Preliminary Experiments Involving Salmonella-immune Lymphokine In Vivo Effects On Neutrophil Function In Weaned Pigs And Salmonella Choleraesuis Organ Invasion In Neonatal Pigs

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Using knowledge gained from the administration of Salmonella enteritidis-immune lymphokines (ILK) derived from the t cells of S. enteritidis-immune chickens to neonatal poultry, the idea of immunopotentiation of the immune response of immune-compromised animals was applied to swine. A S. enteritidis-immune lymphokine (PILK) was isolated from the splenic t cells of S. enteritidis-immune pigs. Pilk was then administered orally to weaned pigs, consid-
ered to possess deficient immune responses, which were subsequently challenged with both lethal and non-lethal doses of the swine pathogen S. choleraesuis (SC). PILK-treated pigs were shown to have a 50-60% decrease in organ invasion and a similar reduction in cecal colonization by SC. PILK also enhanced growth performance in both SC challenged and nonchallenged pigs, with PILK-treated pigs gaining an average of 5 pounds more than both nonchallenged controls and SC challenged controls. PILK also significantly reduced morbidity and mortality as compared to control pigs. Neutrophils isolated from the peripheral blood of PILK-treated pigs exhibited increased functional capabilities when compared to control pigs. Significant increases in the oxidative burst, adherence to nylon wool and bovine serum albumin-coated slides, and increased movement towards chemotactic stimuli were shown by neutrophils from PILK-treated pigs when compared to neutrophils from control pigs. We have shown that PILK protects pigs from SC organ invasion, cecal colonization and enhances growth performance and neutrophil functions in weaned pigs.

Introduction

The mammalian neutrophil and its avian counterpart, the heterophil, represent a first line of nonspecific, innate host defense against invasive pathogens. These leukocytes circulate in the peripheral blood and, upon stimulation by inflammatory signals, begin to adhere to the endothelial cells lining blood vessels in a process termed margination. Eventually, the neutrophils cease rolling due to firm attachments with endothelial cells and emigrate from the vasculature into tissues, drawn to particular sites by concentration gradients of chemotactic molecules. Once at a site of tissue damage and bacterial invasion, neutrophils phagocytize and kill bacteria. The mechanisms neutrophils employ to phagocytize and kill bacteria involve cell surface molecules which allow the leukocytes to adhere to and engulf bacteria, along with oxidative and protein-based antimicrobial substances for killing. Both the process of adherence to endothelial cells in the vasculature and the process of adherence and phagocytosis of bacteria involve the CD18/CD11b cell surface molecule. Researchers have identified this molecule as playing an important role in both neutrophil/endothelial cell adherence and neutrophil adherence to bacteria.(1) Once a neutrophil has phagocytized a bacterium, one of the bactericidal mechanisms employed is the production of oxygen radicals through the oxidative burst mechanism. The oxidative burst is initiated by the activation of NADPH-oxidase, with subsequent production of oxygen radicals, hydrogen peroxide, and, in mammals, the production of hypohalide ions. These reactive oxygen radicals kill bacteria by oxidizing their proteins as well as enhancing the activities of antimicrobial enzymes within lysosomes.

Neonates from mammalian and avian species have long been known to suffer from a variety of developmental deficiencies of the innate and acquired immune re-
sponses.(2,3,4,5-13) Developmental deficiencies of neutrophil/heterophil function have been observed in avian, human, equine, bovine, and porcine neonatal ani-
imals.(2,3,4,5-13,14)

The deficient neutrophil function contributes to an increased susceptibility of neonatal animals to infections. Addressing this issue, Kogut and colleagues developed a Salmonella enteritidis-immune lymphokine (ILK) from the splenic T cells of mature chickens.(15-18) Administration of ILK to day-old chickens and turkeys caused a significant increase in the number of circulating heterophils and in their functional activities, with a subsequent correlation with increased protection against organ invasion by S. enteriti-
dis.(18) The protection against S. enteritidis organ was largely due to the early promotion of adult levels of heterophils the first five days of life.(2,3,4,19) Therefore, ILK administration to day-old chicks and turkeys increased
heterophil function during the period when they are most susceptible to infections, and then declined at the time when the young chickens and turkeys immune systems become functional and can defend the host against infections.

Neonatal and weaned pigs have been shown to develop deficiencies in innate and acquired immune functions.(20,21) Data from experiments with avian - derived ILK lead us to investigate the concept of applying porcine - derived ILK (PILK) to young pigs in hopes to enhance early protection through mediation of their neutrophils. Therefore, the present investigations involve the in vivo effects of the administration of PILK to both neonatal and weaned pigs on organ invasion and neutrophil functions. Three aspects of neutrophil function in weaned pigs were examined: 1) adherence to nylon wool and BSA-coated slides; 2) production of an oxidative burst; and 3) chemotaxis and random migration.

**Material and Methods**

Experimental animals

Neonatal piglets (Yorkshire/Landrace x Hampshire/Duroc) were obtained from a commercial swine operation within the first 24 hours after birth. Piglets were ear-tagged, given iron injections, and raised in commercial nursery units (eight piglets per unit). Neonatal piglets were fed a milk replacer formula ad libitum and had unlimited access to water during these experiments. Fourteen day-old piglets were obtained from a commercial swine producer, ear-tagged and placed in pens (8 piglets/pen, total 150 piglets) with added heat sources. Piglets had ad libitum access to a nipple watering system and a pig starter. All piglets were determined to be Salmonella species-free using established methods.(21) Weaned pigs 14-17 days old were also raised under similar conditions and were fed a pig grower diet.

Bacteria—A porcine isolate of Salmonella choleraeus (SC) var. kunzendorf was selected for novobiocin-nalidixic acid (NONA) resistance in our laboratory and maintained in tryptic soy broth (TSB). Inocula for challenge with SC was prepared using sterile phosphate-buffered saline (PBS) and adjusted to a stock concentration of 10^9 colony forming units (CFUs) per milliliter using a spectrophotometer with a 625 nm reference wavelength. The viable cell concentration of the inocula was determined by colony counts on brilliant green agar (BGA) with NONA. The 10^9 stock of SC was serially diluted to 1 x 10^7 CFU/ml using PBS.

Salmonella-immune lymphokine production—Two gilts (Landrace x Yorkshire) weighing approximately 81.82 kg each, and determined to be Salmonella species-free were used for immune lymphokine (PILK) production. Pigs were fed a nonmedicated finisher diet ad libitum. Pigs were challenged three times weekly for four weeks with 10 ml of 10^9 CFU Salmonella enteritidis (SE). Five ml of SE were given by oral gavage and 5 ml were given intranasally. During the fifth week pigs were not challenged with SE, and at the end of the fifth week pigs were euthanized and spleens were obtained aseptically. Splenic T cells were isolated as previously described.(23) T cells were placed in 175 cm^2 T flasks at a concentration of 5 x 10^6 cells/ml in serum-free RPMI 1640 with 7.5 μl of concanavalin A (Con A) and incubated for 48 hours at 37 °C in a 5% CO₂ incubator. After incubation, supernatants were collected and centrifuged at 2000 g for 15 minutes to remove all cells. Supernatants were treated with β-mannopyranosidase to inactivate any residual Con A and concentrated five-fold using YM-100 and YM-10 membranes. The retentate was then sterile filtered using 0.22 μm filters and stored at -20°C.

Organ invasion by S. choleraeus in neonatal and weaned pigs

Ten neonatal piglets were divided into two groups: 1) control pigs receiving 1 x 10^7 CFU of S. choleraeus 1 hour after the administration of 3mls of RPMI 1640 medium orally and, 2) pigs given 3mls of PILK orally and 1 hour after treatment, a challenge with 1 x 10^7 CFU of S. choleraeus. Four days after challenge, all ten piglets were sacrificed and the following tissues were cultured using established culture methods for the presence of S. choleraeus: palantine tonsil, liver, lung, spleen, ileocolic lymph nodes, and cecal contents. Results from each sample from each pig were recorded as being either positive or negative for the presence of S. choleraeus. Weaned pigs received the same challenge dose, but were cultured 14 days after challenge. Some groups received PILK only once over 14 days (PILK 1X) or 3 times over 14 days (PILK 3X).

Isolation of neutrophils—Blood from weaned pigs was collected from the anterior vena cava into tubes containing 10 % EDTA. After collection, neutrophils were isolated as follows: Blood from animals in the same treatment groups were pooled and centrifuged at 1000 x g for 20 minutes. The resulting plasma and buffy coat layers were discarded. Erythrocytes were removed from the cell pellet by the addition of 2 volumes of cold phosphate-buffered (0.0132 M, pH 7.2) distilled water for 50 seconds, followed by the addition of 1 volume of cold phosphate-buffered (0.0132 M, pH 7.2) distilled water with 2.7% NaCl. The lysate was then centrifuged at 300 x g for 10 minutes and the cell pellet was resuspended in Ca²⁺/Mg²⁺ free Hank's Balanced Salt solution. The cell suspension was then layered over a discontinuous Histopaque 1.077/1.119 density gradient and centrifuged for 45 minutes at 200 x g. The buffer, buffy coat, buffer/1.077 interface and 1.077 layers were discarded and an equal volume of RPMI 1640 was added to the remaining 1.119 layer, which was then centrifuged at 300 x g with the resulting pellet containing neutrophils. Neutrophils were then resuspended in RPMI and counted using a hemocytometer and adjusted to 2.5 x 10^6 cells/ml. Neutrophil purity was assessed by cytofin preparations viewed under a light microscope (x 100) and determined to be greater than 95% pure. Cell viability was determined by trypan blue-
exclusion and found to be greater than 95%.

Adherence to nylon wool—Neutrophil adherence to nylon wool was assessed as previously described.(24) Briefly, 2.0 x 10^6 neutrophils were added to a tuberculin syringe containing 75 g of nylon wool. Columns were incubated for 10 minutes at 39°C in 5% CO₂. Cells passed through the columns by gravity flow and were collected into siliconized 13 x 100 mm glass tubes. The neutrophils in the effluent were determined using a hemocytometer and the data are expressed as the percentage change from the adherence of neutrophils from control pigs.

BSA Adherence Assay—Tissue culture chamber slides were coated overnight with 500 μl of a 1% BSA solution in PBS to provide a protein matrix for neutrophils to adhere to. Before adherence, each well was rinsed with RPMI 1640. One hundred microliters of neutrophils (1x10^6 cells) from each group were added to respective individual chambers in quadruplicate and the chamber slide was then incubated for 1 hour at 39°C. After incubation, non adherent cells were removed by gently rinsing the chambers two times with warm RPMI. For the third rinse, the plastic chambers were removed from the slide and the slide was gently rinsed. The slide was allowed to air dry and then was fixed and stained using the Hema 3 system. Adherence was assessed microscopically by counting cells contained in 5 fields /chamber using an ocular grid under oil immersion (x100).

Chemotaxis Assay—Neutrophil random migration and chemotactic movement were quantitated using Modified Boyden blind well chemotaxis chambers. Control wells were filled with 27μl of HBSS. Chemotactic movement was assessed using 27μl of pooled porcine serum placed in the bottom of some wells in the chambers while other wells were filled with 27μl of a 1:100 dilution of a 10μg/ml stock solution of recombinant human IL-8 (rHu IL-8) (R&D Systems Inc., Minneapolis, MN). All solutions were incubated at 39°C for 30 minutes to standardize temperatures. To the top portion of the chambers 45μl of a 2x10^6 neutrophils/ml suspension in RPMI 1640 were added, separated from the chemoattractants by a polycarbonate membrane with 3μm pore size (Nucleopore Corp., Pleasanton, CA). Chambers were incubated at 39°C in 5% CO₂ for 45 mins. After incubation, filters were removed, air-dried and stained with Hema 3 stain. Random and chemotactic movement of neutrophils on the filter membranes were quantitated microscopically using oil immersion (x 100). Five fields were scored and summed per well with 4 wells per treatment group on each chamber. Values were expressed as the average number of cells per well per group.

Oxidative burst assay—Neutrophil oxidative burst was measured using luminol-dependent chemiluminescence (LDC) as previously described.(25) One hundred microliters of the 2.5 x 10^6 cell suspension and luminol (0.1M) were added to polypropylene scintillation vials and stimu-

lated with opsonized zymosan (OZ). OZ was prepared by boiling Zymosan A in 0.9% saline for 20 minutes and washing twice. The zymosan was then suspended (20mg/ml) in pooled pig serum and opsonized for 30 minutes at 39°C on a rotary shaker, washed twice with Ca²⁺/Mg²⁺-free HBSS and stored at 4°C in HBSS at the same concentration until used. OZ was used at a working concentration of 2mg/ml in the experiments.

Results

*S. choleraesuis* organ invasion in neonatal and weaned piglets—Organ invasion data are presented in Figure 1. In both the control and PILK-treated groups the presence of *S. choleraesuis* was not detected in the cecal contents of any pig. Control pigs were found to have increased incidence of *S. choleraesuis* in all other tissues collected compared to PILK-treated pigs except the liver (both groups were 1/5). PILK-treated pigs had only 1 incidence of *S. choleraesuis* in any of the liver, lung, or spleen samples, while control pigs had 1/5, 2/5, and 2/5 pigs positive for the respective tissues. Weaned pigs treated with PILK had significant reductions (Figure 2) in SC organ invasion (liver, lung, spleen) and cecal colonization when compared to control pigs (P < 0.05).

Adherence to nylon wool—A significant increase in adherence of neutrophils from PILK-treated pigs at 30 days of age was observed (Figure 3), with a biological increase at day 21 of age (all at 24 hours post-PILK administration). Neutrophils isolated from both 21 and 30 day-old pigs of both groups showed no differences in adherence when neutrophils were isolated 4 hours post-PILK administration (data not shown).

Adherence to BSA-coated slides—An almost three-fold increase in the number of adherent neutrophils from PILK-treated pigs was observed in comparison to control neutrophils (Figure 4).

Oxidative burst—There was a significant (P < 0.05) increase in the peak oxidative burst activity noted in neutrophils from 30 day-old PILK-treated piglets compared to 30 day-old control pig neutrophils, as measured by LDC (Figure 5).

Chemotaxis Assay—A significant increase in chemotactic movement towards both porcine serum and rHu IL-8 was noted (Figure 6) in control and PILK groups (P < 0.05). There was also a significant increase in PILK-neutrophil movement towards serum and IL-8 when compared to control neutrophils (P < 0.05).

Weight Gain—Pigs receiving PILK (both infected and noninfected) over a 14-day study showed significant (P< 0.05) increase in weight when compared to control pigs infected with SC and noninfected control pigs (Figure 7).
Discussion

Overall, organ invasion by *S. choleraesuis* was decreased in neonatal and weaned pigs treated with PILK as compared to control pigs. At 30 days of age, PILK-treated piglets showed dramatic increases in both adherence and oxidative burst activities. These preliminary results demonstrate that PILK effectively reduces organ invasion by *S. choleraesuis* in both neonatal and weaned pigs. In older, weaned piglets, PILK appears to enhance the functional activities of neutrophils from treated pigs.

Previous work in our laboratory in neonatal chickens and turkeys has shown that the administration of *Salmonella*-immune lymphokines (ILK) protects these birds from *S. enteritidis* organ invasion and that this protection is correlated with an increase in peripheral blood heterophil numbers.(17) In addition, ILK administered to day-old chicks and turkeys increased heterophil function, including chemotaxis, adherence, and the phagocytosis and killing of *S. enteritidis*. The effects of ILK in neonatal poultry led us to believe that PILK might function in a similar manner, activating neutrophils in neonatal piglets which would subsequently mediate the protection against *S. choleraesuis* organ invasion observed in weaned piglets.

In conclusion, the present investigation suggests that PILK does protect neonatal and weaned pigs from *S. choleraesuis* organ invasion, increases weight gain in treated piglets, and does enhance some neutrophil functions in weaning-age piglets. Further investigations are needed to determine if the effect on neutrophil functions in weaned piglets is also observed in neonatal piglets.

References