The University of Calgary

The Interaction between Alpha Interferon and *Pseudomonas Aeruginosa*

By

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "The Interaction between Alpha Interferon and Pseudomonas Aeruginosa" submitted by Christine M. Zeindler in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

The prognosis of cystic fibrosis patients is closely related to chronic pulmonary infection, especially with *Pseudomonas aeruginosa*. It is evident that exoenzymes of *P. aeruginosa* play a significant role in exacerbations of respiratory symptoms during chronic *P. aeruginosa* lung infections. Thus, it is of significant importance to determine those lung environmental factors which may lead to up-regulation of *P. aeruginosa* exoenzymes and the concomitant exacerbations of respiratory disease in cystic fibrosis. In the present studies we have demonstrated that one of those lung environmental factors which may regulate the production of *P. aeruginosa* exoenzymes is human alpha interferon. Exposure of *P. aeruginosa* to clinically relevant concentrations of human recombinant interferon resulted in significant alterations in the levels of exotoxin A, phospholipase C, exoenzyme S and proteolytic enzyme activity detected in the supernatant fluids of these cultures. It was also determined that this regulation of *P. aeruginosa* exoenzyme expression was mediated through a specific IFN receptor which was demonstrated to be present on *P. aeruginosa* outer membranes. Further evidence from combined *P. aeruginosa*-RSV infection in animals demonstrated that a possible synergistic relationship exists between these two microorganisms which significantly exacerbates the level of lung injury over that observed with either organism alone. Administration of IFN directly to chronically *P. aeruginosa* infected animals demonstrated similar pathology as that observed during *P. aeruginosa-RSV coinfection. This supports our hypothesis that* IFN alpha interacts with *P. aeruginosa* to increase exoproduct expression which subsequently results in an exacerbation of pulmonary injury.

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TO MY PARENTS

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LIST OF ABBREVIATIONS

ADP	...adenosine diphosphate
anova	...analysis of variance
A_{nm}	...light absorbance at a particular wavelength (nm)
CF	...Cystic Fibrosis
con	...control
DNA	...deoxyribonucleic acid
dsDNA	...double stranded deoxyribonucleic acid
dsRNA	...double stranded ribonucleic acid
DSS	...disuccinimidyl suberate
EDTA	...ethylenediaminetetraacetic acid
eEF-2	...eukaryotic elongation factor-2
g	...gram
G	...acceleration of gravity (9.8 m/s/s)
h	...hour
HSA	...human serum albumin
hu-IFN-alpha	...human interferon alpha
hu-IFN-beta	...human interferon beta
IFN	...interferon
IFN-f	...carrier free interferon alpha
IL-1	...interleukin one
IL-2	...interleukin two
INTRON A	...alpha interferon with human serum albumin
IU	...international unit
Kd	...kinetic dissociation constant
kDa	...kilodalton
l	...litre
mA	...milliamps
mCi	...millicurie
MEP	...mucoexopolysaccharide
min	...minute(s)

µg	...microgram
µl	...microlitre
µM	...micromolar
mg	...milligram
ml	...millilitres
mM	...millimolar
mRNA	...messenger ribonucleic acid
Nad	...nicotinamide adenine dinucleotide
ng	...nanogram
nm	...nanometre
nM	...nanomolar
pl	...isoelectric point
PBS	...phosphate buffered saline
pg	...picogram
SDS	...sodium dodecyl sulphate
SDS-PAGE	...SDS-polyacrylamide gel electrophoresis
RSV	...respiratory syncytial virus
TMM	...tryptose minimal medium
vol	...volume

1.0

INTRODUCTION

1.1 Cystic Fibrosis

Anderson (1938) described two pathophysiologic problems associated with the genetically-linked disease, cystic fibrosis (CF): pancreatic insufficiency and chronic airway infections. However, chronic lung infection and not the pancreatic disorder has been implicated in abbreviating the lifespan of CF patients. Further studies have demonstrated that lung infection resulting in progressive lung deterioration and followed by pulmonary dysfunction is responsible for 90% of CF deaths (Hoiby, 1982, Fick, 1989, Gilligan, 1991).

A number of organisms have been associated with chronic lung infection in patients with CF. Two of the most prominent bacterial respiratory pathogens of CF are *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Gilligan, 1991). However, with the advent of antimicrobial therapy, *P. aeruginosa* has been isolated from respiratory tract cultures with greater frequency than *S. aureus* (Thomassen *et al.*, 1987). Furthermore, greater than 70% of CF patients are infected with *P. aeruginosa* and this organism is believed to be involved in the respiratory failure that ultimately leads to morbidity of all CF patients (Pier, 1985).

1.2 *Pseudomonas aeruginosa*

The principal lung bacterial pathogen in the CF lung is *Pseudomonas aeruginosa;* greater than 90% of CF patients are colonized by this organism (Wood, 1976). *P. aeruginosa,* through the production of a number of pathogenic factors, has been associated with respiratory failure (Pier, 1985). These factors include both cell-associated and extracellular products.

1.2.1 Attachment

The initial event of infection is adherence of microorganisms to mucosal epithelial cells (Johanson *et al.*, 1980) Adherence of *P. aeruginosa* to the oropharyngeal mucosal surface may be mediated by a number of mechanisms. Polar pili, present on the outer surface of *P.*

aeruginosa, have been well characterized and their role in attachment of this organism to buccal epithelial cells well established (Doig *et al.*, 1990; Ramphal *et al.*, 1984; Woods *et al.*, 1980) It has been further suggested that the extracellular mucoexopolysaccharide (MEP) of *P. aeruginosa* may also bind to tracheobronchial mucin (Ramphal *et al.*, 1987). However, non-mucoid strains appear to be initial colonizers in CF, signifying the importance of pili in the initial adherence of *P. aeruginosa* (Woods *et al.*, 1980). Recently, an ADP-ribosyl transferase produced by *P. aeruginosa*, exoenzyme S, has also been implicated in adhesion (Woods *et al*, 1991). Exoenzyme S has been found on the outer surface of *P. aeruginosa* where it may function as an adhesin. These studies also indicate that exoenzyme S binds to similar glycosphingolipids which have been identified as receptors for *P. aeruginosa*. This evidence, as well as studies which demonstrate that antibodies to exoenzyme S inhibit *P. aeruginosa* attachment to buccal cells, support a role for this protein in adherence (Baker *et al.*, 1991). Further studies on the binding characteristics of exoenzyme S suggests that this molecule may have two receptor binding specificities: one for carbohydrates and one for the *Helicobacter pylori* receptor glycerolipid (Lingwood *et al.*, 1991).

1.2.2 Pathogenesis

P. aeruginosa produces both cell associated and extracellular compounds which may potentially contribute to the pathogenesis of this organism. As a gram-negative bacterium, *P. aeruginosa* possesses lipopolysaccharide (LPS). However, this LPS has been identified as relatively nontoxic in comparison to that of other gram-negative species (Dyke and Berk, 1973). Alginate, the major component of MEP, is also a cell associated virulence factor. This compound may contribute to pathogenesis by inhibiting the binding of *P. aeruginosa* to macrophages and scavenging hypochlorite, an oxidant generated by phagocytes (Krieg *et al.*, 1988; Learn *et al.*, 1987). *P. aeruginosa* flagella may also function as a cell-associated virulence factor. It has been demonstrated that an

isogenic mutant, lacking flagella was less virulent than the parent strain (Montie *et al.*, 1982).

The virulence of *P. aeruginosa* has been attributed to a number of extracellular pathogenic factors. These substances, which have been demonstrated to contribute to pathology both *in vitro* and *in vivo* include exotoxin A, exoenzyme S, proteases, and phospholipase C (Woods, 1987).

Two ADP-ribosyltrasferases are produced by *P. aeruginosa*, exotoxin A and exoenzyme S. These enzymes differ in their antigenic and structural characteristics. Exotoxin A is secreted as a proenzyme with a molecular weight of approximately 66 kDa (Callahan, 1976) which may be cleaved to form both an enzymatically active 26 kDa and nonenzymatically active fragment (fragment A and B, respectively; Vasil, 1977). Toxicity of exotoxin A is due to its ability to inhibit protein synthesis. This is accomplished by an NAD-dependent ADP-ribosylation of eukaryotic elongation factor 2 (eEF2; Iglewski, 1977). It has been demonstrated that following administration of purified exotoxin A, lethality in a number of animals may ensue (Liu, 1966). Moreover, rats chronically infected with a *P. aeruginosa* exotoxin A-producing strain demonstrated intra-alveolar mononuclear cell infiltration similar to those symptoms observed in human *P. aeruginosa* infections (Woods *et al.*, 1982). Further evidence indicates that mutant strains of *P. aeruginosa* which produce either inactive toxin or no exotoxin A are less virulent than those which secrete exotoxin A. However, as the presence of exotoxin A itself does not insure virulence, the contribution of other pathogenic factors must also be acknowledged (Nicas and Iglewski, 1985).

A second ADP-ribosyltransferase, Exoenzyme S, has been purified. It consists of four products ranging in molecular weights from 20-64 kDa. The protein with the most enzymatic activity has a molecular mass of 50 kDa (Woods and Que, 1987). Differences between exotoxin A and exoenzyme S include: exoenzyme S is not neutralized by antiexotoxin A, exoenzyme s is more heat stable than exotoxin A and does not ribosylate eEF2 (Iglewski

et al., 1978). Exoenzyme S modifies a number of eukaryotic cell proteins including ATPase, cAMP-dependent protein kinase and DNAse. Administration of purified exoenzyme S into rat lungs resulted in grossly observable damage. This damage was similar to histopathological observations described for *P. aeruginosa* infection. This suggests that the pathological changes observed in a clinical *P. aeruginosa* lung infection may be reproduced by a single exoproduct, exoenzyme S (Woods *et al.*1988).

Morihara *et al* (1965) originally characterized two proteases produced by *P. aeruginosa*: an alkaline protease and an elastase. Since then, an additional lysine specific protease has been identified (Elliot and Cohen, 1986). Alkaline protease has a molecular mass of 48.4 kDa and is produced by 90% of *P. aeruginosa* strains (Inove *et al*, 1963). Mutants that are deficient in alkaline protease have been demonstrated to be less virulent than the parental strains (Nicas and Iglewski, 1985). Possible mechanisms of pathogenesis include: inhibition of ciliary function (Hingley *et al*, 1986), alteration of natural killer cell function (Pederson and Kharazami, 1987), and proteolysis of human gamma interferon and tumour necrosis factor (Parmely, 1990).

P. aeruginosa elastase is produced by 85-90% of the strains examined (Nicas and Iglewski, 1985) and has a molecular mass between 20-40 kDa (Morihara *et al.*, 1965). Woods *et al* (1982) demonstrated that a mutant deficient in elastase was less virulent than the parental elastase-positive strain. Substrates of elastase include immunomodulators such as complement and cytokines (Schultz and Miller, 1974; Parmely, 1990), tissue components (Morihara, 1964), and iron binding compounds (Doring *et al*, 1988). Because elastase degrades such a large range of substrates, its pathogenic role may be considered equally as vast.

P. aeruginosa lysine specific protease has a molecular mass of 30 kDa. Only the peptide, ester and amide bonds containing the carbonyl group of lysine are hydrolysed by this enzyme. Fibrinogen has been demonstrated to be a substrate of this enzyme (Elliot and Cohen, 1986).

Phospholipase C is a haemolysin produced by *P. aeruginosa*. It is a lecithinase which liberates phosphorylcholine from lecithin (Berka and Vasil, 1982). This enzyme has the potential to contribute to lung pathogenesis by degrading lung surfactant which is composed of lecithin (Liu, 1974). It has been demonstrated that phospholipase C plays a role in *P. aeruginosa* colonization in the lungs of CF patients; strains producing this protein are less efficiently cleared from the lung (Woods *et al.*, 1986).

1.3 Respiratory syncytial virus

Respiratory syncytial virus (RSV) is a member of the Paramyxoviridae family, and the Pneumovirus genus. Its size ranges from 150-300 nm in diameter and its virions are membrane-bound particles. The nucleic acid of RSV is a single stranded, nonsegmented RNA of negative polarity. During infection, 10 distinct mRNAs are encoded, each of which encodes a single protein. It has been determined that the L gene codes for viral RNA polymerase. The virus obtains its envelope by budding from the host's plasma membrane. Unlike other members of this family, neuraminidase and hemagglutinin are not expressed on the RSV envelope (McIntosh and Chanock, 1985).

RSV has been identified as the most important cause of viral lower respiratory tract disease in infants and children (Tellez *et al.*, 1990) It outranks other microbial pathogens as a cause of pneumonia and bronchiolitis in infants under 1 year of age (McIntosh and Chanock, 1985). It initially replicates in the nasopharynx and then, via the respiratory epithelium, spreads to the lower respiratory tract (Hall *et al.*1981). Cell injury is caused by fusion of infected cells with infected or uninfected cells, resulting in syncytium formation. This cytopathology is directly responsible for most of the symptoms observed with pneumonia and bronchiolitis. Ultimately these processes result in obstruction of the small bronchioles (McIntosh and Chanock). Death due to this infection is rare with medical intervention in normal individuals. However, cases of

fatal RSV infection have been reported in infants or adults who lack cell-mediated immunity (Fishaut *et al.*, 1980).

1.4 Interferon

Interferon was first described by Isaacs and Lindenmann in 1957. This molecule was identified by its ability to interfere with influenza virus multiplication in chick chorioallantoic membranes. Since then interferon has been traditionally characterized as an antiviral substance. However, many other immunological and cellular regulatory functions have been attributed to this molecule or molecules.

1.4.1 Protein structure

Interferons are classified into 3 categories based upon antigenic and structural differences (Wills, 1990). Interferon alpha is produced by the following cells: B lymphocytes, null lymphocytes and macrophages. It can be induced by foreign, virus-infected, tumour or bacterial cells. Seventeen different human interferon-alpha genes produce the 15 different subtypes of this molecule (Baron *et al*, 1991). The molecular sizes of these molecules range from 17.5 - 23 kDa and contain 165 -166 amino acids which have approximately 80% homology. Generally, this group of interferons have 4 cysteine residues which are involved in disulphide bonds. Human interferon-alpha (HuIFN-alpha) does not contain N-linked glycosylation sites and tends to be acidic with isoelectric points of 5.7-7.0. Interferon alpha has been efficiently synthesized from expression plasmids in *Escherishia coli* (Lengyel, 1982). Employing these recombinant molecules, structure-function relationships have been elucidated. Experimental data have been obtained which suggest a role for the amino acid sequence of HuIFN-alpha-2 for antiviral activity (Eichmann *et al.*, 1990).

In contrast to alpha interferon, human beta interferon exists as a single species expressed from a single gene (Baron *et al*, 1991). This molecule is produced by fibroblasts, epithelial cells and macrophages and may be induced by viruses and foreign nucleotides. It is secreted as a

glycoprotein with a molecular size of 23 kDa and it contains 166 amino acids. Interferon beta and alpha share 29% structural homology. Three cysteine residues are present in interferon beta and the isoelectric point is between 6.8-7.8 (Wills, 1990).

Interferon-gamma shares no significant homology with interferon-alpha or beta. It is produced from activated T lymphocytes which have been induced by foreign antigens (Wills, 1990). Human interferon-gamma is expressed from a single gene and depending on the extent of glycosylation the molecular size may vary from 15-25 kDa (Pestka *et al*, 1987). Higher molecular weights have also been reported for interferon-gamma suggesting possible oligomerisation of this molecule. There are two cysteine residues in interferon-gamma and it is acid labile with an isoelectric point of 8.6-8.7 (Wills, 1990).

1.4.2 Biological Activities

Interferons were originally believed to be specific antiviral agents which did not have any effect on normal cell metabolism (Isaacs and Lindenmann, 1957). However, in the last 10 years interferon has become recognized as a prototype of a family of cytokines that all have regulatory affects on cell division, metabolism, and functions (Gresser, 1990). The actions of alpha and beta IFN (type 1 IFN) are quite similar, thus they are generally classified together in terms of their activities. However, the gene products of gamma IFN (type 2 IFN) only partially overlap those of alpha and beta IFN; and thus, this molecule is classified separately. Although type 1 IFN and type 2 IFN act via different mechanisms their actions may often produce additive or synergistic effects (Pestka and Langer,1987).

A role for type 1 interferons has been recognized as a rapidly activated response against viral infections (Gresser, 1984). They may also be involved in resistance to some tumours and in resistance to particular bacterial and parasitic infections (Balkwill, 1985; Hess *et al*, 1989). Immunoreactive IFN alpha has been isolated, under physiological

conditions, from nearly all human tissue except that of the brain, renal cortex and medulla (Khan *et al.*, 1989). These findings imply that the production of IFN-alpha may be stimulated by factors other than viral infection. These low levels of IFN-alpha may have an early role in host defense against viral infection and neoplasia (Gresser, 1990). However, this function of IFN-alpha does not preclude the possibility of this molecule modulating cell growth and a further immune response (Khan *et al*, 1989).

Although type 2 interferons have antiviral effects, they are not as efficient at inhibiting viral replication as the type 1 interferons (Pestka and Langer, 1987). IFN-gamma has pleiotropic effects in lymphokine immune reactions. Administration of IFN-gamma results in antitumour effects in some cancers (Pestka *et al.*,1987). This IFN can also promote efficient killing of bacteria and parasites through activation of macrophages (Knop, 1990).

1.4.3 Mechanism of action - antiviral mechanisms

IFNs are produced in response to induction in a large number of cell types; however, not all cells produce IFN. Also, as indicated previously, some cell lines produce small amounts of IFN constitutively. The inducers of IFN include viruses, mycoplasmas, bacteria, protozoa, natural or synthetic dsRNA, endotoxins, polysaccharides, and foreign antigens (Lengyel, 1982).

Several DNA sequences have been found to be required for IFN, type 1, production and regulation. In the case of Hu-IFN-beta the -77 to -19 region with respect to the mRNA cap site is required for both constitutive and induced expression (Goodbourn *et al*, 1985). Similarly, experiments with Hu-IFN-alpha genes indicate that the -117 to -74 region is required for its expression (Ragg and Weissmann, 1983). Induction of the type 1 IFN has been demonstrated to require a transcriptional binding factor. Further, DNA footprinting experiments have indicated that repressor molecules are released upon induction from the IFN- alpha and beta gene

(Pestka *et al.*, 1987, Taylor and Grossberg, 1990).

IFN acts directly upon virus-susceptible cells to induce a viral-resistant state. Following binding to specific receptors, IFN induces synthesis of specific proteins which result in the biological actions of IFN. Generally, the stage of virus multiplication inhibited by type 1 interferons is the synthesis of viral macromolecules. This synthesis is inhibited at the level of translation of viral mRNA into viral polypeptides (Samuel, 1988). Three IFN-inducible pathways, which are involved with inhibition of viral translation, have been well characterized: protein kinase, 2'5'-oligo-A synthetase and Mx protein systems (Baron *et al.*, 1991).

Virus translation is dependant on eukaryotic elongation factors. Through the induction of a 67-69 kDa protein kinase, termed p1, the action of eukaryotic initiation factor-2 alpha (eIF-2 alpha) is blocked, thereby decreasing the efficiency of initiation of protein synthesis (Taylor and Grossberg, 1990). The mechanism of action of this IFN-induced enzyme is as follows: the enzyme is activated by double- stranded RNA, phosphorylated and then catalyses the phosphorylation of the alpha subunit of eIF-2. Because p1 is activated by dsRNA, this inhibition pathway is specific only for those viruses which utilize dsRNA (Samuel, 1988). Further, this pathway is regulated by a phosphatase which removes the phosphate from the eIF-2 alpha thus freeing eIF-2 to initiate translation once again (Taylor and Grossberg, 1990).

The 2'5'-oligo synthetase system indirectly exerts its antiviral effect by enzymatically degrading viral RNA. This enzyme is also activated by dsRNA. It in turn, via catalysis of adenosine triphosphate (ATP) into 2'5'-linked oligomers of adenylate, activates an endogenous endonuclease (Baron *et al.*, 1991) . This endonuclease (endonuclease L or F) cleaves both messenger and ribosomal RNA resulting in degradation of the viral RNA. The 2'5'-A oligomers are then hydrolysed by a 2'5'-A phosphodiesterase. This action may reduce or inactivate a continued

endonuclease response (Taylor and Grossberg, 1990).

Another IFN-inducing pathway, involved in the inhibition of translation, is mediated via the Mx protein. This protein was originally described in a population of mice which were resistant to an influenza virus infection (Baron *et al.*, 1991). In mice, it is an IFN-inducible protein with a molecular size of 72 kDa. The mechanism of action of this molecule has not been well characterized. However, speculation suggests that the Mx proteins, following their induction by IFN, affect virus growth by directly inhibiting the synthesis, processing and functional utilization of viral transcripts. An alternative explanation hypothesizes that Mx proteins act indirectly against viruses by altering cellular functions required for intracellular transport of viral proteins (Arnheiter and Meier, 1990).

1.4.4 IFN receptors

A binding site was initially postulated for IFN due to the high specific activity of IFN and the minute quantities present for activity (Rubinstein and Orchansky, 1986). The existence of these receptors was later borne out by experimental evidence. As IFN-alpha and beta share both similar DNA sequence homology and biological activities it is not surprising that these molecules interact with cells through the same receptor. However, IFN-gamma has a distinct receptor. It was further determined that the receptors for IFN-alpha and beta and IFN-gamma were distinct based on biochemical and genetic analyses (Langer and Pestka, 1988). The characteristics of the IFN-alpha/beta receptor follow.

In general, IFN-alpha and beta bind to a single class of high affinity binding sites on human cells (Pestka *et al.*, 1987; Rubinstein and Orchansky, 1986). Depending on the cell type, a low number of (300-20 000) of high affinity (Kd value of 10^{-9} to 10^{-11} M) receptors are present (Rubinstein and Orchansky, 1986). It has been suggested that complete occupation of these receptors with IFN is not necessary for IFN induced activity. However, some stimulation through the receptor is required;

antibodies to the IFN inhibit the development of an antiviral response (Pestka *et al.*, 1987). Scatchard analyses, which determines the interaction between a ligand and its cellular receptor, resulted in curvilinear plots. A number of interpretations may be made from these data, the most likely of which is that there is a second class of binding sites which do not induce any biological activity. This is supported by evidence which demonstrates that the second binding site is very abundant and has a very low affinity (Rubinstein and Orchansky,1986).

Cross-linking studies have allowed for the identification of the IFN-alpha/beta receptor. Bifunctional cross-linking agents such as disuccinimidyl suberate (DSS) were used to bind ^{125}I-IFN to human cell lines. This cross-linked complex migrated at 150 kDa on SDS polyacrylamide gels, resulting in a molecular weight of the receptor of approximately 130 kDa. Following neuraminidase treatment, this complex migrated faster in the polacrylamide gel suggesting that this IFN-binding component may be a glycoprotein (Joshi *et al.*, 1982). Higher molecular size complexes have also been identified by similar procedures. These complexes may contain multiple subunits of the 130 kDa receptor (Langer and Pestka, 1988).

1.5 Bacterial-viral coinfection

Bacterial adherence to mucosal surfaces is the initial step of colonization and infection. As indicated earlier, bacteria, particularly *P. aeruginosa*, have a number of mechanisms which facilitate adherence. However, mechanical factors in the normal lung are efficient in removing these aspirated bacteria. It has been thus suggested that insult to the tracheobronchial tree predisposes an individual to bacterial respiratory infection. The following factors have been associated with provoking the appropriate insult to this area: viral infection, endotracheal intubation and chemical injury.

A number of examples demonstrate that following viral infection, a secondary bacterial infection may occur. For example, the oropharyngeal

colonization of gram negative bacilli increases 3-fold during episodes of upper respiratory infection (Ramirez-Ronda, 1981). Also, bacterial tracheitis is thought to be secondary to a primary viral respiratory infection, usually due to parainfluenza (Donnelly, 1990). These viral infections may result in mucosal cell changes which may further lead to an increased adherence of selected bacteria. Greater numbers of *S. pneumoniae, H influenzae,* and *P. aeruginosa* have been demonstrated to adhere to pharyngeal cells obtained during an experimental influenza virus infection (Fainstein *et al,* 1980). Such an influenza infection produces a desquamation of the tracheal cell surface. It has been shown that *P. aeruginosa* adheres to these desquamating cells but not to normal mucosa, demonstrating that damage to the trachea facilitates adherence of *P. aeruginosa* (Ramphal, 1980).

Another mechanism of viral predisposition may involve changes in the cells involved in immune surveillance. Impairment in alveolar macrophage phagocytosis has been correlated with suppression of pulmonary antibacterial defences in both an influenza virus model of upper respiratory tract infection and an influenza virus model of pneumonic involvement (Nickerson, 1990). Similar studies have demonstrated that both the intracellular killing and the phagocytic ability of neutrophils were compromised, following a parainfluenza infection (Surh, 1984). Impairment of these phagocytic cells may partly account for bacterial superinfection of the infected areas.

An endobronchial infection is considered an integral component in symptomatic patients with CF. At birth, the histopathological data suggest that the infant lungs are normal (Berossian *et al,* 1976). Infants who were diagnosed with CF were followed for one year by Ambman and colleagues (1988). It was determined that the most likely agent to cause severe respiratory illness in these infants was respiratory syncytial virus (RSV). Thus, infection in the CF lung appears to be initiated by viruses.

From the above evidence, it may be concluded that following the onset of a viral infection, bacterial invasion may follow. As noted by many investigators the lungs of older CF patients are colonized by bacteria, predominantly *P. aeruginosa* (Mearns, 1980; Hoiby, 1982; and Pier, 1985). However, a role aside from inititiating infection in the CF lung, may be attributed to viruses in the older CF patient.

Viral infection in CF patients may be associated with fluctuations of illness in these individuals. It has been noted by Marks (1984) that the respiratory-virus season is often heralded by an increase in the frequency of pulmonary exacerbations in CF patients. Associations have been established between these periodic viral infections and deterioration of lung function (Wang *et al.*, 1984) or mortality of the patient (Abman *et al.*, 1988). Wright *et al.* (1976) while investigating the effectiveness of Amantadine-HCl as an antiviral drug, observed that a substantial proportion of exacerbations were associated with viral influenza A infections. These findings have been supported by other studies which determined that 20 - 29% of exacerbations were correlated with viral infections (Efthimiou *et al.*, 1984; Petersen *et al.*, 1981). Recently, a study of 159 adult patients with CF was completed. Three percent of these patients, who were already colonized with *Pseudomonas* species, became infected with varicella-zoster. It was observed that this viral infection triggered significant pulmonary exacerbations, ultimately resulting in lung deterioration (Ong *et al*, 1991). Furthermore, an apparent relationship between viral and atypical infections and *Pseudomonas* related exacerbations has been described (Ong *el al.*, 1989) This relationship has also been suggested to be one of synergism between viruses and subsequent bacterial colonization (Abman *et al.*, 1988). The above observations indicate a possible relationship between viral infection and exacerbations of lung disease in CF.

1.6 Research project

It is evident that there is a relationship between viral infection

and episodes of acute pulmonary exacerbation in the lungs of CF patients, who are chronically infected with *P. aeruginosa*. Possibly, it is not only the virus itself, but also the host's immunological reaction to the virus which triggers the onset of these exacerbations. We hypothesize that the IFN produced in an attempt to halt viral replication interacts with *P. aeruginosa* to up-regulate the expression of specific virulence determinants produced by this organism. This alteration in *P. aeruginosa* virulence may contribute to exacerbations of pulmonary symptoms in CF patients.

The overall objective of this work was to characterize the interaction between Human alpha IFN and *P. aeruginosa* both *in vitro* and *in vivo*. To accomplish this a number of specific aims were identified and included the following:

1. In a dose-response manner, the effects of IFN-alpha on *P. aeruginosa* virulence determinants including exoproducts (exotoxin A, exoenzyme S, total protease and phospholipase) and surface determinants were assessed.
2. The specificity of action of the alpha IFN - *P. aeruginosa* interaction. The effects of incubating these cultures with other IFN species and other cytokines on exoenzyme S were examined.
3. The mechanism of the alpha IFN - *P. aeruginosa* interaction was characterized. The saturability and specificity of binding were determined.
4. The effect of IFN-alpha induction on the chronic rat lung infection model was determined. The pathology of lungs coinfected with RSV and *P. aeruginosa* was assessed.

2.0

METHODS AND MATERIALS

2.1 Bacterial strains

Pseudomonas aeruginosa strains DG1, PAO and PA103, which have been previously characterized (Cash *et al.*, 1979; Holloway *et al.*, 1979; and Liu, 1974) were employed for the following studies. The rationale for employing these strains was based upon the differences in the phenotypic expression of exoenzymes by these organisms. *P. aeruginosa* DG1 produces high levels of exoenzyme S and moderate levels of exotoxin A, phospholipase C and proteases. *P. aeruginosa* PAO produces moderate levels of exoenzyme S, exotoxin A, phospholipase C and proteases. *P. aeruginosa* PA103 produces high levels of exotoxin A and was used only in the examination of the effect of IFN alpha on exotoxin A expression. Prior to use, the strains were stored at -70° C in 1% glycerol.

2.2 Cytokines

Two preparations of recombinant human alpha interferon-2b (IFN alpha) were used for these studies. Protein carrier-free IFN (IFN-f) was a generous gift from P. Sorter (Hoffman LaRoche, N.J.) and was received in a reconstituted form with an activity of 2.2×10^9 international units (IU)/ml. The second preparation of IFN, *INTRON A*, was purchased in a lyophilized form from Schering-Plough, Quebec. *INTRON A* was reconstituted with 1 ml of sterilized dH_2O and had a final activity of 5×10^6 IU/ml (1 ug of protein had an activity of 6.7×10^4 IU). A carrier protein, human serum albumin (HSA), was present in the *INTRON A* preparation; this protein supplied stability to the IFN. Although there was no discernable physical difference between the two preparations, there was a difference in their ability to induce *P. aeruginosa* exoenzyme release. IFN-f, presumably due to the mode of transport, was unable to affect the expression of *P. aeruginosa* exoproducts.

Rat IFN and IFN-beta were purchased from Lee Biomolecular, Ca. in a lyophilized form. Rat IFN was reconstituted to an activity of 1.5×10^8 and the activity of IFN-beta was 5×10^4.

Interleukin 1 and interleukin 2 (IL-1 and IL-2) where purchased from

ICN Fine Chemicals, Ont. in a lyophilized form. IL-1 was reconstituted to an activity of 2 X 10^4 IU/ml and IL-2 was reconstituted to an activity of 1 X 10^4 IU/ml.

2.3 Enzyme assays

2.3.1 Growth conditions for enzyme assays

Organisms were initially grown in 10 ml of defined medium for 18 h at 32° C or 37° C. Aliquots (100 ul) of these cultures were reinoculated into flasks containing 10 ml of fresh media and the indicated concentration of *INTRON A*. All experiments were performed in triplicate and duplicate assays were completed. Two culture controls were also included: (1) Control flasks were initially incubated with 1 ug/ml of HSA to control for the presence of this carrier molecule in the *INTRON A* preparations. Neither the growth nor the enzyme activity of these cultures were different in comparison to those cultures grown without cytokine or without HSA. Thus, the *P. aeruginosa* culture controls reported in the following experiments were flasks incubated without cytokine and without HSA. (2) The second culture control was completed to insure that *INTRON A* alone did not demonstrate any activity in the *P. aeruginosa* exoenzyme assays. *P. aeruginosa* strain DG1 was incubated in S medium, as described below. Following 18 hours of incubation the supernatants were removed and differing concentrations of *INTRON A* were added to these supernatants. Exoenzyme S activity was determined and those supernatants containing *INTRON A* were compared to those which did not contain *INTRON A*.

2.3.2 ADP-ribosyl transferase activity

ADPR transferase activities were measured in the cell-free supernatants of organisms grown at 32° C as previously described (Woods *et al.*, 1986). *P. aeruginosa* strains PA103 and PAO were grown in a chemically defined medium described by Blumenthal *et al* (A medium, 1987), and toxin A activity measured. Strains DG1 and PAO were grown in S medium, and the exoenzyme S activity measured (Woods and Sokol, 1985).

Following an 8 h incubation, 100 µl aliquots were removed and the cell-free supernatants were obtained by centrifugation (10 000 X g, 20 min). Wheat germ extract was the substrate for both exotoxin A and exoenzyme S activities. The reaction mixture for exoenzyme S assay was as follows: 10 µl culture supernatant, 25 µl buffer (50 mM Tris [pH 7.2], 0.1 mM EDTA, 40 mM dithiothreitol), 25 µl wheat germ extract and 5 µl (^{14}C)NAD (specific activity, 280 mCi/mM; 25 µCi/ml: Amersham Corp., Arlington Heights, IL). This mixture was incubated at 25° C for 30 min, and addition of 10% trichloroacetic acid terminated the reaction. The precipitates were collected by filtration and counted as described by Chung and Collier (1977). Exotoxin A activity was determined as outlined for exoenzyme S, except that the supernatants were activated with 4 mM urea and 1% dithiothreitol for 20 min at 24° C prior to assay (Bjorn *et al.*, 1979).

2.3.3 Proteolytic activity

The proteolytic activity was measured in *P. aeruginosa* cell culture (strains DG1 and PAO) supernatants which were grown in P medium (Jensen *et al.*, 1980) at 37° C. Total proteolytic activity was quantitated by using Hide powder azure (Sigma Chemical Co., St. Louis, MO) as previously reported by Woods *et al.* (1986). Culture supernatants, 1.5 ml, were incubated with 7.5 mg of Hide powder azure at 37° C for 1 h with continuous agitation. The undigested substrate was removed by centrifugation (3 000 X g, 10 min). The protease activity of the supernatants was reported as absorbance at 595 nm.

2.3.4 Phospholipase activity

The phospholipase C activity was determined by the method of Berka and Vasil (1982). *P. aeruginosa* strains DG1 and PAO were grown in tryptose minimal media (TMM) at 32° C. Following an 8 h incubation, cells were removed by centrifugation (10 000 X g, 20 min) and 10 mg of decolorizing carbon was added to 1 ml of culture supernatant. Further centrifugation (5 000 X g, 10 min) removed the carbon and 10 µl of the supernatant fluid was added to 90 µl of a solution containing 250 mM Tris

buffer (pH 7.2), 60% glycerol, 1.0 µM $ZnCl_2$ and 10 mM para-nitrophenylphosphorylcholine (Sigma). The reaction was completed in individual wells of a microtiter test plate (96 well per plate). These plates were incubated at 37° C for 1 h, and the A_{405} was measured spectrophotometrically in a MicroElisa Autoreader.

2.4 Protein determinants

The effect of *INTRON A* on the expression of *P. aeruginosa* strain DG1 protein determinants was examined. Following an 18 h incubation in S medium at 37° C, 100 µl aliquot of strain DG1 was removed and reinoculated into fresh S medium containing either, 5 X 10^4 IU of IFN-f, 5 X 10^4 IU of *INTRON A*, or 0.01 ug/ml of HSA. The cells were incubated at 37° C and then at the indicated time, 100 µl of the culture was removed and centrifugated (10 000 X g, 20 min). The cell pellet was dissolved in solution containing 0.05 M Tris (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% (vol/vol) glycerol, 0.001% bromophenol blue and 1% (vol/vol) 2-mercaptoethanol. These samples were boiled for 1 min and then layered on a 5-15% gradient SDS-polyacrylamide gel and electrophoresis was conducted as described by Laemmli (1970). After electrophoresis at a constant current of 50 mA for 3 h, the gel was silver stained with Biorad silver stain reagents. Visual comparison between the different samples was then completed.

2.5 Binding characterization

2.5.1 Iodination of interferon

Iodobeads (Pierce Chemicals, Rockford, IL) were utilized to iodinate both IFN-f and *INTRON A*. The iodobeads were initially washed with 1 ml of 10 mM phosphate buffered saline (PBS) per bead. These beads were then added to a solution containing approximately 100 µg of IFN and 0.1 mCi of ^{125}iodine (Amersham Corp., Oakville Ont., {3.7 GBq/ml, 100 mCi/ml, NaOH solution [pH 7-11]}). Following a 15 min incubation at room temperature, the beads were removed. IFN-bound label was separated from unbound label by employing membrane ultrafiltration units (Ultrafree-MC, Millipore

Corp., Beford, MA.) as described by Lipford *et al.* (1990). The protein concentration of the ^{125}I-IFN was determined by the use of a BioRad (Richmond, CA) protein assay procedure.

2.5.2 Outer membrane isolation

To determine the site of binding of IFN, binding kinetics were calculated on both *P. aeruginosa* strain DG1 whole cell and outer membrane preparations. Outer membrane preparations were isolated using an altered technique which was initially described by Hancock and Nikaido (1978). Cells were grown in 500 ml of S medium, and collected by centrifugation (10 000 X g, 20 min). The cells were washed with Tris-HCl (pH 8.0), resuspended in 20 ml of 20% sucrose/Tris buffer which also contained 1 mg each of DNase and RNase, sonicated and treated with lysozyme and phenylmethyl sulfonyl fluoride as indicated by Sokol and Woods (1983). The cell debris was removed by centrifugation (1000 X g) and the supernatant was layered onto a two-step sucrose gradient (60-70%). These preparations were centrifuged (30 000 rev/min, SW41 Tr rotor) and the resulting lower bands, containing the outer membranes, were collected and stored at -70° C in 20% sucrose/30 mM Tris-HCl.

2.5.3 Preparation of P. aeruginosa whole cells

P. aeruginosa cells were removed from -70° C, plated on M9 medium (Sokol and Woods, 1986) and plates were incubated overnight at 37° C. Cells were then inoculated into 10 ml of S medium, grown for 18 h at 32° C in a shaker bath, collected by centrifugation (10 000 X g, 20 min) and resuspended in PBS to an A_{540} of 0.35 (approximately 3 X 10^9 cells/ml). As determined by a BioRad protein assay this quantity of organisms had a protein concentration of 76 µg/ml.

2.5.4 Binding kinetics

Similar IFN binding protocols were employed for both *P. aeruginosa* whole cell and outer membrane preparations. Adsorption isotherms of binding were obtained by incubating a constant cell or outer membrane concentration with increasing concentrations of ^{125}I-IFN as indicated by

Morgensen *et al.* (1981). One hundred µl aliquots of 76 µg/ml of either DG1 whole cells or DG1 outer membranes were incubated with the labelled IFN for 2 h at 4° C. The cell or outer membranes were washed three times with cold 10 mM PBS and the cell-bound and free ^{125}I-IFN was counted in a gamma counter. Triplicate assays were conducted for these experiments. Similar binding protocols were utilized for both IFN-f and *INTRON A*.

The saturation and affinity of binding was estimated according to the method of Scatchard (1949). Where linear plots resulted, the X-intercept was used to determine the number of binding sites and the slope identified affinity of binding. Where curvilinear plots resulted, analysis was completed as indicated by Morgensen *et al* (1981).

2.6 Cleavage of IFN by *P. aeruginosa* proteases

To determine the ability of *P. aeruginosa* alkaline protease and elastase to digest IFN a procedure similar to that of Parmley *et al.* (1990) was used. Both IFN-f and *INTRON A* were treated with purified *P. aeruginosa* alkaline protease and elastase (Nagase Biochemicals Ltd., Japan) under similar conditions. Concentration of 110 µg/ml of *INTRON A* and 11 µg/ml of IFN-f were prepared in 10mM PBS. The IFN was combined with an equal volume of protease in the same buffer at enzyme-substrate weight ratios of 0.001 to 1.0. Following 4 h of incubation at 37° C the reaction mixtures were applied to either 15% SDS-PAGE or 16.5% tricine-SDS-PAGE (Schagger and Jagow, 1987). Proteins were electrophorectically transferred to nitrocellulose (BioRad) with a transblot apparatus at 20 mA. The nitrocellulose was blocked with 1% skim milk in 10 mM PBS for 1 hour at 37° C. Polyclonal antibody (1:100 dilution), produced by immunization with *INTRON A*, was then incubated with the blot for 2 h at 37° C. Following washing the blot was incubated with a 1:500 dilution of horseradish peroxidase conjugated protein A for 2 h at 37° C and then was developed with 4-chloro-1-naphthol substrate (Sigma) containing 0.2% hydrogen peroxide.

2.7 *In vivo* experiments

2.7.1 Induction of IFN

Experiments were conducted, *in vivo*, to determine the effect of IFN induction on lung pathology. Respiratory syncytial virus (RSV) was used to induce IFN production. All *in vivo* experiments were performed in a model of chronic lung infection in rats as described by Cash *et al.* (1979). Male rats weighing 200-220 g each were inoculated with 0.05 ml containing a bead suspension of either *P. aeruginosa* DG1, 5 X 10^4 PFU of RSV, or DG1 and 5 X 10^4 PFU of RSV. The organisms were administered via the trachea and were placed in the left lung. Ninety days following inoculation, bronchial lavage fluid was obtained by flushing the lungs with 7.5% saline. This fluid was then measured for IFN levels by plaque inhibition assays. The lungs were also removed, formalin fixed, sectioned and assessed for pathology.

2.7.2 Direct administration of IFN

Rats were chronically infected with *P. aeruginosa* DG1 as indicated above. Five weeks following this inoculation, 1 X 10^3 IU/ml to 1 X 10^6 IU/ml of *INTRON A* was administered, via the trachea into the left lobe of the infected rats. The lungs were excised, 48 h later. Sectioning and assessment of pathology was also performed on these lungs.

2.8 Statistics

The data were subjected to either unpaired Student's T-tests or one way analysis of variance, as indicated by figure legends. Statistical analyses were conducted using a PC software package (Instat).

3.0

RESULTS

3.1 *P. AERUGINOSA* EXOENZYME ASSAYS

3.1.1 Effect of IFN on P. aeruginosa growth

The effect of *INTRON A* on the growth of *P. aeruginosa* DG1 (Table 1A) and PAO (Table 1B) cultures was determined. Cells were incubated with 5 X 10^4 IU/ml of *INTRON A* for 24 h. As a control for the presence of carrier albumin in the *INTRON A* preparation, separate cultures were grown with 0.01 µg/ml of HSA. These two cultures were compared to flasks of DG1 which were grown in the absence of IFN. The growth of the organisms was not significantly different under the different culture conditions. These *P. aeruginosa* cells also appeared to be at similar stages of growth as assessed by comparison of the absorbance at the various time points.

3.1.2 Effect of IFN on exoenzyme expression

The effect of increasing concentrations of INTRON A on *P. aeruginosa* exoenzyme S activity of *P. aeruginosa* strain PAO is demonstrated in Figure 1. A partial dose-response relationship was observed between increasing concentrations of *INTRON A* and resulting increased expression of exoenzyme S, relative to untreated control flasks. However, at an *INTRON A* concentration of 5 X 10^4 IU/ml, a threshold response was seen where greater concentrations of *INTRON A* did not reflect a greater increase in exoenzyme S activity. Thus, for the following assays only this concentration of *INTRON A* (5 X 10^4) was used.

P. aeruginosa PA103 exotoxin A expression, in the presence of 5 X 10^4 IU/ml of *INTRON A*, was not significantly different from that of untreated control cultures (Table 2a). This result was consistent throughout the 20 h incubation period. There was, however, a significant decrease in exotoxin A expression in those *P. aeruginosa* PAO cultures which were exposed to *INTRON A* (Table 2B). This decrease in exotoxin A activity, relative to the untreated control flasks, was recorded after 8 and 20 h incubation. Whereas the exotoxin A activity of strain PAO peaked after 8 h of incubation, that of strain PA103 continually increased.

TABLE 1A. The effect of *INTRON A* on *P. aeruginosa DG1* growth.

Time[a]	Control (A_{540})[b]	Albumin (A_{540})[c]	IFN (A_{540})[d]
4	0.052 +/- .001	0.054 +/- .006	0.006 +/- 0.001
8	0.11 +/- .01	0.12 +/- .02	0.13 +/- .01
12	0.16 +/- .02	0.16 +/- .01	0.19 +/- .01
16	0.40 +/- .01	0.42 +/- .06	0.39 +/- .02
20	0.84 +/- .01	0.83 +/- .24	0.92 +/- .13
24	1.42 +/- .01	1.36 +/- .20	1.49 +/- .10

[a]Cells were incubated the indicated time, measured in hours.

[b]These cells were incubated in S medium.

[c]These cell were incubated in S medium with the addition of 0.01 µg/ml of HSA.

[d]These cells were incubated in S medium with the addition of 5 X 10^4 IU/ml of *INTRON A*.

TABLE 1B. The effect of *INTRON A* on *P. aeruginosa* PAO growth.

Time[a]	Control (A_{540})[b]	Albumin (A_{540})[c]	IFN (A_{540})[d]
4	0.059 +/- .01	.07 +/- .01	.07 +/- .01
8	0.13 +/- .001	0.14 +/- .03	0.16 +/- .02
12	0.17 +/- .04	0.22 +/- .05	0.28 +/- .05
16	0.47 +/- .18	0.72 +/- .35	0.93 +/- .23
20	1.12 +/- .08	1.53 +/- .15	1.53 +/- .15
24	1.71 +/- .10	1.71 +/- .07	1.71 +/- .03

[a]Cells were incubated the indicated time, measured in hours.

[b]These cells were incubated in S medium.

[c]These cell were incubated in S medium with the addition of 0.01 µg/ml of HSA.

[d]These cells were incubated in S medium with the addition of 5×10^4 IU/ml of *INTRON A*.

FIGURE 1. The effect of IFN on *P. aeruginosa PAO* exoenzyme S expression. The cells were grown overnight in S medium at 32° C. 100 µl aliquots were then inoculated into fresh S medium (con) or S medium to which increasing concentrations (1 X 10^3 - 1 X 10^5 IU/ml) of *INTRON A* were added. These cultures were incubated at 32° C. At the indicated time points, 100 µl aliquots were removed and the exoenzyme S activity was quantitated by ADP-ribosyl transferase activity. This activity was reported as counts per minute (CPM).

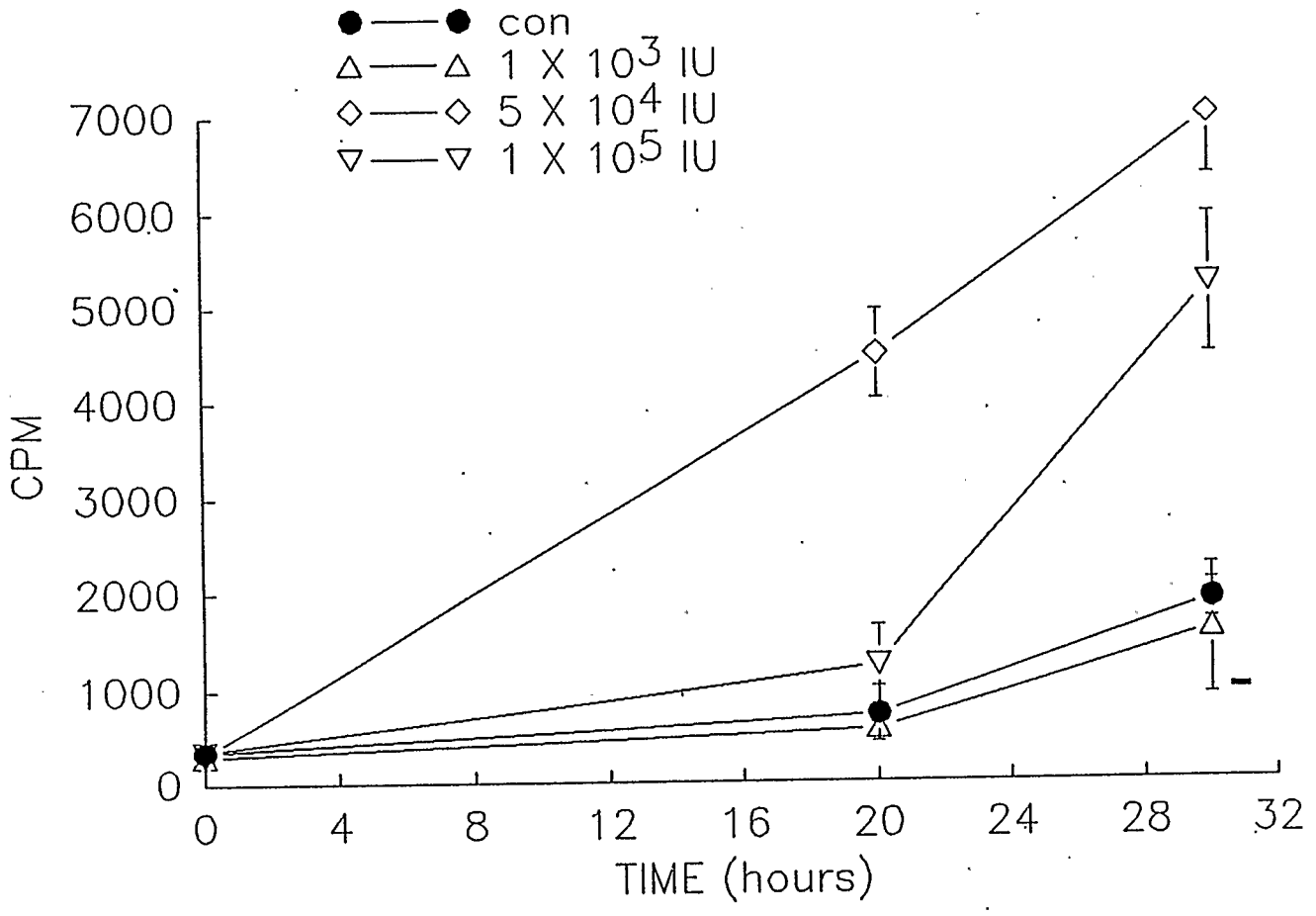

con
1 X 10^3 IU
5 X 10^4 IU
1 X 10^5 IU
CPM
7000
6000
5000
4000
3000
2000
1000
0
0
4
8
12
16
20
24
28
32
TIME (hours)

TABLE 2A. The effect of IFN on *P. aeruginosa* PA103 exotoxin activity.

Time (h)[a]	Control (CPM)[b]	IFN (CPM)[c]
0	1279 +/- 19	1266 +/- 27
8	4459 +/- 314	4184 +/- 356
20	5407 +/- 451	4709 +/- 141

[a]Time following inoculation into A medium.

[b]Organisms were inoculated into untreated A medium. A activity was measured by ADP-ribosyl transferase activity. The mean activity and SEM are reported as counts per minute (CPM).

[c]Organisms were inoculated into A medium containing 5 X 10^4 IU/ml of *INTRON A*. Activity was measured by ADP-ribosyl transferase activity. The mean activity and SEM are reported as counts per minute (CPM).

TABLE 2B. The effect of IFN on *P. aeruginosa* PAO exotoxin activity.

Time (h)[a]	Control (CPM)[b]	IFN (CPM)[c]
0	1138 +/- 26	1125 +/- 5
8	1849 +/- 160	1387 +/- 45*
20	775 +/- 62	427 +/- 107*

[a]Time following inoculation into A medium.

[b]Organisms were inoculated into untreated A medium. A activity was measured by ADP-ribosyl transferase activity. The mean activity and SEM are reported as counts per minute (CPM).

[c]Organisms were inoculated into A medium containing 5 X 10^4 IU/ml of *INTRON* A. Activity was measured by ADP-ribosyl transferase activity. The mean activity and SEM are reported as counts per minute (CPM).

*Significantly different from control ($p < 0.05$).

Following the addition of 5 X 10^4 IU/ml of *INTRON A*, exoenzyme S activity was significantly altered. *P. aeruginosa* DG1 cultures exposed to *INTRON A* demonstrated an increased exoenzyme S expression at 8 and 16 h of incubation, relative to untreated control cultures (Table 3A). A similar increase in exoenzyme S activity was observed for strain PAO (Table 3B). Those cultures which were exposed to *INTRON A* displayed an increased exoenzyme S activity after 16 h incubation. Although, the exoenzyme S expression increased with time in both P. aeruginosa strains, *INTRON A* appeared to have a greater effect on strain DG1 than PAO. This is demonstrated by a significant difference between the slopes representing the exoenzyme activity of the two strains over time.

The addition of 5 X 10^4 IU/ml of *INTRON A* to *P. aeruginosa* DG1 and PAO cell cultures altered the total proteolytic activity expressed by these cells (Tables 4A and 4B). Both strains, when grown in the presence of *INTRON A*, exhibited elevated total protease activity relative to untreated cultures after 7 h of incubation. This increase in expression occurred only in the initial phase of growth.

Those *P. aeruginosa* cultures which were incubated with 5 X 10^4 IU/ml of *INTRON A* expressed significantly less phospholipase C activity then those cultures grown without *INTRON A*. P. aeruginosa DG1 demonstrated this decrease following 24 and 40 h incubation (Table 5A) and the activity of strain PAO was decreased following 16 and 24 h incubation with *INTRON A* (Table 5B). Whereas the phospholipase C expression of the control DG1 culture plateaued at 40 h, that of the PAO culture continually increased.

Experiments were conducted to determine the effect of multiple additions of *INTRON A* on *P. aeruginosa* DG1 exoenzyme S expression (Table 6). One hundred µl of overnight DG1 cultures were subcultured into control S media or S media to which 5 X 10^4 IU/ml of *INTRON A* was added. These cultures were incubated for 8 h at which time exoenzyme S activity was assayed and 5 X 10^4 IU/ml of *INTRON A* was once again administered.

TABLE 3A. The effect of IFN on *P. aeruginosa* DG1 exoenzyme S activity.

Time (h)[a]	Control (CPM)[b]	IFN (CPM)[c]
0	541 +/- 21	540 +/- 36
8	934 +/- 15	1200 +/- 62**
16	5316 +/- 819	13 430 +/- 563**
24	16 243 +/- 661	16 778 +/- 124

[a]Time following inoculation into S medium.

[b]Organisms were inoculated into untreated S medium. S activity was measured by ADP-ribosyl transferase activity. The mean activity and SEM are reported as counts per minute (CPM).

[c]Organisms were inoculated into S medium containing 5 X 10^4 IU/ml of *INTRON A*. Activity was measured by ADP-ribosyl transferase activity. The mean activity and SEM are reported as counts per minute (CPM).

*Significantly different from control ($p < 0.01$).

TABLE 3B. The effect of IFN on *P. aeruginosa* PAO exoenzyme S activity.

Time (h)[a]	Control (CPM)[b]	IFN (CPM)[c]
0	355 +/- 31	373 +/- 21
8	1061 +/- 142	1397 +/- 163
16	5509 +/- 299	7714 +/- 384**
24	9860 +/- 380	9437 +/- 571

[a]Time following inoculation into S medium.

[b]Organisms were inoculated into untreated S medium. S activity was measured by ADP-ribosyl transferase activity. The mean activity and SEM are reported as counts per minute (CPM).

[c]Organisms were inoculated into S medium containing 5 X 10^4 IU/ml of *INTRON A*. Activity was measured by ADP-ribosyl transferase activity. The mean activity and SEM are reported as counts per minute (CPM).

**Significantly different from control ($p < 0.01$).

TABLE 4A. The effect of IFN on *P. aeruginosa* DG1 protease activity.

Time (h)[a]	Control (A_{595})[b]	IFN (A_{595})[c]
0	0.95 +/- .04	0.93 +/- .03
7	1.13 +/- .02	1.32 +/- .02**
22	1.36 +/- .02	1.41 +/- .12
28	1.48 +/- .28	1.29 +/- .04

[a]Time following inoculation into P medium.

[b]Organisms were inoculated into untreated P medium. Protease activity was measured by Hide powder azure assay. The mean activity and SEM are reported as absorbance at 595 nm (A_{595}).

[c]Organisms were inoculated into P medium containing 5 X 10^4 IU/ml of *INTRON A*. Activity was measured by Hide powder azure assay. The mean activity and SEM are reported as absorbance at 595 nm (A_{595}).

**Significantly different from control ($p < 0.01$).

TABLE 4B. The effect of IFN on *P. aeruginosa* PAO protease activity.

Time (h)[a]	Control (A_{595})[b]	IFN (A_{595})[c]
0	0.74 +/- .04	0.76 +/- .02
7	1.01 +/- .03	1.34 +/- .10*
22	1.31 +/- .01	1.35 +/- .06
28	1.30 +/- .15	1.31 +/- .01

[a]Time following inoculation into P medium.

[b]Organisms were inoculated into untreated P medium. Protease activity was measured by Hide powder azure assay. The mean activity and SEM are reported as absorbance at 595 nm (A_{595}).

[c]Organisms were inoculated into P medium containing 5 X 10^4 IU/ml of *INTRON A*. Activity was measured by Hide powder azure assay. The mean activity and SEM are reported as absorbance at 595 nm (A_{595}).

*Significantly different from control ($p < 0.05$).

TABLE 5A. The effect of IFN on *P. aeruginosa* DG1 phospholipase C activity.

Time (h)[a]	Control (A_{405})[b]	IFN (A_{405})[c]
16	0.08 +/- .02	0.04 +/- .02
24	0.11 +/- .01	0.06 +/- .01*
40	0.20 +/- .03	0.03 +/- .02**
48	0.08 +/- .01	0.05 +/- .04

[a]Time following inoculation into TMM.

[b]Organisms were inoculated into untreated TMM. The mean activity and SEM are reported as absorbance at 405 nm (A_{405}).

[c]Organisms were inoculated into TMM containing 5 X 10^4 IU/ml of *INTRON A*. The mean activity and SEM are reported as absorbance at 405 nm (A_{405}).

*Significantly different from control ($p < 0.05$).

**Significantly different from control ($p < 0.01$).

TABLE 5B. The effect of IFN on *P. aeruginosa* PAO phospholipase C activity.

Time (h)[a]	Control $_{(A405)}$[b]	IFN (A_{405})[c]
16	0.35 +/- .03	0.14 +/- .02*
24	0.36 +/- .01	0.20 +/- .03*
40	0.42 +/- .05	0.23 +/- .01
48	0.76 +/- .38	0.21 +/- .03

[a]Time following inoculation into TMM.

[b]Organisms were inoculated into untreated TMM. The mean activity and SEM are reported as absorbance at 405 nm (A_{405}).

[c]Organisms were inoculated into TMM containing 5 X 10^4 IU/ml of *INTRON A*. The mean activity and SEM are reported as absorbance at 405 nm (A_{405}).

*Significantly different from control ($p < 0.05$).

TABLE 6. The effect of multiple additions of 5 X 10^4 IU/ml of *INTRON A* on *P. aeruginosa* DG1 exoenzyme S activity

Time of assay (h)[a]	Addition of IFN (h)[b]	Control (CPM)[c]	IFN (CPM)[d]
8	0	748 +/- 113	3146 +/- 453*
16	0	5128 +/- 942	14008 +/- 59*
16	0,8		16556 +/- 1395**
24	0	22743 +/- 586	20129 +/- 3168
24	0,8		21336 +/- 1377
24	0,16		18656 +/- 1020

[a]Time following inoculation into S medium.

[b]Time at which 5 X 104 IU/ml of *INTRON A* was added.

[c]Organisms were inoculated into untreated S medium. S activity was measured by ADP-ribosyl transferase activity. The mean activity and SEM are reported as counts per minute (CPM).

[d]Organisms were inoculated into S medium containing 5 X 10^4 IU/ml of *INTRON A*. Activity was measured by ADP-ribosyl transferase activity. The mean activity and SEM are reported as counts per minute (CPM).

*Significantly different from control ($p_{corrected} < 0.05$).

**Significantly different from control ($p_{corrected} < 0.01$).

Following another 8 h incubation, S activity was measured and 5 X 10^4 IU/ml *INTRON A* was again administered. This procedure was repeated one more time, resulting in 3 exoenzyme S assays, and a total of 4 multiple *INTRON A* administrations. One way analysis of variance (ANOVA) was used to examine the differences between the various groups. These analyses indicated that after 8 h of incubation those cultures to which *INTRON A* had been added at 0 h, had a significantly greater exoenzyme S activity than those untreated cultures. At time 16 h, the analysis established that those cultures which received *INTRON A* at time 0 h and time 8 h had significantly increased exoenzyme S activity relative to the untreated cultures. There was no significant difference in the exoenzyme S activity between the two *INTRON A* incubated cultures. At time 24 h, as indicated by the ANOVA results, there were no significant difference between any of the groups.

P. aeruginosa supernatants were incubated with increasing concentrations of *INTRON A* to determine the effect of IFN on the exoenzyme S assay (Table 7). The cultures were incubated for 18 h in S media, the supernatants were obtained, *INTRON A* was added and exoenzyme S assays completed. The results were subjected to ANOVA analysis. For both *P. aeruginosa* strains DG1 (Table 7A) and PAO (Table 7B) the difference among the groups was not significant.

3.1.3 The effect of other cytokines on the expression of P. aeruginosa DG1 exoenzyme S

The effect of IL-1 alpha and IL-2 beta on *P. aeruginosa* DG1 exoenzyme S expression is demonstrated in Table 8. Overnight cultures of DG1, grown in S media, were subcultured into fresh media or media containing 500 ng/ml of either IL-1 or IL-2. Similar concentrations of IL were demonstrated to bind to *E. coli* surfaces (Denis *et al.*, 1991). The exoenzyme S activity of the supernatants were measured and interleukin-treated cultures were compared to non-treated cultures. The exoenzyme S activity of the DG1 cultures increased with time in a comparable fashion

TABLE 7A. The effect of increasing concentrations of *INTRON A* on *P. aeruginosa* DG1 supernatants.

Concentration of IFN (IU)[a]	Mean S activity (CPM)[b]
0 (Control)	18 836 +/- 1253
5×10^3	18 303 +/- 1940
5×10^4	18 989 +/- 1005
1×10^5	19 358 +/- 66

[a]The concentration of *INTRON A* added to the supernatants.

[b]The cultures were grown for 18 h in S media and *INTRON A* was added to the supernatants. Exoenzyme S activity was determined by ADP-ribosyl transferase activity. The mean activity and SEM are reported as counts per minute (CPM).

TABLE 7B. The effect of increasing concentrations of *INTRON A* on *P. aeruginosa* PAO supernatants.

Concentration of IFN (IU)[a]	Mean S activity (CPM)[b]
0 (Control)	992 +/- 229
5 X 10^3	1024 +/- 217
5 X 10^4	1368 +/- 264
1 X 10^5	1095 +/- 270

[a]The concentration of *INTRON A* added to the supernatants.

[b]The cultures were grown for 18 h in S media and *INTRON A* was added to the supernatants. Exoenzyme S activity was determined by ADP-ribosyl transferase activity. The mean activity and SEM are reported as counts per minute (CPM).

TABLE 8. The effect of interleukin 1 and interleukin 2 on *P. aeruginosa* DG1 exoenzyme S expression.

Time (h)[a]	Control (CPM)[b]	IL-1 (CPM)[c]	IL-2 (CPM)[d]
4	518 +/- 27	508 +/- 58	479 +/- 58
8	995 +/- 103	1075 +/- 250	1000 +/- 184
24	17662 +/- 1955	16265 +/- 2125	13665 +/- 771

[a]Time following inoculation into S medium.

[b]Organisms were inoculated into untreated S medium. S activity was measured by ADP-ribosyl transferase activity. The mean activity and SEM are reported as counts per minute (CPM).

cOrganisms were inoculated into S medium containing 500 ng/ml of Il-1. Activity was measured by ADP-ribosyl transferase assay. The mean activity and SEM are reported as counts per minute (CPM).

[d]Organisms were inoculated into S medium containing 500 ng/ml of Il-2. Activity was measured by ADP-ribosyl transferase assay. The mean activity and SEM are reported as counts per minute (CPM).

to that of the control. No significant differences were observed.

Comparisons were made between the effect of *INTRON A*, IFN-beta and rat IFN on *P. aeruginosa* DG1 exoenzyme S expression (Table 9). Concentrations of 5 X 10^4 IU/ml of these IFNs were administered, at time 0 h, into separate DG1 cultures. The exoenzyme S expression of these cultures was compared during a 24 h incubation. In comparison to the exoenzyme S activity of the control culture at 4 h, that activity of both the *INTRON A* and rat IFN cultures was significantly increased. This significant increase in activity was still observed after 8 h of incubation. At this time the exoenzyme S activity of the IFN beta culture was also significantly elevated in comparison to that of the control. After 22 h of incubation the exoenzyme S production of only the *INTRON A* culture remained augmented, relative to that of the control flask.

3.2 PROTEIN DETERMINANTS

The two different IFN preparations were subjected to SDS-PAGE analysis, as demonstrated in Figure 2. That IFN preparation (*INTRON A*) which contained the carrier molecule of HSA is present in lanes 1 and 2. The HSA is clearly visible at a molecular weight of 66.2 kDa and the IFN at a weight of 18 kDa. In lanes 3 and 4 only the 18 kDa IFN protein is present, thus establishing that the carrier-free preparation (IFN-f) is pure.

The expression of *P. aeruginosa* DG1 protein determinants was determined following incubation with IFN. Following incubation, DG1 cells were pelleted and separated on an SDS-PAGE as illustrated in Figure 3. The cells were incubated in: S media, (Figure 3A), S media containing 5 X 10^4 IU/ml of IFN-f (Figure 3B), or S media containing 5 X 10^4 IU/ml of *INTRON A* (Figure 3C). Upon visual inspection, there did not appear to be any significant difference in expression of DG1 proteins between the various groups.

3.3 BINDING CHARACTERIZATION

3.3.1 Binding of INTRON A to P. aeruginosa whole cells

TABLE 9. The effect of alpha, beta and rat IFN on *P. aeruginosa* DG1 exoenzyme S expression.

Time (h)[a]	Control (CPM)[b]	Alpha IFN (CPM)[c]	Beta IFN (CPM)[d]	Rat IFN (CPM)[e]
4	323 +/- 30	2047 +/- 150**	462 +/- 56	680 +/- 52**
8	698 +/-43	4546 +/- 198**	900 +/- 50*	1153 +/- 13**
22	11 786 +/- 400	20 233 +/- 1509**	10 474 +/- 943	13 657 +/- 3164

[a]Time following inoculation into S medium.

[b]Organisms were inoculated into untreated S medium. S activity was measured by ADP-ribosyl transferase activity. The mean activity and SEM are reported as counts per minute (CPM).

[c]Organisms were inoculated into S medium containing 1 X 10^4 IU/ml of *INTRON A*. Activity was measured by ADP-ribosyl transferase assay. The mean activity and SEM are reported as counts per minute (CPM).

[d]Organisms were inoculated into S medium containing 1 X 10^6 IU/ml of IFN beta.

[e]Organisms were inoculated into S medium containing 1 X 10^6 IU/ml of rat IFN.

FIGURE 2. SDS-PAGE of *INTRON A* and IFN-f. Samples (5 µg) were dissolved in 5 X sample buffer (Laemmli, 1970), boiled, and electrophoresed in a 15% SDS-PAGE gel. Molecular weight markers (BioRad) are present at the left. Lanes 1 and 2, *INTRON A* (5 µg/lane); lanes 3 and 4, IFN-f (5 µg/ml).

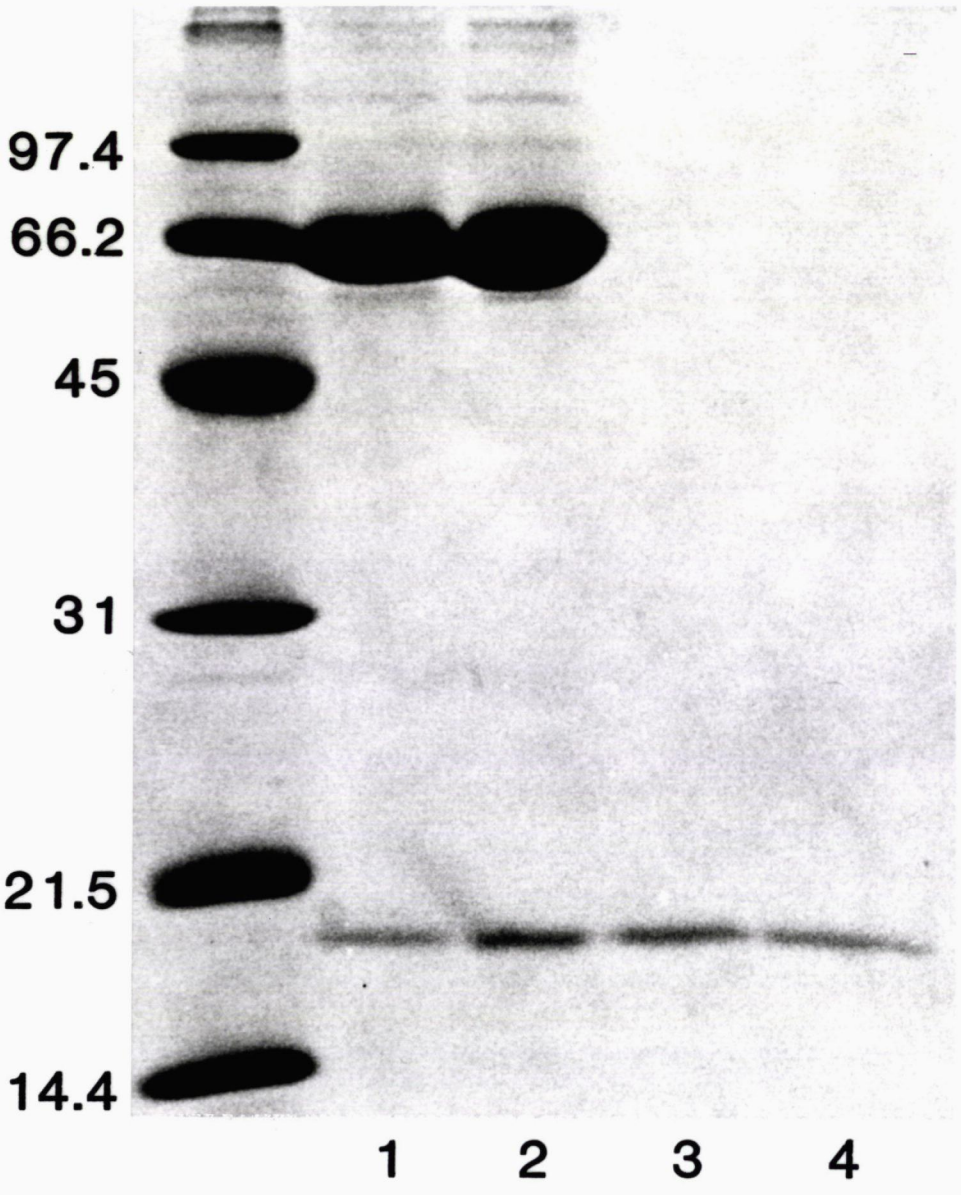
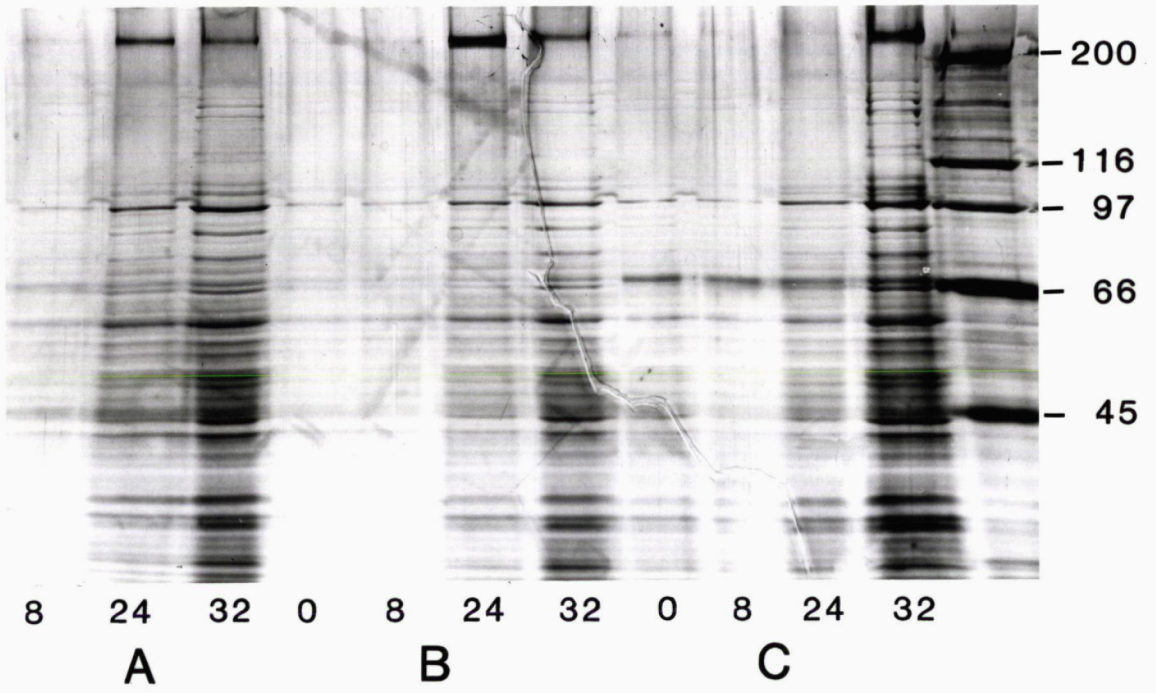

97.4
66.2
45
31
21.5
14.4
1
2
3
4

FIGURE 3. SDS-PAGE of *P. aeruginosa* DG1 surface determinants following IFN incubation. DG1 cells were incubated in either: S media (A), S media containing 5 X 10^4 IU/ml of IFN-f, or S media containing 5 X 10^4 IU/ml of *INTRON A*. The cells were incubated for 32 h, and the time of incubation is indicated at the bottom of the lanes. Following incubation the cells were centrifugated and the pellet was dissolved in 5 X sample buffer (Laemmli, 1970). The samples were layered on a 5-15% gradient gel and electrophoresed. The gel was then silver stained (BioRad). The molecular weight markers are present on the right.


200
116
97
66
45
8 24 32 0 8 24 32 0 8 24 32
A
B
C

The binding of ^{125}I-IFN A to *P. aeruginosa* DG1 was observed to be a saturable phenomenon, indicating that a finite number of specific binding sites for ^{125}I-IFN exist on the *P. aeruginosa* cell surface (Figure 4). Saturation occurred at approximately 2 µg/ml of ^{125}I-IFN. There were approximately 6.9 X 10^9 cells/ml; thus, 2.9 X 10^{-4} pg of ^{125}I-IFN bound to an individual organism. Based upon an estimated molecular weight of 18 kDa for IFN, calculations indicate that there are approximately 9 X 10^3 IFN binding sites per *P. aeruginosa* cell. Calculations performed from the data obtained form the Scatchard plot (determined by extension from initial portion of the curve) and specific activity measurements gave a value of approximately 1 X 10^4 IFN binding sites per *P. aeruginosa* cell and a Kd = 1.7 X 10^{-10}.

3.3.2 Binding of IFN-f to P. aeruginosa whole cells

The binding of ^{125}I-IFN-f to *P. aeruginosa* DG1 cells at 4° C, is demonstrated in Figure 5A. The binding similar to that of *INTRON A*, is saturable (at 3 µg) and can be inhibited by an excess of unlabelled IFN-f (top line). The binding which is not inhibited, is linear with respect to dose, and represents the nonspecific binding of IFN-f (bottom line). The isotherm is then calculated by subtracting the background counts from the total counts (dashed line).

The Scatchard plot for the data illustrated in Figure 5A is demonstrated in Figure 5B. The slope of line obtained from linear regression analysis is significantly different from 0. The reciprocal of the slope gives a dissociation constant of 1.17 X 10^{-11} M. This line intercepts the abscissa at a value of 220 732 CPM, indicating that there are 220 732 CPM of ^{125}I-IFN-f binding to 3 X 10^9 cells. From these values the number of IFN receptors per DG1 cell approximates 1.7 X 10^4.

3.3.3 Binding of ^{125}I-IFN-f to P. aeruginosa outer membranes

The binding of ^{125}I-IFN-f is saturable on *P. aeruginosa* DG1 outer membrane preparations (Fig. 6A). This saturation occurred at approximately 3 µg/ml of ^{125}I-IFN-f.

FIGURE 4. Binding of ^{125}I-*INTRON A* to *P. aeruginosa* DG1 cells. Following overnight incubation in S media at 32° C the culture was centrifuged and the cell pellet was resuspended in 10 mM PBS to an A_{540} of 0.3 nm. 100 µl aliquots were then incubated with increasing concentrations of ^{125}I-*INTRON A* for 2 h at 4° C. The amounts of free ^{125}I-*INTRON A* in the 100 µl supernatant, and cell-bound ^{125}I-*INTRON A*, were determined. The Scatchard plot for the binding data is shown in the insert.

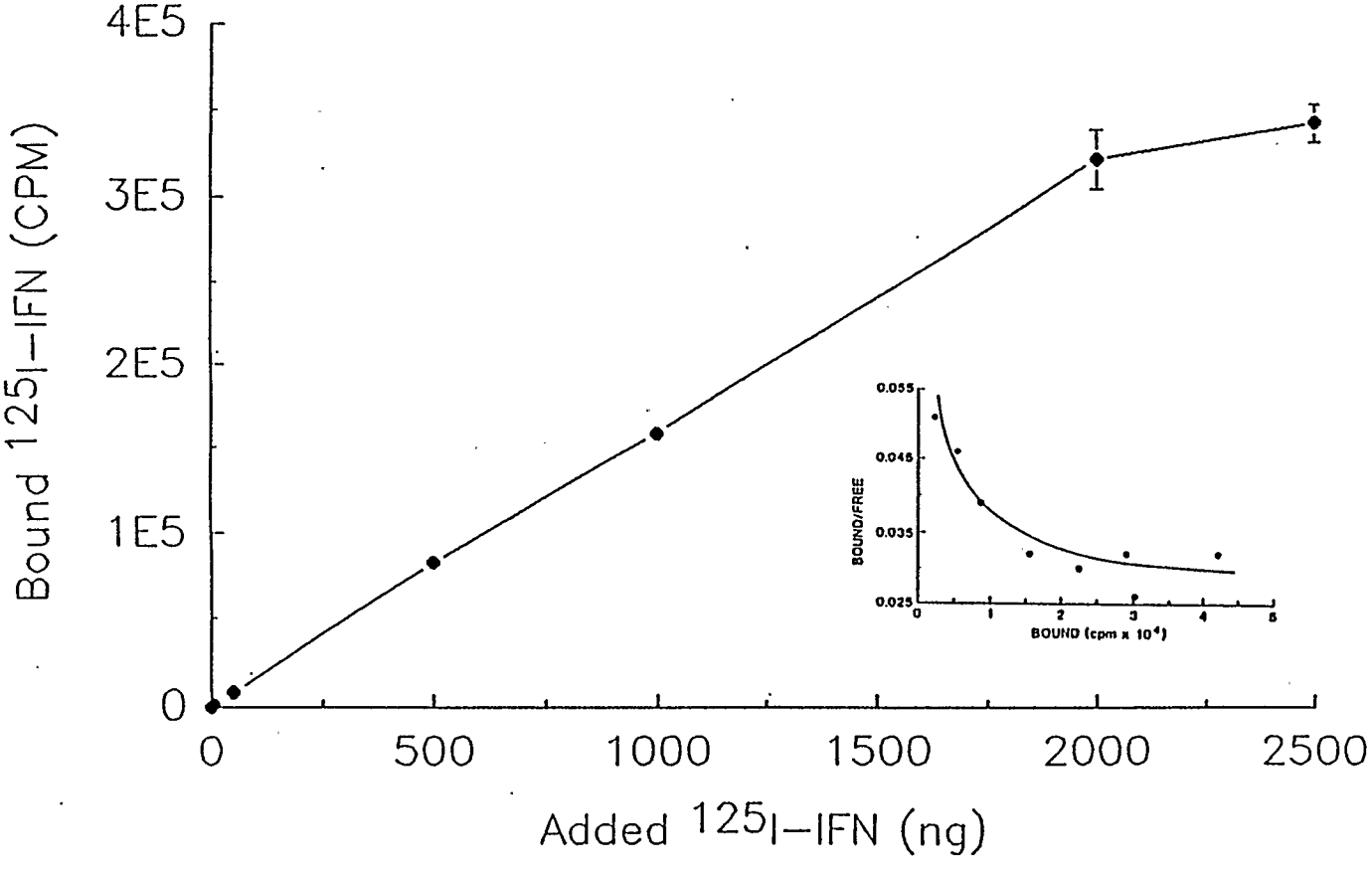

4E5
3E5
2E5
1E5
0
Bound 125I–IFN (CPM)
0
500
1000
1500
2000
2500
Added 125I–IFN (ng)
0.055
0.045
0.035
0.025
BOUND/FREE
0
1
2
3
4
5
BOUND (cpm x 10⁴)

FIGURE 5A. Binding of ^{125}I-IFN-f to *P. aeruginosa* DG1 whole cells. DG1 cells were grown overnight in S medium, centrifuged, and resuspended in PBS. These cells were incubated with either increasing concentrations of ^{125}I-IFN-f (closed circle) or a combination of increasing concentrations of ^{125}I-IFN-f and 30 fold excess unlabelled IFN-f (open circles). The dashed line represents the difference between the two.

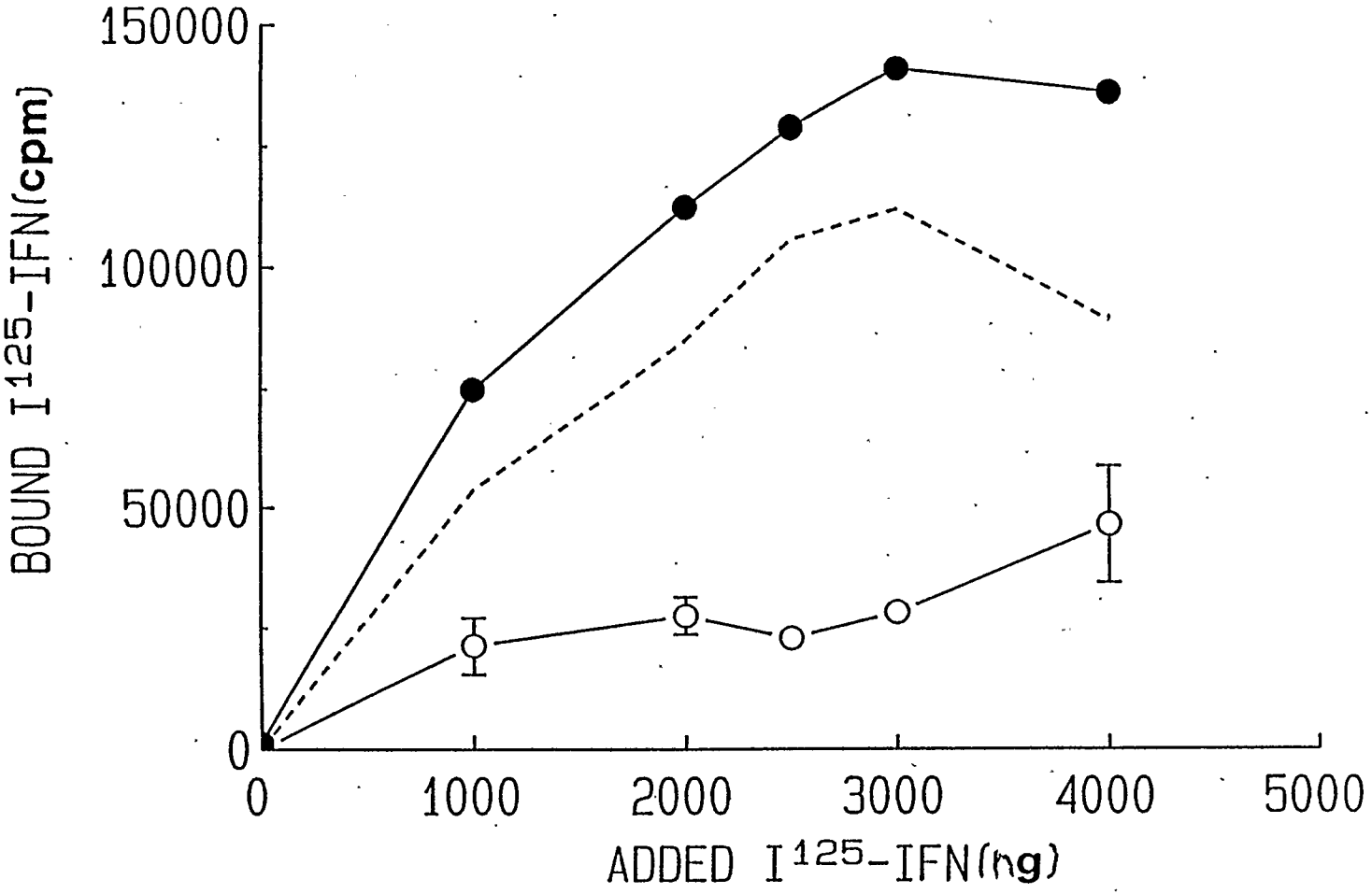

150000
100000
50000
0
BOUND I125-IFN(cpm)
0
1000
2000
3000
4000
5000
ADDED I125-IFN(ng)

FIGURE 5B. Scatchard plot for the binding of ^{125}I-IFN-f to *P. aeruginosa* DG1 whole cells. Reciprocal of the slope Kd = 1.17 X 10^{-11} M. Intercept: 220 732 CPM for 3 X 10^{9} cells.

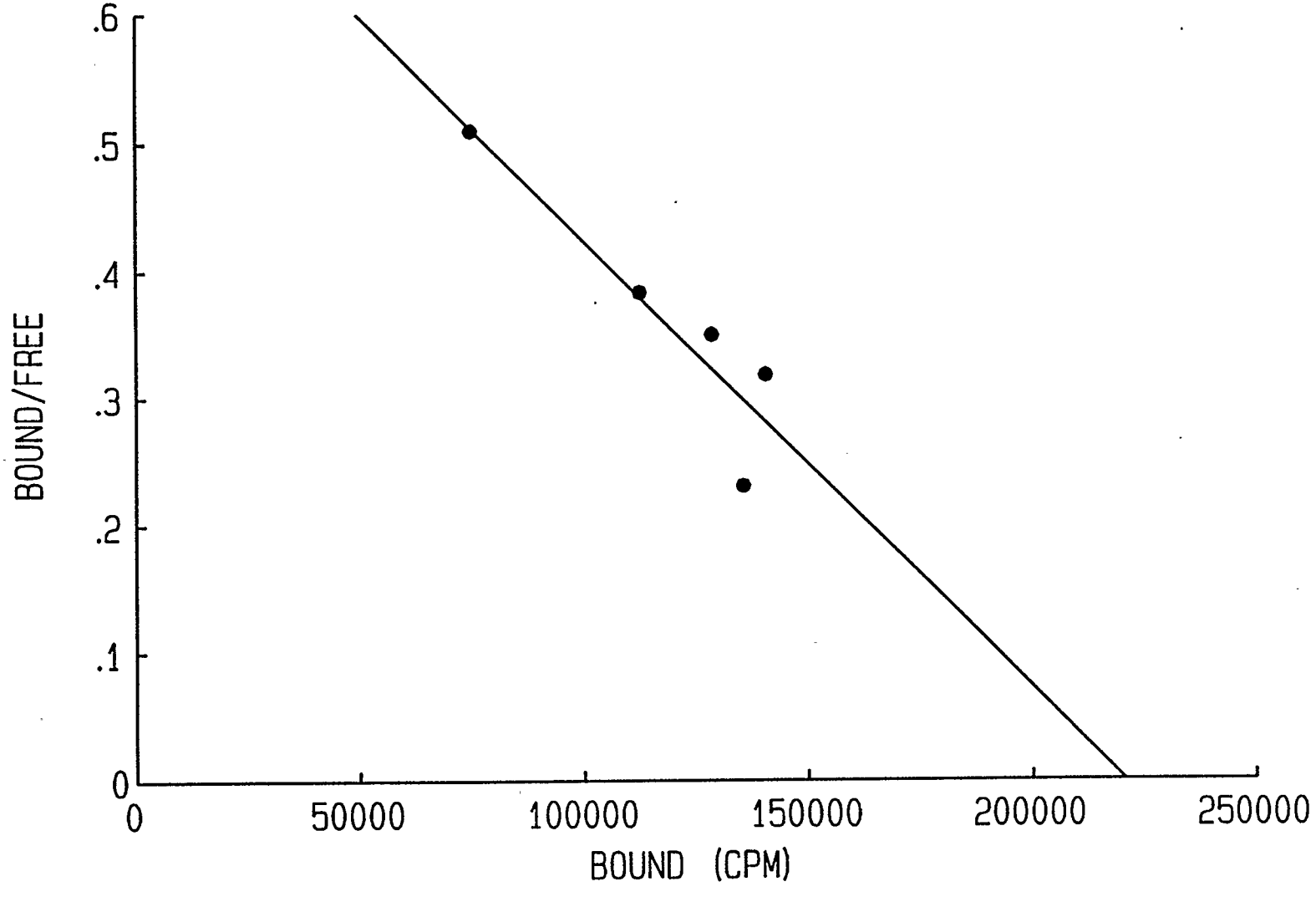
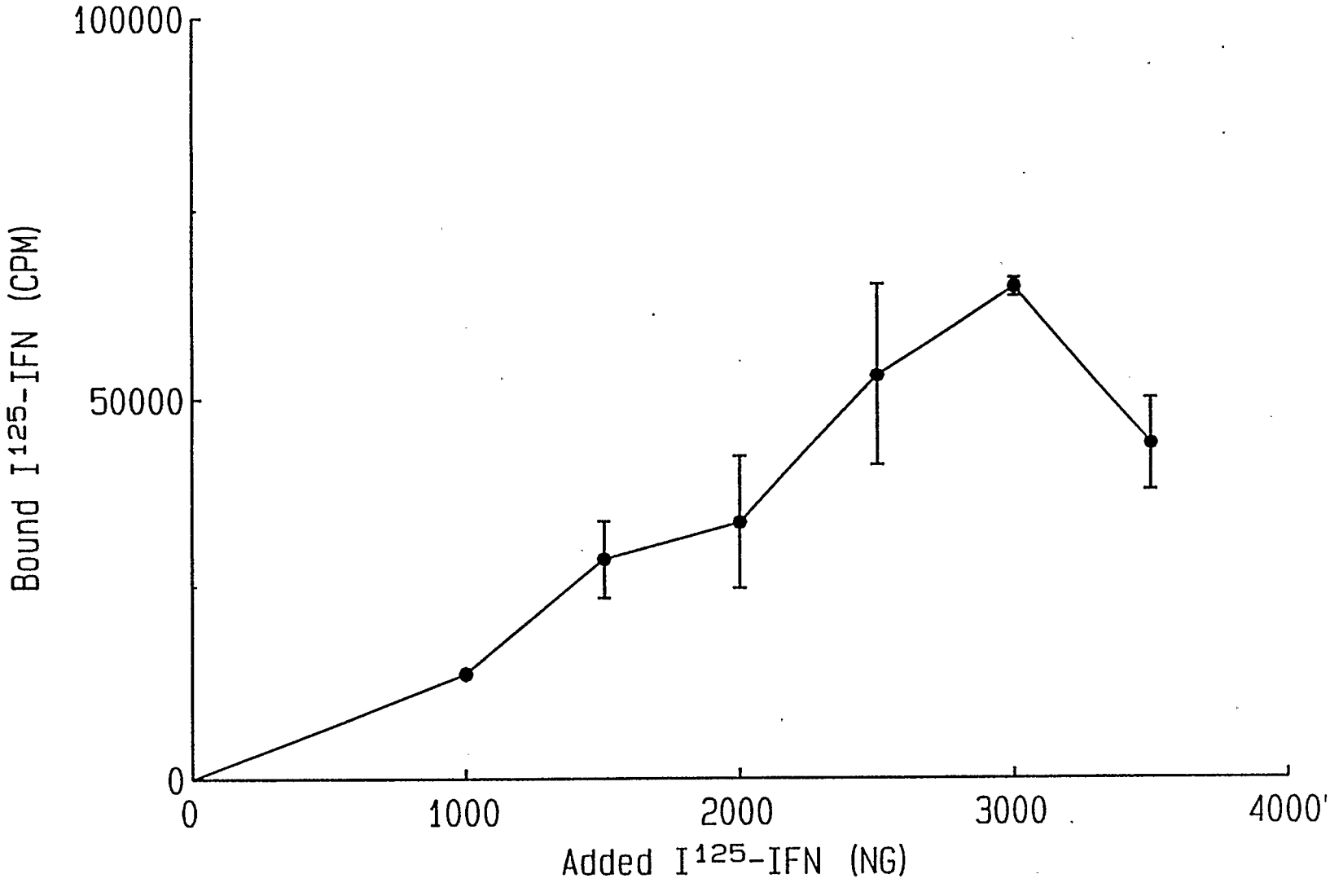

.6
.5
.4
.3
.2
.1
0
BOUND/FREE
0
50000
100000
150000
200000
250000
BOUND (CPM)

FIGURE 6A. Binding of ^{125}I-IFN-f to *P. aeruginosa* DG1 outer membranes. DG1 outer membranes were isolated (Hancock and Nikaido, 1978), and incubated with increasing concentrations of ^{125}I-IFN-f. Cell bound CPM was counted in a gamma counter.


100000
50000
0
Bound I125-IFN (CPM)
0
1000
2000
3000
4000
Added I125-IFN (NG)

The Scatchard plot from the data in Fig 6A is demonstrated in Figure 6B. Linear regression analysis suggests that this plot is non-linear.

3.4 CLEAVAGE OF IFN BY P. AERUGINOSA PROTEASES

3.4.1 Cleavage of INTRON A by P. aeruginosa proteases

The effects of treating *INTRON A* with *P. aeruginosa* alkaline protease and elastase are represented in Figure 7. Loss of reaction with the polyclonal anti-*INTRON A* antibodies at 18 kDa is correlated with loss of activity of *INTRON A* (Parmely *et al.*, 1990). Loss of *INTRON A* bioactivity occurred at elastase-*INTRON A* weight-ratios of 0.05, 0.5 and 1.0. Degradation of HSA by increasing elastase concentrations is also visible. Similar *INTRON A* inactivation occurred at alkaline protease-*INTRON A* ratios of 0.01, 0.05, 0.1, 0.5 and 1.0. At these protease ratios no bands are apparent, which suggests that the *INTRON A* has been completely degraded.

3.4.2 Cleavage of IFN-f by P. aeruginosa proteases

The degradation of IFN-f by *P. aeruginosa* alkaline protease is demonstrated on a 16.5% tricine SDS-polyacrylamide gel (Fig. 8A). A cleavage fragment with an approximate molecular weight of 14.4 kDa is apparent at enzyme-substrate ratios of 0.05 and 0.01. These lower molecular weight species do not react with polyclonal anti-*INTRON A* antibodies (Fig. 8B), which suggests that they are inactive.

Three fragments of different molecular weights result following the degradation of IFN-f by *P. aeruginosa* elastase (Fig. 9A). At enzyme-substrate ratios of 0.001, 0.05, 0.1 the IFN-f fragments have a molecular weight of approximately 7.0 and 4 kDa. The 7 kDa fragment is also present at a ratio of 1.0, however a novel fragment of an approximate molecular weight of 3.0 kDa is also visible. It is interesting to note that the IFN protein (molecular weight of 18 kDa) is not present at enzyme-substrate ratios of 0.001 and 1.0. As previously reported, the lower molecular weight fragments do not react with anti-*INTRON A* antibodies (Figure 9B).

FIGURE 6B. **Scatchard plot for the binding of ^{125}I-IFN-f to *P. aeruginosa* DG1 outer membranes.** Bound and free CPM were counted by the use of a gamma counter.

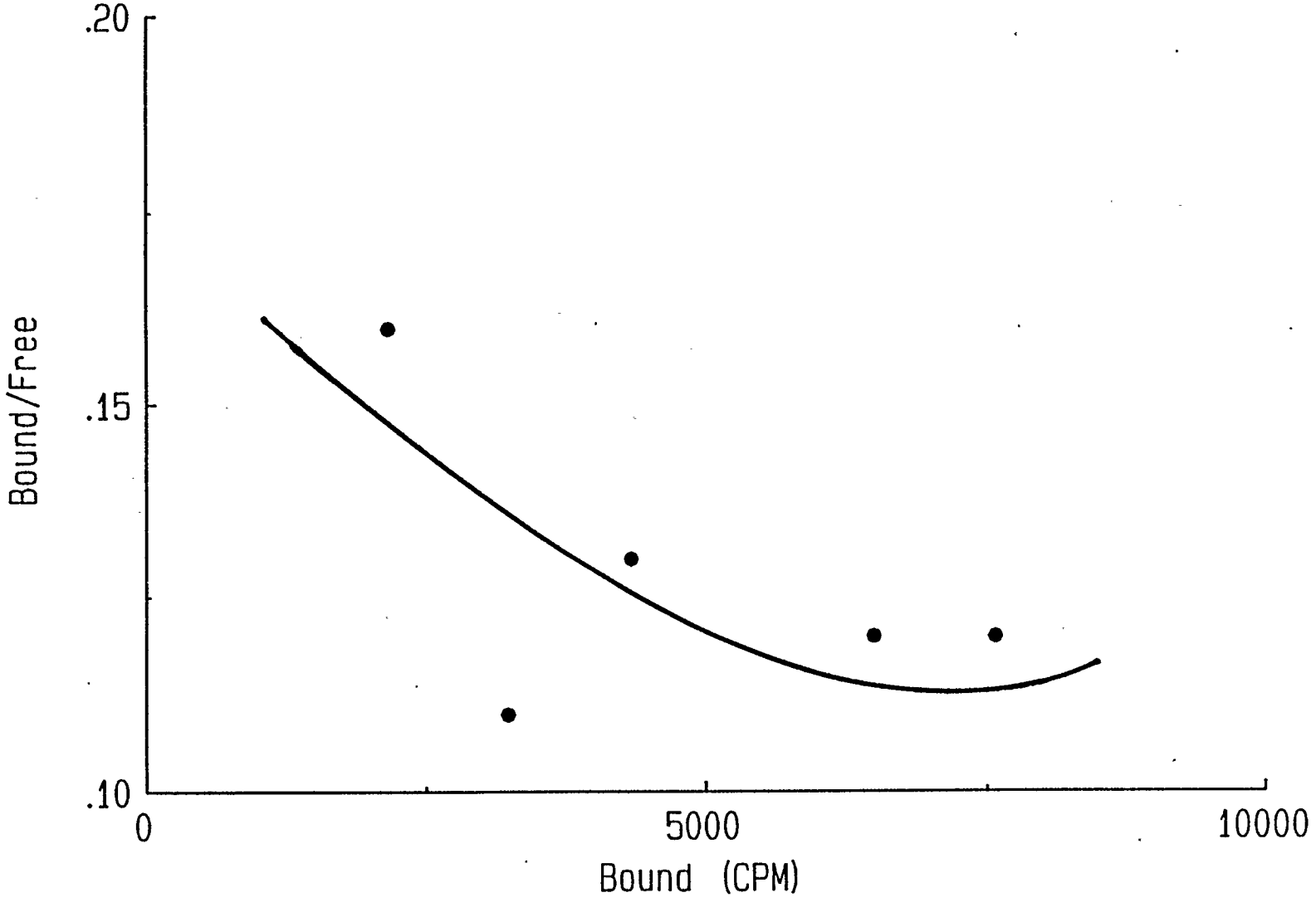

.20
.15
.10
Bound/Free
0
5000
10000
Bound (CPM)

FIGURE 7. **Inactivation of *INTRON A* by *P. aeruginosa* alkaline protease and elastase.** *INTRON A* was treated with either alkaline protease or elastase at various enzyme-substrate ratios for 4 h at 37° C. Shown are the Western immunoblots, from 12.5% SDS-PAGE, which were reacted with polyclonal anti-*INTRON A* antibodies. Samples were incubated with the following elastase-*INTRON A* ratios: lane 1, 0.001; lane 2, 0.01; lane 3, 0.05; lane 4, 0.1; lane 5, 0.5; and lane 6, 1.0. In addition other samples were incubated with the following alkaline protease-*INTRON A* ratios: lane 7, 1.0; lane 8, 0.5; lane 9, 0.1; lane 10, 0.05; lane 11, 0.01; lane 12, 0.001; and lane 13, 0.5. 40 µg/ml of HSA was incubated with alkaline protease-substrate ratio of 0.05 (lane 14) and elastase-substrate ratio of 0.05 (lane 15). *INTRON A* preparation without protease treatment is shown in lane 16. HSA is indicated by the asterix and has a molecular weight of 62.5 kDa. IFN is indicated by the arrow and has a molecular weight of 18 kDa.

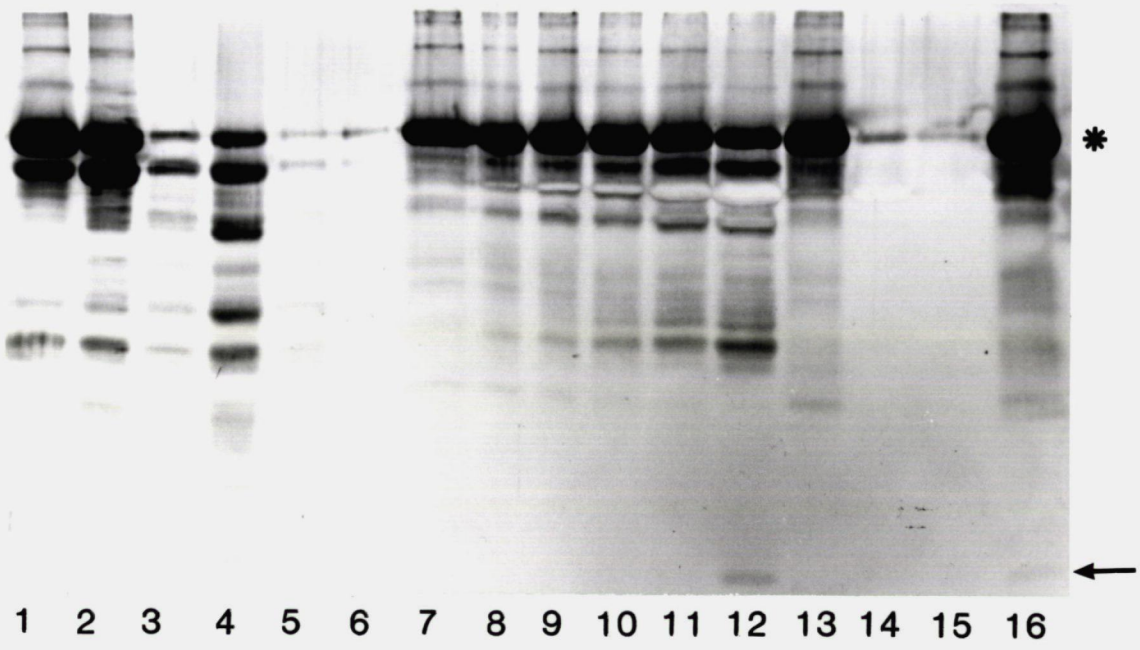

*
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

FIGURE 8A. Degradation of IFN-f by *P. aeruginosa* alkaline protease. Shown is the 16.5% tricine SDS-polyacrylamide gel of these products. Samples were incubated with the following protease-IFN-f ratios: lane 1, 0.01; lane 2, 0.05; and lane 3, 0.1. A fragment of an approximate molecular weight of 14.4 kDa is indicated by the arrow. IFN-f (18 kDa) is visible in lanes 1-3. Lanes 4 and 5 are 20 µg/ml of purified alkaline protease. Lane 6 is 20 µg of untreated IFN-f. The molecular weights are indicated on the left.

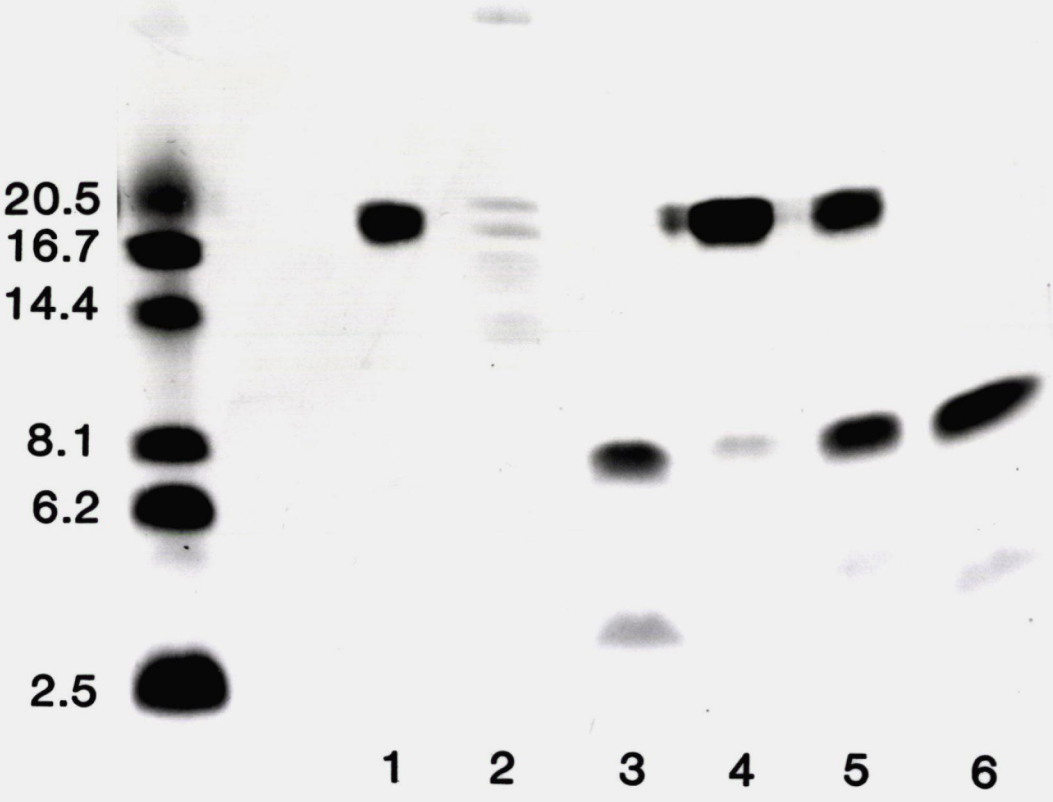

20.5
16.7
14.4
8.1
6.2
2.5
1
2
3
4
5
6

FIGURE 8B. Western immunoblot of Figure 8A. Samples were electrophoresed on a 16.5% tricine SDS-polyacrylamide gel, transferred to nitrocellulose, and then reacted with polyclonal anti-*INTRON A* antibodies. Lanes 1 and 3 are samples which were incubated with the following alkaline protease-IFN-f ratios: 0.01 and 0.1. Lane 6 is a 20 µg sample of untreated IFN-f. The antibodies react with the 18 kDa IFN-f protein.

1 3 6

FIGURE 9A. Degradation of IFN-f by *P. aeruginosa* elastase. Shown is the 16.5% tricine SDS-polyacrylamide gel of these products. Samples were incubated with the following protease-IFN-f ratios: lane 6, 0.001; lane 5, 0.05; lane 4, 0.1; and lane 3, 1.0. Fragments of the approximate molecular weights of: 7.0 and 4.0 kDa are present in lanes 4-6. Fragments of the molecular weights of 7.0 and 3.0 kDa are present in lane 3. IFN-f (18 kDa) is present in lanes 4 and 5. Lane 2 is a sample of 20 µg/ml of purified elastase. Lane 1 is 20 µg of untreated IFN-f. The molecular weights are indicated on the left.

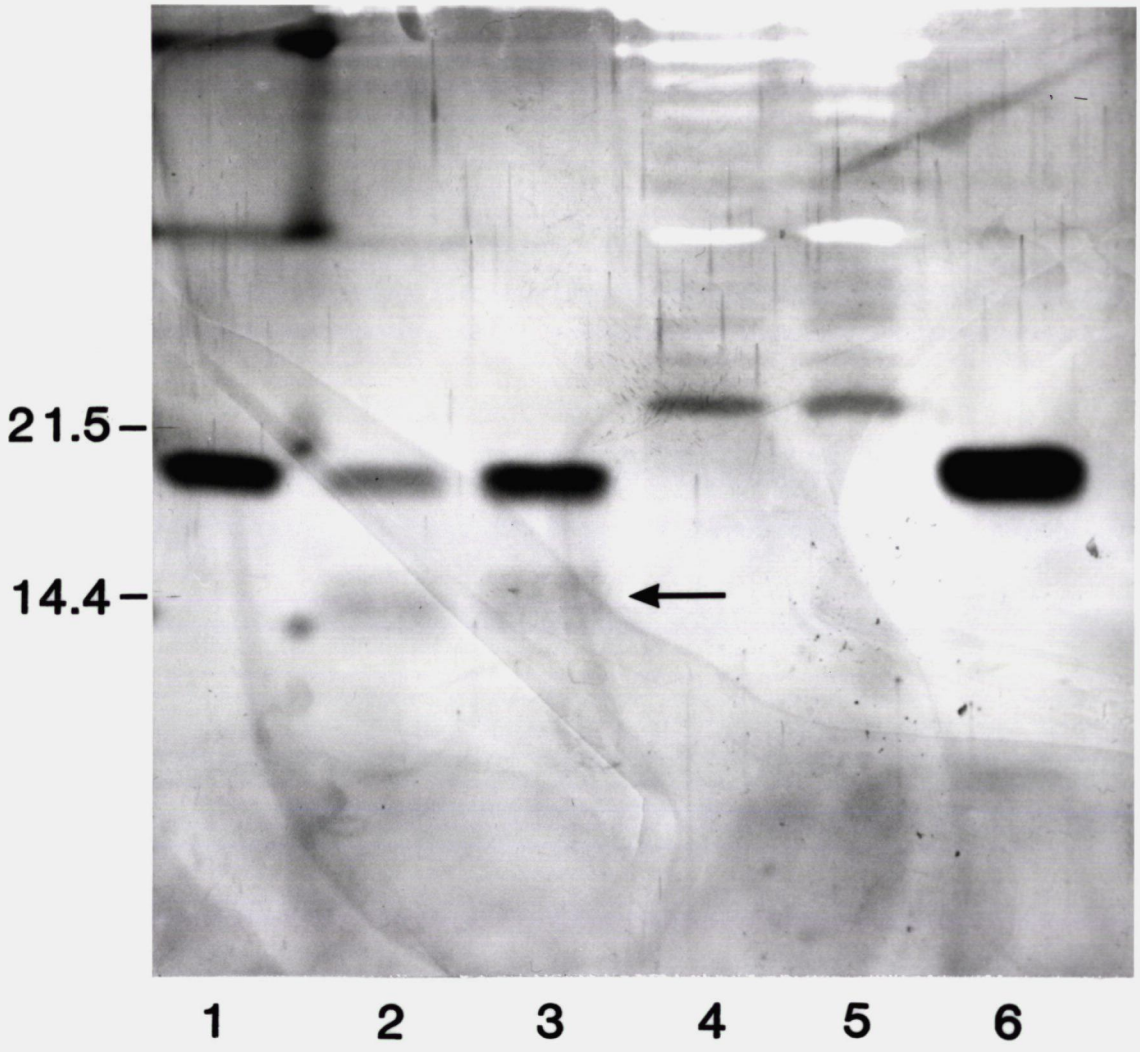
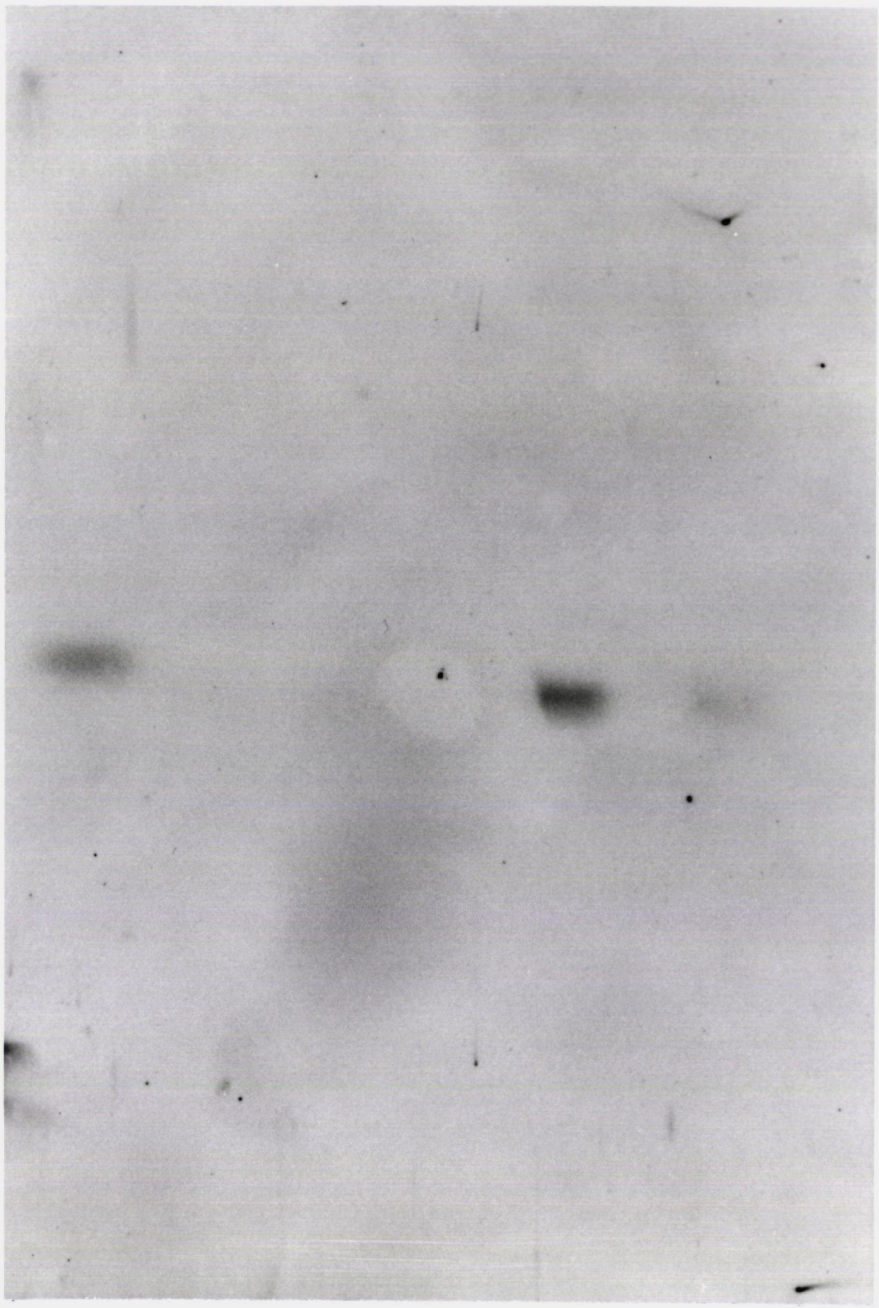

21.5
14.4
1
2
3
4
5
6

FIGURE 9B. Western immunoblot of Figure 9A. Samples were electrophoresed on a 16.5% tricine SDS-polyacrylamide gel, transferred to nitrocellulose, and then reacted with polyclonal anti-*INTRON A* antibodies. Lanes 4 and 5 are samples which were incubated with the following elastase-IFN-f ratios: 0.01 and 0.5. Lane 1 is a 20 µg sample of untreated IFN-f. The antibodies react with the 18 kDa IFN-f protein.



1 4 5

3.5 *IN VIVO* EXPERIMENTS

3.5.1 Induction of IFN

The IFN concentration in the lungs of the chronically infected rats are indicated in Table 10A. Plaque forming units are inversely proportional to IFN concentration: the greater the IFN concentration the greater the viral inhibition; thus, less virus plaques are present. From these data it is apparent that *P. aeruginosa* DG1 and RSV induced similar quantities of IFN. However, augmented IFN concentrations are induced with in animals who are coinfected with both DG1 and RSV.

There is a correlation between IFN induction and subsequent lung pathology as demonstrated in Table 10B. Lung pathology is assessed relative to the whole lung and is reported as a percentage (pathological index). RSV infection resulted in greater lung damage than did *P. aeruginosa* DG1. However, the greatest lung injury occurred in those animals who were coinfected with both DG1 and RSV.

3.5.2 Direct administration of IFN

The effect of administering increasing concentrations of *INTRON A* to chronically *P. aeruginosa* DG1 infected rats is reported in Table 11. Only injection of 1 X 10^5 IU/ml of *INTRON A* to chronically infected animals resulted in significantly greater lung pathology than that observed with *P. aeruginosa* infected animals. Also greater lung injury was observed with a combination of *INTRON A* injection and *P. aeruginosa* infection, than *INTRON A* administration alone.

TABLE 10A. The induction of IFN in infected rats.

Group	PFU[b]
Control[a]	296 +/- 5
DG1[c]	245 +/- 23
RSV[d]	232 +/- 25
DG1 and RSV[e]	141 +/- 15

[a]Medium control, L-2 cells were incubated with PBS and the number of plaques was assayed.

[b]Plaque forming units, an indirect assay for IFN activity. A reduction in the number of PFU indicates IFN activity.

[c]IFN activity of the lung lavage fluid from animals which were chronically infected with *P. aeruginosa* DG1, for 90 days. 10 µl of this fluid was incubated with L-2 cells, which were subsequently challenged with 150 pfu of MHV-A59.

[d]IFN activity of the lung lavage fluid from animals which were chronically infected with 5 X 10^4 PFU of RSV. PFU were determined as previously stated.

[e]IFN activity of the lung lavage fluid from animals which were chronically infected with *P. aeruginosa* DG1 and RSV. PFU were determined as previously stated.

TABLE 10B. Pathology of infected rats.

Group	Pathological Index (%)[a]
DG1[b]	10.3 +/- 2.7
RSV[c]	54.9 +/- 4.8
DG1 and RSV[d]	88.6 +/- 9.9

[a]The pathology of the lungs was determined and expressed as a ratio of damaged/healthy lung.

[b]Lung pathology of *P. aeruginosa* DG1 chronically infected animals.

[c]Lung pathology of RSV chronically infected animals

[e]Lung pathology of animals chronically infected with both *P. aeruginosa* and RSV.

TABLE 11. Pathology of direct inoculation of IFN into rat lungs

Group	Pathology Index (%)[a]
1 X 10^6 IU IFN[b]	11 +/- 9
DG1[c]	11 +/- 3
1 X 10^3 and DG1[d]	12 +/- 9
1 X 10^5 and DG1	47 +/- 9*
1 X 10^6 and DG1	19 +/- 5

[a]Lung pathology as expressed as a ratio of damaged/healthy lung.

[b]Rats were administered *INTRON A*, via the trachea, into the left lung.

[c]Five weeks following a chronic infection with *P. aeruginosa* DG1, the rat lungs were excised. Pathology was then determined.

[d]Animals were chronically infected with *P. aeruginosa* DG1. Five weeks following this inoculation, increasing concentrations of *INTRON A* were administered into the left lung of these rats. 48 h later, the lungs were excised and pathology was determined.

*Significantly different from DG1 infected animals ($P_{corrected} < 0.05$).

4.0

DISCUSSION

The prognosis of cystic fibrosis patients is closely related to chronic pulmonary infections, particularly with *P. aeruginosa* (Gilligan, 1991). Recently, it has been recognized that the production of extracellular enzymes such as exotoxin A, exoenzyme S, proteases and phospholipase C by *P. aeruginosa*, may be as important as replication of the organism. These *P. aeruginosa* exoproducts have been implicated in precipitating lung injury in CF patients (Woods and Sokol, 1986). This suggestion has been supported from data obtained during antimicrobial treatment of CF individuals. Therapy with antipseudomonal antibiotics during exacerbations of disease lead to improved clinical status. However, this improvement occurred in the absence of a significant or sustained bacteriological response (Schaad *et al.*, 1987). This has been confirmed by further studies which demonstrate that suppression of *P. aeruginosa* exoenzymes by subinhibitory concentrations of antibiotics may limit the progressive lung damage which occurs during chronic *P. aeruginosa* lung infections (Grimwood *et al.*, 1989). Thus, it is evident that *P. aeruginosa* exoenzymes play a significant role in exacerbations of respiratory symptoms during chronic *P. aeruginosa* lung infection. It is therefore vital, for the CF patient, to identify those environmental factors which may contribute to the up-regulation of these exoenzymes and the subsequent exacerbations of pulmonary injury.

In the present studies, it was demonstrated that interferon (IFN), was an environmental factor which may regulate the production of *P. aeruginosa* exoproducts. Exposure of three *P. aeruginosa* strains to clinically relevant concentrations of human alpha IFN resulted in significantly altered exoenzyme expression. The effects of IFN on these three strains were consistent. However, the *P. aeruginosa* exoenzymes were not affected uniformly by this molecule: both exoenzyme S and proteolytic expression were increased and the activity of both exotoxin A and phospholipase was decreased. This result, although interesting, is not

entirely unexpected. Evidence indicates that unique regulatory pathways function for each *P. aeruginosa* virulence determinant (Vasil *et al.*, 1986). Perhaps, in light of our data, IFN could be employed to further elucidate *P. aeruginosa* exoenzyme regulation.

It is our hypothesis that increases in *P. aeruginosa* exoenzyme expression following IFN exposure would lead to exacerbation of lung injury. Our *in vitro* studies indicated that this interaction results in increases only in the production of exoenzyme S and proteases. It is probable, however, that the up-regulation of both these enzymes in response to IFN may have a profound effect upon the progression of lung injury during chronic *P. aeruginosa* infections. *In vivo* studies of an animal model of chronic P. aeruginosa lung infection demonstrated that both exoenzyme S and *Pseudomonas* proteases are significant pathogenic determinants (Grimwood *et al.*, 1989). Moreover, examination of clinical *P. aeruginosa* isolates from CF patients for phenotypic expression of exoenzymes *in vitro* also identified exoenzyme S and proteases as *P. aeruginosa* virulence factors. (Woods and Sokol, 1986). Finally, our IFN induction data established that there was an association between IFN induction, *P. aeruginosa* infection and lung pathology: those animals which were coinfected with *P. aeruginosa* and RSV, and had the greatest bronchial lavage IFN concentrations, also demonstrated the most lung injury.

It is apparent that IFN regulates *P. aeruginosa* exoenzyme expression. From our data, the mode of this IFN *P. aeruginosa* exoenzyme regulation may be partially explained. It was demonstrated that IFN did not exert its effects by altering the growth of *P. aeruginosa*: the growth of *IFN-treated P. aeruginosa* cultures were not significantly different from that of untreated *P. aeruginosa* cultures. An increase in exoenzyme S activity was not observed when IFN was introduced into cultures at later incubation times. Increases in this expression only occurred when IFN and *P. aeruginosa* cells were added concomitantly. Also, multiple additions of

IFN into the same cultures did not result in a further increase in exoenzyme S activity. These results suggest that *P. aeruginosa* cells are susceptible to the effect of IFN only at a particular time within their growth cycle. These results imply that as the cells mature, they lose their sensitivity to IFN. It is interesting to note that a similar development-dependant response to IFN has also been reported for eukaryotic cells (Langer and Pestka, 1988). Perhaps, further comparison between eukaryotic IFN regulation systems and that of *P. aeruginosa* may lead to a greater understanding of how IFN regulates *P. aeruginosa* exoenzyme expression.

To determine the specificity of the alpha IFN - *P. aeruginosa* interaction, the effects of rat and human beta IFN on the activity of *P. aeruginosa* exoenzyme S were determined. Although exposure to both these molecules also resulted in increased *P. aeruginosa* exoenzyme S expression, maximal effect was observed with IFN alpha. As previously indicated IFN alpha and IFN beta share homologous DNA sequences and therefore an IFN beta - *P. aeruginosa* interaction may have been anticipated. Rat IFN is a preparation containing alpha, beta and gamma IFN. It may be postulated that the increased exoenzyme S expression observed following addition of this preparation to *P. aeruginosa* cultures was largely due to exposure to IFN alpha and partially in response to IFN beta.

Whereas IFN species other than alpha IFN were able to somewhat modify *P. aeruginosa* exoproduct expression, this was not the case for other classes of cytokines. The effects of IL-1 and IL-2 on *P. aeruginosa* exoenzyme S expression were examined. Exposure of *P. aeruginosa* to these interleukins did not result in altered exoenzyme S expression in comparison to untreated organisms. It is thusly apparent that the effect of IFN on *P. aeruginosa* occurs through a specific interaction.

The initial dose-response relationship between IFN concentration and *P. aeruginosa* exoenzyme S activity suggested that IFN alpha may have mediated its effects through a specific receptor present on the surface of

P. aeruginosa. Results indicated that a threshold concentration of 1 X 10^3 IU/ml of IFN was required for an effect on exoenzyme concentration. Conversely, a concentration of 1 X 10^5 IU/ml had a maximal effect; greater IFN concentrations did not further increase interferon-mediated exoenzyme expression. These results suggest that the interferon-mediated alteration in exoproduct expression was a saturable phenomenon, indicating the involvement of a ligand specific receptor (Eidels *et al.*, 1983). The studies which were conducted to determine if IFN effected surface determinant expression demonstrate that this specific moiety itself was not IFN inducible.

The interaction of a ligand with its cellular receptor can be studied by measuring the amount of bound ligand as a function of its concentration (Rubinstein and Orchansky, 1986). This was done by the use of ^{125}I labelled ligand. Experiments with ^{125}I-IFN demonstrated that the binding of IFN alpha to *P. aeruginosa* DG1 was saturable. To insure that carrier molecules did not interfere with binding, both HSA containing and carrier free preparations of IFN were employed for these binding experiments. Both these preparations exhibited saturation of binding at approximately 3 ug of protein for 3 X 10^9 DG1 cells. It was unfortunate that the IFN-f preparation was not biologically active. This molecule was donated to us as a frozen preparation. Presumably, the exposure of the preparation to extreme cold resulted in its loss of activity. However, because the saturation values of *INTRON A* and IFN-f were comparable, it was assumed that although the IFN-f preparation was not biologically active, its binding capacity was still intact.

Scatchard plot analyses enabled further interpretation of the binding kinetics of IFN alpha to *P. aeruginosa* (Scatchard, 1949). This analysis allows for the calculation of both the affinity constant and the number of binding sites per cell. The Scatchard plot which was generated from the *INTRON A* data described a curvilinear relationship between bound and bound/free ^{125}I-IFN. However, the Scatchard plot from the IFN-f binding

data was linear. The *INTRON A* Scatchard plot may be interpreted such that one class of high affinity receptors is present for IFN on the *P. aeruginosa* cell surface (represented as the initial portion of the curve) and there was also nonspecific interaction occurring between IFN and *P. aeruginosa* (represented as the latter portion of the curve). If this approach is taken, the binding association and the number of IFN receptors per *P. aeruginosa* cell is similar for both the *INTRON A* and IFN-f preparations. These data provide compelling evidence that a single, specific class of receptors for IFN alpha exists on the *P. aeruginosa* cell surface.

The above binding data was calculated for *P. aeruginosa* DG1 whole cells. To identify the location of the receptor, similar binding experiments were performed on *P. aeruginosa* outer membranes. It was demonstrated that ^{125}I-IFN-f saturated on *P. aeruginosa* outer membranes at an approximate concentration of 3 μg of protein. These results are similar to that binding of both *INTRON A* and IFN-f to DG1 whole cells. The similarities between the binding of IFN-f to *P. aeruginosa* outer membranes and those binding data of IFN to *P. aeruginosa* whole cells suggest that the different IFN molecules are interacting with analogous receptors which are present on *P. aeruginosa* outer membrane preparations.

The above results indicate that *P. aeruginosa* has evolved a mechanism to bind and exploit a host-derived molecule. This binding and manipulation of eukaryotic derived molecules is not unprecedented for prokaryotic organisms. Recently, the growth enhancement effects of Il-1 and Il-2 on *Escherichia coli* isolates was described (Denis *et al.*, 1991; Porat *et al.*, 1991). Only those *E. coli* organisms which were pathogenic were susceptible to these cytokines. It was suggested that these effects were mediated by a specific interleukin receptor present on the *E. coli* surface. Oestrogen receptors have also been reported for *Pseudomonas* which have been isolated from female urinary tracts (Rowland *et al.*, 1989). Once again, the isolation of these receptors was correlated with

pathogenic organisms. It is apparent that a relationship between the ability to utilize host derived molecules and an oraganism's pathogenicity exist: the more proficient an organism is at utilizing available molecules for its advantage, the greater the chance of survival.

P. aeruginosa produces a number of proteases for which a broad range of substrates have been identified (Morihara, 1963). Inactivation of cytokines, by such *P. aeruginosa* proteases has previously been demonstrated (Parmely *et al.*, 1990). Cleavage of IFN gamma in these studies was associated with inactivation of this molecule. Our studies indicate that IFN alpha interacts with *P. aeruginosa in vitro* to alter exoenzyme expression. It was of interest therefore, to determine if *P. aeruginosa* proteases would also inactivate IFN alpha and thereby possibly negate the effect of this cytokine on *P. aeruginosa* cultures. Our experiments demonstrated that *P. aeruginosa* proteases also cleaved IFN alpha. We demonstrated that the resulting fragments did not react with polyclonal anti-IFN antibodies, which suggested that there may be loss of IFN bioactivity. However, studies to determine if this decrease in activity were not conducted. Perhaps, the smaller fragments would also bind to the *P. aeruginosa* cell surface and elicit alterations in exoproduct expression.

We hypothesized that the result of increasing *P. aeruginosa* exoenzyme expression following IFN administration would result in an exacerbation of respiratory illness *in vivo*. Production of IFN in the lungs was induced in rats following intratracheal RSV administration. Chronic *P. aeruginosa* infection also resulted in a modest IFN production. The greatest IFN concentrations were measured in rats who were coinfected with both RSV and *P. aeruginosa*. This suggests a possible synergistic effect on IFN induction following bacterial/viral coinfection. Although IFN is thought to be produced to inhibit infection of microorganisms, in our model the presence of this molecule was correlated with greater pulmonary injury. In experiments where IFN was directly administered to

either chronically infected or noninfected rat lungs the greatest pathology was observed in animals who were infected with *P. aeruginosa*. This observed lung damage was greater than those animals who only received either IFN injection or *P. aeruginosa* infection. From these data the following associations may be made: substantial IFN was produced in coinfected animals; this IFN production precipitated lung injury in coinfected rats; and finally, administration of IFN to *P. aeruginosa* infected animals resulted in similar damage. Furthermore, these associations suggest that there is an overall relationship between *P. aeruginosa* infection, the presence of IFN and the exacerbation of lung pathology.

We have suggested that viral infection in CF patients results in the production of IFN which interacts to up-regulate the expression of *P. aeruginosa* exoproducts. To further demonstrate the importance of IFN in CF chronic *P. aeruginosa* infection it is important to determine the IFN concentrations in CF patients. RSV has been identified as an important viral pathogen in younger CF individuals during times of exacerbation. Yet, it has also been demonstrated that high levels of IFN are not present in the sera of these patients. These results conflict with our theory of the importance of IFN in exacerbations of illness. It may be however, that high concentrations of IFN are produced in the lung which are not present in sera. Studies have indicated that IFN is produced locally and is extremely sensitive to serum proteases. Thus, until more accurate measurements of IFN concentrations are possible, our evidence implicates the production of IFN as a determinant in exacerbations of respiratory illness.

In summary, human IFN alpha binds to the outer membrane of *P. aeruginosa* in a saturable, specific manner with an association constant of $1 \times 10^{-10} - 1 \times 10^{-11}$. This binding alters the *in vitro* expression of *P. aeruginosa* exoenzymes in a dose-related fashion. We would hypothesize that up-regulation of *P. aeruginosa* exoenzyme S and proteolytic enzyme

activity following exposure to IFN, produced in response to viral infection in CF patients, could exacerbate pulmonary symptoms in these patients. Further evidence from combined *P. aeruginosa*-RSV infection in animals demonstrated that a possible synergistic relationship exists between these two microorganisms which significantly exacerbates the level of lung injury over that observed with either organism alone. Administration of IFN directly to chronically *P. aeruginosa* infected rats demonstrated similar pathology as that observed during *P. aeruginosa*-RSV coinfection. This supports our hypothesis that IFN alpha interacts with *P. aeruginosa* to increase exoproduct expression which subsequently results in an exacerbation of pulmonary injury.

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