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**Influence of recombinant bovine gamma interferon on neutrophil
function**

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Iowa State University, 1987

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Influence of recombinant bovine gamma
interferon on neutrophil function

by

Marla Jean Steinbeck

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
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For the Graduate College

Iowa State University
Ames, Iowa
1987

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GENERAL INTRODUCTION

Respiratory disease in cattle typically occurs shortly after calves have been weaned, transported, and placed in a feedlot (this is referred to as shipping fever) (Jensen, 1976). The clinical signs, lesions, and death loss in shipping fever usually can be attributed to bacterial pneumonia due to colonization of the lung by resident flora, Pasteurella hemolytica, Pasteurella multocida, or Hemophilus somnus. The causative factors are believed to include: 1. stress-induced release of glucocorticoids leading to immunosuppression and 2. mycoplasma and/or viruses which cause a transient infection and often decrease the lung's resistance to bacterial infections (e.g., Infectious Bovine Rhinotracheitis, Bovine Respiratory Syncytial Virus, Bovine Viral Diarrhea Virus and/or Parainfluenza-3 virus). Suppression of the function of phagocytic cells in the lungs (both alveolar macrophages and neutrophils) is a primary factor in predisposing to bacterial infection (Roth, 1984).

One of the potential host responses to the causative agents (virus, bacteria) of shipping fever is the local production and release of gamma interferon from antigen activated T lymphocytes (Friedman and Vogel, 1983). Gamma interferon is known to have immunomodulatory activities. One of three main goals of this research involved looking at the influence of gamma interferon on neutrophil function. This glycoprotein is available in a highly pure form produced through recombinant DNA

technology. It was hoped that if gamma interferon was found to activate peripheral blood neutrophil function, it might be utilized as an immunomodulator to decrease initial viral infection or lessen secondary bacterial pneumonia. A review of the literature on the influences of the available recombinant cytokines on neutrophil function is presented in section I. Work on the influences of recombinant bovine gamma interferon on bovine neutrophil function is presented in section II.

The second part of this research involved the evaluation of the molecular events (RNA, protein synthesis, arachidonic acid metabolism) involved in the activation of neutrophil cytotoxicity and the inhibition of random migration by recombinant gamma interferon. The ability of gamma interferon to activate neutrophils and the molecular events involved in this process were also examined in neutrophils isolated from cattle immunosuppressed by glucocorticoid treatment (dexamethasone). This work is presented in section III.

The third part of the research for this dissertation involved comparing the ability of bovine gamma interferon to activate bovine neutrophils to have enhanced antibody-independent cytotoxicity and the apparent lack of activation of human neutrophils by human gamma interferon to have this activity. This work is presented in section IV.

Explanation of the Dissertation Format

An alternative format was used in this dissertation. There are four sections after the General Introduction, each of the sections is an individual paper. The first section is a review that will be submitted for publication, the second section has been published in Cellular Immunology, the third section has been submitted for publication, and the fourth section will be submitted for publication. Finally, a general summary and discussion of the dissertation is included.

SECTION I:

A REVIEW OF THE ACTIVATION OF NEUTROPHILS
FOR ENHANCED FUNCTION BY RECOMBINANT
CYTOKINES

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The neutrophil fulfills an important role in the response of the host to inflammatory stimuli by participating in the first line of defense against pathogenic bacteria and parasites. The importance of the neutrophil is dramatically shown by conditions of neutropenia (reduced numbers of circulating neutrophils) (Dale et al., 1979) or severe neutrophil dysfunction (reviews: Hill, 1984; Roberts and Gallin, 1983; Gallin et al., 1983; Gallin, 1981; Malech and Gallin, 1987; Rotrosen and Gallin, 1987). Either condition will result in severe, recurrent microbial infections. Clinical disease manifestations include: ulcers of the mucous membranes, otitis in childhood, frequent skin infections, severe gingivitis, periodontal disease, (Quie, 1982; Buescher and Gallin, 1983; Gallin, 1985b; Van Dyke, Levine, and Genco, 1985; Charon, Mergenhagen, and Gallin, 1985; White and Gallin, 1986) and recurrent bouts of pneumonia and other types of life-threatening bacterial and fungal infections (review: Rotrosen and Gallin, 1987).

The mature neutrophil (polymorphonuclear leukocyte or neutrophilic granulocyte) is completely differentiated and incapable of proliferation (Altman, 1978). In the peripheral blood, these cells are distributed between freely circulating and marginal (adhered to vascular endothelium) pools (Cartwright, Athen, and Wintrobe, 1964), with approximately 50% in each pool. The half-life of the neutrophil in peripheral blood is on the average of 6-7 hours, which is a relatively short-time period compared to other circulating white blood cells, i.e., the half-life of lymphocytes or monocytes (Craddock, Perry, and Lawrence, 1956). At some point during senescence, the neutrophils are removed from the blood by macrophages in

the spleen (Wade and Mandell, 1983). Alternatively, the marginated neutrophil can migrate into the tissues where it functions as a mobile phagocyte, surviving up to 4 to 5 days (Craddock, 1972). This directed migration into tissues occurs in response to a variety of inflammatory stimuli: infectious agents, formylated peptides (in some species), and host products generated in response to invading microorganisms.

It is now recognized that neutrophils can be activated by cytokines to have enhanced function. This indicates that neutrophils serve as effector cells involved in cell-mediated immune responses. Cell-mediated immune reactions are mediated by mononuclear cells in response to specific antigens on the surface of infected or abnormal host cells. The antigen-stimulated cells release monokines and lymphokines that bind to specific receptors on the neutrophil and induce its activation. Activation enhances the ability of neutrophils to localize at sites of inflammation and to produce and release products that function to kill bacteria, virus-infected cells, neoplastic cells, (Babior, 1984; Clark, 1983), and hyphal forms of certain fungi (Diamond, Krzesicki, and Jao, 1978). The purpose of this review is to summarize the information available on activation of neutrophil function by cytokines. Since it is difficult to judge the purity of biochemically purified cytokines, this review will discuss data on recombinant cytokines.

NEUTROPHIL STIMULATION

The function of the neutrophil involves several different events and can be divided into two stages of stimulation. Stage I includes events that take place in response to low concentrations of soluble, inflammatory stimuli: granulocytopoiesis, adherence, diapedesis, emigration, and chemotaxis. Stage II events include attachment and ingestion (phagocytosis), degranulation, metabolic burst activity, killing and digestion which take place in response to higher concentrations of soluble mediators or phagocytosable particulates (Snyderman and Pike, 1984). The biological responses of the neutrophil induced after chemoattractant binding may be the result of increases in the intracellular free Ca^{2+} concentration, alone or in conjunction with the formation of 1,2-diacylglycerol which can activate protein kinase C (review: Snyderman, Smith, and Verghese, 1986; Snyderman and Pike, 1986; Rotrosen and Gallin, 1987). The activation of protein kinase C (phosphorylation) does not appear to be as important in neutrophil degranulation responses (Sha'afi et al., 1986; Salzer, Gerard, and McCall, 1986; Berkow, Dodson, and Kraft, 1987), but may play a role in superoxide anion production initiated by stimuli other than chemoattractants (Gerard et al., 1986; Berkow, Dodson, and Kraft, 1987).

For the purpose of this review we will define neutrophil stimulators as those factors which cause a direct response from the neutrophil such as chemotaxis, ingestion, induction of the respiratory burst, or degranulation. We will define neutrophil activators as those substances

which enhance neutrophil responses to a stimuli.

Granulocytopoiesis is the process of production and delivery to the bloodstream of an adequate number of mature granulocytes (neutrophils, eosinophils, and basophils). This process is normally regulated by hematopoietic growth factors called colony-stimulating factors (Stanley and Jubinsky, 1984) and/or interleukin-1, which has been reported to play a role in the increased release of mature neutrophils from the bone marrow during an acute-phase reaction (Craddock, Perry, and Lawrence, 1956).

The circulating neutrophils and vascular endothelial cells are capable of responding to very low concentrations (~ 1 nM) of soluble inflammatory stimuli. These mediators (chemoattractants) bind to specific receptors (high-affinity) on the neutrophil membrane and initiate various biochemical events culminating in adherence, diapedesis, emigration, and directed migration into the infected tissues. The mature neutrophil is capable of increasing the surface expression of adherence proteins (iC3b receptor family) in response to low concentrations of chemoattractants (Todd et al., 1984). These proteins are in part responsible for weak adhesion to the vascular endothelium (margination) which must take place before directed migration can occur (Wilkinson, 1974; Keller et al., 1979; Dahlgren, 1982). Investigation of patients with neutrophil iC3b deficiency has highlighted the importance of adherence for the biological responses of diapedesis, emigration, and chemotaxis. These patients are deficient in the beta-subunit that is common to iC3b receptor/Mol, LFA-1 and p150,95, a family of structurally

and functionally related glycoproteins that mediate neutrophil adherence (Springer et al., 1984; Anderson et al., 1985; Buescher et al., 1985). Other adherence-related neutrophil functions such as the attachment stage of phagocytosis and antibody-dependent cell-mediated cytotoxicity are also impaired (Kohl et al., 1984; reviews: Anderson and Springer, 1984; Buescher et al., 1985; Anderson et al., 1985). Patients with neutrophils lacking these receptors are susceptible to increased severe and recurrent infections (review: Anderson and Springer, 1984).

Diapedesis or migration between endothelial cells of the capillary venules follows the adherence of the neutrophil to the endothelium. Diapedesis is assisted by the action of vascular permeability factors (e.g., histamine, serotonin, bradykinin, and activated-complement components, particularly C5a) released or formed during the inflammatory process (Wilkinson, 1974). Emigration through the vascular basement membrane is the final step in the process of entering the tissues. The neutrophils may affect this process by selectively releasing proteases (Olsson and Venge, 1980; Cochrane, 1977). After entering the tissues, directed migration of the neutrophil against a concentration gradient (chemotaxis) towards the site of chemoattractant production.

STAGE II OF STIMULATION

As the neutrophils approach the origin of chemoattractants in the inflamed tissue, they are bombarded by a multiplicity of stimuli in addition to elevated levels of chemoattractants. The chemoattractants include soluble products of complement activation (particularly C5a) (Muller-Eberhard, 1981), soluble bacterial products (Schiffmann et al., 1975), host generated arachidonate metabolites (leukotrienes) (Robinson, Curran, and Hamer, 1982), and platelet activating factor (Naccache et al., 1986). At elevated concentrations the chemoattractants bind to low-affinity receptors (Koo, Lefkowitz, and Snyderman, 1982; Naccache et al., 1986; Goldman et al., 1987). In addition, phagocytosable particulate stimuli such as immune-complexes (Cochrane, 1977; Johnson and Ward, 1982) and antibody and complement-coated particles (opsonized particles) stimulate the neutrophil. Some soluble products in high concentrations can initiate specific functions of the neutrophil, (review: Spitznagel, 1983) but generally the chemoattractants serve as activators, enhancing neutrophil responsiveness to a second particulate stimulus. Whereas, stimulation by particulate stimuli triggers neutrophil function without the need for an activator molecule.

Stage II of stimulation results in induction of neutrophil phagocytosis, degranulation, and metabolic burst activity. Phagocytosis is a two stage process whereby the neutrophil selectively binds and then ingests the bound particle (Zakhireh, Block, and Root, 1979). Opsonization or coating of particles with antibody and/or complement

fragments (C3b, iC3b) allows the neutrophil to selectively bind these particles via specific receptors. Both the Fc (e.g., FcR_{low} a low-affinity receptor which recognizes antigen-bound or aggregated IgG_1) and C3b receptors (CR1) are present on the mature neutrophil surface (Roos et al., 1981). Once bound via the receptor, the particle triggers local infolding of the neutrophil membrane as a result of the contraction of actin and myosin filaments attached to cytoplasmic microtubules, and draws the particle into an enclosed vacuole called a phagosome. It has been suggested after observations of some patients with neutrophil iC3b receptor (CR3) deficiency that the CR3 receptor may play a role in the modulation of cytoskeleton assembly and therefore effect the associated function of other cell surface receptors (i.e., C3b and Fc) (Arnaout et al., 1983). Very soon after or simultaneously with the attachment of the particle to the neutrophil, several morphological and biochemical events are set into motion which may result in the destruction of the ingested microorganism. These post-phagocytic events involve: 1. delivery of the hydrolytic enzymes and cationic proteins into the phagosome, a process termed degranulation, 2. formation of highly reactive oxygen radicals through a coordinated series of metabolic events termed the "respiratory burst", and 3. actual killing and digestion of the microbe. A variety of antimicrobial mechanisms are involved in this final step and indicate that neutrophils have an appreciable reserve capacity if one mechanism is impaired (Zakhireh, Block, and Root, 1979).

Degranulation refers to the release of granule contents either extracellularly or into the phagosome from two types of granules (in most

species that have been studied). Neutrophils from some species contain a third type of granule, which has been defined in the bovine and other ruminants to contain predominantly cationic proteins (Gennaro et al., 1983). In human neutrophils a gelatinase containing tertiary granule has been identified (Dewald et al., 1982). The two most characterized granules are the primary (azurophil) and secondary (specific) granules, literally, the first and second granule types made at early stages of neutrophil differentiation (Bainton, Ulliyot, Farquhar, 1971). Primary and secondary granules are morphologically distinct, contain some common but generally unique components, and each granule type releases its contents under separate control mechanisms (Bainton and Farquhar, 1968; Bainton, 1973). Primary granules contain myeloperoxidase, a variety of acid hydrolases, and neutral proteases involved in the killing and digestion of phagocytosable organisms or the destruction of nonphagocytosable fungi or abnormal cells (Goldstein, 1976). Other components of human neutrophil primary granules that may have direct microbicidal activity, include cationic proteins which bind to or permeabilize membranes (Zeya and Spitznagel, 1966; Odeberg and Olsson, 1975; Drazin and Lehrer, 1977; Weiss et al., 1978) and lysozyme (Strominger and Ghuyssen, 1967; review: Baggiolini, 1972). Approximately one-half to two-thirds of the lysozyme is contained in the secondary granules. This enzyme digests the peptidoglycan portion of the bacterial cell wall (review: Spitznagel et al., 1974). Adult bovine neutrophils are devoid of granules containing measurable lysozyme activity, although neutrophils isolated from young calves (10 days to 6 weeks of age)

apparently contain this activity (Bielfeldt-Ohmann and Babiuk, 1984). Secondary granules are often called specific granules because they are characteristic of the mature neutrophil and aid in identification of the neutrophilic granulocyte after Wrights-Giemsa staining. These granules also contain lactoferrin (a microbistatic protein that chelates iron which is required by some bacteria and fungi for growth) (Baggiolini, de Duve, and Masson, 1970) collagenase, and vitamin-B₁₂ binding protein (whose function is unknown). In addition, the specific granules contain monocyte chemotactic molecules and some substance(s) capable of generating the active complement fragments C3a and C5a.

It was originally believed that secondary granules were also the intracellular store for the adherence proteins (CR3) and other surface receptors (e.g., CR1). However, degranulation of secondary granules does not take place at the low chemoattractant concentrations that cause up-regulation of these receptors (Burger et al., 1984; Petrequin et al., 1986; O'Shea et al., 1985; Parkos et al., 1985). Nor do the kinetics of translocation of these molecules to the cell surface fit within the time frame of degranulation of secondary granules. More recently, another granule-like compartment has been separated from human neutrophils by density centrifugation. This mobilizable, intracellular compartment fuses with the surface neutrophil membrane and up-regulates surface alkaline phosphatase activity in response to nanomolar concentrations of chemoattractants.

Therefore, there may be yet another compartment containing alkaline phosphatase activity that serves as the intracellular store for iC3b, C3b, and other surface receptors (Borregard et al., 1987).

The killing and digestion by activated neutrophils can be divided into oxygen-independent and -dependent mechanisms, which are separate but co-functioning processes (Klebanoff, 1975; Klebanoff et al., 1985).

i. Oxygen-independent killing effectors include constituents of both primary and secondary granules, e.g., proteases, lysozyme, lactoferrin, and cationic proteins (defensins and bactericidal permeability inducing protein). Patients with chronic granulomatous disease have various inherited severe defects in the oxidative metabolic effector system and their neutrophils affect killing only through oxygen-independent mechanisms (reviews: Rotrosen and Gallin, 1987; Malech and Gallin, 1987). The neutrophils from these patients are still capable, although to a variable extent, of killing microorganisms (Spitznagel, 1977; review: Root and Cohen, 1981). The CGD patients are often afflicted with increased frequency, severity, and duration of infections with catalase-positive bacteria and fungi (Gallin and Fauci, 1983; Gallin et al., 1983; Tauber et al., 1983). The increased duration of infections is the result of defective neutrophil resolution of the inflammatory process, which is dependent on oxidative product inactivation of soluble inflammatory stimuli including prostaglandins, formyl peptides, C5a, and leukotrienes (Clark and Klebanoff, 1979; Henderson and Klebanoff, 1983). Defective oxygen-independent killing occurs in neutrophils from Chediak-Higashi syndrome patients (human and animal) as a result of defective packaging

and/or release of granule constituents (Klebanoff and Clark, 1978).

Humans with specific granule deficiencies due to production defects also have decreased oxygen-independent killing (reviews: Malech and Gallin, 1987; Rotrosen and Gallin, 1987). The CHS and specific granule deficient patients have depressed inflammatory responses and suffer from severe recurrent bacterial infections (Gallin, 1985a). Therefore, both oxygen-dependent and -independent killing are required for a complete arsenal against all invading microorganisms.

ii. Oxygen-dependent killing depends on the initial generation of superoxide anion by the neutrophil during the respiratory burst. Molecular oxygen is converted by NADPH-oxidase (an intergal-membrane, multi-component enzyme complex) to superoxide anion: $2O_2 + NADPH_2 \rightarrow 2O_2^{\cdot -} + NADPH + H^+$. Superoxide anion itself has little direct toxic effect on microorganisms, (Goldstein et al., 1977; Babior, 1978) but acts as a precursor from which hydrogen peroxide, hydroxyl radicals, and singlet oxygen are derived. The formation of these direct microbicidal effectors occurs via the Haber-Weiss reaction: $O_2^{\cdot -} + H_2O_2 \rightarrow {}^1O_2 + OH^{\cdot} + OH^-$ (Root and Metcalf, 1977; Klebanoff, 1975). Most of the generated hydrogen peroxide is utilized in the MPO- H_2O_2 -halide system. The MPO- H_2O_2 -halide system affects a part of the oxidative killing of bacteria, fungi, viruses, mycoplasma, and tumor cells (Klebanoff, 1967; Klebanoff and Clark, 1978). Myeloperoxidase binds available hydrogen peroxide and oxidizable halide cofactors to the microbial target surface. The MPO-catalyzed reaction: $halide(^-) + H^+ + H_2O_2 \rightarrow (halide)HO^+ + H_2O$ generates toxic hypochlorous acid ($HOCl^+$) (Harrison and Schultz, 1976) or damaging

iodination of viccinal proteins of the bacterial cell wall, if iodide is available (Babior, 1978; Klebanoff, 1967). Myeloperoxidase deficiencies in human phagocytes result in a slower rate of microbicidal activity (review: Rotrosen and Gallin, 1987).

IMMUNE CELL INTERACTION AND ACTIVATION OF NEUTROPHILS BY CYTOKINES

Cytokines include factors produced and released by cells of the immune system activated during cell-mediated immune responses. Some of these factors are also released by nonimmune cells in the immediate area of inflammation and in the bone marrow stroma. Because activated immune cells generally secrete more than one cytokine in response to an appropriate stimulus, it has been difficult to evaluate the direct effects of the individual cytokines. Recombinant DNA technology has provided the ability to clone the gene for each protein and allowed for the evaluation of the direct effect of each factor. The availability of recombinant cytokines has also allowed the use of a defined mixture of cytokines to determine the potential synergy in their abilities to modulate immune cell functions and the immune response. The cytokine genes that have been cloned from cells of one or more animal species include: colony-stimulating factors, interleukins, tumor-necrosis factors, and interferons. This part of the review will cover what is known about the modulatory effects of the individual recombinant cytokines on mature neutrophil microbicidal and cell-mediated functions.

COLONY-STIMULATING FACTORS

The growth of hematopoietic progenitor cells in semisolid media lead to the discovery that cell division and differentiation were regulated by specific protein factors (Pluznik and Sachs, 1965). These specific proteins were called hematopoietic colony-stimulating factors (CSFs) because their activity was identified in cell culture colony formation assays (Ichikawa, Pluznik, and Sachs, 1966; Bradley and Metcalf, 1966; Clark and Kamen, 1987). As mentioned earlier in this review, colony-stimulating factors and interleukin-1 are involved in normal and acute-phase reaction induced granulocytopoiesis. In the low picomolar range, all of the CSFs stimulate hematopoietic progenitor cells to form colonies in soft agar culture. In the murine system, four major types of CSFs have been identified, granulocyte (G)-CSF, monocyte (M)-CSF, granulocyte-monocyte CSF (GM)-CSF, and multi-CSF (IL-3), and analogous factors have been described in the human system (Pluznik and Sachs, 1965; Metcalf, 1985; Metcalf, 1986; Yang et al., 1986) with the possible exception of multi-CSF (Nicola, 1987). These are not the only hematopoietic growth factors, although these are all that are known to influence granulocytopoiesis.

Granulocyte (G)-CSF and monocyte (M)-CSF (CSF-1) are postulated to support the growth (survival), proliferation, and differentiation of only relatively late progenitor granulocytes or monocytes already committed to their respective lineages (Metcalf and Nicola, 1983; Stanley and Heard, 1977). Granulocyte-monocyte (GM)-CSF or CSF-2 interacts with myeloid

precursors which are still able to differentiate into neutrophils, eosinophils or monocytes (Metcalf, 1986). Multi-CSF, interleukin-3 (IL-3), or CSF-3 is a multipotent CSF, which controls the differentiation of all cells of the immune system (Schrader, 1986).

The individual CSF mRNAs can be detected by hybridization techniques using cDNA probes and are found to be selectively expressed in several different cell types after activation. Monocytes activated by endotoxin or interferon-gamma (released during CMI responses) express high levels of mRNA for G- and M-CSF but not GM-CSF (Ramaldi, Young, and Griffin, 1987). Antigen-stimulated T cells contain relatively high levels of GM-CSF mRNA (Wong et al., 1987) and perhaps low levels of IL-3 mRNA (Yang et al., 1986) but do not express the mRNAs for either G- or M-CSF (Ramaldi, Young, and Griffin, 1987). Other cytokines released from monocytes activated during immune responses, interleukin-1 and tumor necrosis factor-alpha, activate cells present in the bone marrow stroma to express G- and GM-CSF mRNA (review: Clark and Kamen, 1987).

The genes encoding the four major murine and human myeloid growth factors have all been cloned in mammalian cells and sequenced (Nicola, 1987; Clark and Kamen, 1987). Approximately 70% cross-species homology in nucleotide sequence of the protein-coding region of the murine (mu) GM-CSF cDNA and human (hu) GM-CSF cDNA exists (Wong et al., 1985). Whereas, murine IL-3 and human IL-3 lack sequence homology, but have similar structural properties (Yang et al., 1986; Miyatake et al., 1985).

Human GM-CSF is an acidic glycoprotein with a relative molecular mass (Mr) of 22,000 (Gasson et al., 1984). Similarly, IL-3 is a glycoprotein

with a Mr of 28,000 (Ihle et al., 1982). Human GM-CSF and IL-3 have some common biological activities, some common structural features, and are both expressed in activated T cells, but no significant nucleotide sequence homology exists for these two products (Clark and Kamen, 1987). Immunochemical analysis of natural huGM-CSF implies that recombinant factors produced in mammalian cells (chinese hamster ovary cells or monkey COS-1 cells) have structures very similar to the natural molecules. Both natural and recombinant huGM-CSF (Donahue et al., 1986) or huIL-3 (Clark and Kamen, 1987) expressed in monkey COS-1 cells are extremely heterogeneous in size. The different molecular weight forms of these proteins can be accounted for by varying degrees of glycosylation. Interestingly, the higher the degree of glycosylation the less active the rhuGM-CSF molecule in in vitro assays (Clark and Kamen, 1987). In vivo glycosylation appears to affect the rate and extent of distribution throughout the fluids and tissues of the rat, but does not appear to play any role in the rate of clearance of GM-CSF.

Granulocyte-CSF is less glycosylated and therefore less heterogeneous in size distribution (Clark and Kamen, 1987). When expressed in E. coli, huG-CSF lacks carbohydrate additions and is biologically active both in vivo and in vitro (Welte et al., 1987).

Murine multi-CSF and murine or human GM-CSF receptor numbers are low and decrease to a few 100/neutrophil during cell maturation (Gasson et al., 1984; Nicola and Metcalf, 1986). Even though there are low numbers of receptors, GM-CSF binding to the neutrophil plasma membrane has been shown to be specific. Receptors for murine and human G-CSF are specific,

have a single affinity, and occur on all cells of the neutrophilic granulocyte cell series. Murine neutrophil G-CSF receptor numbers increase during cell maturation to a few 100/cell (Byrne, Guilbert, and Stanley, 1981; Nicola and Metcalf, 1985) and human neutrophil receptor numbers decrease slightly with cell maturation (Gasson et al., 1986; Clark and Kamen, 1987). Uniquely among the four murine CSFs, G-CSF for man or mouse binds to receptors on cells from either species, which has allowed for the definition of G-CSF receptor distribution in human cells (Nicola, Begley, and Metcalf, 1985). Colony-stimulating factor binding to specific receptors leads to a decrease in cell surface receptor number as the CSF-receptor complex is internalized and degraded. In addition, the binding of some murine CSFs to their specific receptors decreases the number of available receptors for other CSFs as well (trans-modulation) (Nicola, 1987). Less work has been done to examine trans-modulation of CSF receptors on human neutrophils, although one report suggests a similar process takes place (Nicola, Vadas, and Lopez, 1986). Trans-modulation occurs at moderate to high concentrations of a CSF and may account for the induction of biological activities other than those attributed to the bound CSF (Zachary and Rozengurt, 1985; Walker et al., 1985). Nicola (1987) recently reviewed the trans-modulation of CSF receptors and offered several possible explanations of the biological significance.

In general, the biological activities induced by recombinant huG-CSF and GM-CSF are in agreement with those of the natural products (Clark and Kamen, 1987). Recombinant huGM-CSF stimulates the proliferation of human

granulocyte-monocyte progenitor cells, leading to typical colony formation in semi-solid agar cultures (40-200 U/ml) (Metcalf et al., 1986b). This cytokine also increases the survival of mature neutrophils by several hours (Lopez et al., 1986). In addition, rhuGM-CSF up-regulates the surface expression of the adherence glycoproteins, GFA-1, GFA-2, and Mol without changing the expression of beta₂-microglobulin within an hour after rhuGM-CSF addition (80 U/ml) (Lopez et al., 1986). The selective up-regulation does not result in increased adherence of rhuGM-CSF treated neutrophils to either plastic or human umbilical vein endothelial (HUVE) cells. These authors did not address the apparently contradictory findings. Superoxide anion production in response to FMLP was increased (as determined by the cytochrome c assay) in neutrophils pre-incubated in rhuGM-CSF (Weisbart et al., 1985; Lopez et al., 1986, 1987). Pretreatment of the neutrophils for 120 minutes with rhuGM-CSF before adding the FMLP was necessary to see a maximal increase in superoxide anion production. Other effects of rhuGM-CSF addition (8 to 80 U/ml) included a dose-dependent increase in phagocytosis of Saccharomyces cerevisiae in a one hour assay (Metcalf et al., 1986b; Lopez et al., 1986, 1987). An increase in induced lysozyme release occurred only after pretreatment with cytochalasin B, followed by the simultaneous addition of 80 U/ml of rhuGM-CSF and FMLP (Lopez et al., 1986). Recently, questions have been raised as to whether the addition of cytochalasin B may affect neutrophil function directly, making the interpretation of these results difficult. There was little direct increase in the MPO-H₂O₂-halide activity (iodination assay) induced by

rhGM-CSF (80 U/ml) or unopsonized zymosan but together they enhanced iodination to a significantly greater value than either alone (Lopez et al., 1986). Recombinant huGM-CSF enhanced neutrophil ADCC activity against ^{51}Cr -labeled p815 mouse mastocytoma cells in a 2 to 2.5 hour assay (Metcalf et al., 1986b; Lopez et al., 1986, 1987). The activities attributed to the recombinant GM-CSF expressed in E. coli were not due to a contaminating E. coli cell wall component (LPS) which was present at < 0.2 ng/ml in the assays after dilution. The only effect of a higher concentration of exogenous E coli LPS (1 ng/ml) on neutrophil function was an increase in adherence which was not measurable after rhGM-CSF addition.

Compared to GM-CSF, relatively little research has been conducted on the influence of the other CSFs on neutrophil function. Recombinant human granulocyte (G)-CSF exhibits colony-stimulating activity in agar cultures and induces granulocyte differentiation of premature neutrophils (1×10^5 U/ml) (Souza et al., 1986). Little work has been done or published on the effects of G-CSF on mature neutrophil function from any species. Recombinant primate multi-CSF (IL-3) induces granulocyte colonies but has thus far proved to be an activating factor for only mature human eosinophils and not neutrophils (ingestion, ADCC, superoxide anion production) (Lopez et al., 1987). Murine IL-3 induces granulocyte colonies but had no effect on the activity of either mature murine eosinophils or neutrophils (Metcalf et al., 1986a).

In vivo administration of rhGM-CSF to neutropenic patients with AIDS resulted in a dose-dependent increase in the white blood cell count,

correcting the neutropenia (Clark and Kamen, 1987). The highest doses actually caused leukocytosis. The neutrophil functions were not examined. In contrast, rhuG-CSF administration to normal monkeys twice daily via a subcutaneous route increased the white blood cell count but required continuous administration. No increase in neutrophils was observed, but there was an increased number of circulating T cells (Welte et al., 1987). Neutrophils from the rhuG-CSF treated monkeys demonstrated an enhanced ability to kill and phagocytose bacteria in vitro.

Table 1. Influence of recombinant GM-CSF on neutrophil function
in vitro

Increases:

^h Proliferation of granulocyte-monocyte progenitors	(Metcalf et al., 1986b)
^h Neutrophil survival	(Lopez et al., 1986)
^h Expression of GFA-1, GFA-2, and Mol	(Lopez et al., 1986)
^h Superoxide anion production in response to stimulus	(Weisbart et al., 1985; Lopez et al., 1986, 1987)
^h Phagocytic activity	(Metcalf et al., 1986b; Lopez et al., 1986)
^h Lysozyme release	(Lopez et al., 1986)
^h MPO-H ₂ O ₂ -halide activity in response to stimulus	(Lopez et al., 1986)
^h Cytotoxicity (ADCC)	(Metcalf et al., 1986b; Lopez et al., 1986)
^h Neutrophil numbers in peripheral blood after <u>in vivo</u> admin. to AIDS patients	(Clark and Kamen, 1987)
^h Phagocytic and bactericidal activity after <u>in vivo</u> admin. to monkeys	(Welte et al., 1987)

Unaffected:

^h Expression of beta ₂ -microglobulin	(Lopez et al., 1986)
^h Adherence	(Lopez et al., 1987)
^{mo} Neutrophil numbers after <u>in vivo</u> admin. to monkeys	(Welte et al., 1987)

(superscripts indicate neutrophils isolated from (h)humans and (mo)monkeys treated with GM-CSF from the same species)

INTERLEUKIN-1

The term interleukin (IL) was first proposed in 1979 to describe two secreted leukocyte products with similar Mr of 15-17,000 (review: Dinarello and Mier, 1986). The term interleukin was adopted since both IL-1 and IL-2 were produced by and acted on leukocytes. Interleukin-1 (IL-1) was the name given to the macrophage product, called T and B lymphocyte-activating factor, which has a number of biological activities including modulation of neutrophil function.

Production of IL-1 is part of the host's response to challenges, such as microbial invasion, injury, immunological reactions, neoplastic changes, and inflammatory processes. During these responses IL-1 is released from a variety of cells and has multiple effects on a broad range of cell targets (Root and Wolff, 1968; Atkins et al., 1972; Gery, Gershon, and Waksman, 1972; Gery and Waksman, 1972; Unanue, 1978; Rosenthal, 1980; Oppenheim and Gery, 1982). IL-1 induces fever (pyrogen) and it can mediate several components of the host's systemic acute-phase responses to a variety of challenges. These responses are characterized by alterations in metabolic, endocrinologic, neurologic, and immunologic functions (Kampschmidt and Pulliam, 1978; Rosenwasser and Dinarello, 1981). It has been suggested that IL-1 is the factor responsible for the neutrophilia associated with the acute-phase reaction (Craddock, Perry, and Lawrence, 1956). This results from an increased release of mature neutrophils from the bone marrow reserve (Kampschmidt, 1984).

Interleukin-1 is secreted in two forms with different isoelectric

points (pIs) of 7.0 (IL-1 beta) (Auron et al., 1984; March et al., 1985; Gray et al., 1986) and 5.0 (IL-1 alpha) (Lomedico et al., 1984). The IL-1s are products of two separate genes, bearing little homology at the protein or DNA level, yet possessing highly conserved biological activities and structural features (Dinarello, Goldin, and Wolff, 1974; Bodel, 1978; Cebula et al., 1979; Rosenwasser, Dinarello, and Rosenthal, 1979; Lachman and Metzger, 1980). Across species, murine and human IL-1 alpha share 80% homology at the amino acid level (Auron et al., 1985). Resting macrophages are devoid of IL-1 mRNA and protein but upon stimulation with endotoxin or after adherence in vitro, there is a transient one hour to five day increase of IL-1 alpha and beta mRNAs to similar levels (Fuhlbrigge et al., 1987). By four hours, IL-1 can be found as an intracellular, membrane, and extracellular protein (Hanson, Murphy, and Windle, 1980; Kurt-Jones et al., 1985; Kurt-Jones, Virgin, and Unanue, 1986; Matsushima et al., 1986).

Recombinant huIL-1 beta induces lymphocyte activation, but has no effect in several assays of mature neutrophil function. It is important to point out that the neutrophils did respond normally to other stimulants. Recombinant huIL-1 beta did not act as a chemotactic factor for neutrophils nor did it alter the neutrophil chemokinetic activity. Superoxide anion generation was not directly stimulated by 1, 10, or 100 ng/ml of rhuIL-1 beta (cytochrome c assay) (Georgilis et al., 1987). At the same concentrations rhuIL-1 beta was unable to directly stimulate the release of lysozyme or enhance phagocytosis of S. aureus at a 20:1 ratio (number of bacteria per neutrophil) over a time period of 5, 10, 20, or

30 minutes. The physiological dose range for IL-1 is below 10 ng/ml, suggesting that sufficient concentrations of IL-1 were added to the assays. Therefore, most of the activities originally ascribed to IL-1 activity on PMN function in nonhomogenous preparations were probably due to contamination with tumor necrosis factor, which enhances phagocytosis (Shalaby et al., 1985) and is a weak, direct stimulus of the respiratory burst and induced lysozyme release (Klebanoff et al., 1986). It has also been reported that IL-1 affects the intracellular cytosolic-free calcium concentration $[Ca^{++}]_i$ and might stimulate neutrophils via this second messenger. Using Quin 2 fluorescence as a measure, resting $[Ca^{++}]_i$ was unaffected by rhuIL-1 beta at concentrations of 1, 10, 100 ng/ml (Georgilis et al., 1987). In vivo, both forms of human recombinant IL-1 produce a typical monophasic fever at rabbit pyrogenic doses of 50 to 200 ng/kg (Dinarello, 1986; Dinarello et al., 1986). It therefore appears that the IL-1s have biological activity but do not effect neutrophil functions as evaluated by these particular assays.

In vivo recombinant human IL-1 alpha has been shown to increase the survival rate of mice challenged with Pseudomonas aeruginosa or Klebsiella pneumoniae (Ozaki et al., 1987). Based on the rhuIL-1 beta results, this is probably not due to a direct effect of IL-1 alpha on neutrophil function. This does not rule out the possibility that neutrophils were activated by another cytokine whose induction was mediated by IL-1 alpha.

Table 2. Influence of recombinant IL-1 beta on neutrophil function
in vitro

Increases:

^mBody temperature after in vivo admin. to mice (both IL-1 beta and alpha) (Ozaki et al., 1987)

^mSurvival after bacterial challenge (in vivo admin. to mice) (Ozaki et al., 1987)

Unaffected:

^hIntracellular free Ca⁺⁺ concentration (Georgilis et al., 1987)

^hChemokinesis (Georgilis et al., 1987)

^hSuperoxide anion production (Georgilis et al., 1987)

^hPhagocytic activity (Georgilis et al., 1987)

^hLysozyme release (Georgilis et al., 1987)

(superscripts indicate neutrophils isolated from (h)humans and (m)mice treated with IL-1 beta from the same species)

TUMOR NECROSIS FACTORS

Tumor necrotizing factor activity was first described by O'Malley et al. (1962) in the serum of mice challenged with endotoxin obtained from Serratia marcescens. In 1975, Carswell et al. injected bacillus Calmette-Guerin (BCG) and later endotoxin into mice, rats, and rabbits and described a similar tumor necrosis factor activity in serum. This factor appeared in the serum within hours, caused regression of some transplanted tumors in vivo, and was cytostatic or cytotoxic to some tumor cells in culture but had no effect on normal human fibroblasts. The mediator was thought to originate from macrophages and was termed tumor necrosis factor (TNF) also identified as cachectin (Buetler, Milsark, and Cerami, 1985; Buetler et al., 1985).

Two TNFs have now been described: 1. TNF-alpha (cachectin) a soluble protein (17kD) product secreted predominantly from macrophages activated by endotoxin, BCG, lectin, or IL-2, and to a lesser extent activated NK cells, natural cytotoxic cells and IL-3 dependent bone marrow derived mast cells and 2. TNF-beta (also called lymphotoxin), a secreted glycoprotein (25kD) product of lymphoblastoid cells (Granger et al., 1970; Pennica et al., 1984; Gray et al., 1984; Aggarwal et al., 1984; Aggarwal, Moffat, and Harkins, 1985) and antigen- or mitogen-activated T cells (Granger and Kolb, 1968). The TNFs are products of different genes, produced by different cell types, but with approximately 30% amino acid homology and similar functions (Pennica et al., 1984).

Human TNF-alpha and -beta and murine TNF-alpha have been produced in

highly purified forms by recombinant DNA technology (Pennica et al., 1984; Gray et al., 1984; Wang et al., 1985; Pennica et al., 1985). Natural TNF-alpha is not glycosylated, in contrast to TNF-beta which is glycosylated. Glycosylation of the recombinant TNFs is absent if E. coli is used as an expression vector. However, the lack of glycosylation appears to be unimportant for biological activity. Tumor necrosis factor activity is usually expressed as units of antitumor activity. The biological assay for TNF cytolytic activity is calculated as a titer of TNF induced cytolytic activity for actinomycin D-treated L929 mouse fibroblast cells (Aggarwal et al., 1984).

The action of TNF on neutrophils appears to require initial binding to surface receptors (Rubin et al., 1985; Kull et al., 1985). The binding of ^{125}I -rhuTNF-alpha to human neutrophils reaches maximal values in 30 minutes at 37°C (Shalaby et al., 1987). There are approximately 6000 receptors per cell (Scatchard analysis) and the dissociation constant (Kd) is 1.37 nM. Neutrophils appear to express a single class of specific, high-affinity receptors that are probably internalized and degraded after TNF binding (Tsujimoto, Yip, and Vilcek, 1985; Shalaby et al., 1987). More recent observations indicate that rhuTNF-alpha and -beta compete for the same cellular receptor (Aggarwal, Eessalu, and Hass, 1985; Shalaby et al., 1987).

The antitumor activity of TNF has been well established, although, the exact mechanisms mediating the in vitro and in vivo cytolytic activities of TNF-alpha and -beta are not well characterized. The ability of these cytokines to activate neutrophils and increase their

adherence and post-adhesion activities on vascular endothelial cells has been implicated as one possible mechanism that mediates a part of the hemorrhagic necrosis of solid tumors and the associated inflammation (Klebanoff et al., 1986; Shalaby, Pennica, and Palladino, 1986; Naworth and Stern, 1986; Shalaby et al., 1987; Palladino et al., 1987).

Recently, the effects of both recombinant human TNF- α and - β on the mature neutrophil have been published. In general, rhuTNF- α enhances the adhesion of human peripheral blood neutrophils to human umbilical-vein-derived endothelial (HUVE) cell monolayers in culture (Gamble et al., 1985). In addition, it has been reported that rhuTNF- α activated neutrophils disrupt HUVE cells and cause inhibition of their proliferative activities (Shalaby et al., 1987), which could be important in tumor necrosis and inflammatory reactions. The increased TNF-mediated adherence is possibly due in part to translocation of the iC3b (CR3) receptor/adherence glycoprotein from intracellular stores to the surface membrane of neutrophils and in part to TNF effects on the endothelial cells (Todd et al., 1984; Gamble et al., 1985). This correlates with the fact that TNF effects on neutrophils are rapid (maximal within 5 minutes) and appear to be independent of protein and nucleic acid synthesis (Gamble et al., 1985). Neutrophils from individuals with deficiencies in the surface-adherence antigens do not adhere well to HUVE cells, indicating that these molecules are important for optimal adherence (Harlan et al., 1985). The increased expression of surface adherence proteins on neutrophils by rhuTNF- α treatment was detected with murine IgG monoclonal antibodies (mAb) 60.1 and 60.3

(Beatty et al., 1983; Klebanoff et al., 1985) which recognize different epitopes on the CR3 surface-antigen complex. Addition of either of these mAbs to neutrophils blocks resting or TNF-stimulated adherence, but has no effect on endothelium (HUVE cells lack antigen recognized by mAb 60.3). Neutrophils incubated with 100 U/ml of rhuTNF- α have a 2-fold and 1.5-fold increase in expression of surface antigen recognized by mAbs 60.1 and 60.3, respectively (Gamble et al., 1985), as determined by flow cytometry. Recombinant huTNF- α inhibits the migration of neutrophils under agarose at 100 U/ml of antitumor activity, the same concentration required for the increase in expression of the CR3 complex and within the range of the dose-dependent increase in adhesion (1×10^{-4} U/ml); these could be related events (Gamble et al., 1985; Shalaby et al., 1987).

Recombinant huTNF- α has been reported to be a weak but direct stimulus of the neutrophil MPO-H₂O₂-halide activity (Klebanoff et al., 1986) and superoxide anion production of the neutrophil (Shalaby et al., 1987). The addition of a second stimulus, unopsonized zymosan or FMLP, to the iodination assay or cytochrome c assay, respectively, resulted in greater than additive increases in both activities. The concentration range of TNF- α used was from 2 to 200 U/ml, these concentrations resulted in a dose-dependent increase in neutrophil respiratory burst activities in the presence of unopsonized zymosan. Monoclonal Abs 60.1 and 60.3 (Beatty et al., 1983; Klebanoff et al., 1985; Arnaout et al., 1984) inhibit the combined TNF, unopsonized zymosan induced enhancement of the MPO-H₂O₂-halide activity of neutrophils by inhibiting adhesion and subsequent phagocytosis of unopsonized zymosan. Monoclonal antibodies to

other neutrophil surface antigens had no effect on MPO-H₂O₂-halide activity under these assay conditions.

Pretreatment of neutrophils for 15 minutes with rhuTNF-alpha or 20 minutes with rhuTNF-beta resulted in increased phagocytic activity of the neutrophils. The increase in phagocytosis by rhuTNF-alpha (50 U/ml) was impressive, ingestion increased from 6 zymosan particles/100 neutrophils to 395 after 20 minutes and 464/100 neutrophils after one hour (Klebanoff et al., 1986). At 10 to 100 U/ml both recombinant or biochemically purified TNF-beta significantly increased, in a dose-dependent manner the ingestion of 1.5 um fluoresceinated latex beads by neutrophils as determined by flow cytometry (Shalaby et al., 1985).

Recombinant huTNF-alpha (50 U/ml) increased secondary degranulation directly as measured by the release of lysozyme in the absence of another stimulus and greater than additive with zymosan (Klebanoff et al., 1986). Direct TNF induced release of MPO or beta-glucuronidase was equivalent to the release of LDH, a cytosolic enzyme that correlates with cell lysis.

Shalaby et al. (1985) have also reported that rhuTNF-alpha or -beta (100 U/ml) increased neutrophil-mediated ADCC against chicken erythrocytes (CRBC) after a two hour pretreatment. However, Perussia et al. (1987) reported that 40 or 80 U/ml of rhuTNF-beta were toxic for human neutrophils and only 5 U/ml were required to increase neutrophil ADCC using p815y targets in a 3 hour ⁵¹Cr-release assay. In their assay, no pretreatment was needed and the enhanced activity was lost by 18 hours after the start of treatment. In addition, this enhancement apparently did not require the induction of FcR_{high} (high-affinity receptor binds

monomeric IgG) receptors that have been proposed to play a role in enhancement of neutrophil ADCC activity (Perussia et al., 1987). More work needs to be done on the effects of rhuTNF-beta on neutrophil function and the possible toxicity at 40 or 80 U/ml needs to be further evaluated.

Different cytokines have been used in combination to look for synergy in the enhancement of neutrophil function. Recombinant huTNF-alpha (Perussia et al., 1987) does not enhance ADCC by itself after 18 hours of incubation. Pretreatment of the neutrophils for 18 hours with rhuIFN-gamma (200 U/ml) followed by washing and addition of rhuTNF-alpha into a 3 hour assay lead to a greater enhancement than with rhuIFN-gamma alone.

The possibility of contaminating molecules in the recombinant TNF preparations, in particular E. coli LPS, being responsible for the enhancement of neutrophil function has been eliminated in several different ways: 1) The TNF was heated to destroy TNF activity without destroying contaminating LPS (~5 ng/ml original product) activity, 2) the direct effects of exogenous E. coli LPS on various neutrophil functions was examined, and 3) polymyxin B which selectively binds LPS was added along with exogenous LPS or TNF. Heating rhuTNF-alpha to 100°C for 5 to 15 minutes destroys the TNF induced neutrophil adhesion activity (Shalaby et al., 1987) and also abolishes its effects on the MPO-H₂O₂-halide activity (Klebanoff et al., 1986). Exogenous LPS (100 ng/ml) does not duplicate the rhuTNF-alpha effects on MPO-H₂O₂-halide activity even in the presence of unopsonized zymosan. In addition, LPS has no effect on the p815y target ADCC assay in the 5ng to 5 ug/ml concentration range

(Perussia et al., 1987). LPS does stimulate an increase in neutrophil ADCC of chicken red blood cells (CRBC), however, polymyxin B (10 ug/ml) inhibits this increase, yet polymyxin B has no effect on rhuTNF-beta-mediated enhancement (Shalaby et al., 1985) or rhuTNF-beta induced increase in ADCC activity (Perussia et al., 1987). Polymyxin B (1 or 10 ug/ml) has no direct effect on the adherence assay (Shalaby et al., 1987) nor does it effect rhuTNF-alpha dependent neutrophil MPO-H₂O₂-halide activity (Klebanoff et al., 1986)

Anti-TNF monoclonal antibodies were used to determine the specificity of TNF induced enhancement of neutrophil activity. Random migration was not inhibited by TNF-alpha when monoclonal antibodies to TNF-alpha were included in the assay. The lack of random migration inhibition was not due to a direct effect of the antibody on the neutrophil (Shalaby et al., 1987). Preincubation of rhuTNF-alpha with anti-TNF monoclonal antibodies totally inhibited TNF-stimulated adherence. Addition of the antibodies 5 or 15 minutes after rhuTNF-alpha resulted in progressively less inhibition of adherence. In addition, mAbs to TNF inhibited the rhuTNF-alpha induced MPO-H₂O₂-halide activity and the rhuTNF-beta-mediated increase in ADCC activity (Shalaby et al., 1985; Perussia et al., 1987).

Little work has been done with TNF in vivo that did not involve looking for a direct cytotoxic effect of TNF on solid tumors. But it is known that intradermal injection of TNF into human beings results in a lesion that resembles a delayed skin test and TNF injection in murine skin results in neutrophil accumulation at the site of injection and erythema (Ruddle, 1987).

Table 3. Influence of recombinant TNF-alpha on neutrophil function
in vivo

Increases:

^h Expression of iC3b	(Gamble et al., 1985; Shalaby et al., 1987)
^h Adherence	(Gamble et al., 1985)
^h Disruption of endothelial cell monolayers	(Shalaby et al., 1987)
^h Superoxide anion production	(Shalaby et al., 1987)
^h Phagocytic activity (TNF-alpha or -beta)	(Klebanoff et al., 1986; Shalaby et al., 1985)
^h Lysozyme release in response to stimulus	(Klebanoff et al., 1986)
^h MPO-H ₂ O ₂ -halide activity	(Klebanoff et al., 1987)
^h Cytotoxicity (ADCC) (TNF-alpha or -beta)	(Shalaby et al., 1985; Perussia et al., 1987)

Decreases:

^h Proliferation of endothelial cells in culture	(Shalaby et al., 1987)
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Unaffected:

^h Expresson of FcR by TNF-beta	(Perussia et al., 1987)
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(superscripts indicate neutrophils isolated from (h)humans treated
with TNF-alpha or -beta from the same species)

INTERFERONS

Interferon (IFN) was originally described as a protein mediator released from virus-infected cells which prevented viral replication in a target cell (Friedman and Vogel, 1983). It was soon realized that antiviral activity was due to a heterogeneous family of proteins which could be divided into two types of interferon. Type I, originally referred to as viral IFN, included both IFN-alpha and -beta that were produced by a variety of cells in response to virus infections but were first described as products of leukocytes and fibroblasts, respectively. Sequence determinations of cloned cDNAs have revealed more than a dozen members of the huIFN-alpha gene family and at least one and possibly two for huIFN-beta. Type II interferon, also called immune interferon or IFN-gamma, is induced by specific antigens, mitogens, or other stimuli and is the predominant form produced by stimulated lymphocytes. It has been realized that the interferons can be produced in response to not only viruses, but to cells infected with facultative intracellular bacteria and other bacterial antigens by a variety of cell types (Nakane and Minagawa, 1983; Stefanos et al., 1985; Weigent, Baron, and Stanton, 1983). Interferon alone is not viricidal or bactericidal but plays an immunoregulatory role, affecting the microbicidal and cytotoxic functions of both lymphoid and myeloid cells (Friedman and Vogel, 1983).

Interferon gamma shares several characteristic activities with IFN-alpha and -beta, but both IFN-alpha and -beta have greater antiviral activity than IFN-gamma (Friedman and Vogel, 1983). Interferon-gamma has

been reported to have a greater ability to inhibit proliferative and differentiation of hematopoietic precursor cells, than either alpha or beta (Broxmeyer et al., 1983). It has since been shown, however, that even the most purified preparations of IFN-gamma were contaminated with lymphotoxins (Stone-Wolff et al., 1984). Recombinant IFN-gamma without lymphotoxin exerts only a minimal inhibition of colony formation even at doses up to 1000 U/ml (Friedman and Vogel, 1983). Like IL-1, identification of the specific functions of IFN-gamma had to await cloning and production of a single protein. In addition to differences in biological activity and the stimuli that induce the production of IFN-gamma, this type of interferon can be distinguished from IFN-alpha and -beta in several other ways. Interferon-gamma is of higher Mr (20-25,000), is acid-labile (pH 2.0), in man is sensitive to 56°C, and is the product of only one gene that codes for a glycosylated protein (review: Trinchieri and Perussia, 1985). Recently, antigenic differences between the three forms of IFN have been detected by monoclonal neutralizing antibodies.

Interferons exert their actions by binding to membrane receptors (Trinchieri and Perussia, 1985; Zoon, Zur Nedden and Arnheiter, 1986). At 37°C human IFN bound to its receptor and was internalized and ultimately degraded (Zoon et al., 1983; Zoon, Arnheiter, and Fitzgerald, 1986), an event often referred to as down-regulation of receptors. Interferon-alpha and -beta share a receptor which is different from the receptor for IFN-gamma (Branca and Baglioni, 1981), although one report indicates that IFN-gamma treatment of cells induces down-regulation of

IFN-alpha/beta receptors (Zoon and Arnheiter, 1984). The number of IFN receptors per cell varies, but is generally less than 5000 (Zoon, Zur Nedden, and Arnheiter, 1986). The affinity of IFN for its receptor is very high, the apparent dissociation constants (Kd) are on the order of 10^{-10} to 10^{-11} M. A strong synergy has been described between IFN-gamma and -alpha/-beta in inducing antiviral activity (Fleischmann et al., 1979), suggesting that the molecular events leading to this biological response may not be completely identical.

The binding of interferons to specific receptors on the surface of mature neutrophils activates various aspects of neutrophil function. Recombinant huIFN-gamma increased the phagocytic activity of mature human neutrophils; 10 or 100 U/ 10^6 neutrophils resulted in increased phagocytosis of 1.5 um latex beads as detected by flow cytometry (Shalaby et al., 1985). Approximately 82% of nonactivated neutrophils can phagocytose and kill Candida albicans but these activities decrease in vitro over an 18 hour incubation to 43% (Perussia et al., 1987). Addition of rhuIFN-gamma did not enhance but maintained these activities at 82%. Neutrophil fungicidal activity and C3-opsonized zymosan stimulated phagocytosis were also maintained after an 18 hour incubation in the presence of rhuIFN-gamma.

Recombinant huIFN-gamma increased the cytotoxic activity of the mature neutrophil. There was an increased percentage of ^{51}Cr -release in a four hour ADCC assay (optimal effector to target ratio 50:1, suboptimal ratio 5,10,25:1) against ^{51}Cr -labeled CRBC following a one hour pretreatment of neutrophils at 1-100 U/ml of huIFN-gamma (Basham, Smith,

and Merigan, 1984). Shalaby et al. (1985) found similar enhancement using slightly different assay conditions; effector to target ratio of 4:1 and after one hour of pretreatment with lower doses of rhuIFN-gamma (0.01 to 1.0 U/ml). Apparently, higher doses also increased the neutrophil cytotoxic activity against CRBC but not in a dose-dependent manner (10^3 to 10^4 U/ml) (Basham, Smith, and Merigan, 1984). In a three hour ADCC assay against ^{51}Cr -labeled p815y both biochemically pure and rhuIFN-gamma (200 U/ml maximal) increased human PMN cytotoxic activity (Perussia et al., 1987). The neutrophils were pretreated with rhuIFN-gamma for varying times up to 48 hours prior to addition to the assay. Activity was increased after 4 hours and maximal after 18 hours, activity continued for 24 to 48 hours. The enhancement of neutrophil ADCC activity after 18 hours was not due to induction of TNF since antibodies to TNF, which completely blocked the TNF-mediated enhancement of ADCC, had no effect on the ability of IFN-gamma to enhance the ADCC activity. Recombinant or natural huIFN-alpha and -beta at doses up to 1000 U/ml did not induce any enhancement of neutrophil ADCC against p815y target cells (Perussia et al., 1987). Higher doses of rhuIFN-alpha_A or -beta (500 U/ml) have been reported to increase neutrophil ADCC activity towards CRBC targets to approximately the same extent as rhuIFN-gamma (Basham, Smith, and Merigan, 1984). These conflicting results might be explained by the different cells chosen as targets, which may vary in their abilities to be lysed by the neutrophil.

The enhancement by rhuIFN-gamma of neutrophil ADCC has been proposed in part to be dependent on an IFN-induced expression of high-affinity

receptors (FcR_{high}) for monomeric IgG (Trinchieri and Perussia, 1985; Perussia et al., 1987; Shen et al., 1987). Normally, neutrophils do not express FcR_{high} receptors but, as a process of differentiation, they acquire low-affinity (FcR_{low}) receptors that bind aggregated IgG. Recombinant huIFN-gamma induced or enhanced the expression of high-affinity Fc receptors on mature neutrophils and this correlated with the enhancement of ADCC activity (Perussia et al., 1987; Shen et al., 1987). This increase was dependent on neutrophil de novo synthesis of RNA and protein, determined by use of inhibitors of these cellular processes (Trinchieri and Perussia, 1985). The fact that rhuIFN-alpha did not increase the ADCC activity against the p815y targets or the FcR_{low} , or high expression supported this conclusion (Perussia et al., 1987). However, these findings are in direct conflict with the reported enhancement of ADCC activity against CRBC after rhuIFN-alpha treatment of human neutrophils (Basham, Smith, and Merigan; 1984). A recent report by Perussia et al., (1987) emphasizes the expression of FcR_{high} is not absolutely required, but plays a part in the enhanced ADCC activity.

Neither rhuIFN-alpha nor rhuIFN-gamma affected CR3 density on the neutrophil surface over a treatment period varying from 3 to 18 hours (Perussia et al., 1987). This receptor, as previously stated, plays a part in neutrophil adherence and adherence-related activities. Two adherence related functions, phagocytosis and ADCC, were enhanced by the addition of rhuIFN-gamma, despite the apparent lack of increase in CR3 expression (Perussia et al., 1987). These findings suggest that without overt deficiencies in CR3 expression, adherence-related activities can be

enhanced via other cellular processes.

As mentioned previously, E. coli LPS is a contaminant of the recombinant products. Addition of exogenous E. coli LPS to a final concentration of 50 ng/ml stimulated neutrophil phagocytosis of latex beads 3-fold, and enhanced the ADCC activity against ^{51}Cr -labeled CRBCs (Shalaby et al., 1985). A LPS concentration of 50 ng/ml was probably in excess of the contaminating concentration in the assays, which has been reported to be < 5 ng/ml after dilution. Others have reported no enhancement of ADCC against CRBC by LPS 0.1 to 100 ng/ml (Shen et al., 1987). Polymyxin B addition, similar to the TNF experiments, blocked the LPS-induced but not cytokine-induced activity in both assays. Addition of a mixture of two monoclonal antibodies that recognize IFN-gamma, but not an irrelevant I1-2 monoclonal antibody, completely blocked the IFN-gamma-induced enhancement of ADCC (10^{-3} neutralizing U/ml).

Treatment of human neutrophils with a combination of rhuIFN-gamma and natural huTNF-beta exceeded activation induced by either agent alone. TNF-beta (10 U) plus IFN-gamma (1 U) enhanced phagocytosis of latex beads maximally at these combination doses, at higher concentrations of IFN-gamma no further increase was noted (Shalaby et al., 1985). In addition TNF-beta (0.1 or 1 U/ml) plus IFN-gamma (1 U/ml) increased the ADCC activity to a greater extent than either cytokine alone. The ADCC activity against p815y targets was increased after an 18 hour pretreatment with rIFN-gamma and to an even greater extent if during the three hour assay rhuGM-CSF, LT or rTNF-alpha was added (alone these cytokines could minimally enhance ADCC) (Perussia et al., 1987).

Recombinant IFNs from other species have been examined to determine the specific effects on neutrophil function. Although recombinant murine IFN-gamma (rmuIFN-gamma) is available, very little work has been reported concerning its effect on murine neutrophil function. One report indicated that rmuIFN-gamma did not significantly enhance murine peritoneal neutrophil fungicidal activity against C. albicans but it did increase activity against Blastomyces dermatitidis (Morrison et al., 1987). Neutrophil fungicidal activity against phagocytosable C. albicans was significantly higher ($71.3 \pm 17.4\%$) than against nonphagocytosable Blastomyces dermatitidis (extracellular killing). It was interesting, therefore, that a one hour pretreatment of neutrophils with rmuIFN-gamma (3×10^{-5} to 3×10^5 U/ml) increased the fungicidal activity against B. dermatitidis. Maximal fungicidal activity against B. dermatitidis resulted in only 21% killing even at an elevated concentration of rmuIFN-gamma, 3×10^4 U/ml. Exogenous LPS at levels corresponding to those in the recombinant product preparations did not enhance neutrophil fungicidal activity. Pretreatment of the IFN with rabbit anti-IFN antiserum before addition to the neutrophils eliminated the enhancement of fungicidal activity by effective doses of IFN.

Peripheral blood neutrophils from cattle have also been treated with a recombinant bovine IFN-gamma (rboIFN-gamma) preparation in vitro. Recombinant boIFN-gamma (50 U/ml) pretreatment for as little as five minutes or for longer time periods resulted in decreased random migration (Steinbeck, Roth, and Kaeberle, 1986; Bielfeldt Ohmann and Babiuk, 1986; Steinbeck and Roth, 1987) and decreased directed migration under agarose,

the decreases induced by higher concentrations of interferon were not linear (1 to 10^4 U/ml) (Bielfeldt Ohmann and Babiuk, 1986). The rboIFN-gamma itself was not chemotactic. After a 2.5 hour pretreatment with rboIFN-gamma, there was no enhancement of superoxide anion production by the neutrophils in the cytochrome c assay in the presence of C3-opsonized zymosan (Steinbeck, Roth, and Kaeberle, 1986; Bielfeldt Ohmann and Babiuk, 1986). This was also true for neutrophils that were washed free of IFN before being added to the cytochrome c assay (Bielfeldt Ohmann and Babiuk, 1986). These authors found that at a concentration of 1×10^4 U/ml, rboIFN-gamma actually inhibited the production of superoxide anion. Recombinant boIFN-gamma (50 U/ml) after a 2.5 hour pretreatment enhanced neutrophil ADCC activity in a two-hour ^{51}Cr -labeled CRBC (10:1) target assay (Steinbeck, Roth, and Kaeberle, 1986; Steinbeck and Roth, 1987). Interestingly, the neutrophil antibody-independent lysis of the CRBC targets was also enhanced after pretreatment with rboIFN-gamma. Both the inhibition of migration and the enhancement of AINC required RNA and protein synthesis as determined by the use of inhibitors (alpha-amanitin (RNA) or puromycin or cycloheximide (protein)) of these metabolic events (Steinbeck, Roth, and Kaeberle, 1986; Steinbeck and Roth, 1987). The ADCC enhancement was not affected by these inhibitors. The enhancement of AINC was also dependent on arachidonic acid metabolism as determined by the use of two inhibitors of the lipxygenase enzyme (Steinbeck and Roth, 1987).

The neutrophils isolated from cattle immunosuppressed by intramuscular dexamethasone administration (0.04 mg/kg body wt) generally

have impaired functions. The pretreatment of these neutrophils in vitro with rboIFN-gamma (50 U/ml) resulted in the restoration of both random migration and ADCC activities to control values. Recombinant boIFN-gamma pretreatment of these neutrophils did not enhance the AINC activity, other activities were not evaluated. Immunosuppressed animals are more susceptible to microbial infections, and rboIFN-gamma may be a useful immunomodulator to reverse the effects of immunosuppression on neutrophil function.

Both random migration and migration directed towards zymosan activated serum components were inhibited after 30 or 60 minutes of pretreatment with rboIFN-alpha ($5 \times 10^{0-4}$ U/ml) (Bielfeldt-Ohmann and Babiuk, 1984; Bielfeldt Ohmann and Babiuk, 1986). Recombinant boIFN-gamma inhibited directed and random migration to a greater extent than rboIFN-alpha₁ at any of the concentrations tested (Bielfeldt Ohmann and Babiuk; 1986). Recombinant boIFN-alpha₁ (1000 U/ml) pretreatment for 30 minutes enhanced bovine neutrophil bacterial uptake (phagocytosis) of antiserum-opsonized Staphylococcus aureus or E. coli (1:1) after three to four hours at 37°C (Bielfeldt-Ohmann and Babiuk, 1984). At lower doses of IFN the phagocytosis values were zero and at higher doses values were lower than at the optimal 1000 U/ml dose. Like rboIFN-gamma, rboIFN-alpha (3000 U/ml maximal) did not affect superoxide anion production (Bielfeldt-Ohmann and Babiuk, 1984; Bielfeldt Ohmann and Babiuk, 1986) and was inhibitory at higher concentrations under the same assay conditions. Hydrogen peroxide production within 30 minutes of treatment was maximally enhanced by 3000 U/ml of rboIFN-alpha (Bielfeldt-Ohmann and

Babiuk, 1984). Pretreatment of greater than one hour resulted in the loss of consistent effects on hydrogen peroxide production, which might be a result of a toxic effect of hydrogen peroxide on the neutrophil. Since the production of H_2O_2 is directly dependent on the initial synthesis of superoxide anion, it can only be concluded that the assays vary in their sensitivity of detection. Lysozyme activity, which is not measurable in neutrophils from adult cattle, is present in neutrophils isolated from young cattle (10 days to 6 weeks of age). Treatment of neutrophils from young cattle for four hours with rboIFN- α ($15 \times 10^{1-5}$ U/ml) decreased the presence of this enzyme (Bielfeldt Ohmann and Babiuk, 1986). This IFN over the same concentration range had no effect on the beta-glucuronidase activity of the neutrophils. One interpretation of these results might be that lysozyme expression is regulated by the environment of the bone marrow and this environment for whatever reason changes with the age of the cattle.

Bielfeldt Ohmann and Babiuk (1986) reported that i.v. or i.m. administration of 10^4 or 10^6 U of rboIFN- α or - γ altered neutrophil functions as measured in vitro. Depending on the doseage, IFN, route, or time after administration, the neutrophils from treated cattle had decreased random and directed migration and stimulated superoxide anion production. In addition, an increase in circulating neutrophil numbers occurred within the first 24 hours after IFN administration. The time that it took to see an effect and the effect itself was not dose-dependent and varied considerably. This can probably be explained by the fact that the cytokines can bind to numerous cell

types and their effects would be dependent on the cells contacted in the local area of administration.

Table 4. Influence of recombinant IFN-gamma on neutrophil function
in vitro

Increases:

^b Superoxide anion production after <u>in vivo</u> admin. to cattle	(Bielfeldt Ohmann and Babiuk, 1986)
^{h,m} Phagocytic activity	(Shalaby et al., 1985; Morrison et al., 1987)
^m Fungicidal activity	(Morrison et al., 1987)
^h Expression of FcR _{high}	(Trinchieri and Perussia, 1985; Perussia et al., 1987)
^{h,b} Cytotoxicity (ADCC)	(Basham et al., 1984; Shalaby et al., 1985; Steinbeck, Roth, and Kaeberle, 1986; Bielfeldt Ohmann and Babiuk, 1986; Perussia et al., 1987; Steinbeck and Roth, 1987)
^b Cytotoxicity (AINC)	(Steinbeck, Roth, and Kaeberle, 1986; Steinbeck and Roth, 1987; Morrison et al., 1987)
^b Cytotoxicity (ADCC) (neutrophils from immunosuppressed cattle)	(Steinbeck and Roth, 1987)

Decreases:

^{h,b} Random migration	(Steinbeck, Roth, and Kaeberle, 1986; Steinbeck and Roth, 1987; Bielfeldt Ohmann and Babiuk, 1986)
^b Directed migration	(Bielfeldt Ohmann and Babiuk, 1986)
^b Random migration (neutrophil from immuosuppreessed cattle)	(Steinbeck and Roth, 1987)
^b Lysozyme activity, of neutrophils from young calves	(Bielfeldt Ohmann and Babiuk, 1986)

Unaffected:

^h Expression of CR3	(Perussia et al., 1987)
^{h,b} Superoxide anion production	(Perussia et al., 1987; Bielfeldt Ohmann and Babiuk, 1986; Steinbeck, Roth, and Kaeberle, 1986)
^b Phagocytic activity	(Steinbeck, Roth, and Kaeberle, 1986)
^h Phagocytic activity was main- tained after 18 hours in culture	(Perussia et al., 1987)

(superscripts indicate neutrophils isolate from (h)human, (b)bovine,
and (m)murine) treated IFN-gamma from the same species)

Table 5. Influences of recombinant IFN-alpha on neutrophil function
in vivo

Increases:

- | | |
|---|-------------------------------------|
| ^b Neutrophil numbers in peripheral blood after <u>in vivo</u> admin. to cattle | (Bielfeldt Ohmann and Babiuk, 1986) |
| ^b Superoxide anion production after <u>in vivo</u> admin. to cattle | (Bielfeldt Ohmann and Babiuk, 1986) |
| ^b Phagocytic activity | (Bielfeldt-Ohmann and Babiuk, 1984) |
| ^b Hydrogen peroxide production | (Bielfeldt-Ohmann and Babiuk, 1984) |
| ^h Cytotoxicity (ADCC) against CRBC targets (IFN-alpha and -beta) | (Basham et al., 1984) |

Decreases:

- | | |
|---|---|
| ^b Random migration | (Bielfeldt-Ohmann and Babiuk, 1984;
Bielfeldt Ohmann and Babiuk, 1986) |
| ^b Directed migration | (Bielfeldt-Ohmann and Babiuk, 1984;
Bielfeldt Ohmann and Babiuk, 1986) |
| ^b Lysozyme activity in neutrophils from young calves | (Bielfeldt-Ohmann and Babiuk, 1984) |

Unaffected:

- | | |
|---|--|
| ^h Expression of CR3 | (Perussia et al., 1987) |
| ^{h,b} Superoxide anion production in response to stimulus | (Perussia et al., 1987)
(Bielfeldt-Ohmann and Babiuk, 1984;
Bielfeldt Ohmann and Babiuk, 1986) |
| ^b Beta-glucuronidase activity in neutrophils from young calves | (Bielfeldt-Ohmann and Babiuk, 1984) |
| ^h Expression of FcR _{high} | (Perussia et al., 1987) |
| ^h Cytotoxicity (ADCC) for p815y targets (IFN-alpha or -beta) | (Perussia et al., 1987) |

(superscripts indicate neutrophils isolated from (h)human, and (b)bovine treated with IFN-alpha from the same species)

SUMMARY

Interest in the use of recombinant cytokines for immunotherapy is increasing as more information becomes available on the influences of these products on effector cells (e.g., neutrophils) involved in the resistance of the host to disease. This review summarizes the information available on in vitro and in vivo activation of neutrophils by recombinant cytokines. The emerging theme is that various cytokines (colony-stimulating factors, tumor necrosis factors, interferons) have direct and often synergistic abilities to activate the neutrophil to have enhanced cytotoxic activity towards microbial and cellular stimuli. Equally as important (although less well studied) cytokines have been shown in some cases to activate neutrophils isolated from immunosuppressed animals. These neutrophils have depressed function and influences of cytokines on these cells may be different from their influences on normal neutrophils. We chose to discuss only recombinant cytokine data in this review because of the confusion caused by protein contaminants which may be present within biochemically purified preparations.

SECTION II:

ACTIVATION OF BOVINE NEUTROPHILS BY
RECOMBINANT GAMMA INTERFERON

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ABSTRACT

The effect of recombinant bovine gamma-interferon (rboIFN-gamma) on neutrophil functions was investigated and compared to the effects of an unpurified lymphokine preparation. Incubation of purified bovine neutrophils with rboIFN-gamma or antigen-induced lymphokine for 2.5 hours at 37°C resulted in impairment of the ability of neutrophils to migrate under agarose, and an enhancement of their ability to mediate antibody-dependent and antibody-independent cell-mediated cytotoxicity against chicken erythrocytes (CRBC). Neither the lymphokine preparation nor the rboIFN-gamma had any influence on Staphylococcus aureus ingestion, or iodination by neutrophils. The lymphokine preparation enhanced cytochrome C reduction by neutrophils and was weakly chemotactic, whereas the rboIFN-gamma had neither of these effects. Only five minutes of rboIFN-gamma preincubation with neutrophils were needed to trigger protein synthesis by the neutrophils resulting in inhibition of random migration. Therefore, recombinant gamma-interferon acts as a neutrophil migration inhibition factor and a neutrophil activation factor resulting in enhanced neutrophil mediated antibody-dependent and -independent cell-mediated cytotoxicity. Many, but not all, of the in vitro effects of an unpurified lymphokine preparation on neutrophil function can be attributed to the gamma interferon contained in the lymphokine.

INTRODUCTION

We have previously reported that an antigen-induced bovine lymphokine, containing measurable interferon activity, enhanced neutrophil Staphylococcus aureus ingestion, NBT reduction, and antibody-dependent cell-mediated cytotoxicity (ADCC), and impaired neutrophil migration under agarose (Lukacs, Roth, and Kaeberle, 1985). We also reported the apparently novel finding that lymphokine treatment caused neutrophils to be cytotoxic for xenogeneic (but not allogeneic) erythrocytes in the absence of antibody (antibody-independent neutrophil cytotoxicity) (AINC) (Lukacs, Roth, and Kaeberle, 1985). The enhancement of AINC but not ADCC required a 2 hour period of preincubation and was dependent on RNA and protein synthesis by the neutrophil.

In other experimentation we found that the administration of an interferon inducer (avridine) counteracted most of the effects of dexamethasone on neutrophil function (Roth and Kaeberle, 1985). Neutrophils were obtained from cattle given avridine 24 hours prior to receiving dexamethasone and the results were compared to neutrophils obtained from cattle given dexamethasone only. Neutrophils from avridine pre-treated cattle had enhanced S. aureus ingestion, oxidative metabolism, and ADCC and a reduction in random migration under agarose when compared to neutrophils from cattle treated with dexamethasone only. Since the avridine probably induced a variety of potential biologic response modifiers, it could not be assumed that the avridine effects

were due to interferon only.

Both the lymphokine preparation in vitro and avridine administration in vivo enhanced bovine neutrophil activity. The lymphokine contained gamma interferon, and avridine is reported to induce interferon in vivo, therefore, it was of interest to determine the effects of purified gamma interferon on neutrophil function. Recombinant DNA technology has made available several different interferon molecules for use in research. Other investigators have used recombinant interferons (human IFN-alpha, beta, gamma and bovine IFN-alpha₁ & ₂) and have shown that these interferons are biologically active in various assays (Basham, Smith, and Merigan, 1984; Bielfeldt-Ohmann and Babiuk, 1984). In recent reports, recombinant interferon has been shown to enhance human neutrophil ADCC (Basham, Smith, and Merigan, 1984) and neutrophil phagocytosis (Bielfeldt-Ohmann and Babiuk, 1984). A rboIFN-alpha also inhibited random migration of neutrophils under agarose (Bielfeldt-Ohmann and Babiuk, 1984). Our previous work and work of others lead us to investigate the effect of recombinant bovine gamma-interferon (rboIFN-gamma) on neutrophil function and to compare it to the effects of unpurified antigen-induced lymphokine.

MATERIALS AND METHODS

Neutrophil preparation. Neutrophils were separated as previously described (Lukacs, Roth, and Kaerberle, 1985). Briefly, peripheral blood was collected from healthy cattle into acid-citrate-dextrose solution, centrifuged, and the plasma and buffy coat layer were discarded. The packed erythrocytes were lysed by brief exposure to hypotonic conditions and the remaining cells, which generally consisted of greater than 90% polymorphonuclear leukocytes (PMNS), were washed in 0.015 M phosphate buffered saline solution (PBSS) (pH 7.2) and suspended in Medium 199, containing 25 mM HEPES buffer, to a concentration of 1.0×10^8 PMN/ml.

Lymphokine and rboIFN-gamma. The lymphokine was induced in vitro by stimulating purified mononuclear cells obtained from cattle which had been infected with infectious bovine rhinotracheitis (IBR) virus (herpesviridae) with heat-inactivated IBR virus. This method has been described elsewhere in detail (Lukacs, Roth, and Kaerberle, 1985). The bovine rboIFN-gamma was supplied by Genentech Inc., South San Francisco, CA (Lot number 2433/21,33,95) with a specific activity of $\geq 1.0 \times 10^6$ U/mg (MDBK-VSV).

Treatment of PMN with lymphokine or rboIFN-gamma. Three hundred microliters of a 1.0×10^8 /ml suspension of PMN were incubated with 300 ul of different dilutions of rboIFN-gamma or lymphokine (diluted in Medium 199 with HEPES) for 2.5 hours at 37°C. The PMNs were then used in the functional assays without washing. Control PMN preparations were incubated with Medium 199 with HEPES.

Evaluation of neutrophil function. The procedure for evaluation of random migration under agarose, chemotaxis, S. aureus ingestion, iodination, ADCC and AINC have previously been described (Lukacs, Roth, and Kaeberle, 1985)

Cytochrome c reduction. The standard reaction mixture contained 0.075 ml cytochrome c (538 μ M in Hanks balanced salt solution without phenol red (HBSS), 0.025 ml PMNs (5.0×10^7 /ml), 0.025 ml opsonized zymosan preparation (5 mg/ml HBSS), and 0.05 ml of HBSS. The zymosan suspension was replaced with HBSS for the determination of resting cytochrome c reduction. All reactants were added to wells in a 96 well millititer filtration plate (SV, Millipore Corp., Bedford, MA) which had been pre-wetted with 0.5% bovine serum albumin. The reaction was started by the addition of PMNs and the plate was incubated at 37°C for 30 minutes with constant agitation. The reaction was stopped by vacuum filtration and collection of the filtrate in a flat bottom microtiter plate (millipore vacuum holder, Millipore Corp., Bedford, MA). The PMNs trapped on the filter were washed twice by filtration with 0.05 ml of HBSS and those filtrates were collected in the same microtiter plate. The filtrate optical density (OD) at 550 nm was determined. The results were expressed as OD/ 1.25×10^6 PMNs/30 minutes reaction time.

Inhibitor studies. Alpha-amanitin and puromycin (Sigma Chemical Co., St. Louis, Mo.) were used to inhibit RNA and protein synthesis respectively by PMNs during the incubation of PMNs with rboIFN-gamma. PMNs were first preincubated with alpha-amanitin (50 μ g/ml) or puromycin (10 μ g/ml) or both for 30 min at 37°C; rboIFN-gamma containing the same

concentration of inhibitor(s) was then added to the PMNs (without washing) and the incubation continued for 2.5 hours.

Incorporation of L-[³⁵S]methionine into acid-insoluble proteins. Purified PMNs (5.0×10^6) were plated in 16 mm tissue culture dishes (24 well Costar 3524, Costar, Cambridge MA) in 150 μ l of RPMI 1640 medium lacking methionine but supplemented with 0.1% (w/v) bovine serum albumin in an atmosphere of 5% CO₂/95% air at 37°C either in the presence or absence of cycloheximide (10 μ g/ml) or puromycin (10 μ g/ml) (protein synthesis inhibitors). After 30 minutes the cultures received 150 μ l of medium containing 120 uCi of L-[³⁵S]methionine with or without lymphokine (1:5) and + inhibitor. The cells were resuspended by gentle pipetting 2.5 hours later, washed twice by centrifugation in cold tris-buffered saline, and solubilized in 100 μ l of lysis buffer containing 1mM phenylmethylsulphonyl fluoride and 1mM aprotinin. An equal volume of cold 20% trichloroacetic acid (TCA) was added, and the resulting precipitate was collected on glass fiber filters (GC filters Whatman, Inc., Clifton, NJ). The filters were washed with 10% TCA, dried, and counted in a toluene-based scintillation fluid (Liquifluor, New England Nuclear, Boston, MA) in a liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL).

Data analysis. Statistical significance was determined using a Dunnett's multiple comparison test of two means (untreated and treatment), this procedure uses conservative degrees of freedom in determining the level of significance. The individual PMN preparations were used as blocking factors in the analysis of variance in order to

remove the animal to animal variability from statistical consideration of the treatment effect (i.e., the value for a treated PMN preparation was compared to the value from an aliquot of that same PMN preparation that was untreated).

RESULTS

The results in Table 1 indicate that both lymphokine and rboIFN-gamma treatment of PMNs enhanced the percentage of ^{51}Cr release from the CRBCs as compared to the untreated neutrophils incubated in medium alone. The elevated ^{51}Cr release from the CRBCs was evident in the presence (ADCC) and absence of antibody (AINC). In both assays the rboIFN-gamma activity decreased in a concentration dependent manner, showing measurable activity out to a dilution of 5×10^{-10} gm/ml. Included in Table 1 are the results of the random migration under agarose assay. Both the rboIFN-gamma and lymphokine treatments (preincubation 2.5 hrs) inhibited PMN random migration with the rboIFN-gamma demonstrating concentration dependence.

The results for S. aureus ingestion, iodination, and unstimulated cytochrome c reduction in Table 2 were similar in the untreated and treated neutrophils. The lymphokine treatment did cause a small but significant enhancement of cytochrome c reduction by zymosan-stimulated neutrophils. The rboIFN-gamma did not have this same effect.

Table 1. IBR virus-induced lymphokine and recombinant bovine gamma-interferon effects on bovine neutrophil cytotoxicity in the presence and absence of antibody and on neutrophil random migration

ASSAY	Pretreatment of Neutrophils					
	Medium 199	IBR virus-induced lymphokine	Bovine gamma interferon (gm/ml)			
			5×10^{-8}	5×10^{-9}	5×10^{-10}	5×10^{-11}
Antibody-independent neutrophil cytotoxicity (% release of ^{51}Cr)	18.7 \pm 3.6	41.1 \pm 4.3 ^a	31.9 \pm 3.8 ^a	32.0 \pm 4.5 ^a	27.4 \pm 3.9 ^a	22.0 \pm 3.2
Antibody-dependent neutrophil cytotoxicity (% release of ^{51}Cr)	54.4 \pm 2.8	64.0 \pm 2.2 ^a	62.6 \pm 2.1 ^a	61.7 \pm 2.8 ^a	59.3 \pm 2.7 ^b	58.3 \pm 2.7
Migration under Agarose (mm ²)	21.9 \pm 2.1	8.8 \pm 1.7 ^a	7.1 \pm 1.2 ^a	8.9 \pm 1.2 ^a	14.0 \pm 1.8 ^a	18.7 \pm 2.3

^ap < .01;

^bp < .05; the level of statistical significance for the difference between the indicated value and the corresponding value of neutrophils preincubated in Medium 199. Data expressed as the mean \pm SEM (n=15).

Table 2. IBR virus-induced lymphokine and recombinant bovine gamma-interferon effects on bovine neutrophil functions

ASSAY	Pretreatment of Neutrophils					
	Medium 199	IBR virus-induced lymphokine	Bovine gamma interferon (gm/ml)			
			5×10^{-8}	5×10^{-9}	5×10^{-10}	5×10^{-11}
<u>S. aureus</u> ingestion (%) n=19	26.6 \pm 1.7	26.4 \pm 1.2	27.3 \pm 1.3	27.6 \pm 1.5	25.8 \pm 1.4	26.1 \pm 1.7
Iodination n=18 (nmole NaI/10 ⁶ PMNs/hr)	30.3 \pm 4.3	31.9 \pm 3.5	31.8 \pm 3.7	31.7 \pm 4.4	30.6 \pm 3.9	29.8 \pm 3.9
Cytochrome c reduction (unstimulated) n=20 (OD/5x10 ⁶ PMNs/30 min)	0.11 \pm .01	0.11 \pm .02	0.10 \pm .01	0.10 \pm .02	0.11 \pm .02	0.11 \pm .01
Cytochrome c reduction (stimulated) n=20 (OD/5x10 ⁶ PMNs/30 min)	0.38 \pm .02	0.42 \pm .02 ^a	0.38 \pm .01	0.37 \pm .02	0.39 \pm .02	0.36 \pm .02

^ap < .01; the level of statistical significance for the difference between the indicated value and the corresponding value of neutrophils preincubated in Medium 199.

To evaluate the kinetics of rboIFN- γ interaction with PMNs, the PMNs were preincubated with 5×10^{-8} gm/ml rboIFN- γ at 37°C for 5, 30, or 150 minutes then washed to remove unbound rboIFN- γ . Controls were incubated in medium for 150 minutes then washed. All cells were incubated for a total of 150 minutes (combination of before and after washing) before being added to the wells in the migration plates. The migration inhibition results for the 5, 30, and 150 minute time periods were 77.3 ± 5.0 , 73.5 ± 4.8 , and 67.3 ± 5.7 (mean \pm SEM) (n=6) respectively, expressed as a percentage of the value for control PMNs. Therefore, 5 minutes of exposure of neutrophils to rboIFN- γ was all that was required to trigger migration inhibition.

The need for RNA and protein synthesis for the rboIFN- γ inhibition of neutrophil migration was investigated using alpha-amanitin (50 ug/ml) and puromycin (10 ug/ml). At these concentrations the inhibitors did not affect cell viability as determined by trypan blue exclusion (> 95% viable). Inhibition of either RNA or protein synthesis partially blocked the effect of rboIFN- γ on random migration (Table 3a). The combination of both RNA and protein synthesis inhibitors was capable of completely blocking the rboIFN- γ inhibition of PMN migration.

The amount of L-[35 S]methionine incorporation into protein by PMNs was determined by TCA protein precipitation on glass fiber filters and liquid scintillation counting. Purified PMNs were cultured in the presence of RPMI 1640 medium containing 60 uCi of L-[35 S]methionine. Incorporation of L-[35 S]methionine was detectable in both untreated

and lymphokine-treated PMN fractions (Table 3b). The incorporation of radiolabel was inhibited >90% in both PMN fractions when either cycloheximide (10 ug/ml) or puromycin (10 ug/ml) were included during the 2.5 hour incubation. Incorporation of L-[³⁵S]methionine was similar in rboIFN-gamma-treated PMNS (data not shown).

The chemotactic activities of various dilutions of lymphokine and rboIFN-gamma for PMNS are shown in Table 4. The undiluted lymphokine preparation was weakly chemotactic for neutrophils. It was not chemotactic at a dilution of 1:10. Recombinant IFN-gamma was not chemotactic for neutrophils over a concentration range of 1×10^{-6} to 10^{-11} gm/ml.

Table 3a. Effect of inhibitors of protein and RNA synthesis on rbo-IFN-gamma migration inhibition activity for neutrophils

Pretreatment of PMNs ^a	Area of random migration (mm ²)	
	PMNs + medium	PMNs + IFN (1x10 ⁻⁸ gm/ml)
Medium 199	44.9 _± 9.5	18.0 _± 3.7 ^b
alpha-amanitin	40.3 _± 8.4	33.1 _± 6.7
Puromycin	43.3 _± 10.5	38.4 _± 8.4
alpha-amanitin + puromycin	40.7 _± 9.5	40.1 _± 9.2

^aPMNs were preincubated with 50 ug/ml alpha-amanitin and/or 10 ug/ml puromycin for 30 minutes at 37°C, and then for 2.5 hours in the presence or absence of 1x10⁻⁸ gm/ml rboIFN-gamma + inhibitor before being assayed. Results are the mean _± SEM (n=9)

^bp < .01; the level of statistical significance for the difference between the indicated value and the value for medium 199-treated neutrophils.

Table 3b. Effect of inhibitors of protein synthesis on L-[³⁵S]-methionine incorporation into acid-precipitable proteins

Pretreatment of PMNs ^a	L-[³⁵ S]methionine (cpm X 10 ⁴)	
	PMNs + RPMI 1640	PMNs + lymphokine
RPMI 1640	7.92	8.08
Cycloheximide (10 ug/ml)	0.62	0.69
Puromycin (10 ug/ml)	0.60	0.56

^aPMNs (5 X 10⁶ PMN) were pretreated by incubation in RPMI 1640 medium + 10 ug/ml cycloheximide or 10 ug/ml puromycin for 30 minutes at 37°C, and then for 2.5 hours in the presence of lymphokine or medium (+ inhibitors) containing a final concentration of 60 uCi of L-[³⁵S]-methionine before being assayed. The values presented are from a single experiment (triplicate cultures); comparable results were obtained in subsequent experiments.

Table 4. The effects of rboIFN-gamma, lymphokine, and zymosan activated serum on neutrophil chemotaxis under agarose

Chemotactic factor	Chemotactic index
Zymosan activated serum 1:2	1.46 \pm .03 ^a
IBR-virus induced lymphokine 1:1	1.13 \pm .02 ^a
1:10	1.03 \pm .03
Bovine gamma interferon 10 ⁻⁶	1.02 \pm .01
10 ⁻⁷	1.06 \pm .01
10 ⁻⁸	1.04 \pm .03
10 ⁻⁹	1.02 \pm .03
10 ⁻¹⁰	0.92 \pm .02
10 ⁻¹¹	0.96 \pm .02

^ap < .01; the level of statistical significance for the difference between the indicated value and the value for medium 199 as the control chemotactic factor. Data are expressed as mean \pm SEM (n=6).

DISCUSSION

These data indicate that many but not all of the effects of the unpurified lymphokine preparation on neutrophils can be attributed to gamma interferon. Both the lymphokine preparation and rboIFN-gamma inhibited random migration and enhanced AINC and ADCC activity compared to the non-treated neutrophils (Table 1). The lymphokine, however, enhanced the neutrophil oxidative metabolism and was chemotactic whereas the rboIFN-gamma did not have these effects. It is possible that other molecules within the lymphokine preparation are active either alone or in combination with gamma-interferon. Others have noted that a 'synergy' exists; the lymphokines work most effectively in combinations or in sequence rather than individually (Hamblin, 1985). The observed differences between the lymphokine preparation and the rboIFN-gamma could also be due to a difference in IFN-gamma concentration or to differences in the biologic activity of natural and recombinant gamma interferon.

The rboIFN-gamma did activate the neutrophil to have enhanced cytotoxic activity in both the presence and absence of specific antibody. We have previously reported the enhancement of ADCC and AINC activity of neutrophils using antigen-induced lymphokine preparations (Lukacs, Roth, and Kaeberle, 1985). The lymphokine activated PMNs had enhanced AINC activity against heterologous erythrocyte systems including chicken, turkey, and human, but not homologous bovine erythrocytes. The lymphokine itself was not

cytotoxic for the CRBCs (Lukacs, Roth, and Kaeberle, 1985). In addition, the cytotoxic activity was not due to increased phagocytosis by the neutrophil as neither lymphokine nor rboIFN-gamma treated neutrophils had enhanced phagocytic activity (Table 2). Basham et al. (1984) recently reported an enhancement of neutrophil ADCC using human rboIFN-gamma and CRBCs as target cells. Antibody-independent cytotoxic activity has previously been described for normal human peripheral blood neutrophils (Banerjee et al., 1981; Ohara et al., 1983) toward rabbit erythrocytes (RRBC) in the presence of gamma-globulin depleted human serum (Banerjee et al., 1981). In that system the AINC activity required cell to cell contact and was specific for RRBCs. Chicken RBCs were not lysed when co-incubated with the RRBCs. The supernatant removed from the lysed RRBCs was not cytotoxic for fresh RRBC. This activity did not involve phagocytosis, cytophilic antibody absorption to the surface, nor the Forssman heterophile antigens (Banerjee et al., 1981; Ohara et al., 1983). Human peripheral blood granulocytes also are known to have the capacity to recognize and selectively destroy human tumor cells in vitro in the absence of antibody (Gerrard, Cohen, and Kaplan, 1981; Chee et al., 1978). The lymphokine-induced enhancement of AINC by bovine PMNs required at least 2 hours of preincubation with lymphokine; and either RNA or protein synthesis inhibitors could prevent the lymphokine enhancement (Lukacs, Roth, and Kaeberle, 1985). The enhancement of natural killer cell cytotoxicity by human leukocyte interferon also requires RNA and protein synthesis (Bishop and Schwartz, 1982). RNA

and protein synthesis by the PMNs were not required for enhancement of ADCC by bovine lymphokine. The AINC activity was inhibited by superoxide dismutase, catalase, mannitol, azide, colchicine, and cytochalasin B; indicating that protein synthesis, oxidative metabolism, microfilament function, and microtubule function are important in the AINC activity (personal communication).

We found that the rboIFN- γ was not chemotactic for neutrophils, which was similar to the results found with human rIFN- α_2 (Farr et al., 1983). The undiluted lymphokine preparation, however, did possess weak chemotactic activity (Table 4). Neutrophil migration under agarose was markedly inhibited by rboIFN- γ (Table 1). The migration of bovine neutrophils was previously reported to be inhibited by bovine rboIFN- α_1 (Bielfeldt-Ohmann and Babiuk, 1984) and by lymphokine (Lukacs, Roth, and Kaeberle, 1985). Our results indicate that only 5 minutes of preincubation in the presence of rboIFN- γ was necessary to cause an inhibition of neutrophil random migration. Apostolov and Barker (1981) reported that cell membrane buoyant density results did not differ if mouse L cells were incubated in the continual presence of interferon or washed after 1 hour. They concluded that the initial interferon concentration determined the reaction of the cell and it was not a continual action of interferon. In addition, the continued presence of interferon on human amnion (WISH) cells was not required since the cells could be washed after overnight interferon treatment with no loss of antiviral activity (Blalock and Stanton, 1980).

The short 5 min preincubation with rboIFN-gamma apparently triggered protein synthesis by the neutrophils since both RNA and protein synthesis were necessary for optimal inhibition of migration by rboIFN-gamma. We have previously reported that neutrophils incubated with bovine lymphokine must be capable of protein synthesis in order to be fully activated in AINC (Lukacs, Roth, and Kaeberle, 1985). In accordance with this data, a measurable amount of L-³⁵S]methionine was incorporated into newly synthesized protein in both untreated and lymphokine treated neutrophils (Table 3b). The incorporation was inhibited >90% by protein synthesis inhibitors, cycloheximide and puromycin. The incorporation of [³H]leucine, [³H]uridine (Granelli-Piperno, Vassalli, and Reich, 1979), and L-³⁵S]methionine (Blowers, Jayson, and Jasani, 1985) into untreated and dexamethasone treated human peripheral blood neutrophils has been previously reported.

In summary, the data presented here suggests a role for neutrophils as effector cells in T cell directed immunity (gamma interferon is produced by antigen-stimulated T cells). The inhibition of neutrophil migration by rboIFN-gamma may be important for retention of neutrophils at the site of interferon production. The rboIFN-gamma can also act as a neutrophil activation factor resulting in the enhancement of both AINC and ADCC activity.

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SECTION III:

MOLECULAR EVENTS IN GAMMA INTERFERON-INDUCED
ACTIVATION OF BOVINE NEUTROPHILS

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ABSTRACT

Bovine neutrophils were preincubated with recombinant bovine gamma interferon (rboIFN-gamma) and the molecular events leading to enhanced antibody-dependent (ADCC) and antibody-independent (AINC) neutrophil-mediated cytotoxicity and impaired random migration under agarose were investigated. Addition of alpha-amanitin, puromycin, or cycloheximide (RNA and protein synthesis inhibitors) during preincubation and assaying prevented the rboIFN-gamma enhancement of AINC and inhibition of random migration, but did not prevent the ADCC enhancement. In addition, rboIFN-gamma treatment of neutrophils consistently increased the synthesis of a 60 and a 93-94 kilodalton (kD) protein after 60 minutes but within 2 hours of incubation. Neutrophils purified from dexamethasone-treated cattle also had increased synthesis of a 60 kD protein, enhanced ADCC and inhibited migration following rboIFN-gamma treatment but did not have enhanced AINC. Dexamethasone has been shown to decrease the formation of arachidonic acid lipoxigenase products by bovine neutrophils. Addition of lipoxigenase inhibitors (NDGA and BW755C) to neutrophils from nondexamethasone-treated cattle following rboIFN-gamma preincubation (i.e., after protein synthesis had occurred) eliminated the enhancement of AINC. This suggests that dexamethasone does not affect the rboIFN-gamma induced protein synthesis required for migration inhibition or AINC enhancement, but acts to inhibit the production of arachidonic acid lipoxigenase products which are also required for the AINC activity.

We have concluded that the enhancement of the AINC activity and inhibition of random migration (but not the enhancement of ADCC) required both RNA and protein synthesis which resulted in the increased expression of at least one protein. Only the enhanced AINC activity required the generation of lipxygenase products of arachidonic acid.

INTRODUCTION

We have previously reported that after a 2.5 hour preincubation with rboIFN-gamma, bovine neutrophils had enhanced antibody-dependent (ADCC) and -independent (AINC) neutrophil-mediated cytotoxicity and impaired random migration (Lukacs, Roth, and Kaeberle, 1985). The neutrophils did not show any consistent enhancement of oxidative metabolism, degranulation, or ingestion activities. Shalaby et al. (1985) have also reported human neutrophil ADCC enhancement and impaired random migration after a 2 hour preincubation with human rboIFN-gamma.

We are interested in the potential use of rboIFN-gamma as an immunomodulator in immunosuppressed cattle. Intramuscular administration of dexamethasone to cattle (0.04 mg/kg body weight) has been reproducibly used to induce immunosuppression. This synthetic glucocorticoid reduces neutrophil oxidative metabolism, iodination, and antibody-dependent cell-mediated cytotoxicity and enhances neutrophil random migration (Roth and Kaeberle, 1985). Dexamethasone treatment of cattle also leads to a decrease in arachidonic acid release from bovine neutrophil membrane phospholipids after in vitro opsonized-zymosan stimulation (Webb and Roth, 1987). The arachidonic acid released is converted by the lipoxygenase pathway into two major products, leukotriene-B₄ and 5-HETE. These products are necessary for optimal iodination and oxidative response by bovine neutrophils but are less important for random migration, ingestion, and ADCC (Webb and

Roth, 1987). We have previously reported the need for neutrophil protein synthesis for the inhibition of neutrophil random migration by rboIFN-gamma (Steinbeck, Roth, and Kaeberle, 1986). Enhancement of AINC by an antigen-induced lymphokine also required the bovine neutrophil to synthesize RNA and protein (Lukacs, Roth, and Kaeberle, 1985). In contrast, the ADCC enhancement by lymphokine was not decreased by inhibitors of RNA and protein synthesis. This correlates well with the observation that enhancement of ADCC did not require any preincubation with lymphokine (Lukacs, Roth, and Kaeberle, 1986). Protein synthesis has been reported to be unnecessary for enhancement of bovine neutrophil ADCC by other researchers. Unpurified immune interferon enhanced neutrophil ADCC against chicken red blood cell (CRBC) and infectious bovine rhinotracheitis (IBR)-infected targets in the presence of protein synthesis inhibitors (Wardley, Babiuk, and Rouse, 1976). Monocyte factors also enhanced neutrophil ADCC against CRBC targets in the presence of inhibitors of protein synthesis (Frank and Roth, 1986).

The purpose of the research reported here was to investigate the activity of rboIFN-gamma on neutrophils from normal and dexamethasone-treated cattle and to determine the differential roles of RNA transcription, protein synthesis, and arachidonic acid metabolism in the rboIFN-gamma induced inhibition of random migration and enhancement of ADCC and AINC activities.

MATERIALS AND METHODS

Animals. Twenty healthy, one to two-year-old Holstein steers were used in this study. Dexamethasone (Azium, Schering Corp, Kenilworth, NJ) (0.04 mg/kg) was administered intramuscularly 24 hours before the blood was collected for neutrophil isolation. Control animals received no treatment.

Neutrophil preparation. Neutrophils were separated as previously described (Lukacs, Roth, and Kaeberle, 1985). Briefly, peripheral blood was collected from healthy cattle into acid-citrate-dextrose solution, centrifuged, and the plasma and buffy coat layer were discarded. The packed erythrocytes were lysed by brief exposure to hypotonic conditions and the remaining cells, which generally consisted of greater than 90% polymorphonuclear leukocytes (PMNS), were washed in 0.015 M phosphate buffered saline solution (PBS) (pH 7.2) and suspended in Medium 199, containing 25 mM HEPES buffer, to a concentration of 1.0×10^8 PMN/ml.

Treatment of PMNs with rboIFN-gamma. Three hundred microliters of a 1.0×10^8 /ml suspension of PMN were incubated with 300 μ l of rboIFN-gamma (5.0×10^{-8} gm/ml final concentration) (diluted in Medium 199 with HEPES) for 2.5 hours at 37°C. The PMNs were then used in the functional assays without washing. Control PMN preparations were incubated with Medium 199 with HEPES. The bovine rboIFN-gamma was supplied by Genentech Inc., South San Francisco, CA. (Lot number 2433/21,33,95) with a specific activity of $\geq 1.0 \times 10^6$ U/mg (MDBK-

VSV).

Evaluation of neutrophil function. The procedure for evaluation of random migration under agarose, ADCC and AINC have previously been described (Lukacs, Roth, and Kaeberle, 1985).

Inhibitor studies. Alpha-amanitin and puromycin or cycloheximide (Sigma Chemical Co., St. Louis, Mo.) were used to inhibit RNA and protein synthesis respectively during the incubation of PMNs in the presence or absence of rboIFN-gamma. PMNs were first preincubated with alpha-amanitin (50 ug/ml), puromycin (10 ug/ml) or cycloheximide for 30 min at 37°C; medium ± rboIFN-gamma containing the same concentration of inhibitor(s) was then added to the PMNs (without washing) and the incubation continued for 2.5 hours.

Incorporation of L-[³⁵S]methionine and 5,6-³H-uridine. Purified PMNs (1.0×10^7) were plated in 22 mm tissue culture dishes (12 well Costar 3512, Costar, Cambridge, MA) in 100 ul of RPMI 1640 medium (Select-amine kit 300-7402, Gibco, Santa Clara, CA) supplemented with 50 uM methionine in an atmosphere of 5% CO₂/95% air at 37°C either in the presence or absence of RNA or protein synthesis inhibitors. After 30 minutes the cultures received 300 ul of the same medium containing 150 uCi of L-[³⁵S]-methionine or 10 uCi of 5,6-³H-uridine with or without rboIFN-gamma and with or without inhibitor. The cells were resuspended by gentle pipetting 3.5 hours later, centrifuged to remove conditioned medium, washed twice by centrifugation in cold PBS containing 10 mM EDTA, and solubilized in 200 ul of lysis buffer (0.5% Nonidet 40, 20 mM HEPES, 1mM EDTA) plus 1mM phenylmethylsulphonyl

fluoride and 1mM aprotinin (100 U). Nuclei were pelleted by centrifugation (2500 x g, 15 min, 4°C, Jouan Cr.411 E4 rotor). The fractions (conditioned medium, nuclei, and postnuclear supernatants) were subjected to tri-chloroacetic acid (TCA) precipitation and part of each sample was prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-page).

Kinetic labeling and PMN fractionation. L-[³⁵S]-methionine labeling conditions were as described above. The cells were resuspended by gentle pipetting at either 60, 120, or 180 minutes later, centrifuged at 4°C to remove conditioned medium, washed twice by centrifugation in cold PBS containing 10 mM EDTA, and mechanically disrupted through a 20 gauge needle (20 strokes) in cold 10 mM PIPES buffer, pH 7.0 containing 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, 1 mM phenylmethylsulphonyl fluoride and 1 mM aprotinin (100 U). Nuclei were pelleted as described above, then granules and plasma membranes were pelleted sequentially from the remaining supernatant by centrifugation (20,000 x g, 15 minutes and 122,000 x g, 60 minutes, 4°C, Beckman airfuge-ultracentrifuge A-100/18 rotor). The final supernatant (cytosol) and the granule pellet were then subjected to SDS-page.

SDS-page and X-ray film detection of L-[³⁵S]-methionine-labeled proteins. SDS-page (linear gradient of 7.5 to 15% acrylamide) was performed according to a modified Laemmli procedure previously described in detail (Nilsen-Hamilton and Hamilton, 1979). Sample preparation was also described (Nilsen-Hamilton and Hamilton, 1979).

Detection of [^{35}S] in polyacrylamide gels by scintillation autoradiography (fluorography) using X-ray film was performed according to the procedure of Bonner and Laskey (1974).

TCA precipitation of radiolabeled molecules. An equal volume of cold 1.0 M Tris-HCl (pH 10.7) and sample were combined, the mixture was incubated for 15 min at 37°C and applied (2.5 μl) to the bottom of a (5 x 1 cm) polysilicic acid impregnated glass fiber strip (chromatography medium c4943-32, American Scientific Products, McGraw Park, IL). Separation by ascending thin layer chromatography for 10 min took place in a covered glass chamber containing 0.5 ml of solvent (30% v/v methanol, 20% w/v TCA, 10% v/v glacial acetic acid in water). Nonincorporated L-[^{35}S]-methionine migrated with the solvent front and was discarded by cutting the strip cross-wise (1.5 cm from the bottom). The TCA precipitated proteins remained at the application site (0.8 cm from the strip bottom), the strip was dried, and counted in a toluene-based scintillation fluid (Liquifluor, New England Nuclear, Boston, MA) in a liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill).

Arachidonic acid lipxygenase inhibitors. BW755c was a gift from Wellcome Research Laboratory (Kent, United Kingdom) and nordihydroguaiaretic acid (NDGA) was purchased from Sigma (Sigma Chemical Co., St. Louis, MO). Both compounds were initially dissolved in dimethyl sulfoxide (DMSO) immediately before use and diluted in 0.015 M phosphate buffered saline solution (pH 7.2) (PBS) to a final concentration of 1.0 mg/ml. These inhibitors were then added to the

PMNs after incubation with rboIFN-gamma (in M199) to give a final concentration of 50 ug/ml immediately before the cells were added to the functional assays. The DMSO was present at a 0.05% concentration; this concentration of DMSO in PBS was found to have no effect on neutrophil function.

Data analysis. A PMN preparation from each animal was aliquoted to receive all in vitro control and inhibitor treatments in both the presence and absence of rboIFN-gamma. Statistical significance was determined by using a paired comparison analysis. The value for a PMN preparation in the presence of rboIFN-gamma was compared to the value from an aliquot of that same PMN preparation similarly treated (+ inhibitor) in the absence of rboIFN-gamma. The individual PMN preparations were used as blocking factors in the analysis of variance in order to remove the animal to animal variability from statistical consideration of the treatment effect.

RESULTS

Neutrophils (PMNs) were isolated from both dexamethasone-treated and nontreated cattle (control) and half of each aliquot of cells was incubated (2.5 hr) in medium with or without rboIFN-gamma before being added to the assays. The PMNs from dexamethasone-treated cattle had greatly enhanced random migration under agarose as compared to the PMNs from untreated cattle (control) (Figure 1a). The pretreatment of PMNs from either control or dexamethasone-treated cattle with rboIFN-gamma inhibited neutrophil random migration under agarose. The rboIFN-gamma was able to reduce random migration by PMNs from dexamethasone-treated cattle to values similar to those of PMNs from control cattle.

Pretreatment of control PMNs with rboIFN-gamma also resulted in an increased percentage of ^{51}Cr release from antibody-coated chicken erythrocytes (CRBCs) in the antibody-dependent cell-mediated cytotoxicity (ADCC) assay (Figure 1b). Intramuscular injection of dexamethasone reduced the neutrophil ADCC, but this activity was restored to control values following in vitro rboIFN-gamma treatment. Similarly, the ^{51}Cr release from nonantibody-coated CRBCs (AINC) was increased after rboIFN-gamma pretreatment of control PMNs (Figure 1c). The PMNs from dexamethasone-treated cattle, however, had a complete lack of AINC activity (~1.0%) which was not increased by rboIFN-gamma pretreatment (Figure 1c).

The need for RNA and protein synthesis by neutrophils pretreated

with rboIFN-gamma for the inhibition of random migration and enhancement of AINC was investigated using alpha-amanitin (50 ug/ml), cycloheximide (5 ug/ml), and puromycin (10 ug/ml). At these concentrations the inhibitors did not affect PMN viability as determined by trypan blue exclusion (>95% viable). Inhibition of RNA and protein synthesis by alpha-amanitin and puromycin, respectively, blocked the inhibition of random migration by rboIFN-gamma (Figure 2a). Cycloheximide at a 5 ug/ml concentration partially blocked the inhibition of neutrophil random migration by rboIFN-gamma, a concentration of 10 ug/ml was required to block this activity completely (data not shown). These inhibitors were also able to block the enhancement of AINC activity by rboIFN-gamma (Figure 2c). Figure 2b indicates the ADCC enhancement by rboIFN-gamma was not affected by the presence of RNA or protein synthesis inhibitors.

The amount of L-[³⁵S]-methionine incorporation (3.5 hr) into new protein by PMNs was determined by TCA protein precipitation. The incorporation of radiolabel was inhibited by 90% in both rboIFN-gamma treated and nontreated control PMN fractions that received either cycloheximide (5 ug/ml) or puromycin (10 ug/ml) (Table 1). The specific incorporation of L-[³⁵S]-methionine into protein was verified by adding radiolabeled methionine, stopping the reaction immediately (zero-minute incubation), and determining the TCA precipitable cpms. Table 1 also shows that addition of alpha-amanitin reduced the incorporation of 5,6-³H-uridine into TCA-precipitable RNA by approximately 95%.

Figure 1. Effects of in vitro incubation with rboIFN-gamma on random migration and cytotoxicity of PMNs from dexamethasone-treated and nontreated cattle (control). PMNs were purified from control and dexamethasone-treated cattle, then incubated in Medium 199 for 2.5 hours in the presence or absence of 5×10^{-8} gm/ml rboIFN-gamma before being added to the functional assays. Results are the mean \pm SEM (n=6). ^a P < 0.01; ^b P < 0.05; the level of statistical significance for the difference between the in vitro rboIFN-gamma treatment value and the value for the same neutrophils in the absence of rboIFN-gamma. Separate analyses on dexamethasone-treated and control cattle were performed

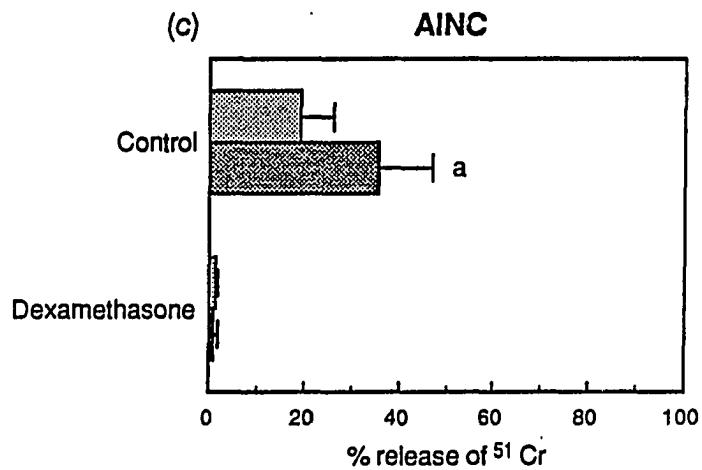
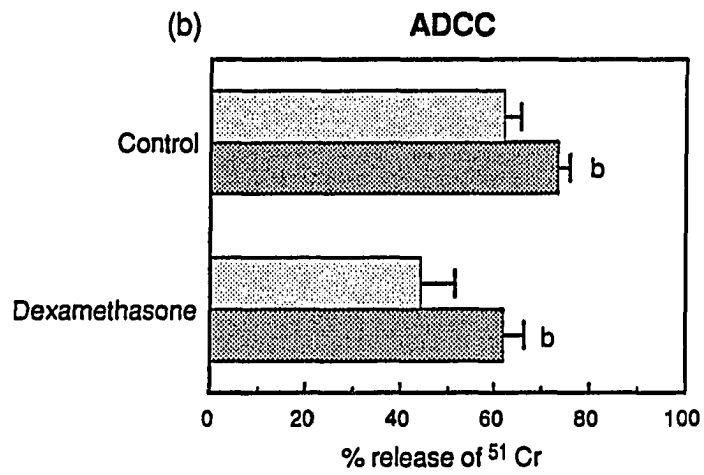
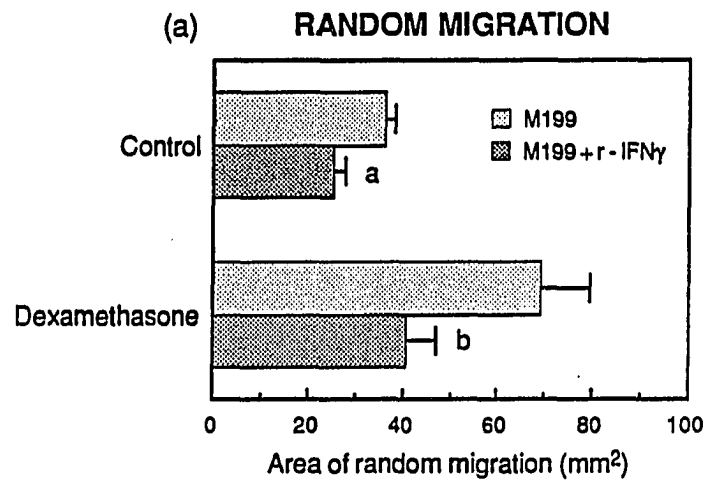


Figure 2. Effects of inhibitors of RNA and protein synthesis on the inhibition of neutrophil random migration and the enhancement of neutrophil cytotoxicity by rboIFN-gamma. PMNs were preincubated with 25 ug/ml alpha-amanitin, 10 ug/ml puromycin, or 5 ug/ml cycloheximide for 30 minutes at 37°C, and then for 2.5 hours in the presence or absence of 5×10^{-8} gm/ml rboIFN-gamma + inhibitor before being added to the functional assays. Results are the mean + SEM (n=6). ^a P < 0.01; ^b P < 0.05; the level of statistical significance for the difference between paired bars

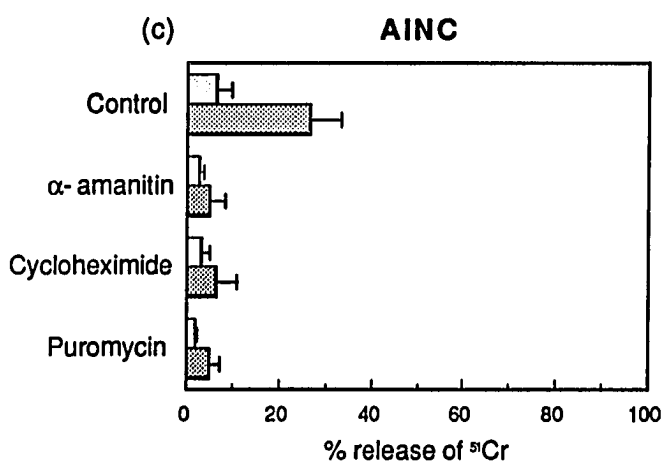
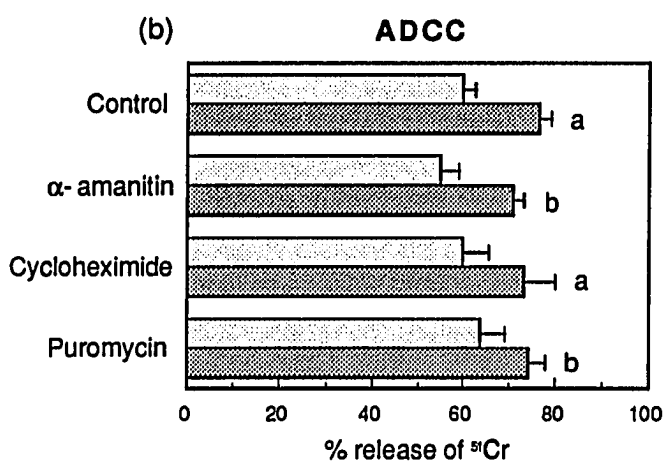
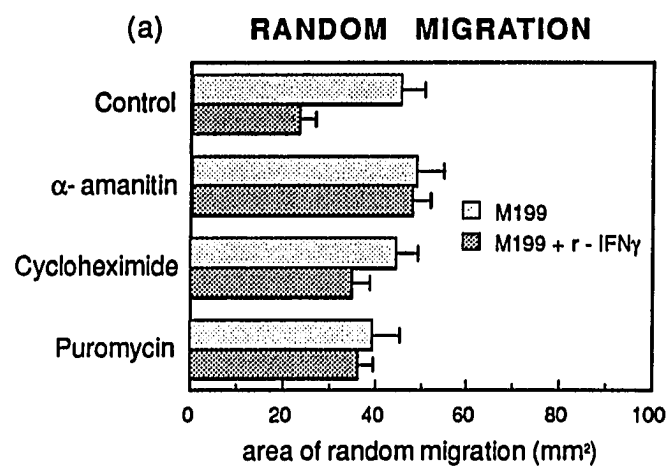


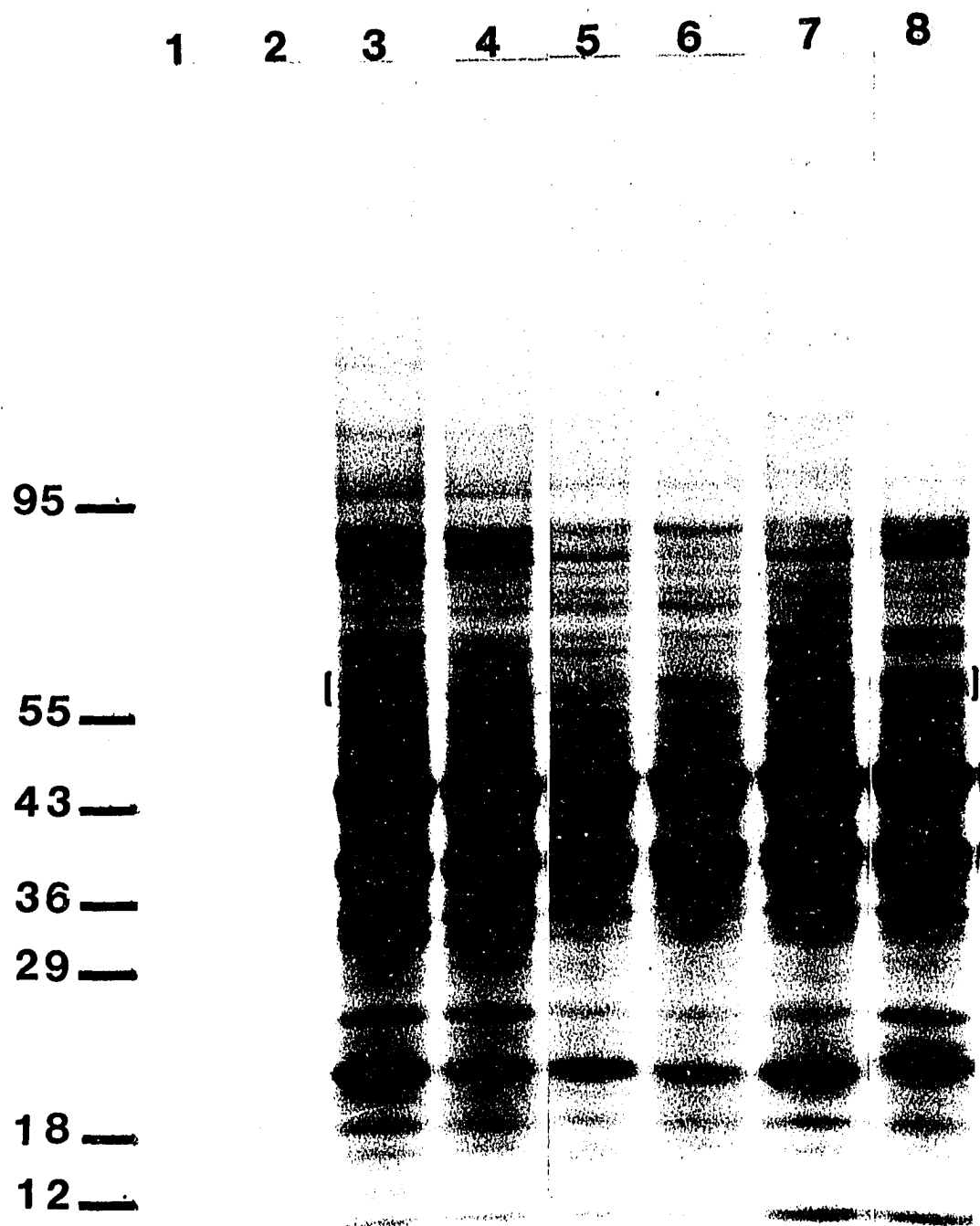
Table 1. Effect of inhibitors of RNA and protein synthesis on [5,6-³H]-uridine or L-[³⁵S]-methionine incorporation into acid-precipitable RNA or protein

	L-[³⁵ S]methionine (cpm X 10 ⁵)		[5,6- ³ H]uridine (cpm X 10 ⁴)	
	PMN + RPMI	PMN + IFN	PMN + RPMI	PMN + IFN
Pretreatment of PMNs ^a				
RPMI 1640	4.80	3.90	2.40	3.10
zero-labeling time	0.51			
Cycloheximide (5 ug/ml)	0.49	0.43		
Puromycin (10 ug/ml)	0.43	0.48		
alpha-amanitin (25 ug/ml)	1.50	1.50	0.05	0.16

^aPMNs (1.0 X 10⁷ PMN) were pretreated by incubation in RPMI 1640 medium + 5 ug/ml cycloheximide, 10 ug/ml puromycin, or 25 ug/ml of alpha-amanitin for 30 minutes at 37°C, and then for 3.5 hours in the presence or absence of rboIFN-gamma (5 X 10⁻⁸ gm/ml + inhibitors) containing a final concentration of 50 uCi of L-[³⁵S]methionine or 7.5 uCi [5,6-³H]uridine before being assayed. The values presented are the average of 3 animals. There was no significant difference between the values in the presence or absence of rboIFN-gamma.

PMNs from dexamethasone-treated and nontreated cattle were incubated in the presence of L-[³⁵S]-methionine for 3.5 hours with and without added rboIFN-gamma. No protein bands were detectable on fluorograms in either the presence of puromycin or after a zero-minute incubation time with L-[³⁵S]-methionine (Figure 3). Equal numbers of PMNs were radiolabeled, solubilized in NP40 lysis buffer, and cell fractions were separated by centrifugation. The supernatants remaining after the nuclear material was centrifuged out were separated on SDS-polyacrylamide linear gradient (7.5-15.0%) gel electrophoresis in the presence of 2.0% 2-mercaptoethanol. Approximately equal TCA-precipitable cpm's were loaded onto the gel for both rboIFN-gamma treated and nontreated PMNs and compared. Only one major quantitative protein change was apparent in the postnuclear fraction after rboIFN-gamma treatment (typical data for 6 different animals analyzed on separate days). The PMNs labeled in the absence of rboIFN-gamma had a protein triplet with apparent molecular weights 59, 60, and 62 kilodaltons. Whereas, only the protein band with an apparent molecular weight of 60 kilodaltons was increased in the presence of rboIFN-gamma (Figure 3). PMNs from both dexamethasone-treated and nondexamethasone-treated (control) cattle showed the same triplet in the absence of rboIFN-gamma and the 60 kilodalton protein induction in the presence of rboIFN-gamma.

Figure 3. Representative fluorogram (of 6 replicates) of PMN L-[³⁵S]-methionine-labeled proteins subjected to SDS-polyacrylamide, linear-gradient (7.5-15%) electrophoresis. PMNs (1.0×10^7) from dexamethasone-treated and nontreated cattle were pretreated by incubation in RPMI 1640 medium + 10 ug/ml puromycin or 25 ug/ml of alpha-amanitin for 30 minutes at 37°C, and then for 3.5 hours in the presence or absence of rboIFN-gamma (5×10^{-8} gm/ml + inhibitors) containing a final concentration of 50 uM cold methionine and 50 uCi of L-[³⁵S]-methionine before being prepared for SDS-PAGE. The lanes contain NP40-solubilized postnuclear fractions from: Lane 1, non-treated cattle (control) PMNs in the presence of puromycin; lane 2, control PMNs subjected to a zero-minute labeling incubation time; lane 3, control PMNs; lane 4, control PMNs treated with rboIFN-gamma; lane 5, control PMNs in the presence of alpha-amanitin; lane 6, control PMNs treated with rboIFN-gamma and alpha-amanitin; lane 7, PMNs from dexamethasone-treated cattle; and lane 8, PMNs from dexamethasone-treated cattle treated with rboIFN-gamma in vitro



Alpha-amanitin decreased the amount of L-[³⁵S]-methionine incorporation into PMN proteins (Table 1). In addition, the protein labeling was less intense on the fluorogram in both control and rboIFN-gamma treated PMNs (Figure 3). There was no increase in the 60 kd protein with rboIFN-gamma treatment in the presence of alpha-amanitin.

The control cattle PMNs were then incubated in the presence of L-[³⁵S]-methionine with and without added rboIFN-gamma for various lengths of time. After incubation for 60, 120, or 180 minutes, the PMNs were fractionated so that changes in cytosolic proteins could be evaluated. Figure 4 represents typical kinetic data (6 different animals analyzed on separate days) the kinetics of the rboIFN-gamma induced increase in a cytosolic 60 kd protein. No increase in this protein was detected at 60 minutes, by 120 minutes the increase was apparent and the increase in expression continued through 180 minutes of incubation with rboIFN-gamma.

Figure 5 represents the kinetics of the rboIFN-gamma induced increase in expression of the 60 kd protein in the granule/granule-associated fraction from the above mentioned cells. The increase in the 60 kd protein occurred between 120 and 180 minutes of incubation with rboIFN-gamma. Recombinant IFN-gamma also induced the expression of a protein in the granule/granule-associated fraction with an apparent molecular weight of 93-94 kd. The induction of this protein occurred between 60 and 120 minutes of incubation with rboIFN-gamma. The 93-94 kd protein was also visible in the cytosolic fraction

(Figure 4) after a similar incubation period. Both the 60 and 93-94 kd proteins were present at a greater percentage of the total protein in the granule/granule-associated fraction (Figure 5) than they were in the cytosolic fraction (Figure 4).

The addition of inhibitors of arachidonic acid lipxygenase metabolism (NDGA and BW755C) after rboIFN-gamma pretreatment or to the control PMNs had no statistically significant effect on random migration (Figure 6a) or ADCC activities (Figure 6b). However, these inhibitors decreased the background AINC activity in the absence of rboIFN-gamma and markedly decreased the AINC activity in the presence of rboIFN-gamma (Figure 6c). The NDGA was more effective than the BW755c at the same concentrations.

Figure 4. Representative fluorogram (of 6 replicates) of radiolabeled neutrophil cytosolic proteins loaded onto an SDS-polyacrylamide, linear-gradient gel (7.5-15%). PMNs (1.0×10^7) from nontreated cattle were incubated in RPMI 1640 medium containing 50 μ M cold methionine and 50 μ Ci of L- 35 S-methionine for 60, 120, or 180 minutes at 37°C in the presence or absence of rboIFN- γ (5×10^{-8} gm/ml). The cells were fractionated and approximately equal acid-precipitable cpm's of cytosolic protein were loaded onto the gel. The lanes contain the labeled cytosolic fractions from PMNs labeled for 60 minutes in the absence or presence of rboIFN- γ (lanes 1 and 2), PMNs labeled for 120 minutes in absence or presence of rboIFN- γ (lanes 3 and 4), and PMNs labeled for 180 minutes in the absence or presence of rboIFN- γ

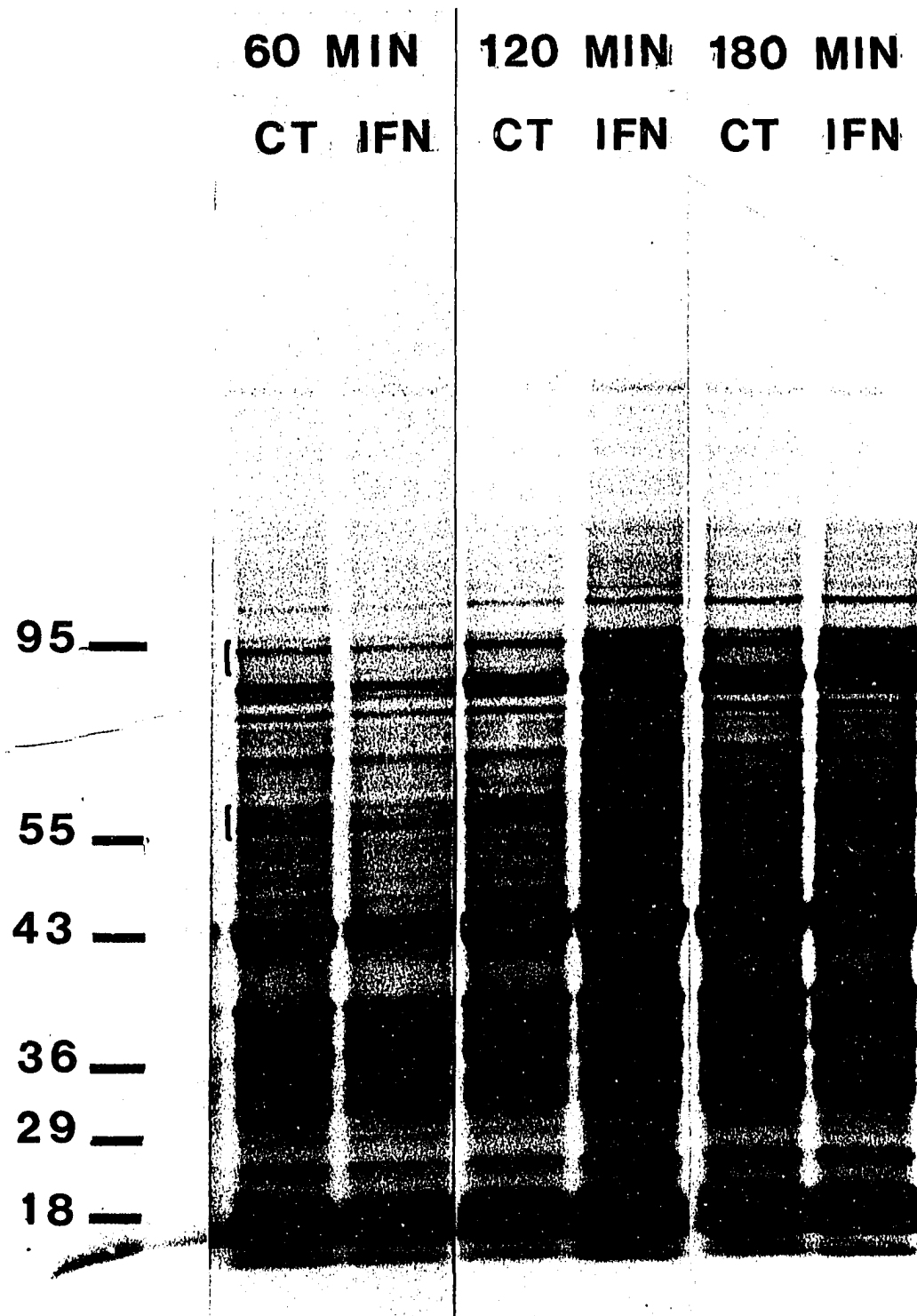


Figure 5. Representative fluorogram (of 6 replicates) of radiolabeled neutrophil granule/granule-associated proteins loaded onto an SDS-polyacrylamide, linear-gradient gel (7.5-15%). PMNs from nontreated (control) cattle were incubated in RPMI 1640 medium containing 50 uM cold methionine and 50 uCi of L-[³⁵S]-methionine for 60, 120, or 180 minutes at 37 °C in the presence or absence of rboIFN-gamma (5 X 10⁻⁸ gm/ml). The cells were fractionated and approximately equal TCA-precipitable cpm's were loaded onto the gel. The lanes contain the labeled granule/granule-associated fractions from PMNs labeled for 60 minutes in the absence or presence of rboIFN-gamma (lanes 1 and 2), PMNs labeled for 120 minutes in absence or presence of rboIFN-gamma (lanes 3 and 4), and PMNs labeled for 180 minutes in the absence or presence of rboIFN-gamma

60 MIN		120 MIN		180 MIN	
CT	IFN	CT	IFN	CT	IFN

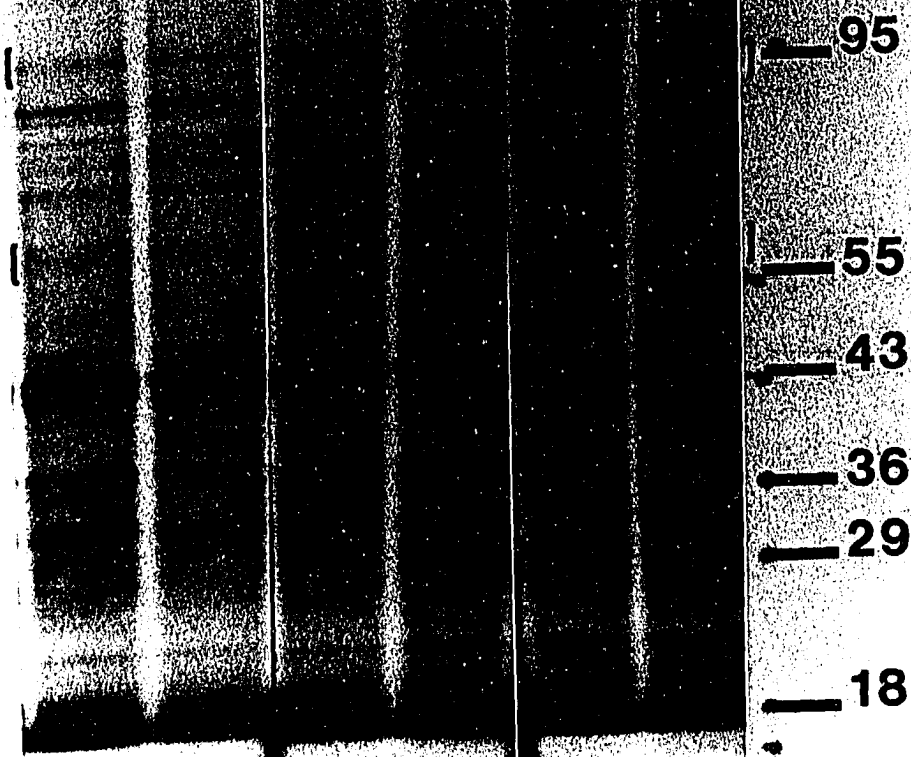
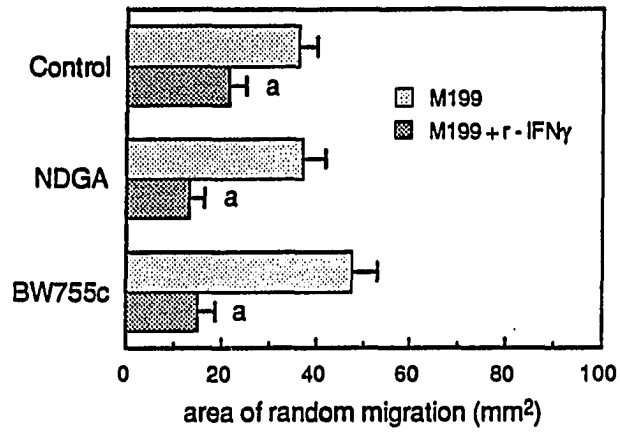
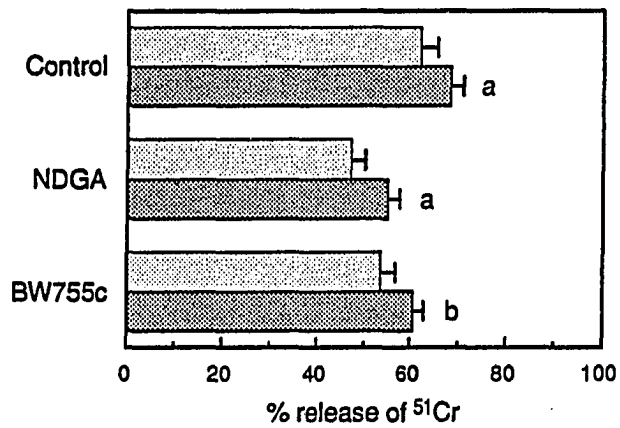


Figure 6. Effects of inhibitors of arachidonic acid metabolism on the inhibition of neutrophil random migration and enhancement of cytotoxicity. PMNs were incubated 2.5 hours in the presence or absence of 5×10^{-8} gm/ml of rboIFN-gamma, and just before being assayed the cells were incubated with either 50 ug/ml BW755c, 25 ug/ml NDGA, or M199 medium without inhibitors. Results are the mean + SEM (n=6).
^a P < 0.01; ^b P < 0.05; the level of statistical significance for the difference between paired bars

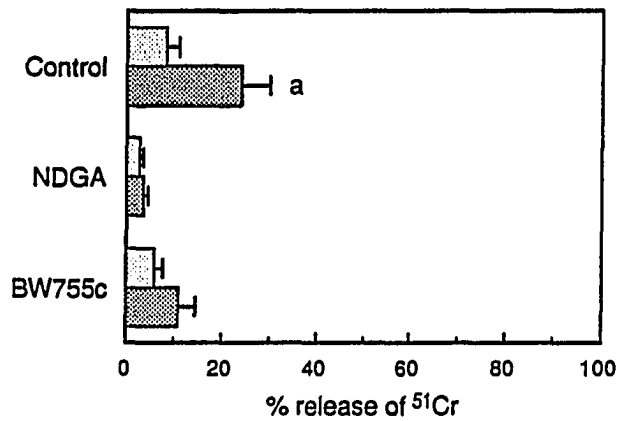
(a) **RANDOM MIGRATION**



(b) **ADCC**



(c) **AINC**



DISCUSSION

Preincubation with rboIFN-gamma in vitro (2.5 hr) reversed the in vivo dexamethasone effects on random migration and ADCC (Figures 1a, 1b) to approximately control values. Previous reports indicate that corticosteroids inhibit lymphokine production (e.g., gamma interferon) (Cesario et al., 1986). These results suggest that rboIFN-gamma might be useful as an immune enhancing agent in glucocorticoid-induced immunosuppression. However, there was no enhancement of AINC after rboIFN-gamma pretreatment of PMNs from dexamethasone-treated cattle (Figure 1c). This result was in direct contrast to the enhancement of the AINC activity of control PMNs pretreated with rboIFN-gamma (Figure 1c). In fact, the dexamethasone treatment reduced the AINC to baseline level and rboIFN-gamma was unable to raise these values. The difference in the rboIFN-gamma activation of PMNs from these two sources provided a means of determining the molecular events required for the enhancement of AINC; events not required for the enhancement of the ADCC activity.

We have previously reported the need for RNA and protein synthesis for the inhibition of neutrophil random migration by rboIFN-gamma (Steinbeck, Roth, and Kaeberle, 1986). In addition, our laboratory has reported that inhibition of RNA and protein synthesis with actinomycin D and puromycin decreased a lymphokine induced enhancement of AINC but had no effect on ADCC (Lukacs, Roth, and Kaeberle, 1985). The use of RNA and protein synthesis inhibitors demonstrated the need

for these molecular events in the rboIFN-gamma enhancement of AINC (Figure 2c) but not ADCC (Figure 2b) activity. Protein synthesis could be necessary for induction and expression of adherence molecules (involved in target cell binding) or for a protein(s) that plays a role in the enhancement of the AINC activity (cytotoxic factor).

The rboIFN-gamma treated PMNs had an increase in a 60 kd cytosolic or granule/granule-associated protein. The importance or function of this protein is unknown, but represents a consistent change after rboIFN-gamma treatment. In the presence of alpha-amanitin there was no increase in the 60 kd protein made by rboIFN-gamma treated PMNs (Figure 3). This suggests that RNA synthesis must take place prior to a detectable increase in this protein. The kinetics indicate that the expression of this intracellular protein was not increased until after 60 minutes of incubation with rboIFN-gamma and before 120 minutes. This time period was consistent with the time required for AINC enhancement (Lukacs, Roth, and Kaeberle, 1985). However, the presence of this protein alone was not enough to enhance the AINC activity of PMNs from dexamethasone-treated cattle.

Inhibitors of the formation of arachidonic acid lipooxygenase products (NDGA and BW755c) also inhibited the enhancement of AINC by rboIFN-gamma (Figure 5c). These inhibitors were added after the rboIFN-gamma pretreatment to allow the essential protein synthesis to take place. Dexamethasone is known to induce the synthesis of lipomodulin in PMNs from several different animal species (Hirata et al., 1980; Blackwell et al., 1983) which then acts to inhibit

phospholipase A₂ and ultimately the release of arachidonic acid from membrane phospholipids. The released arachidonic acid is normally converted in nontreated bovine neutrophils via the lipoygenase pathway into two major metabolic products, leukotriene B₄ and 5-HETE (Webb and Roth, 1987). Both leukotriene B₄ and 5-HETE can act as chemotaxins for bovine neutrophils (Palmer et al., 1980). Leukotriene B₄ can also induce increased Ca²⁺ mobilization and degranulation (Naccache et al., 1984; Palmer and Salmon, 1983). In addition, 5-HETE can be reincorporated into membrane phospholipids and cause membrane fluidity changes (Bonser et al., 1981; Stenson and Parker, 1979). Leukotriene B₄ and 5-HETE are essential for an optimal iodination and oxidative response by bovine neutrophils to opsonized-zymosan stimulation (Webb and Roth, 1987).

It has been reported by several research groups, that oxidative metabolism, extracellular degranulation, and Ca²⁺ play a major role in antibody-dependent and -independent neutrophil-mediated cytotoxicity. Oxidative metabolism was necessary for ADCC activity against tumor cells (Clark and Klebanoff, 1977; Hafeman and Lucas, 1979) and human RBCs (Borregaard and Kragballe, 1982) but oxidative metabolism was not required for killing of antibody-coated, virus-infected cells (Siebens, Tevethia, and Babior, 1979), CRBC (Cordier and Revillard, 1980), and lymphocytes (Katz et al., 1980). Cationic proteins from bovine neutrophil granules have been implicated in the ADCC activity against IBR-infected target cells (Thorne et al., 1984). Antibody-independent killing by oxidative mechanisms has been reported in

lectin-induced neutrophil lysis of CRBC (Simchowicz and Spilberg, 1979) and PMA-induced neutrophil lysis of tumor cells (Clark and Szot, 1981) and human erythrocytes (Weiss, 1980). In addition, a membrane-bound neutral proteinase (300 kd) released from PMA-activated human neutrophils was found to work in conjunction with oxidative metabolites to lyse ox erythrocytes (Pontremoli et al., 1986). Dallegri et al. (1984) reasoned that the target cell lysis depended on both intact oxidative and nonoxidative mechanisms, but could be influenced by the ability of the target to overcome the effects of the oxidative products. The arachidonic acid lipoxxygenase products are essential for optimal production of both oxidative and nonoxidative (degranulation) factors which are effectors of the cytolytic activity (primarily AINC). The arachidonic acid products do not seem to play a role in either random migration enhancement by dexamethasone or inhibition by rboIFN-gamma (Figure 5a).

In addition to the above mentioned cytolytic factors, surface adherence proteins are also required for normal ADCC activity (Kohl et al., 1984; Lopez et al., 1985). The adherence of PMNs is mediated by a family of surface glycoproteins, LFA-1, iC3b/Mol/OKM-1/Mac-1, and P150,95. These proteins have a common 95 kd beta subunit and variable alpha subunits. GFA-2 is another glycoprotein belonging to this family that is expressed only on mature PMNs, and consists of a single 95 kd subunit (Lopez et al., 1985). Binding of a monoclonal antibody directed against GFA-2 results in degranulation of cytochalasin B-pretreated PMNs. Gamma interferon is known to augment human PMN

adherence (Seow and Thong, 1986), presumably by effecting the expression of these surface glycoproteins. It is possible that the 93-94 kd protein induced by rboIFN-gamma (Figures 4, 5) is an adherence glycoprotein involved in both migration inhibition (inversely related functions) and AINC PMN-target cell binding.

In summary, the metabolic events, associated with neutrophil activation by rboIFN-gamma, are different for the three functions measured by these assays. Migration inhibition required both RNA and protein synthesis, but did not require arachidonic acid metabolism. Enhancement of ADCC did not require mRNA transcription, protein synthesis, or arachidonic acid metabolism. On the other hand, AINC enhancement required mRNA transcription, protein synthesis, and arachidonic acid metabolism. Clearly, neutrophils are metabolically active cells which can be stimulated to even greater functional activity by rboIFN-gamma. These data help to explain the mechanisms by which rboIFN-gamma enhances nonspecific resistance to infectious disease.

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SECTION IV:

HOMOLOGOUS GAMMA INTERFERON ENHANCES ANTIBODY-
INDEPENDENT CYTOTOXICITY FOR BOVINE BUT NOT
HUMAN NEUTROPHILS

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ABSTRACT

Recombinant bovine gamma interferon (rboIFN-gamma) at 10 or 50 units/ 5.0×10^7 neutrophils enhanced bovine neutrophil cytotoxicity against ^{51}Cr -labeled chicken erythrocytes (CRBCs) in the presence and absence of anti-CRBC antiserum. Both concentrations of rboIFN-gamma also inhibited bovine neutrophil random migration under agarose. Human gamma interferon (huIFN-gamma) at 10 units/ 5.0×10^7 cells, inhibited human neutrophil random migration and enhanced neutrophil-mediated lysis of CRBCs, but only in the presence of anti-CRBC antiserum. Human IFN-gamma, at 50, 500, or 1000 units, did not affect bovine neutrophil cytotoxic activity or random migration. Interestingly, the enhancement of bovine antibody-independent neutrophil cytotoxicity (AINC) by rboIFN-gamma, but not the enhancement of antibody-dependent cell-mediated cytotoxicity (ADCC), was influenced by the medium chosen for pretreatment and assay. Bovine neutrophils were preincubated for 2 hours and then assayed in either M199, MEM, or RPMI 1640 with or without 10 units of rboIFN-gamma. The mean increase in AINC due to rboIFN-gamma treatment was significantly greater in M199 than in MEM, in RPMI 1640 there was little or no increase in this activity. Addition of three components unique to M199 components (ATP, ascorbic acid, and tween 80), which are known to affect other neutrophil functions, to both MEM or RPMI 1640 did not significantly affect the enhancement of AINC. In conclusion: 1) human neutrophils are not induced by human gamma interferon to have AINC activity under the same conditions in which rboIFN-gamma enhances bovine neutrophil AINC, 2)

huIFN-gamma has no effect on bovine neutrophil random migration or cytotoxicity, and 3) medium components are important for the maximal enhancement of bovine neutrophil AINC activity by rboIFN-gamma.

INTRODUCTION

We have previously reported the enhancement of bovine neutrophil cytotoxicity and the impairment of random migration after 2 hours of rboIFN-gamma pretreatment (Steinbeck, Roth, and Kaeberle, 1986). The ability to activate bovine neutrophils to lyse chicken erythrocyte (CRBC) targets in the absence of anti-CRBC antiserum (AINC) has been of particular interest (Lukacs, Roth, and Kaeberle, 1985; Steinbeck, Roth, and Kaeberle, 1986). Separate molecular events are involved in the activation of neutrophils for lysis of cell targets in the presence and absence of target specific antiserum (Steinbeck and Roth, 1987). Peripheral blood (resting) neutrophils have the ability to lyse antibody-coated CRBCs, although to a lesser extent than activated neutrophils. However, without being activated, neutrophils are generally not able to lyse chicken erythrocyte (CRBC) targets to a significant extent in the absence of anti-CRBC antiserum. Richter et al. (1981) have reported naturally occurring cytotoxic activity of human neutrophils against rabbit, sheep, and ox erythrocyte targets in gamma-globulin depleted serum. This cytotoxic activity of nonactivated human neutrophils was directed against erythrocyte targets from several species, and it was suggested that factors other than immunoglobulins were present in the serum that opsonized these targets. The human neutrophils in the study by Richter et al. (1981) did not lyse chicken erythrocyte targets in the presence of gamma-globulin depleted serum. The ability of activated human neutrophils to lyse chicken erythrocytes was not investigated.

Shalaby et al. (1985) have looked at the ability of gamma interferon-activated human neutrophils to lyse antibody-coated CRBC targets in RPMI 1640, and similar to our present findings, have not reported lysis in the absence of antibody in this medium. Richter et al. (1981) assayed for, cytolytic activity of nonactivated human neutrophils in M199, which is the medium used in the cytotoxic assays performed on bovine neutrophils. We had never assayed for the bovine cytotoxic activity in RPMI 1640 medium or MEM, which are media generally used in human neutrophil cytotoxic assays. Therefore, the difference in the ability of gamma interferon-activated neutrophils from the two species to lyse the same target cell under various assay conditions was investigated. This was of particular interest, because M199 has several components that are not present in RPMI 1640 or MEM. In addition, RPMI 1640 has higher Ca^{++} , Mg^{++} , and glucose concentrations than the other two media. Some of the components present in M199 were added to MEM and RPMI 1640 to determine possible effects on bovine neutrophil cytotoxicity. Assaying for random migration under agarose was used as a measure of neutrophil activation by gamma interferon (neutrophil migration inhibition factor) and neutrophil viability (Glasser, Fiederlein, and Huestis, 1985). Finally, the ability of human gamma interferon to activate bovine neutrophils was evaluated under optimal conditions (M199).

MATERIALS AND METHODS

Donors. Bovine neutrophils were obtained from twenty healthy, one to two-year-old Holstein steers. Human neutrophils were obtained from healthy human volunteers.

Neutrophil preparation. Bovine neutrophils were separated as previously described (Lukacs, Roth, and Kaeberle, 1985). Briefly, peripheral blood was collected from healthy cattle into acid-citrate-dextrose solution, centrifuged, and the plasma and buffy coat layer were discarded. The packed erythrocytes were lysed by brief exposure to hypotonic conditions and the remaining cells, which generally consisted of greater than 95% polymorphonuclear leukocytes (PMNS), were washed in 0.015 M phosphate buffered saline solution (PBS) (pH 7.2) and resuspended in Medium 199, MEM, or RPMI 1640 to a concentration of 1.0×10^8 PMN/ml. Human peripheral blood was collected into acid-citrate-dextrose solution, centrifuged at 1000g x 20 minutes, and the plasma and upper layer of buffy coat were discarded. The packed erythrocytes were lysed (2x) in a 2-fold volume of ice-cold NH_4Cl solution (155 mM NH_4Cl , 10 mM KHCO_3 , and 0.1 mM EDTA, pH 7.4 at 0°C) as previously described (Roos and de Boer, 1986). The remaining cells, which consisted of greater than 90% neutrophils, were washed in 0.015 M phosphate buffered saline solution (pH 7.2) containing 1.0% albumin, and resuspended in Medium 199, MEM, or RPMI 1640 (Grand Island Biologics Co., Grand Island, NY) containing 0.5% albumin to a concentration of 1.0×10^8 PMN/ml.

Treatment of neutrophils with gamma interferon. Three hundred

microliters of a 1.0×10^8 /ml suspension of PMNs were incubated with 300 μ l of human gamma interferon. To each medium the gamma interferons were added to the final concentration of 10 or 50 units of bovine interferon/ 5.0×10^7 PMNs and 10, 50, 500, or 1000 units of human interferon/ 5.0×10^7 PMNs. The PMNs were incubated in the various interferon containing media for 2 hours at 37°C in a humidified atmosphere of 5% CO_2 . Noninterferon-treated PMNs were incubated under the same environmental and media conditions as the interferon-treated neutrophils. The PMNs were then used in the functional assays without washing. The rboIFN-gamma was supplied by Ciba-Geigy, Basel, Switzerland (Lot number 3229/38) with a specific activity of $\geq 1.2 \times 10^6$ U/mg (MDBK-VSV). Human interferon was obtained from Interferon Sciences, Inc., New Brunswick, New Jersey with a specific activity of 0.2×10^6 U/mg (HEp-2-VSV). Other media components added to both MEM and RPMI 1640 included ATP (1.0 mg/l), ascorbic acid (0.05 mg/l), and tween 80 (20 mg/l) (Sigma Chemical Co., St. Louis, MO).

Neutrophil random migration. Evaluation of random migration under agarose was performed as previously described (Roth and Kaeberle, 1981). Agar consisted of bicarbonate-buffered Medium 199 with Earle's salts containing 0.8% agarose, 10% fetal bovine serum, and 1% penicillin (Grand Island Biological Co., Grand Island, NY) in 60- x 15-mm tissue culture grade Petri plates (No. 1007 Falcon, Oxnard, CA). Cells were placed into wells cut into the agar, and the Petri plates were placed into an incubator with a humidified 5% CO_2 atmosphere. Eighteen hours later the cells were fixed with 8% glutaraldehyde for 30 minutes, the

agar removed, and the cells adherent to the plastic were stained with modified Wright's stain. The area of migration was determined and expressed in square millimeters.

Cytotoxicity assay. The procedures for evaluation of antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-independent neutrophil-mediated cytotoxicity (AINC) have previously been described (Lukacs, Roth, and Kaeberle, 1985). Briefly, the ADCC and AINC assays were performed in standard microtiter plates utilizing ^{51}Cr -labeled chicken red blood cells (CRBC) as target cells. The standard reaction mixtures contained 1.25×10^5 CRBC and 1.25×10^6 granulocytes (effector-to-target ratio of 10:1) in 150 μl of medium with or without 2.5% bovine anti-CRBC serum. Human PMNs were capable of binding to and lysing bovine antibody coated CRBCs, so rabbit anti-CRBC antiserum was not used. The AINC assay is performed in the absence of added serum or albumin. The medium used in the pretreatment step was also the medium used in the cytotoxicity assays. Triton-X controls, antibody controls, and granulocytes controls were included. After 2 hours of incubation at 37°C in a humidified 5% CO_2 atmosphere, the supernatant was collected for gamma counting by using the Skatron Supernatant Collection System (Skatron, Inc., Sterling, VA). The results were expressed as the percentage of specific ^{51}Cr release.

Data analysis. A PMN preparation from each donor was aliquoted to receive all in vitro treatments in both the presence and absence of gamma-interferon. Statistical significance was determined by using a paired comparison analysis. The value for a PMN preparation in the

presence of IFN-gamma was compared to the value from an aliquot of that same PMN preparation in the absence of IFN-gamma. The individual PMN preparations were used as blocking factors in the analysis of variance in order to remove the animal to animal variability from statistical consideration of the treatment effect.

RESULTS

Preincubation of bovine neutrophils for 2 hours with recombinant bovine gamma interferon (rboIFN-gamma), but not human gamma interferon, (huIFN-gamma) enhanced neutrophil cytotoxicity in vitro. The enhanced cytotoxic activity against ^{51}Cr -labeled chicken erythrocytes (CRBCs) took place in both the presence and absence of anti-CRBC antiserum. This increase is represented in Figure 1 as the difference in the percent of ^{51}Cr released from CRBCs by neutrophils after IFN-gamma pretreatment (activated) as compared to nontreated neutrophils from the same animal. The enhancement of antibody-dependent (ADCC) and -independent (AINC) neutrophil-mediated cytotoxicity was induced by 10 or 50 units (per 5.0×10^7 neutrophils) of rboIFN-gamma (Figure 1). Either dose of rboIFN-gamma enhanced AINC or ADCC to approximately the same value. Under similar conditions, 50, 500, or 1000 units of huIFN-gamma failed to affect the cytotoxic activity of bovine neutrophils (Figure 1).

Random migration of bovine neutrophils under agarose was inhibited after a 2 hour pretreatment with 10 or 50 units of rboIFN-gamma (Figure 2). Although there was a tendency for random migration to be decreased by the higher concentrations of huIFN-gamma the differences were not statistically significant ($P > 0.05$) (Figure 2).

Figure 1. Influence of in vitro incubation with bovine and human gamma interferon on bovine neutrophil cytotoxicity in Medium 199. PMNs were purified and then incubated in Medium 199 for 2 hrs in the presence or absence of 10 or 50 units/ 5.0×10^7 PMNs of rboIFN-gamma or 50, 500, or 1000 or units/ 5.0×10^7 PMNs before being added to the AINC and ADCC assays. Results are the mean differences (+ SEM) in the percent of ^{51}Cr release between interferon treated and nontreated PMNs isolated from the same animal (n=7). ^a $P < 0.01$; ^b $P < 0.05$; the level of statistical significance for the difference between the interferon-treated and nontreated values

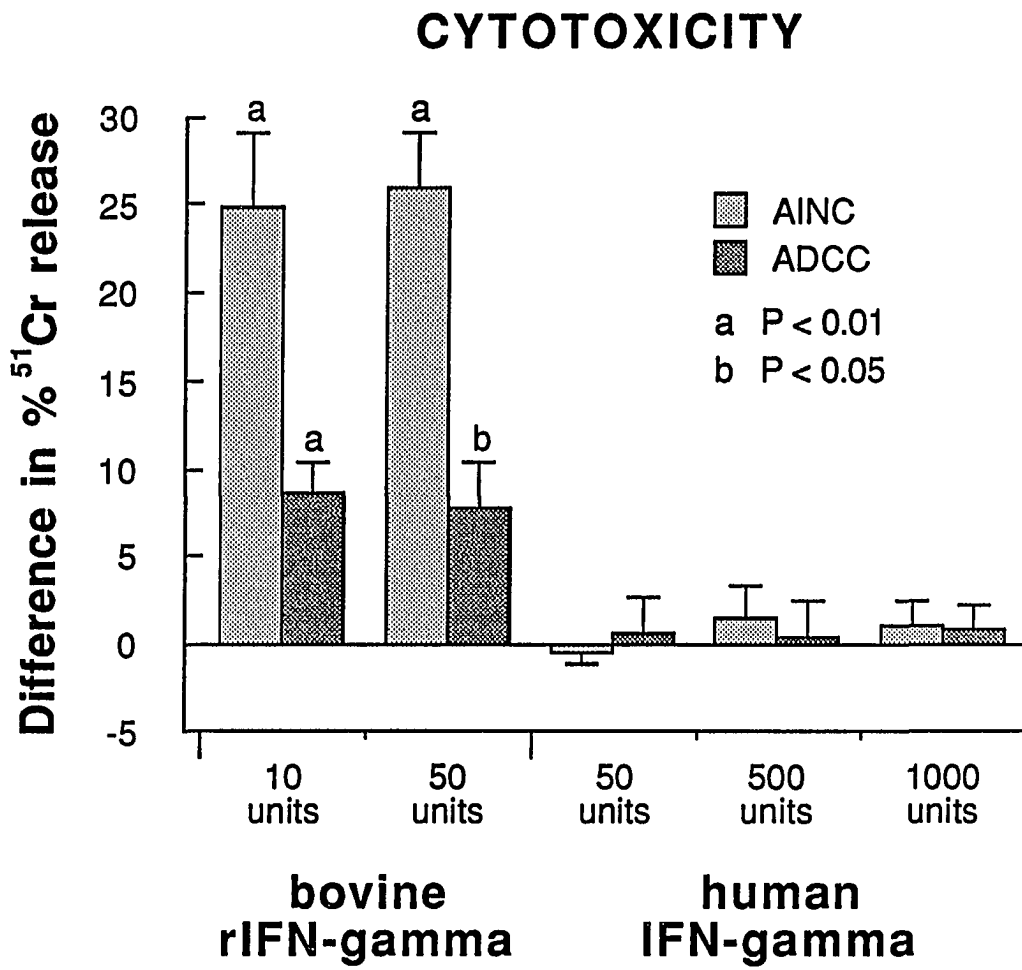
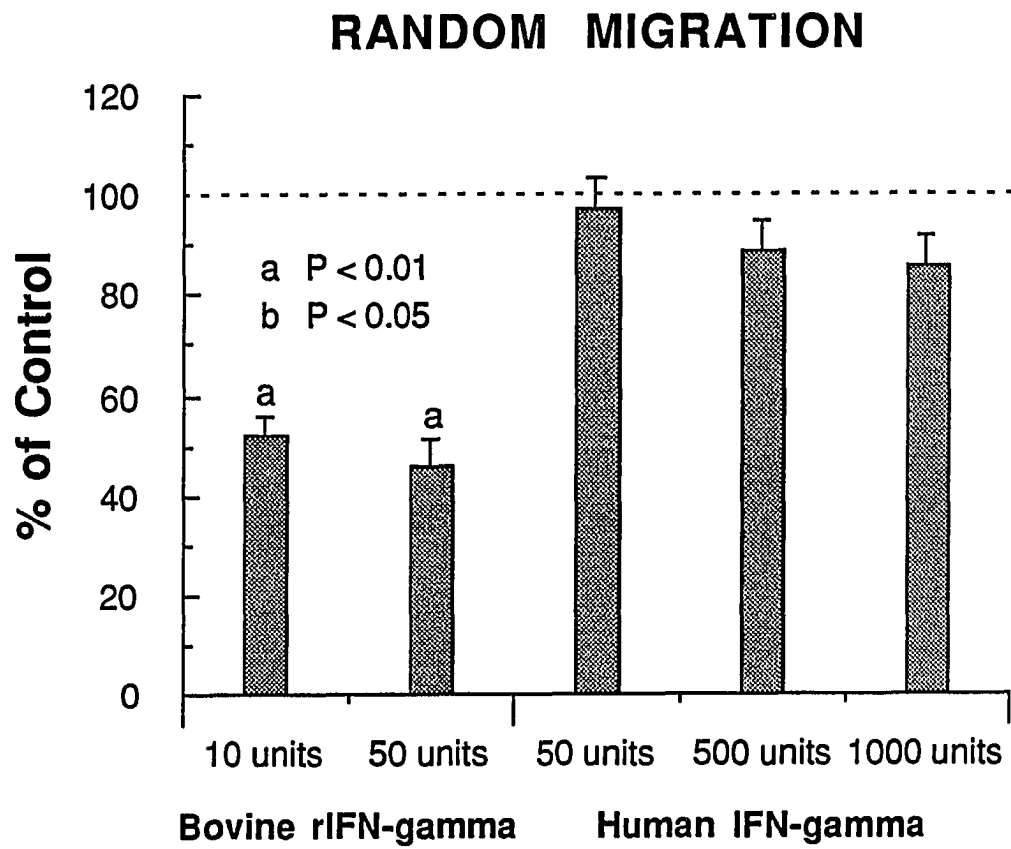


Figure 2. Influence of in vitro incubation with bovine and human gamma interferon on bovine neutrophil random migration in Medium 199. PMNs were purified and then incubated in Medium 199 for 2 hrs in the presence or absence of 10 or 50 units/ 5.0×10^7 PMNs of rboIFN-gamma or 50, 500, or 1000 units/ 5.0×10^7 PMNs before being added to the random migration assay. Results for the interferon-treated and nontreated neutrophils are the expressed as mean percent of nontreated-neutrophil random migration (+ SEM) by PMNs isolated from the same animal (n=7).
^a P < 0.01; ^b P < 0.05; the level of statistical significance for the difference between the interferon treated and nontreated values



Interestingly, the medium chosen for pretreatment and assay affected the amount of enhancement of bovine neutrophil AINC activity by rboIFN- γ . Three commercially prepared media, often used in neutrophil cytotoxic assays, were compared for their abilities to support the induced AINC activity. Neutrophils from the same animals were incubated and assayed in all three media on the same day, under the same environmental conditions. Nonactivated neutrophil AINC values were minimal and nearly the same in all three media. There was significant enhancement of neutrophil AINC activity in M199 or MEM after a 2 hour pretreatment containing 10 units of rboIFN- γ (Figure 3). The enhancement of AINC tended to be higher when performed in M199 than in MEM (Figure 3). Little or no increase in the AINC activity of neutrophils occurred after pretreatment and assay in RPMI 1640, several values were less than those of the nontreated neutrophil. A comparison of the AINC values for the rboIFN- γ treated neutrophils in the three media indicated that the percent of ^{51}Cr release in RPMI 1640 was significantly less ($P < 0.05$) than in the other two media.

The values ADCC by noninterferon-treated bovine neutrophils was the same in M199 and MEM. The ADCC values for nontreated PMNs incubated in RPMI 1640 were significantly higher than those in M199 ($P < 0.01$) and tended to be higher than those in MEM. The ADCC values for the gamma interferon-activated neutrophils were approximately the same, regardless of the assay medium. The differences in the percent ^{51}Cr release between rboIFN- γ -treated and nontreated neutrophils in the ADCC assay performed in RPMI 1640 (Figure 3) were significantly ($P < 0.05$)

lower than in either M199 or MEM. This was due to higher values for nontreated neutrophil ADCC activity in RPMI 1640. The inhibition of random migration under agarose (Figure 4) was the same in all three media.

Figure 3. Influence of in vitro incubation with bovine gamma interferon on bovine neutrophil cytotoxicity in Medium 199, MEM, and RPMI 1640. PMNs were purified and then incubated in medium for 2 hrs in the presence or absence of 10 units/ 5.0×10^7 PMNs of rboIFN-gamma before being added to the AINC and ADCC assays. Results are the mean differences (\pm SEM) in the percent of ^{51}Cr release between interferon-treated and nontreated PMNs isolated from the same animal (n=6). ^a $P < 0.01$; ^b $P < 0.05$; the level of statistical significance for the difference between the gamma interferon treated and nontreated values

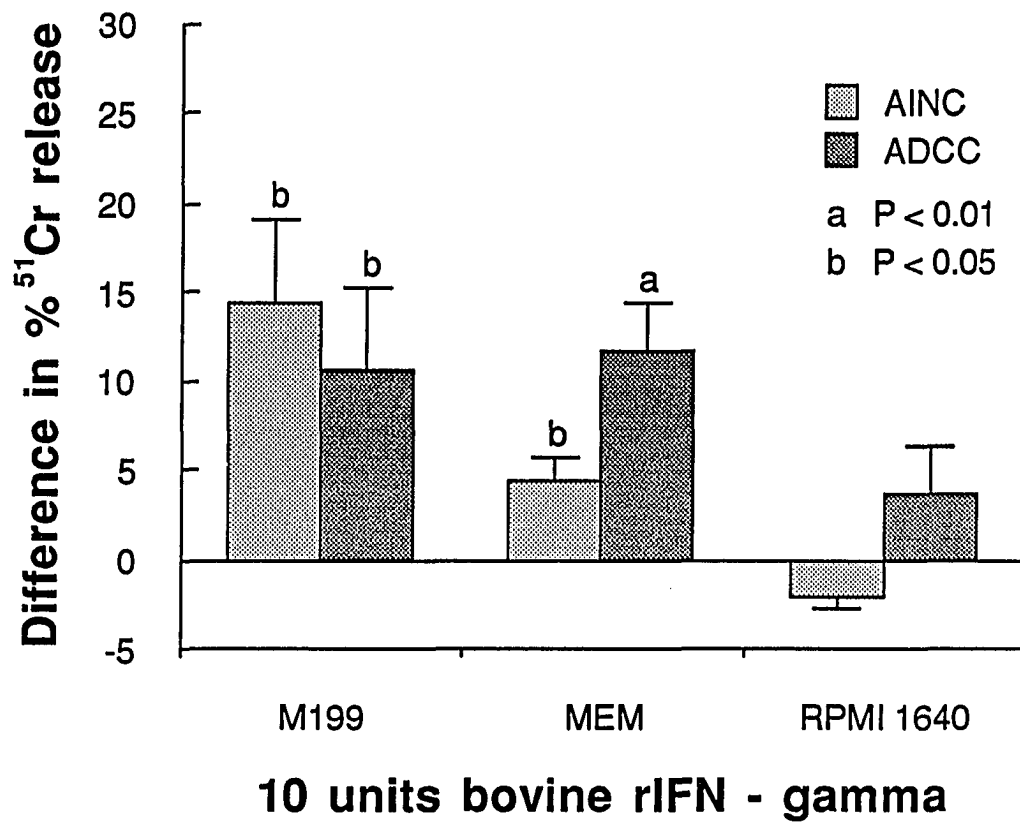
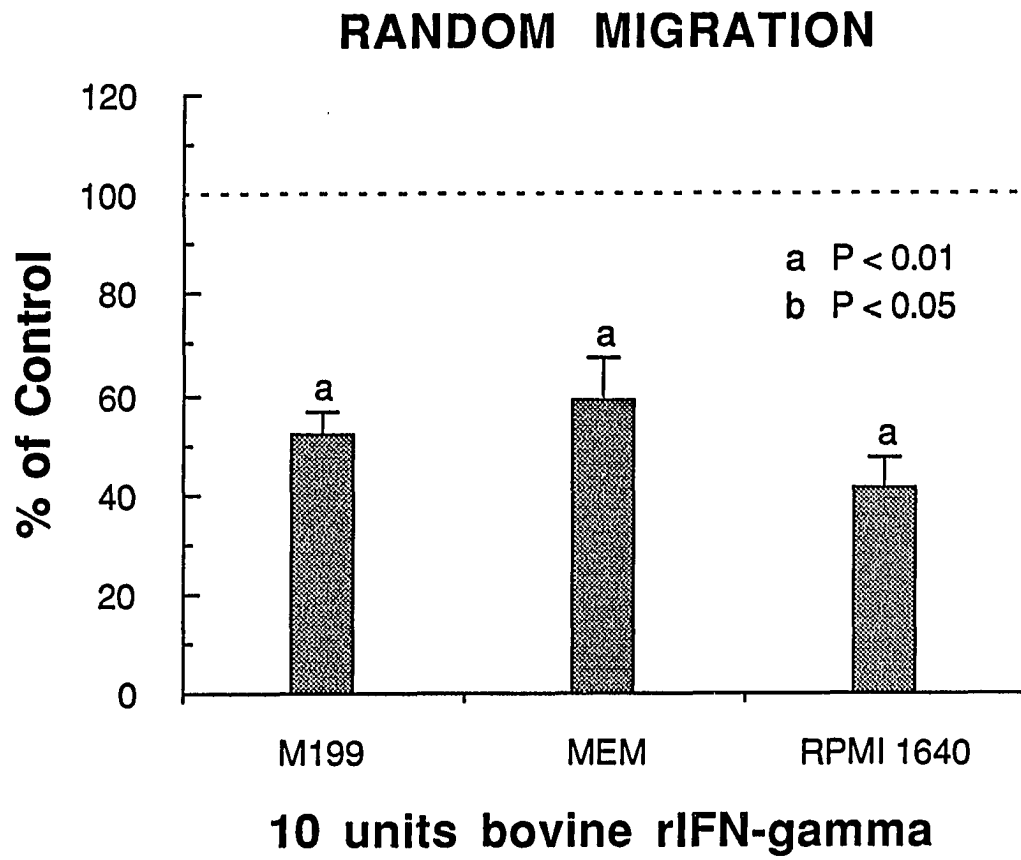
CYTOTOXICITY

Figure 4. Influence of in vitro incubation with bovine gamma interferon on bovine neutrophil random migration in Medium 199, MEM, and RPMI 1640. PMNs were purified and then incubated in medium for 2 hrs in the presence or absence of $10/5.0 \times 10^7$ PMNs of rboIFN-gamma before being added to the random migration assay. Results for the interferon-treated neutrophils are expressed as the mean percent of nontreated-neutrophil random migration (± SEM) by PMNs isolated from the same animal (n=7).
^a P < 0.01; ^b P < 0.05; the level of statistical significance for the difference between the interferon treated and nontreated values



Neither RPMI 1640 nor MEM offered optimal conditions for the rboIFN-gamma-treated bovine neutrophil to express AINC activity. Three components unique to M199 composition were added to both RPMI 1640 and MEM to evaluate possible effects, if any, on the cytotoxic activities. Figure 5 contains the results of the addition to MEM of adenosine triphosphate (1.0 mg/l) and ascorbic acid (0.05 mg/l), either alone or in combination. Neither additive had any significant ($P > 0.05$) enhancing effect on neutrophil AINC or ADCC activities in the presence or absence of rboIFN-gamma. In fact, activated neutrophils had less ADCC activity in MEM supplemented with ATP, but this difference was not statistically significant ($P > 0.05$). Addition of tween 80 (20 mg/l) to MEM lowered the AINC ($P < 0.05$) and ADCC ($P < 0.01$) values for nontreated neutrophils. The enhancement of neutrophil AINC after rboIFN-gamma activation in both MEM and MEM containing tween 80, was approximately the same (Figure 5).

The addition of either adenosine triphosphate or ascorbic acid to RPMI 1640 had little or no effect on AINC or ADCC activities in the presence or absence of rboIFN-gamma (Figure 6). Adding the combination of ascorbic acid and ATP to RPMI 1640 increased the rboIFN-gamma-treated neutrophil AINC and ADCC activities above those of the nontreated neutrophils (Figure 6). The rboIFN-gamma-treated neutrophil values in this medium tended to be greater than those in RPMI 1640 medium alone.

Tween 80 addition to RPMI 1640 lowered ($P < 0.05$) the values for the AINC activity of neutrophils treated with rboIFN-gamma. This additive

also had a tendency to lower the ADCC values for the nontreated neutrophils, a response very similar to the addition of this molecule to MEM. However, the difference in percent of ^{51}Cr release in the ADCC assay between treated and nontreated neutrophils was similar in RPMI 1640 with or without added tween 80 (Figure 6).

Figure 5. Influence of in vitro incubation with bovine gamma interferon on bovine neutrophil cytotoxicity in MEM and RPMI 1640 supplemented with ascorbic acid (0.05 mg/l), ATP, (1.0 mg/L), or tween 80 (20 mg/l). PMNs from each animal were purified, aliquoted into each of the media, and incubated for 2 hrs in the presence or absence of 10 units / 5.0×10^7 PMNs of rboIFN-gamma before being added to the AINC and ADCC assays containing the same medium in which they were preincubated. Results are the mean differences (\pm SEM) in the percent of ^{51}Cr release between interferon-treated and nontreated PMNs isolated from the same animal (n=12). ^a P < 0.01; ^b P < 0.05; the level of statistical significance for the difference between the gamma interferon treated and nontreated values

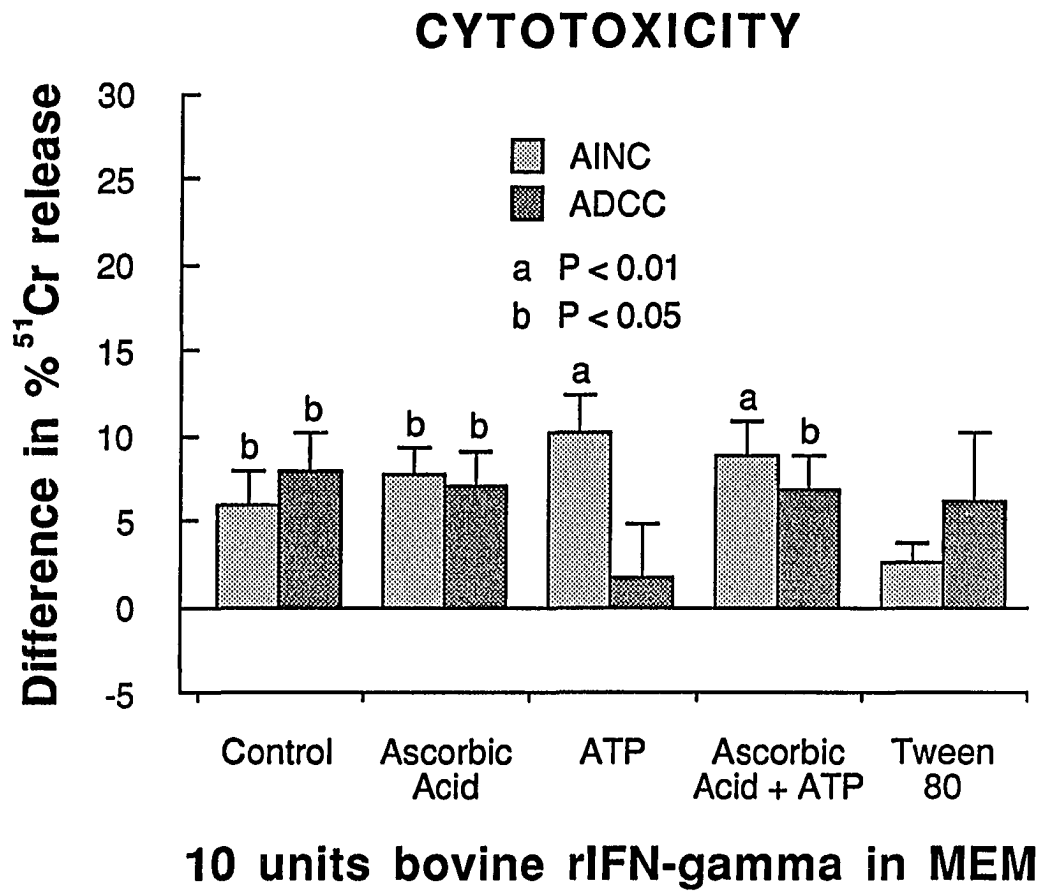
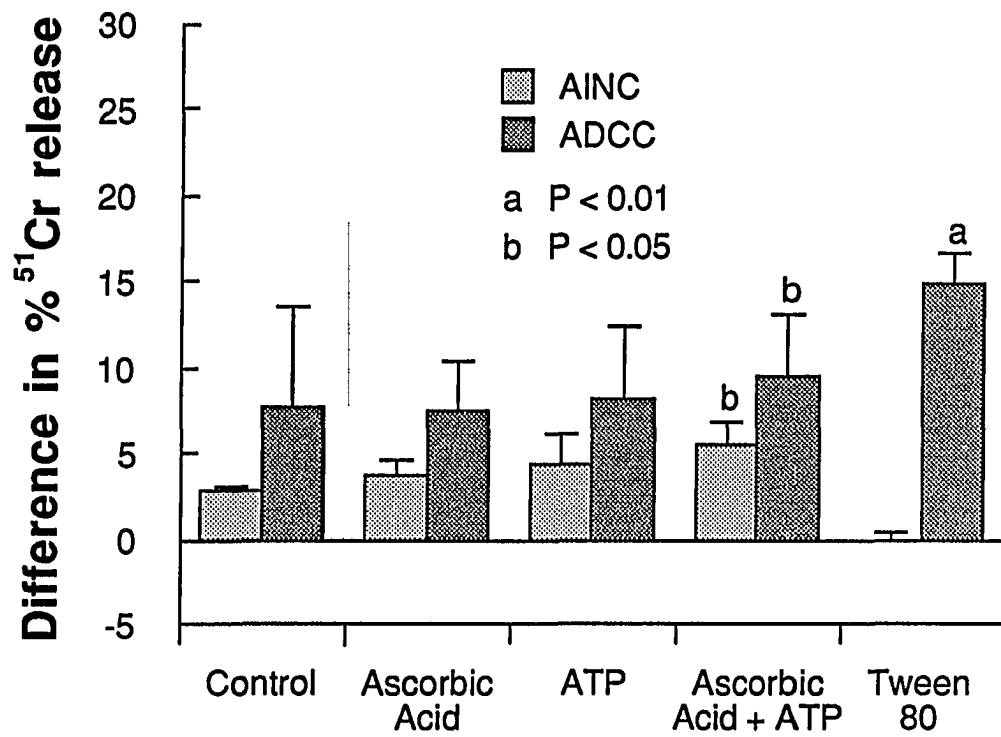


Figure 6. Influence of in vitro incubation with bovine gamma interferon on bovine neutrophil random migration in MEM and RPMI 1640 supplemented with ascorbic acid (0.05 mg/l), ATP, (1.0 mg/L), or tween 80 (20 mg/l). PMNs from each animal were purified, aliquoted into each of the media, and incubated for 2 hrs in the presence or absence of 10 units / 5.0×10^7 PMNs of rboIFN-gamma before being added to the random migration assay. Results for the interferon-treated neutrophils are expressed as mean percent of nontreated-neutrophil random migration (+ SEM) by the PMNs isolated from the same bovine (n=12). ^a P < 0.01; ^b P < 0.05; the level of statistical significance for the difference between the interferon treated and nontreated values

CYTOTOXICITY

10 units bovine rIFN-gamma in RPMI 1640

Pretreatment of human neutrophils with 10 units/ 5.0×10^7 cells of human gamma interferon (huIFN-gamma) for 2 hours resulted in an increase in the ADCC activity in both M199 and RPMI 1640 (Figure 7). The percent of ^{51}Cr released from antibody-coated CRBCs was not significantly increased in MEM. No background AINC activity, nor enhancement after activation, was observed with human neutrophils against ^{51}Cr -labeled CRBC targets under any of the assay conditions (Figure 7). Human neutrophil random migration was inhibited to approximately the same extent in all three media by 10 units of huIFN-gamma (Figure 8).

Figure 7. Influence of in vitro incubation with human gamma interferon on human neutrophil cytotoxicity in Medium 199, MEM, and RPMI 1640. PMNs were purified and then incubated in medium for 2 hrs in the presence or absence of 10 units/5.0 x 10⁷ PMNs of huIFN-gamma before being added to the AINC and ADCC assays. Results are the mean differences (+ SEM) in the percent of ⁵¹Cr release between interferon-treated and nontreated PMNs isolated from the same donor (n=6). ^a P < 0.01; ^b P < 0.05; the level of statistical significance for the difference between the gamma interferon treated and nontreated values

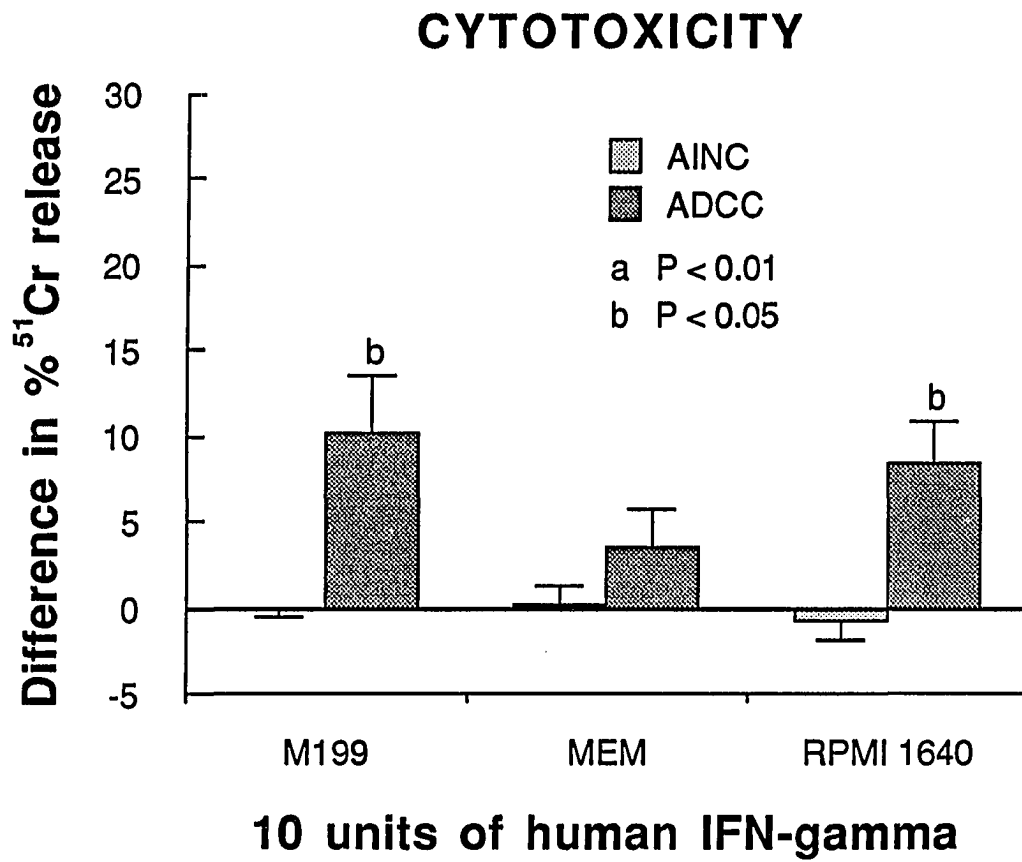
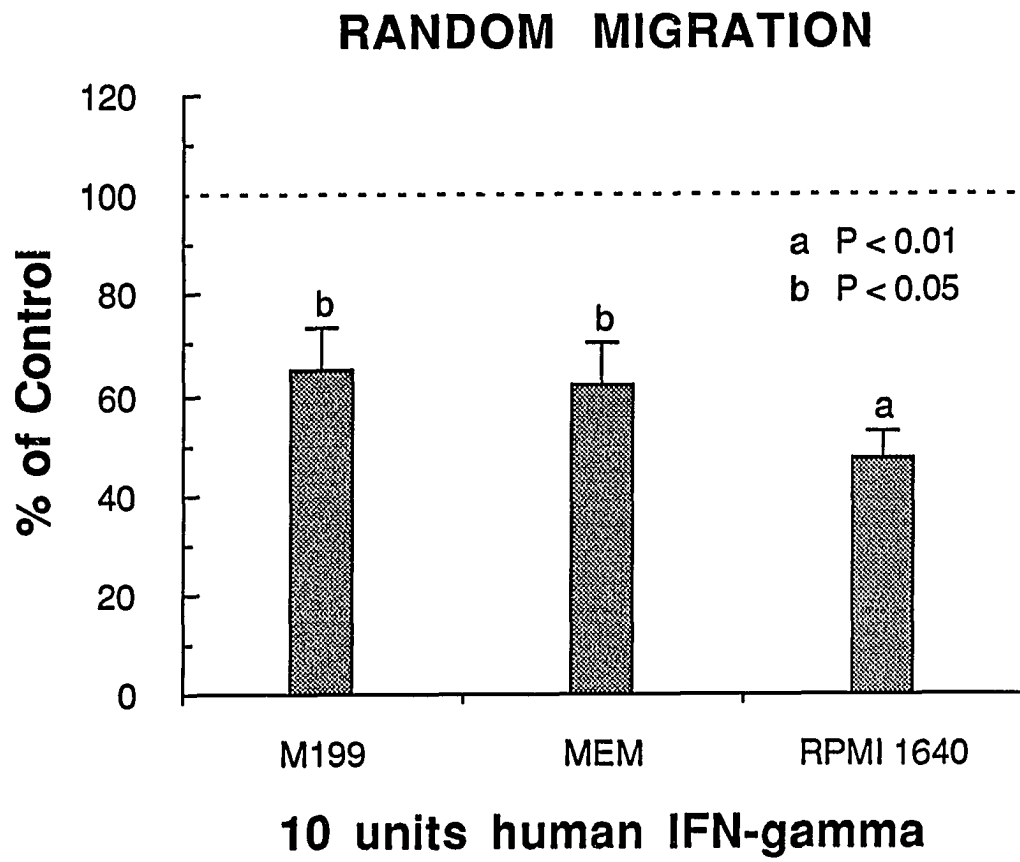


Figure 8. Influence of in vitro incubation with human gamma interferon on human neutrophil random migration in Medium 199, MEM, and RPMI 1640. PMNs were purified and then incubated in medium for 2 hrs in the presence or absence of $10/5.0 \times 10^7$ PMNs of huIFN-gamma before being added to the random migration assay. Results for the interferon-treated neutrophils are expressed as mean percent of nontreated-neutrophil random migration (\pm SEM) by PMNs isolated from the same donor (n=6).
^a P < 0.01; ^b P < 0.05; the level of statistical significance for the difference between the interferon treated and nontreated values



DISCUSSION

We have previously reported that recombinant bovine gamma interferon activates bovine neutrophils to have enhanced antibody-dependent (ADCC) and -independent (AINC) cytotoxicity and impaired migration under agarose in M199 (Steinbeck, Roth, and Kaeberle, 1986). Increased ADCC activity has also been reported for human neutrophils after human gamma interferon treatment in RPMI 1640 (Basham, Smith, and Merigan, 1984; Shalaby et al., 1985; Perussia et al., 1987). Perussia et al. (1987) have reported the lack of induction of human neutrophil AINC against p815y cells by rhuIFN-gamma. The AINC activity against CRBC targets has also been reportedly absent for human neutrophils treated with rhuIFN-gamma. This apparent difference in neutrophil function between human and bovine neutrophils led us to further investigate the AINC activity of neutrophils from each species. We found that human neutrophils activated by huIFN-gamma had enhanced ability to lyse CRBC in the presence of bovine anti-CRBC antiserum in RPMI 1640 (Figure 7), but did not have enhanced AINC activity. This agrees with a previous report by Shalaby et al. (1985). The possibility that human IFN-gamma could activate bovine neutrophils was also investigated. Human IFN-gamma treatment of bovine neutrophils in M199 did not influence AINC, ADCC, or random migration (Figures 1, 2).

Since the media used on human neutrophils in previous reports has been RPMI 1640 and the media used with bovine neutrophils has been M199, it was decided to investigate the influence of various media on neutrophil activation. The inclusion of rboIFN-gamma in either MEM or

M199 resulted in the enhancement of bovine neutrophil AINC activity, (Figure 3) although the increase was not as dramatic in the MEM medium. The AINC activity of activated bovine neutrophils was not measurable in RPMI 1640 (Figure 3). Results for the bovine neutrophil ADCC were the same in both M199 and MEM in presence of rboIFN-gamma, variable results were obtained in RPMI 1640 (Figures 3, 6). Random migration of bovine neutrophils was inhibited to the same degree by rboIFN-gamma in all three media (Figure 4).

The difference in the abilities of the three media to support bovine neutrophil AINC induced by rboIFN-gamma was investigated further. MEM contains similar (but not identical) concentrations of inorganic salts, amino acids, and vitamins as M199, but lacks some of the other supplements. MEM served as a base medium to which M199 components could be added and their effects evaluated. RPMI 1640, like MEM, is a minimal essential medium used routinely on human neutrophils. It contains twice the concentration of Ca^{++} , Mg^{++} , and glucose found in MEM or M199. The ability of M199 and MEM to support AINC activity was not due to any common medium supplement in these two media that was absent from RPMI 1640. Three components present in M199 (ascorbic acid, ATP, tween 80) were selected to be added to MEM and RPMI 1640 because they have been reported to affect other neutrophil functions. Ascorbic acid at a relatively high concentration (5×10^{-3} M) has been reported to enhance guinea pig peritoneal neutrophil chemotaxis (Dallegrì, Lanzi, and Patrone, 1980). With lower concentrations (1×10^{-3} M) of ascorbic acid, there was an increase in neutrophil chemokinesis and inhibition of

chemotaxis. The effect on neutrophil cytotoxic activity was not evaluated. M199 also contains several nucleic acid molecules, some of which are known to have inhibitory effects on neutrophil function. Guanine and adenine, at concentrations similar to those found in M199, are capable of producing a slight inhibition of bovine neutrophil MPO- H_2O_2 -halide activity (Chiang, Kaerberle, and Roth, 1986; Canning et al., 1985). ATP has been reported to inhibit lysosomal enzyme secretion (beta-galactosidase) by mouse macrophages (Riches et al., 1985), but not bovine neutrophil iodination at concentrations found in M199. Tween 80 (20 mg/l), a fatty acid derivative found in M199, was also added to RPMI 1640 and MEM. Even though tween 80 is present at a low concentration, it might effect the membrane properties of bovine neutrophils or CRBC targets.

Neither the addition of ascorbic acid (0.05 mg/l) nor ATP (1 mg/l) at the concentrations found in M199 had any major effect on the bovine neutrophil antibody-independent (AINC) cytotoxicity after pretreatment with 10 units of rboIFN-gamma in the same medium. This was true whether the components were added alone or in combination to MEM or RPMI 1640. Although, values for ADCC activity were generally lower in MEM supplemented with ATP. The inclusion of ATP or ascorbic acid, either alone or in combination, did not affect the inhibition of bovine neutrophil random migration by rboIFN-gamma (data not shown). Medium supplemented with tween 80 lowered the nontreated neutrophil values in both of the cytotoxic assays, without altering the neutrophil ADCC activity in the presence of rboIFN-gamma in either MEM or RPMI 1640. In

general, the inclusion of these components (ATP, ascorbic acid, or tween 80) had little affect on the cytotoxic activity of the bovine neutrophil. These commercially prepared media have also been evaluated for their ability to maintain human neutrophil function during long term incubation (Glasser, Fiederlein, and Huestis, 1985). There were no apparent differences in the maintenance of neutrophil function in any of the media.

Apparent media differences for the enhancement of bovine neutrophil AINC activity led to an investigation of the ability of huIFN-gamma in either M199 or MEM to enhance human neutrophil AINC activity. However, the choice of medium did not make a difference, human neutrophils apparently lack the ability to lyse CRBCs in RPMI 1640, M199, or MEM in the absence or presence of human gamma interferon (Figure 7). The enhancement of ADCC by the human neutrophils treated with huIFN-gamma was approximately the same in either RPMI 1640 or M199, and was present although to a lesser extent in MEM (Figure 7). Random migration was similarly inhibited in all three media (Figure 8). These findings confirm previous reports and imply that in addition to granule component and surface receptor differences the neutrophils from these two species have target cell recognition or cytotoxic activity differences as well.

In conclusion, the ability of the bovine neutrophil to be activated by rboIFN-gamma to have enhanced antibody-independent cell-mediated cytotoxicity against ^{51}Cr -labeled CRBCs is apparently not a function shared by human neutrophils treated with huIFN-gamma. Interestingly, the amount of AINC activity against CRBC targets by bovine neutrophils

dependent on assay conditions. Medium 199 was the optimal medium for expression of the AINC activity and, although lower, values in MEM were enhanced ($P < 0.05$). RPMI 1640, (a medium containing twice the Ca^{++} , Mg^{++} , and glucose concentrations) did not support the bovine neutrophil AINC activity, and bovine neutrophil ADCC activities were more variable in this medium. RPMI 1640 does not lack any components that are contained in the other two media which support AINC activity. Three unique M199 components (ATP, ascorbic acid, and the fatty acid derivative, tween 80) known to have effects on neutrophil function and added to either MEM or RPMI 1640 were not instrumental in changing the AINC activity of bovine neutrophils in these media. It is quite possible that a more complete medium containing a mixture of these and other components, (in vivo conditions) is required before optimal activity can be attained.

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SUMMARY AND DISCUSSION

The first part of this study addressed the influence of recombinant bovine gamma interferon (rboIFN-gamma) on in vitro functions of bovine neutrophils. Neutrophils pretreated with rboIFN-gamma were found to have enhanced ability to lyse target cells in the presence (ADCC) and absence of target specific antibody (AINC). The enhanced ability of the neutrophil to act as an effector cell against virus-infected cells may be important in decreasing the incidence of bacterial pneumonia in bovine respiratory disease. Neutrophil random migration was inhibited by rboIFN-gamma treatment, while the other neutrophil functions (MPO-H₂O₂-halide activity, ingestion, superoxide anion production) were not consistently affected. Inhibition of neutrophil random migration may be important in retaining the tissue neutrophils at the site of infection. The process of inhibiting random migration required both RNA and protein synthesis by the neutrophil. This was determined by the addition of inhibitors of these cellular processes during pretreatment and assay. Only brief exposure, five minutes, to rboIFN-gamma was required for the inhibition of neutrophil random migration.

In order to better understand the activation process, the molecular events involved in the enhancement of neutrophil cytotoxicity were investigated in the second part of this study. The rboIFN-gamma treated and nontreated neutrophils were both capable of RNA and protein synthesis as determined by the incorporation of ³H-uridine and ³⁵S-methionine into acid precipitable macromolecules and by inhibitors of these cellular processes (alpha-amanitin, puromycin, cycloheximide). These events were

processes (alpha-amanitin, puromycin, cycloheximide). These events were only required for the induction of AINC activity and were not required for the enhancement of ADCC by rboIFN-gamma. Recombinant boIFN-gamma-treated neutrophils had enhanced expression of two major proteins, Mr=60,000 and 94,000 by SDS-polyacrylamide, linear-gradient gel electrophoresis. The kinetics of induction of these two proteins followed the time required for neutrophil activation and the expression of AINC activity. Both proteins were located in the neutrophil cytosol and were associated with the granule fractions. Neutrophils isolated from cattle immunosuppressed by intramuscular administration of glucocorticoids (dexamethasone) had depressed AINC and ADCC activities and enhanced random migration. Interestingly, rboIFN-gamma treatment (in vitro) did not enhance AINC, but reversed the suppression of ADCC and inhibited random migration to approximately normal levels. The protein synthesis patterns in the presence and absence of rboIFN-gamma were similar to those of neutrophils from nontreated cattle. It has been previously reported that arachidonic acid metabolism is suppressed in bovine neutrophils after in vivo administration of dexamethasone, specifically products of the 5-lipoxygenase pathway (leukotriene B₄, 5-HETE) were decreased (Webb and Roth, 1987). Inhibitors of arachidonic acid metabolism, nordihydroguaiaretic acid and BW755c, also inhibited the ability of rboIFN-gamma to induce neutrophil AINC activity, but did not affect enhancement of ADCC or inhibition of random migration. It appears that both the enhancement of AINC activity and inhibition of random migration by rboIFN-gamma require RNA and protein synthesis by

the neutrophil. The AINC activity is also dependent on the ability of the neutrophil to metabolize arachidonic acid. Whereas the enhancement of the ADCC activity by rboIFN-gamma did not require any of these cellular processes.

The final part of this study evaluated the reported species difference in the ability of bovine and human neutrophils to express AINC activity after homologous gamma interferon treatment. Pretreatment and assay conditions utilized in previous human neutrophil studies (Basham, Smith, and Merigan, 1984; Shalaby et al., 1985) and bovine neutrophil studies (Steinbeck, Roth, and Kaeberle, 1986) were reevaluated. The findings reaffirmed those of others, additionally we reported that human gamma interferon did not influence bovine neutrophil function. Since the medium conditions used on the neutrophils from both species differed, media normally used on human neutrophils (RPMI 1640, MEM) was tested on bovine neutrophils. Values for AINC activity were enhanced by rboIFN-gamma when neutrophils were incubated in MEM although to a lesser extent than in M199, but RPMI 1640 did not support this activity. Three components found in M199 were added to RPMI 1640 and MEM (both of these media are minimum essential media). These components (ATP, ascorbic acid, and tween 80), at higher concentrations, are known to have effects on other neutrophil and macrophage functions (Dallegrì, Lanzi, and Patrone, 1980; Canning et al., 1985; Chiang, Kaeberle, and Roth, 1986). None of these components directly influenced the AINC results in either RPMI 1640 or MEM. Apparently no one component but the entire mixture of supplements are required for optimal AINC activity.

much as possible. The fact that there was a medium difference led us to reevaluate the ability of human neutrophils to express the AINC activity in all three media. The human neutrophils did not express enhanced AINC activity after homologous gamma interferon treatment in any of the media tested. Bovine and human neutrophils have differences in enzyme activities and surface receptor expression; the difference in induction of AINC activity by gamma interferon is another example of a species difference.

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