EFFECT OF CHICKEN INTERFERON ON UPTAKE OF *SALMONELLA TYPHIMURIUM* BY AVIAN MACROPHAGES

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CHAPTER I

INTRODUCTION

The magnitude of salmonellosis as a worldwide human health problem has initiated great interest in the pathogenesis of the disease. *Salmonella typhimurium* has a wide range of susceptible hosts and is important economically and zoonotically. It causes clinical gastroenteritis and bacteremia, and may also be maintained subclinically in carrier animals. In poultry, especially broiler flocks, infection caused by *S. typhimurium* is frequently diagnosed. It is usually clinically inapparent in adult birds and is of significance largely from the standpoint of contamination of carcasses for human consumption. However, it can cause severe losses in young birds. The disease is known as paratyphoid and manifests itself as enteritis, diarrhea, and septicemia. It can also cause arthritis in ducks and swelling of the wing joints and even death in pigeon squabs. Most infections are derived from contamination of feed, although the organism may rarely localize in the ovary and be transmitted in the egg.

Salmonella species are considered by most investigators to be facultative intracellular pathogens, able to survive and multiply in phagocytic cells. Despite contradictory evidence to dispute that macrophages are the major site for multiplication *in vivo* (Lin et al, 1987; Hsu, 1989), the most popular view is that after ingestion and penetration of intestinal epithelial cells, *S. typhimurium* organisms localize and replicate in submucosal macrophages (Finlay & Falkow, 1989), in which they are transported to extraintestinal sites (Dunlap et al, 1992). The invasiveness and translocation of *S. typhimurium* are thought to contribute to the development of systemic disease and possibly establishment of the carrier state (Barrow et al, 1987; Wells et al, 1988).

Numerous experiments using epithelial cells have shown that interferons (IFNs) inhibit cellular invasion and intracellular replication by facultative intracellular bacteria. In

macrophages, IFN-α and IFN-β stimulate phagocytic activity, in contrast to IFN-γ which appears to depress phagocytosis (Degre et al, 1981). However, IFN-γ does enhance oxygen-dependent killing mechanisms in macrophages (Nathan et al, 1983). Thus, IFNs, and especially IFN-γ, could play a role in decreasing survival rate of *S. typhimurium* in phagocytic cells and this would, in turn, reduce invasiveness of the disease and development of subclinical carriers. Therefore, the purpose of the present study was to examine the effect of various dilutions of virus-induced chicken interferon on the uptake of *Salmonella typhimurium* by chicken macrophages *in vitro*.

CHAPTER II

LITERATURE REVIEW

Salmonella species Interaction with Epithelial Cells

All diseases caused by *Salmonella* species share a common route of infection, viz., ingestion followed by penetration of the intestinal epithelium. The ability of *Salmonella* organisms to invade, survive, and replicate in eukaryotic cells is essential for successful infection. Using transmission electron microscopy, Finlay et al (1992) have shown that invasive *Salmonella* species adhere to the apical surface of microvilli within 30 minutes of infection. By one hour after exposure, these bacteria have passed into the intestinal epithelium and are found in membrane-bound vesicles which begin to coalesce. After a 4-hour lag period, virulent *Salmonella* organisms begin to multiply within this large vacuole, and by 24 hours the vacuole is filled with bacteria, and the host cell lyses. The organisms may enter the lamina propria and cells of the mononuclear phagocyte system (Takeuchi, 1967) and from here may be disseminated to other parts of the body.

Before adherence of *Salmonella* species to host cell surfaces, the bacteria must collide with cells. Uhlman & Jones (1982) showed that the frequency of collision and hence attachment, was increased if *Salmonella* organisms were attracted by diffusible taxins released when cells were damaged. These taxins are thought to be amino acids, sugars and/or tricarboxylic acid intermediates. One has been tentatively identified as glycine. It was suggested that factors increasing cell permeability and the presence of dead and dying epithelial cells at the villus tips where invasion takes place, would attract *S. typhimurium* to host cells *in vivo*.

Only viable and metabolically active *Salmonella* species can adhere to host cell surfaces, in contrast to other invasive bacteria such as *Yersinia* species. Adherence is

followed very rapidly by invasion, as is seen with *S. typhimurium* infection of HeLa cell monolayers where most organisms are internalized within 20 minutes (Finlay et al, 1992). Adherence and invasion appear to be inseparable properties (noninvasive *Salmonella* mutants are also nonadherent). Active invasion requires both bacterial RNA and *de novo* protein synthesis with at least six different genetic loci, including a hyperinvading locus in *S. typhimurium* (Lee et al, 1992) being involved. Recently, Altmeyer et al (1993) identified a highly conserved gene that is necessary for efficient adherence and entry into cultured epithelial cells. The function of the proteins is still being characterized. However, protein synthesis appears to be regulated by the microenvironment, the growth phase, and the epithelial cell surface.

Internalization of *Salmonella* organisms is associated with disruption of the brush border and an increase in permeability of the tight junctions to small molecules. Invading *Salmonella* species have been shown by confocal and light immunofluorescence microscopy to induce rearrangements of host actin filaments and other cytoskeletal proteins. Upon binding, *Salmonella* organisms transduce an "uptake signal" (tyrosine protein kinases appear to be involved) to the host cell which causes recruitment of the cytoskeletal elements. These accumulate around the bacterium and then dissociate shortly after internalization. The bacterial factors involved have yet to be defined (Finlay et al, 1992), although it has recently been established that stimulation of the epidermal growth factor receptor is involved in *S. typhimurium* invasion (Galan et al, 1992). The host cell's responses used for bacterial uptake appear to be analogous to receptor-mediated endocytosis. Transmission electron microscopy has shown clathrin-coated membrane domains associated with penetrating *Shigella* species (Clerc et al, 1988). The protein, clathrin, is the major component of coated pits and vesicles in receptor-mediated endocytosis. Other studies done on invasive *Shigella* species show that they do not perforate the plasma membrane, but are observed within membrane-bound vacuoles shortly after entry (as is *S. typhimurium*), and that energy is required by the host cell for the

process. These would imply phagocytosis, yet this had only been described in cells such as macrophages. Hence, there has been considerable debate as to whether receptor-mediated endocytosis or a phagocytic-like process is involved in bacterial uptake. The process is now often referred to as parasite-mediated endocytosis.

Intracellular multiplication of *S. typhimurium* is an essential component of its pathogenicity. *Salmonella* organisms multiply inside vacuoles of the host cell. This behavior contrasts with that of other invasive bacteria, such as *Yersinia* species which survive in vacuoles but do not replicate, and *Shigella flexneri* which escapes the vacuole and replicates in the host cell cytoplasm. The microenvironment of the vacuole is one of low pH, low oxygen levels, and low free Mg^{2+} and Fe^{2+} concentrations (Finlay et al, 1992). Yet, *Salmonella* organisms survive and multiply in vacuoles, where they seem to modify the vacuolar membrane so that it can expand and enhance incorporation of specific growth-promoting factors from the cytoplasm (Clerc et al, 1988). Survival within the cell appears to involve blockage of phagosome-lysosome fusion.

Salmonella species Interaction with Phagocytes

Intracellular pathogens have evolved means to evade killing by professional phagocytes, thereby allowing them to survive in phagocytic cells (Suter, 1956). There has since been found to be a correlation between the ability of organisms to survive in macrophages *in vitro* and their ability to cause invasive disease (Williams et al, 1991). Fields et al (1986), by using an *in vitro* assay for survival within macrophages, identified *S. typhimurium* mutants with a decreased capacity for intracellular survival that were less virulent than the parent strain *in vivo*, demonstrating that survival within the macrophage is essential for virulence. *Salmonella typhimurium*, a facultative intracellular pathogen (Finlay & Falkow, 1989), has been well characterized physiologically and genetically, but only limited data are available on the mechanisms employed for survival within the

macrophage. In fact, certain researchers state that there has never been indisputable experimental evidence of intracellular survival and multiplication of *Salmonella* species within host phagocytes (Hsu, 1989; Lin et al, 1987). However, there is still persistent inference to survival and growth in phagocytes, and it has been postulated by Wells et al (1988) that motile submucosal macrophages phagocytose intestinal bacteria and fail to carry out intracellular killing. The bacteria survive and grow within this "safe-site" intracellular location during the first 24 hours after infection, and are then liberated at extraintestinal sites, where they may again be phagocytosed by other macrophages (Dunlap et al, 1992). This movement to extraintestinal sites is known as bacterial translocation, and occurs most readily with those bacteria classified as facultative intracellular bacteria. These are the only bacteria known to translocate reliably after oral inoculation into experimental animals. After oral inoculation, *S. typhimurium* has been found in the liver (Helmuth et al, 1985), spleen (Dunlap et al, 1992), mesenteric lymph nodes, mesentery (Que & Hentges, 1985), and Peyer's patches (Hohmann et al, 1978) of mice, and in the mesenteric lymph nodes, liver, and spleen of rats and piglets (Tlaskalova-Hogenova et al, 1983). There has also been the repeated observation by light and electron microscopy of bacteria within intestinal epithelial cells and mucosal phagocytes. Popiel & Turnbull (1985) photographed macrophages containing *Salmonella* species passing through breaks in the basement membrane and in the lamina propria of the intestinal mucosa of newborn chickens. Translocation occurs within hours after ingestion and the rate can be altered by agents that affect immune (including phagocytic) functions.

Once *S. typhimurium* has been phagocytosed, microtubules direct the movement of the phagocytic vesicle or phagosome within the macrophage. Carroll et al (1979) report that the phagosome then fuses with a lysosome to form a phagolysosome and it is here that survival and replication of *S. typhimurium* takes place. However, other reports suggest that survival in the phagosome of the macrophage appears to involve prevention of phagosome-lysosome fusion (Finlay et al, 1992). Nevertheless, the capacity for

intracellular survival and growth of *Salmonella* species within phagocytes appears to be related both to nutritional, especially folic acid, requirements, and to virulence factors (Lindberg, 1980; Falkow & Mekalanos, 1990).

Lipopolysaccharide (LPS) is the most extensively characterized virulence factor of *Salmonella* species. The oligosaccharide composition of the LPS appears to affect the ability of organisms to survive and replicate in macrophages, although most of this work has been conducted using *Haemophilus influenza* Type b (Williams et al, 1991). The lipid A component of LPS (endotoxin) activates macrophages resulting in fever, leukocytosis and shock, although it is unclear whether this benefits the organism. Another important virulence factor is the surface O antigen. It is not certain whether this surface antigen acts by preventing phagocytosis (through inhibition of complement-mediated opsonization) or by preventing subsequent destruction within macrophages. Recently, Stinavage et al (1990) identified an outer membrane protein which protects *Salmonella* species from oxidative killing by polymorphonuclear cells, and Kim et al (1988) identified a specific protector protein which inhibits enzyme activation by a thiol/Fe(III)O_2 mixed function oxidation system, which in turn protects *Salmonella* species from non-oxidative killing by polymorphonuclear cells. A further virulence factor is the flagellum. Flagella are thought to contribute either to enhanced resistance to macrophage killing or are necessary for intracellular multiplication in macrophages (Weinstein et al, 1984). Mutants that have lost flagella show a decreased capacity for survival and growth in macrophages. Most *Salmonella* species contain a large (50-100 Kb) plasmid that is essential for virulence, but the nature of this plasmid-encoded virulence property is unknown. Strains that have lost the plasmid enter epithelial cells and macrophages normally but do not survive and replicate. Recently, however, Riikonen et al (1992) found that the virulence plasmid does not contribute to growth of *Salmonella* species in cultured murine macrophages.

Experimental Methods used to Study Survival and Multiplication of *Salmonella* species in Macrophages

Various methods have been employed to study survival and proliferation of *Salmonella* species in host phagocytes. Cell culture techniques have so advanced over the last 30 years that they provide a valuable means by which direct interactions between virulent bacteria and isolated host cells can be studied. Various experiments have been conducted that use coverslips, culture tubes or culture plates for cell culture. Different staining techniques or viable intracellular bacterial assays have been used for bacterial quantitation. Many *in vivo* experiments have also been conducted. However, Hsu (1989) and others claim that the experimental evidence for survival and multiplication of *Salmonella* species in macrophages is disputable because basic criteria in experimental design have not been fully met. In addition, Hsu (1989), using electron microscopy, and Briles et al (1981) and van Zwet et al (1975) have shown destruction of virulent *Salmonella* species in macrophages.

One of the methods used to determine whether virulent *Salmonella* species will grow in macrophages is the cell culture technique. A disadvantage of this method is that it employs an artificial environment, where the host cells are never replenished and the medium differs from that of an *in vivo* inflammatory reaction. Some of the earliest work was conducted by Gelzer and Suter (1959), who cultured rabbit peritoneal exudative macrophages infected with *S. typhimurium* on cover slips. Mitsuhashi et al (1961) and Sato et al (1962) also used cover slips to culture various macrophages with *S. enteritidis*. At intervals the slides were removed, fixed and stained. The authors concluded that the virulent strain multiplied intracellularly while the avirulent strain did not. However, this method of microscopic counting of stained bacteria did not distinguish live from dead organisms, and therefore may not have reflected multiplication but merely an accumulation of bacteria. Better staining techniques have more recently been developed. Goldner et al

(1983) used a fluorescent acridine orange stain technique with extracellular quenching on gram-positive bacteria, and Miliotis (1991) performed a similar experiment using gram-negative bacteria. This technique not only differentiated between intra- and extracellular bacteria in cultured cells, but also between viable (green-fluorescing) and nonviable (red) intracellular bacteria (West, 1969).

Methods to assay viable intracellular bacteria have also been developed. Furness (1958) and Morello & Baker (1965), using individual culture tubes of infected mouse peritoneal macrophages, recovered viable intracellular *S. typhimurium* by lysing the macrophages with distilled water. Numbers of colony-forming units (CFU) were then counted on bacteriological media. Niesel et al (1985) quantitated bacteria in an infected cell monolayer by overlaying the cells with a distilled water-agarose-agar mixture. Microscopically, it has been shown that distilled water does not disrupt cells to release individual bacteria. Therefore, using these methods, bacterial counts may not represent the viable bacterial population but rather the infected cell population. Chemical detergents, such as sodium deoxycholate, are more effective than water in disintegrating mammalian cells, but are inadequate for recovery of *S. typhimurium* as the organism is sensitive to most of them (Hsu & Mayo, 1973). Amongst the physical methods of lysing host cells, sonication has been found to recover more *Salmonella* organisms than either water or chemicals (Baron & Proctor, 1984; Hsu & Radcliffe, 1968).

A controversial issue in cell culture experiments is the use of antibiotics in the culture medium. Antibiotics are usually used to control the extracellular bacterial population, both to prevent a continuation of phagocytosis after a certain time and to ensure an accurate CFU count of only those bacteria that are intracellular. It has been suspected, although never proved, that some antibiotics may penetrate the host cells and interfere with the host-parasite interaction. Patterson & Youmans (1970) attributed the suppressed multiplication of *Mycobacterium tuberculosis* within cultured immune macrophages to an increased penetration of streptomycin. Prolonged exposure of cultured cells to antibiotics

inhibited the multiplication of and later eradicated *S. typhosa* (Hopps et al, 1961). Both penicillin (Eagle, 1954) and streptomycin (Bonventre & Imhoff, 1970) have been shown to bind to cultured mammalian cells, but their activity against intracellular bacteria has not been established. Lobo & Mandell (1973) and Mandell (1973) claim that intracellular bacteria are protected from the bactericidal actions of non-lipid-soluble antibiotics, e.g. gentamicin. However, Kihlstrom (1977) found that the fraction of surviving bacteria decreased with increasing gentamicin concentration. This could have been due to the inability of the lower drug concentrations to gain access to all extracellular membrane-attached bacteria, or to the effect of higher drug concentrations on intracellular bacteria.

In vivo experiments to show that bacteria survive and multiply in macrophages have also been conducted. Numbers of viable intracellular bacteria were estimated by homogenizing liver or spleen of infected mice at various intervals after inoculation and then culturing the homogenates on bacteriological media (Collins, 1974). This method does not disrupt all infected cells nor effectively disperse intracellular bacteria for quantitation. As the liver and spleen are part of the mononuclear phagocyte system, it is often assumed that bacteria only multiply in resident macrophages. However, they just as readily replicate in the sinusoids and intercellular spaces (Lin et al, 1987; Wang et al, 1988). Therefore, an increase in bacterial population in the liver and spleen would not necessarily represent intracellular multiplication.

Effect of Interferon on *Salmonella* species Infection

Interferon was first described by Issacs & Lindenmann in 1957. They observed that virus-infected cell cultures produced a protein that acted on cells so that they became resistant to infection by many viruses (Baron et al, 1991). We now know that interferons are one of the body's natural defense mechanisms. They not only have antiviral action, but

also play a role in combating other microorganisms and tumors, and in regulating immunity.

Types of Interferon

Currently, three types of interferon are recognized; interferon alpha (IFN-α), interferon beta (IFN-β), and interferon gamma (IFN-γ). All IFNs are relatively small proteins with molecular weights ranging from 20-25 kD. Interferon production is genetically controlled and can be induced in most body cells by various stimuli.

There are at least 17 different human IFN-α genes. When production of IFN-α is induced in B-lymphocytes and other leukocytes (null lymphocytes and macrophages), a number of these genes are expressed resulting in different IFN-α subtypes. There is some speculation as to whether different leukocytes and/or the stage of leukocyte differentiation and/or the type of inducer used are relevant with regard to the subtype of IFN-α produced (Kirchner, 1986). IFN-αs are induced by virus-infected cells, viral envelopes, bacterial cells and their products, tumor cells, and activated natural killer (NK) cells, as well as synthetic polynucleotides.

In contrast to IFN-α, there appears to be only one human IFN-β gene, although data suggesting that there are two genes have been presented (Merigan, 1983). There are thought to be two genes in cattle. The gene coding for IFN-β displays 30-35% homology with those coding for the IFN-α family (Taniguchi et al, 1980). They are located on the same chromosome, and in the mouse, are usually expressed simultaneously, although Brehm et al (1986) showed that an IFN inducer, CMA (10-carboxymethyl-9-acridanone), induces only IFN-β in the mouse macrophage. These IFN-α and IFN-β families are referred to as Type I IFNs. Interferon-β is formed by virus- or synthetic polynucleotide-stimulated fibroblasts, epithelial cells, and macrophages.

Interferon-γ, or immune IFN, is produced by foreign antigen- or mitogen-activated T-lymphocytes and by activated NK cells. Taylor et al (1984) have shown that a B-lymphoblastoid cell line produced IFN-γ upon "heat shock", but this has yet to be confirmed. As IFN-γ is produced exclusively by lymphocytes, it is also a lymphokine. The gene coding for IFN-γ shows no homology with the genes for IFN-α and -β and contains introns that are absent in the other IFN genes (Gray & Goeddel, 1982). Hence, it is referred to as a Type II IFN and is less stable than Type I IFNs in an acid environment. The gene of the lymphokine, interleukin-2 (IL-2), has been reported to share a certain degree of homology with the IFN-γ gene.

The classification of IFNs into types α, β, and γ, is generally applied to mammals, but Dijkmans et al (1990), have found that this classification may not necessarily be applicable in birds. Human, baboon, and mouse spleen cells have been shown to be good producers of IFN-γ upon stimulation with mitogens, but similarly stimulated cultured chicken splenocytes produced a protein which was indistinguishable from IFN-α/β, and which was completely neutralized by anti-IFN-α/β antiserum. Similar results were obtained by Kohase et al (1986), who found that only one type of IFN (α or β) was produced in chick cells when induced with viruses or synthetic polynucleotides. Thus, the ability of chicken cells to produce IFN-γ has not been confirmed.

Mechanisms of Action

Many mechanisms of action of IFN have been identified. This research is focused on the defense against bacteria, but other mechanisms, namely antiviral and antitumor activity, and interaction with intercellular signaling substances (cytokines), are important and worthy of mention. All three types of IFN activate their target cells by binding to specific receptors on the cell surface. These receptors are of high affinity and selectivity. One class of high affinity sites appears to bind the various IFN-α subtypes and IFN-β,

while another recognizes IFN-γ only (Kirchner, 1986). This binding results in transmembrane signaling and induces synthesis of intracellular proteins which mediate the different actions of IFNs.

Over twenty IFN-induced proteins have been identified, including eIF-2a protein kinase, 2',5'-oligo-A synthetase and Mx protein, which are important in antiviral pathways. Antiviral action of IFN can occur at different stages of viral replication. The protein kinase system, activated by double-stranded RNA, reduces the translation of viral proteins by phosphorylating an initiating factor and decreasing the efficiency of protein synthesis initiation. The 2',5'-oligo-A synthetase system, which is also activated by double-stranded RNA, enzymatically degrades viral RNA so that it is no longer available for translation into viral proteins (Pestka et al, 1987). The Mx protein confers resistance against influenza virus and is thought to inhibit transcription (Arnheiter & Meier, 1990). Immunoregulatory actions of IFNs, such as macrophage activation, NK and cytotoxic T cell regulation, cytokine induction, and expression of major histocompatability (MHC) antigens may also affect viral replication.

Interferons exhibit antiproliferative effects on tumor cells, primarily by increasing the length of the cell multiplication cycle, repressing certain oncogenes, depleting essential metabolites, and by cytotoxic action. They also have indirect effects via enhanced expression of MHC antigens and tumor necrosis factor (TNF) receptors leading to better recognition and killing of tumor cells, increased induction of antibodies to tumors, and enhancement of tumor cytotoxicity by macrophages, NK cells and T lymphocytes.

The interaction of IFNs with cytokines is not yet completely understood, but IFN actions appear to be extensively modulated by cytokines. For example, IFN-γ activates macrophages by inducing them to produce TNF (Philip & Epstein, 1986), and IL-2 produced in T lymphocyes induces IFN-γ, which in part, increases NK cell activity (Baron et al, 1987).

During experimental infection with pathogenic protozoa and bacteria, IFN production has been observed. Numerous studies have shown that bacteria and/or bacterial products induce IFN production in certain leukocyte populations and, more recently, in fibroblasts, which produce IFN-α and IFN-β in response to intracellular bacteria (Hess et al, 1989).

The role of IFNs in modulating host defense responses is further illustrated by the observation that host resistance to various pathogens can be enhanced by treating infected animals with IFN. Furthermore, treatment with IFN-specific antibodies decreases host resistance (Baron et al, 1991). Defense mechanisms induced against bacterial pathogens involve both direct effects on nonphagocytic cells to inhibit invasion and replication, and indirect immunoregulatory effects, mainly through activation and increased phagocytic activity of macrophages.

Effect of Interferon on Uptake of *Salmonella* species

Numerous experiments have shown that pretreatment of epithelial cells with IFN inhibits cellular invasion and intracellular replication of facultative intracellular bacteria. It has been reported that pretreatment with homologous IFNs reduced the invasiveness of *Salmonella typhimurium* and *Salmonella paratyphi-B* in HEp-2 cells, and *Shigella flexneri* in cell culture models not susceptible to shiga toxin (Bukholm & Degre, 1983, 1985; Degre & Bukholm, 1988; Degre et al, 1989; Niesel et al, 1986). Furthermore, similar pretreatment reduced *in vivo* invasiveness of *S. typhimurium* in a mouse model (Bukholm et al, 1984). This response appeared to involve a reduced ability of the bacteria to enter IFN-treated cells, although the mechanism is not completely understood.

In macrophages, phagocytic activity is stimulated by IFN-α and IFN-β, but it appears to be depressed by IFN-γ (Degre et al, 1981). Interferons (more recently found to be mainly IFN-γ) also enhance the bacteriostatic and bactericidal activity of macrophages *in*

vitro (Patterson & Youmans, 1970; Fowles et al, 1973) and *in vivo* (Donahoe & Huang, 1976). Both an increase in phagocytic activity and intracellular killing appear to be major mechanisms of host defense, and aid in inhibiting the spread of pathogens in the host. As previously described, facultative intracellular bacteria are believed to survive and grow in macrophages where antibodies cannot confer protection against them. During the course of an infection, the cell-mediated immune response is stimulated and T lymphocytes release IFN which induces macrophage activation. This involves an increase in size, an increase in mobility (enhanced spreading, pinocytosis and phagocytosis) and an increase in metabolic activity (secretion of lysosomal enzymes and IL-1, membrane ruffling, oxidative metabolism, microbicidal activity, and tumoricidal activity). Mechanisms are not completely understood, but Kagaya et al (1989), using recombinant IFN-γ to activate peritoneal macrophages, suggested that enhanced *Salmonella*-killing activity was due to increased phagosome-lysosome fusion followed by oxygen-independent killing. Oxygen-independent killing mechanisms include stimulation of the enzymatic degradation of extracellular tryptophan (Byrne et al, 1986), limiting the availability of iron (Byrd & Horwitz, 1987), and production of microbicidal proteins belonging to the histone family (Hiemstra et al, 1993). These microbicidal proteins kill *S. typhimurium* as well as other bacteria. However, other authors have found that oxygen- (respiratory burst-) dependent mechanisms resulting in release of toxic oxygen intermediates, such as superoxide anion, hydrogen peroxide, and hydroxyl radical, play a primary role (Murray & Cohn, 1979, 1980; Edwards et al, 1992; Nathan et al, 1985). Both oxygen-independent and oxygen-dependent mechanisms are largely IFN-γ induced and can be stimulated by recombinant IFN-γ alone (Murray, 1988).

It is well documented that IFN-γ has differences in biological activities to IFN-α and IFN-β, i.e. structure, cellular sources, stimuli that induce secretion, physicochemical properties, cell surface receptors, antiviral efficacy, and antiproliferative activity. However, with the recent availability of pure recombinant IFN preparations, other

differences, most notably the ability to stimulate bactericidal activity of macrophages, have been shown. Interferon-γ is known to be the predominant lymphokine that activates macrophages for enhanced secretion of hydrogen peroxide (Nathan et al, 1983; Sharp & Banerjee, 1986). It appears to be far more efficient in this regard than IFN-α or -β (Pace et al, 1983), which have been found to be poor activators, or possibly even deactivators, of intracellular killing by macrophages (Nathan et al, 1985; Yoshida et al, 1988). Furthermore, experimental evidence by von Bulow et al (1984) suggested that virus-induced interferons (IFN-α/β) were 20-30 times less effective as macrophage intracellular killing activators than stimulators of antiviral activity. Speert & Thorson (1991) also found that monocyte-derived macrophages cultured in the presence of recombinant IFN-γ exhibited enhanced capacity to produce superoxide anion. Yet, despite this, and the fact that greater number of bacteria (*Pseudomonas aeruginosa*) were bound via the Fc receptors, receptor-mediated phagocytosis was inhibited, and therefore, killing capacity was compromised. This reaffirms that phagocytic activity appears to be depressed by IFN-γ (Degre et al, 1981), and may be important with intracellular bacteria which must gain access to the intracellular environment to survive. Bacteria which are already ingested would be killed more efficiently and those which are uningested would be denied the environment they need for survival and replication. These results differed from other investigators conducting similar experiments (Edwards et al, 1988; Kemmerich et al, 1987), who demonstrated an increased capacity of IFN-γ-treated neutrophils, monocytes or alveolar macrophages to kill *P. aeruginosa*, *S. aureus*, or *L. monocytogenes*. This may have been due to inherent differences in the types of phagocytic cells used.

The quiescent macrophage usually requires 24-72 hours of continuous *in vitro* exposure to IFN-γ before optimal activation is achieved (Nathan et al, 1983). Pulse exposure was far less effective (von Bulow et al, 1984). The activated state decreased in 2-3 days after removal of IFN-γ, in contrast to monocytes which, after brief exposure at relatively low concentrations, showed persistent activation for up to 7 days (Murray et al,

1987). The events at the plasma membrane and intracellular molecular level that lead to IFN-γ-induced macrophage activation are poorly understood. Surface receptors are important (Celada et al, 1986), and calcium levels and protein kinase C activity may also play a role (Celada & Schreiber, 1986).

CHAPTER III

EXPERIMENTAL OBJECTIVES

The overall objective of this experiment was to examine the effect of virus-induced chicken interferon on the uptake of *Salmonella typhimurium* by chicken macrophages. In addressing this goal, a method for cultivation of chicken macrophage (HD11) cells in tissue culture plates had to be developed and the activity of the interferon preparation needed to be confirmed. Thus, the study was conducted in three phases, as described by the following specific objectives:

Phase I: To develop a tissue culture method for the cultivation of HD11 macrophages.

Phase II: To confirm the activity of chicken embryo cell interferon (CEC-IFN) in HD11 cells by performing a yield reduction assay using vesicular stomatitis virus.

Phase III: To determine the effect of various CEC-IFN dilutions on the uptake of *Salmonella typhimurium* by HD11 cells, using a modification of an assay for viable intracellular bacteria described in Bukholm & Degre (1985) and Bukholm et al (1990).

CHAPTER IV

MATERIALS AND METHODS

Phase I: Tissue Culture Methodology

The HD11 cell line was provided by Dr. K. Klasing (Department of Avian Sciences, UC-Davis, Davis, CA). This chicken macrophage cell line was developed by transformation of chicken bone marrow cells using a replication-defective retrovirus, MC29 (Beug et al, 1979). These cells possess normal macrophage functions and markers, including phagocytosis of bacteria, macrophage surface antigens, Fc receptors, esterase and ATPase activity, and production of monokines when stimulated by heat-inactivated *Staphylococcus aureus* (Klasing and Peng, 1987, 1990).

The HD11 cells were provided in a 25cm^3 flask filled with Roswell Park Memorial Institute 1640 medium (RPMI 1640) and 5% fetal bovine serum (FBS). The cells were rounded and covered 80% of the flask surface. A small percentage of cells were free-floating. The medium was removed and replaced with 6 ml fresh RPMI 1640 (Curtin Matheson Sci., Carrollton, TX.), L-glutamine (200mM, 29.2 mg/ml) (JRH Biosciences, Lenexa, KS) at 10 ml/L, penicillin/streptomycin solution (JRH Biosciences, Lenexa, KS) at 10 ml/L, and 5% FBS (Endlo) (JRH Biosciences, Lenexa, KS). The flask was incubated overnight at 39.5 C under 5% CO_2. The medium was then removed, and the flask was scraped using a cell-scraper (Costar, Charlotte, NC). The cells were split 1:4 and transferred to a 75 cm^3 Costar canted-neck flask. Fresh medium and 10% FBS were added, and the flask was reincubated. Within 5 days, cells covered approximately 80% of the flask surface. The medium was removed, the cells scraped, split 1:6, and placed in new 75 cm^3 flasks with fresh medium containing 5% FBS, and reincubated. The fluid was renewed every third day, and the cells split 1:6 once a week (doubling time is

approximately 18 hours) throughout the following experiments. At no time did the cells cover >80% of the flask surface and the attached cells were never confluent. At approximately 80% coverage, the cells began detaching in large numbers.

For cultivation of HD11 monolayers in 24-well tissue culture plates (Phases II and III), a flask with approximately 80% attached cells was split 1:3. One third was diluted to 24 ml with RPMI 1640 and 5% FBS. One ml of this dilution was slowly pipetted into each of the 24 wells and incubated at 39.5 C under 5% CO_2 for 72 hours. Again, not more than 80% of the well surface was covered with attached cells.

As a 100% confluent monolayer was never achieved, a preliminary study was conducted to indicate whether any large differences existed between the number of cells/well or the pH of each well of a plate incubated for 72 hours. The number of viable cells/well (ml) was obtained by scraping the cells from each of 12 randomly chosen well surfaces. The cells were diluted with a 1:10 dilution of 0.25% trypan blue solution and counted in a Neubauer hemocytometer.

Throughout Phases II and III, avian cells were cultured at 39.5 C under 5% CO_2 and mammalian cells at 37 C under 5% CO_2.

Phase 2: Assay of CEC-IFN Activity in HD11 Cells

Previous observations had determined that HD11 cells began to detach when approximately 80% of the flask surface was covered with attached cells which were not confluent. As a result of this phenomenon, direct titration for viral plaques on HD11 cells was not possible. Therefore, a yield reduction assay to titrate for viral plaques was used.

Preparation of HD11 Cell Monolayer: Monolayers of HD11 cells in tissue culture plates were prepared as described in Phase I.

Preparation of Madin Darby Bovine Kidney Cell Monolayer: One 75 cm^3 flask of Madin Darby Bovine Kidney (MDBK) cells was split 1:10. One tenth was diluted to 24 ml with Minimum Essential Media (MEM) and 5% FBS. This step was repeated twice more and 1 ml added to each of 24 wells of 3 tissue culture plates. The plates were incubated for 3 days. At this time, the cells were approximately 90% confluent.

Interferon: A stock solution of Chicken Embryo Cell Interferon (CEC-IFN) (Batch #: RBI7-1, 7-27-88) of 16000 Units/ml (U/ml) was obtained from Dr. Phillip Marcus, Department of Molecular and Cellular Biology, University of Connecticut, Storrs, CT. The CEC-IFN was derived from primary chicken embryo cells that were exposed to UV-inactivated avian reovirus (Sekellick and Marcus, 1986). Six fourfold dilutions of CEC-IFN stock were made (1:100 through 1:102400).

Virus: A vesicular stomatitis virus (VSV SP5, 9-6-83) stock solution (Fulton et al, 1986) was diluted so as to obtain 10^3 plaque-forming units (PFU)/ml to be used as a challenge virus in HD11 cultures.

Yield Reduction Assay: A plaque-reduction assay was conducted to detect the effect of various dilutions of CEC-IFN on VSV-SP5. After the medium had been aspirated from the wells containing HD11 cells, 1 ml of each of the six CEC-IFN dilutions, plus 1 ml of control medium, were placed in duplicate wells and incubated for 24 hours. The CEC-IFN and medium were aspirated from all the wells and 0.1 ml of 10^3 PFU/ml VSV-SP5 was added to each well and incubated for 40 minutes. One milliliter of each of the appropriate CEC-IFN dilutions (and control medium) was replaced on each well and reincubated for 24 hours. The plate was frozen at -70 C and thawed when ready for use. Two samples of each dilution and 2 control samples were pooled and again frozen in tubes at -70 C until titration.

Pooled virus samples were thawed and ten-fold dilutions were made. One hundred microliters of each dilution (10^{-3} through 10^{-6}) of each sample were placed on duplicate MDBK wells from which medium had been aspirated. These plates were incubated for 40 minutes, and after adsorption, overlayed with 1 ml MEM methyl cellulose and reincubated for 48 hours.

An acetic acid : acetone : formalin fixative was placed in each well and left for at least 1 hour, and then each well was stained for 10 minutes with crystal violet. Plaques were scored, and the CEC-IFN titer determined by the probit method and expressed as the reciprocal of the highest CEC-IFN dilution producing 50% reduction in the number of plaques as compared to virus controls. The experiment was repeated once.

Phase 3: Effect of CEC-IFN on Uptake of *Salmonella typhimurium* by HD11 Macrophages

Cultivation of HD11 Cell Monolayers: Tissue culture plates were prepared as described in Phase I.

Preparation of Bacterial Inocula: *Salmonella typhimurium* (ATCC 14028, obtained from Dr. J.K. Skeeles, University of Arkansas, Fayetteville, AK) was streaked for isolated colonies on one blood agar plate and incubated at 37 C for 24 hours, after which the culture was visually checked for purity. From this culture, a new blood agar plate was streaked for confluent growth and incubated at 37 C for 24 hours. The confluent growth of *S. typhimurium* was then harvested with 5 ml phosphate buffered saline (PBS) (0.10M, pH 7.28). An absorbance of the harvest was determined photometrically at 650nm, and the number of colony-forming units/ml (CFU/ml) was estimated using a standard curve previously constructed (Figure 1). The harvest was then diluted with sterile PBS to an approximate concentration of 2.5×10^7 CFU/ml (inoculum).

Preparation of Chicken Interferon Dilutions: Four dilutions of CEC-IFN (0 U/ml, 10 U/ml, 100 U/ml and 1000 U/ml) were made using RPMI 1640 with 2% FBS and no antibiotics. They were stored at -4 C until used. After the HD11 cell tissue culture plate had incubated for 72 hours, the medium was aspirated from each well, and 1 ml of each of the four CEC-IFN dilutions was added to each of six wells on the plate and incubated for 24 hours. Thus each treatment was replicated six times on each plate. Five plates were used.

Challenge of HD11 cells with *S. typhimurium*: After incubation for 24 hours with CEC-IFN, the medium was removed from the wells using an aspirator, and the wells were washed once with 1 ml sterile PBS/well. One milliliter of fresh medium without antibiotics was added to each well and incubated for 1 hour. Thereafter, 200 ul of *S. typhimurium* inoculum was added to each well and incubated for 3 hours before estimation of intracellular bacterial numbers.

Intracellular Bacterial Count: Wells were washed once with 1 ml sterile PBS/well to remove most extracellular, unattached bacteria. One milliliter of medium with gentamicin (Gentamycin Solution, 50 mg/ml) (JRH Biosciences, Lenexa, KS) was then added at 50 ug/ml to each well to kill the remaining unattached and adherent extracellular bacteria, and the plate was incubated for 1 hour. The wells were washed twice with 1 ml sterile PBS/well, and then 1 ml sterile double-distilled water/well was added to lyse the macrophage cells. The plate was incubated at 37 C for 45 minutes. Two tenfold dilutions of supernatant were made, and blood agar plates were spotted with 6 x 0.1 ml for each of the dilutions (10^{0}, 10^{-1}, 10^{-2}) from each of the 24 wells. The blood agar plates were incubated at 37 C for 18 hours and the colony-forming units (CFU) were then counted.

Statistical Analysis: Bacterial counts were log transformed to ensure equality of variances. Data were initially analyzed using an analysis of variance with assays (plates) as blocks and pairwise comparisons of least squares treatment means were conducted using Scheffe's Test. Thereafter, data were reanalyzed using a nested design to test for significant interactions between interferon treatments and assays, and each assay was then analyzed independently. Interferon treatment means within each independent assay were separated by the LSD method. Differences were considered significant at the $P<0.05$ level.

CHAPTER V

RESULTS

Phase I: Tissue Culture Methodology

Growth and attachment of HD11 cells was adequate for the purposes of this study. The doubling time for the macrophages in RPMI 1640 and 5% FBS at 39.5 C, 5% CO_2 was 18-24 hours. Therefore, splitting the cells 1:6 at weekly intervals was sufficient to maintain the cell culture. Most of the cells remained adherent until they covered approximately 80% of the flask surface. Thereafter, the cells began to detach rapidly.

The preliminary study conducted to indicate whether any differences existed between the number of cells/well or the pH of each well of a plate incubated for 72 hours showed no marked differences between wells at the edges and corners of the plates compared to those in the center. For 12 randomly chosen wells, the pH range was 7.00-7.05, and the average number of cells/well (ml) was 8.35×10^5 cells/ml (range of 7.125×10^5 - 1.020×10^6 cells/ml).

Phase 2: Assay of CEC-IFN Activity in HD11 Cells

Two plaque reduction assays conducted to detect the effect of various dilutions of CEC-IFN in HD11 cultures challenged with VSV-SP5 indicated that CEC-IFN was indeed active. Increasing CEC-IFN concentrations caused a reduction in the number of viral plaques scored on MDBK cultures, as compared to CEC-IFN-free controls (Table I). The CEC-IFN titer producing 50% reduction in viral plaques as compared to CEC-IFN-free controls in both assays (calculated using the probit method) was 2839 U/ml and 549 U/ml, respectively.

Phase 3: Effect of CEC-IFN on Uptake of *Salmonella typhimurium* by HD11 Macrophages

Initial analysis using an analysis of variance with assays as blocks indicated that exposure of HD11 cells to CEC-IFN significantly increased the recovery of *S. typhimurium* at concentrations of 10 U/ml and 1000 U/ml CEC-IFN (Tables II & III). Lower concentrations of CEC-IFN had more effect than higher concentrations, as illustrated by the probabilities of making a Type 1 error on the conservative Scheffe's Test (Table IV). However, when the interaction between assays and interferon treatments was tested, a significant result suggested that responses of HD11 cells to interferon treatments was not consistent in all assays. Thus, assays were analyzed independently using one-factor analyses of variance, which revealed significant differences between treatments in only two of the five assays. In both assays showing significant differences, control (interferon-free) means were lower than interferon treatment means.

CHAPTER VI

DISCUSSION

It has long been recognized that *S. typhimurium,* which is regarded by most investigators to be a facultative intracellular parasite, has the ability to survive within macrophages. This phenomenon is thought to contribute to the invasiveness of enteric disease in chickens. In the present experiment, addition of chicken IFN enhanced recovery of *S. typhimurium* from chicken HD11 macrophages after 3 hours of incubation, although this effect was clearly evident in only 2 of the 5 assays conducted. Whether the intracellular organisms still survived and replicated after 3 hours was not addressed in this experiment.

Close examination of the relative magnitudes of treatment means calculated from pooled data (Table III & IV) suggested that although the highest concentration of CEC-IFN tested also caused an increase in the number of bacteria recovered from macrophages, this response was less marked than that caused by the lower concentration of CEC-IFN. Similar rankings in treatment means were found in 4 of the 5 assays conducted (Table III). This observation could be explained by a possible increase in intracellular killing at the higher concentration, which obscured the increased phagocytosis evident at lower concentrations. Chicken IFN concentrations higher than 1000 U/ml would be needed to determine whether the number of viable bacteria continued to decrease until the rate of phagocytosis was equalled or surpassed by the rate of intracellular killing.

The chicken IFN used in this experiment was induced from "aged" monolayers of primary chick embryo cells by UV-irradiated avian reovirus. Residual IFN-inducing particles were removed from the samples by using 6% FBS as a carrier to precipitate inducer virus and non-IFN macromolecules in the presence of perchloric acid (Sekellick & Marcus, 1986). This acidification process would leave only acid-stable IFN which is

presumed to be IFN-α/β. Using a yield reduction assay, this CEC-IFN did stimulate antiviral activity in the HD11 cell line. An IFN-α/β-like protein with antiviral effects was first described in chickens by Issacs & Lindenmann (1957) and later by Kohase et al (1986), but its primary sequence has yet to be elucidated and hence positively identified as a homologue of mammalian IFN-α or IFN-β. It is known that phagocytic activity of macrophages is stimulated by mammalian IFN-α and IFN-β. In the present experiment, results suggest that lower concentrations of CEC-IFN similarly stimulated phagocytic activity of the HD11 macrophage cell line, as indicated by increases in the number of viable bacteria present intracellularly after 3 hours of incubation, an interval previously shown to be sufficient time for adherance and uptake of bacteria, but not long enough for multiplication of surviving organisms (Finlay et al, 1992). However, mammalian IFN-α and IFN-β are poor activators of hydrogen peroxide-mediated bactericidal activity of macrophages (Nathan et al, 1985; Yoshida et al, 1988). Therefore, possible increases in intracellular killing ability of chicken macrophages at higher concentrations of CEC-IFN cannot be explained by IFN-α/β activity, assuming that CEC-IFN is functionally similar to mammalian IFN-α/β.

As yet, no molecular homologue with a similar range of actions to mammalian IFN-γ has been isolated from birds. Dijkmans et al (1990) found that mitogen-stimulated avian splenocytes contained a protein with antiviral activity as well as protein(s) with macrophage-activating activity as demonstrated by H_2O_2 production. The latter had physicochemical properties, e.g., acid resistance, different from mammalian IFN-γ but similar to IFN-α/β and could be neutralized by antiserum against chicken embryo cell IFN. This phenomenon could indicate that birds possess only one type of IFN that possibly also has macrophage-activating capabilities (von Bulow et al, 1984) or that macrophage-activating activity is induced by a second cytokine. It is also possible that birds have two types of IFN, where the IFN-γ equivalent is not produced by culture cells used thus far or is produced in quantities too low to be identified. In the present experiment, the number of

viable bacteria increased when low levels of IFN were added to chicken macrophages. However, despite an increase with higher concentrations of IFN, the effect was not as marked as that resulting from the low concentration. If, as Dijkmans et al (1990) suggested, birds only have one type of IFN which also possesses macrophage-activating capabilities or if they possess two types of IFN, of which the homologue of mammalian IFN-γ is acid resistant, this could account for the decreased intracellular survival when higher concentrations of IFN were added. Phagocytosis would be increased but the increasing number of viable bacteria would be offset by an increase in intracellular killing. Further study to differentiate between the number of bacteria phagocytosed and the number of bacteria killed at increasing IFN concentrations would be necessary, and such information may in turn aid in further elucidation of the chicken IFN system.

In summary, CEC-IFN increased recovery of *S. typhimurium* from HD11 macrophages, although this was only conclusively evidenced in 2 of the 5 assays conducted. This increased recovery was thought to result from enhanced uptake of bacteria, probably by phagocytosis. Examination of treatment means calculated from pooled data and ranking of treatment means in 4 of the 5 assays conducted, suggested that the increase in number of viable bacteria was not as marked at higher concentrations as that observed at lower concentrations, which could have been indicative of an increase in intracellular killing. Although speculative, this increase in phagocytic intracellular killing, together with recent evidence that high IFN concentrations protect chicken intestinal epithelial cells against *S. typhimurium* invasion (Fulton et al, submitted for publication), could decrease the spread of *Salmonella* organisms from the intestine to extraintestinal sites. Such decreased spread of organisms would reduce severe losses characteristic of *S. typhimurium* infections in young chickens and presumably could also play a role in preventing the establishment of a carrier state in chickens.

REFERENCES

Altmeyer, R.M., McNern, J.K., Bossio, J.C., Rosenshine, I., Finlay, B.B. & Galan, J.E. (1993) Cloning and molecular characterization of a gene involved in *Salmonella* adherence and invasion of cultured epithelial cells. Molecular Microbiology, 7(1), 89-98.

Arnheiter, H. & Meier, E. (1990) Mx proteins: antiviral proteins by chance or by necessity? New Biologist, 2, 851-857.

Baron, E.J. & Proctor, R.A. (1984) Inefficient *in vitro* killing of virulent or nonvirulent *Salmonella typhimurium* by murine polymorphonuclear neutrophils. Canadian Journal of Microbiology, 30, 1264-1270.

Baron, S., Dianzani, F., Stanton, G.J. & Fleischmann, W.R. Jr. (1987) The Interferon System: A Current Review to 1987. Austin: University of Texas Press.

Baron, S., Tyring, S.K., Fleischmann, W.R. Jr., Coppenhaver, D.H., Niesel, D.W., Klimpel, G.R., Stanton, G.J. & Hughes, T.K. (1991) The Interferons. Mechanisms of Action and Clinical Applications. Journal of the American Medical Association, 266 (10), 1375-1382.

Barrow, P.A., Huggins, M.B., Lovell, M.A. & Simpson, J.M. (1987) Observations on the pathogenesis of experimental *Salmonella typhimurium* infections in chickens. Research in Veterinary Science, 42, 194-199.

Beug, H., von Kirchbach, A., Doderlein, G., Conscience, J.F. & Graf, T. (1979) Chicken hematopoietic cells transformed by seven strains of defective avian leukemia viruses display three distinct phenotypes of differentiation. Cell, 18, 375-390.

Bonventre, P.F. & Imhoff, J.G. (1970) Uptake of (^{3}H)dihydrostreptomycin by macrophages in culture. Infection and Immunity, 2, 89-95.

Brehm, G., Storch, E. & Kirchner, H. (1986) Characterization of interferons induced in murine macrophage cultures by 10-carboxymethyl-9-acridanone. Natural Immunity and Cell Growth Regulation, 5 (1), 50-59.

Briles, D.E., Lehmeyer, J. & Forman, C. (1981) Phagocytosis and killing of *Salmonella typhimurium* by peritoneal exudate cells. Infection and Immunity, 33, 380-388.

Bukholm, G., Berdal, B.P., Haug, C. & Degre, M. (1984) Mouse fibroblast interferon modifies *Salmonella typhimurium* infection in infant mice. Infection and Immunity, 45, 62-66.

Bukholm, G. & Degre, M. (1983) Effect of human leukocyte interferon on invasiveness of *Salmonella* species in HEp-2 cell cultures. Infection and Immunity, 42, 1198-1202.

Bukholm, G. & Degre, M. (1985) Effect of Human Gamma Interferon on Invasiveness of *Salmonella typhimurium* in HEp-2 cell cultures. Journal of Interferon Research, 5, 45-53.

Bukholm, G., Degre, M. & Whitaker-Dowling, P. (1990) Interferon Treatment Reduces Endocytosis of Virus and Facultatively Intracellular Bacteria in Various Cell Lines. Journal of Interferon Research, 10, 83-89.

Byrd, T.F. & Horwitz, M.A. (1987) Intracellular multiplication of *Legionella pneumophilia* in human monocytes is iron-dependent and the capacity of activated monocytes to inhibit intracellular multiplication is reversed by iron-transferrin (Abstract). Clinical Research, 35, 613.

Byrne, G.I., Lehmann, L.K. & Landry, G.J. (1986) Induction of tryptophan catabolism is the mechanism for gamma-interferon-mediated inhibition of intracellular *Chlamydia psittaci* replication in T24 cells. Infection and Immunity, 53, 347-351.

Carroll, M.E.W., Jackett, P.S., Aber, V.R. & Lowrie, D.B. (1979) Phagolysosome formation, cyclic adenosine 3':5'-monophosphate and the fate of *Salmonella typhimurium* within mouse peritoneal macrophages. Journal of General Microbiology, 110, 421-429.

Celada, A., Allen, A., Esparza, I., Gray, P.W. & Schreiber, R.D. (1986) Demonstration and partial characterization of the interferon-gamma receptor on human mononuclear phagocytes. Journal of Clinical Investigation, 76, 2196-2205.

Celada, A. & Schreiber, R.D. (1986) Role of protein kinase C and intracellular calcium mobilization in the induction of macrophage tumoricidal activity by interferon-gamma. Journal of Immunology, 137, 2373-2379.

Clerc, P., Baudry, B. & Sansonetti, P.J. (1988) Molecular Mechanisms of Entry, Intracellular Multiplication and Killing of Host Cells by Shigellae. Current Topics in Microbiology and Immunology, 138, 3-13.

Collins, F.M. (1974) Vaccines and cell-mediated immunity. Bacteriological Review, 38, 371-402.

Degre, M. & Bukholm, G. (1988) Interferon effects on infection with enteroinvasive bacteria. In Byrne, G.I. and Turco, J. (Eds.) Interferon and Nonviral Pathogens. (pp. 295-310). New York, NY: Marcel Dekker, Inc.

Degre, M., Bukholm, G. & Czarniecki, C.W. (1989) *In vitro* treatment of HEp-2 cells with human tumor necrosis factor-α and human interferons reduces invasiveness of *Salmonella typhimurium*. Journal of Biological Regulators and Homeostatic Agents, 3 (1), 1-7.

Degre, M., Sonnenfeld, G., Rollag, H. & Morland, B. (1981) Effect of gamma interferon preparations on *in vitro* phagocytosis and degradation of *Escherichia coli* by mouse peritoneal macrophages. Journal of Interferon Research, 1, 505-512.

Dijkmans, R., Creemers, J. & Billiau, A. (1990) Chicken macrophage activation by interferon: do birds lack the molecular homologue of mammalian interferon-γ? Veterinary Immunology and Immunopathology, 26, 319-332.

Donahoe, R.M. & Huang, K.-Y. (1976) Interferon preparations enhance phagocytosis *in vivo*. Infection and Immunity, 13, 1250.

Dunlap, N.E., Benjamin, W.H. Jr., Berry, A.K., Eldridge, J.H. & Briles, D.E. (1992) A 'safe-site' for *Salmonella typhimurium* is within splenic polymorphonuclear cells. Microbial Pathogenesis, 13, 181-190.

Eagle, H. (1954) The binding of penicillin in relation to its cytotoxic action. III. The binding of penicillin by mammalian cells in tissue culture. Journal of Experimental Medicine, 100, 117-124.

Edwards, C.K., Ghiasuddin, S.M., Yunger, L.M., Lorence, R.M., Arkins, S., Dantzer, R. & Kelley, K.W. (1992) *In Vivo* Administration of Recombinant Growth Hormone or Gamma Interferon Activates Macrophages: Enhanced Resistance to Experimental *Salmonella typhimurium* Infection is Correlated with Generation of Reactive Oxygen Intermediates. Infection and Immunity, 60 (6), 2514-2521.

Edwards, S.W., Say, J.E. & Hughes, V. (1988) Gamma interferon enhances the killing of *Staphylococcus aureus* by human neutrophils. Journal of General Microbiology, 134, 37-42.

Falkow, S. & Mekalanos, J. (1990) The enteric bacilli and vibrios. In Davis, B.D., Dulbecco, R., Eisen, H.N., and Ginsberg, H.S. (Eds.). Microbiology. Fourth edition. (pp. 576-579). Philadelphia: J.B. Lippincott Co.

Fields, P.I., Swanson, R.V., Haidaris, C.G. & Heffron, F. (1986) Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proceedings of the National Academy of Sciences USA, 83, 5189-5193.

Finlay, B.B. & Falkow, S. (1989) *Salmonella* as an intracellular parasite. Molecular Microbiology, 3, 455-457.

Finlay, B.B., Leung, K.Y., Rosenshine, I. & Garcia-del Portillo, F. (1992) *Salmonella* Interactions with the Epithelial Cell. A model to study the biology of intracellular parasitism. American Society for Microbiology News, 58 (9), 486-489.

Fowles, R.E., Fajardo, I.M., Leibowitch, J.L. & David, J.R. (1973) The enhancement of macrophage bacteriostasis by products of activated lymphocytes. Journal of Experimental Medicine, 138, 952-964.

Fulton, R.W., Burge, L.J. & McCracken, J.S. (1986) Effect of recombinant DNA-derived bovine and human interferons on replication of bovine herpesvirus-1, parainfluenza-3, and respiratory syncytial viruses. American Journal of Veterinary Research, 47, 751-753.

Fulton, R.W., Morton, R.J., Burge, L.J., Short, E.C. & Payton, M.E. (Submitted for publication) Antiviral, Antibacterial, and 2',5'-Oligoadenylate Synthetase Activities in Interferon Treated Quail Cells.

Furness, G. (1958) Interaction between *Salmonella typhimurium* and phagocytic cells in cell culture. Journal of Infectious Diseases, 103, 272-277.

Galan, J.E., Pace, J. & Hayman, M.J. (1992) Involvement of the epidermal growth factor receptor in the invasion of cultured mammalian cells by *Salmonella typhimurium*. Nature, 357, 588-589.

Gelzer, J. & Suter, E. (1959) The effect of antibody on intracellular parasitism of *Salmonella typhimurium* in mononuclear phagocytes *in vitro* : prolonged survival of infected monocytes in presence of antibody. Journal of Experimental Medicine, 110, 715-730.

Goldner, M., Farkas-Himsley, H., Kormendy, A. & Skinner, M. (1983) Bacterial Phagocytosis Monitored by Fluorescence and Extracellular Quenching: Ingestion and Intracellular Killing. Laboratory Medicine, 14 (5), 291-294.

Gray, P.W. & Goeddel, D.V. (1982) Structure of the human interferon gene. Nature, 298, 859.

Helmuth, R., Stephan, R., Bunge, C., Hoog, B., Steinbeck, A. & Bulling, E. (1985) Epidemiology of virulence-associated plasmids and outer membrane protein patterns within seven common *Salmonella* serotypes. Infection and Immunity, 48, 175-182.

Hess, C.B., Niesel, D.W. & Klimpel, G.R. (1989) The induction of interferon production by invasive bacteria: A comparison of *Shigella* and *Salmonella* sp. Microbial Pathogenesis, 7, 111-120.

Hiemstra, P.S., Eisenhauer, P.B., Harwig, S.S., van den Barselaar, M.T., van Furth, R. & Lehrer, R.I. (1993) Antimicrobial proteins of murine macrophages. Infection and Immunity, 61 (7), 3038-3046.

Hohmann, A.W., Schmidt, G. & Rowley, D. (1978) Intestinal colonization and virulence of *Salmonella* in mice. Infection and Immunity, 22, 763-770.

Hopps, H.E., Smadel, J.E., Bernheim, B.C., Danauskas, J.X. & Jackson, E.B. (1961) Effect of antibiotics on intracellular *Salmonella typhosa*. II. Elimination of infection by prolonged treatment. Journal of Immunology, 87, 162-174.

Hsu, H.S. (1989) Pathogenesis and Immunity in Murine Salmonellosis. Microbiological Reviews, 53 (4), 390-409.

Hsu, H.S. & Mayo, D.R. (1973) Interactions between macrophages of guinea pigs and salmonellae. III. Bactericidal action and cytophilic antibodies of macrophages of infected guinea pigs. Infection and Immunity, 8, 165-172.

Hsu, H.S. & Radcliffe, A.S. (1968) Interactions between macrophages of guinea pigs and salmonellae. I. Fate of *Salmonella typhimurium* within macrophages of normal guinea pigs. Journal of Bacteriology, 96, 191-197.

Issacs, A. & Lindenmann, J. (1957) Virus interference. I. The interferon. Proceedings of the Royal Society of London. Series B: Biological Sciences, 147, 258.

Kagaya, K., Watanabe, K. & Fukazawa, Y. (1989) Capacity of Recombinant Gamma Interferon to Activate Macrophages for *Salmonella*-Killing Activity. Infection and Immunity, 57 (2), 609-615.

Kemmerich, B., Rossing, T.H. & Pennington, J.E. (1987) Comparative oxidative microbicidal activity of human blood monocytes and alveolar macrophages and activation by recombinant gamma interferon. American Review of Respiratory Diseases, 136, 266-270.

Kihlstrom, E. (1977) Infection of HeLa Cells with *Salmonella typhimurium* 395 MS and MR10 Bacteria. Infection and Immunity, 17 (2), 290-295.

Kim, K., Kim, I.H., Rhee, S.G. & Stadtman, E.R. (1988) The isolation and purification of a specific 'protector' protein which inhibits enzyme activation by a thiol/Fe(III)O_2 mixed-function oxidation system. Journal of Biological Chemistry, 263,4704-4711.

Kirchner, H. (1986) The interferon system as an integral part of the defense system against infections. Antiviral Research, 6, 1 - 17.

Klasing, K. & Peng, R. (1987) Influence of cell sources, stimulating agents, and incubation conditions on release of interleukin-1 from chicken macrophages. Developmental and Comparative Immunology, II, 385-394.

Klasing, K. & Peng, R. (1990) Monokine-like activities released from a chicken macrophage line. Animal Biotechnology, 1, 107-120.

Kohase, M., Moriya, H., Sato, T.A., Kohno, S. & Yamazaki, S. (1986) Purification and Characterization of Chick Interferon Induced by Viruses. Journal of General Virology, 67, 215-218.

Lee, C.A., Jones, B.D. & Falkow, S. (1992) Identification of a *Salmonella typhimurium* invasion locus by selection for hyperinvasive mutants. Proceedings of the National Academy of Sciences USA, 89, 1847-1851.

Lin, F.R., Wang, X.M., Hsu, H.S., Mumaw, V.R. & Nakoneczna, I. (1987) Electron microscopic studies on the location of bacterial proliferation in the liver in murine salmonellosis. British Journal of Experimental Pathology, 68, 539-550.

Lindberg, A.A. (1980) Bacterial virulence factors with particular reference to *Salmonella* bacteria. Scandinavian Journal of Infectious Diseases Supplement, 24, 86-92.

Lobo, M.C. & Mandell, G.L. (1973) The effect of antibiotics on *Escherichia coli* ingested by macrophages. Proceedings of the Society of Experimental Biological Medicine, 142, 1048-1050.

Mandell, G.L. (1973) Interaction of intraleukocytic bacteria and antibiotics. Journal of Clinical Investigation, 52, 1673-1679.

Merigan, T.C. (1983) Human interferon as a therapeutic agent - current status (Editorial). The New England Journal of Medicine, 308, 1530-1531.

Miliotis, M.D. (1991) Acridine Orange Stain for Determining Intracellular Enteropathogens in HeLa cells. Journal of Clinical Microbiology, 29 (4), 830-831.

Mitsuhashi, S., Sato, I. & Tanaka, T. (1961) Experimental salmonellosis: intracellular growth of *Salmonella enteritidis* ingested in mononuclear phagocytes of mice and cellular basis of immunity. Journal of Bacteriology, 81, 863-868.

Morello, J.A. & Baker, E.E. (1965) Interaction of *Salmonella* with phagocytes *in vitro*. Journal of Infectious Diseases, 115, 131-141.

Murray, H.W. (1988) Interferon-Gamma, the Activated Macrophage, and Host Defense Against Microbial Challenge. Annals of Internal Medicine, 108 (4), 595-608.

Murray, H.W. & Cohn, Z.A. (1979) Macrophage oxygen-dependent antimicrobial activity. I. Susceptibility of *Toxoplasma gondii* to oxygen intermediates. Journal of Experimental Medicine, 150, 938-949.

Murray, H.W. & Cohn, Z.A. (1980) Macrophage oxygen-dependent antimicrobial activity. III. Enhanced oxidative metabolism as an expression of macrophage activation. Journal of Experimental Medicine, 152, 1596-1609.

Murray, H.W., Scavuzzo, D., Jacobs, J.L., Kaplan, M.H., Libby, D.M., Schindler, J. & Roberts, R.B. (1987) *In vitro* and *in vivo* activation of human mononuclear phagocytes by interferon-γ: Studies with normal and AIDS monocytes. Journal of Immunology, 138, 2457-2462.

Nathan, C.F., Horowitz, C.R., de la Harpe, J., Vadhan-Raj, S., Sherwin, S.A., Oettgen, H.F. & Krown, S.E. (1985) Administration of recombinant interferon-γ to cancer patients enhances monocyte secretion of hydrogen peroxide. Proceedings of the National Academy of Sciences USA, 82, 8686-8690.

Nathan, C.F., Murray, H.W., Wiebe, M.E. & Rubin, B.Y. (1983) Identification of interferon-γ as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. Journal of Experimental Medicine, 158, 670-689.

Niesel, D.W., Chambers, C.E. & Stockman, S.L. (1985) Quantitation of HeLa Cell Monolayer Invasion by *Shigella* and *Salmonella* species. Journal of Clinical Microbiology, 22 (6), 897-902.

Niesel, D.W., Hess, C.B., Cho, Y.-J., Klimpel, K.D. & Klimpel, G.R. (1986) Natural and recombinant IFN inhibit tissue culture cell invasion by *Salmonella* and *Shigella.* Infectious Immunology, 52, 828-833.

Pace, J.L., Russell, S.W., Schreiber, R.D., Altman, A. & Katz, D.H. (1983) Macrophage activation: Priming activity from a T-cell hybridoma is attributable to interferon-γ. Proceedings of the National Academy of Sciences USA, 80, 3782-3786.

Patterson, R.J. & Youmans, G.P. (1970) Multiplication of *Mycobacterium tuberculosis* within normal and "immune" mouse macrophages cultivated with and without streptomycin. Infection and Immunity, 1, 30-40.

Pestka, S., Langer, J.A., Zoon, K.C. & Samuel, C.E. (1987) Interferons and their actions. Annual Review of Biochemistry, 56, 727-777.

Philip, R. & Epstein, L.B. (1986) Tumor necrosis factor as immunomodulator and mediator of monocyte cytotoxicity induced by itself, γIFN and interleukin-1. Nature, 323, 86-89.

Popiel, I. & Turnbull, P.C.B. (1985) Passage of *Salmonella enteritidis* and *Salmonella thompson* through chick ileocecal mucosa. Infection and Immunity, 47, 786-792.

Que, J.U. & Hentges, D.J. (1985) Effect of streptomycin administration on colonization resistance to *Salmonella typhimurium* in mice. Infection and Immunity, 48, 169-174.

Riikonen, P., Makela, P.H., Saarilahti, H., Sukupolvi, S., Taira, S. & Rhen, M. (1992) The virulence plasmid does not contribute to growth of *Salmonella* in cultured murine macrophages. Microbial Pathogenesis, 13 (4), 281-291.

Sato, I., Tanaka, T., Saito, K. & Mitsuhashi, S. (1962) Inhibition of *Salmonella enteritidis* ingested in mononuclear phagocytes from liver and subcutaneous tissue of mice immunized with live vaccine. Journal of Bacteriology, 83, 1306-1314.

Sekellick, M.J. & Marcus, P.I. (1986) Induction of High Titer Chicken Interferon. Methods in Enzymology, 119, 115-125.

Sharp, A.K. & Banerjee, D.K. (1986) Effect of gamma interferon on hydrogen peroxide production by cultured mouse peritoneal macrophages. Infection and Immunity, 54, 597-599.

Speert, D.P. & Thorson, L. (1991) Suppression by Human Recombinant Gamma Interferon of In Vitro Macrophage Nonopsonic and Opsonic Phagocytosis and Killing. Infection and Immunity, 59 (6), 1893-1898.

Stinavage, P.S., Martin, L.E. & Spitznagel, J.K. (1990) A 59 kilodalton outer membrane protein of *Salmonella typhimurium* protects against oxidative intraleukocytic killing due to human neutrophils. Molecular Microbiology, 4, 283-293.

Suter, E. (1956) Interaction between phagocytes and pathogenic microorganisms. Bacteriological Reviews, 20, 94-132.

Takeuchi, A. (1967) Electron microscopic studies of experimental *Salmonella* infection. I. Penetration into the intestinal epithelium by *Salmonella typhimurium*. American Journal of Pathology, 50, 109-136.

Taniguchi, T., Mantei, N., Schwarzstein, M., Nagata, S., Muramatsu, M. & Weissmann, C. (1980) Human leucocyte and fibroblast interferons are structurally related. Nature, 285, 547.

Taylor, M.W., Long, T., Martinez-Valdez, H., Downing, J. & Zeige, D. (1984) Induction of γ-interferon activity by elevated temperatures in human B-lymphoblastoid cell lines. Proceedings of the National Academy of Sciences USA, 81, 4033-4036.

Tlaskalova-Hogenova, H., Sterzl, J., Stepankova, R., Dlabac, V., Vetvicka, V., Rossmann, P., Mandel, L. & Rejnek, J. (1983) Development of immunological capacity under germfree and conventional conditions. Annals of the New York Academy of Sciences, 409, 96-113.

Uhlman, D.L. & Jones, G.W. (1982) Chemotaxis as a Factor in Interactions between HeLa Cells and *Salmonella typhimurium*. Journal of General Microbiology, 128, 415-418.

van Zwet, T.L., Thompson, J. & van Furth, R. (1975) Effect of glucocorticosteroids on the phagocytosis and intracellular killing by peritoneal macrophages. Infection and Immunity, 12, 699-705.

von Bulow, V., Weiler, H. & Klasen, A. (1984) Activating effects of interferons, lymphokines and viruses on cultured chicken macrophages. Avian Pathology, 13, 621-637.

Wang, X.M., Lin, F.R., Hsu, H.S., Mumaw, V.R. & Nakoneczna, I. (1988) Electronmicroscopic studies on the location of salmonella proliferation in the murine spleen. Journal of Medical Microbiology, 25, 41-47.

Weinstein, D.L., Carsiotis, M., Lissner, C.R. & O'Brien, A.D. (1984) Flagella Help *Salmonella typhimurium* Survive Within Murine Macrophages. Infection and Immunity, 46 (3), 819-825.

Wells, C.L., Maddaus, M.A. & Simmons, R.L. (1988) Proposed Mechanisms for the Translocation of Intestinal Bacteria. Reviews of Infectious Diseases, 10 (5), 958-979.

West, S.S. (1969) Quantitative microscopy in bacteriology. Annals of the New York Academy of Sciences, 157, 111-122.

Williams, A.E., Maskell, D.J. & Moxon, E.R. (1991) Relationship between Intracellular Survival in Macrophages and Virulence of *Haemophilus influenzae* Type b. The Journal of Infectious Diseases, 163, 1366-1369.

Yoshida, R., Murray, H.W. & Nathan, C.F. (1988) Antagonist and agonist effects of interferon α and β on activation of human macrophages. Journal of Experimental Medicine, 167, 1171-1185.

APPENDICES

APPENDIX A

TABLES

TABLE I

VIRAL PLAQUES SCORED ON MADIN DARBY BOVINE KIDNEY CULTURES AFTER EXPOSURE TO VESICULAR STOMATITIS VIRUS (VSV) DERIVED FROM CEC-IFN-TREATED HD11 CULTURES

		VSV dilutions				
	CEC-IFN	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Experiment I	1:100	TNTC	107	17	0	-
	1:400	TNTC	TNTC	85	8	-
	1:1600	TNTC	TNTC	TNTC	30	-
	1:6400	TNTC	TNTC	TNTC	61	-
	1:25600	TNTC	TNTC	TNTC	36	-
	1:102400	TNTC	TNTC	TNTC	21	-
	Control	TNTC	TNTC	TNTC	77	8
Experiment II	1:100	TNTC	139	12	2	-
	1:400	TNTC	TNTC	63	4	-
	1:1600	TNTC	TNTC	TNTC	18	-
	1:6400	TNTC	TNTC	TNTC	17	-
	1:25600	TNTC	TNTC	TNTC	21	-
	1:102400	TNTC	TNTC	TNTC	20	-
	Control	TNTC	TNTC	TNTC	16	4

TNTC = Too numerous to count

TABLE II

NUMBERS OF COLONY-FORMING UNITS RECOVERED FROM HD11 MACROPHAGES EXPOSED TO IFN-FREE BUFFER (CONTROL) OR DIFFERENT CONCENTRATIONS OF CEC-IFN (1,000, 100, OR 10 U/ML) AND THEN INCUBATED WITH *SALMONELLA TYPHIMURIUM*

	Well replicates					
INF (U/ml)	1	2	3	4	5	6
Assay 1						
Control	267000	252000	302000	292000	257000	195000
1000	302000	278000	223000	303000	198000	337000
100	303000	218000	248000	242000	242000	258000
10	315000	295000	268000	275000	333000	262000
Assay 2						
Control	152000	168000	258000	200000	272000	210000
1000	203000	242000	160000	178000	165000	160000
100	212000	150000	210000	155000	155000	140000
10	287000	160000	173000	305000	147000	267000
Assay 3						
Control	63300	83300	121000	66700	100000	58300
1000	187000	102000	138000	118000	95000	117000
100	109000	88300	105000	108000		113000
10	150000	132000	105000	121000	137000	162000
Assay 4						
Control	12500	36700	65000	13300	50000	41700
1000	28100	50000	35800	29800	32300	36000
100	26800	29900	51600	31900	65000	93300
10	65000	28900	75000	36700	53300	63300
Assay 5						
Control	40800	36500	39100	55700	45000	35100
1000	102000	80000	68300	73300	81700	63300
100	78300	80000	95000	65000	88300	63300
10	63300	63300	71700	60000	51700	66700

TABLE III

MEAN (± SD) NUMBERS OF *SALMONELLA TYPHIMURIUM* COLONY-FORMING UNITS CALCULATED FOR EACH IFN TREATMENT IN EACH OF THE ASSAYS

	Interferon treatments			
	Control	1000 U/ml	100 U/ml	10 U/ml
Assay 1	260833 ± 37765	273500 ± 52888	251833 ± 28315	291333 ± 28218
Assay 2	210000 ± 47699	184667 ± 32469	170333 ± 31979	223167 ± 70712
Assay 3	82100 ± 24463	126167 ±* 33307	104660 ±* 9583	134500 ±* 20305
Assay 4	36533 ± 20668	35333 ± 7847	49750 ± 25969	53700 ± 17764
Assay 5	42033 ± 7542	78100 ±*† 13608	78317 ±*† 12519	62783 ±* 6723
Mean	126300	139553	130979	153097
SE	102734	93266	80961	102958

* Significantly different from control value

† Significantly different from 10 U/ml

TABLE IV

SCHEFFE'S TEST - PAIRWISE COMPARISON PROBABILITIES

	Probabilities			
CEC-IFN	Control	1000 U/ml	100 U/ml	10 U/ml
Control	1.0000			
1000 U/ml	0.0416*	1.0000		
100 U/ml	0.0606	0.9994	1.0000	
10 U/ml	0.0009*	0.6498	0.5800	1.0000

*Significant

APPENDIX B

FIGURE

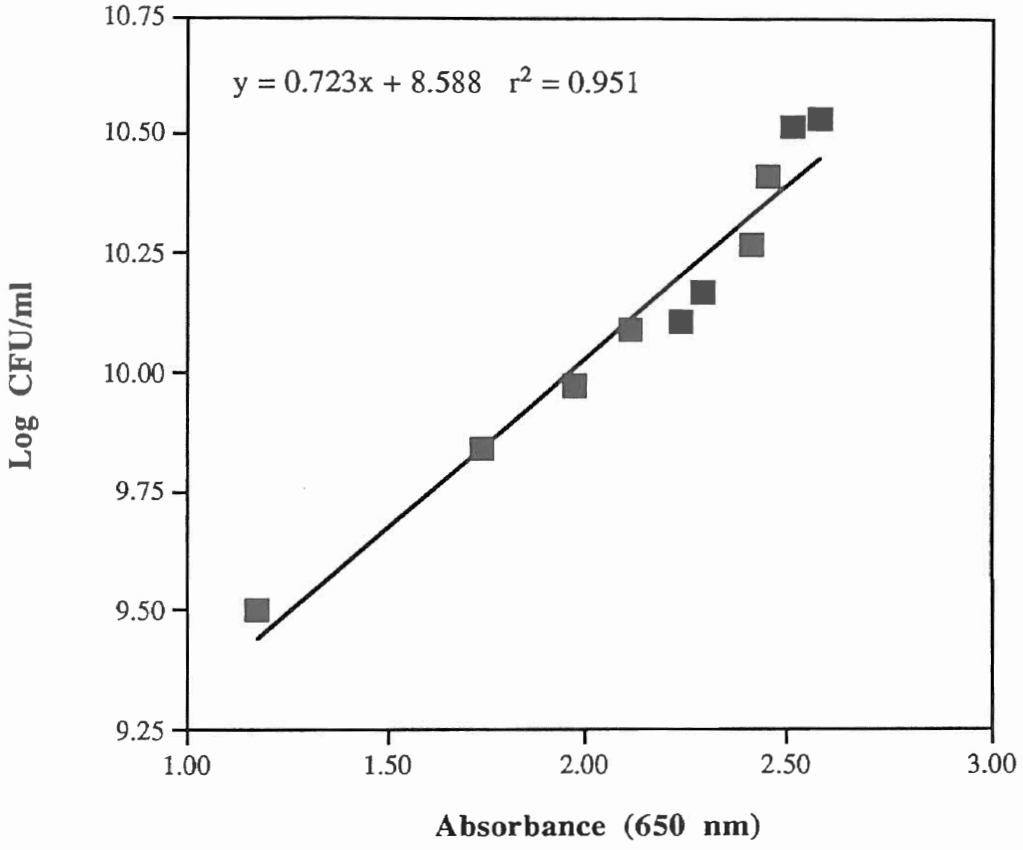

Figure 1. Standard Curve of Absorbance at 650 nm Versus Logarithm of CFU of *Salmonella typhimurium*/ml

VITA

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