THE SUPPRESSIVE EFFECT OF PYOCYANIN ON THE HUMORAL IMMUNE RESPONSE OF MICE

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ABSTRACT

THE SUPPRESSIVE EFFECT OF PYOCYANIN ON THE HUMORAL IMMUNE RESPONSE OF MICE

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Controlling the development or progression of disease within a host involves the activity of both humoral and cell-mediated immunity. This includes the recruitment of cells involved in antigen processing and presentation as well as those which promote the secretion of cytokines and immunoglobulins. *Pseudomonas aeruginosa* is a gram negative, opportunistic bacteria implicated in the morbidity and mortality of immunocompromised individuals such as those with cystic fibrosis, cancer, and burns. This bacteria produces a variety of metabolites, some of which have been characterized as toxic in hosts. Phenazine pigments, which are secondary metabolites unique to *Pseudomonas*, have not been well characterized in terms of their toxicity and potential to contribute to virulence of infections. Pyocyanin, a low molecular weight phenazine pigment has previously been found to interfere with mitogen stimulated lymphocyte blastogenesis as well as inhibit the secretion of IL-2, a cytokine required for the proliferation of T-lymphocytes. However, the effects of pyocyanin on the humoral immune system or cytokines potentially involved in the induction of humoral immunity have not been well characterized.

Pyocyanin was isolated and purified from phenazine methosulfate (PMS). The purification and structural analysis was checked by proton NMR and spectrophotometric analysis. Initial studies on mice treated with 1.0 μg, 10.0 μg, and 100.0 μg pyocyanin revealed that pyocyanin at a 1.0 μg concentration injected intravenously into the tail veins

of mice resulted in significant suppression (74% reduction) of plaque forming units (PFUs) as measured by the hemolytic plaque assay. Trypan blue exclusion confirmed that the decrease in PFUs was not due to a decrease in cell viability, as the percent of viable cells remained above 87%. This indicated that the pigment, when administered at the same time as sheep red blood cells (SRBC), had a suppressive effect on the ability of the animal to mount an antibody response to SRBC. Pyocyanin (1.0 μg) was then injected prior to, at the same time as, and subsequent to SRBCs. The greatest suppression in PFUs occurred when pyocyanin was injected 12h prior and 12 h subsequent to injection with SRBC, with a percent reduction in PFUs of 83% and 79% respectively. These values were statistically different from the number of PFUs obtained when pyocyanin was injected 6 h prior or subsequent to SRBCs, or when the pigment was administered at the same time as the SRBCs.

The results generated from this study indicate that pyocyanin has a definite suppressive effect upon the formation of PFUs in response to a mitogenic stimulus. The decrease in PFUs was not found to be attributed to cell death from toxicity of the pigment, thus suggesting that pyocyanin interferes with the humoral immune response mechanisms of infected hosts. Preliminary data suggests that pyocyanin could also interfere with the activation of cells and/or subsequent release of cytokines from stimulated immune cells. However, before any final conclusions can be made, further experimentation needs to be conducted to specifically delineate the exact mechanism of pyocyanin's action on the immune system.

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LIST OF ABBREVIATIONS

APC	antigen presenting cell
DEPC	diethylpyrocarbonate
cDNA	copied DNA
CF	cystic fibrosis
Con A	concanavalin A
EDTA	ethylenediaminetetraacetic acid
FCS	fetal calf serum
HBSS	Hanks balanced salt solution
LPS	lipopolysaccharide
MEM	Minimal essential medium
MEN	MOPS, EDTA, Sodium acetate
NMR	nuclear magnetic resonance
PCR	polymerase chain reaction
PHA	phytohemagglutinin
PMS	phenazine methosulfate
RPMI	Roswell Park Memorial Institute
RT	reverse transcriptase
SRBC	sheep red blood cells
TBE	Tris, Borate, EDTA
h	hour
min	minute
ml	milliliter
μl	microliter

THE SUPPRESSIVE EFFECT OF PYOCYANIN ON THE HUMORAL IMMUNE RESPONSE OF MICE

CHAPTER I

INTRODUCTION

The Immune Response

Controlling the development or progression of disease within the body involves the activation of the immune system. This elaborate network consists of both primary and secondary defense mechanisms (55). Initial protection or innate immunity, is afforded by cough reflex, stomach acid, mucociliary clearance, and the epithelial barrier, while the second line of defense involves specific branches of the immune system called humoral and cellular immunity (55).

For survival, the host depends on an elaborate network of communication between cells of the immune system for protection from invasion by microorganisms. This internal network helps to enhance or suppress regulation of humoral and cellular immunity by interaction with cytokines, or protein mediators which are released from and communicate with cells of the immune system (47, 55). The humoral and cellular immune branches, which include T and B-lymphocytes, phagocytic cells, antibody, and complement, work in conjunction to destroy pathogens and keep the host free from infection (29, 83).

Phagocytic cells are important for defending the body against extracellular pathogens in both the first and second lines of defense (29, 51, 69, 83). Phagocytosis includes the ingestion of foreign particles bound by specific receptors and destroyed by oxidative and/or non-oxidative mechanisms (29, 69). There are several categories of phagocytic cells including mononuclear phagocytes and polymorphonuclear leukocytes (PMN's) (29, 51, 69).

Mononuclear phagocytes are a group of cells consisting of circulating monocytes and adherent macrophages (29, 69). These cells are involved in the first line of defense, but

they also play an important role in the induction of the cellular and humoral immune responses (51, 83). Neither of these phagocytic cells exhibit antigenic specificity but they have the ability to bind and digest foreign particles and antigen. For this reason they are called antigen presenting cells (APCs) and have the ability to bind to, process and present antigen resulting in the activation of B and T-lymphocytes (11, 19, 29, 83). T-lymphocytes possess specific receptors which bind to antigen, and in turn, secrete soluble proteins, or cytokines, which act on B-lymphocyte activation and the subsequent production of antibodies (83). It is through this cascade that the humoral immune system can respond to antigen.

Macrophages have two main functions: 1.) to phagocytose and break down materials for excretion and reutilization, and 2.) antigen processing and presentation (51). Macrophages possess the ability to phagocytose and destroy not only bacteria, but viruses, fungi and protozoa as well (51). The occurrence of non-specific phagocytosis by activated macrophages is variably determined by the presence of antibodies to the bacterium or invading pathogen (51). Ingestion of a microorganism is dependent on surface properties and the presence of specific serum ligands, or non-antibody proteins, which interact with receptors on the surface of the phagocytic cell, as well as the presence of antibodies and complement (29, 51). Ingestion of a particular microorganism is mainly a localized response, meaning that not all particles which are attached to the phagocytic cell surface will be ingested (29).

Antigen is processed through partial digestion by lysosomal enzymes within the macrophage, and presentation includes the displaying of antigen on the cell surface in the context of a product encoded by the major histocompatibility complex (MHC). This processed antigen is recognized by the T-cell receptor complex expressed on the surface of CD4+ and CD8+ T- lymphocytes. The T-lymphocyte receptor and antigen bind, thus forming a complex on the surface of the macrophage which then triggers the release of

cytokines by macrophages (83). As a result of cytokines being released, T-lymphocytes become activated and thus synthesize more mRNA specific for the expression of different cytokines which can selectively act upon the growth and differentiation of many immune cells. B-lymphocytes in close proximity to activated T-lymphocytes are acted upon by specific cytokines secreted by T-lymphocytes. The activation of B-lymphocytes leads to their differentiation into antibody-secreting plasma cells. Antibody production is dependent on this interaction between B and T lymphocytes (3).

The two main functions of B lymphocytes in the immune system are, 1) to serve as effector cells, thus integrating the humoral immune system, and 2) to process and present antigen (6). The ability of B lymphocytes to selectively capture antigen and then present it to T lymphocytes for further processing, is the basis of the first line of defense in the body, known as the humoral immune system (6, 8).

Cytokines include biologically potent, soluble protein molecules secreted by lymphocytes and monocytes following stimulation by antigens, lectins, and bacterial products (2, 12). Lectins are carbohydrate-binding proteins which induce certain cell types to proliferate upon stimulation. Substances which stimulate lymphocytes to proliferate are called mitogens, and lectins are a type of mitogen. Concanavalin A (Con A) and phytohemagglutinin (PHA) are also examples of mitogens which non-specifically stimulate mitogens. Cytokines in general share certain attributes including being antigen-nonspecific proteins which are synthesized very rapidly, in small quantities in response to a stimulus (2, 12, 55, 62, 70, 72). Other shared attributes of these protein molecules include having a low molecular weight; often being glycosylated; being very potent, acting at picomolar concentrations, in a short-range paracrine or autocrine function (rather than endocrine); and lastly, cytokines are also produced locally and are transient (2, 12, 62). In addition, cytokines are generally involved in immune regulation as well as the extent and duration of an inflammatory response (62). Cytokines have multiple functions which can overlap and

in some instances, may be contradictory, in addition, several different cytokines may act on the same cell to induce similar functions (2, 8, 62). In addition, some of the interactions on cellular functions may be additive, synergistic or even antagonistic (62).

There are six categories of cytokines including: interferons (IFN-α, β, γ), cytotoxic factors (TNF, LT), interleukins (IL-1 through IL-18), hematopoietic colony stimulating factors (GM-CSF), inflammatory, and transforming growth factors (TGF-β) (2). Cytokines have a variety of functions, some of which may be unrelated, including: inflammation, bone resorption, immunomodulation, hematopoiesis, wound healing, and the ability to induce cachexia and septic shock (2, 62).

Interleukins are a class of cytokines which act on or signal between leukocytes of the immune system. Interleukins are produced by T-cells, monocytes, and macrophages, and have such functions as promoting the secretion of antibodies by B cells, enhancing antigen presentation and phagocytosis by macrophages, as well as functioning as a growth factor and promoting proliferation and differentiation of both B and T lymphocytes (1, 2, 12, 62, 72). The interleukin nomenclature consists of the letters "IL" followed by a number, currently, the interleukins include IL-1 through IL-18.

Interferons are a class of cytokines which are capable of modifying or enhancing the immune response by making T-lymphocytes and macrophages more effective. More generally, interferons are classified together because of their antiviral and anti-proliferative properties (62). The interferons include interferon alpha (IFN-α), interferon beta (IFN-β), and interferon gamma (IFN-γ) (2, 62). IFN-α and IFN-β both induce resistance to viral infection (2, 62). In addition, they both exhibit antiproliferative effects on tumor cells and enhance antigen presentation (2, 62). Better characterized, IFN-γ, synthesized by T-helper lymphocytes, enhances the activities of killer T-lymphocytes, natural killer cells, and

macrophages (1, 2). IFN-γ's immunomodulative effects include increasing B-lymphocyte activation, proliferation, and antibody secretion (1, 2). IFN-γ induces bacterial killing through increasing the fusion of the phagosome to lysosome thus triggering an oxygen-independent killing mechanism as well as the generation of toxic oxygen radicals (17). IFN-γ is a widely studied cytokine for it's antiviral properties, phagocytic cell activation potential, and the affect on tumor necrosis factor production (2, 72).

Tumor necrosis factor alpha (TNF-α), also referred to as cachectin, is synthesized by monocytes, macrophages, and lymphocytes and acts on many cells of the immune system, specifically aiding in the regulation of macrophage activation (17, 72). TNF-α induces cachexia, which is the severe weight loss associated with malnutrition and cancer patients, by stimulating the production of the lipoprotein lipase and lipolysis in adipose cells (1, 2, 55, 72-73). TNF-α is most widely known for its ability to induce the lysis of certain tumor cells *in vitro* and necrosis of certain types of tumors *in vivo* (1, 2, 73).

Immunoglobulins, or antibodies are major glycoproteins of the humoral immune system (19, 33, 56). Immunoglobulins secreted by B-lymphocytes help rid the host of foreign invaders through the neutralization of toxins, opsonization, and activation of complement. Antibodies have two main functions which correspond with the two main regions of the molecule. The Fab region of the antibody, consisting of a variable and constant region each with a heavy and light chain held together by disulfide bonds (55). This region is also referred to as the antigen binding fragment, and appropriately it binds to a certain antigen that it recognizes distinctively (55). The Fc region, which is entirely composed of a constant region, does not have the ability to bind to antibody, but rather binds to complement and mediates immune reactions by binding to host cells including

neutrophils and macrophages (55).

There are five major classes of antibodies: IgG, which accounts for 80% of the total immunoglobulin concentration in the serum followed by, IgA (10-15%), IgM (10%), IgD (less than 1%), and IgE (0.01%) (13, 33, 56). IgG is one of the most biologically active antibody, able to neutralize bacterial toxins (56). PMN's and macrophages possess receptors for IgG which promotes phagocytosis of bacteria (25, 56). IgG is also the only immunoglobulin that can cross the placenta, providing antibody protection to the fetus (25, 56). IgM is the first immunoglobulin synthesized in response to antigen, it is also the largest. IgM can be found in serum in its pentameric form and it also exists as a hexamer. B-lymphocytes express IgM on their surface. IgM is one of the most important immunoglobulins in the immune system. IgA is present in the serum in small amounts, but is also a secretory immunoglobulin. Secretory IgA is the main immunoglobulin present in external body secretions including tears, saliva, urine, and colostrum; it possesses a secretory component which makes it relatively resistant to digestion by proteolytic enzymes which are present on mucosal surfaces (51). Although the anti-bacterial activity of IgA has not been well characterized, it is thought that IgA aids in the inhibition of attachment and subsequent growth of bacterial cells to mucosal surfaces (25, 29-30). Not much is known about the capacity of IgE in host defense, however it's role in type I hypersensitivity reactions (i.e., allergies) has been well characterized (11, 29). It is likely that the release of histamines and other vasoactive substances from mast cells and basophils in a hypersensitivity reaction may function in increasing the concentration of other immunoglobulins to the site of antigenic entry (29, 51).

Upon primary exposure to antigen, B-lymphocytes undergo clonal expansion and differentiate into antibody-secreting plasma cells specific for that antigen (51, 55). The type of immunoglobulin secreted by the B-lymphocyte is dependent on the stimulus received by the cell. Cytokines also have a role in antibody production as they aid in the

isotype switch (switch from production of IgM to IgG, IgA, etc.) of B-lymphocytes (55).

All the components of the immune system have a role in the intricate network which functions in keeping our bodies free from infection. Individuals who have intact immune systems, in the absence of underlying disease, can successfully fight off infection from invading microorganisms. There are those however, who cannot achieve this balance due to severe trauma or illness, and therefore generally succumb to infection by opportunistic pathogens.

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a gram negative bacillus which may be found as a free living saprophyte in soil, fresh or marine water, and other natural ecosystems or may exist as an infectious agent in association with plants, animals, and man (15, 20, 22, 38, 40). *Pseudomonas* possesses ubiquitous growth characteristics which better suites it for survival in harsh environments as compared to the enteric microorganisms (15, 42). Some of these characteristics include the ability to grow at higher temperatures (43 °C), and the ability to survive indefinitely in aqueous environments, including distilled water, at room temperature (20, 42, 57-60, 76). The bacteria's simple nutritional requirements include multiple low molecular weight organic compounds, preferably organic acids, and has even been known to employ certain antimicrobial agents such as phenol and penicillin (22). *P. aeruginosa* possesses a large reserve of enzymes which allows it to use multiple substances as nutrients and adapt to changes in it's environment (15). *Pseudomonas* can also utilize a few monosaccharides, however it cannot use any disaccharides or polysaccharides (22).

Traditionally thought of as obligate aerobes, some species of pseudomonads can use nitrate as the terminal electron acceptor in the absence of oxygen (20, 76). *Pseudomonas* can also make use of the amino acid arginine in anaerobic environments by converting it to

ornithine via the arginine dehydrolase enzyme pathway (76). Although pseudomonads are non-fermentative, the ability to utilize nitrates, and in some instances, arginine, contributes to the ubiquitous growth in soils and even hospital environments (76).

This aerobic, non-spore forming bacteria varies in size from 1-3 μm in length and 0.5-1.0 μm in width (20, 21). The motility of *Pseudomonas* is dependent on the number and position of flagella which differ between strains (22). However, motility is an important factor in *Pseudomonas aeruginosa* infections which become systemic, particularly thermal burn injuries and chronic cystic fibrosis (CF) infections (15). As with most gram negative bacteria, *Pseudomonas* possesses an outer membrane composed of lipoproteins, phospholipids, and lipopolysaccharide (LPS). The outer membrane serves as a protective agent in bacteria acting as a permeation barrier to chemical agents (22). Between the outer membrane and the cell membrane exists the periplasmic space which contains many hydrolytic enzymes. Particular strains of *Pseudomonas aeruginosa* may also have a layer of acetylated exopolysaccharide or alginate layer which is useful in adherence. This mucoid exopolysaccharide layer, composed of mannuronic and guluronic acids, provides a selective growth advantage to *Pseudomonas aeruginosa* by interfering with phagocytosis, specifically, with chemotaxis and ingestion by neutrophils and macrophages (48, 49, 63, 69).

Pseudomonas aeruginosa is often referred to as an opportunistic human pathogen (5, 22, 30-32, 48, 76). It is the leading cause of morbidity and mortality in compromised hosts, including individuals with decreased phagocytic or complement function, as well as those with agammaglobulinemia, hypogammaglobulinemia, or a deficiency in IgM (5, 22, 30-32, 48, 76). It may also cause disease in other vertebrates, invertebrates, and even plants. Fresh fruits and vegetables have been traced as colonization vectors for infected patients in hospitals (22, 38). Although seven to twenty-five percent of healthy individuals carry *Pseudomonas* in their intestinal tract, they do not exhibit clinical symptoms or

significant stimulation of their immune systems (20, 38). Those individuals frequently colonized with *Pseudomonas* are immunocompromised and include thermal burn, cancer and CF patients (5, 15, 16, 22, 30-32, 38-40, 42, 48, 50, 57-59, 63-69, 75-76).

Pseudomonas aeruginosa is a predominant component of burn wound flora, colonizing about sixty percent of patients by the 5th day post burn (15, 22). After a burn injury, proteins which are destined to become part of the protective antibodies of the immune system in these individuals are diverted for more immediate functions, thereby leaving room for colonization by bacteria (22, 44). In addition, various aspects of neutrophil function, including chemotaxis, may be damaged in these patients, leaving them susceptible to infection (66). Colonization and infection by *Pseudomonas aeruginosa* in these patients is a major threat and cause of morbidity and mortality (7, 15, 22, 30-32, 57-59, 63-69).

The frequency of *Pseudomonas* bacteremia is extremely high in cancer patients and accounts for over twenty-five percent of deaths involving children with leukemia (22, 69). These infections are predominantly nosocomial (42, 44, 69). Individuals undergoing chemotherapy, or with acute leukemia, bone marrow cancer, or any other secondary malignancy which leads to neutropenia often have a high incidence of *P. aeruginosa* infection (15, 22, 69). Mortality in cancer patients generally ensues as a result of extreme leukopenia or a severe decrease in the levels of opsonizing antibodies for *Pseudomonas aeruginosa* (30-32). There has been some success in recovery from these infections in some cancer patients, with gamma globulin administration (30-32).

Pseudomonas aeruginosa bacteremia in CF patients is mainly confined to the lung and bronchial area until late in disease progression (7, 57, 58, 65, 69, 76). The bacteria is isolated from the sputum of infected patients approximately ninety percent of the time (57). It is speculated that a possible defect in the mucociliary transport system of CF patients is attributed to the chronicity of *P. aeruginosa* infections (60). Speert (1993), using CF

leukocytes and serum, found that phagocytosis was compromised *in vitro* due to alterations in receptor and opsonic function (69). Isolates recovered from infected CF patients appear to be highly motile (59) and more mucoidal than strains of *Pseudomonas* recovered from other types of infections (7, 15, 22, 76). There has not been a single documented case of full recovery or cure of a CF patient infected with *P. aeruginosa* (57).

It is difficult to characterize the pathogenesis of *Pseudomonas aeruginosa* due to the range of disease it causes from the acute infections seen in burn patients and those with endopthalmitis (an eye infection caused by *Pseudomonas*), to the less frequently seen bone and joint infections, endocarditis, meningitis, and malignant external otitis, to the predominant chronic lung infections of CF patients (15, 76). Although this microorganism infects non-compromised individuals, the highest morbidity and mortality is found in the immunocompromised population, particularly those with CF and cancer (16, 22, 44, 48, 66, 69, 76). More than eighty percent of *Pseudomonas* isolates from CF patients are mucoidal, in contrast to the smooth and rough forms isolated from other infections (76). The mucoidal or alginate form, composed of ß-1,4 linked mannuronic acid and L-guluronic acid, is one of the most important virulence factors of Pseudomonas (15, 69, 76). More than eighty percent of *P. aeruginosa* isolates from chronically infected CF patients possess this mucoidal alginate layer as compared with only 2.5 % of *Pseudomonas* recovered from other types of infections (76). Alginate production is an unstable characteristic in that it is inducible and the moist environment of the lung appears to encourage it's production (76). Alginate production contributes to the pathogenesis of *P. aeruginosa* lung infection similar to that of other polysaccharides in that it helps in the adherence of the bacterium to the moist environment of the lung, and also has anti-phagocytic properties (15, 22). Buret and Cripps (1993) have also found that the alginate layer may interfere with normal opsonin-receptor associations by prevention of antibody-complement coating (16, 38). It has also been suggested that in the presence of calcium, alginate forms a protective gel-like matrix

around the bacterium which shelters it from host defenses and possibly the actions of charged antibiotics (76).

Pseudomonas aeruginosa produces many extracellular products, some very toxic to hosts, and others of little or unknown toxicity. These factors include exotoxin A, phospholipase C, numerous proteases, phenazine pigments, and alginate (15, 22, 36, 39, 57-60, 76). Exotoxin A has been identified as the single most toxic substance, based on weight, produced by *Pseudomonas aeruginosa* during acute infections (57-60, 76). The enzyme activity of exotoxin A is identical to that of diphtheria toxin in that it interferes with protein synthesis (36, 76). More specifically, exotoxin A catalyzes the transfer of the ADP-ribosyl portion of NAD onto eukaryotic elongation factor 2, a substance which is critical to protein synthesis at the ribosomal level (36, 76, 81-82). Moreover, the production of both exotoxin A and diphtheria toxin is regulated by iron concentration, thus when iron levels are insufficient enough to allow for optimal growth of *Pseudomonas*, then exotoxin A is produced (76, 81-82). The effects of exotoxin A *in vitro* includes being cytotoxic to mammalian cells in culture, as well as impairing the ability of human macrophages to exhibit phagocytic activity (81-82). In animal studies, Vasil (1986) found that in the chronically infected rat lung, the production of exotoxin A contributed to virulence of infection, but was not essential for increased pathogenicity (76).

Pseudomonas exoenzyme S functions much in the same manner as exotoxin A in that it affects ADP ribosyl transferase activity (61, 66, 82). Exoenzyme S has not been characterized as well as exotoxin A, but in studies involving the chronically infected rat lung, strains of *P. aeruginosa* that did not produce exoenzyme S were not as virulent as those that did, indicating that exoenzyme S may have a role in the pathogenicity of chronically infected patients (76, 82). Elastase and alkaline proteases, also called aggressins, have been found to contribute to the destruction of lung connective tissue as well as altering the cell receptors for opsonins including antibodies and complement,

contributing to the persistence of infection (61, 66, 76). Elastase and alkaline protease have also been found to degrade IL-2, and to a lesser extent, IFN-γ and TNF-α (15). These toxins have also been found to inactivate the humoral immune response through the proteolysis of IgG and IgA, inhibit natural killer cell activity, decrease the expression of the CD4 molecule on T-helper lymphocytes, and inhibit lymphocyte proliferation (15, 36, 60, 66, 75-76).

Phospholipase C is a heat-sensitive hemolysin produced by *Pseudomonas* (76). Much like exoenzyme S, it has not been studied as carefully as exotoxin A, however, it has been found to hydrolyze certain phospholipids, which are important components of eukaryotic cell membranes (76). Hydrolysis of phosphatidylcholine, a major component of lung surfactant, by phospholipase C could contribute to the virulence of *P. aeruginosa* in the lung (76, 82).

The production of pyocyanin, a blue phenazine pigment, is the distinguishing factor between *P. aeruginosa* and other pseudomonads. Pyocyanin (5-methyl-1-hydroxy phenazium betaine) is only one of several phenazine pigments produced by this bacteria; others include 1-hydroxyphenazine, oxychlororaphine, phenazine-1-carboxylic acid, chlororoapine, and aeruginosin A and B (4, 63). Because pyocyanin is produced by approximately fifty percent of all clinical isolates, it is the only one thought to contribute to the virulence of *P. aeruginosa* and may be linked to this microorganisms ability to acquire certain essential nutrients, including iron (17, 19-20, 37-38, 41, 60, 65, 74).

Pseudomonas aeruginosa is highly resistant to most conventional forms of antibiotic therapy as well as most chemical agents (24, 36, 44). Being one of the microorganisms that possesses inducible ß-lactamases, *Pseudomonas* is resistant to virtually all the ß-lactam antibiotics (i.e., penicillins) (44). The pseudomonads are more resistant to chemical agents as compared to other gram negative organisms possibly due to the outer membrane, which

acts as a permeation barrier to chemical substances. Although all gram negative organisms possess this unique characteristic, the LPS layer, which is one of several components which comprise the outer membrane, of *Pseudomonas aeruginosa* differs from that of *Escherichia coli* in that the composition of the lipid A portion contains a greater array of hydroxy fatty acids (22). Another difference between the two LPS layers is the presence of exotic amino acids and sugar acids in the O-specific polysaccharide region of *P. aeruginosa* in contrast to the simple sugars present in that of *E. coli*. This may contribute to the greater diffusion barrier for pseudomonads (59). *Pseudomonas* is not resistant to all chemical agents, however, it is sensitive to ethylenediaminetetraacetic acid (EDTA) and polymyxin (20).

Phenazine pigment production

Phenazines are low molecular weight compounds containing 3 heterocyclic rings with nitrogen substitutions at the 5th and 10th carbons. These naturally occurring pigments are formed exclusively by bacteria as secondary metabolites, and can be classified into 3 groups according to the number of carbon susbstituents (0, 1, and 2) (68, 74, 77, 79). The relatively inactive parent compound which serves as the basis for many biologically active derivatives, has no end groupings and is uncharged (Fig. 1). It carries a molecular weight of 180.2 and can be reduced in both aqueous and non-aqueous media (68, 74, 77, 79).

Well over 50 phenazine pigments are known representing every color of the visible spectrum. The absorption spectra of phenazines include characteristic peaks, including an intense peak in the 250-290 nm range and a smaller peak at 350-400 nm. Phenazine pigments, excreted by bacteria into medium, are mostly soluble in aqueous and organic solvents (26-27, 68).

Pyocyanin is a phenazine pigment produced mainly by *Pseudomonas aeruginosa* and

by *Streptomyces cyanoflavus* (4, 68). This heat resistant, low molecular weight (210.3) pigment diffuses readily into medium and stains blue in alkaline conditions and deep red in acidic environments. This redox dye is zwitterionic in nature, thus confirming it's solubility in aqueous and organic solvents including: chloroform, hot water, ethanol, acetone, glacial acetic acid, and phenol (68, 74). Pyocyanin is slowly degraded to 1-hydroxyphenazine in the presence of ultraviolet light, and becoming yellow in color when neutralized (68, 74, 77, 79). One-hydroxyphenazine, or hemipyocyanine, is often found in aged *P. aeruginosa* culture supernatants appearing purple-red in alkaline conditions. This low molecular weight (196.2) compound carries no charge.

Pyocyanin

First seen in the 1850's on the dressing of wounds, the characteristic blue-green colored pus was viewed as a sign that an infection was impeding healing (26). Successfully isolated in 1859 by Fordos, the name pyocyanin was given to the pigment, thus leading to the original naming of *P. aeruginosa* as *Bacillus pyocyaneus* (68, 74). Ironically, pyocyanin has been found to possess certain antimicrobial properties, thus allowing for the opportunistic growth of *Pseudomonas aeruginosa* (4, 9-10, 18, 65, 68, 74). The antibiotic activity is directed mainly against gram positive organisms, including common respiratory pathogens such as *Hemophilus influenzae*. Most gram negative organisms and all pseudomonads are resistant to pyocyanin. The antibiotic effect of this bacterium is suspected to be dependent on active bacterial respiration rather than the presence or absence of oxygen as originally thought (9-10, 68). This suggests that pyocyanin may be active at several specific sites of the bacterial respiratory chain.

Armstrong *et al.* (1979) found that 1-hydroxyphenazine inhibited the uptake of oxygen by mouse liver mitochondria at the cytochrome b site (5, 64-68, 71). The suggested mechanism for this is that the outer mitochondrial membrane is permeable to all

molecules with a molecular weight of 10,000 daltons or less. However, most compounds cannot permeate the inner membrane where the respiratory enzymes are housed. Pyocyanin has been found to inhibit reactions necessary for specific host defense including the proliferation of T lymphocytes, production of IL-2 and expression of IL-2 receptors, the activity of cytotoxic cells, and secretion of antibodies (44, 48-49, 59-60, 63-67, 69). Pyocyanin has been identified as the heat resistant compound found to inhibit lymphocyte proliferation when cell-free *Pseudomonas aeruginosa* supernatant was added to lymphocytes in culture (68). Pyocyanin has also been found to inhibit lymphocyte proliferation in response to antigens and mitogens and the expression of IL-2 receptors as well as the secretion of IL-2 (48, 59, 75, 80). The inhibition of mitogen-induced lymphocyte blastogenesis is speculated to occur at a step past the initial increase in free cytosolic calcium (64, 67). There may also be specific cell receptors for pyocyanin on immune cells, however any binding that may occur is probably non-covalent given the fact that the effect of pyocyanin on lymphocytes is reversible after washing (63-64). B-lymphocytes have also been found to be affected by the actions of pyocyanin. The secretion of antibodies by B-lymphocytes have been reduced at concentrations between 0.1 and 0.2 μg/ml (71). It has also been found that mitogenic stimulation of B-lymphocytes can be achieved with concentrations of pyocyanin of 0.2 μg/ml or higher (71).

In vivo, pyocyanin has been found in the sputa of infected CF patients at concentrations as high as 27 μg/ml (34). Pyocyanin has been shown to enhance the oxidative metabolism of neutrophils thus, impeding ciliary beat movement by generation of toxic oxygen radicals through the activation of mucosal neutrophils (34, 60, 80). It has also been previously reported that pyocyanin inhibits the uptake of molecular oxygen by mouse monocytes, HeLa cells, isolated mouse liver mitochondria, cultured BHK cells, and intact guinea pig peritoneal macrophages (5, 45, 49-50, 80). The suspected mode of action is the inhibition of electron transport by action upon the ubiquinone, cytochrome b site

(45).

Research Objectives

Pseudomonas aeruginosa is an opportunistic pathogen implicated in the colonization of burn victims as well as the lungs of individuals with CF. *Pseudomonas* produces several exoproducts many of which aid in the colonization of hosts as well as contributing to the virulence of infection and resistance to antibiotic therapy. The phenazine pigment, pyocyanin, a by-product of *Pseudomonas aeruginosa*, has been widely studied as an inhibitor of not only cellular functioning, but also of certain aspects of the immune response, thus contributing to virulence of infection. Therefore, it was important to focus this research on the effect, if any, of pyocyanin on the humoral immune response of mice.

The primary objective of this research is to identify the effect, if any, of intravenously administered pyocyanin on splenic cells of mice involved in the humoral immune response. Next, experiments will be designed to ascertain the kinetics of this response. For example, is there a difference if pyocyanin is given prior to, at the same time, or after SRBCs. The final objective is to examine the effect, if any, of pyocyanin on specific cytokines involved in the generation and maintenance of a humoral immune response.

CHAPTER II

MATERIALS AND METHODS

Animals

B6D2F_1 (C57BL/6J x DBA/2J) mice of both sexes ranging in age from eight to ten weeks were used in all experiments. Breeder mice were obtained from Jackson Laboratories (Bar Harbor, Maine). BDF_1 mice were bred and raised in the animal housing complex in the Department of Biology at the University of Dayton.

Culture Media

Minimal essential media (MEM-Eagle) and RPMI-1640 (10.39 g RPMI-1640 media, 5.96 g HEPES, 10 ml L-glutamine stock, 2 g $NaHCO_3$, pH 7.2-7.4) were used in all experiments involving injection of animals and culturing of cells. Antibiotics [penicillin (10,000 U/ml), streptomycin (10,000 μg/ml), and gentamicin sulfate (10 mg/ml)] were added to RPMI-1640 used in cell culturing. All media was filter-sterilized using 0.2 μm pore size filters and stored at 4 °C.

Preparation and Purification of Pyocyanin

The photochemical reaction using the phenazine methosulfate (PMS) method of Knight *et al.* (1979) was adapted in order to prepare pyocyanin (37). A 0.5 mg/ml solution of PMS [48 mg PMS in 96 ml of 0.01 M tris(hydroxymethyl)aminomethane buffer, pH 7.0] (Sigma, St. Louis, MO) was illuminated for approximately 18 h at room temperature under an intense fluorescent lamp until the yellow solution turned deep blue. The blue solution was then transferred to a glass lyophilizing flask, shell-frozen with dry ice, and

lyophilized. The lyophilizate was dissolved in 2 ml of methanol, and absorbed onto a silica gel column (Merck, grade 9385, 230-400 mesh, 60 Å). Pyocyanin was eluted with a 20% methanol-chloroform mixture using flash chromatography. The blue fractions were collected in 10 ml quantities, pooled, and evaporated to dryness. Approximately 10 mg of the blue crystals were dissolved in chloroform and the purity was checked by proton nuclear magnetic resonance (NMR). The scan revealed a high water content so the crystals were evaporated for 4 days under vacuum. The crystals (10 mg) were dissolved in 1 ml warmed RPMI-1640 for use in experimentation. The remaining pyocyanin was stored in 1.5 ml eppendorf tubes at 4 °C until further use.

Preparation of Sheep Red Blood Cells (SRBC)

Approximately 2 ml SRBC (Colorado Serum, Denver, CO) in Alsevers solution were washed twice in 0.85% sterile saline at 1,500 rpm and spun in a Beckman centrifuge before each experiment. After the second washing, the pellet was resuspended in RPMI-1640 to a final concentration of 10% for injection into animals, and 12% for use in hemolytic plaque assay.

Injection of Animals

Mice were given intravenous (i.v.) injections of either RPMI-1640, SRBC, or pyocyanin via the tail vein. Initially, mice were divided into five groups, consisting of a positive and negative control, and three experimental groups. Mice receiving media alone served as the negative control, while the positive control consisted of mice injected with a 10% suspension of SRBC. Experimental groups received the SRBC suspension plus either (A) 0.1 ml (1.0 μg) pyocyanin, (B) 0.1 ml (10.0 μg) pyocyanin or (C) 0.1 ml (100.0 μg) pyocyanin. The optimal dose was determined to be 1.0 μg concentration of pyocyanin and was used for all subsequent experimentation. Further experimentation

included mice being divided into five groups, consisting of a positive and negative control, and three experimental groups. Mice receiving media alone served as the negative control, while the positive control consisted of mice injected with a 10% suspension of SRBC. Experimental groups received either: (A) 0.1 ml pyocyanin and SRBC at the same time, (B) 0.1 ml pyocyanin 12 h prior to SRBC, (C) 0.1 ml pyocyanin 6h prior to SRBC, (D) 0.1 ml pyocyanin 6 h post injection with SRBC, and (E) 0.1 ml pyocyanin 12 h post injection with SRBC.

Cell Viability

The spleens of control and experimental animals were removed at different intervals, weighed, and cell viability assessed by trypan blue exclusion. In this experiment, one mouse served as the control and received 0.1 ml MEM i.v., while the 5 experimental mice were injected with 0.1 ml of 10 μg/ml (1.0 μg) pyocyanin i.v. at the same time. At 2, 4, 6, 12, and 24 h intervals, the spleen of the animal was removed and weighed. A single cell suspension was made by gently teasing the spleen to release the cells using 1 x 2 toothed forceps, into approximately 1 ml of media. The cells were washed once in MEM and centrifuged for 10 min at 1,500 rpm, then resuspended in 2 ml MEM. In order to remove the red cells, the cell suspension was pelleted by centrifugation at 1,500 rpm for 10 min and 1 ml red cell lysing buffer (Sigma, St. Louis, MO) was added. The tube was then placed on a Nutator rotator for 1 min. The cells were pelleted again by centrifugation and resuspended in 2 ml MEM. A 1:10 dilution of cells (1 ml) was made using MEM, and 0.5 ml of the diluted cells was transferred to a 12 x 75 mm disposable test tube containing 0.5 ml of 0.2% trypan blue, mixed thoroughly, and the cells were immediately counted using a hemocytometer. The cells which excluded the blue dye were considered viable. The percentage of live cells was derived from the ratio of live to dead cells, then compared against the values from the control animal which were counted at each time interval.

Lymphocyte Proliferation Assay

Three normal animals were sacrificed, their spleens removed, and a single cell suspension made. The cells were washed in RPMI-1640 by centrifugation (10 min at 1,500 rpm), resuspended in 2 ml RPMI-1640, then counted. The cells were resuspended at a concentration of 4 x 10^6 cells/ml in RPMI-1640 supplemented with antibiotics and 4% fetal calf serum (FCS) and 0.1 ml dispensed into individual wells of a sterile 96 well microtiter plate (Corning). Quadruplicate wells received either 0.1 ml RPMI-1640 alone, LPS (1.0 μg/well), concanavalin A (Con A) (0.1 μg/well), or pyocyanin (0.05 μg/well, 0.1 μg/well, 0.5 μg/well, 1.0 μg/well, and 10.0 μg/well). The plates were incubated at 37 °C with 5% CO_2 for 54 h then pulsed for 18 h with 0.1 μCi ^{3}H-thymidine. After 72 h the cells were harvested onto glass fiber filter paper using a cell harvester. The filter paper disks were allowed to dry overnight and were placed in 4 ml of scintillation fluid and counted on a scintillation counter (Beckman LS-3801).

Hemolytic Plaque Assay

The humoral immune response in spleen cells was determined using a standard hemolytic plaque assay (Jerne *et al.*, 1954) (33). In each experiment, mice received either 0.1 ml of a 10% SRBC suspension i.v. on day 0, or 0.1 ml of a 10% SRBC suspension plus different concentrations of pyocyanin. On day 4, the mice were sacrificed and their spleens removed. A single-cell suspension was made from the spleens of the mice and the cells were washed in RPMI-1640 and centrifuged for 10 min at 1,500 rpm. The spleen cells were then resuspended in 2 ml RPMI-1640. Five hundred microliters of 0.6% agarose in RPMI-1640 was dispensed into 12 x 75 mm disposable test tubes and placed in a heated water bath (50 °C). Fifty microliters of a 12% SRBC suspension in RPMI-1640 was dispensed into each of the tubes. Duplicate dilutions (1:10, 1:40, 1:100, and 1:400) of

the spleen cells from control and experimental mice were added to the tubes. The tubes were individually finger vortexed and the contents poured onto pre-coated microscope slides. When the agarose mixture solidified, the slides were inverted onto trays containing a 1:30 dilution of guinea pig complement in RPMI-1640 (Colorado Serum, Denver, CO). The trays were then placed in assay boxes and incubated for 3 h at 37 °C with 5% CO_2.

After the 3h incubation period, the individual slides were immediately examined for plaques. Plaques were defined as being a clear-zone in the agar, and were counted using a magnifying glass. In order to determine the number of plaque forming units (PFUs) per 10^6 cells, the number of plaques on the slides was multiplied by the dilution used, and that value divided by the total number of spleen cells (determined by the cell count).

Isolation of Adherent Mononuclear Cell Population

Spleen cells from control and experimental mice were resuspended in RPMI-1640 and 5% fetal calf serum (FCS) to a final concentration of 1 x 10^6 cells/ml and dispensed into 150 x 15 mm petri dishes (Labcraft). The petri plates were incubated for 1.5 h at 37 °C and 5% CO_2. After incubation, the media containing the non-adherent cells was decanted into 50 ml centrifuge tubes (Labcraft) and the plates were rinsed once with warmed (37 °C) RPMI-1640 to ensure removal of all non-adherent cells. The non-adherent cells were centrifuged for 10 min at 1,500 rpm. Warmed (37 °C) RPMI-1640 was poured into the plates and they were gently scraped using a cell scraper to remove the adherent cell population. The adherent cell population was brought to a final concentration of 1 x 10^7, and total RNA was isolated. This cell population consisted mainly of macrophages and monocytes.

Isolation of Non-adherent Cell Population using a Percoll Gradient

The non-adherent cells recovered from the above procedure were centrifuged and counted. The non-adherent cells were enriched for T-lymphocytes using a Percoll (Sigma) gradient equilibrated at 320 mOsm. A 90.86% working solution of percoll and 10x Hanks balanced salt solution (HBSS) was used to create gradients of 70, 65, 60, 55, 50, and 30% Percoll diluted in 1x HBSS. Two milliliters of each Percoll percentage were gently layered into a sterile 15 ml centrifuge tube (Labcraft) which was pre-coated with FCS. Non-adherent cells (at a concentration of 5 x 10^7) were gently layered onto the gradient and centrifuged at 1,500 rpm for 20 min with the brake off. The cells from fraction 3 (50% percoll), enriched for T cells, were collected using a sterile pasteur pipette. The cells from these fractions were washed twice with RPMI-1640 (10 min at 1,500 rpm), resuspended in 2 ml and counted. The cells were brought to a final concentration of 1 x 10^7 and the total RNA was isolated.

RNA Isolation

Total RNA was isolated from three groups of spleen cells: unseparated, adherent, and non-adherent populations from control and experimental groups including those treated with pyocyanin (1.0 μg). Prior to RNA isolation, the red cells were lysed. The RNAzol B™ (Friendswood, Texas) method was used to isolate total RNA. RNA was extracted from a total of 1 x 10^7 cells in each instance. The cells were centrifuged in a sterile microcentrifuge tube at 400 xg for 10 min (Eppendorf 5415 microcentrifuge). The supernatant was aspirated and the pellet was gently resuspended in 1 ml RNAzol B. One hundred microliters of chloroform was added to the homogenate and the contents were vortexed for 15 seconds then centrifuged at 10,000 xg for 15 min at 4 °C. After centrifugation, two layers formed: an upper aqueous layer containing RNA, and a lower

phenol-chloroform phase which contained the DNA and protein. Approximately 450 ml of the aqueous phase was transferred to a sterile microcentrifuge tube and an equal volume of isopropanol was added. The tube was mixed by inversion and incubated on ice for 2-4 h or placed in -20 °C overnight. After the incubation period, the tube was centrifuged at 10,000 xg for 30 min at 4 °C. The precipitated RNA formed an off-white pellet at the bottom of the tube. The supernatant was carefully removed and the pellet washed with 1 ml 75% ethanol in diethylpyrocarbonate (DEPC) treated water (Sigma) and centrifuged at 10,000 xg for 8 min at 4 °C. The supernatant was again carefully removed and the pellet was allowed to dry. The dried pellet was dissolved in 10 μl of DEPC water. To measure the concentration of the extracted mRNA, 1 μl of the mRNA suspension was placed in 69 μl of DEPC water in a microcuvette, and the absorbance was read at 260 and 280 nm on a spectrophotometer. The concentration was determined using the following formula: A_{260mn} x dilution x 40 = μg RNA/ml. The isolated RNA was stored at -20 °C until further use.

Preparation of cDNA

Two micrograms of RNA was suspended in a total of 9 μl of DEPC-treated H_2O. The samples were heated at 70 °C for 10 minutes in a thermocycler (Thermolyne) then quick chilled on ice for 5 minutes. Ten microliters of the Promega reaction mixture [4 μl M-MLV RT 5x buffer, 2 μl 10 mM dNTP, 2 μl random hexamers, 2 μl M-MLV RT (RNAse minus), 1μl RNase inhibitors] was added to each sample, and incubated at room temperature (approximately 25 °C) for 10 min. The samples were then incubated in the thermocycler at 37 °C for 60 min. After this time, the reaction temperature was increased to 95 °C for 10 min. The reaction mixture was quick chilled again in the thermocycler at 4 °C for 30 min.

Polymerase Chain Reaction (PCR)

A PCR master mix was prepared by multiplying the volume of each reagent by the number of samples plus 1. One microliter of cDNA from each sample was added to new sample tubes along with 49 μl of the master mix (per sample: 5 μl 10 x PCR buffer without Mg^{2+}, 3 μl 25 mM $MgCl_2$, 0.5 μl 10 mM dNTP, 1 μl primer, 0.2 μl Taq DNA polymerase (5000 u/ml), and 39.3 μl DEPC-treated H_2O) all from Promega. The tubes were centrifuged at 10,000 xg for 10 sec in an Eppendorf 5415 microfuge and overlayed with 50 μl of sterile mineral oil (Sigma). The samples were placed in the thermocylcer heated to 95 °C for 5 min, then 55 °C for 1 min, and 72 °C for 1 min followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min.

The PCR products and DNA ladder molecular weight marker were run on a 2% agarose gel (dissolved in 1x TBE) with 1.5 μl of 10 μg/ml ethidium bromide. Sixteen microliters of PCR product and 5 ml of loading dye were loaded and run (700 ml 1x TBE) for 1 hour at 120 volts. The gel was examined under ultraviolet light and photographed.

Statistical Analysis

The mean, standard deviation, and standard error were calculated for each experiment yielding PFUs. The standard error is represented as an error bar in each graph. The statistical significance of the results was calculated using a single factor Anova ($P < 0.05$) to compare the controls to each treatment with pyocyanin, as well as comparing the treatments with pyocyanin to each other. The statistical significance of the results is reported in the figure legend of each graph.

CHAPTER III

RESULTS

Purification of pyocyanin

Pyocyanin is a phenazine pigment produced by *Pseudomonas aeruginosa* thought to contribute to the virulence of infection by this bacterium. Fordos tried to characterize this pigment in 1859 after the appearance of a blue-green stain on the bandages of infected individuals (26, 68). Although pyocyanin exhibits antibiotic action against several gram positive organisms, such as *Staphylococcus aureus*, it also inhibits the growth of cells in culture and at concentrations above 2 mg is lethal to mice upon injection (9-10, 66, 68).

To initiate studies addressing the impact of pyocyanin on immune responsiveness, it was first necessary to obtain a purified preparation of this phenazine compound. Pyocyanin was initially isolated from actively growing *Pseudomonas aeruginosa* cultures using the method of Baron *et al.* (10). This involved growing large amounts (4 L) of bacteria for 96 h, centrifuging out the cells, and extracting the dark purple supernatant several times with chloroform. This procedure yielded only a small amount of pyocyanin, while the majority of the supernatant was 1-hydroxyphenazine. The concentration of pyocyanin was very difficult to determine using the method of Baron *et al.* (10), due to the small quantity. This technique was abandoned after further literature reviews revealed that purification of PMS would provide a more efficient way of obtaining pyocyanin.

PMS was converted to pyocyanin via a photochemical reaction in a method adapted from Knight *et al.* (37). Flash chromatography eluted with a chloroform-methanol solution (20%) was used to purify the pigment, then concentrated during lyophilization. Spectrophotometric analysis as well as a proton NMR confirmed the presence and purity of pyocyanin. Figure 1 illustrates the structures of the parent phenazine compound,

pyocyanin, and 1-hydroxyphenazine the breakdown product of pyocyanin. Figure 2 is the proton NMR of the product obtained from the purification of pyocyanin previously described in the methods section. Ten milligrams of the compound were dissolved in chloroform and analyzed for its structure via proton NMR. The proton NMR reveals that this compound is pyocyanin due to the peaks which appear in appropriate places on the spectrum. Tetramethylsilane (TMS) is a standard used in order to get a first peak reading.

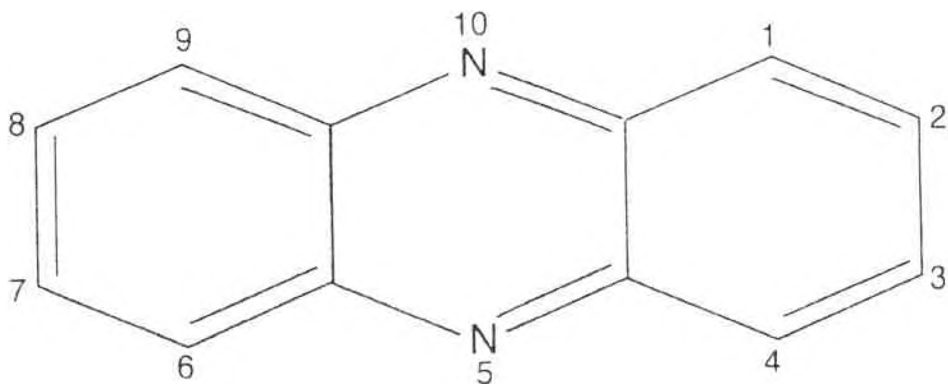
The effect of pyocyanin on the humoral immune response

To determine what effect, if any, this phenazine pigment would have on immune function, different concentrations of pyocyanin (1.0, 10.0, and 100.0 μg) were injected into mice on day 0, simultaneously with a ten percent suspension of SRBC. On day 4 after the injection, the spleens were removed and the number of PFUs were determined by the hemolytic plaque assay adapted from the method of Jerne *et al.* (33) and Mishell *et al.* (45). Pyocyanin (1.0 μg) produced the greatest effect, resulting in a 74% reduction in the number of PFUs (Fig. 3) and was used for all subsequent experimentation.

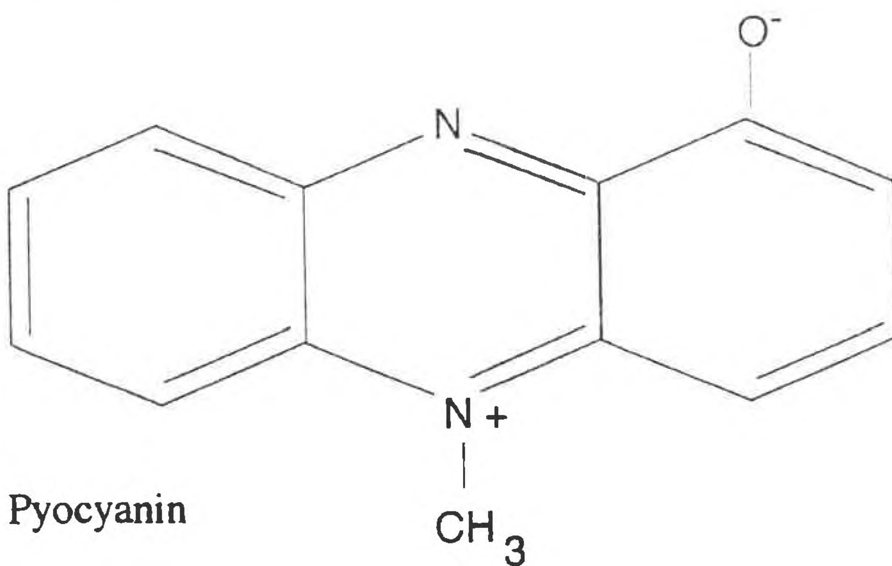
To be certain that the observed suppression in Figure 3 was not due to either toxicity of pyocyanin or endotoxin associated with the purification of pyocyanin, the percent viability of spleen cells obtained from animals treated *in vivo* with pyocyanin was determined by trypan blue exclusion. The percent viability of spleen cells obtained from experimental animals was never less than 87%, which was not significantly different from control animals (Table 1).

Previous research has shown that LPS or endotoxin, at appropriate concentrations, can suppress the immune response. Pyocyanin, obtained from PMS could have been contaminated with LPS or endotoxin during the purification procedure. To address this issue, a lymphocyte proliferation assay was performed, based on the fact that LPS is a known B-cell mitogen. Data in Table 2 illustrates that pyocyanin is not mitogenic at the

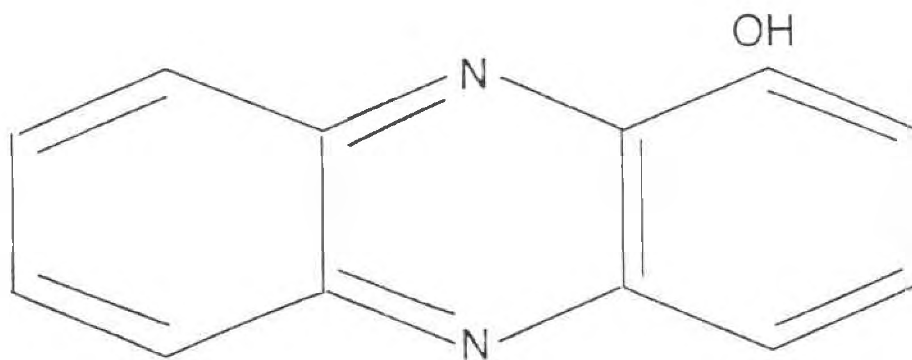
Figure 1. The structures of the phenazine, pyocyanin, and 1-hydroxyphenazine.



Phenazine

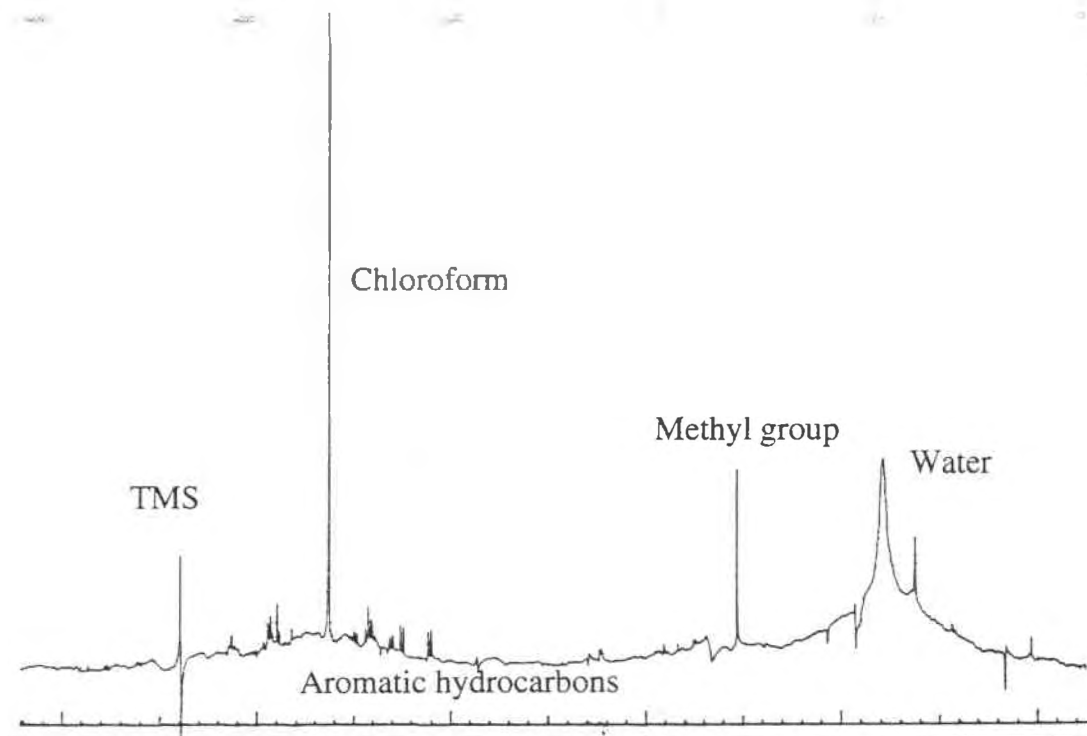


Pyocyanin



1-Hydroxyphenazine

Figure 2. Proton NMR of pyocyanin. Pyocyanin (10 mg) was dissolved in chloroform and TMS was used as a standard.


Chloroform
Methyl group
Water
TMS
Aromatic hydrocarbons

concentrations used. This indicates that the suppression observed in Figure 3 can not be attributed to LPS contamination.

Having ascertained that pyocyanin had a suppressive effect on humoral immunity, and that this suppression was not associated with toxicity or endotoxin contamination, we were interested in determining the kinetics of this suppressive effect. Specifically, does the time at which pyocyanin is administered (i.e., before or after SRBC) alter the suppressive effect. Mice were injected with pyocyanin (1.0 μg), prior to, at the same time, or subsequent to the i.v. injection of SRBCs. Four days later, control and experimental animals were sacrificed, spleens removed, and the number of PFUs quantitated.

Data represented in Figure 4 illustrates that mice receiving pyocyanin before SRBCs also exhibit a suppressive effect. Specifically, when pyocyanin was administered 12 h prior to SRBCs, this resulted in an 83% suppression. This however was statistically different from the 72% suppression observed when pyocyanin was administered 6 h prior to SRBCs, but was not from animals who received pyocyanin at the same time as SRBCs (78% suppression) ($P < 0.05$). Interestingly, the data shown in Figure 5 illustrates that pyocyanin administered 12 h subsequent to injection of SRBCs had the greatest suppression at 79%. This was a dramatic difference in the number of PFUs from animals receiving pyocyanin 6 h subsequent to SRBCs (41% suppression) and pyocyanin at the same time as SRBCs (66 % suppression). These differences were also statistically different ($P < 0.05$) from each other. Overall, when pyocyanin is administered 12 h prior to or subsequent to SRBCs, there appears to be a dramatic suppressive effect which is statistically different from pyocyanin being administered at the same time as SRBCs or 6 h prior to or subsequent to SRBCs. The observed suppression does not appear to be caused by toxicity of pyocyanin.

The effect of pyocyanin on cytokine mRNA expression

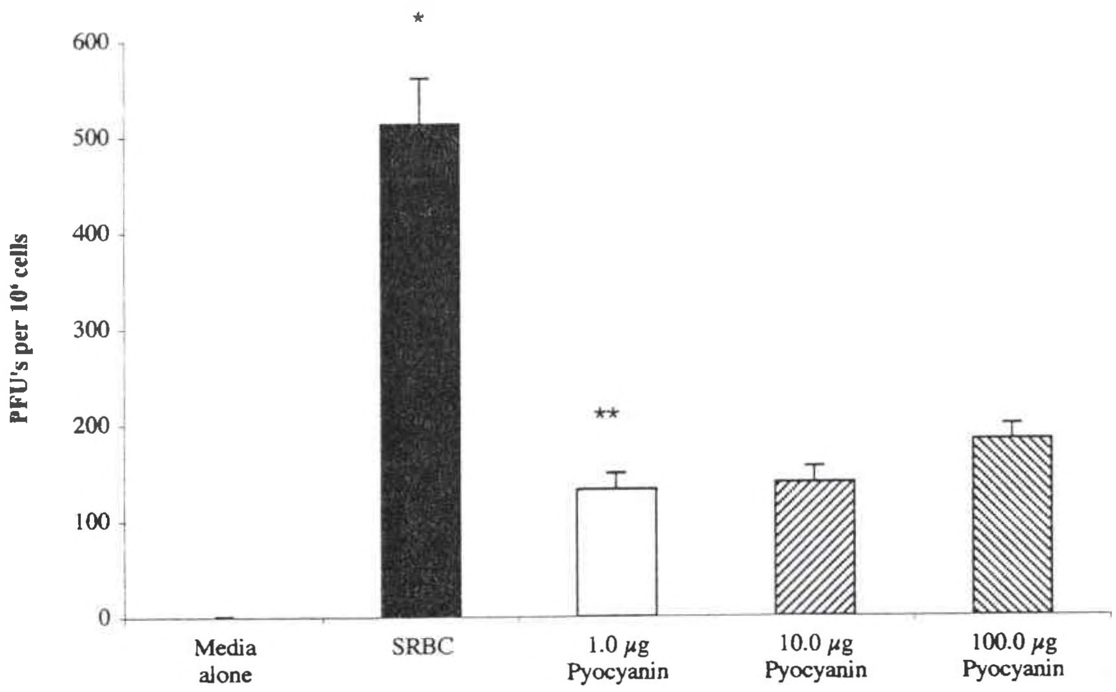
The mechanism of action of the observed suppressive effect of pyocyanin needed to be investigated. One possibility was that pyocyanin had an effect on the ability of immune cells to either produce and/or release cytokines which are necessary for the generation or maintenance of a humoral immune response. To determine the effect, if any, of pyocyanin on the expression of mRNA specific for cytokines, namely, IL-1, IL-4, IFN-γ, and TNF, animals were treated with media alone, SRBC alone, and pyocyanin 12 h prior to SRBC, or pyocyanin and SRBC at the same time. Four days later, the spleens were removed, and cells were enriched for each of the following groups: the unseparated population (US), which consists of a mixture of APC's, neutrophils, NK cells, B and T-lymphocytes; the adherent cell population (A), which is primarily monocytes and macrophages; and the non-adherent cell population (NA), which is primarily T-lymphocytes and some NK cells. Total RNA was isolated from each of the cell populations, and was reverse transcribed and the cDNA amplified using PCR. Primers specific for each cytokine were used in the amplification process. Signals representing the expression of RNA for IL-4 were not apparent for any group of cells. Data in Figure 6 represents the expression of mRNA specific for cytokines in the group of animals treated with media alone, SRBC alone, and those animals treated with pyocyanin and SRBC at the same time. Panel A in Figure 6 represents animals that were treated with media alone. In panel A, bands appear for IFN-γ in both the US population and the A population (Figure 6). Panel B represents data from animals treated with SRBC alone, which was the positive control. Panel B illustrates that in the US population and the NA population, there are faint bands for IFN-γ (Figure 6) . Panel C represents data from animals treated with pyocyanin and SRBC at the same time. Panel C illustrates that there are faint bands visible in both the US and A populations for

TNF, and bands for IFN-γ also appear in both the US and NA populations (Figure 6).

Data in Figure 7 represents the expression of mRNA specific for cytokines in the group of animals treated with media alone, SRBC alone, and those animals treated with pyocyanin 12 h prior to SRBC. Panel A in Figure 7 represents animals that were treated with media alone. Data in panel A demonstrates that there are bands specific for TNF which appear in all three cell populations, US, A, and NA (Figure 7). Panel B represents data from animals treated with SRBC alone, which was the positive control. Data in panel B, Figure 7, shows that there are very faint bands for IL-1 which appear in all three cell populations, and there are also bands that appear for TNF in both the US and A populations. Panel C represents data from animals treated with pyocyanin 12 h prior to SRBC. Data from panel C demonstrates that the only bands visible are for TNF, and these bands appear in all cell populations (Figure 7).

Figure 3. The effect of pyocyanin on the hemolytic plaque forming response when injected simultaneously with antigen at different concentrations (1.0, 10.0, and 100.0 μg). SRBC and pyocyanin were given at the same time (time 0), and spleens were removed four days after treatment. The treatments were as follows: SRBC alone ( ), Pyocyanin (1.0 μg) ( ), Pyocyanin (10.0 μg) ( ), and Pyocyanin (100.0 μg) ( ) all at the same time as SRBC. Each bar represents the mean (n = 6) plus or minus the standard error of the mean. Statistics include a single factor Anova in order to compare differences between groups ($P < 0.05$).

*: indicates that there is a statistically significant difference to all groups
**: indicates that there is a statistically significant difference between this group and 100.0 μg pyocyanin



PFU's per 10⁶ cells
600
500
400
300
200
100
0
*
**
Media alone
SRBC
1.0 μg Pyocyanin
10.0 μg Pyocyanin
100.0 μg Pyocyanin

Table 1. The effect of pyocyanin on the viability of mouse spleen cells[a]

Hours Post Injection	Normal[b] (% Viable)	Experimental[b] (% Viable)
2	96	94.4
4	95	96.8
6	95	87
12	94	87
24	96	88

[a]Mice received an intravenous injection of either RPMI 1640 (normal) or 0.1 ml 10 μg/ml pyocyanin (1.0 μg) (experimental)
[b]Percent viability determined by Trypan Blue exclusion

Table 2. The effect of pyocyanin on mouse spleen cells in a lymphocyte proliferation assay

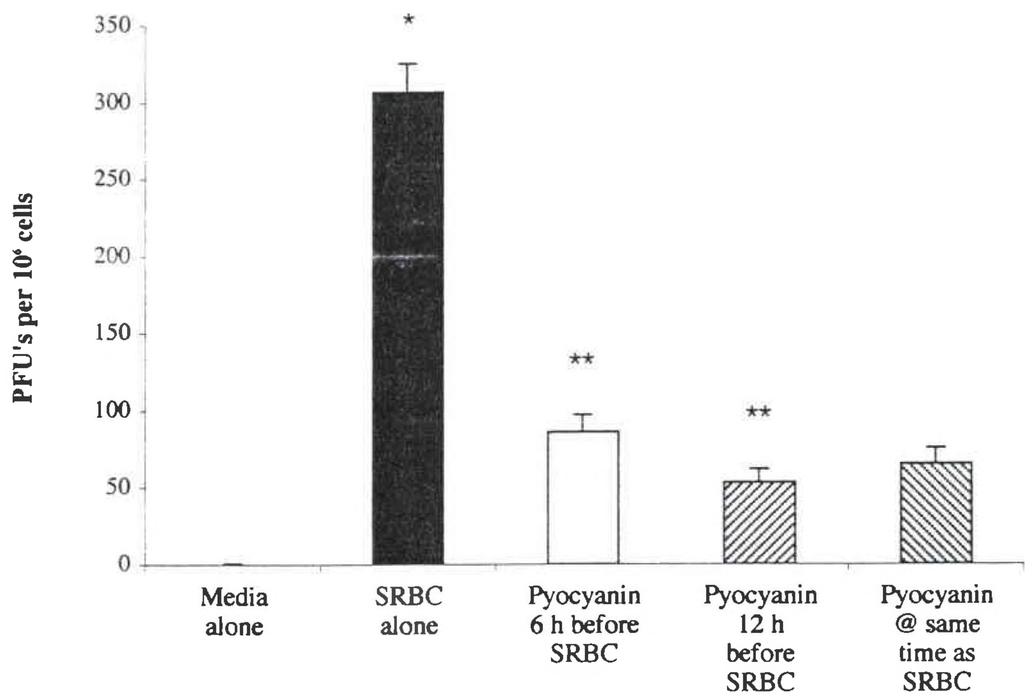
Group	Concentration (μg)[a]	Counts per minute[b]
Control		150
Con A	0.01	23,025 +/- 2000
LPS	0.1	14,001 +/- 3052
Pyocyanin	0.005	119 +/- 49
	0.01	137 +/- 48
	0.05	129 +/- 40
	0.1	73 +/- 19
	1.0	87 +/- 36

[a]Normal mouse spleen cells were cultured at a concentration of 4×10^5 cells/well (100 μl) in the presence of media alone, mitogen (Con A and/or LPS), or different concentrations of pyocyanin
[b]Each number represents the mean of 3 wells, replicated 3 times (n = 9).

Figure 4. The effect of pyocyanin on the hemolytic plaque forming response when injected simultaneously and prior to antigen at the same concentration (1.0 μg). Mice were treated with media alone, SRBC alone ( ), pyocyanin 6 h ( ) and 12 h ( ) before SRBC, and pyocyanin at the same time as SRBC ( ). Each bar represents the mean (n = 6) plus or minus the standard error of the mean. Statistics include a single factor Anova in order to compare differences between groups ($P < 0.05$).

*: indicates that there is a statistically significant difference to all groups

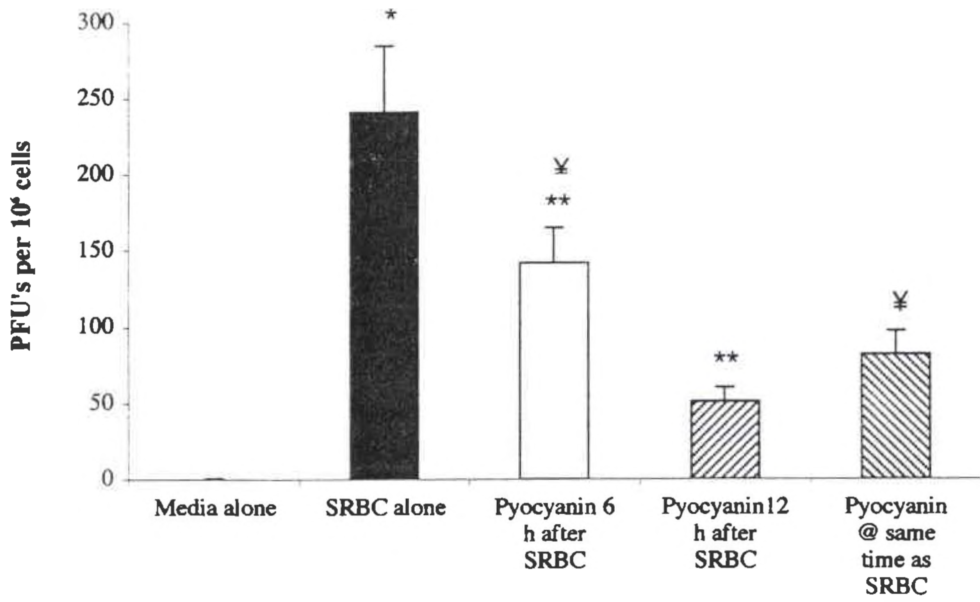
**: indicates that there is a statistically significant difference between these two groups



PFU's per 10^6 cells
350
300
250
200
150
100
50
0
*
**
**
Media alone
SRBC alone
Pyocyanin 6 h before SRBC
Pyocyanin 12 h before SRBC
Pyocyanin @ same time as SRBC

Figure 5. The effect of pyocyanin on the hemolytic plaque forming response when injected simultaneously and subsequent to antigen at the same concentration (1.0 μg). Mice were treated with media alone, SRBC alone (▩), pyocyanin 6 h (□) and 12 h (▨) after SRBC, and pyocyanin at the same time as SRBC (▧). Spleens were removed from the mice 4 days after treatment. Each bar represents the mean (n = 6) plus or minus the standard error of the mean. Statistics include a single factor Anova in order to compare differences between groups ($P < 0.05$).

*: indicates that there is a statistically significant difference to all groups
**: indicates that these groups are statistically different
¥: indicates that these groups are statistically different


300
250
200
150
100
50
0
PFU's per 10^6 cells
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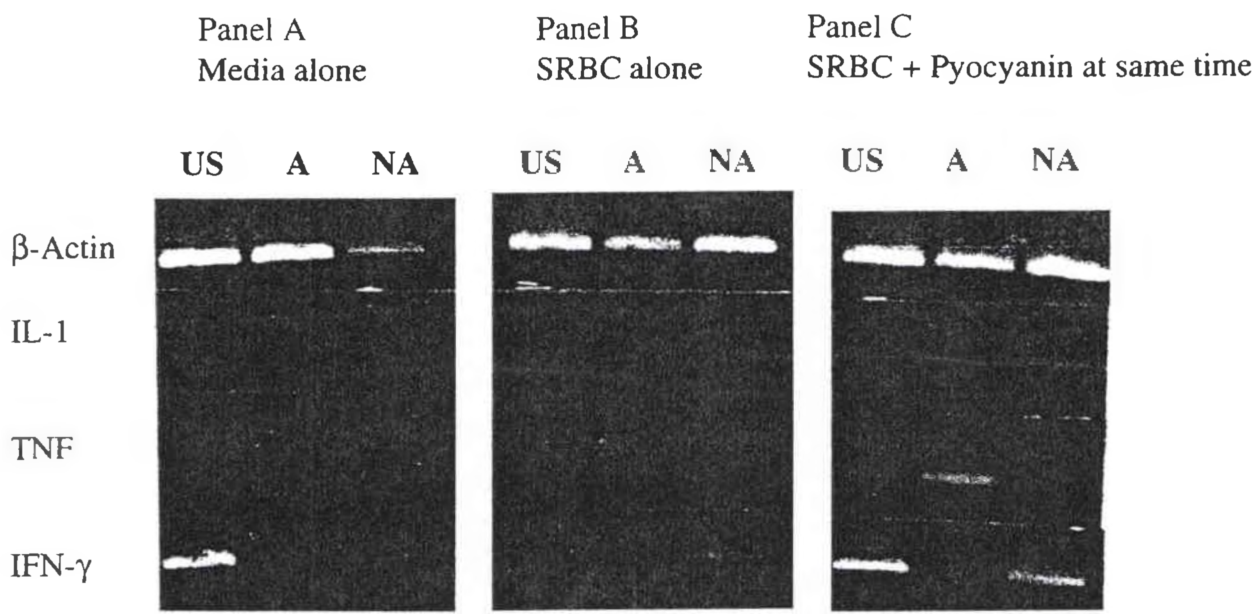
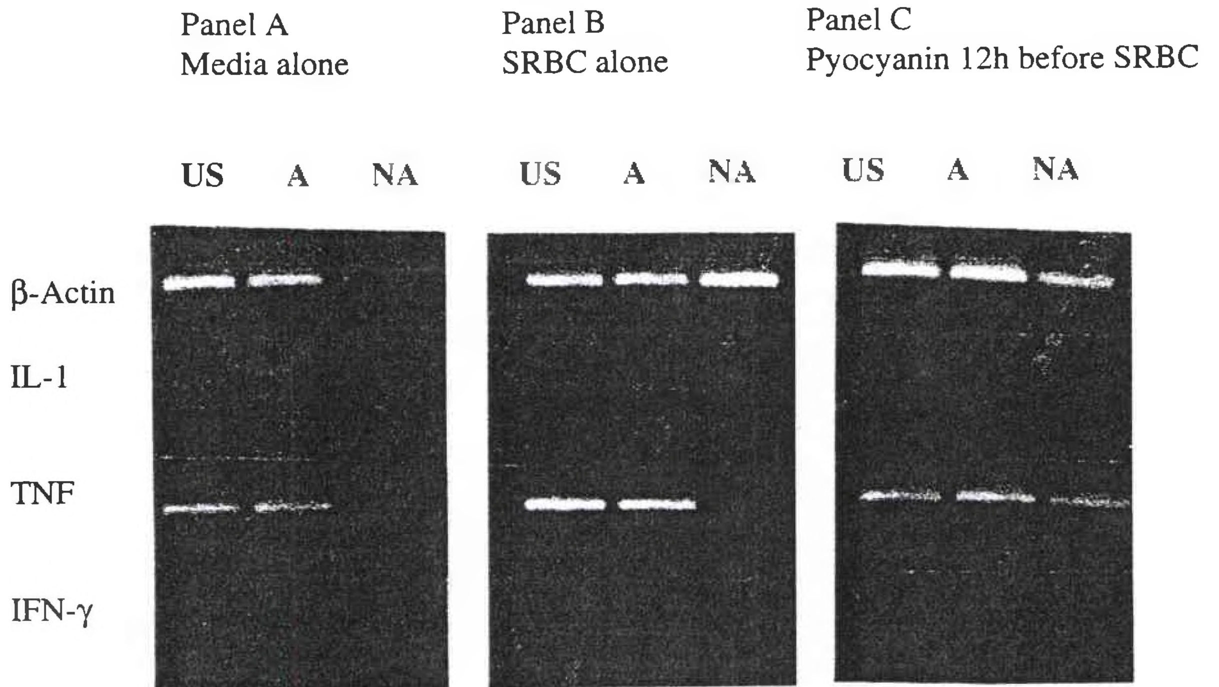
Media alone
SRBC alone
Pyocyanin 6 h after SRBC
Pyocyanin12 h after SRBC
Pyocyanin @ same time as SRBC

Figure 6. The effect of pyocyanin on the expression of mRNA specific for IL-1, TNF, and IFN-γ when injected simultaneously with antigen. Mice were injected with media alone, SRBC alone, and pyocyanin (1.0 μg) at the same time as SRBC. β–Actin was used as the positive control for the PCR reaction. The bands, which represent mRNA specific for a particular cytokine, were compared visually on the basis of intensity.



US-Unseparated cell population
A-Adherent cell population
NA-Non-adherent cell population

Figure 7. The effect of pyocyanin on the expression of mRNA specific for IL-1, TNF, and IFN-γ when injected 12 h prior to antigen. Mice were injected with media alone, SRBC alone, and pyocyanin (1.0 μg) 12 h prior to SRBC. β–Actin was used as the positive control for the PCR reaction. The bands, which represent mRNA specific for a particular cytokine, were compared visually on the basis of intensity.



US-Unseparated cell population
A-Adherent cell population
NA-Non-adherent cell population

CHAPTER IV

DISCUSSION

The primary purpose of this research was to determine if pyocyanin, when given intravenously, has an effect on the humoral immune response of mice. Once the effect of pyocyanin on the humoral immune response of mice was established, studies were designed to ascertain the kinetics of the response. Lastly, preliminary data was obtained about the potential effect of pyocyanin on the expression of mRNA specific for certain cytokines likely to be involved in regulating the development of a humoral immune response.

Pyocyanin is the low molecular weight, blue pigment produced from actively growing cultures of *P. aeruginosa* (4, 9-10). Pyocyanin has been demonstrated to have a variety of effects on both eukaryotic and prokaryotic cells including: the inhibition of ciliary beat function of respiratory epithelia, blocking of nitric oxide release and vasodilation in smooth muscle cells, and the antimicrobial effect on certain gram positive bacteria (i.e., *Staphylococci*) (9-10, 34-35, 39, 77). For the purposes of this research, it was important to examine the possible ways that pyocyanin disrupts normal cell function in order to determine the effect of pyocyanin on the humoral immune response.

Kanthakumar *et. al.* (35), have identified several "ciliatoxins", substances which slow ciliary beat frequency and in some cases, can cause ciliary dyskinesia, a disorganization of ciliary beat patterns. Two of the three toxins identified are pyocyanin and it's breakdown product, 1-hydroyphenazine. In studies conducted by Kanthakumar *et. al.* (1996) (35), it was shown that the onset of the effect of pyocyanin on nasal ciliated epithelium occurs before there is cell damage, and that the slowing of ciliary beat frequency by pyocyanin is associated with a fall in the levels of both cAMP and ATP. This was

especially important as previous studies have shown that cAMP is a regulator of ciliary activity (35). The mechanism of action of pyocyanin on epithelial cells appears to be a reduction in the level of adenosine nucleotides, which in turn reduces the intracellular levels of both cAMP and ATP (35). There are two possible explanations for the mechanism in which pyocyanin and other ciliatoxins affecting epithelial cells can cause a decrease in adenosine nucleotides. The first possibility is that the inhibition of oxidative phosphorylation by toxins could reduce the amount of ATP which is available to generate ciliary movement, which could then downregulate the production of intracellular cAMP (35). Another possibility is that the toxins may be acting directly on the production of cAMP intracellularly which could then affect the generation of ATP by the cell and the utilization of ATP by the cilia (35). 1-Hydroxyphenazine, the breakdown product of pyocyanin, which has a similar effect on cAMP, has been specifically identified at poisoning the electron transport chain within the mitochondria (35). This was interesting as in a previous study conducted by Miller *et. al.* (1987) (45), it was determined that pyocyanin inhibited the uptake of molecular oxygen by mouse monocytes among other cells, and the mechanism of action was that pyocyanin decreased cellular respiration by inhibiting electron transport, specifically at the ubiquinone-cytochrome b site in the electron transport chain (5, 9-10). The mitochondrial outer membrane is permeable to all molecules that are 10,000 daltons or less, which both pyocyanin and 1-hydroxyphenazine are, however, the inner membrane, which houses the electron transport chain is generally impermeable to molecules of this size (68). Somehow, however, pyocyanin is able to penetrate the inner membrane and disrupt electron transport, which makes it feasible that this pigment can potentially penetrate other cellular membranes as well. These findings are important as they provide clues as to the effect of pyocyanin intracellularly.

As previously mentioned, pyocyanin has also been associated with the inhibition of nitric oxide release by and subsequent vasorelaxation by smooth muscle cells (77). The

exact mechanism of action by which pyocyanin interacts with nitric oxide is not known, however there is one possible explanation. In previous studies, it was found that when nitric oxide was passed through a deoxygenated aqueous solution of pyocyanin, a color change occurred from the usual blue color, at neutral pH, to pink, indicative of an acidic pH (77). Mass spectroscopy revealed that the pyocyanin had completely disappeared, and only small amounts of 1-hydroxyphenazine and 1-methoxyphenazine, and two larger ions had remained (77). It was speculated that these two larger ions could have been formed due to the nitrosylation of pyocyanin by nitric oxide (77). This study suggests that a reaction between pyocyanin and nitric oxide may also occur *in vivo*, being a possible explanation for the inhibition of nitric oxide release and subsequent vasodilation (77).

The structural analysis of pyocyanin (Figure 1) may also provide some insight into the mechanism of action of this pigment on cell function. Pyocyanin, a zwitterionic compound, is an electron acceptor and carrier and has the capability of being reduced in an aqueous system (68). In addition, the low molecular weight of pyocyanin (210 daltons) suggest that although not likely to be immunogenic, it may be able to pass through cell membranes with relative ease. Although the biological effects of this compound have not been characterized, some possible effects based upon the chemical structure could include the ability to first attract an electron from a substrate, and then later donate the electron to an acceptor such as oxygen (68). In this scenario, the formation of toxic oxygen radicals could potentially alter important cellular functions thus resulting in tissue destruction and possible immune disruption (68). The possibility that pyocyanin has an effect on immune cell functioning, thereby providing a selective advantage to *Pseudomonas aeruginosa* in colonization of the lung is the primary reasoning behind this research.

Immunoglobulins, released by B-lymphocytes upon antigenic stimulation, are the major glycoproteins of the humoral immune system (56). The hemolytic plaque assay is an indirect method for measuring whether or not immunoglobulins are being produced in

response to SRBC, an antigen. This assay is a useful tool for measuring whether or not particular products, which are not mitogenic or antigenic alone, could influence the ability of a host to produce antibodies to a known antigen. This assay was used to measure the effect of pyocyanin on the ability of mice to produce immunoglobulins to SRBC. Initially, pyocyanin was injected into mice at different concentrations (1.0, 10.0, and 100.0 μg) simultaneously with SRBCs. Pyocyanin at the 1.0 μg concentration proved to have the greatest suppression of PFUs at a 74% reduction as compared with mice injected with SRBC only (Figure 3). This dramatic decrease in the number of PFUs suggested that pyocyanin had a definite suppressive effect on the humoral immune response. Further experimentation was needed to ascertain the kinetics of this effect. Therefore, it was decided that pyocyanin at this concentration would be used for all subsequent experimentation. The viability of mouse splenocytes appeared not to be affected by the injection of pyocyanin (1.0 μg) when compared to mice injected with RPMI-1640 media alone (Table 1). The percentage of viable splenocytes from mice injected with pyocyanin did not fall below 87%. This finding ruled out the possibility that pyocyanin is toxic to cells *in vivo* and any decline in the number of PFUs in the hemolytic plaque assay was unlikely to be caused by cell death as a result of direct toxicity of the pigment.

Next, pyocyanin (1.0 μg) was injected into mice 6 and 12 h prior, at the same time as, and 6 and 12 h subsequent to SRBC. Pyocyanin injected 12 h prior to SRBC resulted in an 83% reduction in the number of PFUs when compared with the number of PFUs from animals who received SRBC only (Figure 4). This reduction was statistically different ($P < 0.05$) from the percent reduction of PFUs obtained from animals injected with the pigment 6 h prior to SRBC (72%), but not from those who received pyocyanin at the same time as SRBC (78%) (Figure 4). What was even more interesting was that when pyocyanin was administered 12 h subsequent to injection with SRBCs, the percent reduction was again dramatic at 79%, and also statistically different ($P < 0.05$) from

pyocyanin 6 h subsequent to or at the same time as SRBC (Figure 5). These kinetic studies indicate that pyocyanin has the greatest suppressive effect on the humoral immune response when administered either 12 h prior or subsequent to SRBC. However, the bottom line is that pyocyanin (1.0 μg), when administered prior, at the same time as, or subsequent to SRBC results in dramatic suppression of PFUs as measured by the hemolytic plaque assay. This suggests that pyocyanin was interfering with the ability of animals to mount a humoral immune response, although the mechanism of action was unclear.

The effect of pyocyanin on the cells primarily involved in the humoral immune system has not been widely characterized, however there is some data that may indicate the mechanism of action of this pigment on these cells. In addition to inhibiting lymphocyte proliferation, pyocyanin has also been implicated in influencing superoxide anion production by neutrophils (9-10, 68-69). However, at lower concentrations of the pigment (less than 5 μM), superoxide production has been shown to be enhanced, thus amplifying tissue destruction (68-69). This dose-dependent phenomenon has also been shown with monocytes and macrophages, that in the presence of certain concentrations of pyocyanin, phagocytosis has actually been enhanced by these cells (49). Conversely, because of the nature of the chemical structure of pyocyanin, being an electron acceptor, it is theorized that perhaps it interferes with the oxidative burst of phagocytes, which is important for antimicrobial defense *in vivo* (49). However, this does not provide a substantial explanation for the suppression of plaque forming cells observed during this research. There are two possibilities for the observed data presented; first, a combined effect was taking place. Perhaps there was cellular damage resulting from superoxide production by neutrophils. Possibly, certain immune cells were also not functioning properly due to the interference of electron transport and cellular respiration. Perhaps many cells died in the process, thus lowering the overall number of plaque forming units, but this is not likely given the cell viability data (Table 1). Secondly, the fact that B-cell responses to SRBC are

controlled by several different factors, including the cytokines IL-1 and IL-2, points to an alternative possibility for our observations (43). In studies conducted by Marrack *et. al.* (43), it was shown that in order to generate an anti-SRBC response resulting in PFUs, it was necessary for certain substances to be present in the media that would act on B-cells to induce this response. In addition, the production of antibodies is dependent on the interaction between B and T- lymphocytes (3). B-cells alone could not induce an anti-SRBC response (43). With this in mind, it was decided that the effect was probably taking place at the molecular level, which could possibly explain the general suppression of PFUs, namely by the involvement of cytokines.

Certain cytokines have important immune functions through the induction of cell activation, growth, and differentiation (2, 12, 48, 62). IL-1 is an important cytokine involved in B-cell activation, and recently, it was demonstrated that IL-1 was an important component in the process by which PFUs are formed in response to SRBC (3). Previous studies have demonstrated that the addition of anti-IL-1 neutralizing antisera to activated B-cells in the presence of *Staphylococcus aureus* Cowan and IL-2, suppressed the formation of immunoglobulin-secreting cells (3). It is possible that pyocyanin inhibits the release of IL-1, thereby resulting in a suppression of PFUs in response to SRBC. Studies have also shown that IFN-γ works in synergy with both IL-1 and IL-2 in response to SRBC *in vitro* (3). If the mechanism of action of pyocyanin was on IFN-γ, then the effect of this pigment was probably additive, therefore affecting the synergistic relationship of different cytokines. Although not studied directly, IL-2 is another cytokine of interest potentially involved in the humoral immune response. IL-2, once thought to act specifically on T-cells, was later found to exert several actions on other cell types, especially B-cells (3). Not only does IL-2 induce the proliferation of B-lymphocytes, but it also induces the

secretion of immunoglobulins from activated B-cells (3). IL-4 is yet another cytokine that stimulates the proliferation and differentiation of B-lymphocytes and the subsequent release of immunoglobulins as well (3). In bacterial infections, the production of TNF-α and IL-1 by immune cells is induced by gram negative bacterial endotoxins, and for this reason, these cytokines may somehow mediate certain aspects of gram negative bacterial pathogenesis (54). IFN-γ has also been shown to activate macrophages and monocytes *in vitro* and *in vivo*, and it may also play an important role in the destruction of intracellular pathogens (54). Inactivation of cytokines such as IFN-γ, TNF-α and IL-1, could lead to the inability of a host to mount an immune response to gram negative bacterial pathogens, such as *Pseudomonas aeruginosa* . This was the rationale for collecting data on the effect of pyocyanin on certain cytokines potentially involved in the humoral immune response, namely IFN-γ, TNF-α and IL-1.

Parmely *et al.* (1990) examined the proteolytic inactivation of IFN-γ, TNF-α and IL-1 by *Pseudomonas aeruginosa* alkaline protease and elastase, however there has not been any research conducted on the inactivation of cytokines by other *Pseudomonas* products, namely pyocyanin (54). As mentioned previously, inactivation of IFN-γ , TNF-α and IL-1 could lead to significant reduction in the antiviral and general immunomodulatory activities (54). The suppressive effect of pyocyanin on humoral immunity, which was not caused by cell death from toxicity or LPS contamination, lead to further exploration of the mechanism of action of suppression by the phenazine pigment. There are two possible ways, among many, to explore the mechanism of action of pyocyanin on the humoral immune response. The first is to investigate the effect of pyocyanin on message specific for those cytokines involved in the humoral immune response. The second is to determine

the direct effect of pyocyanin on the biological activity of any or all immune cells (for example, does pyocyanin affect the antigen presenting ability of monocytes, macrophages, or B-cells). Because cytokines are such an integral part of induction of humoral immune responsiveness, we decided to investigate whether or not the suppressive effect caused by pyocyanin was due to the effect of this phenazine pigment on cytokine production.

In order to address the effect on cytokine production, it was necessary to examine purified cell populations. Spleens taken from mice that were treated with pyocyanin (1.0 μg) prior, at the same time as, and subsequent to SRBCs were removed and enriched for the following cell populations: unseparated population, the adherent mononuclear cell population, and non-adherent cell population. The unseparated population contained a mixture of APC's, neutrophils, B and T-lymphocytes, while the adherent cell population was primarily monocytes and macrophages, and the non-adherent cell population was primarily T-lymphocytes. Once the desired cell populations were obtained, total RNA was isolated, reverse-transcribed, and cDNA amplified by PCR to detect signals specific for the cytokines investigated.

The cytokines investigated include IL-1, IL-4, IFN-γ, because they are all cytokines which in some way affect cells involved in the humoral immune response (macrophages, B and T-lymphocytes) through either activation or promotion of cytokine secretion (2). Signals representing the expression of RNA for IL-4 were not apparent for any of the enriched cell populations (Figures 6 and 7). The trend for the signals for IL-1 in all enriched cell populations in mice that received SRBC only as well as any of the treatment groups appeared to be very faint or non-existent (Figures 6 and 7). IL-1 is generally produced by monocytes and macrophages and promotes T-lymphocyte proliferation (2, 21). Therefore, in normal unseparated populations of cells, one would expect that signals for IL-1 be detected. Signals for TNF were particularly bright in the unseparated and

adherent cell populations in mice receiving SRBCs only, pyocyanin 12 h prior to SRBC, and also pyocyanin and SRBC at the same time (Figures 6 and 7). TNF should be detected because it is also produced by monocytes (2). The signals for IFN-γ were similar to those for IL-1, with the exception of bright bands which appeared in the unseparated and non-adherent cell populations when pyocyanin and SRBC were given at the same time (Figure 6).

In the unseparated population of cells from mice treated with media alone, signals for the expression of IL-1 and TNF are extremely faint, while the signal for IFN-γ was slightly stronger (Figure 6). The signals for IL-1 and TNF would have been expected to be a little brighter as in Figure 7, while the expression of IFN-γ was not expected at all, since media alone does not constitute any mitogenic or antigenic stimulation (Figure 6). The bands for IL-1 and TNF in the adherent populations would also be expected to have been brighter, since the adherent population consists of primarily monocytes and macrophages (Figure 6). Again, there should not have been a signal for IFN-γ. There were no signals detected in the normal, non-adherent cell population which was as expected (Figure 6). A possible explanation for this is that pyocyanin alone is not immunogenic. It does not contain any mitogenic properties nor was it secreted by a bacterial cell *in vivo*. Conversely, LPS from gram negative bacterial cells stimulates the release of IL-1 and TNF-α (21, 62). In the group of animals treated with SRBC alone (Figure 6), one would expect that the signals for each cytokine (IL-1, TNF-α, and IFN-γ) should very bright when compared to the bands which appeared in the group that received media alone due to the fact that SRBCs are antigenic and therefore have a stimulatory effect on mouse splenocytes. A possible explanation for this is perhaps human error in the injection procedure of these animals, or

more likely, the RNA levels isolated were too low to be detected. However, without accurate representation from the positive control group (SRBC), one cannot draw any definite conclusions about the animals that were injected with SRBC and pyocyanin (1.0 μg) at the same time.

Figure 7 demonstrates that the group of animals treated with pyocyanin (1.0 μg) 12 h before SRBC, mediates decreased signals for each of the cytokines in each cell population as compared to SRBC alone, with the exception of TNF-α, which exhibited a strong signal in the non-adherent population of experimental animals. This indicates that perhaps pyocyanin is indeed suppressing cytokines necessary for an anti-SRBC response, namely those which act upon B-lymphocytes. However, again, due to the fact that the RNA results obtained in the control groups were not consistent with the literature or previous research, then a conclusive explanation cannot be drawn.

In order to ascertain whether or not message specific for key cytokines was expressed from enriched cell populations, i.e., macrophages, B cells or T cells, it is critical that pure populations of cells be used in the isolation of RNA. The techniques used in the preliminary study presented, involving either plastic adherence or Percoll gradients, allows for the enrichment of cells, such as monocytes and T lymphocytes. However, one must remember that these in no way represent pure cell populations. It is possible that some of the conflicting data presented could be accounted for by contaminating cells remaining in a specific highly "enriched" cell population. To avoid this problem in the future would require the use of a flourescence activated cell sorter (FACS) and monoclonal antibodies specific for each cell population. Ideally, FACS analysis would avoid some of the pitfalls previously mentioned, and would better answer the question as to whether pyocyanin diminishes the expression of key cytokines involved in the development of a humoral immune response.

An alternative approach to determining the mechanism of suppression observed with pyocyanin would be to evaluate the direct effect of this phenazine compound on normal function of key cell populations. For example, is it possible that pyocyanin, produced during an ongoing infection, might adversely affect the ability of macrophages to process and present antigen to T and B cells? Alternatively, could pyocyanin inhibit the differentiation and proliferation of mature B cells to antibody producing plasma cells? Similar to answering the cytokine question, this approach would require "pure" cell populations. The approach taken would involve obtaining spleen cells from normal mice, and isolating each specific cell type using FACS anaylsis. Having obtained a pure population, i.e., macrophages, these cells would then be cultured in the presence or absence of different concentrations of pyocyanin for varying time periods. Repeat this for each cell type being studied. These "treated" cell populations would then be co-cultured with the remaining two untreated cell populations. To these cell cultures, one would add antigen (S-RBCs) and incubate for four days. Following incubation, a standard hemolytic plaque assay would be performed. The total number of PFUs for each set of cultures would be quantitated, and compared to control values. If treatment of a specific cell population resulted in a reduced number of plaques, this would support the hypothesis that this compound directly inhibits normal cell function, thereby resulting in suppression of a normal immune response.

As an opportunistic pathogen, *Pseudomonas aeruginosa* is a major threat to those individuals whose immune system is compromised, including burn and cancer patients, as well as cystic fibrosis children. It has previously been reported that this organism is capable of producing numerous virulence factors, many of which may play a key role in the genesis of disease. This study illustrates that pyocyanin, a water soluble pigment, has a significant suppressive effect on the development of a humoral immune response. What is unclear is the exact mechanism involved in the observed suppression. Although

preliminary in nature, data presented raises the possiblity that one explanation might involve inhibiting the expression of messenger RNA specific for the synthesis of key cytokines, i.e., IL-1, TNF-α or IL-6. Although not immunogenic, pyocyanin appears to be involved in the pathogenesis of disease caused by this organism, and warrants future investigation. In particular, it would be important to ascertain whether pyocyanin affects both arms of the immune system, i.e., humoral as well as cellular immunity. A suppressive effect on T cell function could alter cytotoxic activity of T cells and macrophages, thereby increasing the incidence of cancer or infectious disease.

CHAPTER V
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