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Maternal 18:3*n-3* favors piglet intestinal passage of LPS and promotes intestinal anti-inflammatory response to this bacterial ligand

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Abstract

We recently observed that maternal 18:3*n-3* increases piglet jejunal permeability. We hypothesized that this would favor intestinal lipopolysaccharide (LPS) passage and alter gut immune system education toward this bacterial ligand. Sows were fed 18:3*n-3* or 18:2*n-6* diets throughout gestation and lactation. In each litter, two piglets were given oral Gram-negative spectrum antibiotic from post-natal day (PND) 14 to 28. All piglets were weaned on a regular diet at PND28. 18:3*n-3* piglets exhibited greater jejunal permeability to FITC-LPS at PND28. Levels of 18:3*n-3* but neither 20:5*n-3* nor 20:4*n-6* were greater in mesenteric lymph nodes (MLN) of 18:3*n-3* piglets. Jejunal explant or MLN cell cytokine responses to LPS were not influenced by the maternal diet. Antibiotic increased jejunal permeability to FITC-LPS and lowered the level of 20:5*n-3* in MLN, irrespective of the maternal diet. At PND52, no long-lasting effect of the maternal diet or antibiotic treatment on jejunal permeability was noticed. 18:3*n-3* and 20:4*n-6* levels were greater and lower, respectively, in MLN of 18:3*n-3* compared to 18:2*n-6* piglets. IL-10 production by MLN cells in response to LPS was greater in the 18:3*n-3* group, irrespective of the neonatal antibiotic treatment. IL-8 secretion by jejunal explants in response to LPS was lower in antibiotic-treated 18:3*n-3* compared to 18:2*n-6* piglets. Finally, proportion of MHC class II^+ antigen-presenting cells was greater in 18:3*n-3* than 18:2*n-6* MLN cells. In conclusion, maternal 18:3*n-3* directs the intestinal immune response to LPS toward an anti-inflammatory profile beyond the breastfeeding period; microbiota involvement seems dependent of the immune cells considered.

Keywords: Intestinal barrier function; Alpha-linoleic acid; Lipopolysaccharide; Microbiota; Gut associated lymphoid tissue

1. Introduction

The intestinal immune system is immature at birth and develops during the first months of life. Early encounter of bacteria is critical for a fine-tuned immune system development and gut homeostasis [1]. Colonization starts at birth and is affected by environmental factors such as delivery mode, diet or antibiotic exposure [2]. Despite variations among individuals, microbiome acquisition is characterized by the succession of different types of bacteria [3]. Gram-negative bacteria expressing lipopolysaccharides (LPS) at their surface are one of the first and most abundant bacteria that colonize the intestine [4]. LPS is a potent inflammatory mediator. At the intestinal level, education of epithelial and immune cells toward LPS that occurs within the first hours/days following birth [5] is critical to avoid over-reaction toward this bacterial compound. Intestinal T cells do not proliferate in response to LPS and have limited secretion of pro-inflammatory cytokines and increased production of anti-inflammatory cytokines.

Intestinal epithelial cells (IEC) lining the intestinal mucosa are the ones constantly facing bacteria. Presence of tight junctions between adjacent IEC creates a relatively tight physical barrier to the entry of bacterial compound such as LPS [6]. Gut inflammation develops when breach in intestinal barrier function happens, though the latter is essential but not sufficient [7,8]. *n-3* polyunsaturated fatty acids (PUFA), especially the long-chain ones, eicosapentaneoic acid (20:5*n-3*) and docosahexaenoic acid (22:6*n-3*) deriving from the precursor α-linolenic acid (18:3*n-3*) have long been considered beneficial to the development of the central nervous system and retina in newborns [9]. *n-3* PUFA are also known as anti-inflammatory nutrients, especially 20:5*n-3* as opposed to the pro-inflammatory *n-6* PUFA arachidonic acid (20:4*n-6*). The effect of *n-3* PUFA on gut immunity is not clear with reports of improvement in inflammatory markers not always followed by clinical improvement [10–12]. Moreover, a recent study reported that although *n-3* PUFA dietary supplementation protected mice against

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Citrobacter rodentium-induced colitis, these mice suffered greater mortality associated with sepsis-related serum factors such as LPS binding protein, IL-15 and TNFα, suggesting enhanced intestinal passage of LPS [13]. Similarly, recent work from Innis *et al.* (2010) showed that supplementation with long-chain *n-3* PUFA of the maternal diet during pregnancy and lactation increased colonic permeability of neonatal rats [14]. This disruption of post-natal development of the intestinal barrier was associated with increased inflammation scores later in life in a model of chemical-induced colitis. Finally, our group recently observed that a diet rich in 18:3*n-3* during pregnancy and lactation increased jejunal permeability in piglets [15]. Our objective was therefore to investigate in the same model if changes in gut permeability induced by maternal 18:3*n-3*-enriched diet affect gut immune system development in piglets. We focused on gut immune response to LPS and investigated first the rate of transepithelial passage of LPS in the jejunum then gut immune response to LPS using cultures of jejunal explant (effector site) and cells isolated from mesenteric lymph nodes (MLN, inductor site). To assess the role of luminal LPS concentration upon the effects of *n-3* PUFA on piglet gut immune system, some of the piglets were given a Gram-negative spectrum antibiotic during the period of time when jejunal permeability was at its highest.

2. Materials and methods

2.1. Animal experimentation

The experimental work on animals was conducted with the approval of Rennes Ethics Committee for Animal Experimentation (CREEA) (File R-2011-GB-03).

Twenty-four Large White × Landrace sows were inseminated with Pietrain semen. They were fed a diet enriched either in extruded linseed (18:3*n-3* diet) or sunflower oil (18:2*n-6* diet) from 28 days after insemination until the end of lactation (Table 1). The amount of linseed in the maternal diet was chosen based on the observation that this amount induced a change in sow milk composition [16] similar to that observed in breast milk of women supplemented daily with flaxseed oil during lactation [17]. The amount of these iso-energetic and iso-lipidic diets given to the sows was adapted to the needs of pregnant and lactating sows (2.8 and 7 kg/day, respectively). Parturitions were not induced. The litters were equalized to 12 piglets 48 h after parturition to ensure equal amount of milk production within litters. Piglets were weaned at post-natal day (PND) 28 and fed a standard weaning diet *ad libitum*.

Table 1
Composition of diets

	18:2*n-6* diet		18:3*n-3* diet	
	Gestation	Lactation	Gestation	Lactation
Ingredients, g/kg				
Wheat	220	234	210	220
Corn	100	120	96	110
Barley	337	263	325	249
Wheat bran	150	100	142	96
Soybean meal	90	212	85	200
Sugar beat pulp	50		48	
Calcium carbonate	18	10	18	10
Dicalcium phosphate	3	12	3	13
Sodium chloride	5	5	5	5
Trace element and vitamin mix[a]	12	24	12	22
Sunflower oil	15	20	0	0
Tradilin 70[b]	0	0	56	75
Lipid content, g/100 g diet	3.9	3.9	3.8	4.2
Fatty acid composition, % of total fatty acids				
SFA	16.5	15.5	16.0	15.0
MUFA	25.2	25.8	18.0	19.5
PUFA	58.3	58.7	66.0	65.5
n-6 PUFA	52.6	54.5	38.0	35.4
18:2*n*-6	51.6	53.8	37.2	35.2
n-3 PUFA	5.0	3.6	27.5	29.8
18:3*n*-3	4.6	3.4	26.6	29.2
18:2*n*-6/18:3*n*-3	11.2	14.9	1.4	1.2

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids.

[a] This premix (Cooperl, Lamballe, France) was supplied per kilogram of diet: 3 mg retinol, 37.5 μg cholecalciferol, 40 mg α-tocopherol, 2 mg menadione, 2 mg thiamine, 4 mg riboflavin, 20 mg niacin, 10 mg D-pantothenic acid, 3 mg pyridoxine, 0.2 mg D-biotin, 3 mg folic acid, 20 μg vitamin B12, 500 mg choline, 80 mg Fe as ferrous carbonate, 10 mg Cu as copper sulfate, 40 mg Mn as manganese oxide, 100 mg Zn as zinc oxide, 0.1 mg Co as cobalt sulfate, 0.6 mg I as calcium iodate and 0.15 mg Se as sodium selenite.

[b] Tradilin 70: 70% extruded linseed+30% wheat bran.

In a first batch of 2×6 sows (experiment 1), one piglet per litter was sacrificed at birth (PND0) before suckling and at PND7, PND14, PND21 and PND28 to measure jejunal permeability in Ussing chamber (see procedure below). In a second batch with the remaining 2×6 sows (experiment 2), two pairs of weight-matched piglets were chosen in each litter at PND14. One piglet of each pair received colistin (100,000 IU; Colipate, Virbac, Carros, France) by daily oro-gastric gavage from PND14 to PND28. Twelve pairs of piglets were slaughtered at PND28 and 12 pairs were slaughtered at PND52.

The animals were slaughtered by electrical stunning and exsanguination. A laparotomy was performed under aseptic conditions. In experiment 1, 15-cm segments of proximal jejunum (20 cm after the Treitz ligament) were rinsed with 0.9% NaCl and placed in Ringer bicarbonate (145 mM Na^+, 128 mM Cl^-, 0.32 mM PO_4^{3-}, 2 mM Ca^{2+}, 1 mM Mg^{2+}, 25 mM HCO_3^-, 1 mM SO_4^{2-} and 6.3 mM K^+) at 4°C for Ussing chamber study. A 2-cm adjacent segment was also fixed in paraformaldehyde for 3 h at room temperature then placed in 0.1% NaN_3–PBS (phosphate-buffered saline) for latter enteric nervous system (ENS) phenotyping.

In experiment 2, MLN draining the proximal part of the intestine were removed aseptically and placed immediately in Hank's solution (Sigma-Aldrich, St. Quentin Fallavier, France) supplemented with 2% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich), 100 IU/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich) (called isolation medium thereafter). MLN were also immediately frozen in liquid nitrogen and kept at −20°C for latter fatty acid composition analysis. Jejunal luminal contents were collected aseptically and immediately frozen in liquid nitrogen for further microbiota analysis. Segments of proximal jejunum (10 cm) were rinsed with 1% DTT (Sigma-Aldrich), 1% FBS–PBS then with PBS and placed in 74% PBS, 25% DMEM (Dulbecco's Modified Eagle Medium, Sigma-Aldrich), 1% FBS, 2 mg/L gentamicin (Sigma-Aldrich) and 5 mg/L amphotericin B (Sigma-Aldrich) for immediate explant culture. Other segments of jejunum were processed as described previously for Ussing chamber study.

2.2. Isolation, purification and MLN cell culture

MLN were dissected under sterile conditions to remove fat and mesentery. MLN cells were isolated by mechanical dissociation before purification over a gradient (Histopaque density of 1.077 g/ml; Sigma-Aldrich), as previously published [18]. MLN cells were placed in FBS–10% DMSO and frozen in liquid nitrogen for latter cytometry analysis. The remaining cells (4×10^6 cells/ml) were resuspended in complete RPMI supplemented with 10% FBS, 100 IU/ml penicillin and 100 mg/ml streptomycin in three different conditions: basal condition (complete RPMI alone), in the presence of LPS (from *Escherichia coli* 055: B5, 100 μg/ml; Sigma-Aldrich) or in the presence of concavanalin A [ConA, a mitogen from *Canavalia ensiformis* (Jack bean), 5 μg/ml; Sigma-Aldrich]. Cell supernatants were harvested after 72 h of incubation at 37°C under a 5% CO_2 atmosphere and frozen at − 20°C for latter cytokine analysis. The proliferative response of MLN cells was quantified in another culture plate by incubation with 18.5 kBq of ^{3}H-thymidine (Perkin Elmer, USA) per well during the last 24 h of culture and further filtration over MultiScreenHTS 96-well-filter plates (Millipore, France) using a MultiscreenHTS Vacuum Manifold (Millipore) following the manufacturer's instructions. Once filters were dried, 35 μl of MicroScint (Perkin Elmer, USA) liquid scintillation was added per well before counting with a TopCount NXT Microplate Scintillation Counter (Perkin Elmer, with permission of Xenoblis SARL, France). Proliferation was expressed as a stimulation index, i.e. the *ratio* between the number of counts per minute (cpm) of stimulated cells and the number of cpm in basal condition.

2.3. Culture of jejunal explants

The fragments of proximal jejunum mucosa were processed as already described [19] and incubated for 20 h in three different conditions: basal condition, in the presence of LPS (100 μg/ml) or in the presence of ConA (5 μg/ml). Supernatants were collected and frozen at −20°C for latter cytokine analysis.

2.4. Determination of cytokine concentrations

Concentrations of porcine cytokines in the culture supernatants were determined by ELISA (R&D Systems Europe, France) and expressed in picograms per milliliter (pg/ml) for MLN cells (IFNγ, TNFα, IL-10) and picograms per milligram (pg/mg) of tissue for explant cultures (TNFα, IL-8).

2.5. Flow cytometry analysis

MLN cells were thawed at 37°C and placed in pure FBS at room temperature before centrifugation (600*g*, 5 min, 4°C). After washing with PBS, flow cytometry analysis on antigen-presenting cells (APC) present in ω3 or ω6 MLN cells at PND52 was performed using specific monoclonal antibodies (mAbs) or isotype-matched mAb (as controls). Cells were incubated with primary mAbs (20 min, 4°C) recognizing pig MHC class II (clone MSA3), pig CD14 (clone CAM36A) and pig CD172α (SIRPα, clone 72-22-15A) all