Pneumolysin’s Role in Pneumococcal Clearance from the Bloodstream of the Alcohol-Ingesting Host

BY

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A THESIS

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Abbreviations

cfu : Colony Forming Units  
CRP : C-Reactive Protein  
HPA : Hypothalamus-Pituitary-Adrenal  
IL-1 : Interleukin-1  
IL-6 : Interleukin-6  
IL-10 : Interleukin-10  
PAF : Platelet-Activating Factor  
PLY- : Pneumolysin Deficient  
PMNL's : Polymorphonuclear Leukocytes  
PNC : Pneumococcus  
RES : Reticuloendothelial System  
SE : Standard Error  
S. pneumoniae : Streptococcus pneumoniae  
TNFα : Tumor Necrosis Factor Alpha
Abstract

Alcoholism increases susceptibility to fatal infections caused by *Streptococcus pneumoniae*, or the pneumococcus (PNC). Alcoholics with PNC pneumonia have an increased likelihood of developing bacteremia, and alcohol ingestion in combination with leukopenia increases the likelihood of fatality due to systemic PNC infection. We have shown previously that ethanol-ingesting rats, like their human counterparts, develop prolonged bacteremia and have a higher mortality after transtracheal PNC infection. All clinical PNC isolates produce the toxin pneumolysin that has been shown to reduce PNC bloodstream clearance. The goal of this study was to determine whether pneumolysin production hampers the removal of PNC from the bloodstream of the alcohol-ingesting host. The pair-feeding regimen employed in our experiments has been shown previously to induce leukopenia in both Ethanol-fed and Pair-fed rats. This enables us to specifically examine the effects of ethanol ingestion in resistance to PNC bacteremia in the leukopenic alcoholic host. Male Sprague-Dawley rats were pair-fed a liquid diet containing 36% of its calories as either ethanol or dextrin-maltose for 7 days. Chow-fed rats were included to control for nutritional deprivation inherent in this feeding model. On day 8, rats were infected intravenously with $2 \times 10^5$ colony forming units (cfu) / ml blood of either a PNC strain producing wildtype pneumolysin (H+C+) or an isogenic pneumolysin-negative mutant (PLY-). Mortality was followed for 14 days. Clearance of the PNC from the bloodstream (as determined by plate counts) was also analyzed at
0.25 to 27 h post-infection. In general, pneumolysin production reduced PNC clearance and increased total mortality significantly for all animals except those in the Chow-fed group. Surprisingly, Ethanol-fed rats cleared both the PNC strains and survived infection at least as well as the Pair-fed and Chow-fed controls. In contrast, Chow-fed rats infected with the H+C+ strain had significantly higher bacteremia counts at 27 h post-infection than rats in the other diet groups. Although nearly all H+C+ infected animals died, the mean day of death for Chow-fed rats was 2.3 ± 0.4 days compared to 6.7 ± 1.4 and 9.2 ± 1.6 days for the Ethanol- and Pair-fed rats, respectively. Furthermore, 86% of the PLY-infected Chow-fed rats died by day 14, whereas at least 80% of both Ethanol- and Pair-fed rats survived PLY-infection. To determine whether pneumolysin production or ethanol ingestion influences the trafficking of PNC through the organs of the reticuloendothelial system, numbers of organisms in the lung, spleen and a lobe of the liver were determined 2 h post-infection. Although there were no differences in organ burdens among the diet groups, each diet group had fewer PLY-organisms than H+C+ organisms in their spleens, possibly suggesting that the PLY-organisms may be more susceptible to killing. In order to further understand the increased susceptibility of the Chow-fed rats to the H+C+ strain, serum levels of TNFα, IL-1β, IL-6 and IL-10 were analyzed by ELISA at 15 min, 2 h and 27 h post-infection. Levels of all four cytokines were minimal early after infection in all feeding groups infected with either strain. Levels of IL-1β, IL-6 and IL-10 were mildly elevated 27 h after infection with the PLY-strain, with all groups having similar levels. By contrast,
27 h after infection with the H+C+ strain, serum levels of TNFα, IL-1β, IL-6 and IL-10 were all significantly higher in Chow-fed than in Ethanol- or Pair-fed rats. This is consistent with the rapid deaths seen in H+C+ infected Chow-fed rats. Since ethanol ingestion and malnutrition have been shown to be associated with increased corticosterone production, we hypothesized that the immunosuppressive effects of this hormone may have suppressed the inflammatory response in the Ethanol- and Pair-fed rats. This would explain why they do not experience the overwhelming lethal cytokine response seen in the Chow-fed rats. To determine if corticosterone differed among the feeding groups, a radioimmunoassay was performed on serum samples taken pre-infection, 2 h post-infection and 27 h post-infection to quantify serum corticosterone levels. Although there were minor and conflicting differences that varied with diet group and infecting strain at 2 h post-infection, there were no significant differences in serum corticosterone levels among the feeding groups pre-infection or at 27 h post-infection. However, both Ethanol-fed and Pair-fed rats had significantly reduced corticosterone levels at 2 h and 27 h post-infection with the H+C+ strain in comparison to their pre-infection levels. In contrast, Chow-fed rats had significantly lower levels of corticosterone 2 h post-infection with the PLY- strain. Because we only have corticosterone levels for three rats per diet group at 27 h post-infection, it is difficult to ascertain the exact role of corticosterone after the initial hours in our model of infection. In conclusion, although pneumolysin production contributed to PNC virulence for rats in all feeding groups, ethanol ingestion was not associated with more severe disease.
Furthermore, Chow-fed rats are inherently more susceptible to PNC bacteremia in general than rats in either of the other diet groups, although the reason for this is not apparent from our results. Our results suggest that caloric restriction may contribute to increased resistance to fatal PNC bacteremia.
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Introduction

"Pneumococcus is an altogether amazing cell. Tiny in size, simple in structure, frail in make-up, it possesses physiological functions of great variety, performs feats of extraordinary intricacy and, attacking man, sets up a stormy disease so often fatal that it must be reckoned as one of the foremost causes of human death. Furthermore, living or dead, whole or in part, on entering the animal body, Pneumococcus starts a train of impulses, stimulating all reactions grouped under those inclusive phenomena known as immunity (White, 1938)."

As Benjamin White wrote in 1938, the pneumococcus (PNC) truly is remarkable and worthy of the research that has focused on this organism since it was first described in 1880. Through the study of this single bacterium, progress has been made not only in treating pneumococcal disease, but also toward a more complete understanding of basic biological principles. In examining lung sections from patients who had died of pneumonia, for example, Christian Gram discovered that the PNC retained the aniline-gentian violet stain. In doing so, he demonstrated the landmark phenomenon that nearly all bacteria are either gram-positive or gram-negative (reviewed in Watson, et al 1993). Possibly even more important than its contribution to the development of the Gram stain, is the role of the PNC in the discovery of DNA as the “transforming principle” (reviewed in Watson, et al 1993). Using rough, unencapsulated, avirulent vs. smooth, encapsulated, virulent strains of *S. pneumoniae*, Griffith discovered that when heat-killed smooth bacteria and live rough bacteria were injected into a mouse,
the mouse died and virulent smooth organisms could be recovered. This suggested that some property of the dead virulent bacteria transformed the avirulent organisms into virulent organisms. Subsequent studies in 1944 by Avery and his colleagues showed chemically that the isolated transforming principle is DNA (Avery, et al, 1944).

Although critical discoveries have been made through experimentation with the PNC, most were probably initiated to understand the virulence mechanisms of the organism with the goal of decreasing its pathogenic potential. Given the fact that S. pneumoniae has the potential to cause a wide spectrum of diseases, there is an obvious need for such studies. In fact, a 1996 estimate from the Centers for Disease Control and Prevention estimated that there are 7,000,000 cases of otitis media, 500,000 cases of PNC pneumonia, 50,000 cases of bacteremia, and 3000 cases of meningitis, annually in the United States (reviewed in Austrian, 1999). Due to the prominence of S. pneumoniae as a cause of disease in humans, the necessity for continued research and a greater understanding of the PNC and its virulence factors is apparent.
THE PNEUMOCOCCUS & ITS VIRULENCE FACTORS

Capsule

*Streptococcus pneumoniae*, also referred to as the pneumococcus (PNC), is a gram positive coccus-shaped bacterium that typically occurs in pairs or short chains and is surrounded by a polysaccharide capsule. In addition to defining the serotype, of which there are greater than 90, the polysaccharide capsule is considered to be the organism's primary virulence factor. In general, PNC with large capsules have greater pathogenic potential than those strains with either small capsules or mutant strains that completely lack a capsule and are, consequently, non-virulent (MacLeod, 1950). The primary advantage conferred by the capsule is protection of the PNC from phagocytosis by leukocytes. The negative charge of the capsule physically hinders opsonophagocytosis by repelling the negatively charged phagocytes. Additionally, the capsule protects the organism from opsonization by shielding the teichoic acid in the cell wall and decreasing its potential to activate the alternative complement pathway. C3b fragments which do manage to bind to the teichoic acid of the cell wall are often covered by the capsule and thus hidden from phagocyte receptors.

Opsonic C3b can also be deposited directly on the capsule surface, where it is cleaved by serum proteases into either iC3b or C3d (Hostetter, 1999). Cleavage patterns differ among serotypes, accounting for differences among strains in susceptibility to phagocytosis and immunogenicity. Serotypes 3 and 4, for example, which are highly resistant to phagocytosis but highly immunogenic,
become coated predominantly with C3d, which is recognized readily by receptors on the B lymphocyte but not by phagocytes. In contrast, serotypes 6A and 14 are readily phagocytosed but poorly immunogenic because they become coated with the iC3b fragment, which is recognized by neutrophil receptors but does not interact well with B lymphocytes.

**Cell Wall**

Interior to the capsule is the cell wall, which is composed of more than a dozen glycopeptides. Antigenic components of the cell wall, particularly teichoic acid, are responsible for eliciting the severe host inflammatory reaction that contributes to tissue damage. In fact, individual components of the cell wall that are released upon lysis of the bacterium are more potent chemotactic factors than the intact cell wall (reviewed in Tuomanen, 1997). Additionally, it is believed that the cell wall contributes to the virulence of the PNC by enabling the organism to attach to host tissues. Lipoteichoic acid within the cell wall has a phosphorylcholine moiety that structurally mimics platelet-activating factor (PAF), allowing the PNC to adhere to and possibly invade endothelial and epithelial cells expressing PAF receptors. The anchored PNC can then either be internalized (Gerard et al, 1994) or can release toxins which damage cell layers so as to facilitate PNC entry between these cells and into the bloodstream.
Pneumolysin

One toxic factor released from PNC within the host is the well-characterized pneumococcal protein, pneumolysin. Pneumolysin, a 53-kDa polypeptide, is a potent thiol-activated hemolytic toxin produced by all clinical PNC isolates. It is synthesized within the cytoplasm and released upon antibiotic-mediated or autolysin-mediated lysis of the bacterium. Autolysin, a cell wall degrading enzyme found in the cell envelope of the PNC, is active during metabolically unfavorable conditions, such as when levels of essential nutrients are depleted. Immunization of mice with purified autolysin conferred a degree of protection against intraperitoneal challenge with wild-type PNC, but it did not provide protection against challenge with a pneumolysin-deficient strain of S. pneumoniae (Lock, 1992). Therefore, it is believed that the primary contribution of autolysin to PNC virulence is in catalyzing the release of pneumolysin.

Although pneumolysin is often referred to as an hemolysin due to its ability to lyse erythrocytes, in vitro experimentation has demonstrated that it is lytic for eukaryotic cells that have cholesterol in their membrane (Mitchell and Andrew, 1997). Despite the fact that there is no definitive proof, the receptor for pneumolysin appears to be cholesterol in the target cells' membranes. In support of this hypothesis, cells without cholesterol in their membrane are not lysed by pneumolysin. In addition, cytolysis by pneumolysin can be inhibited by the addition of free cholesterol (Johnson, et al, 1980 and Rubins and Freiburg, 1994).
It is proposed that the process by which pneumolysin lyses cells is a two-step mechanism. First, monomers of pneumolysin bind to cholesterol on target cells. Then the pneumolysin monomers associate with one another, generating a ring-like structure which forms a pore in the cell membrane. The end result is an influx of water, which causes the target cell to swell and burst (Reviewed in Andrew, et al, 2000).

Various experimental models have demonstrated the deleterious effects of pneumolysin on a variety of cell types. In 1990, using an *in vitro* culture of human nasal epithelium, Feldman et al showed that a concentration of pneumolysin as low as 5 ng/ml caused decreased ciliary action and epithelial disruption (Feldman et al, 1990). By interfering with ciliary beating in this way, the organism is able to evade non-specific mucociliary clearance by the host. Additionally, in cell culture and in isolated perfused rat lungs, pneumolysin was shown to be cytotoxic to alveolar epithelial cells (Rubins, 1993). It also was cytotoxic for pulmonary artery endothelial cells (Rubins, 1994) and human nasal and tracheobronchial ciliated epithelia (Steinfort, et al 1989). Damage to these cells of the alveolar-capillary barrier results in the alveolar flooding and hemorrhage that is characteristic of the early stages of PNC pneumonia and enables the organism to invade the lung tissues and the bloodstream.

Pneumolysin also enhances PNC virulence by its detrimental actions on cells of the immune system. In 1983, Paton and Ferrante treated human polymorphonuclear leukocytes (PMNLs) with purified pneumolysin and found that the toxin inhibits their migration, respiratory burst and bactericidal activity (Paton
and Ferante, 1983). This evidence supported research performed earlier by Johnson, et al (1981) showing that pneumolysin is responsible for inhibiting neutrophil chemotaxis and for lysing neutrophils and platelets. Furthermore, pneumolysin depresses the oxidative burst of monocytes, and consequently reduces their ability to kill PNC (Nandoskar et al, 1986). Other in vitro studies have shown that pneumolysin inhibits the proliferation of lymphocytes in response to concanavalin A, phytohemagglutinin, pokeweed mitogen and protein A (Ferrante, 1984). It also interferes with host immunity by activating the classical complement pathway in the absence of pneumolysin-specific antibodies. This action of the toxin diverts opsonic complement components away from the intact live organisms (Paton et al, 1984). Finally, pneumolysin can stimulate the release of TNFα and IL-1β by human mononuclear phagocytes in vitro, thereby contributing to tissue damage during the inflammatory response (Houldsworth, 1994).

The generation of genetically engineered pneumolysin deficient (PLY-) PNC strains has further elucidated the role of pneumolysin in PNC pathogenesis. Using a type 2 PLY- strain and its isogenic wild-type parental strain, Rubins et al (1995) demonstrated that pneumolysin facilitates intraalveolar replication of PNC, promotes penetration of the bacteria from alveoli into the interstitium of the lung, and enhances dissemination of PNC into the bloodstream during murine experimental pneumonia. Similarly, Berry et al (1989b) showed in a murine model of intranasal infection that mice infected with a PLY- strain survived longer than those infected with a PLY+ strain. Furthermore, when mice were
challenged either intranasally or intraperitoneally, the lethal dose 50 increased 10- to 100-fold for the PLY- strain. The decreased virulence of PLY- organisms in this model correlates with research done by Benton et al. (1995), in which PLY- PNC were more susceptible than wildtype PNC to \textit{in vitro} killing by phagocytes. In summary, pneumolysin is a multi-functional toxin which exerts its detrimental effects on host immune defense directly by acting on pulmonary cells, phagocytes, complement activation and cytokine production.

\textbf{PNEUMOCOCCAL PATHOGENESIS}

Clinically, the organism has a short incubation period of 1-3 days (Mufson, 1981), followed by an abrupt onset of symptoms. Typically, infected individuals experience a severe shaking chill and then a sustained fever of 102°-105°F. Although vomiting and chills are uncommon following the initial onset of symptoms, approximately 75\% of infected people experience a cough associated with rusty-colored sputum. Other symptoms include chest pain, malaise, weakness and dyspnea.

PNC pneumonia usually begins when an individual aspirates respiratory secretions containing the organisms. This must be a rather common event, considering that the PNC colonizes the nose, throat, or both of approximately one fourth of all healthy persons at a given time (Evans, 1998). Although colonization with PNC is more prevalent during the winter season, almost all individuals carry them transiently or periodically sometime throughout the year.
(Evans, 1998). Additionally, it is possible for an individual to harbor up to four different serotypes simultaneously for several months (Cundell et al. 1995b).

PNC can grow as opaque or transparent variants that can be distinguished on agar media by their colony morphology. These phenotypic variations are important to note because they correlate with differences in the ability of the organisms to colonize and survive the host. Only transparent variants are able to colonize the nasopharynx, whereas opaque variants cannot. Nasopharyngeal cells bear a single class receptor, GlcNAcβ3Gal, which is recognized only by the transparent variant (Cundell et al, 1995b). The transparent variants also differ from opaque ones in that their cell walls contain more choline, which acts as a ligand for the PAF receptor. They also contain more choline binding protein A, a surface protein that interacts with cytokine-activated host cells to promote invasion (Ring et al., 1998).

In most healthy individuals non-specific host defenses, such as the cough and epiglottic reflexes and the mucociliary “blanket”, are able to clear aspirated organisms from the lower respiratory tract (Ryan, 1994). These defenses can be compromised, however, in individuals with damaged bronchial epithelia or chronic pulmonary diseases, as well as in alcoholics. In such immunocompromised persons, the PNC can pass through the barriers of the lower airways and reach the lungs. There the bacteria will encounter another major host defense mechanism, the alveolar macrophages. As previously mentioned, the capsule of virulent PNC prevents the organisms from being
phagocytosed effectively by these cells. PNC able to resist killing by macrophages then have the potential to bind to the alveolar epithelium.

Normal lung cells in the resting state, meaning they are not activated by an inflammatory response, have two types of receptors, \textit{GalNAcβ1-4Gal} and \textit{GalNAcβ1-3Gal}. These receptors are recognized by both transparent and opaque PNC. Although PNC have the ability to adhere to these resting epithelial cells, they cannot enter them unless the cells are activated by the inflammatory response (Geelen \textit{et al}, 1993). Lung cells that have been activated by inflammatory cytokines present additional receptors for PAF that recognize only transparent PNC variants (Cundell \textit{et al}, 1995\textsubscript{b}). Therefore, it is the activation of endothelial cells, resulting in \textit{de novo} expression of surface determinants important in leukocyte trafficking, that facilitates PNC binding in the respiratory tract (Cundell \textit{et al}, 1995\textsubscript{A}). PNC then appear to be internalized into the activated cells during PAF receptor uptake and recycling (Gerard and Gerard, 1994).

**Pneumococcal – Induced Inflammation**

Inflammation due to pneumococcal infection follows a classic pattern of events. The first stage is engorgement, which is characterized by the presence of the PNC and a serous exudate in the alveoli after PNC have bound to alveolar cells. This exudate provides the bacteria with nutrients for survival, as well as a means for further dissemination in the lung (Tuomanen \textit{et al}, 1995). Within the alveoli of the lung, the alveolar epithelium is the limiting membrane for capillary water and solute transport. Its function is similar to that of other epithelia in that it
is a barrier which prevents microorganisms from penetrating the tissue. The alveolar epithelium is comprised of two main types of cells: type I and II pneumocytes. Type I pneumocytes, which account for approximately 95% of the alveolar surface, are the cells that sustain the most damage from a variety of blood-borne and inhaled toxins. These cells are thin and elongated, and are connected via tight junctions to form the alveolar barrier. Type II pneumocytes cover only 4-5% of the alveolar surface and are more resistant to injury (Rubins, et al 1993).

When PNC bind to type II pneumocytes, the production of interleukin-1, the separation of endothelial cells from one another, and the accumulation of serous exudate occur. By the second or third day of the disease, the characteristic red hepatization stage results from leakage of erythrocytes into the alveoli (Mufson, 1981). The stage of gray hepatization occurs by the fourth to fifth day, when fibrin is deposited in the alveoli and neutrophils are recruited to the sight of infection. The recruitment of leukocytes is further amplified by the inflammatory nature of the PNC cell wall, which can activate the complement cascade. Nevertheless, activation of the alternative complement pathway in this manner is not highly effective in opsonization of the PNC, for reasons mentioned before. Individuals who have previously developed antibodies against the invading pathogen, however, are able to opsonize the organisms via the classical complement pathway for killing by phagocytes. In most cases, these persons can rapidly resolve their infections. In contrast, infection of immunologically
naive individuals typically will not be resolved until days 5-7, when circulating antibody first appears. (Mufson, 1981).

As pneumococcal multiplication is brought under control by host leukocytes, the dying bacteria release components that amplify the inflammatory and cytotoxic effects of the organism on pulmonary cells. Antibiotic therapy is tremendously useful at this stage, and the outcome of infection depends greatly on the host’s ability to withstand the inflammatory response associated with bacterial lysis (Tumonanen et al, 1995).

Role of Cytokines in Pneumococcal – Induced Inflammation

The immune response to pneumococcal infection is intended to eliminate the organisms and protect the host. However, too weak or too strong an inflammatory response can ultimately prove detrimental to the host. Immunosuppressed individuals are at a disadvantage because they cannot mount a sufficient immune response. Immunocompetent individuals, on the other hand, may actually produce a severe inflammatory reaction resulting in tissue injury, shock or even death. Cytokines, the proteins involved in intercellular signalling, are often responsible for disrupting the precarious balance between a productive and a deleterious response.

In general, the initial inflammatory response is associated with an increase in pro-inflammatory cytokines, such as Tumor Necrosis Factor α (TNFα), Interleukin-1 (IL-1), and Interleukin-6 (IL-6). These cytokines are produced by a variety of cells, as shown in Table 1 (adapted from Roitt, 1997).
Table 1. Cytokines: Their origin and function during infection

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<tr>
<th>CYTOKINE</th>
<th>SOURCE</th>
<th>EFFECTOR FUNCTION</th>
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<tr>
<td>TNFα</td>
<td>Macrophages, T cells</td>
<td>Cachexia; induction of acute phase proteins; anti-viral &amp; anti-parasitic activity; activation of phagocytic cells; induction of IFNγ, IL-1, GM-CSF &amp; IL-6; endotoxic shock</td>
</tr>
<tr>
<td>IL-1</td>
<td>Macrophages, Fibroblasts</td>
<td>Proliferation of activated B- &amp; T-cells; induction of PGE2 &amp; cytokines by macrophages, induction of neutrophil &amp; T-cell adhesion molecules on endothelial cells; induction of IL-6, IFNβ1 &amp; CM-CSF; induction of fever and acute phase proteins</td>
</tr>
<tr>
<td>IL-6</td>
<td>CD4 T cells, Macrophages, Mast Cells, Fibroblasts</td>
<td>Growth &amp; differentiation of B- &amp; T-cell effectors &amp; hematopoietic precursors; induction of acute phase proteins.</td>
</tr>
<tr>
<td>L-10</td>
<td>CD4 T cells, B cells, Macrophages</td>
<td>Inhibits IFNγ secretion, inhibits mononuclear cell inflammation</td>
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In addition to activating lymphocytes and inducing the synthesis of acute phase proteins, these cytokines play a variety of other roles during inflammation (Table 1). Once the invading pathogen has been cleared, however, the inflammatory process needs to be switched off. Interlekin-10 (IL-10) is the main cytokine
involved with downregulation of the inflammatory response and decreased production of pro-inflammatory cytokines.

Van der Poll et al (1997) demonstrated that TNFα produced within the lungs of mice infected intranasally with a type 3 PNC is important in host defense against the infection. Mice treated with an anti-TNFα monoclonal antibody 2 h before infection died significantly earlier and had fourfold more organisms in their lungs 40 h post-infection than control mice. This has been confirmed in similar experiments by O'Brien (1999), who reported that neutralization of TNFα at the time of inoculation increases susceptibility of mice to PNC infection. However, although TNFα is protective within the lungs, high concentrations of this cytokine in the circulation are responsible for most of the tissue injury and organ failure associated with septic shock due to the presence of bacteria or bacterial products in the bloodstream (Hinshaw, et al, 1990, and Tracey, et al, 1987).

IL-6 is another cytokine associated with host defense against S. pneumoniae which is commonly detected in the circulation of patients with pneumonia (Dehoux, et al, 1994, and Puren, et al, 1995). In a murine model, Van der Poll, et al (1997) found that intranasal infection with a type 3 PNC resulted in sustained expression of IL-6 messenger RNA in the lungs. The mice also had increased IL-6 protein concentrations in their lungs and plasma. IL-6 gene deficient mice died significantly earlier and had increased levels of TNFα, IL-1β and IL-10 in their lungs in comparison with wild-type mice capable of producing IL-6. Therefore, IL-6 appears to be important during PNC pneumonia because it controls the activation of both agonist and antagonist cytokine
mediators. Aside from regulating other cytokines, IL-6 is protective in that it reduces PNC growth and prolongs survival.

After the inflammatory response has been initiated and the organisms have been cleared from the host, IL-10 is crucial in downregulating the pro-inflammatory cytokines. IL-10 has profound anti-inflammatory effects and can inhibit further cytokine synthesis and suppress the effector functions of macrophages, T cells and natural killer (NK) cells. Additionally, it contributes to the regulation of B cells, mast cells and thymocytes (Reviewed in Moore, et al 1993). However, in a murine model of intranasal PNC infection, it has been shown that the ability of IL-10 to inhibit pro-inflammatory cytokines impairs host defense during pneumococcal pneumonia (Van der Poll, et al, 1996). When IL-10 was administered in combination with type 3 PNC, it decreased production of TNFα and IFNγ, increased bacterial counts in the blood and lungs, and caused earlier mortality. Alternatively, when mice were treated with an anti-IL-10 monoclonal antibody 2 h pre-infection, they had increased levels of TNFα and IFNγ, reduced bacterial counts in their lungs and plasma, and prolonged survival. Therefore, it appears that IL-10 can be detrimental to the host early in infection in that it inhibits the pro-inflammatory cytokines and prevents them from contributing to effective clearance of the organisms.

Bergeron, et al (1998) characterized the chronology of events and cytokines associated with fatal type 3 PNC pneumonia using a murine model of intranasal infection. At 0-4 h post-infection, alveolar macrophages were able to only partially clear the organisms from the lung. Cytokines were activated at this
stage, including TNFα, IL-6 and IL-1 which were present in homogenized lung
tissue. IL-6 was also present in the serum at this point. Between 4 and 24 h
post-infection, there was bacterial growth in the lungs, accompanied by a
recruitment of neutrophils from the bloodstream, a release of TNFα, IL-6 and IL-1
in the lungs, and a transient spillover of IL-1 into the serum. Between 24 and 48 h
post-infection, TNFα and IL-1 production were downregulated, tissue injuries
became apparent and the organisms progressed from the lungs into the blood.
Between 48 and 72 h, bacteremia correlated with an increase in blood levels of
TNFα and IL-6. Therefore, it appears that pro-inflammatory cytokines in the
lungs help protect the host from the invading PNC. If, however, these cytokines
reach high levels in the blood or are prematurely inhibited by IL-10, the host may
be compromised in its ability to defend itself against this pathogen.

Pneumococcal Bacteremia

If PNC pneumonia is unresolved and the leukocytes are inefficient or do
not succeed in killing the bacteria, the alveolar epithelium of the lung sustains
greater damage and there is potential for the PNC to enter the blood, resulting in
bacteremia. Interaction of the PNC with vascular endothelial cells enables them
to gain access to the blood via the pulmonary capillaries of the cervical lymphatic
system (Cundell et al, 1995b). It is during this stage of bacterial adherence to
lung cells that the cell wall of virulent PNC interacts with the PAF receptor. This
facilitate its entrance into the host cells and aids in the progression from
colonization to pneumonia to bacteremia.
Bacteremia results as a complication from PNC pneumonia in about 25-30 percent of cases. This increases the case fatality rate two to five times higher than for patients who are non-bacteremic. *S. pneumoniae* is the most frequent cause of community-acquired bacteremia and is responsible for 18% of all bacteremias and 61% to 78% of bacteremias in pediatric outpatients (Mufson, 1981). Furthermore, since the development of an effective *Haemophilus influenzae* type b vaccine, *S. pneumoniae* has become the most important bacterial pathogen in children (reviewed in Klein, 2000). Despite the fact that children run an especially high risk of developing PNC bacteremia, they do not have an overall higher mortality than adults. A possible explanation is that the capsular serotypes which tend to infect children differ from those that commonly infect adults. Once acquired, certain serotypes are associated with greater virulence. Two separate studies, for example, have shown that capsular type 3 contributed to a greater risk of death from bacteremic PNC disease than other capsular serotypes (Grandsen, 1985 and Mufson, 1974). Children are normally infected with higher-numbered serotypes, such as 14, 18 and 19 (Burke, et al 1971 and Heldrich, 1970). By contrast, in a review of 325 adults with PNC disease, Mufson *et al* (1974) noted that the capsular serotypes 8, 4, 5, 12, 3, 1, 7, and 9, in decreasing frequency, were responsible for two thirds of all the infections.

In contrast to children, elderly people are particularly likely to succumb to fatal PNC infection. In a retrospective review of medical records from three community teaching hospitals in northeastern Ohio, it was determined that
mortality increased significantly (42%) in patients older than 65 (Watanakunakorn, 1993). Additionally, this study confirmed previous reports that increased mortality due to bacteremia is often associated with congestive heart failure, diabetes mellitus, malignancy and alcoholism or cirrhosis.

**Clearance of PNC from the Bloodstream**

Organisms that have reached the bloodstream are cleared primarily by the spleen and liver. Defects in either organ predispose an individual to more severe PNC bacteremia. Unimmunized individuals who lack a functional spleen due to any cause, such as persons with congenital defects or post-traumatic or therapeutic splenectomy, run an extremely high risk of succumbing to overwhelming PNC sepsis. Although complement activated via the alternative pathway has limited opsonic capabilities, in the healthy unimmunized host a small percentage of PNC can at least be partially opsonized. These “lightly” opsonized organisms are not effectively phagocytosed by hepatic macrophages, but can be cleared by macrophages of the spleen (Reviewed in Schaechter, 1998). Although the reason for differences in the ability of hepatic and splenic macrophages to clear lightly opsonized PNC is unknown, it may be attributed to differences in the structure or blood flow within these organs or to differences in the ability of cells in these organs to recognize opsonic signals (Brown et al., 1981a).

If an asplenic individual is immune due to development of type-specific anticapsular antibodies against the invading strain, the PNC can be completely
and efficiently opsonized via the classical complement pathway. PNC opsonized in such a manner are more easily recognized by hepatic than by splenic macrophages. Using a guinea pig model of intravenous infection with radiolabeled PNC, Brown, et al (1983) demonstrated that recognition by hepatic macrophages causes a shift in sequestration of the organisms in the reticuloendothelial system. In the presence of anticapsular antibody and complement, there was an increase in hepatic sequestration and a decreased reliance on splenic macrophages. Therefore, even an asplenic person who is vaccinated can be fairly well protected against fatal bacteremia (reviewed in Schaecter, 1998), since anticapsular antibody enables effective clearance of PNC even without a spleen. It is therefore recommended that splenectomized individuals be immunized with the PNC vaccine.

The mere presence of anticapsular antibodies does not, however, insure protection against PNC bacteremia. Brown et al (1981a) demonstrated that the presence of an intact classical complement system is necessary for hepatic PNC clearance. This is evidenced by the fact that C4 deficient guinea pigs with an intact alternative complement pathway exhibited decreased PNC clearance in comparison with controls. Therefore, when complement-dependent opsonization is less than optimal, fewer organisms are cleared from the blood by the liver and there is an increased need for the assistance of splenic macrophages.

The importance of the classical complement pathway in PNC clearance also has been demonstrated by examining the role of C-reactive protein (CRP). CRP is as an acute phase protein produced rapidly in response to inflammatory
stimuli. It has been shown that CRP can bind specifically to phosporylcholine in the PNC cell wall (Volanakis and Kaplan, 1971), thereby activating the classical complement pathway even in the absence of antibody (Kaplan et al, 1974). In addition, Mold, et al (1981) reported that a single injection of CRP given to mice prior to infection with either type 3 or type 4 PNC increased their survival time. Furthermore, Horowitz, et al (1987) noted that when xid mice (cannot produce anti-PNC antibodies) were depleted of C3 by cobra venom factor, it abolished the ability of human CRP to promote bacterial clearance and prolong the life of the animals. Therefore, it appears that the benefits of CRP in host defense against PNC disease are dependent upon the activation of the classical complement pathway.

**ALCOHOL-IMPAIRED HOST DEFENSE**

It is well known that alcoholics have an increased susceptibility to severe bacterial infections, including those caused by *S. pneumoniae*. Many factors are involved in their decreased resistance to bacterial infection, including malnutrition, liver cirrhosis, and a delay in seeking therapy (Liu, 1980). Additionally, ethanol ingestion increases susceptibility to infections due to its deleterious impact on innate host defense mechanisms and the acquired immune response (Table 2; Adapted from Cooper, et al, 1988).
Table 2. Effects of Alcohol on Immune Function

<table>
<thead>
<tr>
<th>Immune Defense</th>
<th>Direct Effect of Alcoholism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Local Barrier Defenses</td>
<td>Depressed glottic reflexes</td>
</tr>
<tr>
<td></td>
<td>Aspiration</td>
</tr>
<tr>
<td></td>
<td>Altered ciliary transport</td>
</tr>
<tr>
<td></td>
<td>Diminished pulmonary surfactant</td>
</tr>
<tr>
<td>Humoral Defenses</td>
<td>No Known effect</td>
</tr>
<tr>
<td>Cellular Defenses : Neutrophils</td>
<td>Leukopenia</td>
</tr>
<tr>
<td></td>
<td>Diminished adherence &amp; mobilization</td>
</tr>
<tr>
<td>Reticuloendothelial</td>
<td>Depression of macrophages (hepatic &amp; pulmonary)</td>
</tr>
<tr>
<td>Cell-mediated immunity</td>
<td>Diminished lymphocyte response to plant lectins</td>
</tr>
<tr>
<td></td>
<td>Impaired NK cell function</td>
</tr>
</tbody>
</table>

When an individual is in an intoxicated state of stupor or coma, glottis closure may not occur effectively and the aspiration of oropharyngeal contents can lead to the development of pneumonia. In a rat model, it was shown years ago that the animals were unable to effect glottis closure after receiving large amounts of ethanol (Nungester and Klepser, 1938). Therefore, ethanol-induced impairment of this mechanical barrier appears to increase the incidence of
pneumonia in alcoholics. Furthermore, ethanol suppresses the cough reflex and ciliary transport of mucus, impeding the removal of aspirated organisms (Cooper et al, 1988).

Another factor associated with increased susceptibility to bacterial infections is leukopenia, which is seen commonly in alcoholics (Liu, 1973 and Perlino et al, 1985). Since phagocytes and lymphocytes are the main lines of defense against infections, a reduction in their number or function is detrimental to the host. In a review of pneumococcal bacteremia cases at two different hospitals, Perlino et al found that alcoholism was the only underlying disease significantly associated with leukopenia (Perlino et al, 1985). In that study, the mortality rate for leukopenic patients was dramatically increased (80%) from that of the non-leukopenic group of patients (20.5%). However, bacteremic酒精ics without leukopenia who have normal hepatic function fare no than bacteremic non-alcoholics (Austrian et al, 1964).

In many infections, PMNL's are the primary mediators of bacterial clearance. In order to be effective, they must be able to adhere to the vascular endothelium, undergo chemotaxis and ultimately engulf and kill the pathogen. Alcohol ingestion may contribute to leukopenia in several different ways. Early studies conducted by Pickrell (1938) demonstrated that when alcohol-intoxicated rabbits were injected subcutaneously with PNC, they exhibited defective mobilization and recruitment of granulocytes. In contrast, control animals had a rapid local neutrophil response and did not develop bacteremia. However, subsequent studies have suggested that alcohol intoxication does not prevent
adherence or recruitment of neutrophils but rather impairs the killing mechanisms of neutrophils (Gluckman et al, 1977 and Lister, 1993a). This hypothesis is supported by the work of Jareo et al, (1995 and 1996) which demonstrated that neutrophils from alcohol-fed rats phagocytose bacteria efficiently, but do not kill all strains of PNC effectively.

Macrophage function also is impaired by ethanol. As mentioned before, fixed macrophages in the the spleen and liver are essential in clearance of PNC from the bloodstream. In an assessment of the function of macrophages in the reticuloendothelial system of 12 alcoholic patients without hepatic dysfunction, Liu found that phagocytosis by the macrophages was depressed by acute alcohol ingestion (Liu, 1979).

Acute alcoholism is not known to have a direct effect on humoral host defense. Both human an animal experimental models have shown that the alcoholic host is not deficient in circulating levels of serum immunoglobulins (Caiazza et al, 1976 and Cooper et al, 1988). However, when alcoholism is accompanied by chronic liver disease, the host may have deficiencies in serum bactericidal activites (Fierer and Finley, 1979), as well as complement deficiencies. In contrast, it appears that there is an association between infection and defective cell-mediated immunity resulting from alcohol ingestion. Using an ethanol-fed rat model, Jerrels at al (1989) showed that thymus-derived lymphocytes from the spleens of animals ingesting ethanol were defective in their response to mitogens due to an inability to produce the thymocyte growth factor interleukin-2 (Jerrells et al, 1989). Additionally acute alcohol intoxication was
shown to impair natural killer cell activity (Cooper et al, 1988). In contrast, using a guinea pig model of ethanol intoxication insufficient to cause liver injury or disease, Caiazza et al (1976) stated that impaired immunity is not due to the direct effect of ethanol alone. Instead, both humoral and cell-mediated immunity remained relatively unimpaired in that alcoholic host model as long as the ethanol ingestion did not cause hepatic dysfunction.

Ethanol also exerts its effects on host defenses by suppressing the ability of immune cells to express leukocyte activating cytokines. Standiford and Danforth (1997) demonstrated that alveolar macrophages from rats fed ethanol for two weeks produced decreased amounts of lipopolysaccharide (LPS)-induced TNFα in comparison to controls. It also was shown that short term ethanol ingestion decreased TNFα production by macrophages in response to LPS or S. pneumoniae in vitro (Bleifield and Mellencamp, 1996). Similarly, Szabo et al (1995) reported that acute doses of ethanol decreased human monocyte production of IL-1β, TNFα and IL-6 in vitro in response to staphylococcal enterotoxins. Together these experiments suggest that ethanol diminishes the production of inflammatory cytokines from macrophages and monocytes and may be responsible for the impairment of lung inflammatory responses seen clinically and experimentally during alcohol ingestion.

corticosterone depletes cells from the thymus, spleen and bone marrow and decreases the function of various lymphocytes (Garvey and Fraker, 1991, Han, et al, 1993, Jerrells, et al, 1990 and Rey, et al, 1984). It may, therefore, suppress the innate and acquired immune responses, rendering ethanol-fed animals more susceptible to infectious diseases. In support of this hypothesis, blocking corticosterone production by adrenalectomy partially reverses the loss of cells from the lymphoid organs during chronic ethanol feeding (Jerrells, et al, 1990). Therefore, the reduced inflammatory cytokine response associated with alcohol consumption may be, at least in part, due to suppression by corticosterone.
SUMMARY & PURPOSE

The relationship between excessive alcohol use and increased susceptibility to and mortality from infectious diseases has been well documented. Alcohol impairs host defenses in numerous ways, placing alcoholics at high risk for severe bacterial infections. Alcoholics are particularly susceptible to bacteremia due to *Streptococcus pneumoniae*. Severity of disease and mortality is further exacerbated when accompanied by leukopenia, a condition common in alcoholics. It has been demonstrated that pneumolysin, a toxin produced by all clinical pneumococcal isolates, increases the organism's virulence within the bloodstream. Pneumolysin damages host leukocytes, which may be particularly detrimental to the host who is already leukopenic. However, the specific role of pneumolysin in the bloodstream of the alcoholic or nonalcoholic leukopenic host has yet to be examined. Therefore, our goal was to determine whether pneumolysin production decreases bloodstream clearance and increases mortality following experimental infection in an ethanol-fed rat model. Because this feeding model induces leukopenia in both ethanol- and pair-fed rats (Lister, et al, 1993b) it will enable us to study the roles of pneumolysin and ethanol ingestion in the context of leukopenia.
**Materials and Methods**

**Animals and Feeding Protocol**

Male Sprague-Dawley rats weighing 100-120 grams (Charles River Laboratories; Wilmington, DE) were housed in group cages for 8-10 days and given free access to tap water and rat chow (PMI Nutrition International, Inc.; Brentwood, MO). Rats in the alcohol- and pair-fed groups then were placed in individual cages, their water bottles were removed, and they were fed 80 ml/day of Lieber-DeCarli control diet (DYETS, Inc., Bethlehem, PA), containing 35% fat calories, for 4 days to accustom them to the liquid diet. Those animals then were paired by weight and one rat from each pair was switched to Lieber-DeCarli ethanol diet (DYETS), in which 36% of the carbohydrate calories in the control diet are replaced by ethanol. Each ethanol-fed rat was fed *ad libitum*, whereas his pair-fed counterpart was fed the amount of control diet equal to that consumed by its ethanol-fed counterpart the day before. This pair-feeding protocol was followed for 8 days prior to use of the animals in experiments. Chow-fed control rats receiving rat chow and tap water *ad libitum* also were included in each experiment.
Bacterial Strains and Culture Conditions

To determine the role of pneumolysin production on pneumococcal virulence during bloodstream infections, two isogenic mutants of a type 3 WU2 S. pneumoniae strain were used (kindly provided by Mary K. Johnson, Tulane University). The pneumolysin gene was first excised from the chromosome of the WU2 parent organism. The gene was then re-expressed on the plasmid pVA838, which also carries a gene for erythromycin resistance. The mutant strain designated H+C+ in these studies expresses the wild-type pneumolysin gene and produces pneumolysin with both hemolytic and complement activating activities. The strain designated PLY- carries plasmid pVA838 without an inserted pneumolysin gene, so it does not produce pneumolysin.

Each organism was stored at -80°C in Todd-Hewitt broth (THB; Difco, Detroit, MI) containing 10% glycerol. Fresh cultures were prepared daily by inoculating 75 ml of sterile THB supplemented with 5% heat inactivated rabbit serum (JRH Biosciences, Lenexa, KS) and 10 μg/ml of erythromycin (Abbott Labs, North Chicago, Il.) Cultures were incubated in a candle jar at 37°C until the bacteria reached logarithmic phase (15-17h). The organisms were collected by centrifugation at 18,000 x g for 20 min at 4°C, washed with sterile phosphate-buffered saline (PBS), and re-suspended in 2-5 ml of sterile PBS.
Preparation of Inocula and Infection

To prepare inocula containing known concentrations of organisms, curves of optical density vs. viable count were established for each strain (Figures 1 and 2). Dilutions of organisms, grown as described above, were prepared from the original suspension. The optical density of each dilution was determined at 540 nm using a DU640 spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA). Additional dilutions were made of the suspension and 100μl of each dilution were plated in duplicate on blood agar plates. The plates were then incubated overnight and counted to determine the number of colony-forming units (cfu/ml) in each suspension. The optical density vs. viable counts then were plotted using Pspilot and a best-fit line was calculated by the program. Curves prepared individually for each organism then were used to estimate the number of cfu's in the bacterial suspension with a known optical density.

To prepare the inoculum for each experiment, the organisms were grown, collected and washed as described previously. The bacterial suspensions were diluted with additional PBS to the appropriate optical density to produce the desired viable count.

In initial experiments, the rats were infected to achieve 2 x 10^6 cfu/ml of blood, as confirmed retrospectively via plate count technique. Because all of the animals in all three diet groups died rapidly after infection with this high dose, rats in all subsequent experiments were infected to achieve 2 x 10^5 cfu/ml of blood. To determine the number of organisms needed in the inoculum to achieve this dose, we calculated the number of organisms needed to yield an infectious dose
of 1 x 10^7 cfu/ml blood and then performed the appropriate dilutions. We started with a high inoculum and diluted it because our optical density vs. growth curves did not include values as low as 2 x 10^5 cfu/ml.

First, the average blood volume for each group of rats was determined. (i.e., the inoculum for the larger Chow-fed rats was calculated separately to ensure that all of the feeding groups were infected with the same number of organisms/ml blood.) Because rats have an average of 6 ml of blood / 100 g of body weight, the amount of blood in the rats was estimated as shown below (Harkness et al, 1995).

\[
(Average \ rat \ weight \ (g)) \times \ 6 \ ml \ blood = \frac{Average \ total \ ml \ blood/ \ rat}{100 \ g}
\]

This number was then multiplied by the desired inoculum to determine the total number of cfu's needed to infect each rat. Since 0.2 ml of inoculum are injected into each rat, the inoculum must contain 5 times that number of organisms/ml.

\[
(total \ # \ cfu's \ needed/rat)(5) = \# \ cfu's \ needed / \ ml \ inoculum
\]

Individual syringes were filled with 0.2ml of the bacterial suspension and placed in a beaker containing ice until each rat was infected. The rats were anesthetized lightly with ether (Fisher Scientific Co., St. Louis, MO). The tail was wiped with alcohol, and 0.2 ml of bacterial inoculum was injected into the tail vein. Immediately after infection, a cardiac puncture was performed to remove 0.1 - 0.2ml of blood for confirmation of the actual inoculum received by each rat.
This was performed by serially diluting 50 µl of blood from $10^{-1}$ to $10^{-5}$ and plating 50 µl of each dilution on blood agar plates (Remel, Lenexa, KS). The plates were incubated for approximately 20 h at 37°C in 5% CO₂. The number of bacterial colonies were counted and multiplied by the dilution to determine cfu/ml blood.
Figure 1. H+C+ : Optical Density vs. cfu/ml

Figure 2. PLY- : Optical Density vs. cfu/ml
Survival & Bacterial Clearance

In certain experiments, 0.1-0.2 ml of blood was collected by cardiac puncture at 4 and 21 h post-infection to quantify bacterial numbers in the rats’ blood. In other experiments, blood was collected at 15 min, 2 h or 27 h post-infection. Fifty μl of each blood sample were serially diluted and plated on blood agar plates as described above to determine the number of viable organisms. The rats then were maintained on their appropriate diets for 14 days post-infection to determine survival.

Bacterial Counts in Organs

Rats were anesthetized and infected via the tail vein as described above. Two hours post-infection, the rats were sacrificed by intraperitoneal injection with pentobarbital sodium (Nembutal; Abbott Laboratories, N. Chicago, IL) and exsanguinated by cardiac puncture. Fifty μl of heparinized blood was diluted in PBS to determine the number of cfu’s per ml of blood. The remainder was allowed to clot in an ice bath in vacutainer tubes (Sherwood Medical, St. Louis, MO). The clotted blood was centrifuged for 15 min at 500 x g at 4°C. The serum was then filtered through a 0.22μl syringe filter (Fisher Scientific Co., St. Louis, MO), aliquoted into 1.2 ml cryovials (Corning Costar Corporation, Cambridge, MA), and stored at -80°C for cytokine and corticosterone analysis.

Immediately after exsanguination of each animal, an incision was made from the abdomen to the throat and the chest cavity was opened. To clear the organs of blood, the heart was perfused with 35 ml of cold sterile PBS. This was
accomplished by inserting a 20 gauge needle into the left ventricle of the heart to
drain fluid and then injecting the PBS through a 20 gauge needle into the right
ventricle. The lungs, the upper portion of the left lobe of the liver, and the spleen
then were removed. Each organ was placed into a glass homogenizer (Fisher
Scientific Co., St. Louis, MO) and sufficient PBS was added to produce a total
volume of 5 ml. The organs were homogenized and 50 µl of the original
suspension, as well as serial dilutions down to at least 10⁻⁴ were plated on blood
agar plates to determine viable colony counts.

**Cytokine Assays**

Aliquots of serum samples from blood collected at 15 min, 2 h and 27 h
post-infection were stored at -80°C. Enzyme Linked Immunosorbent Assay
(ELISA) kits were used to determine cytokine levels in each of the serum
samples. Rat cytokine ELISA kits were obtained from the following
manufacturer’s: IL-1β and TNFα from R & D Systems Inc., Minneapolis, MN;
IL-6 from Biosource International, Camarillo, CA; IL-10 from Pharmingen, San
Diego, CA. Serum samples were assayed in duplicate and diluted, as needed, to
obtain a value within the standard curves. For certain samples that were diluted
and assayed multiple times but were still above the level of detection, an
estimated minimal value was calculated by multiplying the highest dilution factor
by the value of the highest standard.
Corticosterone Assay

Serum samples collected pre-infection, 2 h post-infection and 27 h post-infection were assayed for corticosterone using a radioimmunoassay kit according to the manufacturer’s instructions (ICN Biomedicals, Inc., Costa Mesa, CA).

Statistical Analysis

With the exception of the corticosterone results, all values are represented graphically as mean ± SE. Bloodstream clearance results were expressed as mean log numbers of cfu/ml of blood or mean log change in cfu/ml blood. In calculating the mean log cfu/ml of blood, rats that had 0 cfu’s were assigned a log value of 0 for that particular time point. Survival results are expressed as the percentage of rats within a diet group that were alive on day 14 post-infection. Fisher’s Exact (Two-Tailed) Test was performed to determine statistical differences in survival between and among the diet groups. The mean survival time also was calculated for each group of rats, with a value of 15 being assigned to all rats still surviving on day 14. Bacterial counts in organ results are expressed as the mean log cfu/organ or organ lobe. Cytokine values are expressed as mean pg/ml ± SE and corticosterone levels are expressed as ng/ml. Corticosterone levels are expressed as ng/ml. Correlations were determined using Spearman’s RankCoefficient Correlation.

All statistical analyses were performed using SYSTAT 8.0. A probability (p value) of less than 0.05 was considered statistically significant in all of the
tests used. Statistical comparisons for bloodstream clearance, mean survival
time, bacterial counts in organs, cytokine levels and corticosterone levels within a
diet group infected with the two organisms were performed using the Mann-
Whitney U Test. Statistical comparisons among the rats in the different diet
groups infected with the same organism were performed using the Kruskal Wallis
Test. If there was a significant difference among the groups, the values were
assigned a rank order and the Post Hoc Tukey Test was applied to the ranks in
order to determine which of the diet groups differed significantly from one
another.
Results

Bloodstream Clearance

To determine the clearance of *S. pneumoniae* from the bloodstream, rats were infected with $2 \times 10^5$ cfu/ml blood of either the H+C+ strain or the PLY-strain on day eight of pair-feeding. Data were compiled from several separate experiments in which blood was drawn from 5 min to 27 h post-infection. Rats in all three diet groups infected with the H+C+ strain cleared 1.5 - 2 logs of the organisms from their bloodstreams by two h post-infection (FIG. 3A). By four h post-infection, the EtOH-fed rats had cleared more H+C+ bacteria than rats in the other two diet groups, although the difference was only statistically significant for the EtOH vs. Pair-fed rats ($p = 0.03$) due to a wider variance for the Chow-fed animals. Between 4 and 27 h, the number of bacteria rose in the bloodstreams of rats in all of the diet groups. However, by 27 h, the numbers of organisms in the Chow-fed rats was significantly higher (mean log cfu/ml of blood = 8.6 ± 0.1) than that in either EtOH-fed (5.6 ± 0.08) or Pair-fed (6.8 ± 0.5) rats ($p = .005$ and .03, respectively). Additionally, the Chow-fed rats had a significantly greater mean log increase in the # bacteria / ml of blood than either the EtOH-fed ($p = .006$) or Pair-fed rats ($p = .03$ ; Table 3).

Rats in all three diet groups infected with the PLY-strain cleared 2.5 - 3.2 logs of the organisms during the first 2 h post-infection (FIG 3B). However, after two h, the number of organisms then rose within the bloodstreams of rats in each of the diet groups. By 27 h post-infection the EtOH-fed rats had the lowest bacterial counts in their bloodstreams ($3.2 \pm 0.9$ log cfu / ml ) versus $6.0 \pm 0.4$
log cfu / ml for Pair-fed and 5.7 ± 0.7 log cfu / ml for Chow-fed rats. However, these differences were not statistically significant due to a large standard error for the EtOH-fed group.

Comparisons also were made between the two bacterial strains within each diet group in order to determine how pneumolysin production affects bloodstream clearance. Rats in each of the diet groups cleared more of the PLY- than the H+C+ organisms by 27 h post-infection. However, the difference was only statistically significant for the Chow-fed rats ($p = 0.006$ ; Table 3).
FIG. 3  Bloodstream clearance of EtOH-, Pair- and Chow-fed rats infected with either H+C+ (Panel 3A) or PLY- (Panel 3B). All rats (n = 5-10 per group at each time point) were infected intravenously with $2.0 \times 10^5$ of the indicated strains per ml of blood. Data from blood samples taken via cardiac puncture at 5 min, 15 min, 2 h, 4 h, 21 h and 27 h post-infection were compiled from several separate experiments. The number of cfu per ml of blood was determined at each point by plate counts. Results are expressed as the mean log cfu per ml of blood +/- standard error (SE).
3A. H+C+ Strain

- Significantly lower for EtOH-fed than for Pair-fed animals (p=.03)
- **Significantly higher than Pair-fed (p=.03) or EtOH-fed (p=.005) rats.

3B. PLY- Strain
Table 3:  
Mean Log Change in # Bacteria/ml Blood at 27 h

<table>
<thead>
<tr>
<th>Rat Diet Group</th>
<th>H+C+</th>
<th>PLY-</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH</td>
<td>+0.4 ± 0.8</td>
<td>-1.8 ± 1.0</td>
</tr>
<tr>
<td>Pair</td>
<td>+1.6 ± 0.5</td>
<td>+0.3 ± 0.5</td>
</tr>
<tr>
<td>Chow</td>
<td>+3.3 ± 0.1*</td>
<td>+0.4 ± 0.7</td>
</tr>
</tbody>
</table>

*Significantly greater mean log increase in the # bacteria/ml of blood for Chow-fed rats infected with H+C+ strain than those infected with the PLY- strain (p = .006).
†Significantly greater mean log change in the # bacteria/ml of blood for Chow rats than for EtOH-fed (p = .006) and Pair-fed (p = .03) rats infected with the H+C+ strain.
Survival

Survival was recorded for 14 days after the rats were infected with 2 x 10^5 cfu/ml blood of the isogenic mutant strains. Total mortality for rats infected with the H+C+ strain did not differ statistically among the diet groups, as at least 70% of rats in all groups eventually succumbed to the infection (FIG 4A). In contrast, 86% of Chow-fed rats infected with the PLY- strain died by day 14, whereas at least 80% of both the EtOH-fed and Pair-fed rats survived (FIG. 4B). This difference was statistically significant, for comparison of the Chow-fed and Pair-fed groups (p = .01). When the mean survival time was compared among the diet groups (Table 4), Chow-fed rats infected with the H+C+ strain survived a significantly shorter time (2.3 ± 0.4 days) than the Pair-fed rats (9.2 ± 1.6 days ; p = .002). Survival time for the EtOH-fed rats was intermediate (6.17 ± 1.4). The mean survival time for Chow-fed rats infected with the PLY- strain also was significantly shorter (9.3 ± 2.1 days) than that for either the EtOH-fed (14.8 ± 0.2 days ; p = .02) or Pair-fed (14.5 ± 0.5 days ; p = .01) groups (Table. 4).

To determine the contribution of pneumolysin production to mortality in this model of infection, additional comparisons were made of total survival and survival time for rats within each diet group infected with the H+C+ strain vs. the PLY- strain (Table 4). Significantly more rats in both the EtOH-fed and Pair-fed groups survived infections with the PLY- strain than the H+C+ strain (p = .02 and .03, respectively ; FIG. 4). In addition, rats in all diet groups infected with the PLY- strain lived significantly longer than rats in that same diet group infected
with the H+C+ strain ($p = .01$ for EtOH-fed and Pair-fed, $p = .02$ for Chow-fed).

However, although Chow-fed rats infected with the PLY- strain survived significantly longer than those infected with the H+C+ strain, at least 86% of all Chow-fed rats died by day 14 post-infection, regardless of the infecting strain.
FIG. 4. Survival of EtOH-, Pair- and Chow-fed rats infected intravenously with either the H+C+ strain (4A) or the PLY-strain (4B). Rats (n = 5-10 per group) were pair-fed for 8 days before being infected with 2.0 x 10⁵ cfu of the isogenic mutant strains per ml blood. Mortality or survival was determined for 14 days post-infection.
A. H+C+ Strain

% Survival

Days Post-Infection

B. PLY- Strain

% Survival

Days Post-Infection

*Survival significantly greater than for Chow-fed rats infected with the PLY- strain (p = .01)
+Survival significantly greater than for rats in same diet group infected with the H+C+ strain.
**Table 4: Mean Survival Time in Days ± SE**

<table>
<thead>
<tr>
<th>Rat Diet Group</th>
<th>H+C+</th>
<th>PLY-</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH</td>
<td>6.7 ± 1.4</td>
<td>14.8 ± 0.2*</td>
</tr>
<tr>
<td>Pair</td>
<td>9.2 ± 1.6</td>
<td>14.5 ± 0.5*</td>
</tr>
<tr>
<td>Chow</td>
<td>2.3 ± 0.4†</td>
<td>9.3 ± 2.1*‡</td>
</tr>
</tbody>
</table>

Rats alive at 14 days were given a value of 15 for statistical purposes.

* Significantly longer mean survival time for rats infected with the PLY- strain than those in the same diet group infected with the H+C+ strain (p=.01 for EtOH, p=.01 for Pair, p=.02 for Chow).
† Mean survival time significantly shorter than for Pair-fed rats infected with the same strain (p=.02).
‡ Mean survival time significantly shorter than for EtOH-fed or Pair-fed rats infected with the same strain (p = .02 and .01, respectively).
Organ Burden

To determine whether differences in clearance of bacteria from the bloodstream were due to alterations in trafficking of the organisms within the reticuloendothelial system, rats were infected with \(2 \times 10^5\) cfu/ml blood of either the H+C+ or the Ply- strain on day eight of pair-feeding. Two h post-infection, the lungs were removed, along with the spleen and the upper portion of the left lobe of the liver. Plate counts were performed on homogenates of each organ to calculate the number of bacteria per organ or liver lobe. There were no statistical differences among the rat diet groups in the number of H+C+ or PLY- bacteria in any of the three organs. However, within each of the diet groups, there were fewer PLY- than H+C+ organisms in each of the three organs (FIG. 5). These differences were statistically significant in the spleen for all three diet groups (\(p = .02\) to \(.003\)). In addition, there also were fewer PLY- organisms than H+C+ organisms in the livers of the Pair-fed rats (\(p = .009\)) and the lungs of the Chow-fed rats (\(p = .05\)).
FIG. 5. Numbers of organisms deposited in the lung, left lobe of the liver and spleen of H+C+ vs. PLY- infected rats in each of the feeding groups. All rats (n = 9-12 per group) were pair-fed for 8 days before being infected with 2.0 x 10^5/ml blood of the indicated strains. Two hours later, the numbers of cfu per organ or left lobe of the liver were determined by plate counts. Results are expressed as the mean log cfu per organ +/- SE.
A. Ethanol-Fed Rats (n= 10-12)

B. Pair-Fed Rats (n=10)

C. Chow-Fed Rats (n=9)

Mean Log CFU/ Organ +/- SE

Lungs  Liver lobe  Spleen

H+C+  PLY-

P = 0.02

P = 0.009  P = 0.03

P = 0.05  P = 0.02
Cytokines

ELISA's were performed to quantify cytokine levels on serum collected from blood samples drawn at 15 min, 2 h and 27 h post-infection. All samples were assayed for IL-1β, IL-6, IL-10, and TNFα according to the manufacturer's instructions supplied with the kits. At 15 min post-infection, only 13-40% of rats per diet group per organism had detectable levels of IL-1β, IL-6, or TNFα (data not shown). The only cytokine for which levels were above the kits' limits of detection for the majority of animals was IL-10 (Table 5). There were no statistically significant differences between IL-10 levels for H+C+ vs. PLY- organisms within each diet group or among the diet groups infected with either of the organisms.

Of the 60 rats for which blood samples were drawn 2 h post-infection, only 1 rat had a detectable level of TNFα (data not shown). Similarly, less than half of the serum samples assayed had detectable levels of IL-6 (data not shown). In contrast, the majority of rats infected with either strain had detectable levels of IL-1 and IL-10 (Tables 6 and 7). There were no significant differences in the levels of these two cytokines either within a diet group infected with the H+C+ vs. the PLY- strain or among the three diet groups infected with the same strain.

All of the rats had detectable levels of IL-1 and IL-10 at 27 h post-infection. Most of the sera also had detectable levels of TNFα and IL-6, with the exception of the ethanol-fed rats infected with the PLY- strain. For all four cytokines, Chow-fed rats infected with the H+C+ strain had significantly higher levels than either the Ethanol-fed or Pair-fed rats (Tables 8-11). Additionally, Chow-fed rats...
infected with the H+C+ strain had significantly higher levels of all four cytokines than their Chow-fed counterparts infected with the PLY- strain. (Tables 8-11).
Table 5: Serum IL-10 Levels at 15 min Post-infection

<table>
<thead>
<tr>
<th>Diet Group</th>
<th>H+C+</th>
<th>PLY-</th>
<th>H+C+ &gt; PLY-</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH</td>
<td>(3/7) 49 ± 5</td>
<td>(8/8) 36 ± 5.1</td>
<td>—</td>
</tr>
<tr>
<td>Pair</td>
<td>(6/8) 32 ± 2</td>
<td>(6/9) 36 ± 8.6</td>
<td>—</td>
</tr>
<tr>
<td>Chow</td>
<td>(5/8) 42 ± 9.8</td>
<td>(4/10) 49 ± 22.2</td>
<td>—</td>
</tr>
<tr>
<td>Chow &gt; EtOH &amp; Pair</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Table 5. Levels of IL-10 in serum from rats collected at 15 min post-infection. All rats (n = 7-10 per diet group) were pair-fed for 8 days before being infected with 2.0 x 10⁵ of the isogenic mutant strains per ml of blood. ELISA’s were performed on serum samples according to manufacturer’s directions. Results are expressed as mean IL-10 levels in pg/ml of serum ± SE. The numbers in parenthesis are the number of rats with detectable IL-10 levels / out of the total number of rats infected.
Table 6: Serum IL-1β Levels at 2 h Post-infection

<table>
<thead>
<tr>
<th>Diet Group</th>
<th>H+C+</th>
<th>PLY-</th>
<th>H+C+ &gt; PLY-</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH</td>
<td>(6/9) 66 ± 12.7</td>
<td>(7/12) 65 ± 13.8</td>
<td>—</td>
</tr>
<tr>
<td>Pair</td>
<td>(8/10) 55 ± 14.1</td>
<td>(7/10) 86 ± 22.5</td>
<td>—</td>
</tr>
<tr>
<td>Chow</td>
<td>(6/9) 55 ± 11</td>
<td>(8/9) 51 ± 8.4</td>
<td>—</td>
</tr>
<tr>
<td>Chow &gt; EtOH &amp; Pair</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Table 6. Levels of IL-1β in serum from rats collected at 2 h post-infection. All rats (n = 9-12 per diet group) were pair-fed for 8 days before being infected with 2.0 x 10^5 of the isogenic mutant strains per ml of blood. ELISA’s were performed on serum samples according to manufacturer’s directions. Results are expressed as mean IL-1β levels in pg/ml of serum ± SE. The numbers in parenthesis are the number of rats with detectable IL-1β levels / out of the total number of rats infected.
Table 7: Serum IL-10 Levels at 2 h Post-infection

<table>
<thead>
<tr>
<th>Diet Group</th>
<th>H+C+</th>
<th>PLY-</th>
<th>H+C+ &gt; PLY-</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH</td>
<td>(9/9) 53 ± 5.8</td>
<td>(9/12) 41 ± 9.2</td>
<td>—</td>
</tr>
<tr>
<td>Pair</td>
<td>(6/8) 56 ± 11.3</td>
<td>(10/10) 72 ± 19.8</td>
<td>—</td>
</tr>
<tr>
<td>Chow</td>
<td>(9/9) 59 ± 11.8</td>
<td>(9/9) 75 ± 30.7</td>
<td>—</td>
</tr>
</tbody>
</table>

Chow > EtOH & Pair — —

Table 7. Levels of IL-10 in serum from rats collected at 2 h post-infection.

All rats (n = 9-12 per diet group) were pair-fed for 8 days before being infected with 2.0 x 10^5 of the isogenic mutant strains per ml of blood. ELISA’s were performed on serum samples according to manufacturer’s directions. Results are expressed as mean IL-10 levels in pg/ml of serum ± SE. The numbers in parenthesis are the number of rats with detectable IL-10 levels / out of the total number of rats infected.
Table 8: Serum TNFα Levels at 27 h Post-infection

<table>
<thead>
<tr>
<th>Diet Group</th>
<th>H+C+</th>
<th>PLY-</th>
<th>H+C+ &gt; PLY-</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH</td>
<td>(5/7) 195 ± 96</td>
<td>(1/9) 76</td>
<td>N/A</td>
</tr>
<tr>
<td>Pair</td>
<td>(7/8) 128 ± 68</td>
<td>(5/9) 70 ± 20</td>
<td>—</td>
</tr>
<tr>
<td>Chow</td>
<td>(7/7) 1,117 ± 240</td>
<td>(4/8) 36 ± 3.6</td>
<td>+ (p=.008)</td>
</tr>
<tr>
<td>Chow &gt; EtOH &amp; Pair</td>
<td>+ (p =.002)</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

Table 8. Results are expressed as mean serum levels of TNFα in pg / ml of serum ± SE. The numbers in parenthesis are the number of rats with detectable TNFα / out of the total number of rats infected. The rats with detectable TNFα levels were used in statistical analysis. Statistical comparisons within a diet group infected with either of the organisms were performed using the Mann-Whitney U Test. Statistical comparisons among the rats in the different groups infected with the same strain were performed using the Kruskal Wallis Test, followed by the Post Hoc Tukey Test. + or – indicates whether or not the indicated comparison was or was not statistically significant. N/A indicates that there were too few rats for statistical purposes.
Table 9: Serum IL-1β Levels at 27 h Post-infection

<table>
<thead>
<tr>
<th>Diet Group</th>
<th>H+C+</th>
<th>PLY-</th>
<th>H+C+ &gt; PLY-</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH</td>
<td>(7/7)</td>
<td>(9/9)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>1,216 ± 601</td>
<td>586 ± 251</td>
<td>—</td>
</tr>
<tr>
<td>Pair</td>
<td>(8/8)</td>
<td>(9/9)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>1,859 ± 776</td>
<td>929 ± 601</td>
<td>—</td>
</tr>
<tr>
<td>Chow</td>
<td>(7/7)</td>
<td>(8/8)</td>
<td>+ (p = .0001)</td>
</tr>
<tr>
<td></td>
<td>12,579 ± 1,493</td>
<td>510 ± 274</td>
<td>—</td>
</tr>
<tr>
<td>Chow &gt; EtOH &amp; Pair</td>
<td>+</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>(p = &lt;=.02)</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Table 9. Results are expressed as mean serum levels of IL-1β in pg / ml of serum ± SE. The numbers in parenthesis are the number of rats with detectable TNFα / out of the total number of rats infected. The rats with detectable TNFα levels were used in statistical analysis. Statistical comparisons within a diet group infected with either of the organisms were performed using the Mann-Whitney U Test. Statistical comparisons among the rats in the different groups infected with the same strain were performed using the Kruskal Wallis Test, followed by the Post Hoc Tukey Test. + or − indicates whether or not the indicated comparison was or was not statistically significant.
### Table 10: Serum IL-6 Levels at 27 h Post-infection

<table>
<thead>
<tr>
<th>Diet Group</th>
<th>H+C+</th>
<th>PLY-</th>
<th>H+C+ &gt; PLY-</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH</td>
<td>(6/7)</td>
<td>(5/9)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>5,878 ± 4,046</td>
<td>510 ± 318</td>
<td></td>
</tr>
<tr>
<td>Pair</td>
<td>(7/8)</td>
<td>(5/9)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>11,600 ± 25,937</td>
<td>597 ± 432</td>
<td></td>
</tr>
<tr>
<td>Chow</td>
<td>(7/7)</td>
<td>(6/8)</td>
<td>+ (p=.003)</td>
</tr>
<tr>
<td></td>
<td>150,314 ± 90,726</td>
<td>658 ± 322</td>
<td></td>
</tr>
<tr>
<td>Chow &gt; EtOH &amp; Pair</td>
<td>+</td>
<td>(p = ≤.004)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>(p = &lt;.004)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 10. Results are expressed as mean serum levels of IL-6 in pg / ml of serum ± SE. The numbers in parenthesis are the number of rats with detectable TNFα / out of the total number of rats infected. The rats with detectable TNFα levels were used in statistical analysis. Statistical comparisons within a diet group infected with either of the organisms were performed using the Mann-Whitney U Test. Statistical comparisons among the rats in the different groups infected with the same strain were performed using the Kruskal Wallis Test, followed by the Post Hoc Tukey Test. + or – indicates whether or not the indicated comparison was or was not statistically significant.
Table 11: Serum IL-10 Levels at 27 h Post-infection

<table>
<thead>
<tr>
<th>Diet Group</th>
<th>H+C+</th>
<th>PLY-</th>
<th>H+C+ &gt; PLY-</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH</td>
<td>(7/7) 233 ± 145</td>
<td>(9/9) 411 ± 226</td>
<td>—</td>
</tr>
<tr>
<td>Pair</td>
<td>(8/8) 283 ± 159</td>
<td>(9/9) 298 ± 167</td>
<td>—</td>
</tr>
<tr>
<td>Chow</td>
<td>(7/7) 1,764 ± 268</td>
<td>(8/8) 422 ± 230</td>
<td>+ (p=.008)</td>
</tr>
<tr>
<td>Chow &gt; EtOH and Pair</td>
<td>+ (p = ≤.001)</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

Table 11. Results are expressed as mean serum levels of IL-10 in pg / ml of serum ± SE. The numbers in parenthesis are the number of rats with detectable TNFα / out of the total number of rats infected. The rats with detectable TNFα levels were used in statistical analysis. Statistical comparisons within a diet group infected with either of the organisms were performed using the Mann-Whitney U Test. Statistical comparisons among the rats in the different groups infected with the same strain were performed using the Kruskal Wallis Test, followed by the Post Hoc Tukey Test. + or − indicates whether or not the indicated comparison was or was not statistically significant.
Correlations of cfu/ml Blood vs. Cytokine Levels

To determine whether the serum cytokine levels could be predicted by the number of cfu's/ml blood, Spearman's Rank Order Correlations were performed. At 2 h post-infection, there was a significant correlation between the number of cfu's/ml blood and both IL-1β (Fig. 6; \( p < 0.005 \)) and IL-10 (Fig. 7; \( p < 0.02 \)) levels when the rats were infected with the H+C+ strain, but not the PLY- strain. By 27 h post-infection, the number of cfu's/ml blood correlated significantly with TNFα (Fig. 8; \( p < 0.001 \)), IL-1β (Fig. 9; \( p < 0.001 \)), IL-6 (Fig. 10; \( p < 0.001 \)) and IL-10 (Fig. 11; \( p < 0.001 \)) levels in rats infected with either strain. However, it is important to note that the levels of the inflammatory cytokines in rats infected with the H+C+ strain are much higher than those in rats infected with the PLY-strain. Also, at 27 h post-infection when the number of H+C+ organisms approaches 8 logs, the cytokine levels tend to increase (Figs. 8-11).
FIG. 6. Correlation of log cfu/ml blood vs. IL-1β levels at 2 h post-infection with either the H+C+ strain (Panel 6A) or the PLY- strain (Panel 6B). All rats were infected i.v. with $2 \times 10^5$ cfu/ml blood. Correlation coefficient ($r_s$) was calculated by Spearman’s Rank Order Correlation. Note difference in scale for levels in rats in infected with H+C+ vs. PLY- organisms. N.S. Not Significant.
Figure 6. Serum IL-1β Levels at 2 h Post-Infection

6A. H+C+

6B. PLY-

$r_s = 0.529$

$r_s = -0.170$ N.S.
FIG. 7. Correlation of log cfu/ml blood vs. IL-10 levels at 2 h post-infection with the H+C+ strain (Panel 7A) or the PLY- strain (Panel 7B). All rats were infected i.v. with 2 x 10^5 cfu/ml blood. Correlation coefficient (r_s) was calculated by Spearman’s Rank Order Correlation. Note difference in scale for levels in rats infected with H+C+ vs. PLY- organisms. N.S. Not Significant.
Figure 7. Serum IL-10 Levels at 2 h Post-Infection

7A. H+C+

7B. PLY-

$\text{IL-10 (pg/ml)}$

$\text{Mean log cfu/ml blood}$

$r_s = 0.466$

$r_s = 0.068 \text{ N.S.}$
FIG. 8. Correlation of log cfu/ml blood vs. TNFα levels at 27 h post-infection with the H+C+ strain (Panel 8A) or the PLY- strain (Panel 8B). All rats were infected i.v. with $2 \times 10^5$ cfu/ml blood. Correlation coefficient ($r_s$) was calculated by Spearman’s Rank Order Correlation. Note difference in scale for levels in rats in infected with H+C+ vs. PLY- organisms.
Figure 8. Serum TNFα Levels at 27 h Post-Infection

8A. H+C+

8B. PLY-

Log cfu/ml blood

Log cfu/ml blood
FIG. 9. Correlation of log cfu/ml blood vs. IL-1β levels at 27 h post-infection with the H+C+ strain (Panel 19A) or the PLY- strain (Panel 9B). All rats were infected i.v. with 2 x 10^5 cfu/ml blood. Correlation coefficient (r_s) was calculated by Spearman’s Rank Order Correlation. Note difference in scale for levels in rats in infected with H+C+ vs. PLY- organisms.
Figure 9. Serum IL-1β Levels at 27 h Post-Infection

9A. H+C+

9B. PLY-

Log cfu/ml blood

IL-1 (pg/ml)

$\rho_s = 0.675$

Log cfu/ml blood

IL-1 (pg/ml)

$\rho_s = 0.550$
FIG. 10. Correlation of log cfu/ml blood vs. IL-6 levels at 27 h post-infection with the H+C+ strain (Panel 10A) or the PLY- strain (Panel 10B). All rats were infected i.v. with $2 \times 10^5$ cfu/ml blood. Correlation coefficient ($r_s$) was calculated by Spearman's Rank Order Correlation. Note difference in scale for levels in rats in infected with H+C+ vs. PLY- organisms.
Figure 10. Serum IL-6 Levels at 27 h Post-Infection

10B. H+C+

- Pair
- Chow
- EtOH

$r_s = 0.534$

10B. PLY-

- EtOH
- Chow
- Pair

$r_s = 0.776$
FIG. 11. Correlation of log cfu/ml blood vs. IL-10 levels at 27 h post-infection with the H+C+ strain (Panel 11A) or the PLY- strain (Panel 11B).

All rats were infected i.v. with $2 \times 10^5$ cfu/ml blood. Correlation coefficient ($r_s$) was calculated by Spearman’s Rank Order Correlation.
Figure 11. Serum IL-10 Levels at 27 h Post-Infection

11A. H+C+

- Pair
- Chow
- EtOH

\( r_s = 0.758 \)

11B. PLY-

- EtOH
- Chow
- Pair

\( r_s = 0.0770 \)
Serum Corticosterone Levels

A radioimmunoassay to quantify corticosterone levels was performed on serum from 6 random blood samples/group drawn pre-infection and at 2 h post-infection, plus 3 samples/group drawn at 27 h post-infection. Although pre-infection corticosterone levels were generally higher in EtOH-fed rats than either Pair- or Chow-fed rats, this difference did not reach statistical significance (FIG. 12A). At 2 h post-infection, Chow-fed rats infected with the H+C+ strain had significantly higher levels of corticosterone than H+C+- infected EtOH-fed rats (FIG. 12B). There were no significant differences between the diet groups infected with the PLY- strain. Within a diet group, when corticosterone levels were compared for animals infected with different bacterial strains, Chow-fed rats infected with the H+C+ strain had significantly higher levels than those infected with the PLY- strain (p=.02), whereas the opposite was true for Pair-fed rats (p=.02; FIG. 12B). Also, both Ethanol-fed (p=.006) and Pair-fed (p=.02) rats had significantly lower levels of corticosterone at two h post-infection when infected with the H+C+ strain, but not the PLY- strain. In contrast, the Chow-fed rats had a significant decrease in corticosterone levels when infected with the PLY- strain (p=.01), but not the H+C+ strain. Spearman’s Rank Order Correlation was used to determine if corticosterone levels correlated with cfu/ml blood or with IL-1β or IL-10 levels. None of these were significant for either strain (data not shown).

At 27 h post-infection, there were no apparent differences among the diet groups infected with either strain or within a diet group infected with the same
strain (FIG. 12C). Because we were only able to obtain corticosterone levels for 3 animals per diet group at 27 h post-infection, and because the values were so low for all animals tested, we combined the values for all animals within each feeding group. Again corticosterone levels at 27 h were significantly lower for pre-infection levels of both Ethanol-fed ($p=0.004$) and Pair-fed ($p=0.004$) rats, but did not quite reach significance for Chow-fed rats ($p=0.055$).
FIG. 12. Serum corticosterone levels determined by radioimmunoassay on blood collected pre-infection and at 2 and 27 h post-infection. All rats were pair-fed for 8 days and then either sacrificed before infection or infected with $2.0 \times 10^5$ of the isogenic mutant strain / ml of blood and sacrificed at the indicated time point. Each symbol represents the value for a single rat, and the dark line represents the mean level of corticosterone in ng/ml of serum for rats in that group.
A. Pre-Infection Serum Corticosterone Levels

B. Serum Corticosterone Levels 2 h Post-Infection

C. Serum Corticosterone Levels 27 h Post-Infection

* Connected values differ significantly at level indicated in parantheses
Discussion

Alcoholics are more susceptible to a variety of infectious diseases, including those caused by *Streptococcus pneumoniae*. This increased susceptibility is due, at least in part, to ethanol-induced impairment of nonspecific host defense mechanisms which make the alcoholic host more vulnerable to aspiration of PNC organisms. In addition, the function of both macrophages and neutrophils are impaired by ethanol (Gluckman et al, 1977, Lister, et al, 1993a, Jareo, et al, 1995 and 1996 and Liu, 1979). Since neutrophils are the primary mediators of PNC clearance from the lungs, impairment of their function is thought to be particularly detrimental to the alcoholic host with pneumonia. The importance of neutrophils in host defense against PNC bacteremia is less clear cut, but it is known that mortality from bacteremia increases sharply in neutropenic patients. Numerous studies have shown that although ethanol ingestion by itself does not predispose individuals to mortality due to PNC bacteremia, leukopenia in combination with ethanol consumption does increase mortality (Austrian and Gold, 1964, Gransden, et al, 1985, Perlino and Rimland, 1985). Since leukopenia is more common among alcoholics (reviewed in Sternbach, 1990), it is important to consider this element in their defense against *S. pneumoniae*.

In our experiments we used an ethanol-fed rat model to examine the effects of both ethanol ingestion and pneumolysin production on host resistance to PNC bacteremia. This model is appropriate for our needs because it had been
shown that consumption of the liquid diets is sufficient to induce leukopenia in both the ethanol- and pair-fed rats (Lister, 1993b). Since the control pair-fed rats are also leukopenic, we are able to examine the specific role of ethanol alone, while mimicking a confounding common condition seen in alcoholics.

Also, 18% of the energy derived from the liquid ethanol diet comes from protein, with 35% derived from fat, and 36% derived from ethanol. The remaining energy is derived from carbohydrates, fiber, vitamins, and minerals. This is comparable to the high fat content of the American diet. Although the average intake of energy from ethanol amounts to 50% of total energy in the alcoholic human (reviewed in Lieber, 1994) rats will not voluntarily consume a diet with such a high alcohol content. However, this liquid diet is appropriate for our studies because it does not cause the more severe forms of alcoholic liver damage, such as hepatitis and cirrhosis. This enables us to examine the effects of ethanol without the added complications of liver dysfunction.

Because the introduction of ethanol into the diet causes the rats to decrease their overall food intake, pair-fed animals serve as nutritional controls. They receive equivalent amounts of daily calories in a liquid control diet in which carbohydrates isocalorically replace the ethanol. Animals pair-fed the liquid control diet in this way control for nutritional deficiency and allow the effects of ethanol to be examined in isolation (reviewed in Lieber, 1994). Therefore, this model is reflective of human nutrition, and the inclusion of pair-fed animals controls for nutritional variables, enabling us to focus specifically on the effect of ethanol on PNC pathogenesis.
Finally, this model is well-suited to our needs in that it has been shown to produce a fatty liver in the ethanol-fed animals, a complication often observed in alcoholics, (DeCarli, 1967). Rats fed the ethanol diet have blood ethanol levels between 100 and 150 mg/dl, putting them above the levels of legal intoxication (reviewed in Lieber, 1994). Thus, our model is appropriate to study ethanol intoxication and can be used to enhance our understanding of how ethanol affects resistance to PNC infections in the human host.

Although it is clear that the effects of ethanol alone are sufficient to impair mammalian hosts’ antibacterial defense mechanisms, it is also possible that virulence factors unique to *S. pneumoniae* may exacerbate the organism’s pathogenicity in the alcoholic population. One such well-characterized PNC virulence factor is the cytolytic toxin known as pneumolysin, which is produced by all clinical isolates of *S. pneumoniae*. In addition to numerous other detrimental effects, pneumolysin inhibits the bactericidal properties of human neutrophils and macrophages (Paton and Ferrante, 1983, Johnson, et al, 1981, Nandoskar, et al, 1986). It also can inhibit the proliferative response of human lymphocytes to mitogens and activate the classical complement pathway, ultimately diverting complement away from the invading organisms (Ferrante, 1984, Paton, et al, 1984). Pneumolysin, therefore, appears to be extremely well-suited and uniquely designed to impair the very host defenses important for eradication of PNC. In preliminary experiments performed in our laboratory, the concentration of pneumolysin required to inhibit ribosomal respiration in neutrophils from Chow-fed rats was 2- to 3-fold higher than that required for respiratory inhibition of
neutrophils from Ethanol-fed or Pair-fed rats (unpublished observations). Therefore, we hypothesized that pneumolysin production would further exaggerate the defective host defenses of the Ethanol-fed, and perhaps the Pair-fed host, rendering them more susceptible to lethal PNC bacteremia.

We proposed that alcohol ingestion in our model system would decrease the ability of rats to clear PNC from their bloodstreams. To test this hypothesis, the rats were infected intravenously with either a type 3 strain of *S. pneumoniae* that produces wild-type pneumolysin (H+C+) or its isogenic pneumolysin-deficient mutant (PLY-) strain. To our surprise, Ethanol-fed rats cleared both organisms at least as well as both Pair-fed and Chow-fed rats. By 4 h post-infection, Ethanol-fed rats infected with the H+C+ strain actually had cleared the most organisms from their bloodstreams, with the difference being statistically significant in comparison to the Pair-fed controls. However, because rats in all of the diet groups ultimately experienced statistically equivalent mortality rates after infection with the H+C+ strain, it appears that the enhanced early clearance seen in the Ethanol-fed group was not biologically relevant with regard to the final outcome of infection.

An even more unexpected finding in our initial experiments was that by 27 h the Chow-fed rats infected with the H+C+ strain had significantly more organisms in their bloodstreams and a significantly greater mean log increase in the number of bacteria/ml blood than either the Ethanol-fed or Pair-fed rats infected with the same organism. Therefore, contrary to our original hypothesis, alcohol ingestion did not impair the rats' early clearance of PNC from the
bloodstream, and nutritional deprivation, if anything, may have been protective against wild-type PNC.

According to reports from other laboratories (Berry, et al, 1989b, Benton, et al, 1995), mice were able to clear pneumolysin negative organisms from their bloodstreams more effectively than mice infected with a wild-type strain. Therefore, we also predicted that pneumolysin production would decrease the rats' ability to clear organisms from their bloodstreams. Our results are consistent with the previous studies, as the mean log increase in the number of bacteria/ml blood at 27 h post-infection was lower for rats in all three diet groups infected with the PLY- vs. H+C+ strain. However, the difference was only statistically significant for rats in the Chow-fed group due to wide variations in clearance within many of the groups.

Furthermore, the number of organisms in the bloodstreams of rats infected with the H+C+ strain continued to increase after 4 h post-infection, reaching greater than $2 \times 10^8$ cfu/ml blood in the Chow-fed rats by 27 h post-infection. In contrast, the number of organisms in the bloodstreams of rats infected with the PLY- strain remained relatively stable after 4 h post-infection at $<1 \times 10^6$ cfu/ml blood. Previous studies from our laboratory have shown that the H+C+ and PLY- strains have similar growth curves over a 24 hour time period in vitro (Alcantara, et al, 1999). Therefore it is unlikely that these differences are due to a decreased growth rate for the PLY- strain. These results are consistent with those reported by other laboratories. In 1995, Benton, et al noted that a wild-type serotype 2 PNC increased exponentially to $10^9$ to $10^{10}$ cfu/ml in the bloodstreams of mice.
after intravenous infection with $10^4$ cfu/ml blood. Additionally, all mice infected with the wild-type organism died by 24 to 28 h post-infection. In this same study, the number of a pneumolysin negative mutant organisms were maintained at $10^6$ to $10^7$ / ml blood for several days to weeks. Although the exact mechanism remains unclear, the authors of that study suggest that pneumolysin plays a critical role during the first few hours after infection by enabling the PNC to produce an acute lethal sepsis that escapes regulation by the immune system. In the absence of pneumolysin, however, a chronic bacteremia occurs that can delay the death of the mice for at least several days.

In addition to bloodstream clearance, another crucial element of our initial experiments was assessment of mortality. This allowed us to answer what is perhaps the most important question of all: How do ethanol ingestion and pneumolysin production affect the ability of the host to survive a bacteremic infection with *S. pneumoniae*? We found that in addition to having an elevated number of organisms in their bloodstreams at 27 h, Chow-fed rats infected with the H+C+ strain also had a significantly shorter mean survival time than Ethanol-fed and Pair-fed rats infected with the same strain. Also, all Chow-fed rats infected with the H+C+ strain were dead by 5 days post-infection. Furthermore, pneumolysin production significantly decreased the percentage of Ethanol-fed and Pair-fed rats that survived their infections, whereas it did not markedly influence the ultimate outcome of infection in the Chow-fed rats. Only 14% of the Chow-rats infected with the PLY- strain were alive on day 14 post-infection. This suggests that the increased susceptibility of the Chow-fed rats to the H+C+
organism vs. the PLY- organism can not be explained by pneumolysin production alone.

Our results showing that pneumolysin production decreases the mean survival time for rats in all diet groups are supported by studies from other laboratories. Berry (1989b) noted that mice infected intranasally with a type 2 pneumolysin negative PNC strain lived significantly longer and cleared more organisms from the bloodstream than mice injected with an isogenic wildtype strain. When the pneumolysin negative strain was reverted back into a wild-type strain by transformation with the cloned pneumolysin gene, the organism's virulence increased and was similar to the wild-type control, reiterating the importance of pneumolysin production to virulence in this model system. Similarly, in another study, mice infected intravenously with a type 2 pneumolysin negative strain also lived significantly longer and were able to control bacterial growth in their bloodstreams more effectively than mice infected with an isogenic wild-type, pneumolysin producing strain (Benton, 1995). In that study, $10^5$ cfu/ml blood of the wild-type strain killed half of the mice within 24 h, and all of the mice had died by 26 h post-infection. In contrast, none of the mice infected with $10^5$ cfu/ml of the pneumolysin negative strain died prior to 48 h post-infection, and 50% were still alive at 7 days post-infection when the experiment was terminated. This is consistent with our study, in which greater than 50% of our Chow-fed rats infected with the PLY- strain were still alive on day 14. However, a number of deaths in these animals occurred on days 11-14, raising the possibility that more of their mice would have died had their experiment been allowed to proceed as
long as ours. Despite the fact that our experiments were conducted in different rodent models using two different serotypes of PNC, our clearance and survival data in the Chow-fed animals are consistent with those in these previous studies. Chow-fed rats in our experiments cleared the PLY- strain more effectively and died much more rapidly when they were infected with the H+C+ strain.

Contrary to our hypothesis, neither ethanol ingestion nor a dietary regimen that has been shown to result in leukopenia (Lister, et al, 1993b) hindered the ability of the rats to clear PNC from their bloodstreams or to survive an intravenous infection with type 3 S. pneumoniae. Moreover, the addition of ethanol to the liquid diet did not significantly distinguish the Ethanol-fed rats from the Pair-fed rats in these studies. It is possible that the length of ethanol-feeding employed (8 days) was not long enough to impair the systemic immune defenses of the Ethanol-fed rats. However, investigators in other laboratories have reported that 7 days of ethanol-feeding is sufficient to induce immunosuppression that mimics that seen in alcoholics (Jerrells, et al 1990). Additionally, our laboratory has shown that 7 days of ethanol-feeding is sufficient to significantly impair the resistance of rats to a transtracheal infection with another strain of pneumolysin-producing type 3 PNC (Davis et al, 1991). Ethanol-fed rats in that study were more likely to become bacteremic, remained bacteremic longer, and had a significantly higher mortality rate than Pair-fed control rats. This indicates that the host immune defenses impaired by ethanol ingestion may be much more critical for resistance to PNC infections in the lungs than in the peripheral circulation. This is supported by the fact that although S. pneumoniae is a
leading cause of bacteremia during HIV-1 infection, death from HIV-1 associated PNC bacteremia is no more common than that among age-matched seronegative bacteremic patients (Janoff and Rubins, 1997). In our studies, it appears that nutritional deprivation may be protective in some way against bacteremic PNC disease.

The fact that the H+C+ organisms were cleared more effectively from the bloodstreams of the Ethanol-fed and Pair-fed than from the Chow-fed animals suggested that the organisms were being sequestered more efficiently in the reticuloendothelial system (RES) organs of the Ethanol-fed and Pair-fed rats. To test this hypothesis, rats in each diet group were infected with one of the two strains and the numbers of organisms in their lungs, their spleens and one lobe of their livers were quantified. There were no differences among the diet groups infected with the same bacterial strain in the number of live organisms within any of the three organs examined. This suggests that no one diet group was more successful in sequestering or filtering either of the organisms from their bloodstreams. Within each diet group, however, the number of organisms in the three organs was generally lower in rats infected with the PLY- strain than in their counterparts infected with the H+C+ strain. This was statistically significant in the spleens of all diet groups, in the livers of Pair-fed rats, and in the lungs of Chow-fed rats. Therefore, the smaller numbers of PLY- vs. H+C+ bacteria in the bloodstreams of rats in each of the diet groups cannot be explained by increased sequestration of the PLY- bacteria within the organs of the RES. Furthermore, it appears that the PLY- organisms are being killed more efficiently than the
pneumolysin-producing strain within the RES. This is consistent with in vitro killing assays performed by Benton, et al (1995), who found that pneumolysin-negative PNC are more susceptible than pneumolysin-producing PNC to killing by human neutrophils. Those authors suggested that one effect of pneumolysin is to decrease opsonophagocytosis of the organism, a conclusion supported by our studies.

From our bloodstream clearance and survival experiments, it is apparent that host defense against intravenous challenge with PNC is altered by nutritional factors. In order to further elucidate the cellular events associated with PNC bacteremia, we quantified cytokine levels during the course of the infection. This was done for two reasons. First, in our early experiments a number of Ethanol-fed rats infected with the PLY-strain died within 5-30 min of infection (data not shown). We hypothesized that this may have been due to an overwhelming and lethal systemic inflammatory cytokine response in these animals. However, we were unable to detect TNFα, IL-1, or IL-6 in the sera of rats in any of the treatment groups at fifteen min post-infection. Additionally, as our infection technique was mastered, this phenomenon no longer occurred. Inevitably, a small proportion of rats are lost during infection due to the experimental process itself (i.e. excessive ether anesthesia, cardiac puncture). In previous experiments in our laboratory, Ethanol-fed rats have been shown to be more likely to die than Pair-fed or Chow-fed rats during ether anesthesia. Therefore, we believe that this was the primary cause of our original observations, making our original concern unfounded.
The second reason we decided to quantify cytokine levels is that cytokines are important mediators of inflammation. They have the potential to either enhance host defense or, in excess, cause damage to the host. We therefore wanted to determine whether serum cytokine levels could partially explain or further delineate the uncontrolled bacterial growth and rapid deaths exhibited by the Chow-fed rats infected with the H+C+ strain. We chose to examine the levels of TNFα, IL-1β, IL-6 and IL-10 because they have all been shown previously to play a role in sepsis. There is also evidence that their regulation may be altered in the alcoholic host.

Despite the absence of detectable TNFα, IL-1β and IL-6 levels at 15 min post-infection, we were able to detect IL-10 levels in the serum of at least 40% of the rats in each of the diet groups infected with either strain. There were, however, no significant differences among diet groups infected with the same strain or between rats in a given diet group infected with either of the bacterial strains. In previous experiments in our laboratory, serum IL-10 levels were undetectable in uninfected rats within each feeding group (unpublished results). Therefore it appears that the presence of the PNC in the bloodstream rapidly stimulates low levels of IL-10 production independent of pneumolysin production by the organism and host nutritional factors. Our results differ from those reported by Bergeron, et al (1998), who used a murine model to examine the major steps in PNC pathogenesis after intranasal infection with a type 3 strain. In that study, there was a dramatic increase in serum levels of both TNFα and IL-6 at 48-72 h post-infection which coincided with the development of bacteremia.
Since serum levels of these cytokines increased when the organisms reached the bloodstream, we expected that they would be elevated rapidly after intravenous infection in our experiments. However, both TNFα and IL-6 were undetectable at 15 min and even 2 h post-infection. Differences in the appearance of these cytokines in the serum may be related directly to differences in the route of infection. During experimental pneumonia, for example, resident alveolar macrophages are activated to produce cytokines, and it is possible that these are released at high levels into the bloodstream only after the lung has sustained significant damage. In the case of an intravenous infection, however, there is no local site of infection where the production of cytokines is initiated. Ongoing studies in our laboratory are currently examining levels of inflammatory cytokine mRNA production in the spleen, liver and lung tissue, where the organisms are filtered out of the bloodstream of intravenously infected rats.

By 2 h post-infection with either strain, levels of TNFα and IL-6 were still minimal or undetectable in the serum of rats in each of the feeding groups. We were, however, able to detect both IL-1β and IL-10 in the majority of rats in each of the treatment groups. There were no significant differences in the levels of IL-1β or IL-10 among diet groups infected with the same strain or between rats within a given diet group infected with either of the strains. However, there was a significant correlation between the number of H+C+ organisms and serum levels of both IL-1β and IL-10. As the numbers of organisms in the bloodstream increased, levels of these cytokines also increased. In contrast, there was no correlation between the number of PLY- organisms and serum levels of either
cytokine. Therefore, although ethanol ingestion does not appear to affect the early systemic cytokine response to PNC bacteremia, both pneumolysin production and an increased number of organisms seem to contribute to increases in both IL-1β and IL-10.

By 27 h post-infection, all rats had detectable serum levels of IL-1β and IL-10, and at least 50% of the rats in each treatment group had detectable levels of IL-6 and TNFα with the exception of TNFα in Ethanol-fed rats infected with the PLY- strain. Interestingly, the levels of all four cytokines in rats with detectable values correlated significantly with the number of both H+C+ and PLY- organisms. Therefore, as the number of organisms increases, cytokine levels increase as well. However it is important to note that the release of inflammatory cytokines is much more pronounced during infection with the H+C+ strain. Although levels of these cytokines increase with both organisms, a critical distinction is that dramatically higher levels of the inflammatory cytokines occur 27 h post-infection with the H+C+ strain, rather than the PLY- strain. As the number of H+C+ organisms reaches approximately 1 x 10^8 cfu/ml blood, cytokine levels also increase. In contrast, the number of PLY- organisms in the bloodstream remains rather stable and lower levels of inflammatory cytokines are produced 27 h post-infection. Again, there were no significant differences in levels of any of the cytokines between H+C+-infected and PLY^-infected rats in the Ethanol-fed and Pair-fed diet groups or between Ethanol-fed and Pair-fed rats infected with the same bacterial strain. However, the Chow-fed rats infected with the H+C+ strain had dramatically elevated levels of all four cytokines in
comparison to their Chow-fed counterparts infected with the PLY- strain. As previously mentioned, the Chow-fed rats infected with the PLY- strain nearly all died by the end of the experiment, suggesting that something inherent to this diet group renders them more susceptible to PNC, regardless of its ability to produce pneumolysin. However, the number of organisms and cytokine levels in the bloodstreams of the PLY- infected Chow-fed rats do not distinguish them from rats in the other two diet groups infected with the PLY- strain. It is possible that these Chow-fed rats have an increase in the numbers of organisms in their bloodstreams or that massive cytokine production occurs later than 27 hours, accompanying their deaths. Additional experiments with measurements at later time points would be necessary to help explain the high mortality demonstrated by Chow-fed rats infected with the PLY- strain.

At 27 h post-infection, Chow-fed rats infected with the H+C+ strain had significantly higher serum levels of all four cytokines than Ethanol-fed or Pair-fed rats infected with the same strain. Again, this suggests that nutritional deprivation, with or without ethanol ingestion, may account for differences in the ability of the animals to control the number of organisms in their bloodstreams and, indirectly, regulate cytokine activation in response to infection. Although ethanol ingestion does not increase the rats' susceptibility to fatal PNC bacteremia, it does appear that the generation of a massive cytokine release and ultimately, the ability of the host to survive the infection is influenced by pneumolysin production. When Chow-fed rats are infected with the H+C+ strain, a massive and overwhelming cytokine response is generated within 27 h, and the
rats die rapidly thereafter. This type of response is similar to what has been reported in the literature, with excessively high levels of TNFα, IL-1β, IL-6 and IL-10 having been shown to parallel sepsis and increase mortality (Charalambos et al, 2000; Ziegler-Heitbrock, et al, 1992; Friedman, 1997). Furthermore, independent studies have shown that blocking TNFα (Schofield, 1987), IL-1β (Ohlosson et al, 1990), IL-6 (Ziegler-Heitbrock, et al, 1992) and even IL-10 (Van der Poll, 1996) protects against the normal injurious host responses elicited during experimental sepsis or endotoxic shock. It remains to be determined whether blocking one or more of these cytokines in our Chow-fed rats would have a similar protective effect and could preclude the rapid mortality observed after infection with the H+C+ strain.

The very rapid lethal sepsis demonstrated in the Chow-fed rats after infection with the H+C+ strain is moderated somewhat in the Ethanol-fed and Pair-fed rats. One possible reason why the Ethanol-fed rats do not succumb to an early massive and lethal cytokine response is that ethanol or poor nutrition down-regulates the production of certain cytokines. For example, ethanol down-regulates TNFα production in human monocytes (Bikash, et al 1993; Nair, et al, 1994; Szabo, et al 1996), murine alveolar macrophages (Standiford, et al, 1997) and bronchoalveolar lavage fluid (BALF; Nelson, et al, 1989) in response to lipopolysaccharide. Furthermore, IL-6 production is suppressed by ethanol (Szabo, 1995), and acute ethanol treatment in vitro reduces IL-1β production by human monocytes in response to Staphylococcal enterotoxin or to lipopolysaccharide (Szabo, et al, 1995 and 1996). Therefore, it is possible that
the ingestion of ethanol suppressed cytokine production in our infection model. Although this may be detrimental to the host under normal conditions, it may actually be protective during experimental PNC bacteremia in that it precludes the massive outpouring of cytokines initiated by the presence of large numbers of organisms in the bloodstream.

If it was ethanol alone, however, that suppressed the cytokine response, the Pair-fed rats would have experienced an unregulated cytokine response similar to that seen in the Chow-fed rats. There are several other possible explanations for the marked differences seen in the cytokine response and survival between the Chow-fed rats versus the Ethanol-fed and Pair-fed rats. First, nutrition affects almost every facet of host immunity, including cell-mediated immunity, the humoral response, phagocytosis and the complement system (Reviewed in Gross and Newberne, 1980). Although the liquid diets employed in our experiments are nutritionally complete, the rats consume considerably less diet when it contains ethanol. When given control diet ad libitum, the Pair-fed animals will consume as much as 80-120 ml of diet/day. In our studies, however, the Pair-fed animals are given only 25-40 ml of diet/day, depending on the amount eaten by their Ethanol-fed counterpart the previous day. In contrast, Chow-fed rats are given free access to rat chow and water. Therefore, it is possible that differences in PNC resistance observed in the Pair-fed vs. Chow-fed groups could be attributed to nutritional deprivation.

Nutritional deprivation suppresses immunity and decreases host resistance to infection. Surprisingly, however, some studies have reported that
moderate levels of malnutrition can protect the host against infection and, in some cases, actually enhance certain immunologic functions. For example, Sprunt (1941) reported that undernourishment rendered rabbits less susceptible to infection with Vaccinia virus. Years later, a study of patients in Eastern Niger revealed that changing from famine-induced starvation to a near normal diet precipitated attacks of *Plasmodium falciparum* in those with an existing quiescent infection (Murray, et al, 1975). Similarly, Wing and Young (1980) showed that mice starved for 72 h had only a 5% mortality from doses of *Listeria monocytogenes* that killed 95% of fed-mice. Since resistance to *Listeria monocytogenes* is normally mediated by activated macrophages, those investigators compared macrophage activation in starved and fed mice. They found that activation of peritoneal macrophages was enhanced in the starved mice. Therefore, they proposed that nutritional deprivation non-specifically increased the resistance of those animals by activating their macrophages.

Resistance to PNC pneumonia is mediated primarily by neutrophils because alveolar macrophages poorly kill PNC (Jonsson, et al, 1985 and Coonrod, et al, 1982). Therefore, nutritional-deprivation-induced increases in uptake of PNC by alveolar macrophages that cannot kill them effectively could actually exacerbate PNC pneumonia. However the mechanism of resistance to PNC in the bloodstream differs from that in the lung. Since organisms in the circulation are filtered through the RES, activation of macrophages by caloric deprivation in these organs could potentially increase bloodstream clearance of PNC in Ethanol-fed and Pair-fed animals. However, we did not see any significant
differences among the diet groups in the number of organisms in the lung, spleen or liver. This indicates either that RES clearance is not up-regulated in the Ethanol-fed and Pair-fed animals, or that the organisms are being removed from the bloodstream and rapidly killed in these animals.

The differences seen in the susceptibility of our rats to PNC septicemia may not be attributable to caloric deprivation alone. Rather, the differences may be due to alterations in biological mediators as a consequence of nutritional deprivation. For example, Sakai, et al (1991) found that pre-incubating alveolar macrophages with pulmonary surfactant from rats starved for two days enhanced their phagocytic capacity. Further examination revealed that this enhancement was a result of increased protein in the surfactant of the starved rats. They proposed that starvation induces production of glucocorticoids, which bind upstream to the human pulmonary surfactant protein gene and enhance its transcription (White, et al , 1985). Therefore, glucocorticoid release as a result of nutritional deprivation increased the phagocytic capacity of alveolar macrophages in that study. This led us to examine the role of glucocorticoids in the immune response to PNC infection in our model. We proposed that alterations in glucocorticoid production may be involved in the differences observed in our rats for several reasons. Glucocorticoids, including corticosteroids, are hormones essential to metabolism and the body's reaction to stress. They also have been implicated in alterations of host immunity. In addition to depleting cells from the thymus, spleen and bone marrow, high levels of corticosteroids decrease the function of B cells, T cells, and macrophages.
Garvey and Fraker, 1991, Han, et al, 1993, Jerrells, et al, 1990 and Rey, et al, 1984). It is well known that blood glucocorticoid levels increase early during acute starvation in mammals (Sakai, et al, 1991). Furthermore, many of the effects of ethanol consumption on host immunity are similar to the affects of glucocorticoids, specifically corticosterone (Reviewed in Baxter and Harris, 1975). Therefore, it has been suggested that immunosuppression may result from excessive corticosterone production stimulated by nutritional deprivation and ethanol. Additionally, Jerrells, et al (1990) found that preventing the secretion of corticosteroids in rats by adrenalectomy partially reversed the effects of ethanol on thymic atrophy, splenic atrophy and the suppression of lymphocyte proliferation to T-cell dependent antigens. We therefore hypothesized that ethanol ingestion, nutritional deprivation, or both stimulated the production of excess corticosterone levels in Ethanol- and Pair-fed rats, which would help to explain why these animals do not succumb as rapidly as Chow-fed rats to intravenous PNC infection.

When blood samples drawn pre-infection were analyzed for corticosterone levels, we found that the stress of nutritional deprivation did not significantly increase the levels in the Pair-fed rats above those measured for Chow-fed rats. Pre-infection corticosterone levels in Ethanol-fed rats were the highest of all three diet groups, with values for all Ethanol-fed rats being higher than the mean values for the Pair-fed and Chow-fed groups. This difference did not reach statistical significance ($p=.08$) when examined by non-parametric statistics. However, it mirrors what others groups have reported. In rats (Ellis, 1966 and
Rivier, 1984), mice (Han, 1993), and humans (Mendelsen, et al, 1971) ethanol consumption has been associated with activation of the hypothalamic-pituitary-adrenal axis (HPA) and an increased production of corticosteroids. Furthermore, ethanol-induced corticosteroids were shown recently to be partially responsible for a decrease in cell numbers in various lymphoid organs of ethanol-fed rats. Padgett, et al (in print) has showed that Ethanol-fed animals had the highest levels of corticosterone and the greatest reduction in lymphoid organ weight and lymphocyte numbers. In this same study, however, Pair-fed animals also had significantly higher levels of corticosterone than Chow-fed rats, suggesting to those authors that a stress response to food restriction also increases corticosterone levels. This research is supported by Barone et al (1993), who described thymic involution resulting from elevated serum corticosterone levels in protein-malnourished mice.

Additionally, Vinson et al (1998) found that an increase in ethanol-induced endogenous corticosterone may be involved in suppressed recruitment of neutrophils when mice were challenged intraperitoneally with heat-killed Propionibacterium acnes. This study suggests that ethanol-induced corticosterone production caused some of the immunosuppressive effects that render the host more susceptible to infectious diseases. Given the importance of neutrophils in the host defense against PNC pneumonia, increased corticosterone levels in Ethanol-fed rats should impair their ability to survive a transtracheal challenge with this organism. However, since we are using a bacteremic model of infection, the same parameters of the inflammatory
response that are beneficial within the lung may not be applicable to a systemic infection. For example, TNFα produced locally within the lung during PNC pneumonia enhances the host defense, whereas high concentrations of TNFα in the circulation are often associated with a poor prognosis and lethal sepsis.

Therefore, these studies suggest that elevated corticosterone levels can be induced by ethanol ingestion, nutritional deprivation, or both, and that they contribute to decreased immunocompetence in ethanol feeding models of infection. Pair-fed rats in our feeding model were shown previously to be slightly more susceptible than Chow-fed rats to type 3 PNC pneumonia (Lister, 1992). However, in the present studies they showed neither elevated corticosterone levels or increased susceptibility to PNC bacteremia in comparison to Chow-fed controls.

By 2 h post-infection with the H+C+ strain, the differences in corticosterone levels among the feeding groups was reversed. The mean level was highest for the Chow-fed rats, with the value being statistically higher in comparison to that in Ethanol-fed animals. Additionally, corticosterone levels decreased significantly in both the Ethanol-fed (p=.006) and Pair-fed (p=.02) rats 2 h post-infection with the H+C+ strain, but not after infection with the PLY- strain (p=.2 and p=.4, respectively). From our survival experiments we know that both Ethanol-fed and Pair-fed rats have a higher mortality when infected with the H+C+ vs. the PLY- strain. It has been shown that glucocorticoids are protective against murine CMV induced lethality (Ruzek, et al, 1999). In that study, adrenalectomized mice were more susceptible to murine CMV infection, and
resistance to the virus was restored by corticosterone replacement. Therefore, significantly decreased levels of corticosterone at 2 h post-infection with the H+C+ strain could help to explain why these rats are more susceptible to the H+C+ vs. the PLY- strain, although the reason for the decrease in corticosterone remains unclear. In contrast, corticosterone levels in the Chow-fed rats infected with the H+C+ strain remained similar to pre-infection levels (p=.9), whereas their levels decreased significantly when infected with the PLY- strain (p=.01). Since these rats become overwhelmed so rapidly when infected with the H+C+ strain, it is possible that the severity of the infection outweighs any potential benefits of corticosterone. However, a decrease in corticosterone levels during infection with the PLY- strain is only seen with the Chow-fed rats. This is consistent with the data that only the Chow-fed rats are ultimately unable to survive infection with the PLY- strain.

In examining the contribution of pneumolysin production 2 h post-infection, we found that Chow-fed rats had higher corticosterone levels when infected with the H+C+ vs. PLY- strain, whereas Pair-fed rats had higher levels when infected with the PLY- vs. H+C+ strain. However, this does not appear to be biologically relevant as there are no differences by 27 h post-infection.

In our experiments, there were no differences in corticosterone levels among or within diet groups at 27 h post infection. Because we were only able to obtain corticosterone levels for 3 animals per diet group at 27 h post-infection and because the values were so consistently low for all animals tested, we combined the values for all six animals within each feeding group to determine
changes in the values due to infection. We found that the mean corticosterone levels for all three diet groups at 27 h post-infection had decreased from pre-infection levels, although the difference was only statistically significant for the Ethanol-fed (p = .004) and Pair-fed (p = .004) rats. Cytokines such as IL-1, IL-6 and TNFα also can activate the HPA axis (reviewed in Kapcala, et al, 1995). This functions as a counterregulatory response to modulate the activity of cytokines after their initial beneficial effects and to prevent subsequent deleterious actions due to excess cytokine release. Because we do not see a change in the corticosterone levels of Chow-fed rats, however, it does not appear that excessively high cytokine levels in our model stimulate the production of corticosterone. Therefore, the infection remains unregulated and these rats die rapidly. Although we had only a small sampling of corticosterone levels at 27 h post-infection, there were no significant correlations between corticosterone levels and levels of IL-1β and IL-10 at 2 h post-infection. This also suggests that cytokines do not significantly modulate corticosterone levels in our model system.

It is possible that 27 h post-infection was too early a time point to detect the affects of i.v. infection with PNC on glucocorticoid levels. The murine CMV study conducted by Ruzek et al (1999), which noted a peak in serum corticosterone levels 36 h post-infection, used an intraperitoneal method of infection, which could affect the timing of peak glucocorticoid production. The response to infection also may be quite different during viral as opposed to bacterial infections. Because we only have corticosterone levels for 3 Chow-fed rats infected with each strain, it is difficult to accurately assess how these levels
correlate with the number and strain of organisms. An increase in sample size and sampling corticosterone levels at later timepoints could help to clarify why the Chow-fed rats are so susceptible to PNC bacteremia and why pneumolysin production increases their susceptibility.
Summary

Our main objectives were to determine whether ethanol ingestion adversely affects host resistance to a bacteremic PNC infection. Our results show that neither ethanol consumption nor nutritional deprivation inherent to the pair-feeding model rendered rats more susceptible to lethal *S. pneumoniae* bacteremia in our model system. Surprisingly, it appears that ethanol, nutritional deprivation, or both, may actually be protective during PNC bacteremia, although the exact mechanism remains unclear. Additionally, we sought to examine whether pneumolysin production increases the severity of PNC infection by decreasing bloodstream clearance and increasing mortality. Although pneumolysin production did not significantly reduce bloodstream clearance or increase cytokine production in all diet groups, it did increase the PNC virulence in all groups as measured by decreased mean survival time. Contrary to our hypothesis, pneumolysin production appeared to have the most pronounced effect in the Chow-fed rats by significantly decreasing their ability to clear organisms from their bloodstream and regulate cytokine production. These rats therefore became overrun with organisms and produce a massive and lethal cytokine response. However, the majority of Chow-fed rats died regardless of the infecting strain, suggesting that these rats are inherently more susceptible to lethal PNC bacteremia, regardless of pneumolysin production. This was not explained by differences in clearance by the RES of Chow-fed rats, as we saw no differences in the number of organisms sequestered in the organs of the three
diet groups. It is possible that corticosterone levels are involved in the decreased resistance of the Chow-fed rats to PNC bacteremia. However, an increased population size and more time points are necessary to define the role of corticosterone in this model of infection.
Literature Cited


