Studies of Host Defenses in Experimental Intra-Abdominal Sepsis: Effect of Immunomodulating Agents on Polymorphonuclear Leukocyte and Monocyte Function as Measured by Micro-Chemiluminescence and Microbial Killing Assays

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by
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STUDIES OF HOST DEFENSES IN EXPERIMENTAL INTRA-ABDOMINAL SEPSIS: EFFECT OF IMMUNOMODULATING AGENTS ON POLYMORPHONUCLEAR LEUKOCYTE AND MONOCYTE FUNCTION AS MEASURED BY MICRO-CHEMILUMINESCENCE AND MICROBIAL KILLING ASSAYS

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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ABSTRACT

Rat polymorphonuclear leukocytes (PMNL) elicited by an intraperitoneal injection of autoclaved rat caecal contents (ARCC) showed reduced levels of chemiluminescence (CL) and microbial killing when challenged with E.coli and B.fragilis and the E.coli/B.fragilis combination. Rat PMNL, which had been harvested from BCG,-C.parvum-and Levamisole-pretreated rats and elicited by ARCC, showed improved CL and microbial killing as compared to ARCC-elicited PMNL from untreated rats when challenged with E.coli. PMNL which had been harvested from BCG,-C.parvum-and Levamisole-pretreated rats also showed improved CL but were not able to show improved killing when challenged with B.fragilis.

Human peripheral blood PMNL showed high levels of CL when challenged with E.coli, B.fragilis and the E.coli/B.fragilis combination and these PMNL were able to kill both organisms equally well.

Human peripheral blood PMNL were pre-incubated with ARCC, ARCC supernatant, 1 x 10^9 dead E.coli or B.-fragilis, autoclaved human faecal contents (AHFC) OR AHFC supernatant and then challenged with live E.coli or B.-fragilis to determine which of these factors may promote the pathogenesis of intra-abdominal sepsis. PMNL pre-incubated with ARCC or AHFC showed the most inhibition of CL and microbicidal activity when challenged with E.coli or B.fragilis.

Pre-incubation of PMNL with 1 x 10^9 dead E.coli or B.fragilis, AHFC supernatant or ARCC supernatant showed moderate inhibition of CL and killing when challenged with E.coli or B.fragilis.

Rat macrophages, elicited by an intraperitoneal injection of 1% glycogen, show reduced levels of CL as compared to rat or human PMNL when challenged with E.coli, B.fragilis and the E.coli/B.fragilis combination.

Rat macrophages, elicited by glycogen, showed consistent killing of E.coli at one hour but not at two hours without the 10 minute stationary wait in the bactericidal assay. Consistent killing of E.coli at one hour or two hours was shown by glycogen-elicited rat macrophages with the 10 minute stationary wait. Macrophages which had been harvested from Levamisole-pretreated rats and elicited by glycogen showed better killing of E.coli than macrophages elicited by glycogen from untreated animals regardless of the 10 minute wait.

These studies have shown that there is a strong relationship between PMNL chemiluminescence and bactericidal activity. These studies have also shown that particulate components of sterile rat and human colonic material greatly inhibit human PMNL chemiluminescence and bactericidal function. Levamisole, C.parvum and BCG were shown to improve rat PMNL oxidative metabolism in terms of increased CL and these agents also increased rat PMNL bactericidal function when they were challenged with E.coli.

Levamisole also improves rat macrophage bactericidal function as compared to glycogen-elicited macrophages when challenged with *E. coli*.

INTRODUCTION

The events involving host responses to a mixed bacterial infection in intra-abdominal sepsis are not well characterized. Classical clinical teaching has directed physicians toward a mono-etiologic view of infectious disease. This view, however, cannot be applied to the study of intra-abdominal sepsis. In intra-abdominal sepsis, the infection site usually contains a multiplicity of bacterial species representing a simplified version of the initial complex inoculum. Clinical studies of intra-abdominal infections show there are an average of five microbial species at the site of infection with three anaerobic and two aerobic bacterial species present (Gorbach and Bartlett, 1974; Altemeier, 1942).

As well as the identification of the bacterial isolates, the study of a mixed bacterial intra-abdominal infection must involve the interactions of the bacterial isolates with each other and the immune defenses of the host. The lack of knowledge of these interactions has made it difficult for physicians to select the proper chemotherapeutic agents to treat this type of infection. The status of the immune defenses of the host is therefore, a very important factor in the outcome of an intra-abdominal infection.

Phagocytic cells represent the initial cellular response of the host to a bacterial infection. The ability of phagocytic cells to ingest and kill bacteria successfully may be of crucial importance in determining the course of an intra-abdominal infection. The role and microbicidal capabilities of the

polymorphonuclear neutrophil against bacteria have been well documented (Klebanoff, 1975).

The role of the macrophage in cellular immunity and in the resistance to microbial infection is a very complex and diverse one. Macrophages function in the processing of foreign antigens, in intractions with B and T lymphocytes and in tumor immunity. Macrophages also play a role in resisting such pathogens as viruses, protozoans and facultative intracellular bacteria such as Mycobacteria.

Normal, unstimulated macrophages are not able to kill facultative intracellular bacteria which replicate inside the macrophage. However, a population of macrophages exposed to this type of pathogen eventually acquire an enhanced, nonspecific microbicidal capability and are then able to kill many different types of bacteria besides the original intra-cellular pathogen to which they were exposed. Macrophages which display this enhanced microbicidal capability are referred to as activated. Activated macrophages also have the ability to kill neoplastic cells.

Organisms, such as Mycobacterium bovis strain Bacillus Calmette-Guerin (BCG), Corynebacterium parvum, and the antihelminthic drug, Levamisole, have been shown to lead to increased killing of ingested pathogens when administered to the host (Pineiro et al, 1977; Ratzan et al, 1972; Michael and Knight, 1975; Fischer et al, 1976).

Previous work has developed a male Wistar rat model for intra-abdominal sepsis which resembles the human infection in terms of acute peritonitis and subsequent abscess formation

(Weinstein et al, 1974; Onderdonk et al, 1974).

It was also found that a combination of Escherichia coli (facultative aerobe) and Bacteroides fragilis (obligate anaerobe) produced acute peritonitis and subsequent abscess formation in male Wistar rats implanted with this dual inoculum (Onderdonk et al, 1976). This dual inoculum, that produced the clinical disease in the rat, was used as a model for a mixed bacterial intra-abdominal infection in the present study.

Abscess formation, in the male Wistar rat model, can be seen as a host response leading to the localization and containment of an infectious process. Thus, abscess formation may be considered a beneficial host response. It has been shown that phagocytes form a layer in abscesses obtained from male mice (Joiner, 1980). From this evidence, it seems clear that phagocytes play some role in localizing this type of bacterial infection as well as a direct microbicidal role.

Since the biology of host responses to a mixed bacterial intra-abdominal infection is relatively unknown, the aims of the present study are threefold:

1. To investigate the microbicidal activity of the polymorphonuclear neutrophil (PMNL) against components of the infectious inoculum (E. coli and B. fragilis) used in the male Wistar rat with chemiluminescence and bactericidal assays.

2. To investigate the microbicidal activity of the macrophage against components of the infective inoculum used in the male Wistar rat with chemiluminescence and bactericidal assays.

3. To characterize the effects of BCG, Corynebacterium parvum and Levamisole upon the microbicidal activity of the PMN and macrophage against the components of the infective inoculum used in the male Wistar rat.

LITERATURE REVIEW

A. Importance of Host Resistance as a Determinant of Subsequent Infection in Surgical Patients

Despite improvements in surgical techniques, post-operative infection remains an incompletely resolved problem. Approximately 30% of hospital surgical deaths have had an infectious process (Meakins, 1981). Meakins (1981) has reviewed the importance of host resistance to infection in surgical patients in determining the outcome of an infectious episode. There are three determinants in the development of an infection: (1) the micro-organism(s) causing the infection, (2) the environment in which the infection takes place and (3) the host defense mechanisms. There is an interaction between these three factors which will determine whether or not an infection occurs.

In the normal state, the resident microbial flora prevents pathogenic organisms from invading and those that do invade are dispersed by environmental and systemic (immune) responses. Thus, there is a dynamic equilibrium between these determinants. When the equilibrium is upset, infection can be established. This can happen in a general surgical intensive care unit where the patient is exposed to antibiotic resistant bacteria in the setting of many environmental alterations and altered host defenses. Our understanding of the importance of host defenses in the prevention or control of infection is being continually refined.

A good example of the importance of host resistance to

infection is thermal injury. Despite the development of topical agents and new aspects of burn wound care to control the environment in which pathogenic bacteria multiply, patients still die of sepsis. Alexander and Meakins (1972) demonstrated that almost every component of host resistance to infection is affected by the burn injury. Immunoglobulins of all classes have been found in reduced levels following thermal injury in both adults and children (Ritzmann et al, 1969). Cell-mediated immunity is markedly affected after a burn injury. Both man and the experimental animal accepted allografts from unrelated donors and occasionally multiple donors with the grafts lasting as long as eight months (Kay, 1957). Neutrophil function is also altered after a major burn injury. Alexander and Meakins (1972) found that neutrophil bactericidal function is markedly abnormal following thermal injury and may be directly related to the development of septic episodes. Neutrophil chemotaxis was also found to be defective after a burn injury (Warden et al, 1975).

Maclean et al (1975) have documented the status of the immune system of a 13 year old boy who was admitted to hospital two months after surgery for perforated appendicitis and generalized peritonitis. At that time, he suffered from intra-abdominal abscesses requiring drainage, septicemia, respiratory failure and malnutrition. Despite repeated drainage of the abscesses, proper nutritional maintenance, proper antibiotics and the administration of transfer factor, the patient died. Blood cultures revealed the presence of

Staphylococcous epidermidis and Candida albicans. The patient displayed a consistent inability to localize and control infections.

The patient was found to be anergic to recall delayed hypersensitivity (DTH) antigens (mumps, PPD, Trichophyton, Varidase and Candida). Furthermore, the patient also accepted a skin graft without signs of rejection for 44 days and had a serum inhibitor of the mixed lymphocyte culture response of his own lymphocytes to allogenic lymphocytes.

Although the association of the absence of DTH and sepsis could be coincidental in any individual patient, population studies suggest this type of an association.

The data from Pietsch et al (1977) indicates that patients who became anergic (A) or relatively anergic (RA), as shown by a skin reactivity test, at any time during their hospital stay had a markedly increased probability of sepsis and mortality (Table I).

Table I

Sepsis and Mortality Following Initial Skin Test*

Patients Studied	Response	Number	Sepsis	Death
Preoperative (322)	A	21	4 (19.0%)	7 (33.3%)
	RA**	21	5 (23.8%)	7 (33.3%)
	Normal	280	13 (4.6%)	12 (4.3%)
Preoperative, post-trauma (115)	A	71	44 (62.0%)	24 (33.8%)
	RA	25	15 (60.0%)	6 (24.0%)
	Normal	19	5 (26.3%)	1 (5.3%)
No Surgery (83)	A	23	5 (21.7%)	11 (47.8%)
	RA	4	1 (25.0%)	1 (25.0%)
	Normal	57	0 (0.0%)	1 (1.8%)

* Pietsch et al (1977)
** RA = positive skin reaction to only one antigen

This observation has been confirmed by other investigators (McLoughlin et al, 1979; Johnson et al, 1979). However, the results of Brown et al (1982) suggest that the routine use of DTH skin testing in the pre-operative assessment of surgical patients is not justified. The group of patients in this study who had depressed skin reactions did not have significantly higher sepsis or mortality rates when compared with patients who had normal skin reactions.

Delayed hypersensitivity responses to recall antigens are considered to be a reflection of cell-mediated immunity. The infections for which this type of immunity is effective are intracellular parasites such as Salmonella, mycobacteria and Listeria monocytogenes. The organisms that have produced sepsis in anergic patients are common gram-negative and gram-positive bacteria suggesting that other parts of the immune system are affected in the anergic state (Pietsch et al, 1977). Lymphocyte function in terms of mixed lymphocyte culture responses, lymphocyte response to phytohemaglutinin, cell-mediated lympholysis and lymphocyte generation of blastogenic factor was unaffected in anergic patients (Christou and Meakins, 1979a). Neutrophil chemotaxis was reduced in patients with altered skin responses and returns toward normal with the recovery of normal skin responses (Christou and Meakins, 1979b). These authors found that neutrophil chemotaxis was reduced to anergic levels within hours following a major injury. The prompt appearance of defective neutrophil chemotaxis indicated that it may be mediated by a serum factor. Anergic serum was found to

consistently inhibit the chemotaxis of normal neutrophils (Christou and Meakins, 1979c). These authors found that 69% of the 254 surgical patients studied had a poor clinical outcome which correlated with skin test anergy and decreased neutrophil chemotaxis. Lymphocyte chemotaxis is also decreased by anergic serum (Christou and Meakins, 1979a). Superina et al (1980) have produced in vivo evidence which seems to support the in vitro evidence that decreased neutrophil and lymphocyte chemotaxis in anergic patients contributes to the establishment of infection. These authors demonstrated that anergic patients and those with low neutrophil chemotaxis have a reduced delivery of inflammatory cells into a skin window.

Malnutrition has long been associated with decreased host resistance. Studley (1936) observed that patients who had a preoperative loss of more than 20% of their body weight prior to surgery had a postoperative mortality rate ten times that of better-nourished patients. Furthermore, Rhoads and Alexander (1955) showed that hypoproteinemia was associated with an increased incidence of postoperative infections.

Anergy which is associated with malnutrition often is reversible with nutritional therapy (Pietsch et al, 1977). This fact supports the concept that malnutrition is associated with an increased risk of sepsis. Daly et al (1978) found that protein-calorie malnutrition leads to a depression of host cell-mediated immunity in rats. These authors also found that nutritional repletion of the rats initially results in rapid weight gain followed by a more gradual return of immunocompetence.

The study of Law et al (1974) also found depressed cell-mediated and humoral immunity in young adult rats after being fed a protein-free diet for six weeks.

B. Intra-Abdominal Sepsis

1. Natural History of Untreated Disease

Intra-abdominal infection frequently causes prolonged morbidity and high mortality in humans (Gorbach and Bartlett, 1974). The development of an animal model simulating large bowel perforation with subsequent abscess formation is necessary to be able to study intra-abdominal sepsis in terms of bacteriology, immunology, and response to antimicrobial therapy. Early attempts at producing this type of animal model relied on surgical techniques such as devascularization of a segment of intestine or appendiceal ligation (Coridis et al, 1969; Hovhanian and Saddowi, 1972; King et al, 1975; Rosato et al, 1972; Wright et al, 1971).

The problems of these animal models included highly variable results and the uniform mortality produced when the experiment was successful. These experiments were difficult to control and not very reproducible.

Other attempts at producing a successful model have utilized a direct intraperitoneal needle injection of pure bacterial cultures (Browne, 1967; Browne and Leslie, 1976) or faecal material (Deysine et al, 1967; Sleeman et al, 1969; Smith and Hazard, 1970). The problems associated with these techniques centered around the difficulty in determining whether the

injection was placed correctly in the free peritoneal cavity or whether it ended up in the abdominal wall or the intestinal lumen or wall. The injection culture was also not quantified which led to difficulties in reproducing the results.

A male Wistar rat model was developed by Weinstein et al (1974) in which the bacteriological and pathophysiological findings were similar to those observed in patients with intestinal perforation. Gelatin capsules with pooled rat colonic contents and barium sulfate were surgically implanted into the pelvic region of Wistar rats. The inoculum was later refined to a dual inoculum of E. coli and Bacteroides fragilis plus autoclaved caecal contents and barium sulfate (Onderdonk et al, 1976). The consistent results and endpoints produced by this model enable an accurate description of the untreated disease to be made.

The results, using this model, indicate that there are two distinct stages with well defined pathological and bacteriological patterns (Figure I).

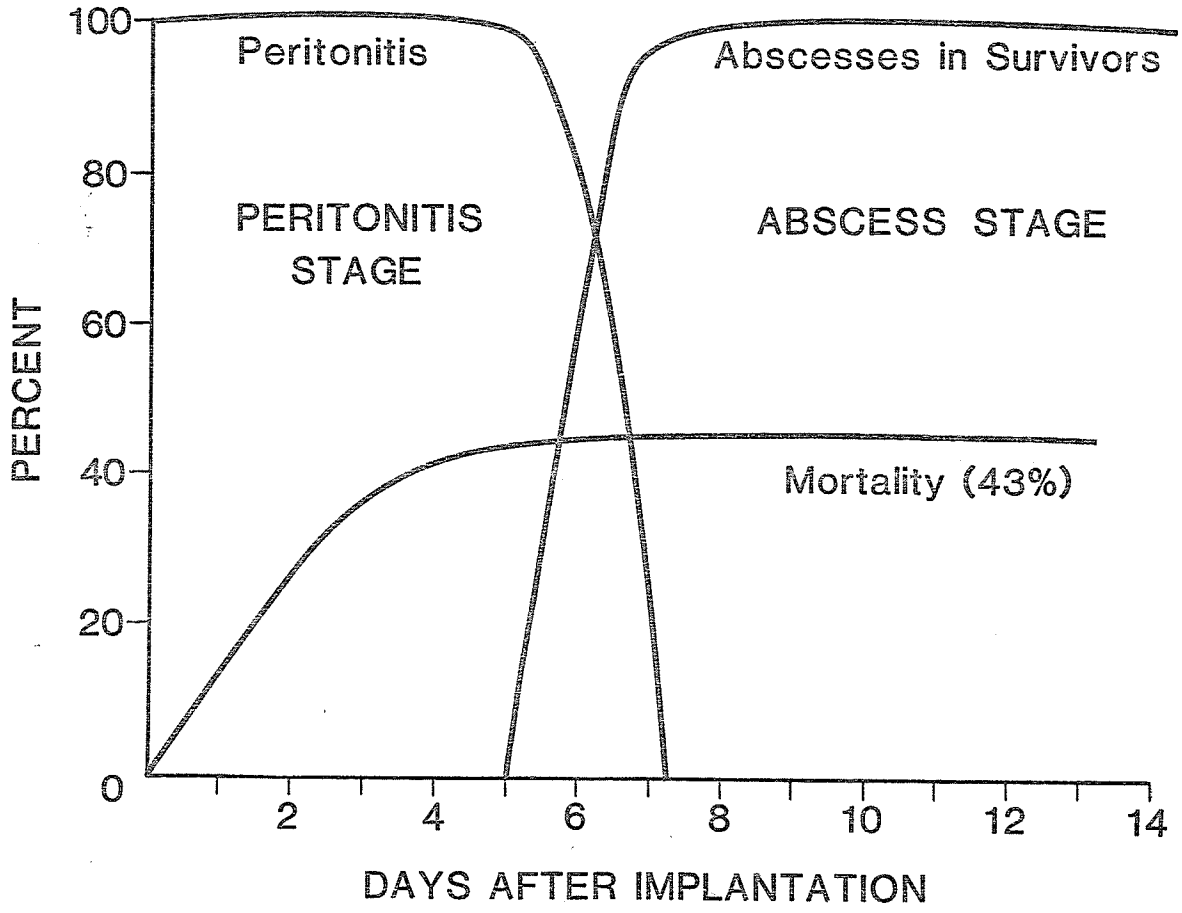


Figure I : Mortality and Abscess Formation in 106 Male Wistar Rats Receiving Inoculum Obtained from Meat-Fed Rats[1]

1. Weinstein et al, 1974

There is an early, acute stage characterized by generalized peritonitis and a high mortality rate during the first three to five days after implantation of the dual inoculum of E. coli and B. fragilis (Onderdonk et al, 1976). The early peritonitis stage was followed in all survivors by an abscess forming stage by the seventh day after implantation. The abscess forming stage followed an indolent, nonlethal course.

Nichols et al (1978) modified the model of Weinstein et al (1974) by using human faecal material instead of rat colonic contents. The authors felt that the use of human faecal material allowed for the development of a wider spectrum of intra-abdominal sepsis more similar to that observed in humans. The authors also felt that the use of human faecal contents in the gelatin capsule more closely approximated the numbers of organisms released when the human colon is perforated.

2. Bacteriology of Intra-Abdominal Sepsis

The bacterial infection that arises from colonic perforation is polymicrobial in nature. Clinical studies of intra-abdominal sepsis show there are an average of five microbial species at the infection site with three anaerobic and two aerobic bacterial species present (Gorbach and Bartlett, 1974; Altemeier, 1942).

Onderdonk et al (1974) have attempted to characterize the bacteria present during both stages of the diesease produced in the intra-abdominal sepsis model of Weinstein et al (1974). During the initial, often lethal, peritonitis stage, Escherichia coli, enterococci, and Bacteroides fragilis were always present. Blood cultures obtained during this stage were

uniformly positive, with E. coli being the main isolate.

The major bacterial isolates in the intra-abdominal abscesses were B. fragilis and Fusobacterium. E. coli and enterococci were also present but in lesser concentrations. Rank order analysis of these four bacterial species showed that the facultative aerobes (E. coli and enterococci) outranked the anaerobes (B. fragilis and Fusobacterium) during the acute peritonitis phase of the disease. The anaerobes were found to outrank the aerobes during the abscess forming stage of the disease. Several bacteria, (Eubacterium, Clostridium perfringens, Lactobacillus, Micrococcus, Corynebacterium and alpha-hemolytic streptococci) which were present in large numbers in the original faecal inoculum, were not isolated after the implantation of the inoculum. This represented a major simplification of the original complex inoculum.

Further work was done by Onderdonk et al (1976) to establish the roles of E. coli, B. fragilis, enterococci, and Fusobacterium varium in intra-abdominal sepsis. All four organisms were tested individually and in combination for their ability to produce abscesses and mortality in male Wistar rats. Mortality was restricted to animals which had received E. coli alone or in combination with the other three species of bacteria. This finding indicated that the acute peritonitis phase of intra-abdominal sepsis was due to gram-negative coliforms such as E. coli. Abscesses were produced in 94% of animals that received the combination of an anaerobe and a facultative aerobe. There were no abscesses formed when any single organism was used or

when the combination E. coli and enterococci was used.

Abscess formation was also detected in only one of nineteen rats receiving B. fragilis and F. varium. These results suggested that synergy was needed between anaerobes and facultative aerobes for intra-abdominal abscess formation.

Subsequent studies (Onderdonk et al, 1977) have shown that the strain of B. fragilis used in studying the Weinstein rat model was unencapsulated. The majority of pathogenic, clinical B. fragilis isolates have been found to possess a lipopolysaccharide capsule (Kasper and Seiler, 1975). The Weinstein rat model, when implanted with an encapsulated strain of B. fragilis (ATCC23745), produced discrete abscesses (Onderdonk, 1977). Inplantation with the nonabscessogenic rat strain of B. fragilis did not result in abscess formation unless it was combined with a facultative aerobe.

Abscesses were also produced when purified, B. fragilis capsular material alone or in combination with the non-abscessogenic rat strain was implanted into male Wistar rats. These results show that the capsular polysaccharide of B. fragilis appears to potentiate abscess formation and may represent a virulence factor in the pathogenesis of intra-abdominal sepsis. The demonstration of serum antibody to B. fragilis capsular polysaccharide in male Wistar rats which were implanted with an encapsulated strain of B. fragilis further supports this idea (Kasper et al, 1977).

3. Factors which Influence Host Response to Intra-Abdominal Sepsis

There is evidence to indicate that the successful outcome of intra-abdominal sepsis depends upon the early, efficient clearance of micro-organisms by phagocytically active cells (Hau et al, 1978).

If the bacterial inoculum is not eliminated, the influx of fluid and cells into the peritoneal cavity can become self-defeating. Substances produced by leukocytes in the peritoneal exudate can actually promote the growth of bacteria if a significant number of organisms survive the initial clearance. Thus, it seems that failure to control peritoneal contamination may result from inefficient delivery of phagocytic cells to the infection site giving rise to a late phase of bacterial proliferation (Flint et al, 1981). Lee and Ahrenholz (1979) showed that hemoglobin and hemoglobin breakdown products prevent phagocytic activity by peritoneal macrophages. Berman et al (1974) reported that soluble factors, possibly endotoxin, within peritoneal exudates contribute to mortality in experimental peritonitis.

Other substances produced by bacteria and cellular components of the inflammatory exudate act as adjuvants and depress chemotaxis of phagocyte cells (Ohlsson, 1976).

Recently, Flint et al (1981) have shown in vitro that sterile human faecal material and filtered peritoneal exudate abolished the microbicidal capabilities of rat, peritoneal phagocytes against E. coli and Staphylococcus aureus. These authors speculated that the filtered peritoneal exudate may contain endotoxin which could inhibit the microbicidal capabili-

ties of rat phagocytes. They also thought that the sterile faecal material may contain endotoxin or may physically inhibit the interaction between phagocyte surface receptors and organisms by allowing bacteria to adhere to faecal particles or by fostering fibrin deposition.

Another factor that may influence host responses to intra-abdominal sepsis was reported by Ingham et al (1977). These authors reported that obligately anaerobic bacteria such as B. fragilis impaired the ability of human leukocytes to deal effectively with facultative bacterial species in an in vitro system. This finding was seen to be an example of synergism between obligately anaerobic bacteria and facultative aerobic bacteria in intra-abdominal sepsis. The work of Kelly (1978) with an in vivo guinea pig model supported the hypothesis of a synergism between anaerobic and facultative aerobic bacteria. Kelly showed that small infective doses of pure E. coli or pure B. fragilis failed to produce inflammation in subcutaneous wounds of guinea pigs while an inoculum containing the same number of organisms, half of which were E. coli and half B. fragilis, produced striking inflammation with the formation of pus.

Reznikov et al (1981), however, were not able to show this synergy in the peritoneal cavity of a mouse. The introduction of B. fragilis into the peritoneal cavity with E. coli did not inhibit clearance of either organism. There have also been investigations of factors that influence subcutaneous abscess formation in the mouse due to B. fragilis or

S.aureus (Joiner et al, 1980). It was shown that the activity of the alternate complement pathway may influence abscess size and that chemotactic factors other than the fifth component of complement (C5) can supervene in C5 deficient mice to produce encapsulated abscesses.

C. Polymorphonuclear Leukocytes

1. Oxidative Metabolism

Babior (1978) has reviewed those aspects of polymorphonulear leukocyte (PMNL) oxidative metabolism which may have a role in bacterial killing by PMNLs. Bacterial killing involves an array of mechanisms which are set into motion by two cellular events: degranulation and the initiation of the respiratory burst. Degranulation is the process of fusion between the primary phagosome and the granules present in the phagocyte cytoplasm. These granules possess substances that participate in bacterial killing and degradation. During degranulation, these materials are discharged into the vesicle containing the ingested microbe. The respiratory burst describes a metabolic pathway, dormant in resting cells, whose function is to produce a group of highly reactive microbicidal agents by the partial reduction of oxygen. Killing is brought about through the actions of both the granule contents and the oxidizing agents provided by the respiratory burst.

Human PMNLs or neutrophils, have three types of granules (Drutz and Mills, 1980). The primary granules contain abundant hydrolytic lysosomal enzymes, large amounts of myeloperoxidase,

lysozyme, elastase and cationic proteins. Secondary granules, which are smaller than primary granules, contain lactoferrin and lysozyme. Tertiary granules have the enzyme acid phosphatase and have no known microbicidal function.

As the phagosome forms during microbial engulfment, secondary granules undergo violent movement in proximity to the phagosome, fuse with the phagocytic vacuole, and disappear from the cytoplasm (degranulate). Somwehere between the stages of attachment and digestion, the membranes of specific granules begin to fuse with nascent phagosomes. When this happens, lactoferrin and lysozyme are allowed to enter the extracellular space. Primary granules fuse with the phagosomes slightly later; the contents of these granules tend to remain in the phagolysosome. Microfilaments and microtubules may be important in the fusion of phagosomes and granules.

There are a series of coordinated metabolic events that take place in the respiratory burst when phagocytes are exposed to appropriate stimuli. This group of events underlies all oxygen-dependent killing by phagocytes. The first important event was the discovery of the sharp increase in oxygen uptake that occurs upon stimulation of the phagocyte (Baldridge and Gerard, 1933). Sbarra and Karnovsky (1959) showed that the oxygen consumed in the respiratory burst was not used for energy production. Iyer et al (1961) demonstrated that part of the oxygen consumed in the respiratory burst was converted into hydrogen peroxide (H_2O_2), which they detected in the medium surrounding the stimulated phagocytes. They suggested that the H_2O_2 was used

by the phagocyte as a bactericidal agent and were the first to draw a connection between the respiratory burst and the microbicidal mechanisms of phagocytes.

There is also an increase in glucose oxidation via the hexose monophosphate shunt during the respiratory burst (Sbarra and Karnovsky, 1959; Iyer et al, 1961). The hexose monophosphate shunt is a metabolic pathway in which glucose is oxidized to carbon dioxide and a five-carbon sugar, with $NADP^+$ serving as an electron acceptor. In the neutrophil, glucose oxidation by this pathway is limited by the rate at which $NADPH^+$ becomes available through the oxidation of NADPH (Beck, 1958). Shunt activation therefore means that the oxidation of NADPH TO $NADP^+$ increases during the respiratory burst.

The last metabolic event that has been discovered has been the production of the superoxide anion (O_2^-) during the respiratory burst (Babior et al, 1973).

There are various agents, both particulate and soluble, which are able to initiate the respiratory burst (Cheson et al, 1977). Particulate activating or initialing agents include opsonized bacteria, zymosan and latex spherules, which do not require opsonization for activity. Some of the soluble activating agents include phorbol myristate acetate, a variety of ionophores, the complement enzyme C5a, and fluoride ion. Activation, which usually follows exposure to the stimulus by thirty to sixty seconds (Root et al, 1975), requires neither phagocytosis nor degranulation, but simply the contact of the stimulus with the phagocyte surface (Goldstein et al, 1975;

Curnutte et al, 1977). The activation of the respiratory burst also appears to be an energy-requiring event (Cohen and Chovaniec, 1978). The molecular basis of activation is not well characterized.

The nature of the enzyme responsible for the primary oxygen-consuming reaction of the respiratory burst has been a very controversial question. There is agreement that its enzyme catalyzes the reduction of oxygen to O_2^- by a reduced pyridine nucleotide. The disagreement centers on whether the physiologic electron donor is NADH OR NADPH. Babior (1978) believes that the evidence favors NADPH as the physiologic electron donor. The evidence for NADPH was first described by Iyer and Quastel (1963). The enzyme that would use NADPH as the electron donor has been given the name "NADPH oxidase". The reaction that it would catalyze is as follows:

$$2O_2 + NADPH \longrightarrow 2O_2^- + NADP^+ + H^+$$

Babior (1978) has termed this reaction as the "O_2^- forming activity". Several studies have yielded indirect evidence which suggests that this activity is associated with the plasma membrane (Goldstein et al, 1977; Briggs et al, 1977).

There are three observations which favor NADPH as the physiologic electron donor. The first is that the Michaelis constant for NADPH is lower than that for NADH, indicating a greater affinity of the enzyme for the former (Babior et al, 1976). The second is that when neutrophils are stimulated, NADPH levels fall and $NADP^+$ levels rise, whereas NADH and NAD^+ levels are unchanged (Selvarij and Sbarra, 1967). The last

observation is that in patients with severe glucose-6-phosphate dehydrogenase (G-6-PD) deficiency, the neutrophil respiratory burst is greatly diminished (Cooper et al, 1972). This observation would be difficult to explain on the basis of an NADH- requiring respiratory burst, since G-6-PD defiency would lead to the serious depletion of NADPH but should have a much smaller effect on NADH, which is mainly provided by glycolysis (Babior, 1978). Studies, which lend support to the idea of an "NADH oxidase" are based on the finding that preparations from cells of patients with chronic granulomatous disease (CGD) showed defective oxidation of NADH (Baehner and Karnovsky, 1968; Baehner and Nathan, 1968; Segal and Peters, 1976). This defect, however, was only partial which conflicts with the complete absence of respiratory burst activity in the intact cells (Babior, 1978). In two of these studies (Baehner and Karnovsky, 1968; Segal and Peters, 1976), the experiments were carried out only with preparations from resting cells raising doubts about the implications of the observed activities in terms of the respiratory burst (Babior, 1978).

2. Oxygen-Dependent Antimicrobial Systems

(i) Superoxide Anion

The discovery that O_2^- was produced by phagocytes gave rise to the idea that this substance might, itself be a microbicidal agent (Babior et al, 1973). This proposition has lost support as the result of investigations on the microbicidal activity of artificial O_2^- generating systems (Babior et al, 1975; Mandell, 1975; Gregory and Fridovich, 1974; Gregory

et al, 1973). These studies showed that, except under unusual circumstances (Gregory and Fridovich, 1974; Gregory et al, 1973), O_2^- contributed very little to bacterial killing by those systems. Babior et al (1975) showed that local rates of O_2^- production in these systems are orders of magnitude smaller than the rates characteristic of stimulated phagocytes and at the very large fluxes generated in the vicinity of the phagosome, O_2^- may be a useful antimicrobial agent.

(ii) Hydrogen Peroxide and Myeloperoxidase

H_2O_2 is one of the oxidizing substances utilized for microbicidal action by phagocytes. This compound is produced by the reaction of O_2^- with itself, either spontaneously or under catalysis by superoxide dismutase:

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

H_2O_2 alone, has some microbicidal potency and Drath and Karnovsky (1974) have proposed that H_2O_2 acts with ascorbic acid and certain metal ions to kill ingested bacteria by non-enzymatic means. The microbicidal capability of H_2O_2 is greatly enhanced through the action of myeloperoxidase which is present in most phagocytic cells.

Myeloperoxidase (MPO), a hemoprotein with a molecular weight of approximately 150,000, is present in the azurophilic granules of neutrophils and monocytes but not eosinophils. MPO catalyzes the oxidation of halide ions to hypohalite ions by H_2O_2:

$$X^- + H_2O_2 \longrightarrow XO^- + H_2O$$

Cl^-, Br^- and I can all be oxidized by this enzyme (Klebanoff, 1967; Harrison and Schultz, 1976). It seems likely that Cl^- is

the physiologic substrate since it is the most abundant halide in the cell. Thyroid hormones are also oxidized by MPO (Klebanoff, 1967).

There are several postulated mechanisms for the bactericidal action of the MPO-halide-H_2O_2 system. The first and most widely studied mechanism involves the halogenation of the bacterial cell wall. Early experiments with an artificial, in vitro MPO-halide-H_2O_2 system, using I^- as the halide ion, showed incorporation of iodine into the bacterial cell wall (Klebanoff, 1967). Later work showed that Cl^- can also be incorporated into bacteria by whole neutrophils and an in vitro MPO-halide-H_2O_2 system (Zgliczynski and Stelmaszynska, 1975). These investigators thought that bacterial death resulted from the loss of integrity of the halogenated cell surface. Some doubt has been cast on this mechanism by the work of McCall et al. (1971) who showed that the correlation between halogenation and bacterial death is only fair.

The second mechanism is based on the finding that the MPO-halide-H_2O_2 system is able to decarboxylate amino acids converting them to aldehydes, CO_2 and NH_3 (Zgliczynski et al, 1968; Strauss et al, 1970):

$$R\text{-}CHNH_2\text{-}COOH \longrightarrow R\text{-}CHO + CO_2 + NH_3$$

Strauss et al (1971) proposes that this mechanism works because the amino acid components of the bacterial cell wall are degraded leading to disruption of the bacterial surface and cell death. Zgliczynski et al (1968) believe that the bacteria are killed by the toxic aldehydes produced in this reaction although

Paul et al (1970) have produced evidence that these aldehydes were not toxic enough to account for the bacterial killing they observed.

The most recent hypothesis proposes that the MPO-halide-H_2O_2 system kills bacteria by means of singlet oxygen.

(iii) Hydroxyl Radical

The hydroxyl radical (OH·) is a highly unstable oxidizing species that reacts almost instantaneously with most organic molecules that it encounters. Phagocytes seem to be able to generate OH· which may participate in the microbicidal activity of phagocytes. The first hint that OH· might be produced by phagocytes arose from studies of bacterial killing by xanthine (or purine) plus xathine oxidase (Babior et al, 1975; Gregory and Fridovich, 1974; Gregory et al, 1973). Xanthine and xanthine oxidase form an enzyme system that produces O_2^- in large amounts and secondarily gives rise to H_2O_2 by the spontaneous dismutation of the enzyme-generated O_2^-. These studies showed that the killing of several bacterial species was abolished by either superoxide dismutase or catalase. This finding indicated that the bactericidal agent was neither O_2^- nor H_2O_2 but rather was the product of a reaction between them since the destruction of either was sufficient to stop killing. Earlier work had suggested that O_2^- and H_2O_2 could react to form OH· by the Haber-Weiss reaction (Beauchamp and Fridovich, 1970):

$$O_2^- + H_2O_2 \longrightarrow OH\cdot + OH^- + O_2$$

On this evidence, OH· was proposed as the bactericidal agent

in the xanthine oxidase system.

Two researchers have more recently presented direct evidence for $OH^{\cdot}$ production by phagocytes (Weiss et al, 1977; Tauber and Babior, 1977). The evidence is based on the fact that $OH^{\cdot}$ reacts with methional to release ethylene (Beauchamp and Fridovich, 1970). It has been shown for both neutrophils and monocytes that ethylene is released from methional during the respiratory burst which suggests that there is $OH^{\cdot}$ produced by the phagocytes. The fact that the generation of ethylene is inhibited by ethanol, mannitol and benzoic acid which are known to react with $OH^{\cdot}$, provides further proof that the oxidation of methional to ethylene by phagocytes is mediated by $OH^{\cdot}$.

The research of Weiss et al (1978) indicates that $OH^{\cdot}$ may be formed in phagocytes by a reaction between O_2^- and a peroxy compound:

$$O_2^- + R\text{-}OOH \longrightarrow OH^{\cdot} + OR^- + O_2$$

The precise role of OH in bacterial killing still remains to be defined.

(iv) Singlet Oxygen

Singlet oxygen ($^1O_2$) is a highly reactive oxygen species which has been implicated in the microbicidal activity of phagocytes. Although $^1O_2$ has the same molecular formula as atmospheric oxygen, it differs in the distribution of electrons around the two oxygen nuclei (Kearns, 1971). In atmospheric oxygen, the electrons form a cylindrical cloud whose axis is the line joining the nuclei while this electron cloud is distorted away from a cylindrical configuration in $^1O_2$. This distortion

makes $^1O_2$ far more reactive than atmospheric oxygen and allows $^1O_2$ to attack a great variety of double bond-containing compounds that are relatively inert to atmospheric oxygen. Thus $^1O_2$ is capable of inflicting lethal damage on biologic systems on contact (Babior, 1978).

There are several agents that are produced in the phagocyte respiratory burst that could react together to form $^1O_2$. OCl^-, a product of the myeloperoxidase-halide-H_2O_2 system, is known to react with H_2O_2 to produce $^1O_2$ (Seliger, 1960):

$$OCl^- + H_2O_2 \longrightarrow {}^1O_2 + Cl^- + H_2O$$

The oxygen produced in the Haber-Weiss reaction is thought to be $^1O_2$ (Kellogg and Fridovich, 1975):

$$O_2^- + H_2O_2 \longrightarrow {}^1O_2 + OH^\cdot + OH^-$$

Khan (1970) believes that the spontaneous (but not enzyme-catalyzed) dismutation of O_2^- gives rise to $^1O_2$ although there is some controversy surrounding this point:

$$2O_2^- + 2H^+ \longrightarrow {}^1O_2 + H_2O_2$$

Although there are many possible sources for the production of $^1O_2$ during the phagocyte respiratory burst, there is no direct, conclusive proof that phagocytes produce $^1O_2$.

3. PMNL Chemiluminescence

Chemiluminescence (CL) is a phenomenon which is related to the respiratory burst in neutrophils. CL is believed to be due to the production of an electronically excited molecular species which upon decay produces a pulse of light. The emission of light by stimulated phagocytes was first noticed by Allen et

al (1972). The light emission, which is at very low levels, can be measured by a liquid scintillation counter operated in the off-coincidence mode. The levels of light emission vary widely from stimulus to stimulus (Babior, 1978).

Respiratory-burst activity is necessary for CL to take place, as indicated by the fact that normal resting cells emit no light, nor is it emitted by either resting or simulated cells of patients with chronic granulomatous disease (Stjernholm et al, 1973). Superoxide dismutase and catalase also abolish light emission by stimulated phagocytes (Webb et al, 1974; Cheson et al, 1976). The mechanism of CL is however, still not clearly defined.

Originally, Allen et al (1972) thought the emission of light by stimulated PMNL meant that they were producing $^1O_2$ during the respiratory burst. $^1O_2$ could cause CL by reverting spontaneously to atmospheric oxygen, a process that releases a pulse of light, or by oxidizing a second molecule to form a light-emitting product. Many oxidizing species, however, could generate CL by the latter process (Babior, 1978). Specifically H_2O_2 and O_2^- are both able to oxidize other substances to form light-emitting species. Thus, the existence of CL does not constitute proof for the production of $^1O_2$ as the properties of the emitted light must also be taken into consideration. Cheson et al (1976) showed that CL occurred at all visible wavelengths in contrast to the red emission characteristic of the $^1O_2$ to atmospheric oxygen transition. These authors also showed that light emission by stimulated PMNL would not occur

unless an oxidizable species was also present. These findings indicate that CL results from the oxidation of the stimulating agent by unspecifiable products of the respiratory burst. The generation of $^1O_2$ is neither proved nor disproved by CL. Light emission by phagocytes is a nonspecific manifestation of the respiratory burst (Babior, 1978).

The CL assay, itself is sensitive to many experimental variables. Andersen and Amirault (1979) have elucidated the effects of some of these experimental variables upon the level of CL generated by dog PMNL. They found that the temperature of the environment during the isolation of the phagocytes had a marked effect of the level of CL. CL was much greater when the PMNL were kept at 4 degrees C during the isolation procedure than when the cells were at 37 degrees C throughout the procedure. The best temperature for the CL assay itself was 37 degrees C.

These authors also found that PMNL aggregation decreases the level of CL generated by the phagocytes. PMNL aggregation was reduced by:

a) reducing the centrifugation and mixing forces used to prepare cell suspensions

b) preparing phagocyte suspensions at 4 degrees C in a Ca^{-+2} and Mg^{-+2} free cell media containing divalent cation chelating agents

c) lysing red blood cells by the NH_4Cl technique

Andersen and Amirault (1979) showed that the type of cell

media used during the CL assay was important. Solutions containing complex mixtures of amimo acids and vitamins gave markedly reduced levels of CL. Tryptophan, ascorbic acid, iron salts and all proteins tested caused significant reduction of CL. Glucose, Ca^{+2} and Mg^{+2} in the final reaction mixture were necessary for maximal CL response.

4. The Relationship of PMNL Chemiluminescence to PMNL Bactericidal Activity

The relationship between neutrophil CL and neutrophil bactericidal activity is a strong, but not absolute one. Horan et al (1982) studied this relationship by interfering with the microbicidal chemistry of neutrophils by exposing them to a variety of pharmacological agents. The anti-inflammatory agent Dimethyl Sulfoxide, showed a concentration-dependent inhibition of PMNL function in terms of CL and bactericidal activity against S.aureus. Phenothiazines, which inhibit O_2^- production, were found to completely inhibit neutrophil CL and killing at a concentration of 0.30 mM. Sulfhydryl group inhibitors (NEME, PCMBSA) produced a noticeable inhibition of PMNL CL and killing when the PMNL were challenged with S.aureus. Cytochalasin-B reduced PMNL phagocytic activity, CL and bactericidal activity. Cyclic AMP regulators (theophyllin and histimine) caused a suppression of PMNL CL and bactericidal activity. A narcotic, Levorphanol, also caused a marked reduction in PMNL CL and bactericidal activity. The authors also showed that relative microbial survival curves increased with decreasing photon yield from phagocytizing PMNL. They concluded from the failure of their

experiments to seperate PMNL CL from PMNL bactericidal activity that the relationship between CL and bacterial killing was a strong one.

Ewetz et al (1981) also evaluated the relationship between PMNL CL and PMNL bactericidal activity. They found that the initial rate of increase of CL and the initial rate of killing of bacteria (S. aureus) were well correlated whereas the correlation was poorer for later stages of the process. When the rate of the bactericidal process was varied by changing concentrations of bacteria and PMNL, directly proportional variations of initial rates of CL increase were observed. The authors thought this last finding was a reflection of an accumulation of oxidizing radicals as the result of a phagocytosis-dependent, gradual activation of the NADPH oxidase system. Ewetz et al (1981) showed that thermal inactivation of PMNL let to diminished CL whereas killing remained unchanged. This finding showed that the two processes could be disassociated.

Grebner et al (1977) found that when serum was heated, chelated with ethylene glycol tetra-acetic acid or adsorbed with zymosan, there was a reduction of bactericidal activity for S. aureus and a comparable reduction of CL activity. In contrast, Salmonella typhimurium was successfully killed in serum treatedwith MgEGTA (complement-alternate pathway intact, classic pathway blocked) and CL was only reduced by twenty percent under the same conditions. Both PMNL CL and killing responses for S. typhimurium were abolished when serum was heated at 56 degrees C or adsorbed with zymosan. There was also a 30% to 50% reduction

in killing of S.aureus and a 50% reduction in CL when PMNL were exposed to S.aureus using EGTA chelated, MgEGTA chelated, zymosan adsorbed or heated serum. The authors showed that very similar conditions are necessary for the successful opsonization of certain bacterial species as well as PMNL CL and bactericidal activity. They suggested that the oxidative metabolisms from which bacterial killing and PMNL CL arise may be closely related or interdependent.

D. Macrophages

1. Oxidative Metabolism

Macrophages are not easy to obtain in high yield and reasonably free of other cell types (Johnston, 1978). Consequently, studies of their oxidative metabolism have lagged behind those of neutrophils. It is known, however, that macrophages undergo a phagocytosis-associated respiratory burst. Early studies indicated there was increased oxygen consumption and hexose monophosphate shunt activation during phagocytosis by guinea pig peritoneal macrophages elicited by intraperitoneal injection of casein or glycogen (Oren et al, 1963; Stahelin et al, 1956). These findings have been confirmed by other investigators with rabbit, guinea pig and mouse peritoneal macrophages (Karnovsky et al, 1975; Rossi et al, 1975).

There has also been increased generation of H_2O_2 during phagocytosis with these same cell types (Nathan and Root, 1977) but this has not been observed in rat or rabbit alveolar macrophages (Biggar and Sturgess, 1978; Tsan, 1977). O_2^-

production has been shown in guinea pig and mouse macrophages (Drath and Karnovsky, 1975; Johnston et al, 1978).

Several investigators have cast doubt on the role of iodination in macrophage microbicidal metobolism, at least in mouse peritoneal and rabbit alveolar macrophages, since these cells lack the capacity to iodinate bacteria they efficiently kill (Bigger et al, 1976; Simmons and Karnovsky, 1973).

Macrophages do generate CL, but the level of light production is much less than that generated by PMNL (Allen and Loose, 1976; Hatch et al, 1978). Superoxide dismutase abolishes the CL generated by phagocytizing guinea pig peritoneal and human alveolar macrophages indicating the importance of O_2^- to CL generation in these cells (Beall et al, 1977; Hatch et al, 1978).

Like neutrophils, guinea pig and rabbit peritoneal and alveolar macrophages contain NADPH oxidase activity in a particulate fraction from cells disrupted during phagocytosis (Rossi et al, 1975). This particulate fraction can generate H_2O_2 in the presence of NADPH. Human monocytes have also been shown to release $OH^{\cdot}$ during phagocytosis (Weiss et al, 1977).

Soluble substances capable of plasma membrane perturbation, such as phospholipase C and phorbol myristate acetate also elicit manifestation of the macrophage respiratory burst (Johnston et al, 1977; Drath and Karnovsky, 1975).

2. Activated Macrophage

The concept of the activated macrophage has developed from

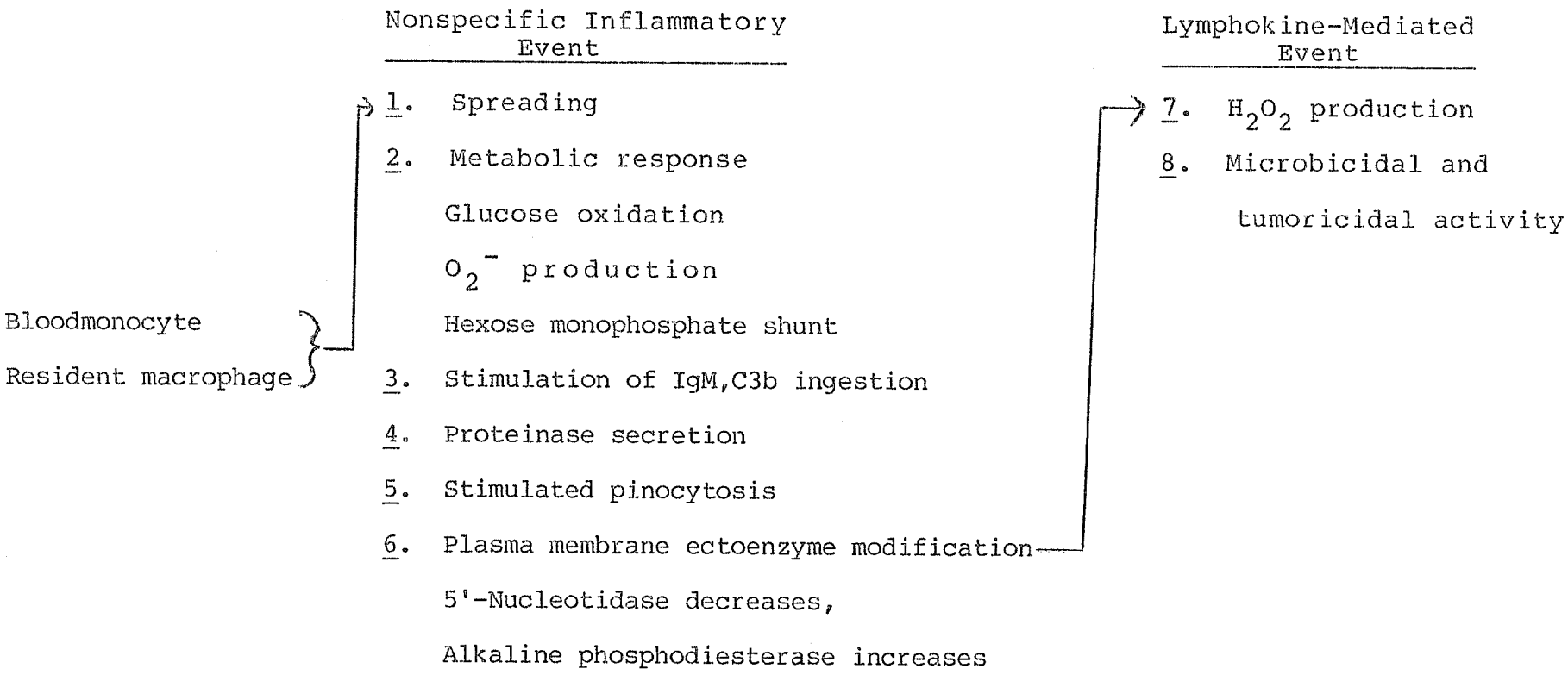
the observation that macrophages from animals infected with certain intracellular pathogens (ex. Listeria monocytogenes) could inhibit the replication of these organisms whereas normal macrophages cannot. It became recognized that activated macrophages had many different properties from normal macrophages. Wing and Remington (1980) attribute the following properties to activated macrophages as compared to normal macrophages:

1. Increased size and spreading
2. Increased ruffled membrane activity
3. Increased adherence to glass
4. Increased glucose utilization through the hexose monophosphate shunt
5. Increased carrier-mediated transport of glucose and amino acids
6. Increased membrane enzyme adenylate cyclase and increased cytoplasmic enzyme lactate dehydrogenase
7. Production and release of collagenase
8. Increased production of plasminogen activator and prostaglandins
9. Increased Ca^{+2} influx
10. Increased in CGMP
11. Increase in the number of cytoplasmic granules
12. Increased phagocytosis of some particles (IgG coated particles) but not others (aggreagated hemoglobin)
13. Phagocytosis of C3b- coated particles
14. Increased pinocytosis

15. Enhanced bacterial killing

16. Tumor inhibition and killing

Macrophages can be activated by certain lymphokines such as MIF (migration inhibition factor), interferon, bacterial endotoxins, and by certain immunomodulating agents such as Bacillus Calmette-Guerin (BCG), Corynebacterium parvum and Levamisole. Cohn (1978) has proposed that macrophages become activated through a series of stages:


Nonspecific Inflammatory Event
Lymphokine-Mediated Event
1. Spreading
2. Metabolic response
Glucose oxidation
O_2^- production
Hexose monophosphate shunt
3. Stimulation of IgM,C3b ingestion
4. Proteinase secretion
5. Stimulated pinocytosis
6. Plasma membrane ectoenzyme modification
5'-Nucleotidase decreases,
Alkaline phosphodiesterase increases
7. H_2O_2 production
8. Microbicidal and tumoricidal activity
Bloodmonocyte
Resident macrophage

The most interesting aspect of the enhanced bactericidal capacity of the activated macrophage is the lack of specificity in its expression. It was found that mice infected with Brucella abortus were highly resistant to Listeria (Mackaness, 1964), while mice that were highly resistant to Salmonella typhimurium were also resistant to Listeria (Blanden et al, 1966). The nonspecificity of this microbicidal activity is even more striking in the fact that totally different types of organisms can elicit nonspecific resistance to bacteria. For example, mice primarily infected with the protozoa Toxoplasma gondii or Besnoitia jellisoni became resistant to Listeria and Salmonella typhimurium (Rirskin et al, 1969).

The mechanisms that allow activated macrophages to kill bacteria more efficiently are still largely undefined although Johnson (1978) felt the increased levels of H_2O_2 and O_2^- produced by BCG activated mouse macrophages as compared to normal, resident macrophages may explain some of the enhanced bactericidal capacity.

3. The Effects of BCG, Corynebacterium parvum and Levamisole on Phagocytes

(i) BCG

BCG allows macrophages to inhibit, nonspecifically the growth of certain intracellular pathogens. Rabbit alveolar macrophages, which were activated by BCG, showed an increased ability to inhibit the growth of intracellular bacteria other than mycobacteria with an accompanying increase in the content of lysosomal hydrolases (Leake and Myrvik, 1968). Heat-killed BCG

are ineffective in the induction of non-specific microbicidal activity; chronic injection with living bacteria is required. BCG increases the random migration, chemotaxis and pinocytosis of macrophages in the guinea pig (Poplack et al, 1976) and increases the chemotaxis of mouse macrophages (Meltzer et al, 1975). BCG-infected animals also clear Salmonella enteritidis more quickly than control animals and breakdown denatured albumin more rapidly. BCG protects against Staphylococcus and Candida in immunosuppressed mice indicating that BCG-activated macrophages are involved (Sher et al, 1975).

Although there is a wide-ranging protective effect against microbes, ranging from bacteria to viruses, BCG does not cause macrophage-mediated protection against all organisms. Myrvik (1972) showed that BCG-activated macrophages, which were capable of killing Listeria, could not kill Yersinia tularensis.

It has also been shown that BCG-activated macrophages are capable of recognizing and killing a variety of tumor cells (Hibbs et al, 1973; Cleveland et al, 1974; Holtermann et al, 1973).

(ii) Corynebacterium parvum

A formalin fixed, washed preparation of this gram positive anaerobic bacillus has been used for immunomodulating studies. This agent can act as a nonspecific stimulant of the reticulo-endothelial system (RES). Halpern et al, (1963) showed in mice that clearance of colloidal particles was increased following C. parvum injection and that RES organs, principally liver and spleen, were increased in weight.

Marcophages from C. parvum treated animals have been shown to be cytostatic (Olivotto and Bomford, 1974) as well as cytotoxic (Basic et al, 1975) to tumor cells.

C. parvum may alter host resistance by inducing clearance of the infection organism or by altering development of the pathological process. Increased resistance to the infectious agent may be due to nonspecific stimulation of the RES. Much research still remains to be done in this area.

(iii) Levamisole

Levamisole (2,3,4,6-tetra-hydro-6-phenylimidazo (2-16) thiazole) is an antihelminthic drug which has found some use in immunotherapy. Symoens and Rosenthal (1977) have reviewed the effects of Levamisole on phagocytic cells. Levmaisole was found to have increased phagocytosis by PMNL or macrophages when added to these cells or given to donor animals and humans. This effect was most pronounced on hypofunctional cells from patients and weak or absent on cells from normal donors. Chemotactic responsiveness of PMNL and monocytes from patients with defective leukocyte motility could be improved by levamisole administered in vivo or in vitro. Leukocyte migration inhibition in response to antigenic stimulation could be restored when levamisole was administered to anergic patients or given to their cells in vitro. Levamisole failed to increase macrophage cytotoxicity to tumor cells in normal mice. There is an increase in cGMP and a decrease in cAMP levels in PMNL from patients after administration of Levamisole. The blood clearance of colloidal particles was enhanced by Levamisole in cases of a relative

deficiency of the RES as found in aged or cortisone-treated animals. Levamisole seemed to restore RES function rather than stimulating it above normal levels of activity.

4. In Vivo Effects of BCG,C.parvum and Levamisole

Workers from the laboratory of Dr. T. Louie (Bourgault et al, unpublished) have investigated the in vivo efficacy of BCG,C.parvum and Levamisole in a male Wistar rat model against an infectious challenge of E. coli ($2x10^6$CFU) and B.fragilis ($1x10^8$CFU). Eight groups of rats were pretreated 18 days and 6 days prior to infection with saline placebo intraperitoneally (i.p.) or subcutaneously (s.c.), C.parvum i.p. or s.c., BCG i.p. or s.c. and Levamisole i.p. or s.c. The acute mortality for the placebo group was 55% while the acute mortalities for the three immunomodulating agents were dramatically reduced when compared to the placebo group. C.parvum given i.p. significantly reduced the presence of abscesses ($p<0.005$) from 91% in the placebo group to 33% in the C.parvum group. Smaller reductions in the number of rats with abscesses were noted with the other immunomodulating agents. It was also noted that the number of intra-abdominal abscesses per rat was reduced by treatment with the three immunomodulating agents. The reduction of mortality in the BCG and C.parvum groups was correlated with a reduction in the frequency of E. coli bacteremia. Rat liver and spleen weights were increased in the BCG and C.parvum groups when compared to the saline control group. The immunomodulating agents reduced the number of positive cultures for E.coli and B.fragilis in abscess

pus and reduced the $\log_{10}$ CFU/ml of bacterial isolates in abscess pus of some animals. Single dose Levamisole treatment (i.p. or s.c.) given 18 and 6 days prior to infection was inadequate to significantly reduce mortality or the frequency of E. coli bacteremia as compared to the saline controls. Liver and spleen weights were similar to those of the saline controls. However, three, daily eight hour injections (i.p. or s.c.) of Levamisole given three days pre-and three days postinfection markedly reduced mortality and the frequency of E. coli bactermia as compared to the saline controls. Liver and spleen weights were also increased. The investigators concluded from these studies that BCG,C.parvum and Levamisole may be useful in augmenting host defenses in intra-abdominal sepsis.

5. Macrophage Bactericidal Assays

There are a number of different bactericidal assays used by researchers to assess the in vitro ability of macrophages to kill a test organism. The most common type of bactericidal assay involves rotating a suspension of macrophages, serum opsonins and test bacteria at 37 degrees C and sampling the mixture at various times to determine the number of viable colony forming units (C.F.U.) by appropriate dilution in distilled water (Zeligs, 1981; Peterson et al, 1977). Appropriate dilutions are plated out onto agar plates to determine the number of viable C.F.U. remaining. Any living intracellular bacteria are obtained by lysis of macrophages in the distilled water. Van Furth et al (1978) has modified this method slightly by lysing macrophages in a freeze-thaw fashion. The cells are lysed by putting them in

-170 degrees C liquid nitrogen and then transferring them to a 37 degree C water bath.

Territo and Cline (1978) have devised a bactericidal assay in which the bacteria and macrophages are incubated in a pellet at 37 degrees C after being centrifuged. The bacteria in the supernatant are subtracted from the bacteria in the whole suspension in order to arrive at the number of bacteria actually associated with the macrophages. This calculation is also used to arrive at the number of organisms killed by the macrophages. Dilutions are made in distilled water and then plated onto agar. Lysis of macrophages is also done in distilled water.

The differing methods of measuring macrophage bacterial killing of test organisms shows that there is no standard assay used by workers in this field. However, there are two unifying principles apparent in all these assays. The first is that successful opsonization of the test organism must occur before successful phagocytosis and killing by macrophages can happen. Thus, serum is employed for opsonization purposes in all macrophage bactericidal assays. The second principle is that the temperature of the test environment must approximate the temperature of the host animal from which the macrophages came for optimal killing of the test bacteria. For this reason, all macrophage bactericidal assays are performed in heated incubation chambers.

A problem of all macrophage bactericidal assays is that the apparent rate of replication of the target organism is often faster than the ability of the macrophages to kill it. Zeligs

(1981) encountered this problem when using a wild-type strain of E.coli. This author dealt with this problem by using a temperature sensitive mutant of E.coli which would not replicate at a temperature of 37 degrees C. The method of Territo and Cline (1978) addresses this problem by getting rid of most of the bacteria not associated with the macrophages. Test macrophages and their associated bacteria are centrifuged into a pellet and the extracellular bacteria in the supernatant are discarded.

One advantage of the rotating macrophage bactericidal assay methods is the speed at which they can be performed. The time-consuming and ponderous nature of the method of Territo and Cline (1978) is its main disadvantage.

Zeligs (1981) and Van Furth et al (1978) maintain that macrophages are harder to lyse than are PMNL. Zeligs suggested leaving macrophages in distilled water for 15 minutes followed by vigorous vortexing while Van Furth et al advocated lysing macrophages by their freeze-thaw method.

Macrophage bactericidal assays are being continually refined in order to improve their efficiency. Cell to bacteria ratios, methods of eliminating extracellular bacteria and methods of speeding up the assay procedures are being refined to allow for a more accurate type of macrophage bactericidal assay.

MATERIALS and METHODS

A. Bacterial Stains

Escherichia coli (strain R4-6) and an encapsulated strain of Bacteroides fragilis (strain R1-1) were isolated from intra-abdominal abscesses of male Wistar rats. This procedure was done in the laboratory of Dr. T. Louie at the University of Manitoba.

B. Growth Media

1. Liquid Media

E. coli was grown in Brain Heart Infusion (BHI) broth (Appendix Ia) in an aerobic incubator at 37 degrees C. B. fragilis was grown in a supplemented form of BHI broth (Appendix Ib) in an anaerobic incubator (10% CO_2, 10% H_2 and 80% N_2) at 37 degrees C. Both organisms were allowed to grow for 18 hours.

2. Solid Media

E. coli was grown aerobically on Trypticase Soy agar (TSA, Appendix II) for 18 hours at 37 degrees C. B. fragilis was grown anaerobically (10% CO_2, 10% H_2 and 80% N_2) on Wilkins - Chalgren agar (WCA, Appendix III) for 48 hours at 37 degrees C. Neomycin (0.10 mg/ml) was added to the WCA to suppress the growth of any E. coli on these plates.

C. Preparation of Autoclaved Rat Caecal Contents and Autoclaved Human Faecal Contents

Rat caecal contents were obtained from the caecae of meat-fed male Wistar rats. One volume of rat caecal contents was diluted in one volume of Peptone-Yeast-Glucose (PYG) broth and

this mixture was then autoclaved for 15 minutes. The autoclaved rat caecal contents was stored in five ml aliquots at -70 degrees C. Autoclaved human faecal contents were obtained from human stool specimens. The method of preparation for autoclaved human faecal contents was the same as for autoclaved rat caecal contents.

D. Technique of Recovering Peritoneal Exudate Cells

Rat PMNL and macrophages were obtained from the peritoneal cavities of male Wistar rats (160 g - 200 g) by peritoneal lavage with Hank's Balanced Salt Solution (HBSS, Appendix IV) containing 0.1% gelatin. Male Wistar rats (Central Breeding Farms, Montreal) were anesthetized with a subcutaneous injection of 0.1 ml of sodium pentabarbital (65 mg/ml) per 100 g of rat body weight. The anterior abdominal walls of the animals were shaved with animal clippers and swabbed with 1% tincture of iodine. All surgical manipulations were performed in a laminar flow hood (Class II-A, Labconoco) to ensure a sterile working environment. In addition, all surgical supplies and instruments used were sterile as well. The anesthetized rats were covered with fenestrated, sterile drapes through which a midline incision was made with a #15 disposable scalple. After widening the initial incision with a Kelly clamp, a purse string 3-0 silk suture was placed around the opening and a peritoneal dialysis catheter was inserted into the peritoneal cavity and the opening was closed. The catheter was shortened to accomodate the small animals and was anticoagulated with 1/1000 solution of heparin in normal saline. Fifty ml of HBSS was flushed into the peritoneal cavity through the catheter by a fifty ml syringe. The abdominal cavity was

gently massaged for 3-4 minutes, following which the contents were drained with the catheter in the dependent position. This allowed 85% - 95% of the original volume to be retrieved.

E. Chemiluminescence (CL) Assay

The assay that was used in this research was obtained from the laboratory of Dr. Paul G. Quie at the University of Minnesota.

1. Preparation of Bacteria

E. coli and B. fragilis were grown up in broth culture for 18 hrs. and then centrifuged at 1500g (International Equipment Co., model PR-2) for 30 minutes. The bacteria were resuspended in HBSS containing 0.1% gelatin and again centrifuged at 1500g for 30 minutes to wash them free of any growth madium. The bacteria were then adjusted to approximately 5×10^9 bacteria/ml by Mcfarland's Standards. Two parts of this bacterial suspension and one part of pooled diluted rat or human serum were combined and rotated on a rotorack (10 rpm) for 30 minutes to pre-opsonize the bacteria.

2. Preparation of Phagocytes

(i) Human PMNL

Forty ml of heparinized human blood (10 v/ml) was obtained from healthy volunteers and was combined with a 6% Dextran -75 solution. Ten ml of blood was mixed with three ml of the Dextran solution in 16 x 125 mm Falcon tubes (Becton - Dickinson California) and the tubes were placed on a 45 degree angle until red blood cell (RBC) sedimentation began. The tubes were then placed in an upright position for the remainder of the 60 minutes settling period. The buffy coat was then drawn off by Pasteur

pipette and centrifuged at 200g (Lourdes Clini-fuge model 10R) for 10 minutes. The remaining RBCs in the leukocyte pellet were lysed by resuspending them in a 0.84% NH_4Cl solution in a 37 degree C water bath for 10 minutes. After this procedure, the cells were diluted further with HBSS and centrifuged at 200 g for 10 minutes. The PMNL were also washed a second time by centrifuging them in HBSS at 200g for 10 minutes. The PMNL were then resuspended and tested for their viability by trypan blue dye exclusion on a hemacytometer. Differential cell counts were done using Tuerk's white cell diluting fluid in 1% glacial acetic acid which highlites the nuclei of the cells, on a hemacytometer. The cells were then suspended to a final concentration of 1.25×10^4 PMNL/ml.

(ii) Rat PMNL and Macrophages

Rat PMNL were induced 24 hours prior to harvesting by peritoneal lavage through an intraperitoneal injection of 0.5 ml of autoclaved rat caecal contents and 0.5 ml of PYG broth. Rat macrophages were induced 72 hours prior to harvesting by an intraperitoneal injection of a 1% glycogen solution (w/v). After harvesting the phagocytes in the fifty ml aliquots of HBSS were centrifuged at 200 g for 10 minutes. Any remaining RBCs were lysed by resuspending the pellet in a 0.84% NH_4Cl solution in a 37 degree C water bath for 15 minutes. The phagocytes were then further diluted by HBSS and centrifuged at 200g for 10 minutes. The cells were resuspended in HBSS and washed a final time by centrifugation at 200 g for 10 minutes. The viability of the phagocytes was tested by trypan blue dye exclusion on a

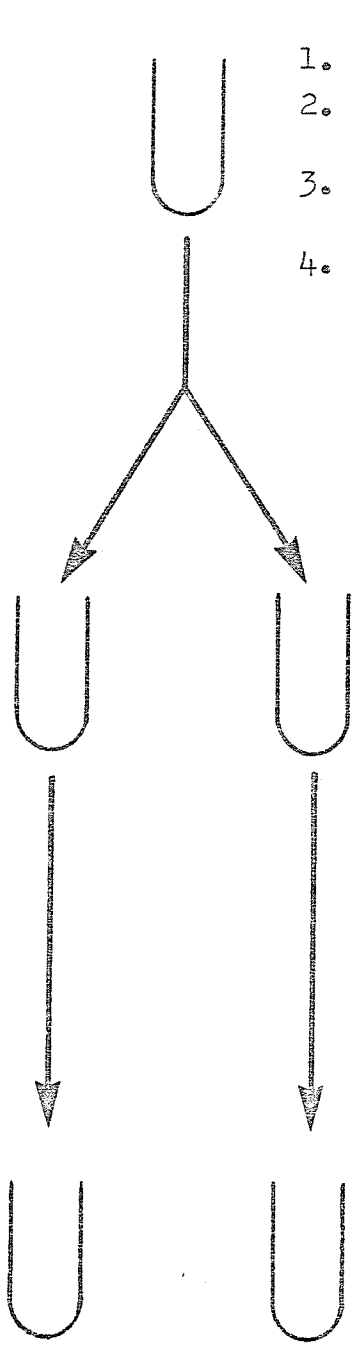
hemacytometer. Differential counts of rat PMNL were done using Tuerk's white cell diluting fluid on a hemacytometer. Differential counts of rat macrophages were performed using Tuerk's white cell diluting fluid on a hemacytometer, the Wright's stain (Appendix V) and the nonspecific esterase stain (Appendix VI). Rat PMNLs were resuspended to a final concentration of 1.25×10^4 cells/ml while rat macrophages were resuspended to a final concentration of 2.5×10^4 cells/ml.

3. Chemiluminescence Procedure

CL was performed on a Beckman LS-100C scintillator in the out-of-coincidence mode with a completely open window ($^3H + ^{14}C + ^{32}P$). The CL scintillation test vials were dark adapted for 6 hours before they were used. Each test vial contained 0.4 ml of an opsonized bacterial suspension (5×10^9), 20ul of luminol (Appendix VII) and HBSS to make a final volume of 4.5ml. Background CL levels were recorded for each vial. At one minute intervals, one ml of prepared phagocytes was added to each vial sequentially. After the addition of phagocytes to a vial, the vial was swirled moderately for 30 seconds and placed back in the scintillation counter to be counted while other phagocytes were added to the next vial. The vials were sequentially counted for one minute periods continuously for one hour and peak CL levels were recorded.

F. Macrophage Bactericidal Assay

The assay (Figure II) that was used in this research was a modification of the assays of the laboratory of Paul G. Quie (University of Minnesota) and of Territo and Cline (1977).



0 Time

1. Sample whole suspension
2. Centrifuge at 200g for 10 minutes
3. Sample supernatant and discard
4. Add new diluent to make final volume of 1.0 ml.

5. Leave pellet stand for 10 minutes at 37 degrees C
6. Resuspend pellet and rotate at 37 degrees C for rest of 60 or 120 minutes
7. After designated time, sample whole suspension
8. Centrifuge at 200g for 10 minutes
9. Sample supernatant and discard

5. Resuspend pellet and rotate at 37 degrees C for 60 minutes or 120 minutes
6. After designated time, sample whole suspension
7. Centrifuge at 200g for 10 minutes
8. Sample supernatant and discard

FIGURE II MACROPHAGE BACTERICIDAL ASSAY

1. Preparation of Bacteria

The method of preparation of E. coli for this assay was the same as that for the CL assay except that the E. coli was not pre-opsonized for 30 minutes with pooled, undiluted serum. The process of opsonization occurred during the actual bactericidal assay.

2. Preparation of Rat Macrophages

The method of preparation of rat macrophages for this assay was the same as that for the CL assay.

3. Macrophage Bactericidal Assay Procedure

The bactericidal assay was performed in Falcon plastic disposable test tubes (12 x 75 mm). Each tube that was to be assayed for macrophage bactericidal activity contained 0.5 ml of rat macrophage suspension of approximately 4×10^6 cells/ml, 0.4 ml of pooled rat serum (1/2 dilution with HBSS), and 0.1 ml of an E. coli suspension of approximately 1×10^8 bacteria/ml to make a final volume of 1.0 ml. The whole test suspension was allowed to sit for five minutes in order to let the bacteria make contact with the rat macrophages. The test suspension was then initially sampled by putting 0.1 ml of the test suspension into 0.9 ml of sterile distilled water. Ten-fold serial dilutions were then made and 0.3 ml of the appropriate dilutions were plated out on TSA plates in six, 0.05 ml drops (Miles and Misra, 1938). Each serial dilution was vortexed vigorously lysing macrophages which freed living intracellular E. coli. After the initial sample of the test suspension was taken, the suspension was centrifuged at 200 g for 10 minutes in

order to sample the supernatant. The pellet was either immediately resuspended and allowed to begin spinning on a rotorack (10 rpm) at 37 degrees C or was allowed to sit on a rotorack at 37 degrees C in a stationary position for 10 minutes before being resuspended and rotated. The initial number of bacteria actually associated with the rat macrophages was calculated by subtracting the number of bacteria in the supernatant from the total number of bacteria in the test suspension. The amount of bacterial killing by the macrophages was determined over periods of one and two hours. Two individual tubes were used for these two determinations. Both tubes were initially sampled and they were then allowed to rotate for one and two hours respectively before the final samplings were done to determine the number of bacteria remaining in both tubes. The final samples were also taken by the same method described for the taking of the initial samples.

G. Treatment of Rats with BCG, C. parvum and Levamisole

1. BCG

Lyophilized BCG (Institute Armand - Frappier, Ville de Laval, Quebec) was prepared in a suspension of physiological saline (0.85%) which was sterile. Sixty gram male Wistar rats were given two weekly subcutaneous injections of this suspension at a dosage of 6 mg/kg of body weight prior to the intraperitoneal injection of the inducing agent.

2. C. parvum

A prepared suspension of C. parvum was obtained from Burroughs and Wellcome Research Triangle Park, North Carolina.

Sixty gram male Wistar rats were given two weekly injections of C. parvum (10 mg/kg of body weight) prior to the intraperitoneal injection of the inducing agent.

3. Levamisole

A suspension of Levamisole (Ortho Pharmaceuticals) was prepared in sterile physiological saline (0.85%) at a concentration of 30 mg/ml. Subcutaneous, 0.1 ml injections of this suspension were given at 8 hour intervals to 160 g male Wistar rats for the length of the injection schedule. Injections were given for three days prior to the induction of rat PMNLs by an intraperitoneal injection of 0.5 ml of autoclaved rat caecal contents. Injections were given for two days prior to and three days after the intraperitoneal injection of 1% glycogen to induce macrophages.

H. Statistical Methods

A Dunett's Test and a one-tailed Student's T test were used used to analyze the research data.

RESULTS

A. Rat PMNL Chemiluminescence and Killing

Peritoneal exudate cells, elicited by an intraperitoneal injection of 0.50 ml of autoclaved rat caecal contents (ARCC) from untreated animals and animals pretreated with BCG, *C.parvum* and Levamisole consisted of 85% - 95% PMNL. PMNL which had been elicited by PYG broth also consisted of 85% - 95% PMNL. All chemiluminescence (CL) values for rat PMNL, human PMNL and rat macrophages were recorded in counts per minute. The mean peak CL values for ARCC-elicited PMNL, Peptone-Yeast-Glucose (PYG) broth -elicited PMNL and PMNL harvested from BCG- and Levamisole-pretreated rats were calculated from three separate individual experiments. Assessment of rat and human PMNL microbial killing using the bactericidal assay adapted from Dr. Paul Quie was carried out concurrently (Louie and Bell, unpublished) with the CL studies to investigate the relationship between PMNL CL and bactericidal activity. Microbial killing was expressed in terms of maximum delta log kill (maximum change in $\log_{10}$ CFU of bacteria). All mean delta log kill values for ARCC-elicited PMNL, PYG-elicited PMNL and PMNL harvested from BCG-, *C.parvum*-and Levamisole- pretreated rats were calculated from three separate individual experiments.

There were no significant differences in peak CL values between *E.coli*, *B.fragilis* and the *E.coli*/ *B.fragilis* combination for any of the rat PMNL tested. However, the peak CL value for the ARCC-elicited PMNL (Table II) when challenged with *B.fragilis* was approximately 45% lower

TABLE II

Bacterial Killing and CL of Autoclaved Rat Caecal Contents Elicited Rat PMNL when Challenged with *E.coli*, *B.fragilis* and the *E.coli*/*B.fragilis* Combination.

Bacteria	Mean CL±S.D.*	Mean delta Log Kill±S.D.
E.coli	**215,960±20,325	-0.55±0.26
B.fragilis	90,497±46,354	-0.24±0.12
E.coli/*B.fragilis* (CL only)	206,012±73,834	---
E.coli/*B.fragilis* (delta log kill only):	----	
a. *E.coli*		-0.52±0.27
b. *B.fragilis*		-0.63±0.09

* S.D. = Standard Deviation
** counts per minute (CPM)

than the values obtained with these PMNL were challenged with E.coli or the E.coli/B.fragilis combination with values approximating E.coli alone in the combination. There was significantly better killing of E.coli as compared to B.fragilis with PMNL harvested from BCG- pretreated rats ($p[0.025$, Table II), PMNL harvested from C.parvum-pretreated rats ($p[0.01$, Table IV), PMNL harvested from Levamisole-pretreated rats ($p[0.025$, Table V) and PYG-elicited PMNL ($p[0.01$, Table VI). There was no significant difference between the killing of E.coli and B.fragilis with ARCC-elicited PMNL (Table II). PMNL obtained from BCG-pretreated rats (Tables VIIa and b) did not give significantly higher peak CL values when challenged with E.coli or B.fragilis as compared to ARCC -elicited PMNL from untreated rats.

Significantly higher peak CL values were obtained with PMNL harvested from C.parvum-pretreated rats ($p[0.01$, Tables VIIa and b) and PMNL harvested from Levamisole-pretreated rats ($p[0.05$, Table VII) than those of ARCC-elicited PMNL from untreated rats with similar bacterial challenges. PYG broth -elicited PMNL (Table VIIa and b) gave similar peak CL values to those of ARCC-elicited PMNL with the same three bacterial challenges.

Only the PYG broth-elicited PMNL killed E.coli significantly better ($p[0.025$) than ARCC-elicited PMNL.

PMNL harvested from BCG-,C.parvum-and Levamisole-pretreated rats killed E.coli better than ARCC-elicited PMNL from untreated rats although the levels of significance were greater than 0.05 (Table VIIa).

TABLE III

Bacterial Killing and CL of PMNL Harvested from Rats Pretreated with BCG when Challenged with *E.coli*, *B.fragilis* and the *E.coli*/*B.fragilis* Combination.

Bacteria	Mean CL±S.D.	Mean delta Log Kill±S.D.	P value for Mean delta Log Kill
E.coli	*260,849±153,870	-1.10±0.50	
			**t=3.096, p<0.025
B.fragilis	229,409±89,182	-0.13±0.19	
E.coli/*B.fragilis* (CL only)	282,522±85,005	----	
E.coli/*B.fragilis* (delta Log kill only):	----		
a. *E.coli*		-0.86±0.55	
b. *B.fragilis*		-0.14±0.05	

* counts per minute (CPM)
** t = calculated value for Students test

TABLE IV

Bacterial Killing and CL of PMNL Harvested from Rats Pretreated with C.parvum when Challenged with E.coli, B.fragilis and the E.coli/B.fragilis Combination

Bacteria	*Mean CL±S.D.	Mean delta Log Kill±S.D.	P value for Mean delta Log Kill
E.coli	355,062±251,824	-1.14±0.18	
			t=4.336, p<0.01
B.fragilis	392,668±204,355	-0.36±0.24	
E.coli/B.fragilis (CL only)	368,451±191,589	---	
E.coli/B.fragilis (delta Log kill only):	---		
a. E.coli		-0.61±0.16	
b. B.fragilis		-0.30±0.29	

* The mean is calculated from two experimental trials for the C.parvum CL values.

TABLE V

Bacterial Killing and CL of PMNL Harvested from Rats Pretreated with Levamisole when Challenged with E.coli, B.fragilis and the E.coli/B.fragilis Combination.

Bacteria	Mean CL±S.D.	Mean delta Log Kill±S.D.	P value for Mean delta Log Kill
E.coli	379,253±124,219	-0.91±0.11	
			t=3.634, P<0.025
B.fragilis	305,895±267,124	-0.50±0.15	
E.coli/B.fragilis (CL only)	411,550±241,992	---	
E.coli/B.fragilis (delta Log kill only):	---		
a. E.coli		-0.67±0.31	
b. B.fragilis		-0.36±0.05	

TABLE VI

Bacterial Killing and CL of Peptone-Yease-Glucose Broth Elicited Rat PMNL when Challenged with *E.coli*, *B.fragilis* and the *E.coli*/*B.fragilis* Combination.

Bacteria	Mean CL±S.D.	Mean delta Log Kill±S.D.	P value for Mean delta Log Kill
E.coli	160,983±26,582	-1.47±0.42	
			t=4.537, p<0.01
B.fragilis	211,431±66,881	-0.26±0.16	
E.coli/*B.fragilis* (CL only)	160,929±25,976	---	
E.coli/*B.fragilis* (delta Log kill only):	---		
a. *E.coli*		-0.56±0.20	
b. *B.fragilis*		-0.61±0.10	

TABLE VIIa

Comparison of the CL and Killing Capabilities of Autoclaved Rat Caecal Contents Elicited PMNL, Peptone-Yeast-Glucose Broth Elicited PMNL and PMNL Harvested from Rats Pretreated with BCG, C.parvum and Levamisole when Challenged with E.coli.

PMNL Source		Chemiluminescence			Microbial Killing	
BRM *	Eliciting Agent	Bacterial Challenge ($5x10^9$CFU)	Mean Peak CL +S.D.	CL P value **	Mean delta Log Kill+S.D. ($1X10^7$CFU)	delta Log Kill P value ****
None	ARCC	E.coli	215,960+20,325	--	-0.55+0.26	--
None	PYG	E.coli	160,983+26,582	NSD***	-1.47+0.42	p<0.025
BCG	ARCC	E.coli	260,849+153,870	NSD	-1.10+0.50	0.05<p<0.10
C.parvum	ARCC	E.coli	355,062+251,824	p<0.01	-1.14+0.18	0.05<p<0.10
Levamisole	ARCC	E.coli	379,253+124,219	p<0.05	-0.91+0.11	0.05<p<0.10

* BRM = Biological Response Modifier

** CL P value = Statistical Comparison as compared to ARCC elicited PMNL

*** NSD = no significant difference

**** delta Log Kill P value = Statistical comparison as compared to ARCC elicited PMNL

Table VIIb

Comparison of the CL and Killing Capabilities of Autoclaved Rat Caecal Contents Elicited PMNL, Peptone-Yeast-Glucose Broth Elicited PMNL and PMNL Harvested from Rats Pretreated with BCG, *C.parvum* and Levamisole when Challenged with *B.fragilis*.

PMNL Source		Chemiluminescence			Microbial Killing	
BRM	Eliciting Agent	Bacterial Challenge ($5x10^9$CFU)	Mean Peak CL ±S.D.	CL P value	Mean delta Log Kill±S.D. ($1X10^7$CFU)	delta Log Kill P value
None	ARCC	*B.fragilis*	90,497±46,354	---	-0.24±0.12	---
None	PYG	*B.fragilis*	211,431±66,881	NSD	-0.26±0.16	NSD
BCG	ARCC	*B.fragilis*	299,409±89,182	NSD	-0.13±0.19	NSD
C.parvum	ARCC	*B.fragilis*	392,668±204,355	$p<0.01$	-0.36±0.24	NSD
Levamisole	ARCC	*B.fragilis*	305,895±267,124	$p<0.05$	-0.50±0.15	NSD

B. Human PMNL Chemiluminescence and Killing

Baseline experiments were performed with human PMNL to determine their CL and killing (Louie and Bell, unpublished) responses when challenged with E.coli, B.fragilis and the E.coli/B.fragilis combination (Table VIII).

All CL and bactericidal experimental means were calculated from three experimental trials. The human PMNL yielded much higher peak CL values than did any of the rat PMNL tested with these bacterial challenges. There was also little difference between any of the peak CL values obtained for these three bacterial challenges. There was substantial killing of both E.coli and B.fragilis by human PMNL.

Table VIII

Bacterial Killing and CL of Human PMNL when Challenged with *E.coli*, *B.fragilis* and the *E.coli*/*B.fragilis* Combination.

Bacteria	Mean CL±S.D.	Mean delta Log Kill±S.D.
E.coli	1,350,266±130,828	-2.14±0.67
B.fragilis	1,283,564±270,953	-1.34±0.62
E.coli/*B.fragilis* (CL only)	1,243,487±186,174	
E.coli/*B.fragilis* (delta log kill only):	---	
a. *E.coli*		-1.61±0.49
b. *B.fragilis*		-1.26±0.57

C. Human PMNL Pre-incubation Experiments

To investigate the effects that individual factors promoting the pathogenesis of intra-abdominal sepsis may have on PMNL, human peripheral blood PMNL were pre-incubated with 0.50 ml of ARCC, 0.50 ml of ARCC supernatant or $1x10^9$ dead E.coli or dead B. fragilis on a rotorack (10 rpm) for 30 minutes at 37 degrees C. The pre-incubated PMNL were washed by centrifugation at 200g and then challenged with live E.coli or B. fragilis.

Normal human PMNL gave significantly higher peak CL values ($p<0.05$, Table IX) than human PMNL pre-incubated with ARCC when challenged with E.coli. Normal human PMNL killed E.coli significantly better than PMNL pre-incubated with ARCC ($p<0.005$) or PMNL pre-incubated with ARCC supernatant ($p=0.05$).

When challenged with B.fragilis, there were no significant differences in peak CL levels between normal human PMNL and PMNL which had been pre-incubated with ARCC or ARCC supernatant (Table X). Normal PMNL however, killed B. fragilis significantly better than PMNL pre-incubated with ARCC ($p=0.05$) although there was no significant difference with PMNL pre-incubated with ARCC supernatant.

Significantly higher peak CL ($p<0.05$) and killing ($p<0.025$) values were obtained from normal PMNL challenged with E.coli when compared to PMNL pre-incubated with dead B.fragilis (Table XI). PMNL, pre-incubated with dead E.coli, had markedly lower but not significantly different peak CL values than normal PMNL when challenged with E.coli. Normal PMNL had

TABLE IX

Bacterial Killing and CL of Human PMNL, Pre-incubated with Autoclaved Rat Caecal Contents or Autoclaved Rat Caecal Contents Supernatant, when Challenged with E.coli

Pre-incubation Condition of PMNL	Mean CL±S.D.	P value for Mean CL	Mean delta Log Kill ± S.D.	P value for Mean delta Log Kill
Normal Control	816,003±134,640		-2.13±0.21	
Autoclaved Rat Caecal Contents Supernatant	783,878±700,914		-0.81±0.04	D=2.473, p<0.05
Autoclaved Rat Caecal Contents	17,226±19,964	D*=2.373, p<0.05	+0.18±1.11	D=4.329, p<0.005

* D = calculated value for Dunett's test

TABLE X

Bacterial Killing and CL of Human PMNL, Pre-incubated with Autoclaved Rat Caecal Contents or Autoclaved Rat Caecal Contents Supernatant when Challenged with B.fragilis

Pre-incubation Condition of PMNL	Mean CL±S.D.	Mean delta Log Kill ± S.D.	P value for Mean delta Log Kill
Normal Control	876,064±559,158	-1.57±0.78	
Autoclaved Rat Caecal Contents Supernatant	697,925±534,736	-0.96±0.79	
Autoclaved Rat Caecal Contents	114,847±165,288	-0.036±0.14	D=2.888, $p<0.05$

TABLE XI

Bacterial Killing and CL of Human PMNL, Pre-incubated with Dead *E.coli* or Dead *B.fragilis* when Challenged with *E.coli*

Pre-incubation Condition of PMNL	Mean CL±S.D.	P value for Mean CL	Mean delta Log Kill ± S.D.	P value for Mean delta Log Kill
Normal Control	991,249±425,654		-2.11±0.37	
Dead *E.coli*	504,005±251,176		-0.89±0.79	D=2.909, p<0.025
Dead *B.fragilis*	416,315±145,573	D=2,366, p<0.05	-0.62±0.14	D=3.561, p<0.025

significantly better killing of E.coli (p[.025) than PMNL which had been pre-incubated with dead E.coli.

PMNL, which had been pre-incubated with dead E.coli or dead B.fragilis had reduced, but not significantly different peak CL values from normal PMNL when challenged with B. fragilis (Table XII). Normal PMNL killed B.fragilis significantly better (p[0.05) than PMNL pre-incubated with dead B.fragilis but this was not the case for PMNL pre-incubated with dead E.coli.

The effect of human faecal material on human peripheral blood PMNL was also tested to simulate the microbial challenge faced by leukocytes that migrate into an area of peritoneal soilage by intestinal contents. This was a corollary experiment to challenge of human PMNL by ARCC. Normal human PMNL had significantly higher peak CL values that either PMNL pre-incubated with autoclaved human faecal contents (AHFC) supernatant (p[0.05) when challenged with E.coli (Table XIII).

Normal PMNL killed E.coli significntly better (p[0.025) than PMNL pre-incubated with AHFC but there was no significant difference with PMNL pre-incubated with AHFC supernatant (Table XIII).

TABLE XII

Bacterial Killing and CL of Human PMNL, Pre-incubated with Dead E.coli or Dead B.fragilis when Challenged with B.fragilis

Pre-incubation Condition of PMNL	Mean CL±S.D.	Mean delta Log Kill ± S.D.	P value for Mean delta Log Kill
Normal Control	1,031,890±276,853	-1.35±0.39	
Dead E.coli	715,543±589,723	-0.65±0.38	
Dead B.fragilis	596,264±500,526	-0.48±0.44	D=2.605, p<0.05

TABLE XIII

Bacterial Killing and CL of Human PMNL, Pre-incubated with Autoclaved Human Faecal Contents or Autoclaved Human Faecal Contents Supernatant, when Challenged with E.coli

Pre-incubation Condition of PMNL	Mean CL±S.D.	P value for Mean CL	Mean delta Log Kill ± S.D.	P value for Mean delta Log Kill
Normal Control	1,044,085±119,058		-1.81±0.54	
Autoclaved Human Faecal Contents Supernatant	515,839±377,569	D=2.828, p<0.05	-1.08±0.66	
Autoclaved Human Faecal Contents	16,687±15,389	D=5.501, p<0.01	-0.20±0.84	D=3.556, p<0.025

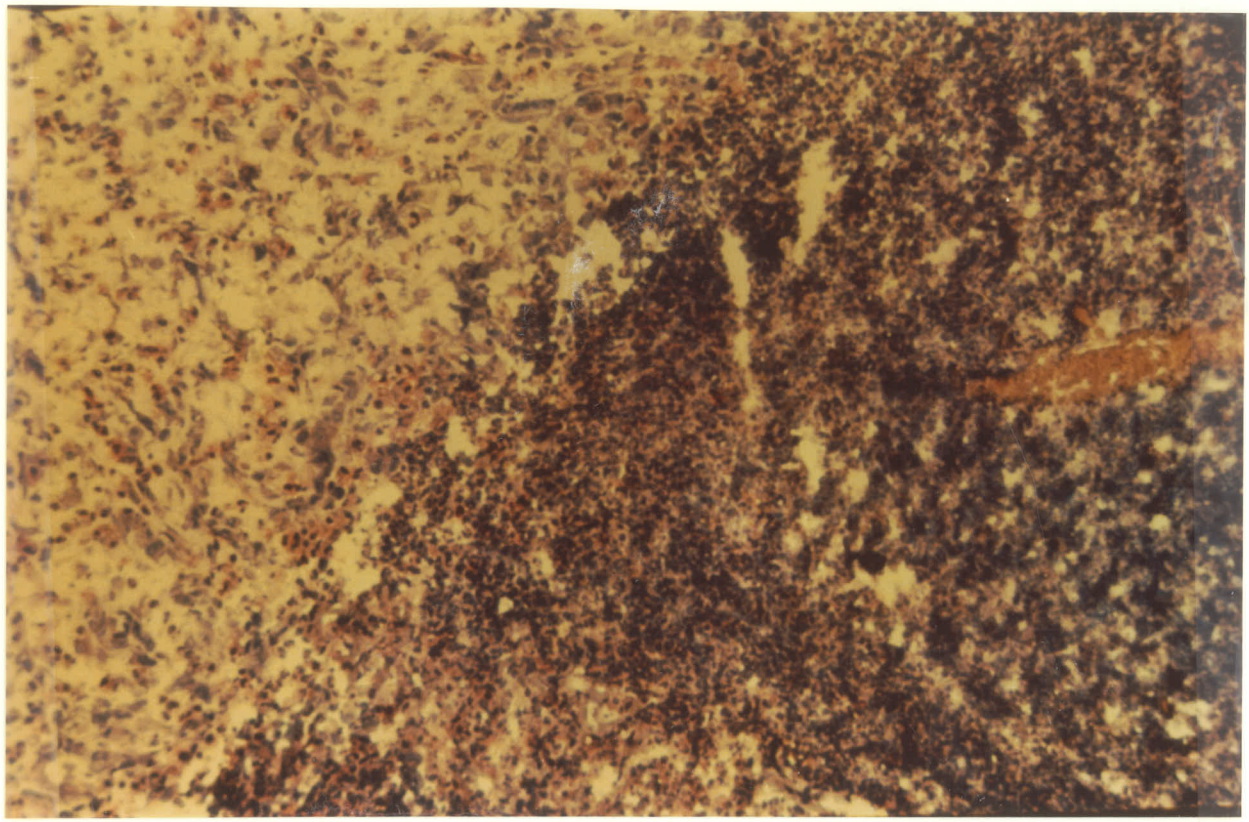
D. Rat Macrophage CL

The glycogen-elicited peritoneal exudate consisted of 54%-63% rat macrophages as determined by the nonspecific esterase stain, 43%-55% macrophages as determined by the Wright's stain and 48%-63% rat macrophages as determined by Tuerk's white cell diluting stain. The rest of this exudate mainly consisted of lymphocytes and there was very little PMNL contamination. The mean peak CL values are replicates of three experimental trials. Glycogen-elicited rat macrophages had peak CL values that were much lower than any human or rat PMNL tested when challenged with E.coli, B.fragilis, and the E.coli/B.fragilis combination (Table XIV). There was also little difference in peak CL values between the three bacterial challenges. The nonspecific esterase stain has shown the presence of rat macrophages in intra-abdominal abscess walls (Figure III).

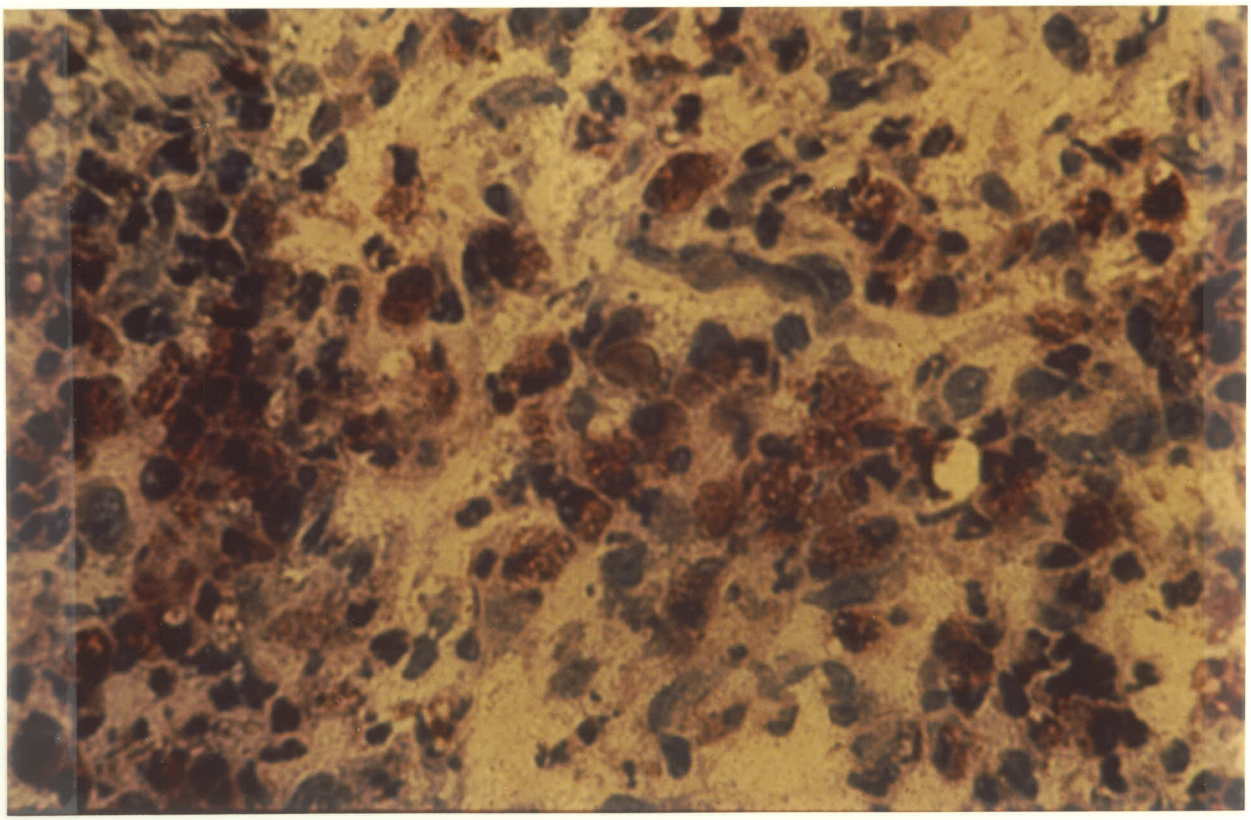
TABLE XIV

Mean Peak CL Values of Glycogen Induced Rat Macrophages when Challenged with E.coli, B.fragilis and the E.coli/B.fragilis combination

Bacteria	Mean ± S.D.
E.coli	24,677±12,437
B.fragilis	35,466±20,462
E.coli/B.fragilis	37,876±17,589



Photomicrograph of rat intra-abdominal abscess stained with non-specific esterase. A is a central zone of non-viable PMN leukocytes, B is an intermediate zone primarily of viable PMN leukocytes and C is the outer zone of mixed cellular response in the capsule of the abscess, consisting of macrophages (stained red) other mononuclear cells and fibroblasts.



Higher magnification of zone C showing esterase positive macrophages in the capsule of the abscess.

E. Macrophage Bactericidal Activity

B.fragilis was not used in this part of the study as it had a highly variable viability during the bactericidal assay. This part of the research centered on the responses of rat macrophages to E.coli.

The PMNL bactericidal assay of Dr. Paul Quie was first used to test the ability of glycogen-elicited rat macrophages to kill E.coli. This assay was unsuccessful as the replicative ability of E.coli allowed it to multiply beyond the original inoculum size making any killing undetectable. This result happened in eight out of eight experimental trials performed and this is illustrated in Figure IV. A modified bactericidal assay was then used to eliminate the extracellular growth of E.coli which rendered the Quie assay ineffective. This was done by discarding the supernatant after sampling the whole solution. There was some killing of E.coli by glycogen-elicited rat macrophages at 60 minutes but the results at 2 hours were inconsistent with only one of three trials showing any killing of E.coli (Table XV). The macrophages were then allowed to sit with the E.coli in a pellet for 10 minutes at 37 degrees C before being resuspended to rotate for the rest of the assay.

The 10 minutes produced consistent killing of E.coli at 60 minutes and 2 hours although the killing results were quite variable at 2 hours (Table XVI). Macrophages harvested from Levamisole-pretreated rats killed E.coli significantly better ($0.05<p<0.10$) than did glycogen-elicited macrophages (with 10 minute wait) at 60 minutes and 2 hours (Table XVII). C.parvum

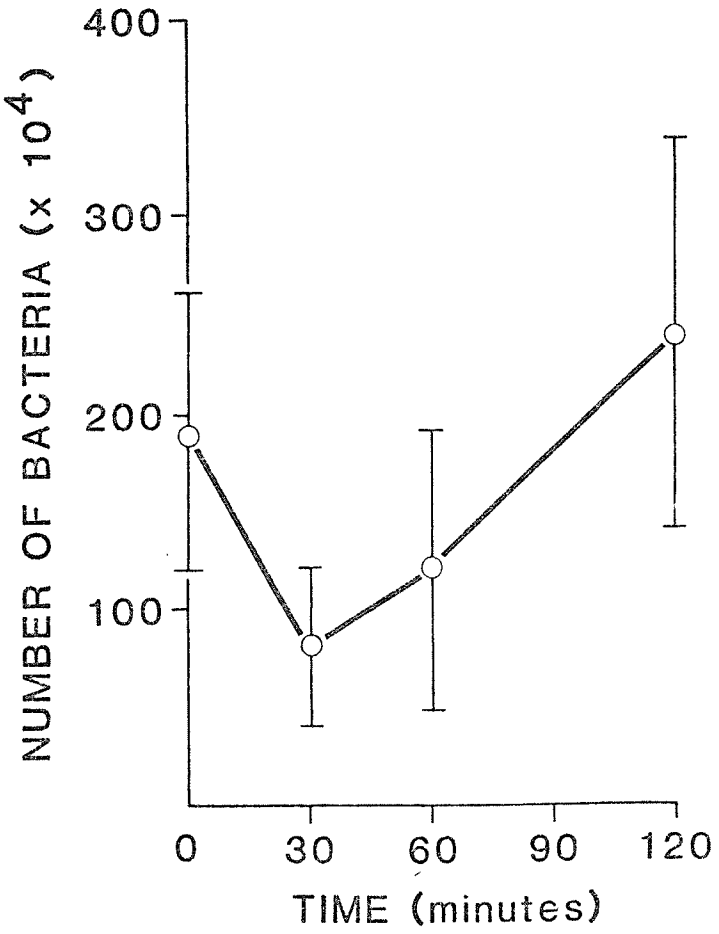


Figure IV: Experiments with the Quie Assay Showing Replicative Ability of E. coli with Glycogen Elicited Rat Macrophages[1]

1. Each point on this graph represents a mean of 8 individual experiments ± the standard deviation.

TABLE XV

Bactericidal Activity of Glycogen Elicited Rat Macrophages when challenged with *E.coli* with no 10 Minute Wait

Original Inoculum Size (bacteria/ml)	60 Minutes (bacteria/ml)	120 Minutes (bacteria/ml)	delta log kill
3.6×10^6	1.24×10^6	---	-0.234
5.0×10^6	8.6×10^5	---	-0.54
5.8×10^6	1.84×10^6	---	-0.396
2.4×10^6	5.6×10^5	---	-0.58
1.0×10^6	---	2.62×10^6	+0.162
3.0×10^6	---	3.6×10^5	-0.94
1.8×10^6	---	2.0×10^6	+0.02

TABLE XVI

Bactericidal Activity of Glycogen Elicted Rat Macrophages when challenged with *E.coli* with 10 Minute Wait

Original Inoculum Size (bacteria/ml)	60 Minutes (bacteria/ml)	120 Minutes (bacteria/ml)	delta log kill
3.8×10^6	9.0×10^5	---	-0.38
7.8×10^6	2.1×10^6	---	-0.57
4.4×10^6	1.0×10^6	---	-0.34
3.7×10^6	---	1.2×10^5	-1.25
5.2×10^6	---	1.36×10^6	-0.384
6.4×10^6	---	4.14×10^6	-0.226

TABLE XVII

Bactericidal Activity of Rat Macrophages Harvested from Rats Pretreated with Levamisole when challenged with E.coli

Original Inoculum Size (bacteria/ml)	60 Minutes (bacteria/ml)	120 Minutes (bacteria/ml)	delta log kill
3.2×10^6	7.0×10^5	---	-0.52
4.0×10^6	7.6×10^5	---	-0.54
1.6×10^6	3.0×10^5	---	-0.76
6.8×10^6	5.8×10^5	---	-1.10
3.8×10^6	---	1.2×10^5	-1.26
2.2×10^6	---	4.4×10^5	-0.68
5.4×10^6	---	5.0×10^5	-1.04

was not used in these experiments as it dramatically reduced the number of macrophages in the peritoneal exudate.

DISCUSSION

A. Rat PMNL Chemiluminescence and Killing

Rat PMNL, which were elicited by ARCC, had reduced levels of killing and chemiluminescence with E.coli, B.fragilis and the E.coli/B.fragilis. These results indicated that ARCC or some component of it may inhibit rat PMNL microbicidal capabilities. Flint et al (1981) showed that sterile human faecal material and filtered peritoneal exudate from rats moribund with bacterial peritonitis inhibited the ability of rat phagocytes to kill E.coli and S.aureus. In contrast, PYG broth-elicited PMNL killed E.coli significantly better ($p<0.025$) than did ARCC-elicited PMNL. However, the CL generated by PYG broth-elicited PMNL when challenged with E.coli was actually slightly lower than that produced by ARCC-elicited PMNL. The fact that ARCC-elicited PMNL harvested from BCG-,C.parvum- and Levamisole-pretreated rats had much better killing and CL results when challenged with E.coli supports the concept that ARCC-elicited PMNL (no pretreatment) have defective bactericidal capabilities. The fact that ARCC-elicited PMNL, which have been harvested 24 hours after the i.p. injection of ARCC, cannot effectively kill E.coli and B.fragilis indicates that ARCC may have persistent effects on rat PMNL. There may also be a mixed population of PMNL in the exudate depending of their time of arrival in the peritoneal exudate. The first initial wave of rat PMNL may be overwhelmed by particulate matter or soluble products derived from ARCC. It is uncertain why there is reduced micro-

bicidal activity and CL of cells harvested at 24 hours. The initial inhibitory 24 hour period of rat PMNL microbicidal activity allows E.coli to replicate, seriously damage host defenses and allows bacteremia to supervene.

B.fragilis was not killed well by any of the rat PMNL tested although PMNL harvested from C.parvum-and Levamisole-pretreated rats gave significantly better CL results with B.-fragilis as compared to ARCC-elicited PMNL. Ingham et al (1977) reported that B.fragilis (obligate anaerobe) impaired the ability of human PMNL to kill facultative aerobic bacteria effectively. The present results indicate that the failure of rat PMNL to kill B.fragilis effectively may help to promote abscess formation in intra-abdominal sepsis in the male Wistar rat model. Onderdonk et al (1977) have shown that encapsulated strains of B.fragilis are capable of causing abscess formation by themselves. The failure of rat PMNL to kill encapsulated strains of B.fragilis could be very important in the process of abscess formation. Reznikov et al (1981), however, reported that B.fragilis did not inhibit the clearance of E.coli from the peritoneal cavity of a mouse. Shapiro et al (1982) have shown that T-cells (Ly^- 1^- 2^+) will prevent mice from developing abscesses when injected with B.fragilis capsular polysaccharide. Thus, the immune response to B. fragilis may involve more than phagocytes.

The immunodulating agents BCG,C.parvum and Levamisole were able to restore the ability of rat PMNL to deal effectively with E.coli in terms of CL and killing. An injection (s.c.)

of C.parvum tended to reduce the number of rat macrophages in a peritoneal exudate from 50% in untreated animals to 25% in C.parvum-pretreated animals.

These results complement those of Bourgault et al (unpublished) which showed that pretreatment of rats with BCG,C.parvum and Levamisole improved their survival rate when exposed to an infectious challenge of E.coli and B. fragilis. However, the failure of the immunomodulating agents to improve upon the killing of B.fragilis in vitro is poorly correlated with the in vivo results in terms of abscess formation. Thus, although microbicidal killing of B.fragilis was not improved significantly, Levamisole and C.parvum decreased abscess formation. This finding may be due to effector mechanisms which are operative as well such as macrophage-T-cell interactions. This was not measured in these experiments.

The effects of BCG,C.parvum and Levamisole on the host immune system are wide ranging. A great deal of research has focused on the effect of these agents on macrophages. Schleupner and Glasgow (1978) showed that macrophages from C.parvum-treated mice gave enhanced CL over controls during the phagocytosis of zymosan particles or yeasts. The present research shows that these immunomodulating agents have restorative or augmenting effects on PMNL as well. The immunomodulating agents seem to have effects on rat PMNL metabolism rather than increasing the numbers of PMNL in the peritoneal cavity. The total numbers and percentages of PMNL in the peritoneal exudates

of BCG,-C.parvum-and Levamisole-pretreated animals were very similar to that of ARCC-elicited PMNL from untreated animals. BCG-,C.parvum-and Levamisole-pretreatment of rats increased the CL and bactericidal capabilities of ARCC-elicited PMNL when they were challenged with E.coli as compared to ARCC-elicited PMNL from untreated rats.

B. Human PMNL Chemiluminescence and Killing

Human peripheral blood PMNL gave uniformly good CL and kill results when challenged with E.coli, B.fragilis and the E.coli/B.fragilis combination in the present study. These results showed no interference by B.fragilis in the killing of E.coli by human PMNL as did the results of Ingham et al (1977). The present in vitro study has failed to demonstrate any synergystic relationship between E.coli and B.fragilis which helps either species to escape killing by human PMNL.

Human peripheral blood PMNL also showed much higher levels of CL than any of the rat PNNL tested with these three bacterial challenges. This may indicate that human PMNL have a greater reliance on oxidative metabolism to kill certain kinds of bacteria than do rat PMNL. B.fragilis may not be sensitive to the microbicidal mechanisms of rat PMNL. This may be strain specific and further testing with other of B.fragilis would shed light on the matter.

C. Human PMNL pre-incubation Experiments

These experiments were conducted with human peripheral blood PMNL in order to determine which factors in rat colonic contents might inhibit PMNL bactericidal capability and allow the estab-

lishment of a serious peritoneal infection. Pre-incubation with ARCC almost completely abolishes any PMNL CL or killing when the phagocytes were challenged with live E.coli or B. fragilis. Pre-incubation with dead E.coli or B.fragilis seemed to have an intermediate inhibitory effect on PMNL CL or killing of live E.coli or B.fragilis. Pre-incubation with ARCC supernatant had the least effect on PMNL CL or killing of these two bacterial species. Human PMNL CL and killing of E.coli was severely inhibited by pre-incubation with autoclaved human faecal contents (AHFC). Pre-incubation with AHFC supernatant also had a significant inhibitory effect ($p<0.05$) on PMNL CL when these cells were challenged with E.coli.

These results support the finding of Flint et al (1981) that sterile human faecal material inhibits the killing of E.coli by rat phagocytes.

Since the sterile faecal material was added during the bactericidal assay, Flint et al (1981) proposed that faecal suspensions may physically inhibit the interaction between phagocyte surface receptors and organisms by allowing bacteria to adhere to faecal particles or by fostering fibrin deposition.

A possible explanation for the inhibitory effect of pre-incubating PMNL with dead bacteria, ARCC or AHFC may have to do with the idea of "overfeeding" the PMNL. PMNL, which have phagocytosed these types of particulate matter will discharge a large portion of their granules into the phagosome. The PMNL, having already discharged the greater portion of their micro-

bicidal granules, will not have enough of these types of granules to kill any live bacteria phagocytosed in a subsequent bacterial challenge. Colonic material could overwhelm the first wave of phagocytes attracted to an area of intestinal perforation allowing a serious bacterial infection to become established.

D. Correlation of Chemiluminescence and Bacterial Killing

In these studies where microbial killing and CL are done in paired experimental trials, there is a direct relationship between microbial killing and the degree of CL in most instances. These trends are present in both the human PMNL and rat PMNL tested. PMNL harvested from BCG,-C.parvum-and Levamisole-pretreated rats and elicited by ARCC show increased CL and killing when challenged with E.coli as compared to ARCC-elicited PMNL from untreated animals. PYG broth-elicited PMNL showed increased killing of E.coli but less CL than ARCC-elicited PMNL. All rat PMNL, harvested from animals pretreated with BCG,C.parvum and Levamisole, showed increased CL when challenged with B. fragilis as compared to ARCC-elicited PMNL from untreated animals. However, the increased CL was not associated with improvement in killing of B.fragilis. Some of these differences may be explained in terms of the CL assay itself. The CL assay is far more sensitive to the number of cells used than is the bactericidal assay and there is still more variation encountered when using a liquid scintillator in the our-of-coincidence mode. As well, the discrepancies may reflect different pathways of microbial killing. Nevertheless, the data from human peripheral blood PMNL shows that both E.coli and

B.frgilis are killed equally well. Both species of bacteria may be sensitive to one or more of the oxygen radicals produced by human PMNL.

High levels of killing and CL were shown by human peripheral blood PMNL when challenged with E.coli, B.fragilis and the E.coli/B.fragilis combination. Pre-incubation experiments with AHFC, ARCC and dead E.coli or B.fragilis decreased both CL and microbial killing when the pre-incubated PMNL were subsequently challenged with live E.coli or B.fragilis.

Differences in statistical significance between paired CL and kill results as compared to a normal PMNL control may be explained by variation in the CL assay itself. These studies have shown that the relationship between CL and bactericidal activity is strong but direct correlations cannot always be made. This observation has also been confirmed by other workers (Ewetz et al, 1981; Grebner et al, 1977). The present data suggests that CL testing should be performed with other techniques and that CL alone could be used as a screening device with an understanding of the limitations of the method.

E. Rat Macrophage Chemiluminescence and Killing

Rat peritoneal macrophage CL levels are much less that those of rat PMNL or human PMNL. This may indicate that oxidative metabolism may not be as important to the macrophage for bactericidal activity as it is for the PMNL. However, macrophages are known to produce some of the same oxygen species as PMNL and Johnson (1978) felt that the increased levels of H_2O_2 and O_2^- produced by BCG-activated mouse macrophages may account

for some of the enhanced bactericidal capacity of these cells.

Macrophages kill E.coli less efficiently than do rat or human PMNL. This is demonstrated by the fact that E.coli was able to replicate beyond its original inoculum size with the Quie assay. The modified assay used in the macrophage bactericidal studies combined the best elements of the Quie assay and the assay of Territo and Cline (1977). The discarding of the supernatant after sampling the whole solution and supernatant eliminated the large amount of extracellular bacteria which are not "cell associated" and which would replicate and obscure any measurable microbicidal activity. At the same time, the speed and flexibility of the Quie assay has been retained in the modified assay. This modified procedure is less cumbersome than the assay of Territo and Cline (1977) and may be more practical. Further testing will be required to confirm this approach to macrophage function testing.

The consistent results that were obtained with glycogen elicited macrophages at 60 minutes regardless of the ten minute wait indicates that glycogen-elicited rat macrophages can kill E.coli. The inconsistent results obtained at 2 hours without the 10 minute wait may possibly be attributed to small amounts of extracellular growth by E.coli. It may also be that macrophages may require close apposition to the target bacteria to effect or enhance phagocytosis and microbial killing. The 10 minute wait with the macrophages and E.coli concentrated in a pellet may give the phagocytes time to phagocytose the E.coli successfully and kill it. Macrophages may only be successful

phagocytes if they are apposed to the target organisms.

Macrophages, which have been elicited by glycogen and harvested from rats pretreated with Levamisole, kill E.coli markedly better ($0.05<p<0.10$) than glycogen-elicited macrophages from untreated rats with or without the 10 minute wait. Thus, Levamisole improves the ability of rat macrophages to process and kill E.coli more efficiently. The role of the macrophage in cell-mediated immunity to intra-abdominal sepsis is still unsettled.

More research must be done on the respective roles of the PMNL, macrophage and the lymphocyte in host resistance to intra-abdominal sepsis in order to achieve an improved understanding of the biology of intra-abdominal infections.

SUMMARY

The main features of the thesis are summarized as follows:

1. ARCC-elicited rat PMNL showed reduced levels of CL and killing when challenged with E.coli as compared to PMNL harvested from rats pretreated with BCG,C.parvum and Levamisole. Peptone-Yeast-Glucose (PYG) broth-elicited rat PMNL showed the greatest killing of E.coli but had a CL level similar to that of ARCC elicited cells.

2. All rat PMNL showed very little killing of B.fragilis. PYG-elicited rat PMNL and PMNL harvested from rats pretreated with BCG,C.parvum and Levamisole did show improved CL levels, however when challenged with B.fragilis.

3. Human peripheral blood PMNL showed both substantial microbicidal activity and CL levels when challenged with E.coli, B.fragilis and the E.coli/B.fragilis combination. The CL levels of human PMNL were higher than any of the rat PMNL tested.

4. Human peripheral blood PMNL which were pre-incubated with ARCC and AHFC showed the most inhibition of CL and microbial killing when challenged with E.coli or B.fragilis.

5. Human PMNL, which were pre-incubated with 1 x 10^9 dead E.coli or B.fragilis or AHFC supernatant, showed moderate inhibition of CL and microbial killing when challenged with E.coli or B.fragilis. PMNL pre-incubated with ARCC showed very little inhibition of CL and killing when challenged with E.coli or B.fragilis.

6. Glycogen-elicited rat macrophages showed much less CL than either rat or human PMNL when challenged with E.coli, B.fragilis or the E.coli/B.fragilis combination.

7. Glycogen-elicited rat macrophages were only able to kill E.coli consistenty at 60 minutes with no 10 minute wait. These macrophages were able to kill E.coli consistently at 60 minutes and 120 minutes with the 10 minute wait in the bactericidal assay.

8. Macrophages which were harvested from rats pretreated with Levamisole were able to kill E.coli better than glycogen elicited rat macrophages regardless of the 10 minute wait.

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Appendix Ia

Brain Heart Infusion Broth (BBL)

Formula per litre distilled water:

Brain Heart Infusion from solids	6.0 g
Peptic Digest of Animal Tissue	6.0 g
Sodium Chloride	5.0 g
Dextrose	3.0 g
Pancreatic Digest of Gelatin	14.5 g
Disodium Phosphate	2.5 g

Appendix Ib

Brain Heart Infusion Broth Supplement

1)	hemin	5 ug/ml
2)	normal or horse serum	10% v/v
3)	peptic digest of sheep - Fildes enrichment	5% v/v

Appendix II

Trypticase Soy Agar (Gibco)

Formula per litre distilled water:

Peptone 140 (pancreatic digest of casein)	15.0 g
Peptone 110 (papaic digest of soy protein)	5.0 g
Sodium Chloride	5.0 g
Agar	15.0 g

Appendix III

Wilkin - Chalgren Agar (Wilkins and Chalgren, 1975)

Formula per litre distilled water:

Trypticase (pancreatic digest of casein, BBL)	10.0 g
Gelysate (pancreatic digest of gelatin, BBL)	10.0 g
Yeast Extract (Difco)	5.0 g
Glucose (Fisher)	1.0 g
Sodium (Fisher)	5.0 g
L-Arginine-free base (Sigma)	1.0 g
Pyruvic acid-sodium salt (Sigma)	1.0 g
Agar	15.0 g
Hemin	5ug/ml
Vitamin K_1	5ug/ml

Appendix IV

Hanks Balanced Salt Solution

Formula per litre of distilled water:

Solution A

NaCl	8.0g
KCl	0.4g
$MgSO_4$ $7H_2O$	0.2g

Solution B

Na_2HPO_4 (anhydrous)	0.048g
KH_2PO_4	0.06g

Solution C

$CaCl_2$	0.14g

1. Dissolve salts completely for each solution in about 300 ml of water each.

2. Autoclave solutions A,B, and C separately.

3. Solutions A and B are combined with constant stirring and then solution C is added.

4. Add 1 ml of 10% Gelatin to every 100 ml of HBSS (final concentration is 0.1%) to ensure bacterial viability in HBSS.

Appendix V

Wright's Stain Procedure

1. Flood a slide with an air-dried smear of cells with the Wright's Stain (Fisher) for 10 minutes.

2. Add a buffer solution consisting of 68.2 ml of KH_2PO_4 (M/15) and 31.8 ml of Na_2HPO_4 (M/15) to the flooded slide until a greenish sheen develops of the Wright's Stain. The slide is then left for seven minutes.

3. The Wright's Stain is then dumped off the slide and the slide is briefly destained in the methanol and then dried.

Appendix VI

Nonspecific Esterase Stain

A. Pararosaniline Solution

1 g of Pararosaniline hydrochloride is dissolved in 25 ml of 2NHCl.

B. Alpha-Napthyl Acetate Solution

50 mg of alpha-napthyl acetate is dissolved in 2.5 ml of ethylene glycol monethyl ether. This solution is unstable and must be made up before use only.

C. Procedure

1. Hexazotized pararosaniline stain is made by adding 1.5 ml of pararosaniline solution to 1.5 ml of 4% sodium nitrite solution.

2. The incubating solution is made by mixing 2.5 ml of the alpha-napthyl acetate solution, 44.5 ml of 1/15 M phosphate buffer solution (pH 7.6) consisting of 87 ml of 1/15 M Na_2HPO_4 and 13 ml of 1/15 M KH_2PO_4 and 3.0 ml of fresh hexazotized pararosaniline solution. The incubating moxture is adjusted to pH 6.1 and filtered into a Coplin jar.

3. The slides are incubated in the incubating solution for 45 minutes, rinsed with distilled water and then counter stained with Rayer's haematoxylin stain for 5 - 10 minutes. This stain is specific for macrophage esterases and will stain the macrophage cytoplasm a bright red color. All nonspecific esterase stains were performed by the Immunopathology Laboratory of Dr. Norman Pettigrew at the Health Sciences Centre in Winnipeg.

Appendix VII

Luminol

A stock solution of luminol (5-amino-2,3-dihydro-1,4-pthalazinedione, Eastman-Kodak) was made by dissolving 19.5 mg of luminol in 20 ml of dimethyl sulfoxide (DMSO). A working solution was made by diluting 0.5 ml of the stock solution in 9.5 ml of DMSO.