Development of a Quantitative Polymerase Chain Reaction Procedure for Analysis of Cytokine Responses to Strongylus Vulgaris in Ponies.

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DEVELOPMENT OF A QUANTITATIVE POLYMERASE CHAIN REACTION PROCEDURE FOR ANALYSIS OF CYTOKINE RESPONSES TO STRONGYLUS VULGARIS IN PONIES

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in

The Interdepartmental Program in Veterinary Medical Sciences through the Department of Veterinary Microbiology and Parasitology

by

Cyprianna E. Swiderski
D.V.M., Virginia Polytechnic Institute and A & M College, 1987
May 1998
DEDICATION

Dedicated to my father, whose tremendous love, support, and thirst for knowledge have always been an inspiration to me. I could not have done this without you - Thank you.
How many people does it take to complete a dissertation? Those who believe it to be the work of one either have not attempted the feat or are not honest. To quote my mentor during my residency, Dr. Jill McClure, it takes a village and to the members of my village I am eternally grateful. These members provided support to the mind and body and without any one the task could not be completed.

I was first introduced to Dr. Horohov by Dr. Jill McClure in the fall of 1989. I had a raw fascination with immunology at that time which stemmed from a case of selective IgM deficiency I had diagnosed while in private practice. Accordingly, I enrolled in the cellular immunology course and was impressed by Dr. Horohov’s ability to teach. Here, I thought, is someone from whom I can learn. Dr. Horohov’s teaching style is well recognized for its efficacy and each year students new to his classroom have always made positive comments to me about this talent. Unfortunately, my foundations in cellular and molecular biology were pitiful at that time after having only two years of college, and so like clockwork, after every few lectures, Dr. Horohov would scan through copious pages of questions that I had formulated from the previous lectures, questions that reflected the huge gaps in my knowledge base, but he patiently answered, allowing me to build the foundation that lead to this study. During those early years I recognized personal qualities in Dr. Horohov that I have always valued and led me to choose him as my Ph.D. advisor. These are: patience, free thought both in his dealings with people and interactions with science, and a willingness to support those people and things in whom/which he believes. This was displayed in his continued support of my
program after the birth of my daughter, Katelyn. Given the other options that life brings, I am sure that without his support I would not have completed my program.

I have also been extremely fortunate to work with Dr. Tom Klei whose work over the last fifteen years provided the foundation for the questions regarding Strongylus vulgaris in this dissertation. In my dealings as an equine internist I have met countless people with tremendous respect and admiration for him and I consider myself among them. Without a doubt my favorite quality in Dr. Klei is that he is so humble in the face of tremendous professional accomplishment. I also appreciate his candor and will never forget the infamous "Flower Garden" speech.

Both Dr. Horohov and Dr. Klei have shown that horses can make real contributions to the field of immunology and I appreciate the confidence that both of them have shown in my ability to contribute to this goal.

Perhaps the first guidance I received at LSU came from Dr. Storz who helped me tremendously with my graduate program during the first months of my residency. His support continued in the form of a departmental stipend after completing my residency. I greatly appreciate this and all other support that he has shown me. I would also like to acknowledge the other members of my committee who have provided various forms of support throughout the years. Dr. Kathy O'Reilly provided the primers used to clone equine IL-10 and I believe that her knowledge base, humanism, and commitment to graduate education are an asset to the department. And the other members of my committee Dr. Tom Gillis, and especially Dr. John Battista, whose choice comment at my proposal seminar "it 'can' be done" while scratching his head (which I have taken to
imply with significant difficulty) was perhaps the most insightful of all as it has both come to haunt me and given me comfort through the difficulties.

It should be noted that this dissertation could not have been completed without the expert assistance of Susan Pourciau, Melanie Chapman, and Dr. Rob Folsom. Their efforts were integral to completing the *Strongylus vulgaris* portions of this work. Thymidine incorporation studies leading to Figure 3.3 were performed by Susan Pourciau and ELISA titers leading to Figure 3.2 were determined by Dr. Rob Folsom with assistance by Ann Chapman. The exploratory laparotomies that yielded abdominal lymph nodes were performed by Drs. Ray McClure and Rusty Moore. The pony manipulations required in the final chapter of this dissertation: vaccinating, challenging, performing surgery, post-operative observations, and necropsy are not small feats and reflect the work of numerous members of Dr. Klei’s research team. They are all gratefully acknowledged, but most significantly Dr. Dennis French, who was also a great help to me during my residency. Many other people either directly contributed to or facilitated this work. These include Tracy DeJean, Jody Territo, Brandye Sawyer, Li Huang, Susan Newman, Sukanya Jayachandra, Tim Foster, Mary Tetzlaf. Thank you

I would like to express my gratitude to the sources that supported this work. Support for the development of cytokine reagents and cytokine quantification assays was provided by an Idea Grant from the National Institute of Health. The QPCR System 5000 was purchased with a grant from the Louisiana Educational Quality Support Fund. This instrument was integral to the quantification of PCR products performed in these experiments. Funding for study of the immune response of ponies to *Strongylus vulgaris* was provided by the United States Department of Agriculture. Equally significant, the

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Grayson Jockey Club Research Foundation provided stipend support in the final years of my program.

Without a doubt my family members have made the greatest sacrifices in allowing me to complete this program. My daughter Katelyn, you have brought me peace with my place in life and shown me how to make a truly significant contribution to the world. My husband Terry, you supported me on this emotional roller coaster even though it took longer than we had envisioned. And to the members of my village who saw to it that the most precious being in my life was cared for to my expectations while my mind and body were in the laboratory: Dad, Mom & Matthew (for making it so that Dad could be with us in the early months), Mrs. Gloria Hall, Elizabeth Hall, Ashley Van Uden, Drs. Jill McClure and Lais Costa and Matthew Bauer, and Mercedes Costa, - Thank you.

Terry, Matthew, Mom and Dad deserve special additional acknowledgment for it was the strong sense of self that you instilled and your grounding me in the truths of life that allowed me to persevere when the going got too tough. Thank you - as a family we pulled together to make this happen in the end.
PREFACE

As I look back over the last five years the convolutions that my program has taken are absolutely amazing to me. After completing a residency in Equine Medicine here at LSU, I began working full time in Dr. Horohov’s laboratory. I had completed the majority of my course work during my residency and so I expected that, working full time in the laboratory, I would complete my Ph.D. in approximately two to three years. I struggled initially to find a project that held my interest. Evie Vandergriff had recently cloned equine IL-2 and IL-4 in our laboratory and interferon-γ was available from the laboratory of Dr. Doug Antczak, thanks to the efforts of his graduate student Gabriel Grunig. With the unique availability of these three reagents, my idea was to determine if horses exhibit cytokine patterns consistent with the T-helper (TH)1/TH2 paradigm. Because of my background in equine medicine and familiarity with the work of other investigators in the School of Veterinary Medicine, I proposed to explore the TH1/TH2 paradigm in three equine diseases that had immunologically well characterized mouse and human corollaries, influenza virus infection, Strongylus vulgaris infestation, and Summer Pasture Associated Obstructive Pulmonary Disease (SPAOPD). There was, and remains to this day, no easy way to identify helper cell phenotypes. There is no magical surface marker that warns the investigator of a TH1 or TH2 response and so I began, a veterinarian whose greatest asset is her power of observation, on the road to quantify messenger RNA in the realm of the unseeable.

After reviewing the literature I hoped to utilize Northern blotting so as to avoid the pitfalls of the polymerase chain reaction (PCR). PCR (dubbed ‘polymerase chain
rubbish' by some) with its logarithmic expansion whose efficiency is limited by mis-priming, primer dimer, secondary structure, magnesium concentration, annealing and extension times, pH, and entering the dreaded plateau phase, was something that as a practicing veterinarian, I surely wanted to stay away from. Besides, people who knew far more about molecular biology than I said they knew graduate students that spent years just standardizing their PCR reactions. And so I forged into the land of Northerns, dot blotting, end-labeling, and random priming, ribo-probing with fluorescent dyes and radioactive poisons determined to avoid PCR. When dot blotting didn't provide a signal, I tried solution hybridization northerns (RNase protection) to tap into their 'greater sensitivity' and stability. But this too did not work. That assay required tremendous amounts of RNA (more than 10 μg of RNA per cytokine), had unlimited opportunities for RNase contamination, and would never be adaptable to large sample numbers. So after almost two years of Northern blotting it was time to try a PCR based method. Southern blotting using fluorescently-labeled random-primed probes was sure to give results. But the blots again had too much background and were too insensitive. So there I was, two years of intense work and no hope of any data. Frustrated and on the verge of quitting, I decided to clone equine IL-10 and β-actin so that I would have something to show for my time, maybe someone would give me my Masters degree if all else failed. Luckily I did manage to clone these genes but expression and bioassay of the cytokine was a little less fruitful. And then it happened, the black box that was to revolutionize quantification of PCR products arrived - the Perkin-Elmer QPCR System 5000.

Still reticent about PCR I abdicated that I would have to perform a PCR based quantification but by now some data was better than no data. And it didn't look as
though I would ever have data. So into the land of quantitative PCR (QPCR) I forged. The company literature and initial presentation made the QPCR System 5000 seem like a Red Rider BB gun. Little did I know just how proficient I would come to be at understanding the functioning and interpretation of luminosity values from the QPCR System 5000. One year after the machine's arrival I finally understood how it really worked (which is actually quite powerful but not exactly in line with my initial understanding) and with the exception of three months for the birth of my daughter, I was ready to 'generate data'.

From my proposal four years earlier, many changes had occurred in the samples I was to analyze. In fact, as I complete my final writing, samples that I collected to answer many of the questions that I have formulated sit dormant at -80 °C as the development of this assay came together too late for me to learn the answers. As for the original questions of a TH1 response for protection against equine influenza virus, which has been documented in other species, I initially proposed looking at changes in the cytokine profile of peripheral blood mononuclear cells derived from horses both before and after vaccination and stimulated in vitro with influenza virus. Though I initially suggested to, I did not believe that we would be allowed to nebulize equine influenza virus to horses in the facilities as they exist at LSU. I noted in my original proposal that this killed virus vaccine, by virtue of it's induction of the humoral immune response, was actually more likely to induce a TH2 response and that our best hope for detecting a TH1 response with equine influenza hinged on assaying the cytokine profile of lymphocytes from the portal of infection (draining the respiratory tract) and comparing this profile to that of a distant lymph node following a live influenza virus challenge.
Though I will not personally get to answer these questions, I have not only collected and frozen RNA to address the vaccination question, but also collected peripheral and respiratory associated lymphocyte samples from ponies challenged with equine influenza virus, and been co-investigator on a funded research proposal to address this question of TH1 responses in live equine influenza challenge of ponies.

My second disease state of interest, Strongylus vulgaris infestation, became a focus of this dissertation. My initial proposal sought to characterize the cytokine response of peripheral blood mononuclear cells (PBMC) from parasite free and chronically S. vulgaris parasitized ponies in response to stimulation with a crude S. vulgaris antigen. As I addressed my committee I noted that data from mice indicated that a protective TH2 response to gastrointestinal nematodes was sometimes restricted to the GI tract and not detectable in PBMC, indicating that lymphocytes draining the GI tract might be more useful in our assays. Over the next few years I managed to collect lymphocytes from blood, as well the peripheral and colonic lymph nodes of close to a hundred ponies with a variety of parasitic conditions. In a moment of humor I suggested that following the cytokine response of parasite free ponies to nematode parasite infestation could be accomplished using serial surgeries. Little did I know this comment would come to fruition as a funded USDA grant and a whole new set of freezer samples that would become the focus of my dissertation.

Being a clinician at heart, my greatest interest lay in the study of horses with SPAOPD. Horses with this disease suffer immensely and factions studying the disease stand divided as to how to determine its cause. My original proposal for these horses was to examine cytokine production from PBMC's collected monthly from horses with
SPAOPD. I further proposed that bronchoalveolar lavage (BAL) lymphocytes be evaluated similarly. My first samples were tracheal aspirates (TTA) and had the copious mucous characteristic of tracheal aspirates from SPAOPD horses. Unfortunately, after developing a method to separate the mononuclear cells from the TTA mucous, the procedure was only repeated once on a limited number of horses. These samples were collected and frozen. However, as of this writing, I am aware that a proposal submitted by Dr. Horohov and Dr. Beadle examining some of the above issues has been funded and hopefully will provide some direction for therapy in this disease.

It is hard to finish when you feel you are finally ready to embark upon your goals of six years prior. This preface is to those who secretly wondered what was taking so long and those who will benefit from my work, ideas, and ‘my legacy’ of frozen RNA samples, so that you will know their origins.
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ABSTRACT

The effect of oral vaccination with radiation-attenuated *Strongylus vulgaris* larvae on the cytokine responses of ponies to oral challenge with *S. vulgaris* was examined. Message was quantified using RT-PCR by interpolation against a plasmid-derived standard curve. PCR product was detected using the Perkin-Elmer QPCR System 5000. Standard curves were analyzed with a two parameter exponential rise to maximum fit of the data. By altering the cycling parameters to maintain β-actin amplification within its exponential phase, β-actin was used as a normalization factor. The assay resolves 2 fold changes in message, has a linear dynamic range on the order of 3 logs, and is precise with errors in the range of 10% for replicate amplifications from the same sample. The error between replicate RNA samples from separate extractions of the same cell population, following β-actin normalization, was 13-27%. Equine Interleukin(IL)-6, IL-10, and β-actin were cloned and sequenced for use in this assay.

10 ponies were reared parasite-free. Four received 500 attenuated *S. vulgaris* L₃, 9 and 6 weeks prior to challenge with 1000 nonirradiated L₃. Four nonvaccinated ponies were also challenged. Control ponies were neither vaccinated nor challenged. Four days prior to challenge, and on days 4, 9, and 14 post-challenge, peripheral blood mononuclear cells (PBMC) and colonic lymph nodes (CLN) were collected. Prior to necropsy on D14, CLN were obtained surgically. PBMC and CLN cells (CLNC) were assayed for IL-2, IL-4, IL-5, IL-10, and IFN-γ. Vaccinates and nonvaccinates demonstrated TH2-like cytokine patterns with significant elevations in IL-4 and IL-5 in response to *S. vulgaris* challenge. Vaccinates produced more IL-4 and IL-5 than...
nonvaccinates prior to challenge (not statistically significant), and significantly more IL-4 in response to challenge, first in CLNC at D4 and then PBMC at D9. IL-4 in CLNC of nonvaccinates increased significantly by D9. Thus, ponies appear to generate TH2-like responses to gastrointestinal nematodes and anamnestic IL-4 production may be one component of the protective immune response generated by vaccination. *Strongylus vulgaris*-specific antibody isotypes and lymphoproliferative responses were also examined.
INTRODUCTION

Cytokine production by helper T-lymphocytes has been divided into two categories based on the relative balance of the cytokines that are produced (Mosmann et al., 1986). T-helper (TH)1 responses, characterized by a predominance of interferon-gamma (IFN-γ), interleukin (IL)-2, and tumor necrosis factor-alpha (TNF-α) production, have been shown to be integral to delayed-type hypersensitivity reactions (DTH), graft rejections, and to the protective immune response to intracellular organisms such as viruses (reviewed in Abbas et al., 1996). TH2 responses, which are characterized by a predominance of IL-4, IL-5, and IL-13 production (as well as IL-6 and IL-10 in the mouse), are integral to the protective immune response to some extracellular organisms including gastrointestinal nematode parasites (reviewed in Finkelman et al., 1997). Because these cytokine profiles have been identified in cell populations that are not composed purely of helper T-cells (Robinson et al., 1992), the terms Type I and Type II responses, respectively, have been coined to describe TH1 and TH2 cytokine profiles in non-T-cell populations or cell populations that are not purely CD4+ T-cells.

The purpose of this dissertation was determine the contribution of Type I and Type II responses to vaccine induced resistance to Strongylus vulgaris infections. S. vulgaris is a highly pathogenic gastrointestinal nematode of horses whose life-cycle, which takes 4 to 6 months to complete, causes severe inflammation in arteries that perfuse the intestine (Ogbourne and Duncan, 1985). These lesions can compromise intestinal perfusion resulting in a syndrome, known as verminous arteritis, that is
characterized by ischemic infarctions of the bowel which result toxemia, abdominal pain, and death in severe cases (White, 1985). However, clinical signs of infection including pyrexia, anorexia, depression, and colic appear within the first week following ingestion of third-stage infective larvae (L3) and are near maximal by 14 days post infection (Klei et al., 1982). Oral immunization with radiation-attenuated L3 produces a strong protective resistance to challenge infection which prevents classical lesions of verminous arteritis and eliminates clinical signs of infection (Klei et al., 1982).

Natural and experimental infections with *S. vulgaris* result in eosinophilia and eosinophilia is known to be an IL-5 (Type II) dependent phenomenon in murine models (Coffman et al., 1989). Furthermore, when compared to parenteral vaccination with adult or larval somatic antigen homogenates which did not confer resistance to challenge infection, the protection observed in ponies vaccinated orally with radiation-attenuated L3 correlated to both pre-challenge anti-*S. vulgaris* antibody titers specific for surface antigens of late L3 stages, and to induction of a post challenge anamnestic-like eosinophilia (Monahan et al., 1994). Infections by *S. vulgaris* have also been shown to activate eosinophils in vitro (Dennis et al., 1988), and eosinophils from *S. vulgaris* primed but not unprimed ponies kill *S. vulgaris* L3 in vitro in an antibody-dependent manner (Klei et al., 1992), indicating that an antibody-dependent phenomenon involving eosinophils may contribute to the resistance seen in immune ponies. In addition, a cytokine or combination of cytokines which exhibits chemotactic activity for eosinophils has been found in the supernatant of *S. vulgaris* stimulated peripheral blood mononuclear cells (PBMC) from immune but not nonimmune ponies (Dennis et al., 1993). A synthesis of these findings suggests that differential cytokine production,
perhaps similar to that seen in classical Type II responses to helminth parasites may play a role in immunity to *S. vulgaris*.

The underlying hypothesis of this study was that in the equine, as in murine species, Type II responses confer protective resistance to gastrointestinal nematode infection. Identifying Type I and Type II cytokine responses requires cytokine quantification and so two major portions of this study were devoted to cloning and sequencing IL-6 and IL-10, which are Type II cytokines in the murine system, and developing a method for quantifying equine cytokine messenger ribonucleic acid (mRNA). This latter step also necessitated the cloning and sequencing of equine β-actin for use as an internal standard in the cytokine quantification assay. Using this assay, the cytokine responses in both peripheral blood mononuclear cells and colonic lymph nodes taken from a region of parasite invasion were examined to elucidate the nature of the cytokine responses in both vaccinated and nonvaccinated ponies.
LITERATURE REVIEW

The TH1 and TH2 Paradigm

The clinical response to infectious organisms, most notably Mycobacterium leprae, provided early and convincing evidence of a dichotomy in the immune response. Patients with the tuberculoid form of the disease demonstrated reduced numbers of organisms in association with strong delayed-type hypersensitivity reactions (DTH) and low levels of antibody, while patients with the lepromatous form displayed large organism burdens, high antibody titers, and a relative lack of DTH (Turk and Bryceson, 1971). Recognition that CD4+ helper T-cells were required for both antibody and DTH responses raised questions as to how a helper T-cell subset that appeared phenotypically homogenous based on surface staining characteristics could present such differing functional capabilities as antibody production and DTH.

The recognition of at least two distinct helper T-lymphocyte responses originated in murine studies of T-cell help for antibody production. Tada et. al. (1978) first used the term "Th₁" cell to describe a subset of carrier-specific mouse Lyt1+,2-,3- helper T-cells which did not adhere to nylon wool, did not express the class II major histocompatibility (MHC) antigen Ia, and provided help for anti-hapten antibody production by B-cells, only if the hapten was linked to a sensitizing carrier on the same molecule. Tada termed this cognate interaction or 'linked' help. In contrast, Tada's "Th₂" cells were nylon wool adherent, Ia⁺, and did not depend upon the relevant carrier being coupled to the hapten in order to provide help for B-cell anti-hapten antibody production (nonlinked help). Studying cloned Lyt1+,2- L3T4a+ helper T-lymphocytes, Kim et al. (1985) also
determined that helper T-cell subsets could be identified by characteristics of the help provided for antibody production. Based on their activity in bulk cultures with B-cells, Kim defined 4 types of helper T-cell clones. "Type 3" clones were unable to provide help for antibody production and some actually suppressed the ability of other clones to provide help, while "Type 4" clones were specific for antibody directed at class II (Ia) antigens. When compared to clones he defined as "Type 1" helper T-cells, Kim's "Type 2" clones resulted in 15 fold greater antibody production, which was predominately of a single idiotype. Limiting dilution analysis determined that this idiotype specificity resulted from both a higher precursor number and greater clonal expansion of the idiotype specific B-cell precursors. Such differences in precursor frequency for a single idiotype could not be attributed to the B-cells which bear the same germ-line encoded idiotype. This lead Kim to postulate that "Type 2", not "Type 1" helper T-cell clones delivered a critical activation signal for the B-cell in the form of either enhanced affinity receptors or more likely via secreted molecules. He suggested that such a molecule from "Type 2" helper T-cells, now known to be interleukin(IL)-4, could act by increasing MHC class II expression on B-cells, thereby allowing B-cells with low MHC II expression to become activated. Collectively these and other early studies indicated that despite fairly uniform surface distribution of CD3 and CD4 antigens, when assessed by functional criteria, at least two types of helper T-cells exist whose activities might be linked to differing patterns of secreted molecules (Waldman, 1977; Swiekosz et al., 1979; Asano et al., 1982; Imperiale et al., 1982; Melchers et al., 1982).

The description by Mosmann et al. (1986) of two classes of antigen-specific mouse helper T-cell clones that could be identified not only by differing modes of B-cell
help, but more significantly by distinct patterns of cytokine production was an
immunologic milestone. Mosmann's "Th2" clones, which secreted IL-3, IL-4, and IL-5,
bound nylon wool, were Ia+, enhanced the production of IgG1 and IgE by LPS
stimulated B-cells, and stimulated the expression of Ia antigen on B-cells. In contrast, his
"Th1" clones which secreted IL-2, interferon-γ (IFN-γ), granulocyte-macrophage
colony stimulating factor (GM-CSF), and IL-3, did not bind nylon wool, were Ia-, and
did not possess IgG1, IgE, or Ia enhancing activities. A second major finding of this
paper was that the "Th" phenotype was dependent upon the antigen used for clonal
stimulation. Chicken RBC-specific T-cell clones (200 in all) from three different mouse
strains exhibited the "Th1" phenotype, while fowl γ-globulin stimulated clones from the
same mice all exhibited the "Th2" phenotype. Interpreted together, these findings
suggest that antigens which induce substantial IgE responses may do so by preferentially
inducing the "Th2" phenotype; whereas antigens that induce high IgM and low IgE titers
may do so by preferentially activating the "Th1" phenotype, which aids in IgM
production and suppress IgE production.

Recognition of distinct cytokine phenotypes in mouse Th1 and Th2 clones lead
to attempts to characterize cytokine production by helper T-cells in experimental animals
and humans in response to a variety of antigenic stimuli, both in vitro and in vivo.
Cloned helper T-cell subsets with characteristic cytokine patterns have been described in
humans (Del Prete et al., 1991; reviewed in Del Prete et al., 1994; Romagnani, 1994),
rats (Druet and Pelletier, 1996), and cows (Brown et al., 1995). However, initial studies
of existing mitogen induced and alloreactive human T-cell clones indicated that most
clones produced combinations of the Th1 and Th2 cytokines (Maggi et al., 1988;
Paliard et al., 1988; Umetsu et al., 1988), leading to scepticism regarding the functional significance of Th1 and Th2 clones in mice (Kelso, 1995). In sharp contrast to the early human clones which were derived from unimmunized or mildly immunized individuals, Mosmann's clones originated from strongly immunized mice which were stimulated for prolonged periods in vitro. Further analysis of cytokine patterns from alloreactive mouse T-cell clones, T-cell clones maintained for a short period in vitro, and T-cell clones from unimmunized mice indicated that these clones also exhibited the unrestricted cytokine patterns of early human T-cell clones (Kelso and Gough, 1988; Mosmann and Coffman, 1989a). Mosmann also observed that the generation of distinct Th1 and Th2 phenotypes was most rapid with cells from mice that had been immunized with antigens that stimulated strong Th1 and Th2 responses in vivo. In support of this observation, the first human Th1 and Th2 clones were generated from normal humans in response to purified protein derivative (PPD) of Mycobacterium tuberculosis and excretory/secretory antigen of Toxocara canis, respectively (Del Prete et al., 1991). These antigens, as will be discussed, originate from classes of infectious organisms, namely intracellular bacteria and helminth parasites, that classically evoke Th1 and Th2 responses, respectively, in vivo. Together these findings indicate that polarization to a Th1 or Th2 response is dependent not only upon the nature of the inciting antigen, but also the duration of exposure to the antigen, with polarization toward the Th1 or Th2 response increasing with the chronicity of exposure. Unrestricted cytokine patterns, termed Th0, therefore reflect an earlier step in the maturation of the antigen-specific immune response, while distinct Th1 and Th2 responses become more pronounced in chronic disease states. The occurrence of clear helper cell phenotypes in association with persistent infections, Th1
(reviewed in Kaufmann, 1993) in association with leishmaniasis (Reiner and Locksley, 1995), listeriosis (Hsieh et al., 1993b), and mycobacterial infections (Yamamura et al., 1991), and Th2 in association with helminth infections (Sher and Coffman, 1992), substantiates this hypothesis.

The characterization of distinct classes of CD4+ helper T-cells whose functions are determined by their cytokine repertoire, termed the Th1/Th2 paradigm, provided an explanation as to how a T-cell subset that appeared phenotypically identical based on surface staining characteristics could present such differing functional capabilities as immunoglobulin production and DTH. Several questions raised by Mosmann's characterization of Th1 and Th2 cytokine phenotypes have led to an explosion of research in order to elucidate the relationship between antigen stimulation, secretion of defined patterns of cytokines by helper T-cells, and generation of the protective antigen-specific immune response. These are: 1) What cytokines define the TH1 and TH2 phenotypes? 2) What is the ontogeny of the TH1 and TH2 phenotypes? 3) How are they differentially regulated? 4) Is selection of the helper T-cell phenotype by antigen real and what determines it? and 5) What is the role of each subset in normal in vivo responses?

**Cytokine Phenotypes of Th1 and Th2 Responses**

Continued investigation into the cytokine repertoires of Th1 and Th2 cells has confirmed that in both human and murine systems, production of IFN-γ, IL-2, and tumor necrosis factor-β (TNF-β) is unique to Th1 cells, while only Th2 cells produce IL-4, IL-5, and IL-13 (Mosmann et al., 1986; Cherwinski et al., 1987; Janeway et al., 1988; Mosmann and Coffman, 1989b; Romagnani, 1991; Romagnani, 1994). However, IL-6
(Romagnani, 1992) and IL-10 (Del Prete et al., 1993), which segregate exclusively with Th2 cells in mice, are produced by both Th1 and Th2 cells in humans.

The nature of the pathogens which incite Th1 and Th2 responses, as well as the immunologic activities of the cytokines produced by each helper cell subset, suggest that Th1 responses provide protection against intracellular pathogens through phagocyte mediated mechanisms, while Th2 responses protect from extracellular pathogens primarily via phagocyte independent mechanisms. IFN-γ has been termed the signature cytokine of the Th1 response (Abbas et al., 1996) and its functions have been reviewed (Trinchieri and Perussia, 1985). Produced by CD8+ T-cells, CD4+ Th1 cells, and NK cells, IFN-γ was initially recognized by its ability to inhibit viral replication in fibroblasts (Wheelock, 1965). IFN-γ is now recognized as a potent activator of macrophage function, greatly augmenting oxidative and nitrogen dependent microbicidal activities (Murray, et al., 1985; Belosevic et al., 1989; Nacy and Meltzer, 1991; Beaman et al., 1992). IFN-γ also modulates expression of class I and II MHC antigens on a variety of cells, potently upregulating class II expression on classical APC and inducing de novo synthesis on a variety of other cells (reviewed in Trinchieri and Perussia, 1985). As will be discussed in more detail, IFN-γ is involved in the differential regulation of Th1 and Th2 responses, augmenting induction of Th1 cells while impeding the induction, proliferation, and effector function of Th2 cells (Mond et al., 1986; Belosevic et al., 1989; Gajewskiet al., 1989; Swain et al., 1991). In the murine system, IFN-γ stimulates the production of complement fixing IgG isotypes, IgG2a and IgG3 (Snapper and Paul, 1987; Coffman et al., 1993), which also bind to the high-affinity Fcγ receptors of phagocytes, making them integral to osponization and phagocytosis of pathogens. In
addition, IFN-γ augments cytotoxic immune mechanisms by directly activating natural killer (NK) and cytotoxic T lymphocytes (CTL) (reviewed in Trinchieri and Perussia, 1985).

IL-2, whose functions have been extensively reviewed (Smith, 1988; Swain, 1991), is well recognized as an autocrine growth factor for T-cells which is especially significant in the development of antigen-stimulated cytotoxic T-lymphocyte precursors into functional cytotoxic effectors, and augmentation of NK responses. However, IL-2 also activates most cells of the immune system. IL-2 is produced primarily by activated T-cells, though B-cells have been induced to secrete IL-2 in vitro. IL-2 activates monocyte mediated cytotoxicity, and increases phagocytosis. In conjunction with other cytokines, IL-2 induces B-cell differentiation and immunoglobulin secretion (Coffman et al., 1988; Loughnan and Nossal, 1989).

The activates of TNF-β have been reviewed (Paul and Ruddle, 1988) and mimic those of TNF-α. Though the two proteins display only 30% amino acid homology, TNF-β can activate both of the TNF-β receptors (Aggarwal and Eessalu, 1987) (whose expression is upregulated by IFN-γ), making TNF-β a mediator of inflammation and graft rejection, able to recruit and activate inflammatory leukocytes. In addition to production by CD4+ T-cells, TNF-β is also produced by activated CD8+ T-cells (Ruddle et al., 1987), and B-cells (Aggarwal et al., 1994). TNF-β was first described as a cytotoxic activity in culture supernates of activated lymphocytes and is one of the cytotoxic mediators involved in CTL, CD4+, NK, and LAK cell killing. TNF-β is directly cytotoxic toward tumors, both in vitro and in vivo, and has been shown to kill antigen presenting B-cells, while also functioning as a B-cell growth factor.
The collective actions of the TH1 cytokines which induce macrophage cytocidal mechanisms, the generation of opsonizing, complement fixing antibodies, and induce lymphocyte mediated killing mechanisms support the notion that Th1 responses are designed to destroy host cells which have been altered either by infection or malignant transformation. Cytokine induced mononuclear phagocyte recruitment and activation lead histologically to inflammatory reactions that are characteristic of delayed type hypersensitivity reactions. In contrast, the signature cytokines of the Th2 response, IL-4 and IL-5, support the destruction of extracellular invaders, primarily via phagocyte independent mechanisms.

IL-4 and IL-13 share many, though not all, functions and will therefore be discussed together. To date at least two mechanisms for this cross reactivity have been documented. Both cytokines utilize the IL-4 receptor-α (IL-4 Rα) subunit in signal transduction (Kotowicz et al., 1996; Andersson et al., 1997, Orchransky et al., 1997), and IL-13 binding to its low affinity receptor induces the expression of a high affinity IL-13 receptor capable of binding IL-4 (Hilton et al., 1996). However, the receptor system for these two cytokines still requires study. Both IL-4 (Howard et al., 1982; Isakson et al., 1982) and IL-13 (McKenzie et al., 1993) stimulate the proliferation of activated B-cells and provide help for immunoglobulin production. In addition to production by Th2 cells, IL-4 is also produced by CD4- CD8- splenic T-cells (Zlotnick et al., 1992), CD3+CD4+ CD8- Thymocytes (Fischer et al., 1991; Bendelac and Schwartz, 1992), some CD8+ clones (Yamamura et al., 1991; Seder et al., 1992a), as well as basophils and mast cells (Plaut et al., 1989). IL-13, in addition to production by T-cells, is produced by mast cells (Krishnaswamy et al., 1997), basophils (Dahinden et al., 1997).
and certain B-cell lines. IL-4 (reviewed in Paul, 1992; Paul and Ohara, 1987) and IL-13 (Defrance et al., 1994) have stimulatory effects on both resting and activated B-cells, including increasing MHC II, and Fcε receptor expression. Two prominent roles of IL-4 which have been substantiated in vivo are the augmentation of IgE production via induction of isotype switching (Kuhn et al., 1991; also reviewed in Finkelman et al., 1990; Schultz and Coffinan, 1992), a function demonstrated in vitro by IL-13 in an IL-4 independent manner (Punnonen et al., 1993), and the ability to drive the Th2 response (Zlotnick et al., 1987; Swain et al., 1990) while inhibiting the generation of the Th1 response (LeGros et al., 1990; Swain et al., 1990). Inhibition of Th1 responses by IL-4 also applies to IL-2 stimulated B-cells whose proliferation is inhibited by IL-4 (reviewed in Paul, 1992). IL-4 also augments the production of non-complement fixing IgG isotypes (Bruggemann et al., 1987; Dangl et al., 1988), IgG1 in mice, and IgG4 in humans. Augmentation of IgG4 production by human B-cells has also been demonstrated by IL-13 (Punnonen et al., 1993). Both IL-4 and IL-13 have also been shown to act as cofactors in the inhibition of B cell apoptosis (Lomo et al., 1997).

Though IL-4 and IL-13 do enhance MHC II expression on monocytes (McKenzie et al., 1993), both cytokines also exhibit potent inhibitory effects on monocytes (Fgdor and te Velde, 1992; de Waal Malefyt et al., 1993; Doherty et al., 1993; Bogdan et al., 1997; Mijatovic et al., 1997). IL-4 and IL-13 inhibit monocyte cytotoxicity by decreasing production of monocyte derived cytotoxic metabolites, such as nitric oxide (Doherty et al., 1993), as well as inhibiting the production of a wide range of monokines, including IL-1, IL-6, IL-8, IL-10, IL-12, GM-CSF, granulocyte colony...
stimulating factor (G-CSF), IFN-α, and TNF-α (de Waal Malefyt et al., 1993). Both IL-4 and IL-13 also increase production of the IL-1 receptor antagonist (IL-1Ra).

Both IL-4 and IL-13 have unique attributes. IL-4 is a growth factor for mast cells (reviewed in Figdor and te Velde, 1992) that also enhances CTL differentiation (Widmer et al., 1987) and has demonstrated potent anti-tumor activity in vivo. IL-4 induces tumor infiltration by cytotoxic eosinophils, followed by a long lived immunity that is mediated by CTLs (Tepper et al., 1989; Bosco et al., 1990; Golumbek et al., 1991; Tepper et al., 1992). In contrast to IL-4, IL-13 does not inhibit production of IFN-γ, RANTES (regulated on activation, normal T expressed and secreted), macrophage inhibitory proteins 1-α and 1-β, or perforin by IL-2 stimulated T cells (Minty et al., 1997). IL-13 functions as a cofactor for dendritic cell differentiation (Piemonti et al., 1995) and like IL-4 induces the expression of VCAM-1 (vascular cell adhesion molecule-1) on endothelial cells (Ying et al., 1997). IL-13 also induces MCP-1 (monocyte chemoattractant protein-1) release from endothelial cells (Goebeler et al., 1997), which in coordination with VCAM-1 upregulation results in recruitment of inflammatory cells including eosinophils, monocytes, and T-cells.

Recent studies have indicated that IL-4 and IL-13 can exhibit paradoxical effects on monocytes, inducing both activation and suppression of monocyte functions (Kanbayashi et al., 1996; Minty et al., 1997). To date the differences in action of these two cytokines have related to the timing of monocyte exposure to IL-4 and IL-13. When monocyte exposure to IL-4 or IL-13 preceded LPS or SAC stimulation, monocyte activation, characterized by increased monokine production, ensued whereas cotreatment with IL-13 or IL-4 and LPS inhibited monocyte activation. This differential
effect is an area of ongoing interest which may have significant ramifications for the modulation of the inflammatory response in the face of ongoing infection.

In vivo neutralization experiments indicate that IL-5 is the predominant regulator of eosinophilia (Coffman et al., 1989; Sher et al., 1990b; Korenaga et al., 1991; Urban et al., 1992) stimulating mature eosinophil progenitors to proliferate and differentiate into mature effector cells (reviewed in Sanderson, 1992). IL-5 was originally described as a B-cell stimulatory factor (reviewed in Takatsu, 1991) and has demonstrated growth and differentiation activities in the murine system, including differentiation of antigen stimulated B cells to plasma cells, and expression of the IL-2 receptor. However, the role of IL-5 in human B-cell growth and differentiation remains controversial (Azuma et al., 1986; Yokota et al., 1986; Clutterbuck et al., 1987).

IL-6 is a Th2 cytokine in the murine system. However, this polarization is not always clear in human CD4+ subsets (Romagnani, 1992). IL-6 was initially described in separate experiments as an antiviral, hybridoma/plasmacytoma growth (Nordan and Potter, 1986; Van Snick et al., 1986), hepatocyte stimulating, and B-cell stimulating factor (Hirano et al., 1985). The biology and functions of IL-6 have been extensively reviewed (Akira et al., 1990; Van Snick, 1990; Hirano, 1992). IL-6 is produced by macrophages, T-cells, B-cells, fibroblasts, and a variety of transformed cells (Hirano et al., 1985; Kawano et al., 1988; Tsato et al., 1988). It is an inflammatory cytokine that demonstrates a broad spectrum of actions, overlapping those of TNF-α and IL-1. Unlike TNF-α and IL-1, which amplify their own production as well as the production of IL-6 (Shalaby et al., 1989), IL-6 suppresses production of both TNF-α and IL-1 by macrophages (Schindler et al., 1990). IL-6 functions in an endocrine manner, traveling
via the bloodstream to induce systemic events collectively termed the acute phase response and characterized by fever, and the hepatic synthesis of proteins including C3, haptoglobin, serum amyloid A, alpha₂-macroglobulin, and fibrinogen (Ramadori et al., 1988). IL-6 induces monocytopenesis (Jansen et al., 1992) and synergizes with IL-3 in the induction of pleuripotent stem cell differentiation (Ikeuchi et al., 1987). IL-6 exhibits a late effect on both B-cell and CTL function, augmenting IgM secretion in proliferating B-cells (Hilbert et al., 1989), and IL-2 expanded CTL function (Takai et al., 1988; Uyttenhove et al., 1988), presumably via induction of serine esterase, and perforin (Liu et al., 1990). IL-6 has exhibited characteristics of a costimulator for T-cells by permitting a growth response to IL-2 (Garman et al., 1987; Stein and Singer, 1992). IL-6 appears to play an important role in the mucosal immune response, functioning as a cofactor in IgA production (Rafferty and Montgomery, 1995; Okahashi et al., 1996; Van Cott et al., 1996; Yamamoto et al., 1996), but does not appear to function as an IgA heavy chain switch factor (Kawanishi and Joseph, 1991).

IL-10 was first characterized independently by two research groups as a factor from murine Th2 clones that inhibited IFN-γ production by Th1 clones (Fiorentino et al., 1989), as well as a factor in B-lymphoma supernates that enhanced the proliferation of murine thymocytes in response to IL-2 and IL-4 (Suda et al., 1990). These activities were later confirmed to be IL-10 (MacNeil et al., 1990). The biologic activities of IL-10 have been reviewed (Moore et al., 1993). IL-10 inhibits cytokine synthesis by murine and human Th1 cells, human Th2 and Th0 cells, NK cells, and macrophages. IL-10 inhibition of cytokine production by Th1 and NK cells is indirect, being macrophage dependent. Similarly, IL-10 also inhibits macrophage dependent T-cell proliferation. The
macrophage cytokine profile inhibited by IL-10 (de Waal Malefyt et al. 1991; Fiorentino et al., 1991; D’Andrea et al., 1993) parallels the inhibitory actions previously described for IL-4 and IL-13, inhibiting TNF-α, IL-1, IL-6, IL-8, GM-CSF, and G-CSF production, while also increasing IL-1Ra production (Pajkrt et al., 1997), and decreasing reactive nitrogen and oxygen intermediates (Bogdan et al., 1991; Cunha et al., 1992; Gassinelli et al., 1992). The effects of IL-10 on macrophages that lead to inhibition of IFN-γ synthesis by T cells are multiple. IL-10 inhibits production of the p40 component of IL-12, which is produced by macrophages primarily in response to bacterial products and has been shown to be required for optimal IFN-γ production in vitro (Trinchieri et al., 1992; D’Andrea et al., 1993). IL-10 also inhibits production of IL-1, which functions as a co-stimulatory molecule for IFN-γ production by T cells (D’Andrea et al., 1993). IL-10 inhibits upregulation of B7 expression, which is significant in macrophage costimulation (Ding et al., 1993). IL-10's inhibition of TNF-α production by macrophages also eliminates a necessary costimulatory factor for IFN-γ induced macrophage activation (Oswald et al., 1992). There is also evidence that IL-10 plays a role in the paradoxical activation and suppression of monocytes induced by both IL-4 and IL-13, with IL-10 levels being elevated in association with antigen/cytokine timing manipulations that result in monocyte suppression and decreased in association with those manipulations that result in macrophage activation (Kambayashi et al., 1996). In vivo experiments support the in vitro findings that IL-10 is a modulator of the inflammatory response. IL-10 protects mice from lethal endotoxic shock mediated by inflammatory cytokines, including TNF-α and IL-1 (Howard et al., 1993a), and
neutralization of IL-10 in vivo results in elevated IFN-γ, TNF-α, and IL-6 (reviewed in Howard et al., 1993b).

One of IL-10's earliest documented activities was the ability to augment the growth and proliferation of murine mast cells lines in response to IL-3 and IL-4. IL-10 also augments the proliferation, differentiation, and immunoglobulin secretion of activated B-cells. In conjunction with IL-6, IL-10 appears to play a significant role in the generation of IgA responses, though not via isotype switching (Jackson et al., 1996; Okahashi et al., 1996; Van Cott et al., 1996; Yamamoto et al., 1996). In addition, IL-10 depleted mice also have decreased serum IgM and IgA, depletion of peritoneal B-cells, and fail to develop in vivo antibody responses to bacterial antigens, highlighting the role of IL-10 in B-cell function.

Ontogeny of Th1 and Th2 Responses

It is now clear that Th1 and Th2 cells originate from antigen naive CD4+ T-cells, termed pre(p)TH (Swain et al., 1991). Upon receptor engagement and costimulation, pTH produce IL-2 but little or no IL-4 or IFN-γ. Upon priming, pTH cells can develop into cells that make IL-4 or IFN-γ, termed effector(e)TH1 and eTH2, respectively. Generally, IFN-γ producers retain IL-2 production while IL-4 producers loose it. pTH cells may traverse through a Th0 like state in which both IFN-γ and IL-4 are produced prior to differentiation into eTH phenotypes, but it is not clear if this state is obligatory. Early support for this single cell progenitor came from 48 hour mitogen stimulated single cell cultures which were split into culture pairs and cultured under conditions favoring IFN-γ or IL-4 production (Röcken et al., 1992). Upon restimulation, twin cultures from the same parent cell produced IL-2 or IL-4, depending upon the culture method.
Differentiation of the naive CD4+ T cells into IFN-γ producing TH1 or IL-4 producing TH2 phenotypes is driven primarily by cytokines which reflect the environment and nature of the inciting antigen. IL-12 drives differentiation into the IFN-γ secreting Th1 phenotype (Hsieh et al., 1993), while IL-4 is the primary driving force for the differentiation of naive CD4+ T cells into IL-4 secreting Th2 phenotypes (LeGros et al., 1990; Hsieh et al., 1992; Seder et al., 1992b).

IL-12, which is produced primarily by activated macrophages and dendritic cells, is potently induced by intracellular pathogens (reviewed in Scott, 1993). IL-12 activates three transcriptional factors: signal transducer and activator of transcription (STAT)1, STAT3, and STAT4, of which STAT4 is exclusively activated by IL-12 (Jacobson et al., 1995). IL-12 (Magram et al., 1996) and STAT4 knock out mice (Kaplan et al., 1996; Thierfelder et al., 1996) display markedly reduced Th1 responses. Prior to the characterization of IL-12 there was some evidence that IFN-γ was the driving force for Th1 differentiation. IFN-γ induced substantial IFN-γ production in polyclonally stimulated mouse CD4+ spleen cells, but unlike IL-4, which potently induced both IL-4 and IL-5 production, IFN-γ only supported low to moderate IL-2 production (Swain et al., 1991). However, more reductionist experiments using murine T-cells with antigen-specific transgenic T cell receptors (TcR) indicated that IFN-γ was not sufficient for IFN-γ priming (Seder et al., 1992b), suggesting that while IFN-γ augments Th1 expansion and possibly differentiation, it is not itself sufficient to bias the immune response toward the Th1 phenotype. IL-12 itself is a primary mediator of IFN-γ production from T-cells and NK cells (Chan et al., 1991; D’Andrea et al., 1992) and IFN-γ promotes TH1 development by enhancing macrophage IL-12 transcription (Hayes...
et al., 1995; Ma et al., 1996) and by maintaining the expression of IL-12 Rβ2 subunit on CD4+ T-cells (Gollob et al., 1997; Szabo et al., 1997). IL-10 (D'Andrea et al., 1993) and IL-11 (Leng et al., 1997) inhibit IL-12 production by macrophages, and IL-12 can induce IL-10 production by human T cell clones (Meyaard et al., 1996). Though the significance of this latter finding has not been substantiated, collectively this suggests a Th1 induction loop. Cytokines induced by intracellular pathogens facilitate pathogen destruction by activating macrophages and then induce negative feedback in the form of IL-10, which downregulates the reaction after the inciting agent is eliminated.

IL-18 is a newly characterized cytokine (Okamura et al., 1995), also termed IFNγ-inducing factor, which like IL-12 may be important in the development of the Th1 response. Studies are limited at this time and have been restricted to the murine system. Like IL-12, IL-18 has been produced by activated macrophages in response to bacterial products (Okamura et al., 1995) and shares IL-12's capacity to induce IFN-γ production by Th1 cells, as well as to induce the production of IL-2 and GM-CSF, while inhibiting IL-10 production (Ushio et al., 1996). IL-18 has been shown to serve as a costimulatory factor for established Th1 clones activated in a variety of manners, as well as antigen stimulated T cell lines, augmenting their proliferation and production of IFN-γ, whereas IL-18 failed to support IL-4 production or proliferation from similarly stimulated Th2 clones (Kohno et al., 1997). IL-18 is a more potent inducer of IFN-γ production than IL-12 and appears to utilize a separate induction pathway (Kohno et al., 1997). Though the role of IL-18 in driving pTh to Th1 cells has not been fully elucidated, recent evidence suggests that IL-18 is dependent upon IL-12 mediated upregulation of the IL-
18 receptor for its activity, making it likely that IL-12 remains the pivotal Th1 inducing cytokine (Ahn et al., 1997).

The mechanisms involved in the generation of Th2 cells are not as clear as those that drive Th1 differentiation. IL-4 is considered to be the primary driving force, though its signaling pathway and origins remain enigmatic. Two transcriptional factors are currently known to be activated by IL-4, STAT6 and insulin response substrate (IRS)-2 (Hou et al., 1994; Ryan et al., 1996). Though the mechanism by which STAT6 induces IL-4 production is not clear, a multimeric STAT6 binding site does exist within the IL-4 promoter (Lederer et al., 1996), suggesting that IL-4 may participate in an autoregulatory mechanism. Prolonged activation of STAT6 is characteristic of populations undergoing Th2 differentiation, and STAT6 is activated in an autocrine manner when differentiated Th2 populations are stimulated by antigen receptor ligation (Lederer et al., 1996). Furthermore, IL-4 (Kuhn et al., 1991; Kopf et al., 1993) and STAT6 knock out mice (Kaplan et al., 1996; Shimoda et al., 1996; Takeda et al., 1996) exhibit deficient Th2 responses. Other transcription factors including NFAT, which is activated in both eTh1 and eTh2 cells but only supports IL-4 production by eTh2 cells (Rincon and Flavell et al., 1997), and NIP45, which has been shown to synergize with NF-AT and proto-oncogene c-Maf to drive IL-4 production in B lymphoma cells (Hodge et al., 1996), suggest that the control of IL-4 upregulation is complex. In light of the potent suppressive effects of IL-4 and its associated Th2 cytokine congeners, intricate regulation of Th2 induction is not so surprising.

Because IL-4 appears to be the earliest signal for TH2 differentiation and it appears to be autoregulated, many theories have been proposed to explain the original
source of IL-4 that drives Th2 differentiation. Naive T cells produce small amounts of IL-4 upon initial activation (Schmitz et al., 1994), and it is proposed that Th2 induction may result from a threshold effect, with IL-4 levels necessary to drive the Th2 response resulting from a collective increase in lymphocyte activation at the local site of antigen presentation. Along these lines, it has been suggested that antigens stimulating Th2 responses may act as superantigens (Paul and Seder, 1994) or be presented in association with nonpolymorphic molecules (Yoshimoto and Paul, 1994) inducing the production of small amounts of IL-4 from a large number of CD4+ T-cells with diverse TcR specificities. Other cells have also been implicated, including mast cells and basophils (Plaut et al., 1989), and NK 1.1 T cells (reviewed in Bendelac et al., 1997). Mast cells and basophils have been discounted by some because immunoglobulin mediated triggering requires prior exposure to the antigen for B-cell priming. However, there is ample evidence that mast cells and basophils can be triggered via nonimmunologic mechanisms as well as via the induction of products of the complement cascade, C3a, and C5a (Rockoff et al., 1970; Findley et al., 1981; Sheffer, 1983; Eggleston et al., 1984).

Though cytokines appear to be the major driving force that determines which Th response will be generated, a number of other factors including co-stimulation by accessory molecules, the dose of the antigen, and the type of antigen presenting cell have been shown to influence pTh differentiation. However, there is conflicting data regarding the role that each of these factors plays in determining the Th phenotype. The best defined co-stimulators are B7-1 and B7-2, whose kinetics have been characterized on B cells and dendritic cells cultured in vitro (Hathcock et al., 1994), but not on traditional...
antigen presenting cells during in vivo immune responses. B7-2 expression, which tends to exceed B7-1 by as much as 100 fold (Inaba et al., 1994), is induced early during activation, followed later by B7-1 expression. However, alterations in the course of B7-2 and B7-1 expression have been characterized (Hathcock et al., 1994; Lenschow et al., 1994; Constant et al., 1995). Both molecules bind to their T-cell receptors, CD28 and CTLA4, with differing avidity (Kariv et al., 1996) and CD28, which is constitutively expressed on all T cells, has a 20 fold lower binding avidity for B7 molecules than CTLA4, which is expressed following activation (Linsley et al., 1991b).

CD28/B7 interactions are required for early IL-2 production during activation of naive T-cells, thereby complicating interpretation of the effects of B7 interactions on Th differentiation (Seder et al., 1994). Blocking of CD28/B7 interactions in Th1 clones, which inhibited IL-2 production but did not affect cytokine secretion by Th2 clones, provided the first evidence of a possible B7 mediated costimulatory effect (Mcknight et al., 1994). Since that time studies of B7 mediated signals have yielded conflicting results. A number of studies using antibodies to B7-1 and B7-2 indicate that B7-1 mediated T-cell stimulation provides co-stimulatory signals for TH1 differentiation, while B7-2 helps to drive TH2 differentiation (Freeman et al., 1995; Kuchroo et al., 1995, Lanier et al., 1995 and reviewed in Constant and Bottomly, 1997). However, there are also conflicting studies that report the reverse effect (Lenshow et al., 1995; Levine et al., 1995; Natesan et al., 1996) or no effect (Lanier et al., 1995 and reviewed in Constant and Bottomly, 1997) on Th differentiation. In contrast to the aforementioned studies which examined the immune responses to systemically administered protein antigens, B7-1 and B7-2 neutralization experiments in a mouse model of gastrointestinal nematode infection in
which Th2 responses are protective showed that blocking both molecules was required to decrease Th2 responses, but there was no elevation in Th1 cytokines (Gause et al., 1997).

The variable effects of anti-B7 antibodies in different disease states have been postulated to reflect the significance of different antigen presenting cells and concentrations of antigen at different stages of an infection (reviewed in Constant and Bottomly, 1997). Differing characteristics of the antigen, including low dosage and/or other characteristics that result in weak TcR signaling, could increase the requirements for costimulation which may be mediated primarily by B7-2 because it is more rapidly upregulated. Whereas, in the case of high antigen density or antigens that for other reasons potently signal via the TcR, the need for co-stimulation may be limited or even bypassed, such that exogenously added blocking antibodies would have little effect on the response. Other authors have suggested that differences in neutralization studies with B7-1 and B7-2 reflect differences in the nature of the immune response to intact pathogens, as compared to protein antigens, with B7-1 being able to provide both a co-stimulatory and a down-regulatory signal (Gause et al., 1997).

Equally confusing and interrelated are the effects of differential B7 signaling through the CD28 and CTLA4 receptors on T cells. However, unlike B7 mediated signaling experiments, a variety of in vitro and in vivo systems suggest that CTLA-4 mediated signaling is integral to the generation of Th2 responses in naive T-cells (reviewed in Gause et al., 1997). Blocking CTL-4 mediated signaling at the beginning of the immune response inhibits the generation of Th2 responses but has no effect if administered following the initiation of the immune response (Corry et al., 1994; Lu et
Taken together with the dependence of Th1 clones and naive T-cells on TcR and CD28 mediated signaling for activation and IL-2 production (Linsley et al., 1991a; Reiser et al., 1992), and the ability of some Th2 clones to utilize IL-4 as an autocrine growth factor (McArthur and Raulet, 1993) or to utilize IL-1 as a cofactor with TcR crosslinking without CD28 costimulation (Lichtman et al., 1988; Weaver and Unanue, 1990; McArthur and Raulet, 1993), CD28/CTLA-4 signaling appears to be required for IL-2 and IFN-γ production in vitro and for the differentiation of naive T-cells into Th2 cells, but not for the activation of differentiated Th2 cells, which appear to be able to utilize other co-stimulatory molecules (reviewed in Gause et al., 1997).

Differential Regulation of Th1 and Th2 Responses

Shortly after characterization of Th1 and Th2 clones it was discovered that IFN-γ could inhibit proliferation and cytokine synthesis of Th2 clones (Fernandez-Botran et al., 1988; Gajewski and Fitch, et al., 1988) and search for a Th2 derived cytokine that inhibited cytokine synthesis of Th1 cells lead to the discovery of IL-10 (Doherty et al., 1993). Continued interest in the differential regulation of these two populations has indicated that cytokines from each Th population inhibit functions of the opposite phenotype. The cross-regulatory mechanisms appear to be quite complex with co-stimulatory molecules and a variety of cytokines being capable of exerting inhibitory roles. Significantly, IL-13 has been shown to inhibit IL-12 production by macrophages (de Waal Malefyt et al., 1993). However, this discussion will be limited to cross-regulatory mechanisms mediated by IL-4, IL-10, IFN-γ and IL-12.

IL-4 has been shown to prevent the generation of IFN-γ producing Th1 cells from accessory cell stimulated TcR transgenic (Hseih et al., 1992; Seder et al., 1992b;
Tanaka et al., 1993) and polyclonally stimulated naive CD4+ T cells (reviewed in Swain et al., 1991). There is also ample evidence that IL-4 inhibits effector functions of Type I populations in vivo. IL-4 inhibited delayed type hypersensitivity reactions and IFN-γ production by lymphocytes draining the site of antigen challenge as effectively as IL-10 (Powrie et al., 1993). In the murine model of Leishmania major infection in which susceptibility is associated with a Th2 cytokine profile and resistant mice exhibit a Th1 cytokine profile, exogenous IL-4 blocks the generation of the IFN-γ producing protective Th1 phenotype in resistant strains (Chatelain et al., 1992), while neutralizing IL-4 at the time of L. major challenge converts susceptible mice to a resistant Th1 response (Heinzel et al., 1989; Saddick et al., 1990; Chatelain et al., 1992). Unlike IL-4 neutralization, neutralizing IL-10 does not change the course of L. major infection in susceptible strains (Coffinan et al., 1991), suggesting that IL-4 may be more potent than IL-10 in the differential regulation of Th phenotype. However, unlike IL-10, IL-4 does not inhibit proliferation or cytokine production by Th1 clones.

Despite the intense interest in Th1 and Th2 responses, the mechanisms by which IL-4 inhibits Th1 differentiation and IFN-γ production are poorly characterized. IL-4 has been shown to down regulate expression of the IL-12 Rβ2 subunit during Th2 differentiation, thereby inhibiting IL-12 signaling that drives Th1 differentiation (Gollob et al., 1997; Szabo et al., 1997). IL-4 is also a potent down regulator of macrophage function and has been shown to reduce CD40 mediated IL-12 secretion by dendritic cells (Kelsall et al., 1996). IL-4 inhibition of IL-2 production and associated priming for IFN-γ production may also play a factor (reviewed in Seder and Paul, 1994), though

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IFN-γ does not appear to be essential for the generation of Th1 responses in all systems (Seder et al., 1993; McKnight et al., 1994).

IL-10 was first recognized as a cytokine produced by mouse Th2 clones that inhibited cytokine synthesis by, but not proliferative responses of, Th1 clones (Fiorentino et al., 1989). However, human IL-10, which is clearly not exclusively a Th2 cytokine, has been shown to inhibit both proliferation and cytokine production by Th1 as well as Th2 cells (Del Prete et al., 1993). In the face of conditions that usually propagate a Th2 response, including in vitro cultures of TcR transgenic mouse T cells (Coffman et al., 1991), as well as in vivo models of murine gastrointestinal nematode infection (reviewed in Urban et al., 1991b), neutralization of IL-10 changes the cytokine pattern to Th1-like. However, as previously noted, IL-10 neutralization is not sufficient to alter the course of L. major infections in susceptible hosts, while IL-4 neutralization will (Coffman et al., 1991).

Early on it was recognized that IL-10's inhibitory effect on cytokine production by Th1 clones required antigen presenting cells and had a delayed onset (8 hours) (Fiorentino et al., 1989), suggesting that IL-10 acted via an indirect APC dependent mechanism. Consistent with these early findings, IL-10 has been shown to inhibit expression of co-stimulators, such as B7-1 on antigen presenting cells (Ding et al., 1993) that are required for up regulation of IFN-γ production by some Th1 clones, and to inhibit the induction of Th1 development from naive T-cells by inhibiting IL-12 production by antigen presenting cells (D’Andrea et al., 1993; Kennedy et al., 1994; Murphy et al., 1994; Minty et al., 1997).
IFN-γ and IL-12 have both been shown to play a role in the down regulation of Th2 responses. IFN-γ inhibits the proliferative response of Th2 cells (Fernandez-Botran et al., 1988; Gajewski and Fitch, 1988; Maggi et al., 1992), as well as their generation from naive CD4+ cells (Fernandez-Botran et al., 1988; Noble et al., 1993). However, this latter effect is dependent upon the degree of IL-4 priming present (Seder et al., 1992b). With optimal levels of IL-4, IFN-γ is not sufficient to inhibit the generation of the Th2 phenotype. However, in the presence of suboptimal IL-4 concentrations, IFN-γ inhibits the generation of IL-4 producing Th2 cells. In vivo findings in the mouse L. major model using IFN-γ supplementation (Scott, 1991) and neutralization (Reiner and Locksley, 1995) substantiate the findings that IFN-γ decreases IL-4 production in susceptible strains, but unlike IL-4 neutralization, IFN-γ administration is not sufficient to induce the protective Th1 phenotype (Sadick et al., 1990; Reviewed in Scott, 1991; Reiner and Locksley, 1995).

The mechanisms by which IFN-γ inhibits Th2 differentiation and effector functions are not clear. CD28 mediated co-stimulation of Th2 cells has been demonstrated to induce IL-4 responsiveness in an IL-1 dependent manner, and IL-1 receptors, which are expressed on T-cells only after primary stimulation (Luqman et al., 1992), are upregulated on Th2 cells following IL-4 exposure (Koch et al., 1992). Accordingly, a growing number of experiments indicate IFN-γ inhibits Th2 proliferation by inhibiting IL-1 co-stimulation (Gajewski and Fitch, 1988; Oriss et al., 1997). Differentiation to Th1 is also associated with loss of the IFN-γ Rβ chain which is maintained on Th0 and Th2 cells (Pernis et al., 1995), and Th2 cells have been shown to transduce signals via this receptor that are lacking in Th1 clones (Pernis et al., 1995;
Groux et al., 1997), suggesting that IFN-γ may provide negative signals that inhibit Th2 differentiation. However, the significance of the IFN-γ receptor on Th2 cells remains enigmatic. Studies of rIL-12 mediated suppression of Th2 responses in murine leishmaniasis (Heinzel et al., 1993) and murine graft vs host disease (Via et al., 1994) indicate that IFN-γ is required for inhibition of IL-4 priming. Accordingly, IFN-γ KO mice on a resistant Th1 background switch to the susceptible Th2 phenotype when challenged with *L. major* (Wang et al., 1994a). However, IFN-γR KO mice effectively mount Th1 responses to pseudorabies (Schijns et al., 1994) and *L. major* (Swihart et al., 1995), even in the presence of IFN-γ neutralizing antibodies.

While it is well accepted that IL-12 inhibits Th2 responses (Manetti et al., 1993), factions stand divided as to the IFN-γ dependence of this inhibition. As previously indicated, IL-12 mediated Th2 inhibition has been shown to be IFN-γ dependent using IFN-γ neutralization experiments in a number of murine Th2 models, including graft vs host disease (Via et al., 1994), the egg granuloma stage of *S. mansoni* (Finkelman et al., 1994; Oswald et al., 1994; Wynn et al., 1994), anti-IgD stimulation (Morris et al., 1994), and leishmaniasis (Heinzel et al., 1993). However, the leishmaniasis model presents a paradox. IL-12 administration at the time of infection protects susceptible strains in an IFN-γ dependent manner (Heinzel et al., 1993), but other experiments support direct inhibition of IL-4 production by IL-12. Exogenous IL-12 decreases IL-4 transcription and protein production, converting susceptible strains to resistant if administered during the first week of infection (Seder et al., 1993; Sypek et al., 1993), a time when both resistant and susceptible strains produce equivalent amounts of IFN-γ (Morris et al., 1992; Reiner et al., 1994). Furthermore, experiments with IFN-γ
neutralization in Balb/c and IFN-γ KO mice indicate that IL-12 can directly inhibit IL-4 transcription independent of IFN-γ (McKnight et al., 1994; Wang et al., 1994b). As with all cytokine treatments, timing appears critical in that rIL-12 cannot suppress established Th2 responses (Heinzel et al., 1993; Finkelman et al., 1994; Via et al., 1994; Wang et al., 1994b), due to a lack of the IL-12R signaling chain on Th0 and Th2 cells (Szabo et al., 1995; Gollob et al., 1997; Rogge et al., 1997). However, the finding that early exposure of Th2 cells to IFN-γ maintains the IL-12Rβ subunit (Szabo et al., 1997) suggests a mechanism by which some CD4+ subpopulations can exhibit IFN-γ independent IL-12 mediated suppression of IL-4 production.

**In Vivo Significance of the Th1/Th2 Paradigm**

Studies of *L. major* in mice were especially significant because they demonstrated for the first time that susceptibility or resistance to disease are attributable to the specific Th1 or Th2 phenotype that is generated during interaction with the pathogen and that endogenous cytokines produced as a result of interaction with the pathogen not only determine the Th phenotype that is generated, but can be manipulated to direct the development of a protective or resistant Th phenotype (reviewed in Reiner and Locksley, 1995). Thus, lymphocytes draining the lesions of disease resistant C57BL/6 and C3H/HeN mice produced Th1 cytokines, while lymphocytes draining the lesions of Balb/c mice, which develop a chronic and eventually lethal systemic disease, produced Th2 cytokines. Furthermore, treatment of resistant mice with anti-IFN-γ or anti-IL-12 ablated Th1 development resulting in a susceptible Th2 expressing phenotype, whereas treatment of susceptible mice with anti-IL-4 or IL-12 supplementation inhibited Th2 differentiation and conferred a resistant Th1 phenotype. Interestingly, IFN-γ
supplementation was not sufficient to drive conversion of susceptible mice to the resistant phenotype, though it did substantially reduce IL-4 production. Further investigation has substantiated a major role for IFN-γ in protection from *Leishmania* in that resistant C57BL/6 mice with disruption of the IFN-γ gene converted to the susceptible Th2 phenotype (Wang *et al*., 1994a). However, other non-IFN-γ dependent mechanisms for control of *Leishmania* appear to exist as IL-12 has been shown to suppress IL-4 transcription even in the absence of IFN-γ (McKnight *et al*., 1994; Wang *et al*., 1994b).

Since the original characterization of Th1 induced resistance to *L. major*, a growing number of studies have supported the notion that Th1 responses, by virtue of their IFN-γ and TNF-α mediated potentiation of macrophage cytocidal activities, play a crucial role in defense against a wide variety of intracellular pathogens, including parasites, bacteria, and viruses (Sher and Coffman, 1992; Kaufman, 1993). Th1 responses have been associated with protection in human leprosy (Yamamura *et al*., 1991b), human lyme disease (Yssell *et al*., 1991), human leishmaniasis (Pirmez *et al*., 1990; Pirmez *et al*., 1993), *Toxoplasma gondii* (Suauki *et al*., 1989), *Plasmodium sp.* (Sedegah *et al*., 1994; Stevenson *et al*., 1995), *Cryptosporidium parvum* (Ungaar *et al*., 1991; Urban *et al*., 1996), *Listeria monocytogenes* (Buchmeier and Schreiber, 1985; Hsieh *et al*., 1993), and murine cytomegalovirus (Orange *et al*., 1996) infections (From Finkelman *et al*., 1997). However, macrophages are certainly not the sole mediators of resistance in Th1 dominated responses *in vivo*, with NK cell, CTL, and cytotoxic antibody responses all being induced or augmented by Th1 cytokines. 

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Th1 responses by virtue of their activation and recruitment of macrophages to the site of infection result in delayed type hypersensitivity reactions. This is especially true for slow growing, persistent organisms, such as *M. leprae*, for which resistance has been associated not only with the development of Th1 responses but also the presence of granulomatous inflammation (Yamamura *et al.*, 1991). However, Th1 responses, by virtue of the toxic side effects of TH1 cytokines and inflammatory mediators from recruited cells, can result in pathological inflammatory reactions. In particular, Th1 cytokines have been linked to diseases characterized by organ-specific autoimmunity (reviewed in Romagnani, 1994), including autoimmune thyroiditis, Crohn's disease, experimental autoimmune uveoretinitis (Dubey *et al.*, 1991), experimental allergic encephalomyelitis (Beraud *et al.*, 1991), insulin-dependent diabetes mellitus (Hahn *et al.*, 1987), and multiple sclerosis (Benvenuto, *et al.*, 1991; Brod *et al.*, 1991). It is certainly tempting to attribute such disease to faulty Th2 cross-regulatory mechanisms, especially in light of the association of lethal exacerbations of pathogen induced inflammatory reactions with disruption of the IL-10 gene (Gazzinelli *et al.*, 1996).

In contrast to intracellular pathogens, studies of parasitic helminths in rodent models indicate that Th2 responses provide protection from extracellular pathogens primarily via phagocyte independent mechanisms. Though many parasites have been studied to determine the Th phenotype induced in susceptible versus resistant hosts, four models are primarily responsible for establishing that Th2 responses are important for protective immunity to nematode parasites. These are *Trichinella spiralis*, *Heligmosomoides polygyrus*, *Nippostrongylus brasiliensis*, and *Trichuris muris* (reviewed in Urban *et al.*, 1992; Finkelman *et al.*, 1997).
The lifecycles of these four parasites have been briefly reviewed (Urban et al., 1992; Finkelman et al., 1997). *H. polygyrus* and *T. muris* have strictly enteral lifecycles. *H. polygyrus* third-stage larvae (L₃) invade the wall of the small intestine, then enter the gastrointestinal lumen approximately 7 days after infection, mature to adults, and remain alive for a period of several months in susceptible (Balb/c) strains. Challenge infections, however, are eliminated more rapidly in immune hosts, especially in rats where the effect is extremely rapid, occurring within 1 hour of challenge. *T. muris*, which infects as L₁ in a resistant egg, parasitizes the large intestine. Infections are chronic in some strains (B10.BR) and more short lived in most inbred mouse strains (Balb/k), which expel the parasite before it develops into egg laying adults.

In contrast to *H. polygyrus* and *T. muris*, the lifecycles of *T. spiralis* and *N. brasiliensis* have visceral migration phases. *T. spiralis* infects its hosts as L₁, matures and mates within 2 days of infection, and then resides within the wall of the small intestine, where females release larvae beginning 4-7 days after ingestion. Larvae migrate through the body via intestinal lymphatics and venules, settling primarily in striated muscle where they encapsulate. Adults are generally expelled from rodents less then 2 weeks following ingestion. *N. brasiliensis* penetrates the skin of hosts as L₃ and migrates to the lungs by 36 hours, where the parasite causes eosinophilic granulomas before being coughed up, swallowed, and maturing to egg laying adults in the small intestine. *N. brasiliensis* have been mouse adapted. In the mouse the prepatent period is 5-6 days. *N. brasiliensis* are expelled by 10 days after inoculation, and challenge infections are characterized by a decrease in the number of larvae that reach the intestine, and markedly reduced or obliterated egg production.
Protective immunity to each of these parasites has been shown to be CD4 dependent (reviewed in Finkelman et al., 1997). Anti-CD4+ mAb treatment causes increased fecundity in primary *H. polygyrus* infections, blocks immunity to *H. polygyrus* challenge infections (Urban et al., 1991a), prolongs primary infections in *T. muris* resistant mice (Koyama et al., 1995), prevents expulsion of *N. brasiliensis* (Katona et al., 1988), and prevents the rapid expulsion of *T. spiralis* in previously infected rats (Vos et al., 1983).

IFN-γ and IL-12 have been shown to inhibit protective immunity to gastrointestinal nematodes. These effects are most evident in *T. muris* and *N. brasiliensis* infections (reviewed in Finkelman et al., 1997). Mouse strains that develop chronic *T. muris* infections produce strong IFN-γ responses as compared to resistant strains which produce a strong IL-4 response (Else and Grencis, 1991; Else et al., 1992). IFN-γ neutralization at the time of *T. muris* inoculation shortens the duration of infection (Katona et al., 1988). IFN-γ (Urban et al., 1993) or IL-12 (Finkelman et al., 1994) supplementation during *N. brasiliensis* inoculation enhances egg production and prolongs the duration of infection. IL-12 administered in this manner will also retard, but not inhibit the Th2 response leading to rapid clearance of challenge infection (Finkelman et al., 1997). IL-12 also increases the susceptibility of resistant hosts to *T. muris* (reviewed in Finkelman et al., 1997). IL-12's effects in enhancing susceptibility have been shown to be IFN-γ dependent using IFN-γ mAB and IFN-γ KO mice (Finkelman et al., 1994). Though IFN-γ was once thought to contribute to *T. spiralis* resistance because of the more rapid expulsion from mouse strains expressing higher IFN-γ (Pond et al., 1989), subsequent studies using IFN-γ neutralizing antibodies (Finkelman et al.,
1997) and adoptive transfer of immune T cells expressing Th1 or Th2 phenotypes (Ramaswamy et al., 1994) now indicate that Th2 phenotypes are significant in protection immune responses directed against this parasite.

Studies of the effect of IFN-γ supplementation on immunity to nematode parasites were also enlightening because they suggested that strongly polarized Th1 or Th2 responses could interfere with the ability to generate the opposite response in vivo. Heat killed *Brucella abortis* injected intravenously, a strong inducer of IFN-γ production, increased *N. brasiliensis* adult worm survival and fecundity in a manner similar to that of exogenous IFN-γ (Urban et al., 1993). These parasite sparing effects were reversed by the administration of anti-IFN-γ and anti-IFNα/β, suggesting that animals with systemic IFN responses may be more likely than healthy animals to develop severe nematode infections. This finding was also consistent with observations from *Eimeria spp.* infected mice, which have enhanced IFN-γ production but demonstrate depressed eosinophil and mast cell responses and increased fecundity in response to infection with *N. brasiliensis* (Upton et al., 1987). Similarly, ongoing Th2 responses induced by helminth parasite antigens have been shown to alter the Th1 phenotype of purified protein derivative (PPD)-specific Th cells to include IL-4 and IL-5 production (Pearlman et al., 1993).

Several other key characteristics of helper T cell phenotypes originated in studies of nematode infections. First, the nature of the helper T-cell response to a given pathogen is, at least for some pathogens, a local phenomenon (Svetic et al., 1993). Perhaps reflecting the totally enteral lifecycle, *H. polygyrus* infected mice displayed large increases in Th2 gene expression in gut wall, Pye'r patch, and mesenteric lymph nodes,
while only minor changes were seen in the splenic expression of these cytokines. A second finding was that the kinetics of Th cytokine expression are not always the same with different pathogens (reviewed in Urban et al., 1992). IL-3, IL-5, and IL-9 were rapidly expressed within 24 hours of infection, followed by IL-4, IL-10, and IL-6 at day 3 post-infection in *H. polygyrus*, whereas IL-3, IL-4, and IL-9 were expressed 3 days post and IL-5 at 8 days post infection with *N. brasiliensis*. *H. polygyrus* infected mice exhibited slight increases in IL-2 and IFN-γ production followed by an IFN-γ decrease below baseline levels, while *N. brasiliensis* infected mice failed to show increases in IL-10 or IL-2 and only exhibited a decrease in IFN-γ production.

Though IL-4 production is a characteristic of nematode infections, its protective effects are best demonstrated in *H. polygyrus* and *T. muris* infections. Neutralization of IL-4 or the IL-4R blocks immunity to challenge infections with *H. polygyrus* (Urban et al., 1991b), and IL-4R neutralization converts *T. muris* resistant to susceptible hosts (Else et al., 1994). However, immunity to *H. polygyrus* appears to be more IL-4 stringent than immunity to *T. muris* in that IL-4 KO mice fail to expel *H. polygyrus* but efficiently expel *T. muris* (reviewed in Finkelman et al., 1997). Thus, IL-4 appears necessary for immunity to *H. polygyrus*, but IL-4 alone has been shown to be insufficient for complete immunity in *H. polygyrus* infected SCID mice (Urban et al., 1995).

IL-4 appears to play a less crucial role in protective immunity against *N. brasiliensis* and *T. spiralis*. IL-4 and IL-4R neutralization do not significantly alter the course of *N. brasiliensis* infection (Madden et al., 1991; Ungar et al., 1991) but sustained IL-4 stimulation via IL-4C (IL-4 complexed 2:1 with its Mab to increase its half-life) cures CD4 depleted and SCID mice (Urban et al., 1995). (This treatment also
cures established *T. muris* and *H. polygyrus* infections in immunocompetent mice (Else et al., 1994; Urban, et al., 1995)). Studies in *T. spiralis* are limited by a lack of IL-4 neutralizing reagents for rats, but IL-4 R neutralization in the mouse model modestly exacerbated *T. spiralis* infections (Finkelman et al., 1997). IgE depleted rats have increased *T. spiralis* larval numbers (Dessein et al., 1981) and rapid expulsion can be transferred with purified IgE (Ahmad et al., 1991), suggesting a protective role for IL-4 or IL-13, since as previously discussed, these cytokines are responsible for IgE isotype switching. The apparently unnecessary but protective effects of IL-4 in *T. muris*, *N. brasiliensis*, and *T. spiralis* have lead to the summary that while IL-4 is required for protective immunity to *H. polygyrus* infections, it appears to provide a redundant mechanism that becomes critical for some parasites when CD4 dependent mechanisms are blocked (Finkelman et al., 1997).

In addition to IgE production, nematode infections are characteristically associated with eosinophilia that is mediated by IL-5 (Coffman et al., 1989; Sher et al., 1990a, b) and mucosal mastocytosis that is mediated primarily but not exclusively by the combined effects of IL-3 and IL-4 (Takeda et al., 1997). Though eosinophils are known to kill some parasites *in vitro*, IL-5 neutralization experiments which obliterate *in vivo* eosinophilia have failed to indicate a necessary role for eosinophils in the immune response to *T. muris* (reviewed in Finkelman et al., 1997), *N. brasiliensis* (Coffman et al., 1989), and *H. polygyrus* (Urban et al., 1991b). However, there is evidence that IL-5 dependent mechanisms may be significant outside the confines of the intestinal tract. Anti-eosinophil serum has been shown to increase *T. spiralis* muscle larval recoveries (Grove et al., 1977), and transgenic mice expressing elevated IL-5 levels exhibit
increased killing of *N. brasiliensis* in lung tissues (reviewed in Finkelman *et al.*, 1997). Additionally, ablation of the eosinophil response with anti-IL-5 enhanced the survival of *Angiostrongylus cantonensis* (Sasaki *et al.*, 1993) and *Strongyloides venezuelensis* (Korenaga *et al.*, 1994) in mouse brain and lung, respectively.

In order to determine how IL-4 controls *H. polygyrus* and *T. muris* infections, studies have focused on the physiological effects of this cytokine that could limit infection (reviewed in Finkelman *et al.*, 1997). Early evidence that IL-4 might have physiological effects came from studies of *T. spiralis* infections in immune rats in which mast cells (Woodbury *et al.*, 1984) and IgE (Ahmad *et al.*, 1991), both regulated by IL-4, have been shown to be strongly associated with the rapid expulsion phenomenon. A localized anaphylactic reaction in which chemical mediators of anaphylaxis, including serotonin, leukotrienes, and prostaglandins released through IgE or IgG mediated mast cell degranulation cause increased smooth muscle contraction and increased fluid secretion may be one mechanism which inhibits larval penetration of the mucosa. Unfortunately, the role of IL-4 in this phenomenon has not been elucidated because IL-4 neutralizing antibodies and IL-4 knockout rats are not available. Like *T. spiralis*, specific antibodies can promote *T. muris* expulsion though mast cells do not appear to be necessary. The role of IL-4 (or IL-13) in *T. muris* expulsion has not been well characterized.

In *H. polygyrus* infections of mice, mAb to the IL-4 R has been shown to block a mast cell and leukotriene dependent, parasite induced, increase in small intestinal fluid influx and smooth muscle contractility during challenge infections. These changes can also be induced in non parasitized mice treated with IL-4C. Though these findings
suggest a mechanism by which IL-4 could be directly responsible for immunity in *H. polygyrus*, there are a number of discrepancies. Changes in smooth muscle contractility with IL-4C treatment lag behind, by 3-5 days, the treatment induced decrease in fecundity. In addition, in the natural immune response to *H. polygyrus*, endogenous IL-4 appears to eliminate early parasite stages, while mechanisms mediated by exogenous IL-4 are directed against adult worms. Studies of IL-4's role in other immune mechanisms including antibody-mediated defenses and alterations in cellular adhesion molecules such as VCAM-1 are an area of continued investigation.

Primary *N. brasiliensis* infections have also been shown to induce changes in intestinal smooth muscle contraction and fluid dynamics, though these changes are B cell, T cell, leukotriene, and mast cell independent. The role of IL-13 in protective immunity to nematodes is an area of great interest at this time because STAT6 KO mice fail to expel *N. brasiliensis* (Finkelman *et al.*, 1997) and STAT6 is only known to be involved in IL-4 and IL-13 receptor transduction (Yssell *et al.*, 1991). Other IL-4 independent mechanisms that induce expulsion of *N. brasiliensis* are an ongoing focus of study and include mucous trapping, antibody dependent mechanisms, and lipid peroxidation (Finkelman *et al.*, 1997).

Like Th1 responses, Th2 responses have also been linked to a number of human diseases (reviewed in Romagnani, 1994). Allergen specific T-cells from atopic individuals preferentially express TH2 profiles, while those from nonatopic donors express Th1/Th0 profiles. Th2 but not Th1 cytokines have been found via in situ hybridization at the site of late phase reactions from atopic patients, bronchial biopsies and BAL fluids from asthma patients, allergen challenged nasal mucosa of allergic
rhinitis patients, and in the BAL fluids of allergic asthmatics. Over production of Th2 cytokines and an associated decrease in Th1 cytokines has also been documented in one person with Omenn's syndrome, a rare immunodeficiency characterized by hypereosinophilia and increased serum IgE.

In the eleven years since the cytokine profiles of mouse helper T cell clones were first characterized, continued study of the in vivo cytokine responses to a variety of stimulating antigens has confirmed that Th1 and Th2 responses are true in vivo phenomena which determine the resistance or susceptibility to pathogens. Noting that some Th2 mediated functions are required for immunity to certain parasites while being redundant for other parasites (eg IL-4 for H. polygyrus vs N. brasiliensis immunity), it has been suggested that Th2 mediated immune mechanisms are generalized responses initiated by attributes that are common to Th2 inducing pathogens (Finkelman et al., 1997). Proteolytic enzymes are one potential common thread among Th2 inducing extracellular pathogens which tend to traverse integumentary surfaces, but the immunologic role of these enzymes has been poorly studied. In the case of Th1 inducing attributes, lipopolysaccharide (LPS) is known to induce IL-12 production (Ozmen et al., 1994) and is common to a number of Th1 inducing organisms. Therefore, it is likely that for both Th1 and Th2 responses, certain attributes are common to the organisms that evoke the respective response. It has further been suggested that the Th1 pathway which protects against intracellular pathogens can be summarized as simply an IL-12/IFN-\(\gamma\)/iNOS mediated pathway, sharply contrasting the diverse mechanisms identified for the control of nematode pathogens. Given the rapid rate of genetic mutations in single celled organisms, it would seem that redundancies in Th1 responses are even more critical to
the survival of the host than Th2 redundancies. Furthermore, redundancies in the Th1 response have been identified. For example, in addition to nitrogen-dependent mechanisms, macrophages possess potent oxidative metabolic pathways which are known to kill pathogens (Jones, 1993). At the cytokine level IL-18, which like IL-12 is also induced by bacterial pathogens, potently induces IFN-γ production, in an IFN-γ independent manner (Kohno et al., 1997). Antibodies induced during Th1 responses may also have phagocyte independent effects, such as virus neutralization and complement mediated cytotoxicity. Thus, it appears that Th1 and Th2 responses are more like sides of the same coin. Each response being induced by pathogen attributes that relegate the pathogen to one of two immunologically relevant groups, with diverse effector mechanisms in each response being redundant for some pathogens but required for others.

**Strongylus vulgaris** a Potential Model for the TH2 Paradigm in Horses

Because of the clear association between TH2 responses and nematode parasite infections, a nematode parasite of horses was studied to determine if cytokine profiles suggestive of the TH2 phenomenon are evident in the equine species. *Strongylus vulgaris* is considered the most pathogenic nematode parasite of horses due to the severe arterial lesions it causes in the cranial mesenteric artery during larval migration. The life-cycle has been extensively reviewed (Enigk, 1950, 1951; Duncan and Pirie, 1972; Ogbourne and Duncan, 1985). Infective third-stage larvae (L₃) ingested from contaminated pasture penetrate the mucosa primarily of the cecum and ventral colon where they migrate for 4-7 days reaching the submucosa. There, larvae molt to the fourth-stage (L₄) and then proceed along arterioles and arteries that supply the intestine toward the root of the cranial mesenteric artery. Upon reaching the
regions of ileo-ceco-colic and cranial mesenteric arteries, larvae cease migration and molt to become immature adults (L₃), causing severe arteritis before returning, again via the vasculature, to complete their life cycle in the large intestine. Arterial lesions include the formation of tortuous subintimal tracts, thrombi, and in severe cases, verminous aneurysms, which can compromise perfusion of intestinal vascular beds. This syndrome, known as verminous arteritis or thromboembolic disease, is characterized by ischemic infarctions of the bowel which result in toxemia, abdominal pain, and death in severe cases (White, 1985). Though the life cycle is prolonged, taking 4-6 months to complete, signs associated with large inoculae of virulent L₃ including pyrexia, anorexia, depression and abdominal pain appear within the first week following L₃ ingestion and are near maximal by 14 days post infection (Klei et al., 1982).

Though the importance of S. vulgaris as an equine pathogen has decreased (Herd, 1990) due to regular use of ivermectin, which is efficacious against adult and migrating larval forms of the parasite (Slocombe et al., 1982; Klei et al., 1984), S. vulgaris provides several advantages as a model in the study of the equine immune response to nematode parasites. Horses can acquire an incomplete resistance to further infection from natural exposure to S. vulgaris L₃ (Amborski et al., 1974; Duncan, 1975; Klei et al., 1986), indicating that immune modulation in order to limit S. vulgaris infestations is likely to be an attainable goal and that elucidation of the mechanisms that limit natural infection could serve as a model to define some aspects of the equine response to nematode parasites that may have more universal applications. Singular infections can be produced by surgical implantation of adults
into parasite free ponies (McClure et al., 1994) and late L₃ and early L₄ have been successfully cultured in vitro (Chapman et al., 1994). This provides both a ready source of feces for collection of infective S. vulgaris L₃ and a source of late L₃ and L₄ for characterization of the host immune response to these forms. While mature L₅ forms can be procured fairly easily from necropsy specimens, the availability of late L₃ and early L₄ forms provides an invaluable tool for the characterization and manipulation of the host immune response to these forms. As early larval forms represent the first line of immune evasion that ultimately leads to a patent and pathogenic S. vulgaris infection, successful vaccine strategies will likely be directed against epitopes of these early larval forms. In support of this, previous studies have demonstrated that oral vaccination with radiation-attenuated S. vulgaris L₃ induces resistance to challenge infection for up to 9 months (Klei et al., 1989) and prevents classical lesions of verminous arteritis (Klei et al., 1982). However, radiation-attenuated S. vulgaris vaccination does have its practical limitations. The protection is species-specific and is influenced by management practices in that vaccinated animals reared without anthelmintic therapy up until the time of challenge are not protected from challenge infections of S. vulgaris, whereas vaccinates reared with anthelmintic therapy are protected (Klei et al., 1989), suggesting that ongoing parasitism interferes with the protective immune response induced by vaccination.

Eosinophils, though not apparently essential in the protective immune response to helminth parasites (Coffman et al., 1989; Sher et al., 1990a; Urban et al., 1991b), are considered a hallmark of helminth infections (Finkelman et al., 1991) and eosinophilia is an IL-5 dependent phenomenon (Coffman et al., 1989). Following
infection with *S. vulgaris*, both circulatory and tissue eosinophilia develop. A primary circulatory eosinophilia occurs 3-5 weeks after infection in parasite free and previously infected animals (Amborski *et al.*, 1974; Duncan and Pirie, 1975; Hofing and Bennett, 1982) and corresponds temporally to establishment of larval infestation in the mesenteric arteries. The timing of peripheral eosinophilia in ponies parenterally vaccinated with larval or adult somatic extracts follows this pattern. However, both irradiated larval recipients (Monahan *et al.*, 1994) and strongyle naive ponies receiving immune serum (Klei, 1992) experience an anamnestic eosinophilia, which begins to rise on week 2 following challenge and reaches a higher maximal value than that of parenterally vaccinated ponies on week 4. Primary eosinophilias have been shown to disappear between 6 and 8 weeks following infection, which was the case for parenterally and orally vaccinated ponies whose eosinophilia appeared to be falling by week 5 post challenge. Depending upon the immune status of the animal, a second peak in eosinophil numbers occurs sometime after the 8th-10th week. This second eosinophilia, which tends to remain until the 20th week, may be delayed in lightly infected animals (Bailey, 1984) and its occurrence in horses with pre-existing infections following experimental challenge is unclear (Amborski *et al.*, 1974; Bailey, 1987). Trickle infections by contrast have been shown to result in sustained increases in eosinophil counts (Drudge *et al.*, 1966; Amborski *et al.*, 1974). Tissue eosinophilia, along with a neutrophilic component, tends to predominate within the submucosa shortly after infection (2 days to 1 week) but subsides until approximately 2-4 months following infection, presumably associated with larval return to the intestine (Duncan and Pirie, 1972, 1975). By week 3 following infection,
inflammatory cells of the arterial intima are primarily plasma cells, lymphocytes, and neutrophils with few eosinophils, though eosinophilic infiltration of the adventitia predominates. In other helminth systems, eosinophilia has been demonstrated in associations with tissue migration, following parasite death in response to anthelmentic therapy, and in response to tissue sequestration of helminth antigens (Walls and Beeson, 1972; Schriber and Zucker-Franklin, 1975).

In addition to the anamnestic eosinophilia associated with challenge infections in ponies rendered immune by radiation-attenuated oral vaccination, *in vitro* determinations of eosinophil functions suggest that eosinophils may play a role in the protective immune response to *S. vulgaris* infection. Eosinophils have been suggested to act as effector cells in anti-parasite immunity, based on their adherence and cytotoxicity toward a variety of nematode parasites, including *Trichinella spiralis*, *Dictyocaulus viviparus*, and *Angiostrongylus cantonensis* (Kazura and Grove, 1978; Wassom and Gleich, 1979; Kazura and Aikawa, 1980; Knapp and Oakley, 1981; Yoshimura *et al.*, 1984). Eosinophils from ponies with *S. vulgaris* induced eosinophilia express more Fc and complement cell surface receptors than do eosinophils from strongyle naive ponies (Dennis *et al.*, 1988), and activated eosinophils have been shown to release cytotoxic compounds including major basic protein and reactive oxygen metabolites (Venge, 1990) that could lead to worm destruction. Serum from strongyle immune ponies has been shown to mediate adherence of eosinophils from strongyle sensitized but not strongyle naive ponies to *L*$_3$, resulting in *L*$_3$ death (Klei *et al.*, 1992), and suggesting that eosinophils become
activated during *S. vulgaris* infections, allowing them to function as mediators of cellular cytotoxicity.

Parasites that exhibit multistage lifecycles complicate investigations of the host's antigen-specific immune response because of the diversity of antigens that each stage presents. Indeed this multistage changing of the parasite antigenic blanket, as well as the presentation of immunodominant antigens that divert the immune response from other epitopes that may be critical to the parasite's survival, are considered virulence factors employed by parasites. Early studies of the lymphoproliferative response to *S. vulgaris* L₄ and L₅ extracts indicated that these extracts were mitogenic and that differences in the proliferative responses of infected and noninfected animals could not be detected (Bailey et al., 1984). Fractionation of these extracts by gel filtration yielded three fractions which specifically stimulated lymphocytes from *S. vulgaris* infected but not helminth free foals (Bailey, 1987; Bailey et al., 1989). One fraction specifically stimulated PBMC from *S. vulgaris* infected ponies 1-3 weeks post infection, but this responsiveness decreased by 7 weeks post infection and was absent by the end of the prepatent period, suggesting that the response was directed at early forms of the parasite. Similar studies of naturally infected equines indicated that the lymphoproliferative responses were inversely correlated to larval burdens, being more responsive in animals with less severe infections, whereas experimental infections did not reveal differences in the proliferative responses of ponies receiving inoculae of 200 or 700 L₃ (Bailey, 1984). Specific proliferative responses to soluble adult *S. vulgaris* antigens (SAWA) have been characterized in ponies with oral radiation-attenuated L₃ vaccine induced resistance to challenge (Klei, 1992). These
initial attempts failed to detect significant differences in the proliferative responses of resistant and non-resistant ponies, both before and after challenge infection. Specific proliferative responses to SAWA have been demonstrated in infected ponies and in ponies receiving trickle infections of 50 L₃ per week for 25 weeks, both with and without the benefit of anthelmintic therapy (Dennis et al., 1992; Klei, 1992).

Proliferative responses of PBMC from ponies receiving trickle infections were examined prior to challenge and on weeks 7, 25 and 27 into challenge. PBMC from all ponies, regardless of anthelmintic therapy, failed to proliferate on week 7 of challenge but developed a significant proliferative response to SAWA by week 25 of challenge, which was decreasing by week 27. Lymphocytes derived from the mesenteric and mediastinal lymph nodes of these ponies at week 27 indicated that mesenteric lymph nodes from ivermectin treated ponies were more reactive to SAWA than nontreated ponies, although this difference was not statistically significant.

Mediastinal lymph node derived lymphocytes from ivermectin treated, untreated, and uninfected control ponies were equally unresponsive, suggesting a compartmentalization of the antigen-specific immune response.

In addition to the specific lymphoproliferative responses of *S. vulgaris* sensitized ponies, liberation of an eosinophil chemotactic factor or factors from SAWA stimulated PBMC of *S. vulgaris* sensitized ponies confirms the generation of cytokines in antigen-specific responses to *S. vulgaris* infections (Dennis et al., 1993). Supernates from SAWA stimulated PBMC of *S. vulgaris* sensitized but not *S. vulgaris* naive ponies exhibited an eosinophil chemotactic activity. This chemotactic activity was specifically stimulated by *S. vulgaris* in that *S. vulgaris* sensitized PBMC...
stimulated with *S. edentatus* antigen preparations did not induce eosinophil chemotactic activity. Though the chemotactic factor(s) was only partially characterized as a protein exceeding 8000 kDa that was heat labile and contained essential carbohydrate moieties, its production following *in vitro* antigen stimulation of PBMC that were sensitized to *S. vulgaris* *in vivo* suggests the presence of an eosinophil chemotactic factor that could contribute to the blood and tissue eosinophilia that is characteristic of *S. vulgaris* infection.

In part due to the complexity of the *S. vulgaris* system, in which antibodies evoked by the different larval stages suggest an array of epitopes that often cross react both with other larval stages of not only *S. vulgaris* but other parasite species as well, the role of antibodies in the protective immune response to *S. vulgaris* has been poorly characterized. Circulating β-globulins increase following *S. vulgaris* infection, primarily due to an increase in IgG(T) production (Patton *et al.*, 1978) and *S. vulgaris*-specific antibodies have been demonstrated in response to infection (Bailey *et al.*, 1989). The nature of the immunodominant proteins associated with infection appears to vary with the type of exposure to *S. vulgaris* (reviewed in Klei, 1992). Western blot analysis of adult antigen preparations identified 110, 100, 88, and 79 kDa proteins as immunodominant in one foal with naturally acquired resistance induced by trickle infectious of *S. vulgaris*, while a 21 kDa protein was recognized with serum from orally vaccinated foals that were protected from challenge infections by ivermectin treatment. 22 and 23 kDa bands were recognized by sera from all ponies vaccinated or previously infected and subsequently challenged with virulent *L*. Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
To date, the reactivity of larval antigen preparations with immune horse serum has not been examined by western blot analysis.

Several findings heighten interest in antibodies specific for early *S. vulgaris* larval stages. When compared to parenteral vaccination with adult or larval somatic antigen homogenates, which did not confer resistance to challenge infection, oral radiation-attenuated L₃ vaccinated ponies which were protected from challenge had higher prechallenge antibody titers specific for L₃ surface antigens, as identified by indirect fluorescent antibody testing, which remained higher than those of parenterally vaccinated groups for two weeks following challenge (Monahan *et al.*, 1994). These L₃-specific antibodies appear to be both species and stage specific in that antibodies from vaccinates did not cross react with L₃ of the closely related *S. edentatus* parasite, but did react with both *S. vulgaris* and *S. edentatus* L₄ (Klei *et al.*, 1993). Antibodies directed at L₄ stages were also higher in irradiated larval vaccinates prior to challenge but converged with those of parenterally vaccinated ponies by 2 weeks following challenge. *In vitro* assays also demonstrate that an anti-L₃ antibody in immune sera promotes adherence of eosinophils from strongyle sensitized but not strongyle naive ponies to *S. vulgaris* L₃, but not L₃ of closely related *S. edentatus*, resulting in L₃ death (Klei *et al.*, 1992) and suggesting that anti-L₃ antibodies may function in antibody dependent cellular cytotoxicity mechanisms. These findings, in conjunction with *in vivo* findings that irradiated L₃ vaccinates lack arterial lesions associated with migration of late larval and adult forms (Klei *et al.*, 1982), while animals acquiring natural resistance sustain small numbers of later stage larval parasites in the face of an immune response that rejects newly acquired infections (Duncan, 1973; Amborski,
Bell et al., 1974; Klei, 1986), suggest that antibody to early larval stages may play a role in the protective immune response generated by both natural immunity and irradiated larval vaccines. However, humoral factors adoptively transferred from immune ponies were not sufficient to protect nonimmune ponies when administered in conjunction with or prior to oral challenge with *S. vulgaris* L₃ (Klei, 1992). In fact, the presence of high antibody titers in these adoptively transferred ponies, as well as titers induced by parenteral immunization with larval or adult somatic extracts in conjunction with Ribi adjuvant (Monahan et al., 1994), which also did not protect from challenge infections, actually resulted in an exacerbation of lesions normally associated with larval migration. This exacerbation of lesions seen with high circulating titers does not necessarily rule out a role for antibodies in the protective response to *S. vulgaris* but may suggest that additional factors, such as timing of the induction of certain epitope-specific antibodies, activation of cellular immune mechanisms, or differences in the local secretion pattern of antibodies within the lumen of the intestines may be significant in the protective immune response to *S. vulgaris*.

Helminth infections are characteristically associated with eosinophilia and elevated IgE production (Finkelman et al., 1991), traits that also characterize the Type II or TH2 response initially described in cloned mouse cells (Mosmann et al., 1986; Mosmann and Coffman, 1989b). Type II responses, identified by the production of IL-4 and IL-5, which are integral to the generation of IgE (Snapper and Paul, 1987) and eosinophilia (Coffman et al., 1989) respectively, have been shown to play a significant role in the protective immune response to metazoan helminths.
(reviewed in Mosmann and Coffman, 1989a; Cox and Lieu, 1992; Urban et al., 1992). Though the role of IgE in equine *S. vulgaris* infection has not been examined because equine-specific reagents have not been made commonly available, *S. vulgaris* infections are characterized by the generation of a peripheral and tissue eosinophilic response. As previously described, following oral vaccination with radiation-attenuated *S. vulgaris*, the eosinophil response is induced in an amanestic fashion and eosinophil chemotactic activity has been found in the supernatants of *S. vulgaris* stimulated PBMC from immune but not nonimmune ponies (Dennis et al., 1993). These findings suggest that differential cytokine production, perhaps similar to that seen in classical Type II responses to helminth parasites may play a role in the immune response to *S. vulgaris*.

**Cytokine Quantitation**

To date, no single surface marker has allowed Th1 and Th2 clones to be distinguished so that identification in cloned cells (Mosmann et al., 1986), purified CD4+ T-cells (Swain et al., 1991), and more recently nonhomogeneous cellular populations (Yamamura et al., 1991; Robinson et al., 1992) has hinged on the ability to detect differing relative amounts of the characteristic Th1 or Th2 cytokines. Early studies examined supernatants from *in vitro* stimulated CD4+ T cells for cytokine activity using bioassays (Mosmann et al., 1986). However, assay cell lines typically respond to more than one cytokine and there are often positive and negative interactions between the activities of different cytokines. Additionally, supernatants from stimulated cells contain a variety of cytokines, many with effects on more than one cell type, making such assays unreliable and, when panels of neutralizing antibodies were used in attempts to eliminate
cytokine cross-reactivity, cumbersome. Thus, rigorously monospecific bioassays for cytokines are difficult to establish.

Enzyme linked immunosorbent assays (ELISAs) are especially useful for cytokine quantification in liquid samples, such as supernates, because they are quantitative, not subject to cytokine cross-reactivity, and easily adaptable to large sample throughput. However, two drawbacks to quantification of cytokine proteins by ELISA are that cytokine-specific antibodies must be generated, and samples for ELISA analysis are most commonly derived from cell culture, which introduces its own set of biases.

Cherwinski et al. (1987) demonstrated that analysis of cytokine mRNA from ConA stimulated Th1 and Th2 clones using northern hybridization correlated with the activity of the cytokine protein as determined in bioassays of supernatants. Furthermore, northern hybridizations were more efficient at detecting differences in cytokine production between Th1 and Th2 clones than previously utilized bioassays. These and other findings confirmed that cytokines are transcriptionally regulated (Brorson et al., 1991; Ye et al., 1997). Additionally, due to the advent of the polymerase chain reaction (PCR), generating molecular reagents for the quantification of cytokine mRNA is much less laborious than generating anti-cytokine antibodies. For these reasons, cytokine mRNA quantification has become the most commonly utilized method to identify cytokine profiles that characterize the Th1 or Th2 phenotype.

A variety of methods have been utilized to quantify cytokine mRNA. These can roughly be divided into northern hybridizations and PCR based methods. A quick scan of the literature suggests that northern hybridization methods, though still utilized, have fallen out of favor. My experience with a variety of northern based methods, including
dot blotting methods using both radio- and fluorescently labeled DNA and RNA probes, as well as the RNase protection assay which is a solution based hybridization method, suggests the following reasons for this trend. First, PCR methods are far more sensitive (in the range of 3-5 molecules) than the most sensitive northerns, which are solution hybridization northerns using $^{32}$P-labelled RNA probes. Second, radioisotopes must be used to achieve the sensitivity required for cytokine quantification using northern analysis but need not be utilized in PCR based quantitative methods. Third, though labor intensive in their own right, PCR based methods require fewer manipulations with RNA which is highly susceptible to environmental RNase contamination. Finally, PCR methods require substantially less RNA, with PCR based methods commonly requiring 1 μg of total RNA for a complementary DNA (cDNA) reaction that allows quantification of a large number of cytokines versus 20 to 100 μg of RNA for the quantification of a single cytokine in northern based methods.

The polymerase chain reaction (Saiki et al., 1985) provides a highly sensitive method for the detection of nucleic acids. In the case of cytokine mRNA quantification, RNA is first reversed transcribed (RT) to cDNA, and cDNA serves as the template for PCR amplification. Barriers to accurate quantification that occur in this step can be due to inaccurate spectrophotometric readings during RNA quantification, including inaccuracies due to sample impurities, and tube to tube variations in RT reaction efficiency. Accordingly, RNA extraction and cDNA synthesis are the first site at which investigators differ in their methods. The quantity and type of RNA, total vs poly A, as well as the nature of cDNA priming can be varied. Many investigators utilize 1 μg of total RNA and Oligo d(T), as opposed to sequence specific priming for their RT
reactions because it allows for the amplification of several cytokines from a single cDNA reaction. Some investigators spike their extractions and/or RT reactions with known quantities of RNA of a specific sequence that contains priming sites common to the PCR target(s) of interest (Wang et al., 1989). Spiking prior to extraction is useful for intersample comparisons in which total RNA entering the cDNA reaction is not quantified. RNA spikes are labor intensive but provide the best control in reverse transcription followed by PCR (RT-PCR) based quantitative PCR (QPCR) systems because the spike serves as an internal control for both the efficiency of the RT reaction and the PCR reaction. However, the internal standard RNA may compete with the target mRNA in the PCR when both are present in disproportionate concentrations in the initial simultaneous RT reaction, necessitating, multiple simultaneous RT reactions with varying amounts of internal standard RNA (Babu et al., 1993).

Following reverse transcription, target cytokines are amplified from RT reactions using PCR. The PCR has three phases: a negligible lag phase in which amplification is initially non-exponential, an exponential amplification phase, and a linear or stationary phase in which the amplification product gradually stops accumulating exponentially (Saiki, 1989). Amplification (N) is characterized by the equation \( N = N_0(1 + \text{efficiency})^n \), where \( N_0 \) is the initial amount of target and \( n \) is the cycle number. Thus, minor variations in starting template or reaction conditions that alter efficiency are magnified during the exponential phase. The onset of the plateau phase appears at different stages depending upon the initial target concentration and characteristics of the primers (Linz et al., 1990; Robinson and Simon, 1991). Though its mechanism is not fully understood (Morrison and Gannon, 1994), the practical consequence of plateau is that samples of differing
initial template concentration can appear equivalent or even inversely related. This does not appear to be as much of a problem for competitive reactions in which co-amplification of different concentrations of different targets into the plateau phase has been shown to result in retention of the initial target:competitor proportions (Morrison and Gannon, 1994) (within certain limits discussed later).

The simplest approach to quantification of RT-PCR products is measurement of the amount of amplified product in the exponential phase by reference to a dilution series of an external standard (Ferre, 1992; Clementi et al., 1993; Foley et al., 1993; Cross, 1995). This technique has two major drawbacks. First, preliminary experiments must be utilized to determine conditions that allow the PCR to remain in its exponential phase, and second, this technique is also the most susceptible to amplification induced errors. Modifications in which internal standards are amplified in the same tube have been utilized to overcome the latter drawback (Coutlee et al., 1995). Limiting dilution techniques have also been applied to QPCR, but these techniques are extremely labor and reagent consuming (Sykes et al., 1992; Luque et al., 1994).

The most accepted PCR based method for cytokine quantification is quantitative competitive RT-PCR (QC-RT-PCR) and is based upon the competitive co-amplification, in a single reaction tube, of the target sequence, together with known concentrations of an added internal standard, resulting in a series of tubes containing a fixed amount of target and varying amounts of internal standard (Wang et al., 1989; Zimmermann and Mannhalter, 1996). The internal standard is designed to share primer recognition sites with the target sequence but differs from the target either by size or an altered restriction digest site that allows it to be distinguished from the wild type target. Following
amplification, the number of target copies in the sample is calculated by determining the equivalence point for competitor and target on a regression plot created by plotting $\log(10)[\text{signal intensity competitor/signal intensity target}]$ vs quantity of internal standard template (Piatak et al., 1993). The term quantitative PCR has come to be almost synonymous with QC-RT-PCR, while all other methods are often considered semi-quantitative.

Once standardized, QC-RT-PCR is quite powerful but has several drawbacks. The internal standard must amplify with the same efficiency as the wild type. The internal standard as well as the target must be able to be analyzed separately, and signal corrections are required as competitors commonly differ in size from wild type (Piatak et al., 1993). QC-RT-PCR is laborious and reagent intensive, requiring a large series of reactions with different competitor concentrations for the quantification of a single sample. Because of this, replicate samples are rarely done. Amplification efficiency of competitors can vary significantly from that of wild type target due to size differences or the formation of competitor/wild type heteroduplexes (Becker-Andre and Hahlbrock, 1989; Kanangat et al., 1992; McCulloch et al., 1995; Henley et al., 1996; Souaze et al., 1996). Most significantly, QC-RT-PCR is limited to a narrow linear dynamic range where the target:competitor ratios remains between 0.66 to 1.5 (Arnold et al., 1992; Raeymaekers, 1993; Souaze et al., 1996).

A number of methods have been utilized to generate competitors for QC-RT-PCR (Zimmermann and Mannhalter, 1996) and are not germane to this discussion. However, in addition to competitors which serve as controls for the errors inherent in the PCR amplification phase of a QC-RT-PCR assay, a "housekeeping gene" is generally
quantified. Housekeeping genes have been shown to be useful as normalization factors for intersample differences in RNA input and RT efficiency during cytokine quantification (Babu et al., 1993). However, corrections utilizing housekeeping genes are error prone due to the presence of processed pseudogenes in the genome, which are related to some of the commonly used transcripts of housekeeping genes (Raff et al., 1997). In theory a housekeeping gene should be constitutively expressed equally among different tissues of an organism, at all stages of development, for both control and experimentally treated cell types. Unfortunately, there is no single RNA species that meets these criteria, and the best compromise is to identify a gene that comes closest to this ideal for the cell types being studied. β-actin was one of the earliest mRNA species utilized as an internal control (Chelly et al., 1988; Kashani-Sabet et al., 1988). It encodes a constitutively expressed, ubiquitous cytoskeletal protein, that is highly conserved in eukaryotes and expressed at moderately abundant levels (0.1% of mRNA or 0.003% of total RNA). β-actin's primary drawback is that it is expressed in much higher levels than cytokine mRNA. In a 10 µg sample of total RNA (approximately 5 x 10^5 cells) there is approximately 300 pg of β-actin mRNA which 100-1000 times the expression of rare (0.3-3 pg/10 ug total RNA or 0.001% of mRNA) mRNA species such as cytokines (Smith, 1995). This translates to approximately 950 molecules of β-actin mRNA per cell ([MW RNA= #nucleotides x 339]/60). Levels of β-actin have been shown to vary in cultured adipocytes, mammary epithelial cell lines, fibroblast cell lines, and cultured human colon carcinoma cells (Dodge et al., 1990; Bonini and Hofmann, 1991; Spanakis, 1993). β-actin mRNA levels have been shown to be higher in normal mouse spleen, brain, ovary and thymus than in liver, lung, and testes (Kimball, 1996).
When compared using a QC-RT-PCR method, β-actin levels in mitogen stimulated human monocytic, promyelocytic, and lymphoma cell lines, were not significantly different from unstimulated cells (Kanangat et al., 1992). Other studies of T cell lines (Melby et al., 1993) and normal human PBMC (McCa irns et al., 1984) have respectively demonstrated a 1.3 and three fold increase in β-actin mRNA following mitogen stimulation.

Other mRNA species that have been utilized as internal controls include glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Dukas et al., 1993), hypoxanthine phosphoribosyltransferase (HPRT) (Murphy et al., 1993), and peptidyl-prolyl cis-trans isomerase (Cyclophilin) (Nast et al., 1994). GAPDH is a key enzyme in glycolysis whose mRNA, like β-actin, is expressed at moderately abundant levels (0.1% of mRNA) (Smith, 1995). GAPDH is constitutively expressed in many tissues, including rat and human muscle and heart (Piechaczyk et al., 1984). However, GAPDH levels show great variability. Levels have been shown to vary with the embryonic stage of the cell and dexamethasone treatment (Oikarinen, et al., 1991), viral or oncogene transformation (Bhatia et al., 1994), as well as during different stages of the cell cycle in skin fibroblasts (Mansur et al., 1993). GAPDH levels have also been shown to vary in smooth muscle cells treated with vasoconstrictors and mitogens (Gadiparthi et al., 1990), as well as in breast cell lines, and adipocytes cultured with insulin (Alexander et al., 1990). GAPDH expression in mouse brain, muscle, heart, and kidney, exceeds that of liver, spleen, lung, embryo, and hypothalamus (Smith, 1995).

HPRT is an enzyme which catalyzes the metabolic salvage of the purine bases hypoxanthine and guanine. mRNA is constitutively expressed in all tissues at low levels.
(Ogasawara et al., 1996). Expression in the order of 1-3 mRNA molecules per cell was found in normal human lymphocytes, amnion, fibroblasts, EBV-transformed lymphoid cell lines, neuroblastoma, glioblastoma, and melanoma cultures (Steen et al., 1990). Brain tissues, especially the basal ganglia, have been shown to express HPRT at a higher level than other tissues (Jiralerspong et al., 1996), and resting lymphocytes have been shown to have expression that exceeds fibroblasts by at least two fold (Steen et al., 1991). Though HPRT expression remains steady in long term lymphocyte cultures, HPRT is not a well suited housekeeping gene for immunologic studies because mitogen stimulation (PHA) has been shown to increase HPRT expression by 10-20 fold in human lymphocytes, with levels returning to within 20% of baseline 24 hours after mitogen removal (Steen et al., 1990, 1991).

Cyclophilin encodes a cytoplasmic protein important in protein folding that is highly conserved. Cyclophilin's expression has been shown to be less abundant than β-actin in all mouse tissues, though it is still fairly abundant, comprising close to 0.1% cytoplasmic RNA (Smith, 1995). Cyclophilin expression in rat brain, spleen, thymus, adrenal glands, ovaries, and testes exceeds that of liver, lung, and anterior pituitary (Danielson et al., 1988). In monkeys, expression has been shown to be lower in skeletal muscle than other tissues (Danielson et al., 1988), and expression in some tumor lines exceeds that of their normal cellular counterparts (Haendler et al., 1990). Despite the variable distribution of cyclophilin within body tissues, cyclophilin is a promising lymphocyte housekeeping gene because levels have not been altered in T-lymphocytes by treatment with mitogens or tumor promoters (Haendler et al., 1990).
Following PCR, PCR targets as well as competitors in QC-RT-PCR must be detected and quantitatively assessed. PCR products can be divided into those that bear no endogenous labels, or products which have been labeled during PCR through the use of radioactive, fluorescent, or biotin-labeled nucleotides or primers. In the most basic method, unlabeled PCR products are separated via agarose gel electrophoresis and stained using ethidium bromide (Piatak et al., 1993; Zimmermann et al., 1994). Bands are then assigned a signal strength using computerized image analysis. Gel electrophoresis allows not only the separation of competitors and targets that vary by size, but also reveals nonspecific bands resulting from PCR. To ensure accurate quantification in the case of competitive PCR, correction factors are applied to account for greater fluorescence emitted by larger fragments. In noncompetitive methods, densitometry of ethidium bromide (EtBr) stained gels is associated with gel to gel variations in staining intensities that can distort interpretations of absolute signal strength. Other methods including capillary gel electrophoresis (Fasco et al., 1995) and high pressure liquid chromatography (de Kant et al., 1994) have been utilized to separate PCR products by size.

Methods in which labels are incorporated during the PCR process have greater sensitivity than methods that utilize EtBr staining. Endogenously labeled PCR products have been analyzed by a variety of methods depending upon the nature of the label. Because PCR products labeled in this manner have the drawback of labeled nonspecific PCR products that erroneously contribute to signal strength, they are generally separated by gel electrophoresis, then quantified either from excised bands using direct assessment of cpm (Scadden et al., 1992; Furtado et al., 1993), or from dried gels via
autoradiography and densitometry (Dostal et al., 1994), or radioimaging (Arnold et al., 1992). PCR products bearing biotin have been similarly separated via electrophoresis, blotted onto filter paper, and quantified using streptavidin conjugated chemiluminescent labels, autoradiography and image analysis (Su et al., 1994). Automated sequencers have also been employed to quantify fluorescently labeled PCR products in polyacrylamide gels (Cottrez et al., 1994; Michael et al., 1995).

To circumvent problems with labeled non-specific PCR products that are generated from labeling during PCR amplification, hybridization with sequence-specific probes followed by probe quantification has been adopted in some methods. These methods either involve membrane transfer of PCR product following electrophoresis (Telenti et al., 1992) or solid phase capture of PCR product onto microplates. Biotinylated PCR product has been captured onto streptavidin coated plates (Jalava et al., 1993; Lehtovaara et al., 1993, Martin et al., 1996), aminated PCR products onto carboxylated plates (Kohsaka et al., 1993), as well as capture via plates bearing covalently bound oligonucleotides (Berndt et al., 1995). Once captured, PCR product is hybridized with radiolabeled or enzymatically labeled oligonucleotides and quantified by measuring incorporated radioactivity, fluorescence, or enzyme activated color change.

Two recent chemiluminescence based adaptations have been directed at automating the detection process in QPCR procedures. Both of these methods have the disadvantage of requiring expensive reagents and machinery that is both expensive and not commonly available. In the first, a biotinylated primer is incorporated into the PCR reaction. An aliquot of the reaction is then hybridized in solution with a tris (2,2'-bipyridine) ruthenium II chelate (TBR)-labeled oligonucleotide probe that is
complementary to the biotinylated strand. The biotinylated PCR product bearing its TBR probe is then captured onto streptavidin coated iron beads and added to reagent assay buffer. In an automated process carried out within a machine known as the QPCR System 5000 (Perkin-Elmer, Foster City, CA), the entire reaction is analyzed and assigned a luminosity value proportional to the amount of TBR-bearing, hybridized, oligonucleotide probe that is detected. The QPCR System 5000 aspirates the iron beads with their attached PCR products onto a working electrode and traps them magnetically. The electrode is washed several times to eliminate unbound TBR-labeled probe and a voltage is applied to the electrode, inducing a series of redox reactions whereby stimulated TBR molecules emit light at 620 nm, which is then detected by a photomultiplier tube. This system has been adapted for use in competitive PCR by the use of two TBR-labeled oligonucleotide probes, one specific for the target, the other for the internal competitor (Wilkinson et al., 1995; Blok et al., 1997). In a modification of this system, TBR-labeled oligonucleotide can also be utilized as a complementary primer to the biotin-labeled primer without a hybridization step, but this method has the disadvantage of label incorporation into nonspecific products of the PCR reaction. Use of the QPCR System 500 has several advantages. The instrument's linear dynamic range has been determined to be between 3 and 4 orders of magnitude (Jessen-Eller et al., 1994) and allows high sample throughput. The QPCR System 5000 has greater sensitivity than $^{32}$P-labeled methods (Zhao et al., 1996) which have been calculated to be $7.4 \times 10^{11}$ mole of PCR product above background (Crivello, 1997), and the ability to determine starting copy number over 10 orders of magnitude in competitive methods (10-10$^{11}$ copies) with 90% accuracy (Crivello, 1997).
The second method referred to as Taq-Man (Perkin-Elmer), is a real time QPCR system (Heid et al., 1996) which exploits the 5' exonuclease activity of Taq (Holland et al., 1991). The reaction is carried out with three primers, two for amplification, and a third that hybridizes internally in the amplified region. A fluorescent dye on the 3' end of the internal primer (such as TAMRA, 6-carboxy-tetramethyl rhodamine) quenches fluorescence from a reporter fluorescent tag on the 5' end (such as FAM, 6-carboxyfluorescein) of the internal primer (Livak et al., 1995). During PCR, the 5' exonuclease activity of Taq degrades the 3' quenching activity on the internal primer, resulting in fluorescent emission which is determined during the PCR process. This approach is less sensitive (limit of detection 10,000 copies) then the QPCR System 5000 (Crivello, 1997) but allows much higher throughput because post PCR manipulations are not required. The system can also be easily adapted for simultaneous detection of competitors through the use of different fluorescent dyes.

The plethora, permutations, and drawbacks of the aforementioned methods should raise one resounding truth about quantitative PCR - that accurate quantification is difficult to achieve and that each method has its drawbacks. QC-RT-PCR, despite its many drawbacks, has the primary advantage of being commonly accepted. Because of the nature of our laboratories' work in which large numbers of samples from a genetically heterogeneous population of horses serve as the source of RNA, a rapid, labor conserving method in which replicate samples could be run became the primary force that governed our assay development. Our system synthesizes the use of a dilution series of an external standard with solution hybridization techniques that minimize errors.
due to nonspecific PCR products. The housekeeping gene β-actin is used to correct for differences in initial RNA concentrations or variations in RT efficiency.

The underlying hypothesis of this study was that horses exhibit cytokine profiles consistent with the TH2 phenotype in response to S. vulgaris infection. The primary aim of this study was to determine if differences existed in the cytokine profiles of lymphocytes derived from immune and susceptible ponies, both in response to vaccination and following challenge. Lymphocytes for examination were collected both from the local site of parasite invasion along the large colon, as well as from peripheral blood, and immunity was induced by oral vaccination with radiation-attenuated S. vulgaris L₃. Characterization of the TH1 and TH2 cytokine profiles in the horse necessitated the development of molecular tools which included the cloning and sequencing of equine β-actin, to be used as a housekeeping gene for quantitative assays, and IL-10, considered a member of the TH2 cytokine family, and most significantly, the development of a PCR based assay for the quantification of equine cytokines.
CHAPTER 1
MOLECULAR CLONING AND SEQUENCING OF EQUINE IL-6, IL-10, AND BETA-ACTIN

Introduction

Equine interleukin-6 (IL-6), IL-10, and β-actin were cloned and sequenced to facilitate their use as immunologic reagents in assays to quantify equine cytokines. IL-6 and IL-10 are produced by Th2 cells in the murine system but this polarization is not always clear in human CD4+ subsets (Romagnani, 1992). IL-6 (Robinson et al., 1993) and IL-10 (McKay et al., 1995) have been examined as inflammatory mediators in the horse. However, neither the significance of the Th1/Th2 paradigm in equine disease, nor the role of IL-6 or IL-10 in Th1 and Th2 responses have been examined in the horse.

Materials and Methods

Preparation of cells

Equine peripheral blood mononuclear cells (PBMC) were isolated from venous blood by differential centrifugation over Ficoll-Pague (Pharmacia LKB Biotechnology, Piscataway, NJ). After washing in calcium and magnesium free phosphate-buffered saline (CMF-PBS), PBMC were suspended in media consisting of RPMI 1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 2-mercapto-ethanol (100 mM), glutamine (2 mM), 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% heat inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT). PBMC were incubated at a concentration of 2 x 10⁶/ml for 72 hours.
with phytohemagglutinin-M (2μg/ml, Sigma) at 39°C in a humidified environment with 5% CO2.

Total RNA isolation and complementary DNA (cDNA) synthesis

Small scale preparations of total RNA were isolated from 2-3 x 10⁶ PHA stimulated PBMC using RNA Stat 60 reagent (Tel-Test, Friendswood, TX) and chloroform extraction as per the manufacturer's directions. 0.30 μg of RNA in a volume of 17.5 μl diethyl pyrocarbonate-treated water were heated to 65°C for 10 minutes to denature the RNA and then chilled in ice and pulse spun in a microcentrifuge at 4°C. 22.5 μl of reagent master mix were then added resulting in a final reaction containing 1X first strand buffer (Gibco BRL, Gaithersburg, MD), 0.5 mM dNTP (Perkin-Elmer, Norwalk, CT), 1.0 μM Oligo-dT (Promega, Madison, WI), 10 mM DTT (Gibco), 0.075 μg/μl BSA (NEB, Beverly, MA), 60 Units RNAsin (Promega), and 400 units Moloney Murine Leukemia Virus Reverse Transcriptase (BRL). The reaction was incubated 1 hour at 40°C, then frozen at -20°C until use.

Equine IL-6, IL-10, and β-actin message amplification

Equine IL-6, IL-10, and β-actin were amplified using the polymerase chain reaction (PCR). All PCRs were performed in 50 μl volumes containing 5 μl cDNA, 1X PCR buffer (Perkin-Elmer), 2.5 units Taq polymerase (Perkin-Elmer), 200 μM dNTP (Perkin-Elmer), and 10 pmoles of each 5' and 3' primer. Primers were derived from phylogenetically conserved regions of the respective genes using published nucleotide sequences (Table 1.1). The amplification steps involved denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1
Table 1.1. Primer sequences used for PCR amplification and sequencing of equine IL-6, IL-10, and β-actin cDNA.

<table>
<thead>
<tr>
<th>cDNA</th>
<th>Coding Strand Primer</th>
<th>Noncoding Strand Primer</th>
<th>Internal Sequencing Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>5'ATGAACTCCTTCTCCACAAG3'</td>
<td>5'TCTAAGCCTCATACTTT3'</td>
<td>543AS: 5'CCACTCATTTACTGTAATGCGACT3'</td>
</tr>
<tr>
<td>IL-10</td>
<td>5'ATGCACAGCTCAGCCTC3'</td>
<td>5'TTCACAGAGAAGCTCATTT3'</td>
<td>S103: 5'AAGAGAAAGGGGTCTACAAGCC3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'ATGATATGATATAGCCG3'</td>
<td>5'TCAGAAACATTTCGGGT GGAGATGGGGCC3'</td>
<td>255S: 5'TCTGGCACCACACTCTTAC3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>510S: 5'CATATTCACTGGTGAGC3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>739AS: 5'TGATCCCTGTGGAC3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>964AS: 5'TGATCTTCACTGGCTGG3'</td>
</tr>
</tbody>
</table>
minute. Reactions were amplified to 40 cycles in a GeneAmp PCR System 9600 Thermal Cycler (Perkin-Elmer).

**Cloning and sequencing**

The equine IL-6, IL-10, and β-actin PCR products were purified by electrophoresis and excision from a 1.2% agarose (Sigma) gel using diatomaceous earth (Prep-a-Gene; Bio Rad, Hercules, CA). Fragments of approximately 670, 740, and 1150 base pairs (bp) respectively were cloned into pCRII vector (Invitrogen, Carlsbad, CA) using the TA cloning method according to the manufacturer's instructions, then transformed into competent *E. coli* DH5αMCR using heat shock (Lederberg and Cohen, 1974). Transformants were selected by ampicillin resistance and loss of β-lactamase gene function, then screened by restriction digest mapping.

The cloned PCR-vector products were purified from overnight cultures using alkaline lysis followed by CsCl gradient separation in the presence of ethidium bromide (Sambrook *et al.*, 1989) or using a commercial miniprep kit (Qiagen, Valencia, CA). Sequencing was accomplished using dideoxynucleotide chain termination from double stranded plasmid DNA. Plasmids containing IL-10 and β-actin were sequenced manually using alkali denaturing according to the Sequenase Version 2.0 kit (United States Biochemical, Cleveland, OH) followed by polyacrylamide gel electrophoresis. IL-6 was sequenced using dye primer cycle sequencing (ABI PRISM Dye Primer Cycle Sequencing Core Ready Reaction Kit with AmpliTaq DNA polymerase, FS; Perkin-Elmer). Sequences were resolved using capillary gel electrophoresis on an ABI Prism 310 Genetic Analyzer (Perkin-Elmer/Applied Biosystems Division). Sequencing primers were -48 (GeneLab, Baton
Rouge, LA) and T7 (Promega, Madison, WI) plasmid primers, and primer pairs used for PCR. Internal primers were generated to span the middle of the IL-6, IL-10, and β-actin fragments as needed from the derived sequences (Table 1.1). The sequence information was entered into the MacVector database program for subsequent alignment with published cDNAs for other species IL-6, IL-10, and β-actin.

Results

Amplification of mononuclear cell mRNA

PCR amplification of cDNA from PHA stimulated PBMC produced distinct 745, 739, and 1128 base pair fragments for IL-6, IL-10, and β-actin respectively (Figure 1.1). Because these fragments were the same size as predicted from sequence analysis of other species, the products were purified and cloned for sequencing.

Sequencing of equine IL-6, IL-10, and β-actin

The nucleotide sequences of the IL-6, IL-10, and β-actin cDNA inserts are shown in Figures 1.2, 1.3 and 1.4, respectively. These sequences have been submitted to GenBank EMBL and bear the following accession numbers: IL-6: U64794, IL-10: U38200, and β-actin: AF035774. All clones of the three cDNA sequences contained a single open reading frame which included a stop codon. The 745 bp IL-6 cDNA included the coding sequence which spans bases 1 to 627, and 118 bases in the 3' nontranslated region. Based upon comparisons to IL-6 sequences of other species, bases 1-84 encode the hydrophobic signal peptide. The 739 bp IL-10 cDNA included a coding sequence from bases 1-537. Bases 1-54 encode the putative signal peptide. The IL-10 cDNA also includes 202 bases in the 3' nontranslated region. The 1128 bp β-actin cDNA encodes only the translated regions of the protein.
Figure 1.1 PCR amplification of equine IL-6, IL-10, and β-actin. Lanes 1 and 8 contain the size marker phage X174 DNA HaeIII digested (the size of the fragments in base pairs is given); lanes 2, 4, and 6 contain the PCR products from PHA stimulated equine PBMC cDNA for IL-6, IL-10, and β-actin, respectively. Lanes 3, 5, and 7 are the respective PCR negative controls which contained water instead of cDNA template.
**Figure 1.2:** Nucleotide sequence of amplified equine IL-6 cDNA. The nucleotide sequence of the cDNA insert was determined by automated cycle sequencing of double stranded plasmid. The coding sequence spans bases 1-627. Bases 1-84 encode the hydrophobic signal peptide, and bases 628-745 are in the 3' nontranslated region. Primer sequences are represented in bold. The stop codon is underlined. The GenBank accession number for this sequence is U64794.

**Figure 1.3.** Nucleotide sequence of amplified equine IL-10 cDNA as determined from double stranded plasmid using manual chain termination sequencing. The coding sequence spans bases 1-537. Bases 1-54 encode the hydrophobic signal peptide, and bases 538-739 are in the 3' untranslated region. The stop codon is underlined. The GenBank accession number for this sequence is U38200.
Figure 1.4. Nucleotide coding sequence of amplified equine β-actin cDNA (top panel) and deduced amino acid sequence (bottom panel). The nucleotide sequence of the cDNA insert was determined by chain termination sequencing of double stranded plasmid. Primer sequences are represented in bold. The stop codon is underlined. The GenBank accession number for this sequence is AF035774.
β-actin is a cellular cytoskeletal protein which is not secreted and therefore lacks a signal peptide. Nucleic acid homology of these equine sequences with the respective sequences in other mammalian species were analyzed using the BLAST program (Basic Local Alignment Search Tool, National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health). These homologies were extensive and are summarized in Table 1.2.

Amino acid analysis

The open reading frames for IL-6, IL-10, and β-actin encode 208, 178, and 375 amino acids, including 28 and 18 amino acid signal peptides for IL-6 and IL-10, respectively. Like the nucleic acid sequences, the predicted amino acid sequences for equine IL-6, IL-10, and β-actin have a high degree of similarity at the amino acid level with the respective sequences of other species as determined using the BLAST program. These similarities are summarized in Table 1.2. The predicted amino acid sequences for IL-6, and IL-10, aligned to the respective human sequences, are shown in Figures 1.5, and 1.6. The amino acid sequence of equine β-actin is shown in Figure 1.4 and is identical to the human sequence.

The open reading frame for equine IL-6 encodes 208 amino acids with a predicted relative mass of 23,383. Comparison to other IL-6 sequences suggests that the first 28 amino acids serve as the signal peptide which is cleaved off during secretion, making proline the amino terminal amino residue of mature IL-6. The mature protein would consist of 180 amino acids and have a predicted relative mass of 20,471. The predicted molecular weights do not take into consideration any post-translational modifications of the polypeptide. Sequence alignments also indicate that
Table 1.2. Comparison of equine IL-6 and equine IL-10 nucleotide coding and predicted amino acid sequences with other mammalian species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Equine IL-6</th>
<th></th>
<th>Equine IL-10</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nucleotide identity (%)</td>
<td>Amino acid similarity (%)</td>
<td>Nucleotide identity (%)</td>
<td>Amino acid similarity (%)</td>
</tr>
<tr>
<td>Human</td>
<td>81.7</td>
<td>77.6</td>
<td>89.3</td>
<td>91.0</td>
</tr>
<tr>
<td>Pig</td>
<td>84.4</td>
<td>82.1</td>
<td>86.0</td>
<td>92.8</td>
</tr>
<tr>
<td>Dog</td>
<td>82.3</td>
<td>83.7</td>
<td>83.5</td>
<td>86.1</td>
</tr>
<tr>
<td>Cat</td>
<td>83.7</td>
<td>81.4</td>
<td>86.5</td>
<td>90.4</td>
</tr>
<tr>
<td>Sheep</td>
<td>80.7</td>
<td>74.9</td>
<td>87.3</td>
<td>92.5</td>
</tr>
<tr>
<td>Cow</td>
<td>80.3</td>
<td>70.7</td>
<td>86.9</td>
<td>90.4</td>
</tr>
<tr>
<td>Red Deer</td>
<td>-</td>
<td>-</td>
<td>96.4</td>
<td>89.7</td>
</tr>
<tr>
<td>Mouse</td>
<td>69.9</td>
<td>64.0</td>
<td>79.7</td>
<td>85.4</td>
</tr>
<tr>
<td>EHV-2 vIL-10</td>
<td>-</td>
<td>-</td>
<td>85.1</td>
<td>83.6</td>
</tr>
</tbody>
</table>

Comparisons were made using the BLAST program of National Center for Biotechnology and Information, National Library of Medicine, National Institutes of Health (http://www.ncbi.nlm.nih.gov/BLAST/)

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Figure 1.5. Deduced amino acid sequence of equine IL-6 aligned with peptide sequence of human IL-6. The putative signal sequences are in italics. Locations of structural α-helices (A-D) determined for human IL-6 are shown (after Bazan, 1990). Cysteine residues are marked (▲) at positions of putative conserved disulphide bridges. Arginine 179 (♦) and other invariant residues (*) are also shown. Amino acids are numbered according to the human sequence (Gen Bank accession M145840. Gaps (-) were inserted to maximize alignment.
Figure 1.6. Deduced amino acid sequence of equine IL-10 aligned with peptide sequence of human IL-10 (GenBank accession M57627). Putative signal sequences are in italics. Locations of predicted structural α-helices (A-D) are shown (after Walter, 1995). Putative receptor binding residues are marked (*). Cysteine residues are marked (·) at positions of putative conserved disulphide bridges.
equine IL-6 possesses the 40 amino acids, including 4 conserved cysteine residues, which are invariant in humans, the domestic animal species, mice, and several sea mammals (King et al., 1996).

The open reading frame for equine IL-10 encodes 178 amino acids with a predicted relative mass of 20,449. The N-terminal amino acid of mouse IL-10 is Gln$^{22}$, while the N-terminus of human IL-10 is Ser$^{19}$. Equine IL-10 has a Ser$^{19}$ and a Gln$^{25}$. Phylogenetic analysis of equine IL-2 indicates that equine and human sequences are more closely related evolutionarily than equine and murine sequences (Vandergriff and Horohov, 1993), making Ser$^{19}$ the more likely N-terminal amino acid in the horse. With Ser$^{19}$ as the N-terminal amino acid, the mature equine IL-10 would consist of 160 amino acids and have a predicted molecular mass of 18,615, without post-translational glycosylation.

The open reading frame for equine β-actin encodes a 375 amino acid protein. Beta-actin is a cytoskeletal protein and therefore lacks a signal peptide. After removal of the initiator methionine, the 374 amino acid equine β-actin protein has a predicted relative mass of 41,603.

Discussion

40 amino acid residues of IL-6 have been shown to be invariant among a wide variety of species and these residues are also conserved in the equine sequence (King et al., 1996). This is in line with observations that cell supernatant from many different mammals can demonstrate biological activity in mouse bioassays. However, mouse IL-6 is unable to activate human cells (Ehlers et al., 1994). The biological activity of IL-6 is mediated through two different receptor components. IL-6 first
binds to a low affinity IL-6R alpha (gp80) which is stabilized when bound by the signal transducing IL-6R beta unit (gp130) (Tage et al., 1989). The carboxy-terminus has been shown to be important for receptor binding of the functional protein (Kruttgen et al., 1990; Brakenhoff et al., 1990). The gp80 binding site (termed site I) is thought to involve an area where the carboxy-terminus of IL-6 and a section of the chain at the beginning of α-helix A are in close proximity (Brahennhoff et al., 1989, 1990). However, Phe$_{78}$ in the loop between the second and third α-helix has been shown to be critical for gp80 binding (Ehlers et al., 1996) and is conserved in the equine sequence. Amino acid deletions at positions 171-179 in the carboxy terminus of the human sequence have demonstrated that residues 177-179 contribute to the formation of this receptor binding site and Arg$_{179}$ is required for biological activity of the molecule in the mouse bioassay (Fontaine et al., 1993). Residues 177-179, Ser, Leu, Arg, which are invariant among domestic and sea mammals are also conserved in the horse. The mouse has a Thr$_{177}$, but this substitution has not been reported to be a cause of species specificity. In fact, the specificity of mouse IL-6 activity has been linked to three of the four domains (van Dam et al., 1993) including residues 50-55 which activate gp130 (Ehlers et al., 1995).

The gp130 binding region of IL-6, involves regions early in the loop between the first and second α-helix, specifically Cys$_{50}$-Glu$_{55}$ (Ehlers et al., 1994), and regions at the end of the third and fourth α-helix, specifically Gln$_{153}$-His$_{165}$ (de Hon et al., 1995). Equine IL-6 shares 100% and 85% similarity to the human sequence in these regions. Determination of the significance of specific residues in this region is an area

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of ongoing study. However, Lys$^{54}$ is known to be critical to gp130 activation (Ehlers et al., 1996) and is conserved in the horse.

IL-6 produced by macrophages (Bauer et al., 1988, Gross et al., 1989), and fibroblasts (Santhanam et al., 1989), as well as recombinant products of eukaryotic expression systems (Tanner et al., 1990; Schiel et al., 1990) has been shown to be variably glycosylated at both N- and O-linked positions. However, recombinant IL-6 expressed in E. coli is biologically active indicating that glycosylation is not required for biological activity (Skelly et al., 1994). Proper protein folding is required for maximal activity and IL-6 mutants lacking the first and second cysteine residues retain activity similar to nonmutated IL-6 while mutants lacking the third and fourth cysteine residues have a significant reduction in biological activity (Snouwaert et al., 1991; Skelly et al., 1994).

IL-10 exists as a homodimer with each monomer composed of six α-helices, two of which intertwine to form the dimer (Walter and Nagabhushan, 1995). The resulting structure forms a four helix bundle motif. The molecule is predominantly α-helical (67%) and the putative helix regions derived from X-ray crystallographic structure of human IL-10 are shown in (Figure 1.6). Human IL-10 lacks detectable glycosylation but murine IL-10 is variably N-glycosylated near its N-terminus (reviewed in Moore et al., 1993). However, glycosylation is not required for activity. IL-10 is expressed (reviewed in Moore et al., 1993) and binds its receptor (Tan et al., 1993) as a noncovalent dimer. Equine IL-10 shares four conserved cysteine residues with human IL-10 and mouse IL-10. The first cysteine residue in the mature peptide pairs with the third and the second with the fourth. The reduced protein
displays no biological activity suggesting that one or both of these bonds is critical for structure and function (Windsor et al., 1993).

Equine IL-10 demonstrates DNA and amino acid homology to an IL-10-like sequence of equine herpes virus-2 and also to a similar IL-10 like motif in the sequence of Epstein Barr virus termed BRCF or viral IL-10 (vIL-10). vIL-10 has been shown to have a specific activity 3-10 times lower than human IL-10 (Moore et al., 1991). Postulated functions of this molecule include the inhibition of virus induced T or NK cell activation, promoting the growth and differentiation of B cells (EBV transforms human B cells), and promoting Fc receptor-mediated uptake of the virus into susceptible cells (reviewed in Moore et al., 1993).

Though the X-ray crystallographic structure of human IL-10 has been resolved (Walter and Nagabhushan, 1995; Zdanov et al., 1996), mutagenesis studies to identify residues critical to receptor binding have not been reported. The 85 invariant amino acid residues in human, BRCF, equine herpes virus-2, murine, and porcine IL-10 described by Walter and Nagabhushan (1995) are also conserved in the horse. Based on the structural similarities between IL-10, IFN-γ, and growth hormone, and the residues critical to receptor binding in these latter two molecules, Ser₁₄₁ is predicted to be a critical receptor binding residue for IL-10. Leu₄₆, Leu₅₃, and Ile₁₄₅ are also predicted to interact with the receptor. Three of these four residues are invariant in the equine with Leu₄₆ being replaced by a biochemically similar Met residue. This residue is not, however, invariant and Met substitution is reported in the equine herpes virus type II sequence. Ser₁₄₁ is also not an invariant residue among the published sequences.
Equine cDNA's with considerable nucleic acid and amino acid homology to
the IL-6 and IL-10 sequences of other species have been amplified using PCR and
primers based on the consensus sequences of these genes in other species. The cloned
equine IL-6 bears 80-84% nucleic acid identity and 71-84% amino acid similarity to
the IL-6 sequences of humans and domestic animal species. Equine IL-10 exhibits 86-
89% nucleic acid identity with these species and 96% with red deer. The predicted
IL-10 amino acid sequence similarity is in the range of 87-92% when compared to the
previously noted species. Though functional assays are required to definitively
determine if the proteins encoded by these sequences are biologically active, the high
degree of sequence similarity suggests that these sequences are indeed equine IL-6 and
IL-10. The cloned β-actin gene shares 100% amino acid identity with human β-actin
and therefore is identifiable as equine β-actin without functional assays of the protein.
CHAPTER 2
A NOVEL METHOD FOR QUANTIFICATION OF EQUINE CYTOKINE MESSENGER RNA USING TARGET SPECIFIC STANDARD CURVES

Introduction

RT-PCR provides a powerful tool for the detection of mRNA which has been widely applied to quantify a variety of transcripts including cytokines. Quantitative competitive RT-PCR (QCRT-PCR) is accepted as a reliable method for quantifying differences in mRNA. However, QCRT-PCR has many drawbacks. The assay is labor and reagent intensive requiring competitor construction and the generation of a large series of reactions with differing competitor:target ratios in order to assay a single sample. Amplification efficiency of competitors can vary significantly from that of wild type target due to size differences or the formation of competitor/wild type heteroduplexes (Becker-Andre and Hahlbrock, 1989; Kanangat et al., 1992; McCullough et al., 1995; Henley et al., 1996; Souaze et al., 1996). Post PCR detection generally requires signal corrections as competitors commonly differ in size from wild type (Piatak et al., 1993). Most significantly, the accuracy of QCRT-PCR is limited to a narrow range where the target:competitor ratio remains between 0.66 to 1.5 (Raeymaekers, 1993; Souaze et al., 1996). Because QCRT-PCR assays are labor intensive, replicate determinations of the same sample are rarely done which can lead to overestimation of the accuracy of this technique. In fact, well established tests of this type by experienced scientists yield errors between 10% and 20% for analysis of replicate portions of the same sample on different occasions indicating that it is preferable to calculate the mean of replicate determinations of the same sample for
reliable discrimination of two fold differences in copy numbers between two samples (Apostolakos et al., 1993; Piatuk et al., 1993; Besnard and Andre, 1994; de Kant et al., 1994; Zimmerman et al., 1994; Xia et al., 1995).

Standard curves are routinely used in a variety of scientific disciplines including immunology to evaluate enzymatically dependent phenomenon characterized by an exponential rise to maximum. Standard curves derived from dilutions of cloned cDNA sequences have been described (Melby et al., 1993; Martin et al., 1995) and RNA standard curves have recently been employed to quantify mRNA in a competitive RT-PCR assay with excellent intra- and inter-assay precision (Tsai and Wiltbank, 1996). We demonstrate, using equine cytokines, that the quantity of mRNA can be determined via RT-PCR using standard curves generated from dilutions of double stranded circular plasmids encoding the transcript of interest and that by decreasing the cycle number and cDNA volume used for β-actin amplification, as compared to cytokine amplifications, β-actin is an accurate normalization factor for the differences that are inherent in the RT step of the assay. Advantages of this method include: (i) the assay has a linear dynamic range on the order of 3 logs; (ii) the target utilized to generate the standard curve is identical to the cDNA target thereby minimizing differences in amplification efficiency commonly reported with the use of competitor molecules; (iii) post PCR detection and analysis does not utilize radioisotopes or require adjustment for size of the reference molecule; (iv) the assay is technically straightforward and easily adaptable to any gene that has been cloned without subcloning or competitor construction; (v) the assay requires fewer reactions
than QCRT-PCR assays (vi) the method accommodates high sample throughput and, (vii) replicate samples are easily generated to allow statistical analysis of data.

Materials and Methods

Preparation of cells

Equine peripheral blood mononuclear cells (PBMC) were isolated from venous blood by differential centrifugation over Ficoll-Pague (Pharmacia LKB Biotechnology, Piscataway, NJ). After washing in calcium and magnesium free phosphate-buffered saline (CMF-PBS), PBMC were suspended in media consisting of RPMI 1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 2-mercaptoethanol (10⁻⁸ M), glutamine (2 mM), 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% heat inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT). PBMC were incubated at a concentration of 2 x 10⁶/ml for 72 hours with phytohemagglutinin-M (PHA, 2 μg/ml, Sigma) at 39°C in a humidified environment with 5% CO₂. Aliquots of PBMC (2 x 10⁶ cells) were frozen at -70°C in RNAStat60 (Tel-Test, Friendswood, TX) until use.

RNA isolation and cDNA synthesis

Samples of PHA stimulated PBMC which had been frozen in RNAStat60 (Tel-test) were defrosted and processed to RNA using chloroform extraction as per the reagent manufacturer’s directions. RNA, which was quantified spectrophotometrically, consistently yielded absorbance ratios (260 nm:280 nm) on the order of 1.9. 0.60 μg of RNA in 35 μl diethyl pyrocarbonate (DEPC)-treated water were heated to 65°C for 10 minutes to denature the RNA then chilled on ice and pulsed in a microcentrifuge at 4°C. 45 μl of reagent master mix was then added resulting in a
final reaction containing 1X first strand buffer (Gibco BRL, Gaitherburg, MD),
0.5mM dNTP (Perkin-Elmer, Norwalk, CT), 120 Units RNAsin (Promega, Madison,
WI), 0.075 µg/µl BSA (NEB, Beverly, MA), 1.0 µM Oligo-dT (Promega), 10 mM
DTT (Gibco) and 800 Units Moloney Murine Leukemia Virus (MMLV)-Reverse
Transcriptase (Gibco). The final reaction volume was 80µl. The reaction was
incubated at 25°C for 10 minutes to facilitate primer annealing, then 40°C for 1 hour,
and then frozen at -20°C until used for PCR. DEPC-treated water was substituted for
MMLV-RT in control reactions in order to detect genomic DNA contamination.

In some experiments cDNA reactions were synthesized from serial two fold
dilutions of RNA ranging from 2 µg to 0.0625 µg per reaction. The RNA from these
reactions originated from the same extraction of PHA stimulated cells. This set of
cDNA reactions is referred to as the RNA dilution series. In other experiments, three
cDNA reactions were synthesized from identical amounts of RNA originating from a
single extraction procedure in order to isolate variability associated with the RT
reaction from that associated with RNA extraction and spectrophotometric
quantification. These reactions are referred to as identical cDNA reactions. In assays
that examine the inter- and intra-assay variation associated with RNA that originates
from separate RNA extractions, a split sample design was utilized. Six aliquots of
cells from a PHA stimulated culture of equine PBMCs were frozen in RNAStat60 at
-80°C. RNA was extracted from three of the six samples and processed to cDNA on
two separate days.
Preparation of plasmid templates and oligonucleotides

Each cDNA encoding equine IL-2, IL-4, IL-5, IL-10, and β-actin was cloned into a plasmid vector and sequenced in our laboratory. The respective GenBank accession numbers are: IL-2: L06009, IL-4: L06010, IL-5: U91947, IL-10: U38200, β-actin: AF035774. Sequences for equine IL-2 (Vandergriff et al., 1993) and IL-4 (Vandergriff et al., 1994) have been published. Equine Interferon-γ (IFN-γ) was a gift from Dr. D. Antczak (James A. Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Ithaca, New York). Plasmids encoding these cDNA sequences were purified from 50 ml cultures using CsCl ultracentrifugation in the presence of ethidium bromide (Sambrook et al., 1989) and quantified spectrophotometrically.

In order to generate standard curves, double stranded plasmids containing the equine cytokines and β-actin were serially diluted in one-half log increments to achieve eight dilutions. Cytokine plasmid dilutions ranged from $1 \times 10^{-19}$ to $1 \times 10^{-22.5}$ moles (approximately 40 to 1.2 x $10^5$ cytokine molecules for double stranded plasmid) of plasmid per PCR reaction. β-actin plasmid dilutions ranged from $1 \times 10^{-16}$ to $1 \times 10^{-19.5}$ moles (approximately 1.6 x $10^4$ to 4.8 x $10^7$ β-actin molecules for double stranded plasmid) per PCR reaction.

Equine-specific PCR primers and oligonucleotide probes for hybridization were selected using ‘Oligo’ primer analysis software (National Biosciences, Plymouth, MA) and were commercially prepared (Genelab, Baton Rouge, LA; Baron Biotech, Milford, CT). Sequences of the PCR primers, hybridization probes, as well as the length of the PCR products are listed in Table 2.1. PCR primers were designed.
Table 2.1 PCR primers, hybridization probes, and expected fragment size for PCR amplification and detection of equine IL-2, IL-4, IL-5, IL-10 and β-actin.

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Positive Strand primer (5′Biotin-3′)</th>
<th>Minus strand primer (5′-3′)</th>
<th>Hybridization probe (5′TBR-3′)</th>
<th>Fragment size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>gcacctacttcaagctctaagag</td>
<td>cacatgtgaacttgcttctgacc</td>
<td>ttcattctgtgctctctgcc</td>
<td>303</td>
</tr>
<tr>
<td>IL-4</td>
<td>ctccagagggaggggaagggaagaat</td>
<td>cacagctacagcaggtccgatc</td>
<td>cagcagcaggctccgagcagacag</td>
<td>213</td>
</tr>
<tr>
<td>IL-5</td>
<td>gcgaacctctgcttcatagggcagag</td>
<td>agacgaatgtagtttggaatgtttggcc</td>
<td>tggacagttcttcctgaatgt</td>
<td>216</td>
</tr>
<tr>
<td>IL-10</td>
<td>gtctttaagggtagctgctcgttgg</td>
<td>tgcttccagttttctttctgctg</td>
<td>gcctgggcaacacactctct</td>
<td>324</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>acacagataggggaggggtgag</td>
<td>cttcgctctcctcagttagct</td>
<td>gcttgctgctgctgtttaa</td>
<td>345</td>
</tr>
<tr>
<td>β-actin</td>
<td>atgagatgatgatagccgccct</td>
<td>aggagtcccccttctcctg</td>
<td>acagtgaggggaaganacgaa</td>
<td>194</td>
</tr>
</tbody>
</table>

The positive strand primers were labeled with biotin at their 5′ terminus. Hybridization probes were labeled with TBR at their 5′ terminus. Labeled oligonucleotides were purified by HPLC to eliminate unlabeled oligonucleotides.
to encompass approximately 250 base pairs of the cDNA sequence (range 194 bp to 345 bp). Positive strand primers were biotinylated on their 5' terminus. Oligomers used for hybridization were complementary to the biotinylated strand and were labelled with tris (2,2'-bipyridine) ruthenium II chelate (TBR) on their 5' terminus. Labelled primers and hybridization probes were purified by HPLC to eliminate non-labelled oligonucleotides.

**Polymerase chain reaction**

Reactions were optimized for the amplification of both cDNA and plasmid in preliminary experiments using commercially prepared buffers (PCR Optimizer Kit, Invitrogen, Carlsbad, CA; 10X PCR Buffer, Perkin-Elmer) in order to achieve a single band of maximal intensity on a 3% NuSeive 3:1 GTG agarose gel (FMC BioProducts) with ethidium bromide staining. The target sequences were amplified in 50µl reactions whose final components were 1X PCR Buffer (Table 2.2), 200µM dNTP (Perkin-Elmer), 2.5 units Taq DNA polymerase (Perkin-Elmer), and 0.2 µM of each sense and anti-sense primer. Unless otherwise noted, 5 µl of the cDNA reaction were used to amplify cytokines and 2µl to amplify β-actin. Cycling parameters were 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 minute except for IL-4 which annealed at 60°C for 30 seconds. Samples were cycled using a GenAmp 9600 (Perkin-Elmer). PCR reactions were performed in triplicate and plasmids constituting the standard curve were included on the same plate with the unknown cDNA reactions using the same PCR reagent master solution.

The optimal number of PCR cycles which would maintain the targeted sample population in the exponential amplification phase of the PCR reaction were
Table 2.2  PCR buffer composition.

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Buffer Composition (5X)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tris-HCl (mM)</td>
</tr>
<tr>
<td>IL-2</td>
<td>300</td>
</tr>
<tr>
<td>IL-4</td>
<td>300</td>
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</tr>
<tr>
<td>IFN-γ</td>
<td>300</td>
</tr>
<tr>
<td>β-actin</td>
<td>250</td>
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</tbody>
</table>

INV(A,G,K, or O) signifies buffer designation in PCR Optimizer Kit (Invitrogen, Carlsbad, CA). 10X PCR buffer with MgCl₂ used to amplify equine IL-5 and equine β-actin, was obtained from Perkin-Elmer (P-E).
determined for each cytokine as well as for β-actin using cDNA from PHA stimulated equine PBMC. A PCR master mix that included cDNA was divided into equal aliquots. Tubes were amplified under conditions previously described and one tube was removed at 15, 20, 25, 30, 35, and in the case of the cytokines at 40 and 45 cycles. Tubes were kept on ice until analyzed.

For plasmid standard curves, plasmids were serially diluted in half log increments to achieve final concentrations of $2 \times 10^{-20}$ to $0.2 \times 10^{-23.5}$ moles/μl for cytokines and $2 \times 10^{-17}$ to $2 \times 10^{-20.5}$ moles/μl for β-actin. 5μl of each plasmid dilution were amplified in each PCR reaction. A standard curve therefore was created by the amplification of eight dilutions of plasmid encoding the cDNA of interest. Plasmid standard curves were always generated in parallel with amplifications from cDNA using the same PCR reagent master mix.

**Quantification of PCR product**

PCR targets were quantified using the QPCR System 5000 (Perkin-Elmer). 5μl of PCR product were hybridized in solution in a 50μl reaction containing 10 pmoles of target-specific TBR labeled oligonucleotide probe and 1X PCR Buffer without MgCl$_2$ (Perkin-Elmer). The reaction was heated to 95°C for 90 seconds followed by a 5 minute hold at 55°C. Biotinylated PCR product was then captured by adding 15μl of streptavidin coated iron beads (Dynabeads, Perkin-Elmer) to the hybridization reaction and incubating at 55°C for 30 minutes. The entire 65 μl reaction was transferred to a 175 mm polypropylene tube containing 335 μl QPCR Assay Buffer (Perkin Elmer) and quantified on the QPCR System 5000. The QPCR System 5000 aspirates the iron beads with their attached PCR product bearing TBR.
labeled probe onto a working electrode. TBR on the target specific probe emits light at 620 nm as a result of a series of redox reactions that are induced when voltage is applied to the electrode. The emissions are detected and quantified by a photomultiplier tube and reported as luminosity units. A plot of luminosity values vs initial template number was derived from plasmid amplifications. Initial template numbers in the cDNA reactions were then interpolated using luminosity values. These values were designated as copy units.

Statistical analysis

Interpolated copy units and luminosity values were compared using one way analysis of variance. Where data were not normally distributed, the analysis was performed on ranks. In the case of amplifications originating from identical cDNA reactions, that is cDNA reactions which were synthesized using equivalent amounts of RNA from the same extraction, interpolated copy units were compared using repeated measures analysis of variance. Post hoc pairwise comparisons were made using the Student-Neuman-Keuls method. Inter-assay sample variation was compared using a paired t-test and the correlation between the predicted cytokine and the predicted β-actin copy numbers derived from the same cDNA sample was determined using Pearsons Product Moment analysis. Unless otherwise noted, differences are considered significant when P < 0.05.

Results

Determination of PCR cycle number

To determine the optimal cycle number which would maintain the amplification of the cytokines and β-actin in their exponential amplification phase, the
transcripts of interest were amplified in duplicate reactions to varying cycle numbers from cDNA reactions and assayed for luminescence with sequence-specific probe. By plotting luminosity vs cycle number, the exponential amplification phase of the reactions could be identified. Cycle numbers 20 and 30 defined the limits of the exponential amplification phase during amplification of equine β-actin (Figure 2.1). Accordingly, 25 cycles was chosen for subsequent β-actin amplifications. By contrast, 35 cycles were found to provide better sensitivity for cytokine amplification while maintaining the reactions in their exponential amplification phase.

**Generation of PCR standard curves**

Using 25 cycles in the case of β-actin and 35 cycles for the cytokines, one-half log dilutions of plasmid ranging from $1 \times 10^{-16}$ to $1 \times 10^{-19.5}$ moles for β-actin and $1 \times 10^{-19}$ to $1 \times 10^{-22.5}$ moles for the cytokines were amplified to generate a standard curve. A plot of luminosity vs template number allowed rapid identification of the exponential amplification and plateau phases of each reaction (Figures 2.2, 2.3, and 2.4). Because the noncoding strand of the plasmid derived sequence serves as a template for the PCR reaction, each molecule of plasmid was considered equivalent to 2 cDNA molecules when labeling the standard curve axis. A linear relationship was maintained for all cytokines from $1 \times 10^{-22.5}$ to $1 \times 10^{-20.5}$ moles of plasmid DNA indicating a three log dynamic range for input DNA. Similarly, post PCR quantification of β-actin also demonstrated linearity of three logs for input DNA ranging from $1 \times 10^{-19.5}$ to $1 \times 10^{-17.5}$ moles. For brevity, amplifications from these series of plasmid dilutions will be referred to as a standard curve.
Figure 2.1. Optimizing cycle number to maintain β-actin amplification in the exponential amplification phase of the PCR. Identical 50 ml PCR reactions containing cDNA from PHA stimulated equine PBMC were amplified to different cycle numbers as indicated. 5 ml of each PCR reaction were hybridized with sequence specific probe, captured onto streptavidin-coated iron beads (Dynabeads, Perkin-Elmer), and assayed using the QPCR System 5000. Reactions amplified between 20 and 30 cycles defined the exponential amplification phase of the reaction.
Figure 2.2. PCR Standard Curves: Beta-actin and Interleukin-2. Luminosity (measured by the Perkin-Elmer QPCR System 5000) vs. differing initial quantities of plasmid encoding beta-actin or interleukin-2 after PCR cycles 25 and 35 respectively. Data points represent mean and standard deviation of three replicate PCR reactions. Regression adjustment represents a two parameter exponential rise to maximum fit of the data defined by the equation $y = a(1-e^{bx})$. 

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Figure 2.3. PCR Standard Curves: Interleukin-4 and Interleukin-5. Luminosity (measured by the Perkin-Elmer QPCR System 5000) vs. differing initial quantities of plasmid encoding interleukin-4 or interleukin-5 after 35 PCR cycles. Data points represent mean and standard deviation of three replicate PCR reactions. Regression adjustment represents a two parameter exponential rise to maximum fit of the data defined by the equation \( y = a(1-e^{-bx}) \).
Figure 2.4. PCR Standard Curves: Interleukin-10 and Interferon-gamma. Luminosity (measured by the Perkin-Elmer QPCR System 5000) vs. differing initial quantities of plasmid encoding interleukin-10 or interferon-gamma after 35 PCR cycles. Data points represent mean and standard deviation of three replicate PCR reactions. Regression adjustment represents a two parameter exponential rise to maximum fit of the data defined by the equation \( y = a(1-e^{bx}) \).
Mathematical characterization of PCR standard curves

To determine the mathematical equation that accurately modeled the standard curves over a wide range of input DNA, IL-4, IL-10, and β-actin were amplified from six cDNA reactions whose RNA content constituted serial two fold dilutions of RNA (2.0, 1.0, 0.5, 0.25, 0.125 and 0.0625 μg). Standard curves encoding the targeted cytokine or β-actin were amplified in the same run.

Several mathematical regression models were applied to the standard curve data which was graphed on combinations of log and linear scales (SigmaPlot™, Jandel Scientific, San Francisco, CA). Luminosity values from the RNA dilution series were then interpolated to copy units using each regression model to determine if two fold differences in message content were predicted (Figures 2.5, 2.6).

Mathematical models of the standard curve included a linear polynomial (y=b+ax) adjustment to data graphed as log luminosity vs log copy units. A logistic mathematical adjustment (y= [(a-d)/(1+(x/c)b)]+d) was applied to data graphed as luminosity vs log copy units where a = signal maximum, b = slope parameter, c = value at inflection point, and d = signal minimum. These analyses of PCR standard curve data have been previously published (Melby et al., 1993; Reyes-Engel et al., 1996). The logistic mathematical equation was also applied to data on a log luminosity vs log copy units scale.

Though the aforementioned adjustments sometimes provided superior correlation coefficients (r) and did predict dilutions of message that approximated two fold at the lower RNA concentrations, a two parameter exponential rise to maximum
Figure 2.5. Mathematical characterization of the PCR standard curves. IL-4 and IL-10 were amplified from a series of cDNA reactions whose input RNA constituted serial two fold dilutions ranging from 2 μg to 0.0625 μg. Luminosity values were interpolated to copy units using four mathematical analyses of the standard curve and plotted according to the predicted fold change. Mathematical analyses included: (•) two parameter exponential rise to maximum \( y = a(1-e^{-bX}) \), (○) linear polynomial \( y = b + ax \) applied to log luminosity vs log copy units, (▼) logistic mathematical \( y = \left(\frac{a-d}{1+(X/c)b}\right) + d \) applied to luminosity vs log copy units and, (▼) the logistic mathematical equation applied to log luminosity vs log copy units. The line (-----) represents the ideal prediction of two fold changes between samples. Data represents the fold change between the mean of triplicate PCR reactions.

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Figure 2.6 Mathematical characterization of the PCR standard curve. β-actin was amplified from a series of cDNA reactions whose input RNA constituted serial two fold dilutions ranging from 2 μg to 0.0625 μg. Luminosity values from the cDNA reactions were interpolated to copy units using four mathematical analyses of the standard curve and plotted according to the predicted fold change. Mathematical analyses included: (●) two parameter exponential rise to maximum \( y = a(1-e^{-bx}) \), (○) linear polynomial \( y = b + ax \) applied to log luminosity vs log copy units, (▼) logistic mathematical \( y = [(a-d)/(1+(x/c)b)] + d \) applied to luminosity vs log copy units and, (▽) the logistic mathematical equation applied to log luminosity vs log copy units. The line (-----) represents the ideal prediction of two fold changes between samples. Data represents the mean of triplicate PCR reactions. (*) indicates replicate beyond the final iteration of the standard curve. Luminosity values for amplifications from 2 mg of RNA were beyond the final iteration of the two parameter exponential rise to maximum analysis.
regression analysis given by the equation \( y = a(1 - e^{-bx}) \) was superior at predicting the expected two fold dilutions (Figure 2.7).

One way analysis of variance of interpolated copy units from this RNA dilution series indicated that the predicted two fold dilutions were significantly different for each of the PCR targets examined \((P < 0.01)\). Pairwise comparison of the RNA dilution groups indicated that differences among all groups were statistically significant with the exception of the \(0.0125 \mu g\) vs \(0.0625 \mu g\) comparison for IL-4 whose t-test approached significance \((P=0.067)\). Data representing IL-4 at \(2 \mu g\) and \(\beta\)-actin at \(1.0 \mu g\) and \(2.0 \mu g\) were not included in the statistical analysis as one or more values of the triplicate were within the plateau phase of the curve and therefore beyond the final iteration of the mathematical model.

**Reproducibility of the RT-PCR process**

To determine the precision of the combined RT-PCR process, IL-2, IL-4, IL-10, IFN-\(\gamma\), and \(\beta\)-actin were amplified using PCR from three identical cDNA reactions. The identical cDNA reactions were synthesized using RNA from a common extraction procedure and a common cDNA reagent master mix. Luminosity values from the cytokine amplifications were interpolated to copy units (Figure 2.8) using the two parameter exponential rise to maximum adjustment of the respective standard curves. Copy units for IL-4 and IFN-\(\gamma\) could not be interpolated because their luminosity values were beyond the final iteration of the standard curve within the plateau phase of the reaction. Luminosity values for IL-4 and IFN-\(\gamma\) amplification are shown in Figure 2.9.
Figure 2.7 Interpolated copy units from cDNA reactions whose RNA content differed by two fold. Following PCR, luminosity values were interpolated to copy units using a two parameter exponential rise to maximum fit of the standard curve given by the equation $y = a(1-e^{-bx})$. The distribution of β-actin values among the cDNA reactions was positively correlated to the distribution of IL-4 and IL-10 values. All 2 µg β-actin replicates were beyond the final iteration of the regression. (*) indicates 1 replicate of the triplicate was beyond the final iteration of the regression.
Figure 2.8. To determine the precision of the RT-PCR assay, IL-2 and IL-10 (top panel) as well as β-actin (bottom panel) were amplified from 3 identical cDNA reactions. Copy units were interpolated from luminosity values using the appropriate standard curves. Data represents the average and standard error of the mean from triplicate PCR amplifications of each cDNA reaction.
Figure 2.9. To determine the precision of the RT-PCR assay, IL-4 and IFN-γ were amplified from 3 identical cDNA reactions. Luminosity values for these amplifications could not be interpolated to copy units because they were beyond the final iteration of the standard curve. Data represent the average and standard error of the mean from triplicate PCR amplifications of each cDNA reaction.
The error, determined by the coefficient of variance associated with replicate amplifications from an individual cDNA reaction ranged from 5-10% for β-actin, and 8-33% for the cytokines (with most values being < 10%). The error in the average predicted copy units from replicate cDNA reactions ranged from 7-13%. When subjected to repeated measures analysis of variance, the interpolated IL-2, IL-10 and β-actin copy units from the 3 identical cDNA reactions were not significantly different (P > 0.1). Similarly, luminosity values following IL-4 and IFN-γ amplification from the identical cDNA reactions were not significantly different (P > 0.08).

Inter and Intra-Assay Variability

To determine the inter- and intra-assay variation associated with RNA that originates from separate RNA extractions, IL-2, IL-4, IL-10 and IFN-γ were amplified in triplicate PCR reactions from six cDNA reactions whose RNA originated from separate extractions of the same culture. As noted in the methods section, one-half of the RT reactions (1-3 vs 4-6) were performed on two separate days. PCR amplification was repeated on a second day. Standard curves were amplified from a common PCR reagent master solution on each day and message from the 6 cDNA reactions was determined by interpolation against the standard curve (Figure 2.10).

To assess inter-assay variability, interpolated copy units from the first day’s amplification were compared to those of the second day for each of the six cDNA reactions using paired t-tests. The interpolated copy units on day 1 and day 2 were not significantly different for IL-2, IL-4, and IL-10. Consistent with prior analyses, IFN-γ luminosity values from the 72 hr PHA stimulated cDNA samples tended to be
Figure 2.10. Comparison of cytokine message amplified on two separate days (■ day 1, and ■■ day 2) from 6 cDNA reactions whose RNA originated from separate extractions of the same culture. Message detected from an individual cDNA reaction did not differ significantly when the two days were compared but there were significant differences in message content among the six reactions. (*) denotes IFN-γ values which had reached the plateau phase of the reaction. Bars represent the mean with standard error of triplicate amplifications.
beyond the final iteration of the standard curve. However, one of the six cDNA reactions had luminosity values that were much lower for each of the transcripts examined than the other 5 cDNA reactions. The IFN-γ luminosity values for this cDNA (#3) did fall within the exponential amplification phase of the standard curve and the interpolated copy units were not significantly different on days 1 and 2.

To assess the intra-assay variability, the interpolated cytokine copy units from the six cDNA reactions were compared. As anticipated, there were significant differences (P<0.01) among the 6 cDNA reactions in the predicted copy units. IL-4 values differed on day 1 and IL-10 on both day 1 and day 2. IL-4 values on day 2 were not significantly different due to a single reaction (#4) whose coefficient of variation for the three replicates was very high (135%). Removal of this cDNA reaction from the statistical analysis restored normality to the sample population and indicated significant differences (P=0.002) between the predicted IL-4 content of the remaining 5 cDNA reactions. Interpolated copy units for IL-2 among the 6 cDNA reactions were not significantly different. The inability to resolve differences in IL-2 input among the 6 cDNA reactions reflected the fact that, as expected for a 72 hour PHA stimulation, IL-2 message was extremely low and approached the lower limit of detection of the assay for all of the 6 cDNA samples.

**β-actin as a Normalization Factor**

The usefulness of β-actin as a house keeping gene for quantitative PCR has been questioned primarily because its expression exceeds that of cytokines by 100-1000 fold such that cycle numbers optimized for detection of cytokines cause β-actin amplifications to enter the plateau phase of the reaction. Accordingly, in order to
maintain β-actin amplification in the exponential amplification phase of the reaction cDNA samples were amplified to only 25 cycles in this protocol whereas cytokines were amplified to 35 cycles. To define the limitations of this approach, β-actin was amplified from the RNA dilutions and 6 extraction cDNA sets. cDNA samples were amplified in conjunction with a standard curve from a common master mix. β-actin copy units from each PCR reaction were interpolated using the standard curve and a normalization factor which corrected each sample to 500,000 copies. (500,000/average copy number of three β-actin replicates) was derived. The β-actin luminosity values from the cDNA reaction that contained 2μg of RNA were beyond the final iteration of the standard curve, within the plateau phase. Due to the magnitude of the β-actin copy numbers, data were log transformed for statistical analysis.

Pearson's product moment analysis of the predicted cytokine and β-actin copy values indicated that the cytokine values for each cDNA were positively and significantly correlated to β-actin values from the same cDNA reaction (Figure 2.7 and 2.11). Thus, there was a statistically significant correlation between predicted cytokine copy units and predicted β-actin copy units over a wide range of input RNA even in the practical situation where PCR reactions resulting from multiple RNA extractions were compared.

To define the conditions for using β-actin as a normalization factor, interpolated cytokine copy units from the previous experiments were multiplied by their respective β-actin normalization factor and compared. In the case of the cDNA series whose input RNA constitute serial two fold dilutions (Figure 2.12), the normalized IL-4 values from each cDNA reaction were not significantly different.
Figure 2.11. Correlation between cytokine and β-actin levels from the same cDNA reaction. IL-2, IL-4, IL-10 and β-actin were amplified in triplicate PCR reactions from six cDNA reactions whose RNA was derived from separate extractions of a single PBMC culture. Cytokine values for each cDNA were positively and significantly correlated to β-actin values from the same cDNA reaction.
Figure 2.12 Cytokine copy numbers normalized for β-actin content of the cDNA reaction. Interpolated IL-4 and IL-10 values derived from PCR amplification of cDNA reactions that contained two fold dilutions of RNA were normalized to 500,000 copies of β-actin and compared. Of the two cytokines only the 1 μg amplification of IL-10 was significantly different from the other cDNA reactions. However, 1 μg β-actin replicates were within the plateau phase of the reaction (*).
However, the normalized IL-10 value for the cDNA reaction that contained 1 μg of RNA was significantly different from the normalized values for the other dilutions. Because two fold dilutions of IL-10 were predicted at all dilutions prior to normalization, this finding suggested that amplification of β-actin from 1 μg of RNA was beginning to exceed the useful limits of the assay.

When normalized cytokine copy units for the 6 cDNA reactions that originated from separate RNA extractions of PHA stimulated cells were compared (Figure 2.13), there were significant differences among the cDNA reactions for IL-2 and IL-10. Normalized copy units were not significantly different for IL-4 but this reflected a large coefficient of variance associated with cDNA #3, a phenomenon previously observed with PCR amplifications on very low template concentrations. Normalized IL-4 values for the remaining cDNA reactions were significantly different when analyzed without data from cDNA #3. Though there was some overlap in the statistical groupings of the six cDNA using pairwise comparisons of the normalized cytokine numbers, there was a tendency for three groupings. Group one consisting of cDNA #6 and #4. Group two consisting of cDNA #1, #2, and #5. and group three having only cDNA #3. Closer scrutinization of the luminosity values revealed an explanation for these findings. Cytokine luminosity values for cDNA #3 were at or below the lowest dilution of the plasmid standard curve, while the luminosity values for β-actin from cDNA #4 and #6 were between the two highest dilutions of the β-actin standard curve at the portion of the curve entering the plateau region. Analysis of the data with and without values from these reactions (#3, #4, #6) determined that values in these ranges could not be used in comparisons of the data (Table 2.3).
Figure 2.13. Cytokine copy numbers normalized for β-actin content of the cDNA reaction. The cytokine content of six cDNA reactions derived from separate RNA extractions of the same culture were normalized to 500,000 copies of β-actin using β-actin values amplified from the same cDNA reaction. Bars represent the mean and standard deviation of triplicate reactions run on (■■) day 1 and repeated on (■■) day 2.
Table 2.3 Effect of β-actin normalization on the coefficient of variation (CV)

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<th>cDNA #1-6</th>
<th>cDNA #1,2,4-6</th>
<th>cDNA # 1,2,5</th>
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<td>IL-2 PCR I</td>
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<td>0.53</td>
<td>1480.2</td>
</tr>
<tr>
<td>normalized</td>
<td>287.5</td>
<td>197.0</td>
<td>0.69</td>
<td>207.2</td>
</tr>
<tr>
<td>IL-10 PCR II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>raw</td>
<td>1144.8</td>
<td>642.3</td>
<td>0.56</td>
<td>1354.4</td>
</tr>
<tr>
<td>normalized</td>
<td>222.9</td>
<td>111.3</td>
<td>0.50</td>
<td>179.8</td>
</tr>
</tbody>
</table>

The average of triplicate normalized cytokine amplifications from 6 cDNA reactions (#1-6) whose RNA originated from separate extractions of the same culture were grouped as noted and analyzed for their effect on the assay error. Removing data derived from the extremes of the standard curves (reactions 3, 4, and 5) decreased the error to 13-27%.
Values at or below the bottom of the cytokine curves and at the top of the β-actin curve overestimated the copy values leading to erroneously high correction factors. When all six reactions were analyzed, normalization did not improve the sampling error as determined by the coefficient of variation (CV) among the average values for the six reactions and actually tended to increase it to unacceptable levels (50-100%). Removing values from cDNA #3 decreased the errors in both normalized and non-normalized samples from the same population but the errors were still up to two fold greater in the normalized than the non-normalized data. However, when the cDNA #4 and #6 were removed from the analysis, the coefficients of variance (CV) prior to normalization ranged from 20-40% and were decreased to 13-27% after normalization. This confirms that when values lying outside the linear portion of the standard curve are excluded, β-actin is a valuable normalization factor and differences of +/- 2 standard deviations about the mean will adequately discriminate significant differences between samples.

Discussion

Amplifications from cloned cDNA of identical sequence to the wild type target have been previously utilized to generate standard curves for the quantification of PCR products (Martin et al.; 1995, Melby et al., 1993). As with our experiments, the standard curve and unknown cDNA samples were amplified using the same PCR reagent master mix and were analyzed in replicates to minimize any tube to tube variation in the PCR reaction. This approach eliminates the need for synthetic templates of different sizes from wild type. This has several advantages. First, because the target and control sample are of identical sequence but amplified in
different tubes, the concerns regarding different amplification efficiencies based on
size differences (Becker-Andre and Hahlbrock, 1989; Kanangat et al., 1992; Souaze
et al., 1996) or the formation of competitor/wild type heteroduplexes are minimized
(McCullough et al., 1995; Henley et al., 1996). This method is less labor intensive
because there is no internal standard to construct nor is there a large series of samples
with differing competitor:target ratios. Varying the competitor:target ratio is required
for accurate quantification using competitive methods (Arnold et al., 1992; Souaze et
al., 1996; Raeymaekers, 1993). Most significantly, this method is easily performed
with very high sample throughput from cells to quantified product in replicates
sufficient for statistical analysis of the data in a single day.

The generation of standard curves and their graphical representation of signal
intensity (luminosity) vs copy number on a linear scale allowed identification of the
exponential amplification and plateau phases of the PCR reaction. It is well accepted
that in noncompetitive methods, samples must remain in the exponential amplification
phase of the reaction for meaningful comparison because all reactions, regardless of
starting template concentration, will reach a maximum defined as plateau (Ferre,
1992; Morrison and Gannon, 1994). Despite occasional reports to the contrary
(Dostal et. al., 1994; Rüster et. al., 1995) competitive reactions can be run to plateau
without adversely affecting target:competitor ratios (Bouaboula et. al., 1992; Siebert
and Larrick, 1992; Couttrez et. al., 1994; Morrison and Gannon, 1994) Decreases in
PCR product following plateau have been described and the reason for this
phenomenon is not known (Personal communication, John Pfeifer, Perkin-Elmer).
The mathematical model applied to these standard curves, a two parameter
exponential rise to maximum given by the equation \( y = a(1-e^{-bx}) \), has the advantage of preventing interpolations beyond the final iteration of the standard curve which lie within the plateau phase of the reaction. The most commonly utilized mathematical model for PCR based quantifications is a linear fit where data is graphed following detection of PCR product as \( \log_{10} \) signal strength vs \( \log_{10} \) copy number (Wang et. al., 1989; Murphy et. al., 1990; Noonan et. al., 1990; Melby et al., 1993). However, a logistic mathematical analysis of signal strength vs \( \log_{10} \) template concentration has been shown to provide a superior fit to the curve over a wide range of template concentration (Reyes-Engel et al., 1996). When these models are applied to noncompetitive methods, because there is no mathematically imposed maximum, the beginning of the plateau is subjectively determined by the investigator. Experience with different graphical representations of our standard curve data indicated that linear adjustments made it difficult to appreciate the onset of plateau.

Unlike other experiments which simply utilize a mathematical adjustment that provides the best fit of the standard curve data, in this instance cDNA reactions created from serial two fold dilutions of RNA, which should therefore contain two fold dilutions of cytokine template, were utilized to determine the mathematical model which most accurately modeled the performance of the PCR amplification. Though the other mathematical adjustments to the standard curves often provided superior correlation coefficients, the two parameter exponential rise to maximum adjustment was chosen because it most closely predicted the anticipated two fold dilutions of message with minimal variation such that the two fold dilutions were significantly different when subjected to analysis of variance. This also indicates that the assay is..
capable of resolving at least two fold differences in message, though the limits of resolution were not determined. Based on the graphical representation of the standard curves and amplifications from diluted RNA, the assay has a linear dynamic range on the order of 3 logs.

To our knowledge this is the first report of the use of double stranded (ds) circular plasmid to generate standard curves used for quantification of PCR product. Other reports have used linearized cDNA plasmid (Martin et al., 1995) or liberated the cDNA fragment using restriction digests (Melby et al., 1993) but use of ds plasmids eliminates the need to purify the fragments from inhibitors of the PCR reaction following digests. Though there are reports of differences in the efficiency of amplification between double stranded and single stranded templates, the curves described here accurately predicted a two fold difference in transcript numbers from cDNA reactions whose input RNA varied by two fold for each of the transcripts examined. This indicates that, at least over the range of these template dilutions which in the case of IL-10 covered most of the linear portion of the standard curve, the amplification efficiency of the target sequences from double stranded circular plasmid and linear single stranded cDNA was equivalent.

A number of housekeeping genes including glyceraldehyde-3-phosphate dehydrogenase (GADPH) (Dukas et al., 1993), hypoxanthine phosphoribosyl-transferase (HPRT) (Murphy et al., 1993) and peptidyl-prolyl-cistrans isomerase (Cyclophilin) (Nast et al., 1994) have been utilized in PCR analysis of cytokine mRNA content. β-actin was chosen as the internal control for this assay because the equine sequence has been determined allowing the generation of sequence-specific
oligonucleotide probes and primers. Additionally, attempts to amplify HPRT using published sequences (Grunig et al., 1992) were variably successful in our hands. While 1.3 (Melby et al., 1993) to three fold (McCairns, 1994) increases in β-actin message have been reported in response to mitogen stimulation using cloned T cells and normal human PBMC respectively, increases in HPRT production on the order of 10-20 fold have been demonstrated in mitogen stimulated human lymphocytes (Steen et al., 1990; Steen et al., 1991) making HPRT a less favorable internal control for immunologic studies. The primary drawback of β-actin is that it is a moderately abundant transcript whose expression is several orders of magnitude greater than cytokines, a problem not significantly different for GADPH and Cyclophylin which are, respectively, moderately abundant and slightly less than moderately abundant species (Smith, 1995).

To address the difference in expression between β-actin and cytokine species, the volume of cDNA and the cycle number used for β-actin amplification were lowered when compared to parameters used for cytokine amplification. The premise that normalization should not be compromised and actually improved by this difference in template concentration cycle number reflects the fact that normalization values are based on relative differences between the samples which can only be resolved in non-competitive methods if all samples are within the exponential amplification phase of the reaction. This is well illustrated by the results of cDNA # 4 and #6 in the six extraction set of cDNA reactions whose β-actin values approached the plateau phase and whose normalized cytokine values were significantly lower than those of the other replicates. 25 cycles have been previously reported for β-actin
amplifications using non-competitive methods but these reports utilized comparatively more RNA per PCR reaction, on the order of 0.5 \( \mu g \) per PCR reaction (Melby et al., 1993). In preliminary experiments, RNA quantities of this magnitude consistently pushed the PCR reaction into the plateau phase which eliminates the ability of \( \beta \)-actin amplifications to resolve the differences inherent in the RT step. The finding in this series of experiments that \( \beta \)-actin values in the region of the standard curve immediately preceding the plateau phase are associated with normalization errors has restricted use of samples whose \( \beta \)-actin values amplify to this level. Negative samples are still accurate but amplifications from samples with positive cytokine values are repeated on dilution. To our knowledge this is the first time that different cycle numbers and cDNA volumes have been used to amplify the housekeeping gene and cytokine targets.

By using RNA from a single RNA extraction in the synthesis of three identical cDNA reactions the precision of the PCR process (given by the error associated with repeated amplifications from the same cDNA reaction) as well as tube to tube differences in the efficiency of the RT reaction, were examined. The precision of this PCR process (10-30\%) is quite good when compared to other investigators whose pre-normalization errors ranged from 20\% to as high as 50\% (Kanangat et al., 1992; Melby et al., 1993; Jessen-Eller et al., 1994). The fact that the average predicted copy numbers from these identical cDNA reactions were not significantly different and varied by only 5-10\% indicates that there was no difference in the tube to tube efficiency of the RT reaction. When amplifications were made from multiple cDNA reactions whose RNA was derived from separate extractions of the same culture, at
least two of the reactions had significantly less message (prior to normalization) than the remaining four reactions and this distribution of message was equivalent for each of the cytokines examined. Taken together these findings suggest that the differences in RT efficiency which are commonly reported (Cone et al., 1992; Jessen-Eller et al., 1994) most likely reflect either errors in RNA quantification or, the presence of RT reaction inhibitors which are copurified with the RNA during extraction.

While the findings from the cDNA reactions created from RNA dilutions and from the identical cDNA reactions synthesized using RNA from a single extraction demonstrated that the use of plasmid standard curves is repeatable and accurately detects two fold changes in input RNA, single RNA isolations do not reflect laboratory practice. Using six separate cDNA reactions whose RNA originated from separate extractions of the same PBMC culture, there was no difference in the predicted copy values for each of the cytokines when amplifications from the same cDNA reactions on two different days were compared. This confirms that plasmid derived standard curves can be used to overcome day to day variability in the PCR reaction. There were however, as previously mentioned, differences in the predicted cytokine values among the six reactions prior to normalization. The relative ranking of the six cDNA reactions based on quantity of transcript was significant and positively correlated with the ranking derived from β-actin amplification for each of the transcripts examined. This confirmed that β-actin amplified under these conditions could be used to determine which reactions had significant differences in the RT step, suggesting that β-actin might be useful as a normalization factor.
When β-actin was examined as a normalization factor several limitations to the assay were identified. While it was previously assumed that the final iteration of the standard curve would prevent errors due to interpolations within the plateau phase, instances did occur in which the final iteration of the standard curve was between the 2 highest dilutions of the standard curve, within the plateau. β-actin luminosity values from cDNA reactions which were in this region were falsely overestimated, resulting in low normalization factors which therefore underestimated the normalized cytokine values. Similarly, cytokine values at or below the bottom of the curve overestimated the copy number causing normalization errors even when β-actin values were derived from the exponential amplification portion of the β-actin standard curve. Samples falling in these regions must therefore be re-amplified using an appropriate cDNA volume adjustment. It is also important to note that the luminosity values derived from negative controls are not equivalent to 0 copy units using this regression model so that comparisons with negative values must be adjusted based on the value interpolated from the negative control. When these contingencies are applied to normalized values both from the RNA dilution set and the set of six separate extractions from a single culture, the corrected copy numbers are not significantly different despite significant differences prior to normalization. These contingencies are not unique but simply reiterate the need for strict adherence to the limits of the exponential amplification phase of the PCR reaction. Normalized values from the nonexponential regions of the curves increased the assay error (defined by the variance when the average cytokine copy units from each cDNA reaction are
compared) from 13-27% to 45-160% for the reactions from separate extractions and from 13% to 21% in the RNA dilution samples.

The objective in the development of this assay was to create a quantitative method for the analysis of cytokine mRNA in equine lymphocytes but not necessarily to achieve the highest sensitivity accessible by PCR. However, this method is readily adaptable to increased or decreased sensitivity by altering cycle number and/or the quantity of RNA utilized based on pilot studies of the population in consideration. This method is precise, repeatable, easily resolves two fold differences in message (and perhaps smaller differences), and has a dynamic range on the order of 3 logs without the use of competitors. By strictly adhering to the limits of the exponential amplification phase of the PCR reaction, a range of +/- 2 standard deviations about the mean will predict significant differences between samples.
CHAPTER 3
VACCINATION AGAINST STRONGYLUS VULGARIS IN HORSES:
COMPARISON OF THE CYTOKINE, HUMORAL AND
LYMPHOPROLIFERATIVE RESPONSES OF VACCINATES AND
NONVACCINATES

*Strongylus vulgaris* is considered the most pathogenic nematode parasite of equidae due to the severe arterial lesions it causes in the cranial mesenteric artery during larval migration. Infective third-stage larvae (L$_3$) ingested from contaminated pasture penetrate the mucosa of the large intestine, molt to fourth-stage larvae (L$_4$) in the submucosa, and then proceed along arterioles and arteries that supply the intestine to the root of the cranial mesenteric artery (Ogbourne and Duncan, 1985). There larvae molt to become immature adults (L$_a$) causing severe arteritis before returning, again via the vasculature, to complete their life cycle in the large intestine. Arterial lesions include the formation of tortuous subintimal tracts, thrombi, and in severe cases verminous aneurysms which can compromise perfusion of intestinal vascular beds. This syndrome known as verminous arteritis or thromboembolic disease is characterized by ischemic infarctions of the bowel which result in toxemia, abdominal pain, and death in severe cases (White, 1985).

Previous studies have demonstrated that oral vaccination with radiation-attenuated *S. vulgaris* L$_3$ induces resistance to challenge infection and prevents classical lesions of verminous arteritis (Klei *et al.*, 1982). When compared to parenteral vaccination with adult or larval somatic antigen homogenates which did not confer resistance to challenge infection, the protection observed in oral radiation-attenuated L$_3$ vaccinated ponies correlated to both prechallenge anti-*S. vulgaris*
antibody titers specific for surface antigens of late L₃ stages and to induction of a post-challenge anamnestic-like eosinophilia (Monahan et al., 1994). Infections by S. vulgaris have been shown to activate eosinophils and neutrophils in vitro (Dennis et al., 1988) and eosinophils from S. vulgaris primed but not unprimed ponies kill S. vulgaris L₃ in vitro in an antibody-dependent manner (Klei et al., 1992), indicating that an antibody-dependent phenomenon involving eosinophils may contribute to the resistance seen in immune ponies.

Helminth infections are characteristically associated with eosinophilia and elevated IgE production (Finkelman et al., 1991), traits that also characterize the Type II or TH2 response initially described in cloned mouse cells (Mosmann et al., 1986; Mosmann and Coffman, 1989b). Type II responses, identified by the production of IL-4 and IL-5 which are integral to the generation of IgE (Snapper and Paul, 1987) and eosinophilia (Coffman et al., 1989) respectively, have been shown to play a significant role in the protective immune response to metazoan helminths (reviewed in Mosmann and Coffman, 1989a; Cox and Liew, 1992; Urban, J. F. et al., 1992). A cytokine or combination of cytokines which exhibits chemotactic activity for eosinophils has been found in the supernatant of S. vulgaris stimulated PBMC from immune but not nonimmune ponies (Dennis et al., 1993). This finding coupled with the anamnestic eosinophilia which is characteristic of immunity to S. vulgaris suggest that differential cytokine production, perhaps similar to that seen in classical Type II responses to helminth parasites may play a role in immunity to S. vulgaris.
Methods

Experimental design

Ten yearling ponies were raised and maintained under parasite-free conditions (Monahan et al., 1997), housed in pairs on wood shavings, and fed a pelleted ration and water ad libitum. The experimental design consisted of three groups. Four ponies in group 1 (vaccinates) were orally vaccinated with 500 S. vulgaris L3 irradiated with 90 kRads 60Co as described (Klei et al., 1982). Vaccination larvae were administered in tap water by syringe behind the tongue, then the syringe was rinsed and the rinsate administered orally as well. Immunization was repeated in this group 3 weeks after the first immunization. Group 2 (nonvaccinates) consisted of 4 ponies and group 3 (controls) consisted of two ponies. Six weeks following the second immunization vaccinates and nonvaccinates were challenged per os with 1000 S. vulgaris L3.

Following challenge ponies were monitored at least twice daily for fever, signs of abdominal pain, depression, and anorexia. Observers were blinded to the nature of the ponies’ treatment and pyrexia was considered to be any rectal temperature above 39°C. Four days prior to challenge (D-4), on the day of challenge (D0), and on days 4 (D4) and 9 (D9) post challenge, exploratory celiotomies were performed to collect lymph nodes from the region of the cecum in close proximity to the ceco-colic fold, termed colonic lymph nodes (CLN). Blood was collected for hematologic evaluation and collection of peripheral blood mononuclear cells (PBMC) at these time points as well as fourteen days following challenge (D14). On D14 necropsy examinations were performed as described previously (Klei et al., 1982) for evaluation of lesions.
collection of CLN, and recovery of larvae from cranial mesenteric artery (CMA) dissections.

**Hematology**

Blood was analyzed in a routine fashion. Total white blood cell counts were analyzed using a Baker 9000 analyzer (Serono-Baker, Allentown, PA). Differential white cell counts were determined by counts of 100 cells examined at 100 x under oil immersion.

**Strongyulus vulgaris larvae and soluble antigen**

Larvae for use in the irradiated immunizations were recovered as L₃ from Baermann sedimentations of fecal cultures from monospecifically infected *S. vulgaris* donor ponies (McClure *et al.*, 1994). Larvae were washed and stored in tap water at 4°C until use. Soluble adult *S. vulgaris* antigen (SAWA) preparations used in ELISA and lymphoproliferation assays were prepared as previously described (Klei *et al.*, 1982) from adult *S. vulgaris* recovered from the intestines of horses.

**Enzyme-linked immunosorbent assay (ELISA)**

Circulating antibody isotypes specific for adult *S. vulgaris* soluble antigens were monitored by ELISA using serum collected on D-4 and D14. Soluble adult *S. vulgaris* antigen was diluted in buffer (0.015 M Na₂CO₃, 0.035 M NaHCO₃, 0.003 M NaN₃) to 5 µg/ml. Antigen preparation was added to wells of 96-well flat bottom polystyrene microtiter plates in 50 µl volumes (Dynatech Laboratories, Chantilly, Virginia) and incubated overnight. Unless otherwise noted, incubations were performed at 37°C in a humidified incubator. Plates were washed three times with a solution of 0.05% Tween 20 in PBS (PBST) and nonspecific binding sites were
blocked using 1% fish gelatin (Sigma, St. Louis, MO) in PBS (PBSG), 100 μl per well, for 1 hour at room temperature. A 1:200 dilution of serum was made in PBSG and added to triplicate wells in 50 μl aliquots. Serum from known high responders and negative responders served as standard positive and negative controls on each plate. Plates were incubated for 90 minutes then washed three times with PBST. Culture supernates from hybridoma lines specific for equine IgA, IgM, IgGa, IgGb, or IgG(T) (Lunn et al., 1996) were diluted 1:100 in PBSG, added to appropriate wells in 50 μl volumes, and incubated for 90 minutes. Following incubation, wells were washed three times with PBST. Affinity-purified, horseradish peroxidase-conjugated goat anti-mouse IgG and IgM, heavy and light chain specific (Jackson Immunoresearch Labs, West Grove, PA) was diluted 1:1000 in PBSG, added to wells in 50 μl aliquots, and incubated for 90 minutes. Plates were washed three times with PBST. The substrate was 3,3',5,5'-tetramethylbenzidine (TMB, Kirkeguard and Perry, Gaithersburg, MD) at 75 μl per well. Reactions were allowed to proceed for 10 minutes for optimum color development. Optical density (OD) was recorded using a Dynatech MR 700 automated microtiter plate reader (Dynatech Industries) with absorbance set at 630 nm.

Preparation of cells

Equine PBMC were isolated from venous blood by density gradient centrifugation over Ficoll Paque (Pharmacia LKB Biotechnology, Piscataway, NJ). After washing in calcium and magnesium free phosphate buffered saline (CMF-PBS), PBMC were suspended in media consisting of RPMI 1640 (Sigma Chemicals, St. Louis, MO) supplemented with 2-mercaptoethanol (10⁻⁴ M), glutamine (2mM), 100
U/ml penicillin, 100 µg/ml streptomycin and 5 % heat inactivated fetal bovine serum (HyClone Laboratories, Logan, UT). Cells were isolated from colonic lymph nodes by disruption through sterile mesh screens. Colonic lymph node derived cells (CLNC) were washed twice in media, resuspended and counted. Aliquots of PBMC and CLNC (2 x 10^6 cells) were frozen at -70°C in RNA Stat 60 (Tel-Test, Friendswood, TX) for use in cytokine quantification assays.

**Lymphoproliferation**

On D-4, D4, D9, and D14, PBMC and CLNC were seeded onto multiple wells of a 96 well round bottomed tissue culture plate (Costar, Assonet, MA) at a density of 2 x10^5 cells and incubated in 200 µl of media containing soluble adult *S. vulgaris* antigen at a final concentration of 4 µg/ml. Proliferation was measured by (^3)H-thymidine incorporation. After incubation for 5 days at 39°C with 5 % CO₂, 50µl (0.5 µCi) of ^3^H-thymidine was added to each well and incubation was continued for 4 hours. The pulsed cells were harvested onto glass fiber filter pads with a Tomtech 96-well Harvester for liquid scintillation counting using a LKB Betaplate counter (Pharmacia).

**Cytokine quantification**

Samples of PBMC and CLNC which had been collected at D-4, D4, D9, and D14 and frozen in RNA Stat 60 reagent were defrosted and processed to RNA using chloroform extraction as per the reagent manufacturer's directions. RNA was quantified spectrophotometrically and the ratio of optical densities at 260nm and 280 nm (O.D.\_{260nm}/O.D.\_{280nm}) was consistently in the range of 1.9. 0.60 µg of RNA in a volume of 35 µl DEPC-treated water were heated to 65°C for 10 minutes to denature

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the RNA, then chilled on ice for two minutes and pulse-spun at 14,000 x g in a microcentrifuge at 4°C. 45 μl of reagent master mix were then added resulting in a final reaction containing 1x first strand buffer (Gibco BRL, Gaithersburg, MD), 0.5 mM dNTP (Perkin-Elmer, Foster City, CA), 1.0 μM Oligo-dT (Promega, Madison, WI), 10 mM DTT (Gibco), 0.075 μg/μl BSA (NEB, Beverly, MA), 120 Units RNAsin (Promega), and 800 units Moloney Murine Leukemia Virus Reverse Transcriptase (BRL). The reaction was incubated 1 hour at 40°C then frozen at -20°C until use.

Equine IL-2, IL-4, IL-5, IL-10, and IFN-γ were quantified with the aid of the polymerase chain reaction (PCR) by interpolation against a standard curve as described in chapter 2. Standard curves were generated for each cytokine by simultaneously amplifying, from a common PCR master mix, known quantities of plasmids which contained the equine cytokine sequence of interest. Briefly, cytokines were amplified in 50 μl PCR reactions from 5 μl of each cDNA reaction using 2.5 units of taq polymerase (Perkin-Elmer), 0.5 mM dNTP (Perkin-Elmer), and species-specific primers at a final concentration of 0.2 μM. Primers were commercially prepared (Genelab, Baton Rouge, LA; Baron Biotech, Milford, CT) from species-specific sequences which were determined using Oligo primer analysis software (National Biosciences, Plymouth, MN). Upstream primers were biotinylated on their 5' terminus. Cycling parameters including buffer (PCR Optimizer, Invitrogen, Carlsbad, CA) and annealing temperatures were optimized for each cytokine. All cytokines were amplified to 35 cycles which was previously determined to maintain the reactions in their exponential amplification phase. β-actin, which was utilized as a
housekeeping gene, was amplified from 2 μl of cDNA using 25 cycles in order to remain within its exponential amplification phase.

PCR products were quantified using the QPCR System 5000 (Perkin-Elmer) as has been described (Zhao et al., 1996; Blok et al., 1997). 5 μl of each PCR reaction were hybridized in a 50 μl reaction containing 1x PCR buffer II (Perkin-Elmer) and 10 of pmoles tris(2,2'-bipyridine) ruthenium II chelate labeled oligonucleotide probe whose sequence was specific to the cytokine targeted by PCR. Oligonucleotide probes were commercially prepared (Baron Biotech, Milford, CT) and purified by HPLC to eliminate non-labeled oligonucleotides. The reaction was heated to 95°C for 90 seconds followed by a 5 minute hold at 55°C. 15 μl of streptavidin-coated iron beads (Dynabeads, Perkin-Elmer) were added to each reaction followed by incubation at 55°C for 30 minutes. The entire 65 μl reaction was transferred to a 175 mm polypropylene tube containing 335 μl QPCR Assay Buffer (Perkin-Elmer) and quantified on the QPCR System 5000 whose output is in luminosity units.

Statistical Analysis

The number of days that anorexia, pyrexia, depression, and abdominal pain were exhibited by ponies in each treatment group in response to oral challenge with *S. vulgaris* larvae were subjected to one way analysis of variance. Lymphoproliferation data was subjected to one way repeated-measures analysis of variance. ELISA OD data was subjected to two way analysis of variance. Due to the wide distribution of cytokine responses displayed by this outbred population of ponies, cytokine data were log transformed for statistical analysis. For each day examined (D-4, D4, D9, and
D14), differences in cytokine production among the three treatment groups were compared using one way analysis of variance while comparisons of daily cytokine production within a treatment group were made using repeated-measures analysis of variance. Daily cytokine production in PBMC was compared to that of CLNC for each treatment group using the Student's t-test. Differences were reported as significant when $P<0.05$.

**Results**

**Clinical signs.**

Ponies in the vaccinal group exhibited significantly fewer episodes of pyrexia, depression, and abdominal pain than nonvaccinates (Table 3.1). Pyrexia, depression, and abdominal pain exhibited by vaccinates was not significantly different from that exhibited by control ponies. There was no difference in the number of days that anorexia was exhibited by ponies in the three treatment groups.

**Larval recoveries**

Irradiated recipients had a 100% reduction of migrating larvae compared with nonvaccinates (Table 3.2). Larval numbers in nonvaccinates ranged from 3 to 28 per pony.

**Eosinophil counts**

Differentials performed on peripheral blood smears from ponies on D-4, D0, D4, D9, and D14 following challenge demonstrated that ponies vaccinated with radiation attenuated larvae developed an eosinophilia by day 4 following challenge (Figure 3.1). In contrast, eosinophil counts from nonvaccinated ponies failed to show significant elevations within the two weeks following challenge.
Table 3.1. Summary of the clinical signs exhibited by vaccinated, nonvaccinated, and control ponies in response to challenge with 1000 *S. vulgaris* L₃.

<table>
<thead>
<tr>
<th>Group</th>
<th>Anorexia</th>
<th>Pyrexia</th>
<th>Depression</th>
<th>Colic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinates</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nonvaccinates</td>
<td>9</td>
<td>5*</td>
<td>3*</td>
<td>3*</td>
</tr>
<tr>
<td>Controls</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Data represent the mean number of days that ponies exhibited the respective clinical sign. Pyrexia was considered any rectal temperatures exceeding 39°C. (*) indicates values are significantly higher than vaccinates and controls.

Table 3.2. Larval recoveries from vaccinated and nonvaccinated ponies following challenge with 1000 *S. vulgaris* L₃.

<table>
<thead>
<tr>
<th>Immunization Group</th>
<th>Average Larval Recovery</th>
<th>Percent Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinates</td>
<td>0</td>
<td>100 %</td>
</tr>
<tr>
<td>Nonvaccinates</td>
<td>16</td>
<td>-</td>
</tr>
</tbody>
</table>

Larvae were recovered from scrapings of the lumen of the CMA and its branches and counted using a dissecting microscope. Larval numbers in nonvaccinates ranged from 3 to 28 per pony.
Figure 3.1. Eosinophil response to *S. vulgaris* challenge in vaccinate, nonvaccinate and control ponies. Mean eosinophil counts for each group were calculated by multiplying the WBC count/µl blood, as determined by automated counting, by the percentage of eosinophils determined by manual counting of 100 cells viewed under oil immersion.
ELISA

Levels of IgM, IgA, IgGa, IgGb, and IgG(T) specific for SAWA were determined using sera collected from vaccinate and nonvaccinate ponies on D-4 and D14 (Figure 3.2). When compared to prechallenge levels from nonvaccinates, levels of SAWA-specific IgGa, IgGb, and IgG(T) were significantly greater in the sera of vaccinates. Following challenge IgG(T) levels of vaccinates remained significantly greater than those of nonvaccinates but did not change significantly from prechallenge levels. Challenge with *S. vulgaris* L₃ resulted in statistically significant increases in IgA in both vaccinates and nonvaccinates with nonvaccinate IgA levels significantly exceeding those of vaccinates following challenge. Nonvaccinates also exhibited a statistically significant increase in IgGa in response to challenge. There were no differences in IgM production within or among the treatment groups prior to or following challenge.

Lymphoproliferation

The proliferative response of PBMC and CLNC from vaccinate, nonvaccinate and control ponies to SAWA was determined using cells collected on D-4, D4, D9, and D14 (Figure 3.3). Vaccinates and nonvaccinates demonstrated a significant elevation in the proliferative response of both PBMC and CLNC to SAWA on D14. Though differences in the lymphoproliferative responses of vaccinates and nonvaccinates were not statistically significant, the proliferative responses of PBMC and CLNC from vaccinates to SAWA exceeded those of nonvaccinates prior to challenge. Proliferation to SAWA in the CLNC of vaccinates continued to exceed that demonstrated by nonvaccinates by as much as five fold on day 4 following challenge.
Figure 3.2. Antibody isotype response of vaccinated and nonvaccinated ponies to *S. vulgaris* soluble adult worm antigen 4 days prior to and 14 days following challenge. Serum samples were analyzed by ELISA in quadruplicate.
Figure 3.3. Lymphoproliferative response of pony PBMC and CLNC to *S. vulgaris* antigen. PBMC (top panel) and CLNC (bottom panel), collected 4 days prior to challenge and on days 4, 9, and 14 following challenge, were incubated for 5 days with 4 μg/ml soluble adult worm antigen. Proliferation was measured by thymidine incorporation. (■ vaccinates, △ nonvaccinates, □ controls)
Cytokine quantification

Differences in the levels of IL-2, IL-4, IL-5, IL-10 and IFN-γ on D-4, D4, D9, and D14 post challenge were compared within each treatment group (Figure 3.4 and 3.5). PBMC from vaccinates demonstrated a significant decrease in IL-10 at D4 and D9 while IL-2, IL-4, IL-5, and IL-10 increased significantly at D14. However, IL-2 and IL-10 did not exceed prechallenge levels (ie. fold increase < 1). PBMC from nonvaccinates demonstrated significant decreases in IFN-γ and IL-10 at D9 followed by a significant rise in IL-10 on D14. Interferon-γ production decreased significantly in the CLNC of both vaccinates and nonvaccinates on D9 followed by a significant increase in both groups on D14. On D9, nonvaccinates also experienced a significant increase in IL-4 and a decrease in IL-10 in their CLNC. Significant increases in IL-4, IL-5, and IL-10 were also observed in nonvaccinates on D14.

Comparisons of cytokine production among the three treatment groups on each day (Figure 3.6) indicated that IL-4 production in vaccinates was significantly greater than in nonvaccinates and controls on D4 in CLNC and on D9 in PBMC.

Comparisons of daily cytokine production in PBMC to that of CLNC for each treatment group indicated that on D9 IL-4 production in CLNC of nonvaccinates significantly exceeded that in PBMC.

Discussion

Differences in postchallenge clinical signs and larval recoveries demonstrated that ponies immunized orally with irradiated L₃ developed a protective response to challenge when compared to nonimmunized ponies. In previous studies, necropsies performed six weeks following challenge with S. vulgaris L₃ demonstrated that ponies
Figure 3.4. Cytokine production in PBMC from vaccinated, nonvaccinated, and control ponies after oral *S. vulgaris* L3 challenge. Cytokine copy numbers were determined using a quantitative RT-PCR assay and were normalized for β-actin content of each cDNA. Data is represented as the fold increase in geometric mean over prechallenge values on the indicated days following oral challenge with 1000 *S. vulgaris* L3. (■ ■ § Day 4, □ □ □ Day 9, □ □ □ Day 14). (*) represents a significant decrease, and (•) a significant increase in cytokine production in relationship to the other days examined.
Figure 3.5. Cytokine production in colonic lymph node derived lymphocytes (CLNC) from vaccinated, nonvaccinated, and control ponies after oral *S. vulgaris* L3 challenge. Cytokine copy numbers were determined using a quantitative RT-PCR assay and were normalized for β-actin content of each cDNA. Data is represented as the fold increase in geometric mean over prechallenge values on the indicated days following oral challenge with 1000 *S. vulgaris* L3 (■ Day 4, □ Day 9, ▲ Day 14). (*) represents a significant decrease, and (▲) a significant increase in cytokine production in relationship to the other days examined.
Figure. 3.6. IL-4 and IL-5 production in vaccinated and nonvaccinated ponies after oral *S. vulgaris* L3 challenge. Copy numbers for each cytokine from PBMC (top panel) and CLNC (bottom panel) as determined using a quantitative RT-PCR assay were normalized for β-actin content of each sample and are represented as the geometric mean of values for ponies in each treatment group on the indicated days following oral challenge with 1000 virulent *S. vulgaris* L3. *Represent significant differences among treatment groups (vaccinates, nonvaccinates, controls).
vaccinated with irradiation-attenuated $L_3$ did not develop lesions of verminous arteritis but instead had significant periportal fibrosis, suggesting that the protective response is generated within the intestinal submucosa and that larval antigens are cleared via the portal system prior to $L_4$ migration into the intestinal vasculature (Monahan et al., 1994). Blocking parasite entrance to the intestinal arterioles in vaccinates should both limit peripheral exposure to parasite antigens and prevent arterial lesions resulting in the significant reductions in fever, depression and abdominal pain we observed in vaccinates relative to nonvaccinates. Massive killing of larvae within the wall of the intestine in vaccinates should, however, be expected to trigger the cascade of inflammatory mediators responsible for pyrexia including tumor necrosis factor-$\alpha$, IL-1, and IL-6. It has been postulated that clearance of killed larvae and inflammatory mediators by the portal system, which is by virtue of its network of vessels of expanding diameter, mechanically less susceptible to procoagulant effects and thrombus trapping than intestinal vasculature, could also contribute to the decrease in both pyrexia and abdominal pain observed in vaccinates as compared to nonvaccinates (Monahan et al., 1994).

Following challenge with $S. vulgaris$, vaccinates developed an anamnestic eosinophilia which began to rise on D4 and appeared to be peaking at D9. The kinetics of this response preceded those previously reported (Monahan et al., 1994) and may be the result of a larval challenge which exceeded previously published doses, or perhaps improved viability of either the vaccinal or challenge larvae leading to more robust stimulation of the eosinophilic response mechanisms. IL-5 is required for the eosinophilic response to helminth parasites (Coffman et al., 1989; Sher et al.).
Levels of IL-5 in both the PBMC and CLNC of vaccinates exceed those of nonvaccinates by at least four fold prior to challenge and on D4 and D9 following challenge. Though not statistically significant, these findings appear to be physiologically significant with greater IL-5 production prechallenge and on D4 post challenge correlating to the more rapid increase in eosinophil numbers observed in vaccinates relative to nonvaccinates.

Though the magnitude of the IL-5 response in PBMC from nonvaccinates on D14 actually exceeded that of vaccinates, this change was not statistically significant. However, analysis of the fold increase in IL-5 produced by PBMC of nonvaccinates over prechallenge levels indicated that the IL-5 response on D14 was statistically significant. Significant increases in IL-5 production were also observed in the CLNC of nonvaccinates on D14. In previous experiments, eosinophil responses in nonvaccinates lagged behind that observed in vaccinates by approximately one week (Monahan et al., 1994). Accordingly, the slight increase in eosinophil counts observed in nonvaccinates on D14 which correspond temporally with significantly elevated IL-5 levels may signal the beginning of the eosinophilic response in this group.

Though cytokine regulation of murine IgG isotypes is well characterized, allowing one to correlate antigen-driven isotype production to cytokine profiles consistent with either Type I or Type II responses, both cytokine regulation of equine immunoglobulin production and the relationship between the equine and murine IgG isotypes have not been characterized. IgE production, a hallmark of helminth
infection, was not quantified because equine-specific reagents have not been made commonly available.

Prior studies have demonstrated that following immunization with radiation-attenuated *S. vulgaris* L₃, vaccinates develop a small increase in total immunoglobulin directed against soluble adult *S. vulgaris* antigen and that following challenge with *S. vulgaris* L₃, this SAWA-specific antibody production displays an anamnestic response, converging with that of nonvaccinates by 3 weeks post challenge (Monahan *et al.* 1994). We examined the differences in immunoglobulin isotypes specific for SAWA in vaccinates and nonvaccinates in response to challenge with *S. vulgaris*. Our data indicate that the previously described increase in SAWA-specific immunoglobulin titers seen in response to vaccination are at least in part due to production of isotypes IgGa, IgGb, and IgG(T) in response to vaccination. Following challenge, SAWA-specific IgG(T) remained significantly greater in vaccinates compared to nonvaccinates but was not significantly different from prechallenge levels. Increases in IgG subclasses in response to *S. vulgaris* infection have been previously demonstrated with IgG(T) showing the greatest increase (Patton *et al.*, 1978). However, to our knowledge, this is the first data to demonstrate the antigen-specificity of this elevation in IgG subclasses. Challenge resulted in significant increases in SAWA-specific IgA production in both vaccinates and nonvaccinates. SAWA-specific IgA titers observed in nonvaccinates following challenge were significantly greater than those of vaccinates indicating that vaccination was associated with a blunting of the antigen-specific IgA response to adult parasite antigen. SAWA-specific IgGa also increased significantly in the sera of nonvaccinates but not
vaccinates following challenge. Though no changes in SAWA-specific IgM were noted, only day D14 serum samples were assayed and given the acute nature of antigen-specific IgM responses, it is likely that earlier samples might have detected a difference in SAWA-specific IgM. Serum from D4 and D9 is available and characterizing SAWA-specific isotype responses on these days is a focus of ongoing study.

Stimulation of PBMC from ponies infected with *S. vulgaris* has produced a specific proliferative response to SAWA that is absent in uninfected ponies (Dennis *et al.*, 1992). However, attempts to demonstrate differences in the proliferative response to adult worm antigens between resistant and non-resistant ponies before and after challenge infection were not successful (Klei, 1992). Though not statistically significant, in this experiment the prechallenge proliferative responses of PBMC and CLNC from vaccinates exceeded those of nonvaccinates suggesting that some component of the larval vaccine was able to prime these individuals for a secondary response to adult antigen. Following challenge, both vaccinates and nonvaccinates respond similarly demonstrating statistically significant increases in the proliferative responses of both PBMC and CLNC that were not significantly different between vaccinates and nonvaccinates.

Statistical analyses of cytokine data was complicated by the wide range of responses seen in this outbred population as well as the limited number of controls. Significant increases in IL-4 production were noted in the PBMC of vaccinates on D14. Unlike IL-5 which tended to increase (though not significantly) in the PBMC of each nonvaccinate pony, IL-4 showed minimal response in the PBMC of
nonvaccinates over the course of the experiment and in fact the small response on D14 reflected that of a single individual with a disproportionately high IL-4 response. In contrast to the IL-4 response of PBMC from nonvaccinates, IL-4 production was significantly elevated on D9 and D14 in nonvaccine CLNC. Vaccinates also demonstrated a daily increase in CLNC IL-4 levels throughout the duration of the experiment but these changes were not statistically significant.

IL-4 was unique among the cytokines in that it's production by vaccinates on D4 in CLNC and D9 in PBMC significantly exceeded that of nonvaccinates and controls. This was not the case for IL-2, IL-5, IL-10 and IFN-γ production. Though not statistically significant, prechallenge IL-4 levels in the CLNC of vaccinates tended to exceed those of nonvaccinates. These differences between vaccinates and nonvaccinates, namely greater IL-4 production in prechallenge CLNC and significantly greater IL-4 production occurring first at the local site of parasite invasion followed by an increase in peripheral IL-4 production by PBMC constitute an anamnestic IL-4 response presumably associated with priming during immunization of vaccinates that is of course absent in nonvaccinates. Nonvaccinates, however, did demonstrate a significant increase in IL-4 production which lagged behind the response of vaccinates temporally, being first detected in CLNC on D9. Though the role of IL-4 in IgE production by memory B-cells is unclear (Le Gros et al., 1996; Levy et al., 1997), IL-4 is required for the generation and maintenance of secondary antigen-specific IgE responses in vivo (Finkelman et al., 1990). Therefore, anamnestic IL-4 production might be associated with an antigen-specific anamnestic IgE response in vaccinates following challenge. Elimination of the IgE response with
anti-IL-4 has been shown to reverse protection in both *Heligmosomoides polygyrus* immune mice (Urban, J. J. *et al.*, 1991b) and primary *Trichuris muris* infections (Else *et al.*, 1994). A synthesis of these findings leads to the hypothesis that anamnestic IL-4 production, possibly leading to an anamnestic antigen-specific IgE response may be one component of the immune response that differentiates *S. vulgaris* immune from susceptible ponies.

Though further characterization of the differences in IL-4 production in CLNC and PBMC associated with vaccination and challenge are warranted, it is clear that both vaccinates and nonvaccinates respond to challenge with *S. vulgaris* by producing IL-4, and that at least in immune animals, this production of IL-4 is not restricted to the CLNC. Though further studies are warranted to confirm that IL-4 production varies in its magnitude and site of initial production in *S. vulgaris* immune ponies, significant increases in IL-4 production in vaccinates relative to nonvaccinates at the CLNC on D4 and PBMC on D9 suggest that the occurrence of a local immune response characterized by the production of the Type II cytokine IL-4 which spreads to a peripheral immune response in which IL-4 also plays a role may differentiate susceptible and immune ponies. This difference in peripheral IL-4 production was unique to the vaccinates and could reflect a peripheral recognition event present in vaccinates but absent in nonvaccinates that contributes to the protective immune response.

We observed statistically significant decreases in IFN-γ on D9 in the CLNC and PBMC of nonvaccinates as well as in the CLNC of vaccinates. Depression of IFN-γ production was also observed in the PBMC of vaccinates though this was not
statistically significant. Nonetheless, unlike the other cytokines assayed, IFN-γ production was consistently lower on D-4, D4, and D9 in the CLNC of vaccinates and nonvaccinates as compared to controls. In light of the association between decreased IFN-γ production and the increased TH2 response, these findings are certainly noteworthy.

In the TH1/TH2 paradigm inhibition of IFN-γ production is often attributed to IL-10. Though IL-10 was originally described as a product of mouse TH2 clones, it is now clear that IL-10 is also secreted by TH1 cells (especially in humans) and activated macrophages (Sornasse et al., 1996; Martinez et al., 1997). IL-10 is a potent inhibitor of IFN-γ production (Fiorentino et al., 1989; Urban, J. J. et al., 1991b). However, in our experiments a temporal relationship between increased IL-10 levels and decreased IFN-γ levels was not clear. All groups exhibited a decrease in IL-10 production following challenge which was significant on D4 and D9 in the PBMC of vaccinates and D9 in the PBMC and CLN of nonvaccinates. Significant increases in IL-10 which were noted on D14 in the PBMC of vaccinates and the PBMC and CLNC of nonvaccinates were relative to the depression on D9 and were not significantly different from prechallenge levels. It is important to remember that while IFN-γ is capable of directly inhibiting the TH2 response, IL-10 inhibition of IFN-γ production is indirect, acting on antigen presenting cells to reduce their ability to stimulate cytokine secretion by TH1 cells. Other cytokines including IL-3 and IL-4 (Liew et al., 1989), IL-11 (Leng and Elias, 1997), IL-13 (Doherty et al., 1993), and TGF-β (Hausmann et al., 1994) have been shown to interfere with macrophage activation. Statistically significant elevations in IL-4 production were observed in the
CLNC of vaccinates on D4 which could account temporally for the significant
decrease in IFN-γ production seen on D9 in these individuals.

IFN-γ and IL-2 tended to increase by D14 in PBMC and CLN of both
vaccinates and nonvaccinates. These increases were significant for IL-2 in the PBMC
of vaccinates, and IFN-γ in the CLNC of vaccinates and nonvaccinates and may
reflect a Type I response associated with an inflammatory response to dead parasites
from the challenge dose.

It appears from this data that the immune response of both naive susceptible
and vaccinated immune ponies to *S. vulgaris* may involve the generation of a classical
Type II response rather than being dramatically polarized as seen in Type I and Type
II responses that define susceptibility and resistance, respectively, to murine helminth
parasites. Both *S. vulgaris* immune and susceptible ponies showed similar
lymphoproliferative and antibody responses to adult worm antigen following
challenge and both groups exhibited characteristics of a Type II response producing
IL-4 and IL-5 in response to challenge. However, the timing of the immune response
to challenge infections of *S. vulgaris* in vaccinates appears to precede that of
nonvaccinates. Natural exposure to *S. vulgaris* L₃ is known to induce resistance to
further infection (Ogborune and Duncan, 1985) and our data support the notion that
oral vaccination with radiation-attenuated larvae may simply induce this resistance
quicker than natural exposure, thereby protecting animals from pathology. Prior to
challenge vaccinates exhibited greater antigen-specific proliferative responses and
produced more IL-4 and IL-5 in CLNC than did nonvaccinates, though these
differences were not statistically significant. Following challenge IL-4 production by
nonvaccinates lagged behind vaccinates. Vaccinates produced significantly more IL-4 in response to challenge than nonvaccinates first in CLNC at D4 and then in PBMC at D9 while IL-4 production in the CLNC of nonvaccinates increased significantly by D9. Thus the ability to generate an anamnestic IL-4 response to *S. vulgaris* either at the site of parasite invasion and/or perhaps in peripheral blood appears to be one component of the protective immune response generated by vaccination with radiation-attenuated *S. vulgaris* L₃.
SUMMARY

The purpose of this dissertation was to create reagents for the study of equine Type I and Type II responses, to develop an assay for the quantification of equine cytokine mRNA, and to apply this assay in order to examine the cytokine responses of control ponies and ponies vaccinated with radiation-attenuated Strongylus vulgaris larvae during the generation of their immune response to challenge with S. vulgaris L₃.

In order to develop reagents for the study of cytokine responses of the horse, equine IL-6, IL-10 and β-actin were cloned from mitogen stimulated equine PBMC using reverse transcription followed by polymerase chain reaction amplification (RT-PCR) with primers based on consensus sequences of these cytokines in other species.

Equine IL-6 cDNA comprises 745 bases including 118 bases in the 3' untranslated region. The cDNA shares 82% nucleotide identity with human IL-6. The open reading frame for equine IL-6 encodes 208 amino acids including a 28 amino acid signal peptide. The mature protein would consist of 180 amino acids and have a predicted relative mass of 20,471, without post-translational modification. The predicted amino acid sequence of equine IL-6 shares 78% similarity with human IL-6 and includes the residues which have been shown by other investigators to be critical for biological activity of the protein (Brakenhoff et al., 1989, 1990; Kruttgen et al., 1990; Fontaine et al., 1993; van Dam et al., 1993; Ehlers et al., 1994, 1995, 1996; de Hon et al., 1995; King et al., 1996). The GenBank accession number for equine IL-6 is U64794.

Equine IL-10 cDNA comprises 739 bases including 202 bases in the 3' untranslated region. The cDNA shares 89% nucleotide identity with human IL-10. The
open reading frame for equine IL-10 encodes 178 amino acids including an 18 amino
acid signal peptide. The mature protein would consist of 160 amino acids and have a
predicted relative mass of 18,615 without post-translational modification. The predicted
amino acid sequence of equine IL-10 shares 91% similarity with human IL-10 and
includes the residues which are postulated to be significant in receptor binding based on
the X-ray crystallographic structure of human recombinant IL-10 (Walter et al., 1995).
Equine IL-10 shares 85% nucleotide identity with an IL-10-like sequence in the genome
of Equine Herpes Virus-2. The GenBank accession number for equine IL-10 is U38200.

The cloned equine β-actin comprises a single open reading frame of 1128 bases.
The cDNA shares 94% nucleotide identity and 100% amino acid identity with human β-
actin. The mature protein has a predicted relative mass of 18,615 without post-
translational modification. The GenBank accession number for equine β-actin is
AF035774.

A RT-PCR based assay was utilized to quantify cytokine message in cDNA
samples by comparison to a standard curve. To generate the standard curve, known
concentrations of plasmid encoding the cDNA of interest were amplified in conjunction
with the unknown cDNA samples in triplicate PCR reactions. PCR product was detected
using a luminescence based assay in which sequence-specific probes were hybridized to
the PCR products in solution and detected using a Perkin-Elmer QPCR System 5000.
The message content of the unknown samples was determined by interpolation against
the standard curve using a two parameter exponential rise to maximum analysis of the
standard curve data. β-actin was used as a normalization factor by altering the cycling
parameters to maintain its amplification within the exponential amplification phase of the
reaction. The assay resolves 2 fold changes in cytokine message, has a linear dynamic range on the order of 3 logs, and is precise with errors most commonly in the range of 10% for replicate amplifications from the same sample. The error between replicate samples whose RNA was derived from separate extractions of the same cell population following β-actin normalization was 13-27%. This assay was utilized to determine the effect of oral vaccination with radiation-attenuated *S. vulgaris* larvae on the cytokine response to oral challenge with *S. vulgaris*.

10 ponies were reared in a parasite free environment and divided into three groups. Four ponies were orally vaccinated with 500 *S. vulgaris* third-stage infective larvae (vaccinates) which had been radiation-attenuated with 90 krads from a $^{60}$Co source. Vaccinations were performed 9 and 6 weeks prior to oral challenge with 1000 non-attenuated $L_3$ on day 0. Four other ponies were not vaccinated (nonvaccinates) and were also challenged with 1000 $L_3$ on day 0. Control ponies were neither vaccinated nor challenged. Four days prior to challenge, as well as on days 4 (D4) and 9 (D9) post-challenge, peripheral blood as well as lymph nodes from the region of the cecum (CLN) were collected. Peripheral blood and CLN were also collected at necropsy on day 14 (D14) post-challenge.

Oral immunization with radiation-attenuated *S. vulgaris* $L_3$ provided protection from challenge. Ponies immunized with irradiated $L_3$ developed anamnestic eosinophilias, had fewer episodes of pyrexia, depression, and abdominal pain, and were 100% protected from larval migration when compared to nonvaccinated ponies.

Soluble adult *S. vulgaris* antigen-specific antibody isotypes from vaccinates and nonvaccinates were compared on D-4 and D14. Vaccination caused significant increases
in SAWA-specific IgGa, IgGb, and IgG(T). IgG(T) remained significantly greater in vaccinates than nonvaccinates on D14 but was not significantly different from D-4 values. Challenge significantly increased IgA in vaccinates and nonvaccinates. Vaccination blunted the SAWA-specific IgA response. IgGa also increased significantly in the sera of nonvaccinates but not vaccinates by D14.

The proliferative response of PBMC and CLNC from vaccinate, nonvaccinate, and control ponies to SAWA was determined on D-4, D4, D9, and D14. Prior to challenge, the proliferative responses of PBMC and CLNC from vaccinates exceeded those of nonvaccinates but these differences were not significant. By D14 vaccinates and nonvaccinates demonstrated a significant elevation in the proliferative response of both PBMC and CLNC to SAWA.

Total RNA from PBMC and CLN derived cells (CLNC) was assayed for IL-2, IL-4, IL-5, IL-10, and IFN-γ message in the aforementioned RT-PCR assay. Prior to challenge, vaccinates produced more IL-4 and IL-5 than nonvaccinates, though these differences were not statistically significant. Following challenge, both vaccinates and nonvaccinates demonstrated a Type II cytokine pattern with significant elevations in IL-4 and IL-5 production. Vaccinates demonstrated significant increases in IL-2, IL-4, IL-5, and IL-10 production in PBMC on D14 post-challenge though IL-10 production initially decreased significantly on D4 and D9. Nonvaccinates demonstrated significant decreases in IL-10 and IFN-γ production on D9 and a significant increase in IL-10 production on D14 in their PBMC. In CLNC, only nonvaccinates demonstrated significant increases in IL-4 and IL-5 production in response to challenge. Vaccinates also demonstrated increases in IL-4 and IL-5 production in CLNC in response to challenge but these
increases were not significant because of the higher pre-challenge levels of IL-4 and IL-5 in their CLNC. Vaccinates did exhibit a significant decrease in IFN-γ production on D9 followed by a significant increase on D14. Nonvaccinate CLNC exhibited significant decreases in IFN-γ and IL-10 message on D9 followed by significant increases in IL-4, IL-5, IL-10 and IFN-γ by D14. When groups were compared, vaccinates produced significantly more IL-4 in response to challenge then nonvaccinates or controls, first in CLNC at D4 and then in PBMC on D9. There was not a significant difference in the production of IL-2, IL-5, IL-10, or IFN-γ between groups.
CONCLUSIONS

Equine IL-6 and IL-10 exhibit significant nucleic acid homology (>80%) and amino acid similarity (>70%) with the respective sequences of other domesticated mammals. The sequences include putative receptor binding residues and are therefore likely to be biologically active. Expression and determination of the biological activity of the recombinant proteins is a focus of continuing research.

Cytokine messenger RNA can be quantified using RT-PCR in the exponential amplification phase of the reaction by interpolation against a plasmid standard curve derived from known dilutions of plasmids encoding the cytokines of interest. Analysis of the standard curve using a two parameter exponential rise to maximum analysis of the data graphed on linear scales, given by the equation \( y = a(1 - e^{bx}) \), provides superior prediction of two fold dilutions in RNA content of cDNA reactions when compared to other published analyses of the standard curve. This also indicates that amplification from double stranded plasmid can accurately model the amplification efficiency of cDNA. Using triplicate amplifications from the same cDNA, the assay resolves 2 fold changes in cytokine message and is precise with errors most commonly in the range of 10% for replicate amplifications from the same sample which is in line with the results achieved with quantitative competitive RT-PCR methods. The dynamic range of this assay is on the order of 3 logs, exceeding the dynamic range achieved by competitive methods.

Despite its 100-1000 fold greater expression, equine \( \beta \)-actin, whose amino acid sequence is identical to that of human \( \beta \)-actin, is useful in normalizing inter-sample
variability in the RNA extraction and reverse-transcription steps of the RT-PCR assay provided its values fall within the exponential amplification portion of the standard curve. Under these conditions the error between replicate samples whose RNA was derived from separate extractions of the same cell population following normalization was 13-27% which is in line with the errors using quantitative competitive methods.

As previously demonstrated, oral immunization with radiation-attenuated *S. vulgaris* L₃ protects ponies from challenge infections, preventing larval migration and significantly decreasing clinical signs associated with challenge including pyrexia, depression, and abdominal pain. Vaccination is associated with the development of an anamnestic eosinophilia which is known to be a Type II dependent response though a temporal relationship between elevated IL-5 production and eosinophilia was not established.

Vaccination induced significant increases in SAWA-specific IgGa, IgGb, and IgG(T). Challenge induced significant increases in IgA in vaccinates and nonvaccinates and significant increases in IgGa in nonvaccinates. Though cytokine regulation of murine IgG isotypes is well characterized, allowing the correlation of antigen-driven isotype production to cytokine profiles consistent with either Type I or Type II responses, both cytokine regulation of equine immunoglobulin production and the relationship between equine and murine IgG isotypes have not been characterized so that the relevance of Type I and Type II cytokine responses to equine isotype production cannot be established.

Though not statistically significant, the prechallenge proliferative responses of PBMC and CLNC from vaccinates exceeded those of nonvaccinates suggesting that
some component of the larval vaccine was able to prime these individuals for a secondary response to adult antigen. Challenge sensitized both vaccinates and nonvaccinates to adult antigen.

Both vaccinates and nonvaccinates exhibited characteristics of a Type II cytokine response producing significant IL-4 and IL-5 in response to challenge and indicating that equine species generate Type II responses to gastrointestinal nematodes. However, the timing of the immune response to challenge infections in vaccinates appears to precede that of nonvaccinates. Thus, the ability to generate an anamnestic IL-4 response either at the site of parasite invasion and/or perhaps in peripheral blood appears to be one component of the protective immune response generated by vaccination with radiation-attenuated \textit{S. vulgaris} L, indicating that the protective immune response to \textit{S. vulgaris} probably involves the induction of a Type II cytokine response.
REFERENCES


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Bosco M, Giovarelli M, Rorni M, Modesti A, Scarpa S, Masueli L, et al. Low doses of IL-4 injected periolympathically in tumor-bearing mice inhibit the growth of poorly and


Druet P, Pelletier L. Th2 and Th1 autoreactive anti-class II cell lines in the rat suppress or induce autoimmunity. J Autoimmunity 1996;9:221-6.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Hahn HJ, Lucke S, Kloting I, Volk HD, Bachr RV, Diamantstein T. Curing BB rats of freshly manifested diabetes by short term treatment with a combination of a monoclonal


Imperiale MJ, Faherty DA, Sproviero JF, Zauderer M. Functionally distinct helper T cells enriched under different culture conditions cooperate with different B cells. J Immunol 1982;129:1843-.


Kawanishi H, Joseph K. IL4, IL5 and IL6-mediated regulation of immunoglobulin (Ig) heavy chain class switching and Ig production by gut-associated lymphoid tissue (GALT) B cells from athymic nude (nu/nu) mice. Immunol Invest 1991;20:605-21.


King DP, Schrenzel MD, McKnight ML, Reidarson TH, Hanni KD, Stott JL, et al. Molecular cloning and sequencing of interleukin 6 cDNA fragments from the harbor seal (Phoca vitulina), killer whale (Orcinus orca), and Southern sea otter (Enhydra lutris nereis). Immunogenetics 1996;43:190-5.


Kotowicz K, Callard RE, Friedrich K, Matthews DJ, Klein N. Biological activity of IL-4 and IL-13 on human endothelial cells: functional evidence that both cytokines act through the same receptor. Int Immunol 1996;8:1915-25.


Lanier LL, O'Fallon S, Somoza C, Phillips JH, Linsley PS, Okumura K, et al. CD80 (B7) and CD86 (B70) provide similar costimulatory signals for T cell proliferation, cytokine production, and generation of CTL. J Immunol 1995;154:97-105.


Levine BL, Ueda Y, Craighead N, Huang ML, June CH. CD28 ligands CD80 (B7-1) and CD86 (B7-2) induce long-term autocrine growth of CD4+ T cells and induce similar patterns of cytokine secretion in vitro. Int Immunol 1995;7:891-904.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


McCairns E, Fahey D, Muscat GE, Murray m, Rowe PB. Changes in levels of actin and tubulin mRNAs upon the lectin activation of lymphocytes. Mol Cell Biol 1984;4:1754-60.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Minty A, Ferrara P, Caput D. Interleukin-13 effects on activated monocytes lead to novel cytokine secretion profiles intermediate between those induced by interleukin-10 and by interferon-gamma. Eur Cytokine Netw 1997;8:189-201.
Monahan CM, Chapman MR, Taylor HW, French DD, Klei TR. Foals raised on pasture with or without early pyrantel tartrate feed additive: comparison of parasite burdens and host responses following experimental challenge with large and small strongyle larvae. Vet Parasitol 1998;in press.


Oswald IP, Wynn TA, Sher A, James SL. Interleukin 10 inhibits macrophage microbicidal activity by blocking the endogenous production of tumor necrosis factor alpha required as a costimulatory factor for interferon gamma-induced activation. Proc Natl Acad Sci USA 1992;89:8676-80.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Rincon M, Flavell RA. Transcription mediated by NFAT is highly inducible in effector CD4+ T helper 2 (Th2) cells but not in Th1 cells. Mol Cell Biol 1997;17:1522-34.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Sher A, Coffman RL, Hieny S, Scott P, Cheever AW. Interleukin-5 is required for the blood and tissue eosinophilia but not granuloma formation induced by infection with Schistosoma mansoni. Proc Natl Acad Sci USA 1990b;87:61-5.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Stein PH, Singer A. Similar co-stimulation requirements of CD4+ and CD8+ primary T helper cells: role of IL1 and IL6 in inducing IL2 secretion and subsequent proliferation. Int Immunol 1992;4:327-35.


Urban JFJ, Katona IM, Paul WE, Finkelman FD. Interleukin 4 is important in protective immunity to a gastrointestinal nematode infection in mice. Proc Natl Acad Sci USA 1991b;88:5513-7.


Wheelock EF. Interferon-like virus-inhibitors induced in human leukocytes by phytohemagglutinin. Science 1965;149:310-.


Wynn TA, Eltoum I, Oswald IP, Cheever AW, Sher A. Endogenous interleukin 12 (IL-12) regulates granuloma formation induced by eggs of Schistosoma mansoni and exogenous IL-12 both inhibit and prophylactically immunized against egg pathology. J Exp Med 1994;179:1551-61.


Cyprianna Ellen Swiderski, the daughter of Matthew and Ellen Hormanski, was born in Baltimore, Maryland, on July 14, 1963. She attended Trinity Preparatory School until 1978 and graduated from Catonsville Senior High School in 1981. While attending the University of Maryland at College Park she pursued a degree in Microbiology and was accepted to the Virginia-Maryland Regional College of Veterinary Medicine in Blacksburg, Virginia, in 1983. During her summers she was involved in research on Potomac Horse Fever in the laboratory of Suskanta Dutta at the University of Maryland. After completing her Doctor of Veterinary Medicine in 1987, she practiced in a 100% equine practice in Virginia Beach, Virginia. However, she found the fact that challenging cases had to be referred for proper management frustrating and applied to the School of Veterinary Medicine at North Carolina State University in Raleigh, North Carolina, for a rotating internship in large animal medicine and surgery. After completing this internship in 1989 she pursued a residency in equine medicine, which she completed in 1992, at the School of Veterinary Medicine, Louisiana State University in Baton Rouge, Louisiana. 1992 was a big year for “Chipper”. She married Dr. Terry Finis Swiderski and also elected to pursue graduate training in immunology. Her interest in immunology stemmed from a registered Arabian horse named “Peter Pickle” with selective IgM deficiency. Her graduate work at the L.S.U.-S.V.M. was guided by Dr. David W. Horohov, professor of immunology. She passed the certifying examination of the American College of Veterinary Internal Medicine in 1995. She was blessed with a beautiful daughter, Katelyn Maye, in 1996, which enriched her life beyond description.
Candidate: Cyprianna E. Swiderski

Major Field: Veterinary Medical Sciences

Title of Dissertation: Development of a Quantitative Polymerase Chain Reaction Procedure for Analysis of Cytokine Responses to *Strongylus vulgaris* in Ponies

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination: December 18, 1997