ELSEVIER

Food Microbiology 24 (2007) 67–74

FOOD MICROBIOLOGY

www.elsevier.com/locate/fm

The presence of endotoxin in powdered infant formula milk and the influence of endotoxin and *Enterobacter sakazakii* on bacterial translocation in the infant rat

Stacy Townsend[a], Juncal Caubilla Barron[b], Catherine Loc-Carrillo[b], Stephen Forsythe[b,*]

[a]*Department of Pathology, Childrens Hospital Los Angeles, Keck School of Medicine, University of Southern California, 4650 Sunset Boulevard MS# 103, Los Angeles, CA, 90027, USA*

[b]*Applied Microbiology and Environmental Biology, School of Biomedical and Natural Sciences, The Nottingham Trent University, Clifton Lane, Nottingham, NG11 8NS, UK*

Received 14 November 2005; received in revised form 17 March 2006; accepted 17 March 2006
Available online 12 June 2006

Abstract

Lipopolysaccharide (LPS) is a heat stable endotoxin that persists during the processing of powdered infant formula milk (IFM). Upon ingestion it may increase the permeability of the neonatal intestinal epithelium and consequently bacterial translocation from the gut. To determine the level of endotoxin present in IFM, 75 samples were collected from seven countries (representing 31 brands) and analysed for endotoxin using the kinetic colorimetric *Limulus* amoebocyte lysate (LAL) assay. The endotoxin levels ranged from 40 to 5.5×10^4 endotoxin units (EU) per gram and did not correlate with the number of viable bacteria. The neonate rat model was used to address the risk of endotoxin-induced bacterial translocation from the gut. Purified *Escherichia coli* LPS was administered to rat pups followed by inoculation with *Enterobacter sakazakii* ATCC 12868. Bacteria were isolated from the mesentery, spleen, blood and cerebral spinal fluid (CSF) of endotoxin-treated rats due to enhanced gut and blood brain barrier penetration. Histological analysis of the colon showed marked distension of the mucosal and muscular layers. It is plausible that the risk of neonatal bacteraemia and endotoxemia, especially in neonates with immature innate immune systems, may be raised due to ingestion of IFM with high endotoxin levels.

Keywords: *Enterobacter sakazakii*; Lipopolysaccharide; Endotoxin; Infant formula milk; Translocation

1. Introduction

Currently the microbiological safety of powdered instant formula milk (IFM) is being revised due to the rare occurrence of neonatal infections caused by *Enterobacter sakazakii* (Iversen and Forsythe, 2003; Pagotto et al., 2003; WHO, 2004). These cases are often low birth weight preterm neonates, who are generally more susceptible to Gram-negative bacterial sepsis and endotoxemia which is associated with necrotizing enterocolitis (Beck-Sague et al., 1994; Stoll et al., 2004). The Joint FAO–WHO workshop (WHO, 2004) considered the bacteria associated with neonatal infections and IFM, but not preformed bacterial toxins. Staphylococcal enterotoxin A does retain its activity during powdered milk manufacture and can cause staphylococcal food poisoning (Asao et al., 2003). Endotoxin is another heat stable bacterial toxin. It is the lipid A core region of lipopolysaccharide (LPS) which forms the outer membrane of Gram-negative bacteria. Endotoxin is heat stable at 100 °C and, therefore, can remain biologically active in powdered IFM that has been heat treated to kill vegetative cells.

Endotoxemia is described as the immunological response to the presence of endotoxin in the blood stream (Hurley, 1995). Symptoms include fever, low blood pressure, leucocytosis, thrombocytopenia, and coagulopathies. Deitch et al. (1987) showed that intramuscular and intraperitoneal (IP) injection of endotoxin into healthy

*Corresponding author. Tel.: +44 115 8483529; fax: +44 115 8486680.
E-mail address: stephen.forsythe@ntu.ac.uk (S. Forsythe).
URL: http://www.foodmicrobe.com.

0740-0020/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fm.2006.03.009

mice caused the translocation of intestinal Gram-negative bacteria to the mesenteric lymph nodes (MLN). Islam et al. (2000) showed that intravenous injection of endotoxin into rats promoted *Salmonella enteritidis* translocation from the gut to the MLN and liver in healthy rats. Endotoxemia has been associated with enteral feeding of pre-term neonates and necrotizing enterocolitis in infants (Scheifele et al., 1985a,b). In addition endotoxin impairs enterocyte migration and epithelial restitution and, therefore, inhibits tissue repair following bacterial infection (Cetin et al., 2004a,b). Studies of human infants found that necrotizing enterocolitis was 20 times more frequent in infants that were fed formula rather than breast milk (Lucas and Cole, 1990). Similarly there is a five-fold increase in the frequency of gastroenteritis in formula fed neonates compared with breast fed babies (Duffy et al., 1986). Levy (2002) reported that low birth weight pre-term neonates have 3–4 times less bactericidal/permeability-increasing protein (BPI) in their neutrophils than term neonates. BPI binds LPS and effectively neutralizes endotoxin, thus this deficiency may contribute to the vulnerability of the immature immune system to endotoxin. Hence, endotoxin present in IFM could be a risk factor in neonatal infections. However, studies concerning endotoxemia in pre-term neonates have not assessed the endotoxin levels in powdered IFM used in enteral feeding.

This study proposes that endotoxin may be present in high levels in IFM and contribute to failure of neonatal intestine integrity therefore facilitating bacterial translocation leading to local and systemic infection. The survey has determined endotoxin levels using the standard LAL endotoxin assay that is applied to clinical samples and pharmaceutical drugs (Hurley, 1995). In addition, a neonatal rat model was used to examine the influence of endotoxin on bacterial translocation across the intestinal epithelium and into deeper tissues.

2. Methods

2.1. Powdered infant milk formula

Seventy-five powdered IFM samples were purchased from food retailers in seven countries and represented 31 brands. They had been manufactured in South Africa, Holland, Spain, Switzerland, USA, Belgium, Ireland, Slovenia and the UK. The samples were a subset of the 82 samples previously analysed for general bacterial flora and members of the Enterobacteriaceae (Iversen and Forsythe, 2004).

2.2. Microbiological analysis

Twenty-five grams of each IFM sample was homogenized in 225 ml buffered peptone water (BPW CM509, Oxoid Ltd.). Decimal dilutions of the homogenate were used to inoculate Plate Count Agar (CM325, Oxoid Ltd.), Violet Red Bile Glucose agar (CM0107, Oxoid Ltd.), *Bacillus cereus* agar (CM0929, SR99 and SR0047, Oxoid Ltd.), Baird Parker agar (PO0168 and SR0054, Oxoid Ltd.), and Tryptone sulphite cycloserine agar (TSCA) (CM0587, SR0088 and SR0047, Oxoid Ltd.). The plates were incubated at 37 °C aerobically for 24 h except the TSCA plates which were incubated anaerobically in a Don Whitley Compact Anaerobic cabinet for 48 h. Identification of the isolates was confirmed using the Gram reaction, catalase reaction, oxidase test and biochemical profiles with reference to standard texts.

2.3. Endotoxin assay

Endotoxin levels were determined using a kinetic colorimetric LAL assay with Endochrome-K (Charles River, UK). The protocol followed manufacturer's (Charles River, UK) standardized protocol for pharmaceutical and clinical sample analysis and hence IFM samples were rehydrated in endotoxin free water as provided by the manufacturer. Samples were analysed in a 96-well format using a Sunrise spectrophotometer (Tecan, Austria) and EndoScan-V endotoxin software (Charles River, UK). One gram quantities of powdered IFM were rehydrated in 9 ml of reagent water (Charles River, UK) and further diluted as appropriate. Endotoxin specific buffer (ESB) was used to prevent interference from glucans (Morita, 1981; FDA, 1987; Cooper et al., 1997). Various water sources which had been boiled and cooled, as recommended for the rehydration of IFM, were also tested.

2.4. Neonatal rat pup model of LPS-induced bacterial translocation

Timed pregnant Sprague-Dawley rats were obtained from Charles River Laboratories (Raleigh, NC) and gave birth after a 21-day gestation period. Pups were kept with the dam in an opaque polypropylene cage under a small animal isolator (Forma Scientific, Inc.). An average of 12 pups were born per litter. Rat pups were anesthetized with isoflurane at two days old. *Escherichia coli* 0111:B4 LPS (Sigma) was injected into the IP cavity at 0.05 and 0.25 mg LPS/kg body weight, using six pups per treatment. Preliminary experiments showed that rat pups given more than 1 mg LPS/kg resulted in 50% pups dying within 24 h and, therefore, this dose was not used in this study. Pups were inoculated with 10^8 cfu *Ent. sakazakii* ATCC 12868 in 10 µl PBS via the oral route of infection 1 h after LPS injection. Controls included pups administered with 0.5 mg LPS/kg body weight ($n = 4$) and pups only inoculated with 10^8 cfu *Ent. sakazakii* ($n = 6$). Two pups treated with PBS were analyzed as negative controls. Rats were euthanized 24 h following inoculation. The liver, spleen, mesentery, and descending stool-filled colon samples were aseptically collected in that order. Tissues were weighed and homogenized aseptically in PBS prior to bacterial enumeration. The limit of detection was 5 cfu per tissue sample and 33 cfu/ml of fluid obtained.