilities of neutrophils to phagocytise and respond to chemokinesis (Tizard, 1995). Thus, the anti-inflammatory action of PGE_2 in relation to its steroidogenesis activity appears to be based on inhibition of neutrophil atachment to vascular endothelial cells prior to migration surrounding tissues.

The third proposed mechanism relates to the possible effect of PGE_2 on a neutrophil glycoprotein expression. Lewinsohn et al. in 1987 showed that MEL-14 antigen, a neutrophil surface glycoprotein defined by the monoclonal antibody MEL-14, inhibits the capacity of neutrophils to migrate from the blood into sites of acute inflammation by blocking the interaction of neutrophils with endothelial cells. Therefore considering the capacity of PGE_2 to suppress neutrophil migration, it is reasonable to

hypothesize that PGE_2 may enhance the expression of the surface MEL-14 antigens on neutrophils.

SUMMARY

My findings support the hypothesis that PGE_2 may be an inhibitor of the inflammatory process in *Trichinella pseudospiralis* , particularly acting on the migration of neutrophils. It is obvious, however, that before a definitive statement can be made with regard to the exact relationship between PGE_2 and neutrophil movement, it will be necessary to conduct additional studies to determine the precise mechanism(s) by which PGE_2 modulates this anti-inflammatory response.

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APPENDIX A

Sep-Pak C_{18} Cartridge Sample Preparation Strategy

After placing the sample on the cartridge:

A. Eliminate high polarity compounds by washing the sep-park cartridge with a polar solvent. The polar solvent should be chosen so that the compound(s) of interest is retained on the cartridge.

B. Remove the compound(s) of interest by washing the sep-park cartridge with a less polar solvent. The cartridge eluent collected can now be brought to a known volume for final analysis.

C. The non-polar compounds still remain on the cartridge. Additional compounds can be eluted with a non-polar solvent, or discarded along with the spent cartridge.

NOTE:	When using the sep-pak C_{18} cartridge with aqueous samples, it is necessary to pre-wet the cartridge with about 5 mL of a water-miscible solvent such as methanol and then with about 5 mL of clean water.

APPENDIX B

Source of Ultrapure Water

Water used to activate C_{18} cartridges and to prepare all EIA reagents and buffer was deionized and free of trace organic contaminants ("Ultra Pure"). Four types of filters were used in the water deionizer:

a. Pre-filter Cartridge
b. Two Ion-exchange (millipore) cartridges
c. Activated charcoal (millipore) cartridge
d. 0.2 micron cross flow filter

The water met the requirements of the College of America Pathology (CAP) and the National Committee for Clinical Laboratory Standards (NCCLS) for resistivity and minimal contamination.

Sybron/Barnstead Deionized water system:

Filter: Barnstead/Thermolyne
Catalog # D0809: Ultrapure DI cartridge
Lot #31 / Expiration Date: 12/95

Rosin description: Di Vinyl Benzene Styrene manufactured by Rohm and Haaf.

APPENDIX C

Definition of Key Terms

Blank: Background absorbance caused by Ellman's Reagent. Even freshly prepared Ellman's Reagent has some measurable absorbance, approximately 0.2 Absorbance Units (A.U.). The blank absorbance should be subtracted from the absorbance readings of all the other wells.

Total Activity: Total enzymatic activity of the acetylcholinesterase-linked tracer. This is analogous to the specific activity of a radioactive tracer.

NSB (Non-Specific Binding): Non-immunological binding of the tracer to the well. Even in the absence of specific antibody a very small amount of tracer still binds to the well; the NSB is a measure of this low binding.

B_0 (Maximum Binding): Maximum amount of the tracer that the antibody can bind in the absence of free analyte.

B/B_0 (Bound/Maximum Bound): Ratio of the absorbance of a sample or standard well to that of the maximum binding (B_0) well.

Standard Curve: A plot of the B/B_0 values versus concentration for a series of wells containing various known amounts of analyte.