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Full Length Research Paper

New mechanism for complement killing of Gram-negative bacteria

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The complement system plays a strategic role in the vertebrate immune system in protecting the host from infection by numerous pathogenic agents. The system consists of at least 30 proteins that orchestrate attack on pathogenic agents. Although the susceptibility of Gram-negative bacteria to complement attack has been under investigation for over 100 years, the mechanism(s) by which the complement directly kills Gram-negative bacteria (*Escherichia coli* J5) is not understood. Inner membrane damage to *E. coli* J5 by complement with the subsequent release of cytoplasmic and periplasmic markers are important for killing. That observation has been extended to show that complement attack on Gram-negative bacteria prevents the detoxification of methylglyoxal, a lethal by-product of glucose metabolism. It is very important to know that lysozyme is not required for killing by C5b-9 complexes. That is, C5b-9 complexes kill bacteria but lysis of the organisms do not occur in lysozyme depleted or lysozyme neutralized serum. Killing is a function of damage or injury to the cytoplasmic membrane in *E. coli* J5 by C5b-9 complexes with the subsequent accumulation of a toxic by-product (methylgloxal) from glucose metabolism.

Key words: Complement proteins, Gram-negative bacteria, cytoplasmic membrane, methylglyoxal.

INTRODUCTION

The complement system is a vital component of the immune system and it consists of three pathways: Classical, Alternative and Lectin. Each pathway has its own unique method of activation. Classical pathway activation requires the presence of antibody-antigen complexes. The alternative pathway is activated by various microbial pathogens, and in fact is a by-pass for the earlier steps needed for classical pathway activation. The lectin pathway is activated by carbohydrates and Toll like proteins and like the alternative pathway it predates the development of the classical pathway (Fujikta, 2002).

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Abbreviations: PNHS, Pooled normal human serum; **Scfu,** surviving colony forming; **cfu,** colony forming units; **J5,** *E. coli* J5; **MG,** methylglyoxal; **µl,** microliter; **MAC,** membrane attack complex; **AL,** anti-human lysozyme.

Activation of each pathway results in the production of the membrane attack complex (MAC) which consists of terminal or late acting complement proteins (C5b-9 complexes). The C5b-9 complexes are needed for direct killing of Gram-negative bacteria and has no effect on Gram-positive bacteria. Gram-positive bacteria have thicker cell walls which prevents C5b-9 complexes to penetrate them and damage their cytoplasmic membranes. However, the exact mechanism(s) by which inner membrane damage to Gram-negative bacteria by C5b-9 complexes leads to cell death is not known. In previous studies it was demonstrated that pores formed by C5b-9 complexes will allow the release of “molecules” of divergent sizes such as rubidium atoms and β-lactamase, a high molecular weight compound of 28,000 daltons (Bloch et al., 1987). In short, the passage of “molecules” of varying molecular weights through C5b-9 pores as a consequence of inner membrane damage contributes to the death of Gram-negative bacteria

through enhance osmotic forces and or reactions with cytoplasmic proteins. To extend this observation we studied the uptake of methylglyoxal, a low molecular weight molecule, through C5b-9 pores as a contributor in the killing of Gram-negative bacteria. Methylgloxal is produced in cells as a by-product of glucose metabolism. Additionally, methylgloxal is an electrophile and a dicarbonyl compound and its detoxification by glutathione-dependent glyoxase I-II pathway is found in the cytosol of cells (Thornalley, 1998).

MATERIALS AND METHODS

Buffers

The following buffer was used in these experiments. 0.01 M phosphate buffered saline, pH 7.2 with 0.15 mM Ca^{2+} and 1.00 mM Mg^{2+}

Bacteria

The J5 strain of *Escherichia coli* was kindly provided by Dr. E. Ziegler (San Diego, CA). This strain produces a rough Rc type LPS, even when grown in the presence of exogenous galactose, due to the presence of both a UDP galactose epimerase mutation and a second undefined defect which precludes incorporation of galactose (Betz and Isliker, 1981). Additionally, *E. coli* J5 directly activates and is killed by the classical complement pathway in the absence of specific antibody. The J5 strain of *E. coli* was inoculated into trypticase soy broth and incubated overnight without agitation at 37°C in a water bath. The next day the bacteria were centrifuged and washed twice in sterile saline (0.86% NaCl). Optical density (O.D.) reading were taken on the concentrated sample of *E. coli* J5. An O.D. of 0.500 at 600 nm corresponds to 6×10^8 colony forming units per ml of *E. coli* J5. In these investigations the bacteria are in stationary phase and used at final concentrations of 1.0×10^7cfu/ml. The four smooth strains used in these studies were obtained from Salmonella genetic stock Centre from the University of Calgary in Alberta, Canada.

(i) 818 (mR595a) *Salmonella entericaserovarminnesota*
(ii) SA 1377 *Salmonella entericaserovartyphimurium*
(iii) 258 (SL1102) *Salmonella entericaserovartyphimurium*
(iv) 4697 (MGH 78578) *Klebsiella pneumonia*

The organisms were grown overnight without agitation in trypticase soy broth, and the next day the stationary phase cultures were washed twice and adjusted to a final concentration of 1.0×10^7cfu for use in this investigation.

Pooled normal human serum (PNHS)

PNHS is obtained by combining serum samples from at least 10 normal volunteers (Bloch et al., 1987, 1997, 2008; Bugla-Plosoriska et al., 2009). The PNHS is absorbed before use with 1.0×10^{10} colony forming units (cfu) of glutaraldehyde-fixed *E. coli* J5 per ml of serum at 0°C for 30 min to remove specific antibody. The organisms are removed by centrifugation at 10, 000 × g for ten minutes at 4°C. The absorbed PNHS is stored at -70°C until needed. Before use in these experiments 20 µl of anti-human lysozyme (AL) is added to 1000 µl of thawed absorbed PNHS for 20 min at room temperature to neutralize serum lysozyme. This is done to demonstrate specific killing of *E. coli* J5 by C5b-9 complexes and not by serum lysozyme. Finally, it has been determined that incubation of 2.4 × 108 cfu/ml of *E. coli* J5 in 10% PNHS will deposit an average of 890 C5b-9 complexes per cfu (Bloch et al., 1987).

Methylglyoxal (mw 70) is obtained as 40% aqueous solution from ICN Biochemicals, Inc. On the day of the experiments the stock reagent (MG) is diluted in sterile buffer to desired concentrations and placed on ice until it is needed.

Frozen stock cultures of *E. coli* J5 are thawed and added to 100 ml bottle of trypticase soy broth, placed in a 37°C water bath and allowed to grow up over night without agitation. The next day the organisms are centrifuged for 30 min at 10,000 × g to remove broth and serum proteins. The organisms are re-centrifuged for an additional 30 min at 10,000 × g and placed on ice. The supernatant is discarded and the bacterial pellets at the bottom of the centrifuge tubes are vortexed and then suspended in cold sterile saline. The resuspended pellets from the tubes are combined into a single tube. An aliquot is removed and a 1:20 dilution is made to determine its optical density. An optical density of 0.5 at 600 nm equals 6×10^8 cfu/ml. *E. coli* J5 is then adjusted to 1.0×10^7 cfu/ml for use in these assays.

Every aspect of the experiments, involving the preparations of the concentrations of *E. coli* J5, percentages of PNHS and varying concentrations of the reactants must be done in ice cold buffer and kept in a mixture of crushed ice and water until needed. This is to ensure that when the reagents are mixed with PNHS as outlined in the protocol the reagents will remain cold and the PNHS (source of complement proteins) will not be activated until the temperature is raised to 37°C. All experiments were done in triplicate and the next day, the surviving colony forming units were enumerated and the standard error of the mean (SEM) was used to evaluate the precision of the data. Determination of log killed and relationship to percent killed.

Example:

Test = 10^8 cfu ; Control = 10^9cfu

Log killed = log control – log test (surviving colony forming units) = 9 – 8 = 1

Percent killed = [1-(test / control)] × (100)

[1 - (0.1)] × (100) = 0.90 × 100 = 90% killed

That is, a one log difference between the control and test = 90% killed.

RESULTS

Four smooth strains of Gram-negative bacteria were employed in these studies. The strains have functional LPS side chains and are resistant to complement killing in the presence of 20% PNHS (Figure 1). These experiments were done to ensure that PNHS as a source of complement proteins were active in killing Gram-negative bacteria (*E. coli* J5). The figure also demonstrates that other “heat stable proteins” present in HI-PNHS were not involved in killing *E. coli* J5 (Figure 2). Pooled normal human serum (PNHS) at 1, 5 and 10% were incubated with 1.0×10^7 cfu/ml. *E. coli* J5 at 37°C to determine which percentage of PNHS would be used as a sub-lethal concentration in these assays (Figure 3). Data not shown for dose response curves for 1 and 5 mM MG. However, it was demonstrated that 1 mM MG was less toxic then 5 mM MG and was chosen as the

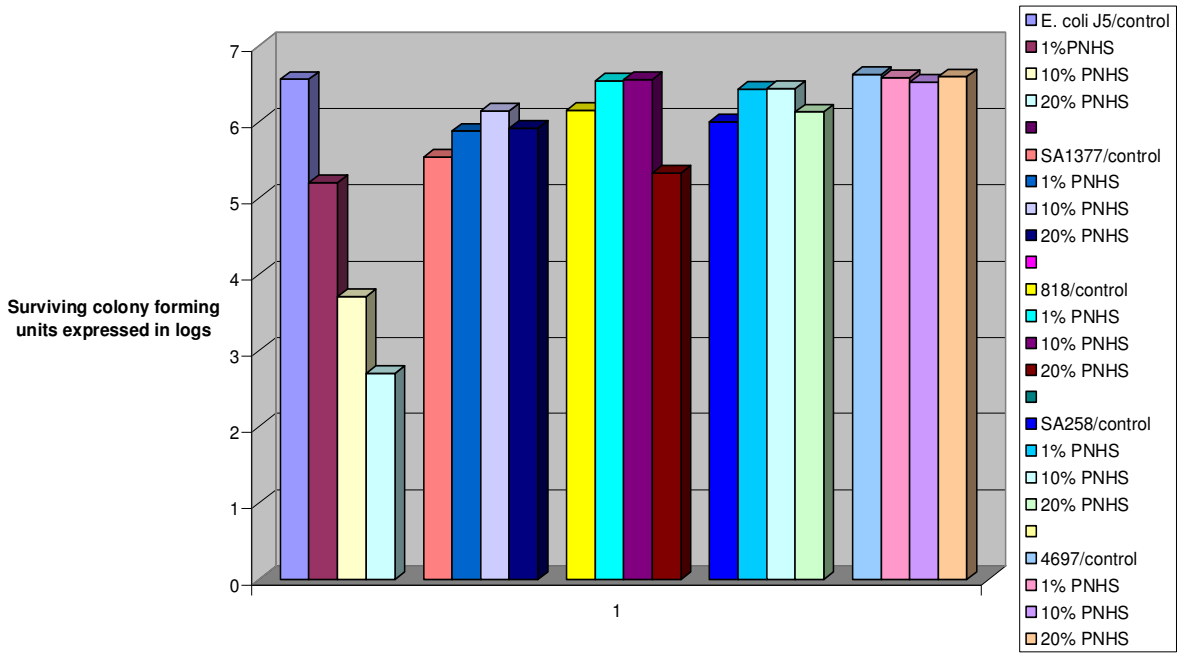

Figure 1. Complement "mediated" killing of selected strains of Gram-negative bacteria. Four *smooth strains of Gram-negative bacteria were employed in these studies and those strains were shown to be resistant to complement killing in the presence of 20% PNHS. The smooth strains do not have defective LPS side chains and thus their functional LPS molecules block the ability of C5b-9 complexes (PNHS) to deposit and damage the bacterial inner membrane. The four strains are resistant to complement killing by C5b-9 complexes as indicated by the surviving colony forming units in the diagram. The experiments were done in triplicate and the next day, the surviving colony forming units were enumerated and the standard error of the mean (SEM) was used to evaluate the precision of the data.

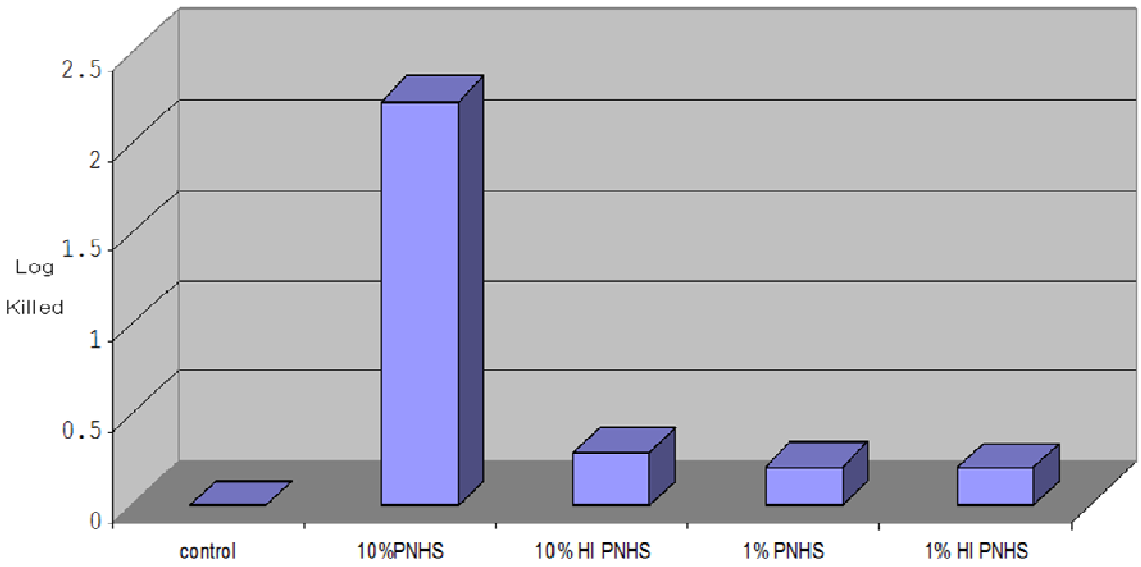

Figure 2. Log killed of *E. coli* J5 with 1 and 10% PNHS. Pooled normal human serum (PNHS) was heated at 56°C for thirty minutes to inactivate complement proteins. Non-heat inactivated serum (PNHS) and heat inactivated serum (HI-PNHS) were used at tenfold serial dilutions and incubated with *E. coli* J5 at 37°C for sixty minutes. These experiments were done to ensurethat PNHS as a source of complement proteins were active in killing our serum sensitive Gram-negative bacteria (*E. coli* J5). The results are expressed in terms of log killed. That is, the log of the control minus the log of the test. Each assay was done in triplicate and the next day the standard error of the mean (SEM) was used to evaluate the precision of the data.

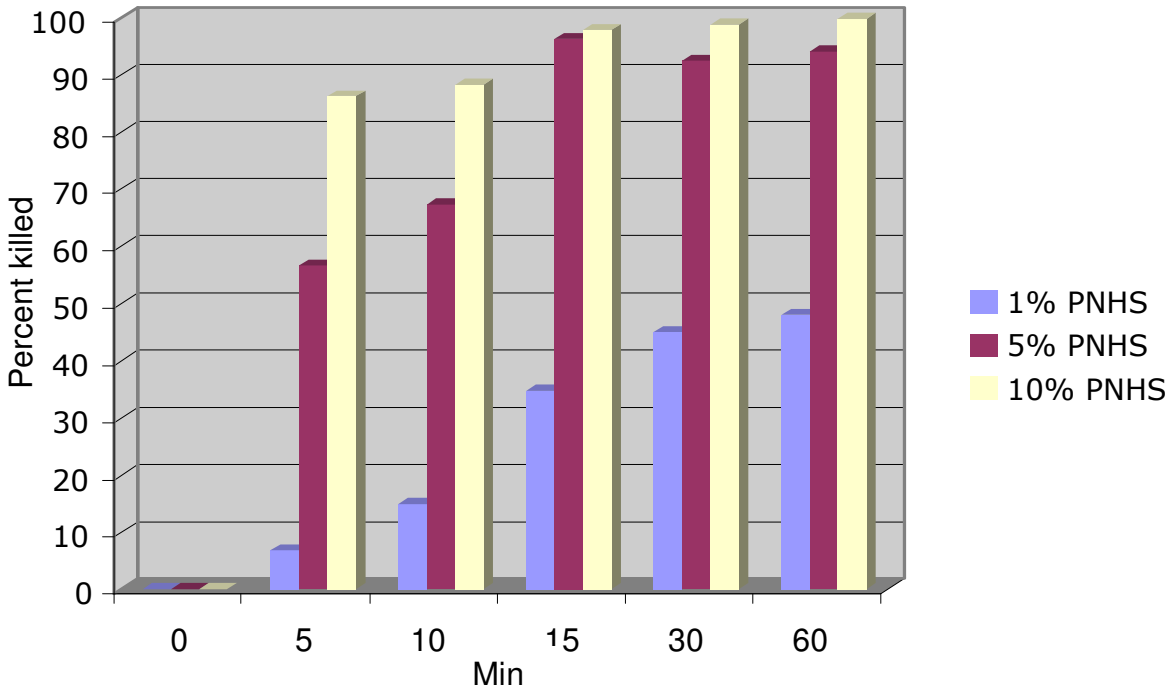

Figure 3. Percent killed of *E. coli* J5 as function of increasing concentrations of PNHS. Pooled normal human serum (PNHS) at a final concentrations of 1, 5 and 10% were separately incubated with 1.0 x 10^7cfu/ml. *E. coli* J5 at 37°C from 5 to 60 min to determine which concentration of PNHS produced the least amount of killing at the designated time points. After the incubation periods, the samples were removed and plated on 1.7% trypticase soy agar plates and incubated over night at 37°C. Each experiment was done in triplicate and the standard error of the mean (SEM) was used to evaluate the precision of the data.

sublethal concentration of the electrophile for use in these experiments (Figure 4).

Sublethal concentrations of PNHS (1%) and MG (1 mM) in the same reaction mixture were added to *E. coli* J5 for incubation periods of 5, 10, 15, 30 and 60 min intervals. The figure illustrates 85% enhanced killing of the bacterial colony forming units at the five and ten minute time periods. The "stimulatory" effects in terms of enhanced killing of the bacteria were not present at the 30 and 60 min incubation periods with both reagents present in the reaction mixture.

DISCUSSION

It has been demonstrated that deposited C5b-9 complexes on Gram-negative bacteria will allow the uptake and release of molecules of divergent sizes (Bloch et al., 1987). In fact, complement mediated lysis of Gram-negative bacteria is the direct result of serum lysozyme (mw 14,400) passage through C5b-9 pores. That is, C5b-9 complexes kill the bacteria but lysis of the organisms do not occur in lysozyme depleted or lysozyme neutralized serum. Since lysozyme can pass through C5b-9 pores, it was anticipated that methylglyoxal, a much smaller molecule should easily enter pores created by deposited C5b-9 complexes. The exclusion of inner membrane damage and death presumably by C5b-9 complexes in killing four smooth serum resistant strains of Gram-negative bacteria were also investigated. The strains used were:

(i) 818 (mR595a) *Salmonella entericaserovarminnesota*
(ii) SA 1377 *Salmonella entericaserovartyphimurium*
(iii) 258 (SL1102) *Salmonella entericaserovartyphimurium*
(iv) 4697 (MGH 78578) *Klebsiella pneumonia*

The figure shows that 20% activated PNHS, as a source of C5b-9 complexes could not damage the inner membrane of the Gram-negative bacteria and kill any of the four serum resistant strains. These experiments emphasized the importance of inner membrane damage in the death of Gram-negative bacteria by deposited C5b-9 complexes on the surface of our target organisms.

On a related issue investigators studied the effect of sub-lethal concentrations of enterocin CRL35, cationic peptide, on the increased uptake of antibiotics (Minahk et al., 2004). Basically, the aim of their study was to evaluate the interaction of several conventional antibiotics with sub-lethal concentrations of enterocin CRL35, a cationic peptide, on *Listeria innocua* 7. The researchers reported that positive interactions between

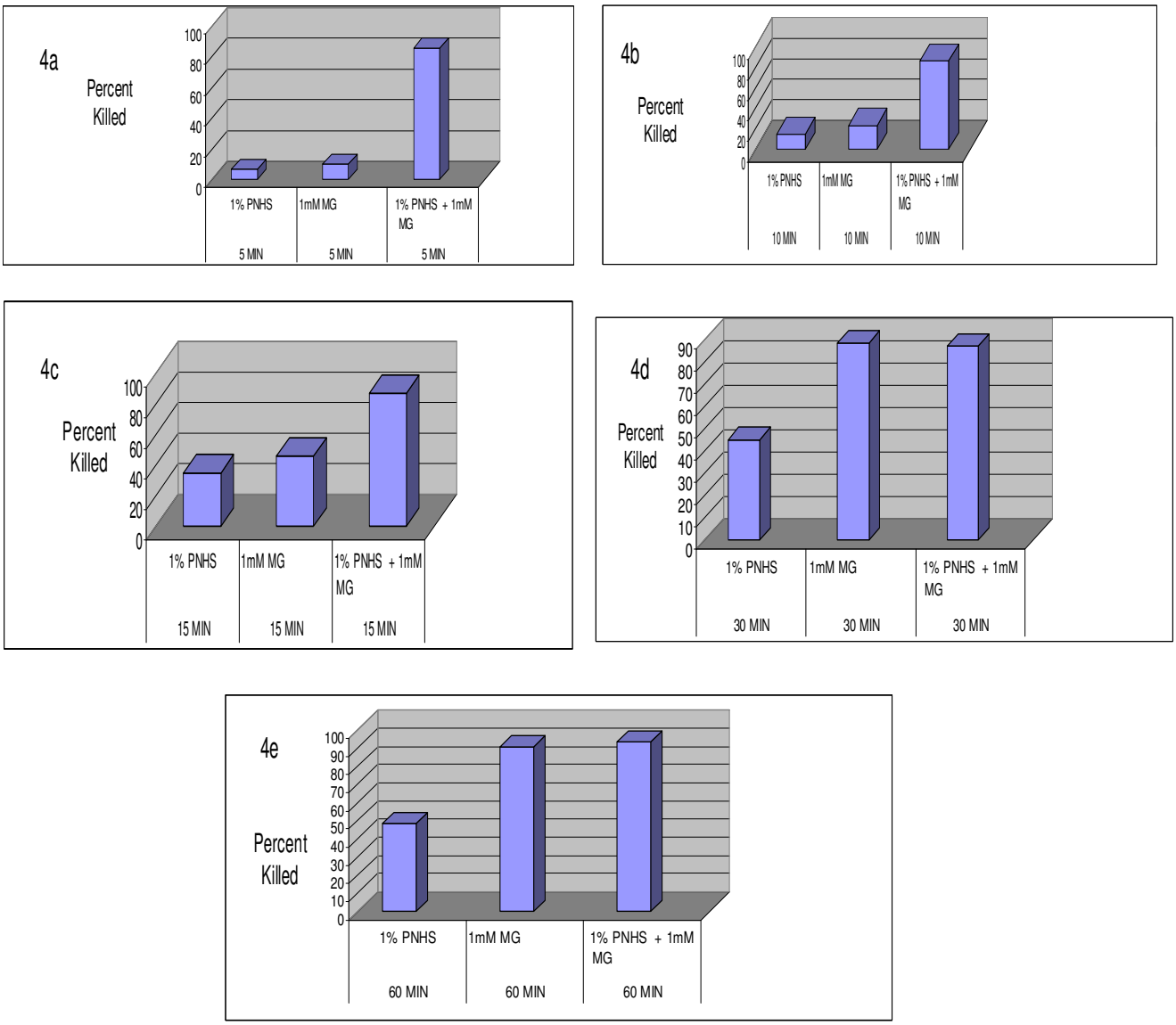

Figure 4. A dramatic effect of combining sub-lethal concentrations of PNHS (1%) and MG (1 mM) in the death of *E. coli* J5 for five, ten, fifteen, thirty and sixty minutes are illustrated in the figure 4a, 4b. 4c, 4d and 4e respectively. The figure illustrates that a significant amount of killing occurs at the five and ten minute periods with a percent killed of 85% when sublethal concentrations of the reagents are present in the same reaction mixture. The stimulatory effect is marginal at the 15 min time period and is completely absent at 30 and 60 min incubation periods. After the incubation periods, the samples were removed and plated on 1.7% trypticase soy agar plates and incubated over night at 37°C. Each experiment was done in triplicate and the standard error of the mean (SEM) was used to evaluate the precision of the data.

the cationic peptide and three conventional antibiotics (tetracycline, erythromycin and chloramphenicol), which are excluded from the cells by efflux pumps are dependent on the membrane proton gradient.

Furthermore, enterocin CRL35 even at sublethal concentrations induced the dissipation of both components of the proton motive force (p), that is, trans-membrane electrical potential and pH gradient and hence the alteration of processes dependent on it.Moreover, they hypothesized that enterocin CRL35 increases the effectiveness of these antibiotics by impairment of the bacterial active efflux systems and the consequent accumulation of these toxic compounds in the cytoplasm. In this study we have demonstrated that sublethal concentrations of PNHS amplifies killing by allowing toxic accumulation of the methylglyoxal in the cytoplasm. Which parallels the above observations that show sublethal concentrations of enterocin amplified killing of Gram-positive bacteria by conventional antibiotics. It is clear in these investigations that complement mediated damage to the bacterial inner membrane increases the toxicities of methylglyoxal. Exactly, how this damage

results in cell death requires further investigation. It has been demonstrated that methylglyoxal damages DNA in a plasmid transformation assay (Ferguson, 1995). As previously indicated methylglyoxal is a very toxic electrophile that can react with RNA and protein macromolecules in the bacterial cell. Additionally, its production and activation has been extensively studied (Booth et al., 2003; Ferguson et al., 1998a, 1998b; Huang et al., 1999; Cooper, 1984; Misra et al., 1995). A consistent finding in this study was amplified killing of *E. coli* J5 when sublethal concentrations of PNHS and MG were present in the same reaction mixture for periods of five to ten minutes. A plausible explanation for the significant killing is that deposited C5b-9 pores are accelerating the uptake of methylglyoxal with a rapid increase in the toxicity of the electrophile. The opposite argument that MG is amplifying killing by C5b-9 complexes is not very likely. As indicated C5b-9 complexes must have access to the inner membrane for killing to occur. Since MG does not form pores on the bacterial surface the high molecular weight C5b-9 complexe (greater than 10^6 daltons) does not have access to the cytoplasmic membrane to amplifying killing. Furthermore, C5b-9 complexes cannot pass through bacterial porins because the porins will only allow molecules with molecular weights of 600 daltons or less to pass through its pores. It must be emphasized that methylglyoxal has a low molecular weight of (mw 70) and should easily pass through the pores in the deposited C5b-9 complexes. However, it is not readily explained why amplified killing of *E. coli* J5 colony forming units are not detected for 30 and 60 min time periods when PNHS and MG are present in the same reaction mixture. Presumably, passive transport of methylglyoxal through porins on *E. coli* J5 is a continuous process that contributes to the killing of the organisms. Nonetheless, it is clear from this investigation that killing Gram-negative bacteria by serum complement is a very complex process that does not involve serum lysozyme. In earlier studies it has been demonstrated that killing Gram-negative bacteria by C5b-9 complexes involves collapse of inner membrane potential with a subsequent loss of periplasmic and cytoplasmic components through the pores in the complex. We have demonstrated in this study that pores formed from deposited C5b-9 complexes on *E. coli* J5 enhances the uptake of methylglyoxal (a toxic metabolite and a by-product of glucose metabolism) and contributes to the killing of serum sensitive strains of Gram-negative bacteria.

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