Journal of Surgical Research **172,** 18–28 (2012)
doi:10.1016/j.jss.2011.04.019

ASSOCIATION FOR ACADEMIC SURGERY

Role of Neutrophils and Macrophages in the Pathogenesis of Necrotizing Enterocolitis Caused by *Cronobacter sakazakii*

Claudia N. Emami, M.D., M.P.H.,*,§,[2] Rahul Mittal, Ph.D.,†,[2] Larry Wang, M.D.,‡ Henri R. Ford, M.D., M.H.A.,*,§ and Nemani V. Prasadarao, Ph.D.*,†,§,[1]

*Department of Surgery, The Saban Research Institute, Children's Hospital Los Angeles, Los Angeles, California; †Division of Infectious Diseases and Department of Pediatrics, The Saban Research Institute, Children's Hospital Los Angeles, Los Angeles, California; ‡Department of Pathology, Children's Hospital Los Angeles, Los Angeles, California; and §Keck School of Medicine, University of Southern California, Los Angeles, California

Originally submitted January 31, 2011; accepted for publication April 7, 2011

Background. ***Cronobacter sakazakii*** **(CS) is a highly virulent gram-negative opportunistic pathogen that has been implicated in clinical outbreaks of necrotizing enterocolitis (NEC). The role of mucosal immune cells in CS infection is not well understood. In this study, we sought to elucidate the role of neutrophils (polymorphonuclear leukocytes; PMNs) and macrophages in the pathogenesis of NEC induced by CS using a novel newborn mouse model.**

Materials and Methods. **PMNs and macrophages were depleted in newborn mice using Gr-1 antibody and carrageenan, respectively, and then infected with 10^3 CFU of CS. The development of NEC in these mice was assessed by a pathologist based on the morphologic changes in the intestine. Cytokine production was determined in the serum and intestinal homogenates of infected mice by enzyme-linked immunosorbent assay (ELISA). Inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production was determined by flow cytometry and Greiss method, respectively.**

Results. **Depletion of PMNs and macrophages in newborn mice led to increased recruitment of dendritic cells (DCs) in the intestine compared with wild-type mice upon infection with CS. PMN- and macrophage-depleted mice showed increased bacterial load, production of pro-inflammatory cytokines, iNOS expression, and NO production in the intestines in comparison to wild-type mice fed with CS. In addition, depletion of PMNs and macrophages prior to infection in mice resulted in severe inflammation, villus destruction, and enhanced enterocyte apoptosis in the intestines compared with CS-infected wild-type mice.**

Conclusions. **Our data suggest that depletion of PMNs and macrophages from the lamina propria (LP) exacerbates experimental NEC, indicating that both of these immunocytes play an important role in the clearance of CS during the initial stages of infection. The increased mucosal cytokine response and NO production in the absence of these immunocytes may be responsible for the observed increase in mucosal injury. Understanding how CS manipulates these cells, employing novel mouse model of NEC reported in this study, will provide significant insights for the development of novel therapeutic and preventive strategies to combat NEC.**

Key Words: Cronobacter sakazakii**; macrophages; PMNs; dendritic cells; nitric oxide; necrotizing enterocolitis.**

INTRODUCTION

Necrotizing enterocolitis (NEC) is the most common life-threatening gastrointestinal surgical emergency encountered in premature infants [1, 2]. Despite recent advances in neonatal medicine, the morbidity and mortality from NEC have remained largely unchanged [3, 4]. In fact, current trends suggest that NEC may soon become the leading cause of mortality for premature infants in the United States. Although multiple risk factors have been implicated in the pathogenesis of NEC, the exact etiology of the disease remains undefined [5, 6]. The only

[1] To whom correspondence and reprint requests should be addressed at, Division of Infectious Diseases, MS #51, Children's Hospital Los Angeles and Keck School of Medicine, University of Southern California, 4650 Sunset Blvd., Los Angeles, CA 90027. E-mail: pnemani@chla.usc.edu.

[2] These authors contributed equally to this work.

0022-4804/$36.00

consistent epidemiologic precursors of NEC are prematurity, bacterial colonization, and enteral alimentation [7, 8]. Bacterial colonization of the immature gut and the resultant bacterial-epithelial interactions appear to play a leading role in the activation of the mucosal immune system that is characteristic of NEC [9–12]. Understanding the role of bacterial infection and how it contributes to disease susceptibility is important for developing novel strategies for the prevention and treatment of NEC.

Cronobacter sakazakii (CS), formerly known as *Enterobacter sakazakii*, is a virulent gram-negative pathogen that has been implicated in clinical outbreaks of NEC in premature infants [13, 14]. Powdered infant formula (PIF) contaminated with CS has been implicated as a putative source of these outbreaks. CS-induced NEC results in a mortality rate of 40%–100% [15]. CS has been designated by the International Commission for Microbiological Specifications for Foods (ICMSF) as a severe hazard for restricted populations, associated with life-threatening chronic sequelae. We have previously demonstrated that CS suppresses the maturation of dendritic cells (DCs) by down-regulating the expression CD40, CD86, and MHC class II antigen [16]. In addition, CS utilizes DCs as a vehicle for propagation and survival, hence evading potential immune surveillance [16].

Neutrophils (polymorphonuclear leukocytes; PMNs) and macrophages play an important role in the clearance of infections [17–21]. Despite the fundamental function of PMNs and macrophages in innate immunity, the role of these immune cells in CS-induced NEC *in vivo* is not known. Here, we report that the oral feeding of CS results in acute intestinal inflammation and death in newborn mouse pups. We also demonstrate that the presence and recruitment of PMNs and macrophages to the lamina propria (LP) is important for clearance of the bacteria during the initial stages of infection. Furthermore, their absence exacerbates mucosal injury by increasing the levels of proinflammatory cytokines and iNOS expression.

MATERIALS AND METHODS

Bacterial Strain

C. sakazakii (strain 51329) was obtained from American Type Culture Collection. CS was transformed with a GFP containing plasmid as previously described [22]. The resulting colonies were selected for ampicillin resistance. For experiments, the bacteria were grown in Luria broth containing ampillicin (50 μg/mL).

Animal Experiments

The animal studies were approved by the institutional animal care and use committee (IACUC) of the Saban Research Institute at Children's Hospital Los Angeles and followed National Institutes of Health guidelines for the performance of animal experiments. Time pregnant C57BL/6 mice were obtained from Charles River at E18. After delivery, the pups were kept with the mothers and housed at CHLA. Three-day-old pups were fed 10^3 CFU of CS in 10 μL of sterile PBS orally and left with the mother. The control animals received sterile PBS at d 3. Blood was collected from the tail or facial vein at different time points post-infection. Dilutions were made and plated on ampillicin LB agar plates to determine the bacteremia levels and success of infection. Mice were perfused with 0.9% saline intracardially at 24, 48, and 72 h post-infection to remove blood and contaminating intravascular leukocytes. Intestines were aseptically removed, weighed, and homogenized in sterile PBS. Bacterial counts were determined by plating 10-fold serial dilutions of intestinal homogenates on ampillicin LB agar plates. The results were obtained from six independent experiments with 15 animals per group.

PMN and Macrophage Depletion

For PMN and macrophage depletion, mice received six injections of Gr-1 antibody (BD Biosciences, San Diego, CA) and carrageenan (Sigma, St. Louis, MO), respectively, in sterile PBS (dose) (50 μL) by intra-peritoneal injections as described previously [23–25]. Gr-1 antibody has been shown to selectively bind to and deplete PMNs [23, 24]. Carrageenan is a sulfated polysaccharide extracted from cell walls of certain algae of Rhodophyta. Due to its unique structure, carrageenan is ingested by macrophages but not by other immunocytes that are not actively phagocytic and lack well developed lysosomal complex. Once ingested by macrophages, carrageenan causes the lysis of phagolysosomes and the release of hydrolytic enzymes, leading to bursting of macrophages. Therefore, carrageenan is widely used to deplete macrophages in animals to study their role during infection [25]. Mice received three injections of Gr-1 antibody or carrageenan 6 h apart on d 1; two injections of Gr-1 antibody or carrageenan 6 h apart on d 2, and final injection of Gr-1 antibody or carrageenan on d 3 prior to infection with CS. Depletion was confirmed using flow cytometry for Gr-1 (specific to PMNs) and F4/80 (specific to macrophages) expression in liver, spleen, and the intestinal homogenized tissue. The results were obtained from five independent experiments with 12 animals per group.

Histopathologic Examination

Intestine, spleen, and liver specimens were collected at 24, 48, and 72 h post-infection from at least 12 mice, fixed in formalin, 3–5 μ sections cut with microtome and stained with H and E [22]. At least 10 sections from each organ were examined for morphologic changes by a pathologist. In addition, intestinal sections were graded microscopically by a pathologist blinded to groups, from grade 0 (normal) to grade 4 (severe), based on pathologic manifestations, including submucosal edema, villus core edema, epithelial sloughing/obliteration, neutrophil infiltration, intestinal perforation, and necrosis [22].

Flow Cytometry

Animals were sacrificed at 24, 48, and 72 h post-infection, the intestines were dissected, Peyer's patches removed, the specimen placed in RPMI medium, and prepared for Percoll gradient. The epithelial and lamina propria (LP) layers were isolated on the Percoll gradient as previously described [26]. Percentage of different immunocytes recruited to the lamina propria (LP) layer was determined by staining with fluorochrome coupled primary antibodies. DCs were identified by CD11c staining (Abcam, Cambridge, MA), PMNs by Gr-1 antibody, and macrophages by F4/80 antibody. Mouse IgG isotype matched antibodies was used as control. Cells were analyzed by four-color flow cytometry using FACSCalibur analyzer (Becton Dickinson, San Diego, CA) and cell Quest Pro software (BD Biosciences, San Diego, CA),