

3rd International Immunonutrition Workshop

Session 7: Prebiotics and probiotics usefulness against pathologies Potential role of the intestinal microbiota of the mother in neonatal immune education*

Anne Donnet-Hughes¹, Pablo F. Perez^{2†}, Joël Doré², Marion Leclerc², Florence Levenez²,
Jalil Benyacoub¹, Patrick Serrant¹, Iris Segura-Roggero¹ and Eduardo J. Schiffrin^{3‡}

¹Nutrition and Health Department, Nestlé Research Centre, Nestec Ltd, PO Box 44, 1000 Lausanne 26, Switzerland

²INRA, CR de Jouy-en-Josas, 78352 Jouy en Josas, France

³Nestlé Nutrition, Nestec Ltd, Avenue Reller 22, CH-1800 Vevey, Switzerland

Mucosal dendritic cells are at the heart of decision-making processes that dictate immune reactivity to intestinal microbes. They ensure tolerance to commensal bacteria and a vigorous immune response to pathogens. It has recently been demonstrated that the former involves a limited migration of bacterially loaded dendritic cells from the Peyer's patches to the mesenteric lymph nodes. During lactation, cells from gut-associated lymphoid tissue travel to the breast via the lymphatics and peripheral blood. Here, we show that human peripheral blood mononuclear cells and breast milk cells contain bacteria and their genetic material during lactation. Furthermore, we show an increased bacterial translocation from the mouse gut during pregnancy and lactation and the presence of bacterially loaded dendritic cells in lactating breast tissue. Our observations show bacterial translocation as a unique physiological event, which is increased during pregnancy and lactation. They suggest endogenous transport of intestinally derived bacterial components within dendritic cells destined for the lactating mammary gland. They also suggest neonatal immune imprinting by milk cells containing commensal-associated molecular patterns.

Breast milk: Bacterial translocation: Microbiota: Lactation: Pregnancy: Neonatal immunity

Understanding of the association and interactions between the intestinal microbiota and the host immune system has advanced greatly in recent years. However, mammals are born sterile and the events shaping the establishment of a flourishing intestinal microbiota remain elusive. It is known that dendritic cells (DC), via pattern recognition receptors such as toll-like receptors, sample the microbes that continuously bombard the intestinal mucosa⁽¹⁾. The end result is tolerance to the normal microbiota and protection against pathogenic attack. Yet, successful, simultaneous deployment of such divergent processes requires highly sophisticated

control mechanisms which are not expected of an inexperienced neonatal immune system. The very survival of the neonate after the massive bacterial insult in the birth canal is testimony to solid regulatory mechanisms which may already operate during fetal life. The subsequent assembly of specific bacterial communities, in the absence of adverse immune responses, is testimony to its long-term effects and robustness. Environmental factors, including the method of delivery and the use of antibiotics, impact the final composition of the neonatal microbiota⁽²⁾. However, differences between breast-fed and formula-fed infants in intestinal

Abbreviations: DC, dendritic cells; MG, mammary gland; MLN, mesenteric lymph nodes; PBMC, peripheral blood mononuclear cells; rDNA, ribosomal DNA; TP, time point; TTGE, temporal temperature gradient-gel electrophoresis.

*This article represents the presentation given at the 3rd International Immunonutrition Workshop; the original article was previously published elsewhere⁽⁴²⁾.

†Present address: CIDCA-Cátedra de Microbiología. Facultad de Ciencias Exactas – UNLP, 47 y 116, La Plata (1900), Argentina.

‡Corresponding author: Eduardo J. Schiffrin, fax +41 21 924 7894, email eduardo.schiffrin@nestle.com

bacterial colonization⁽²⁾ and in susceptibility to intestinal disease or sepsis⁽³⁾ suggest that further education is also acquired by factors in breast milk.

More specifically, breast milk provides the suckling infant with a myriad of soluble factors such as Ig, lactoferrin and lysozyme, which influence the composition of the neonatal microbiota and play a direct role in the protection of the neonatal intestine⁽⁴⁾. Another possibility is that soluble pattern recognition receptors such as CD14 and toll-like receptor 2 present in milk have an indirect effect by instructing the neonatal immune system to recognize and respond appropriately to commensals and pathogens^(5,6). However, there is accumulating evidence that bacteria of maternal origin are transmitted to the infant via colostrum and milk⁽⁷⁾. The majority of milk organisms arise from the mother's skin or the infant's mouth^(7,8), but certain species are suggested to colonize the neonatal intestine⁽⁹⁾. Most studies on the microbiology of human milk have addressed the transmission of pathogens or contaminating commensals in samples meant for milk banks^(8,10,11). However, it has been suggested that breast milk is not sterile, even when collected aseptically⁽¹⁰⁾. This raised the interesting possibility that breast milk harbours a natural bacterial inoculum.

Here, we examined whether some microbial species are indeed intrinsic to human milk. Confirmation of a bacterial inoculum, albeit of low dose, prompted a subsequent investigation into its origin. Since human breast milk leucocytes arise from cells which have migrated from the intestine to the lactating mammary gland (MG) via the lymphatics and the circulation, we considered that this cellular circuitry conveys microbial components in a manner which is not deleterious to maternal health. Consequently, we examined the bacterial profile associated with the cellular compartments of human milk and maternal blood and compared this to the profile in maternal and infant faeces. This was followed by a study of bacterial colonization in various tissue compartments of virgin, pregnant and lactating mice. Taken together, our results suggest that bacterial translocation is a physiological process which is augmented in the pre- and postpartum period, one of the consequences of which is the cellular transfer of bacterial components to the suckling infant via the breast milk. The biological relevance of these phenomena to the mother and to the suckling infant remain a matter of conjecture. However, if one considers the risks associated with such an evolutionary process, it is likely that the motive goes beyond simple colonization of the neonatal intestine by small numbers of milk-borne organisms.

Methods

Human milk, blood and faecal samples

Breast milk was collected from healthy lactating mothers who delivered at term. Foremilk was discarded, the breast cleaned with antiseptic soap, rinsed with sterile distilled water and dried using sterile gauze before aseptic collection using an electric breast pump. As a control, a swab of the areola was taken before milk collection. Samples of whole milk were plated on Man, Rogosa and Sharpe medium containing cysteine, on Eugon Tomato, Drigalski,

Shaedler Neo Vanco or on blood agar (Bio-Mérieux, Marcy l'Etoile, France) and incubated aerobically or anaerobically at 37°C. Cells were collected from the remaining milk by centrifugation and exposed to sterile PBS containing 1% gentamycin to kill extracellular bacteria. Cells were aliquoted and used to make cytopreparations, frozen in RPMI media (Life Technologies, Basel, Switzerland) containing 10% dimethylsulphoxide in fetal calf serum for flow cytometric analysis or lysed using cold sterile distilled water and passed through a sterile needle for plating on bacterial culture media.

Venous blood (10 ml) was collected from eight lactating women at different times postpartum or from five age-matched, non-gravidus, non-lactating women. The blood was centrifuged over Ficoll-Hypaque media (Sigma-Aldrich, St. Louis, MO, USA), washed and then processed as for milk cells. Maternal and infant faecal samples were collected in sterile tubes, aliquoted and stored frozen at -80°C until required. Protocols were approved by an internal ethical committee and the Swiss authorities.

Flow cytometry

Myeloid and lymphoid DC in the peripheral blood mononuclear cells (PBMC) fraction were examined using the FACSCalibur and DC-KIT (Becton Dickinson, Basel, Switzerland). This kit contains fluorescein isothiocyanate-conjugated lineage cocktail with antibodies to CD3, CD14, CD16, CD19, CD20 and CD56; peridinin chlorophyll protein-conjugated anti-human leucocyte antigen-DR, allophycocyanin-conjugated anti-CD11c and phycoerythrin-conjugated anti-CD123. In separate tubes, cells were labeled with fluorescein isothiocyanate-anti-CD11c and PE-anti CD14 (Becton Dickinson) according to the manufacturer's instructions before analysis using the FACSCalibur.

Temporal temperature gradient gel electrophoresis

Total DNA was extracted from 200 mg faecal samples and from milk and blood samples as described previously for faeces and biopsies, respectively⁽¹²⁾, except that for milk and blood, DNA precipitations were done overnight and the pellets centrifuged for 1 h at 4°C.

Isolated DNA were then used as templates to amplify the V6-V8 regions of 16S ribosomal DNA (rDNA), using primers U968-GC-F and L1392-R⁽¹²⁾. Several dilutions of template DNA were tested if the presence of PCR inhibitors was suspected, in order to optimize PCR yield and limit possible bias. The PCR amplification and temporal temperature gradient-gel electrophoresis (TTGE) were performed as before⁽¹³⁾ and Gel Compar II software (Applied Maths) used to compare TTGE profiles. A marker consisting of a PCR amplicon mix of seven cloned rDNA from different bacterial species was used to normalize the profiles.

DNA from milk, maternal blood cells and infant faecal samples at week 4 postpartum were amplified by PCR with primers U350-F and L1392-R. Ligation and cloning in pGEM-T Vector system I (Promega) were performed as described previously⁽¹³⁾. Forty-eight clones per library were sequenced using the primers M13F and M13R. An equal portion of the small subunit rDNA (*Escherichia coli*

position 350 to 1392) was used for the sequence analysis. These sequences were checked manually and contigs were made using BioEdit software. These sequences were submitted to Genbank for preliminary analysis using the Blast programmes (Blast and Megablast) of the Ribosomal Database Project in order to identify putative, close phylogenetic relatives. The sequences were tested for chimera structure using the Ribosomal Database Project analysis service Check Chimera and during manual inspection of alignment. The sequences were aligned to their nearest neighbour with the automated alignment tool of the ARB programme package and the arb database (Technical University of Munich) of January 2004. Mother and infant sequences were compared using the Blast2sequences programme of the National Center for Biotechnology Information.

Bacteria localization in human milk and blood cells

After fixation in absolute ethanol, cytopreparations of human milk and blood cells were incubated for 5 min with 100 mg/ml acridine orange^(14,15), washed extensively, mounted in fluorescent mounting medium (DakoCytomation, DAKO Schwiez, Baar, Switzerland) and analysed by epifluorescence microscopy.

IL-12 and fetal liver tyrosine kinase-3 ligand

IL-12 and fetal liver tyrosine kinase-3 ligand in human serum (1:2 dilution) were assayed by ELISA according to the manufacturer's instructions (Diacclone and R&D Systems, Epalinges, Switzerland).

Animal study

Conventional virgin and pregnant/lactating C57BL/6 mice (Charles River, l'Arbresle, France) were sacrificed (n 10 per group) at 5–6 d before parturition, or at 1–2, 3–4 or 14–15 d postpartum. Samples of blood, intestinal contents, mesenteric lymph nodes (MLN), spleen, liver and MG were aseptically collected for microbiological analysis, fixed in Bouin's fixative before mounting in paraffin blocks and/or mounted in optimal cutting temperature compound and frozen in liquid nitrogen. The experimental procedure was approved by an internal ethical committee and the Swiss authorities.

Bacteria in mouse tissues

Micro-organisms were visualized in tissues using Gram's stain or a rabbit antibody to lipoteichoic acid⁽¹⁶⁾ and tetramethylrhodamine β -isothiocyanate-labelled anti rabbit-IgG (Jackson) as secondary antibody. For microbiological analysis, samples of mouse tissues were homogenized, suspended in sterile PBS, plated onto blood agar (Bio Mérieux) and incubated aerobically or anaerobically at 37°C.

Fluorescent labelling of mouse tissues

Thin sections (5 μ m) of optimal cutting temperature compound-embedded tissues were fixed in cold ethanol and

rehydrated using PBS. Non-specific staining was blocked using 10% fetal calf serum in PBS before labelling with fluorescein isothiocyanate-labelled hamster anti-mouse CD11c or the control isotype hamster IgG1 (Becton Dickinson) according to the manufacturer's instructions.

Statistical analysis

The proportion of animals with bacteria in different tissues was analysed by the Fisher's exact test. Differences in the median percentage of DC in human blood were compared using the Mann–Whitney test. TTGE profiles were compared using Gel Compar II software (Applied Maths, Kortrijk and Belgium). Similarity coefficients (Pearson correlation method) were then calculated for each pair of profiles, yielding a similarity matrix. A dendrogram was constructed from this matrix by using an Unweighted Pair Group Method with Arithmetic Mean algorithm.

Results

Bacterial signatures common to the neonatal microbiota and maternal blood and milk cells

To minimize contamination of expressed milk with skin bacteria or organisms residing in the superficial ducts of the breast, the foremilk was rejected and the breast cleaned with antiseptic soap prior to milk collection. No bacteria were grown from swabs of the nipple areola. Expressed milk contained a total concentration of $<10^3$ cfu/ml bacteria composed of *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Peptostreptococcus*, *Staphylococcus*, *Corynebacterium* and/or an occasional *Escherichia* species.

Since endogenous intracellular trafficking of bacterial components from the intestine to the lactating breast would implicate the lymphatic and systemic circulation, we next used TTGE to examine the bacterial rDNA content in human milk cells and maternal PBMC and faeces during lactation. Furthermore, we examined corresponding infant faecal samples to address a possible transmission of maternal bacteria via milk. The advantages of TTGE analysis lie in its sensitivity and its capacity to detect non-culturable bacteria. Thus, a more extensive number of bacteria can be catalogued.

Maternal faecal samples gave classic TTGE profiles which were specific for each individual and of greater biodiversity than those of infant faeces (Fig. 1). Although breast milk cells harboured a less complex microbiota than in maternal faeces, TTGE revealed a greater biodiversity than previously observed by plating of the milk. Thus, non-culturable bacteria or the DNA from dead bacteria may also be present intracellularly. Fig. 1 shows one mother–infant couple analysed over weeks 1–4 postpartum. Similar TTGE profiles were observed for seven other mother–infant pairs (data not shown). Interestingly, some bacterial signatures (indicated by arrows) were common to the infant faeces and to several samples of maternal origin (Fig. 1). Upon excision from the TTGE gel and sequencing, the lowest of these bands, especially intense in infant faeces and comigrating in maternal faeces and blood, was identified as *Bifidobacterium longum* on the basis of 369 nucleotides. Sequencing of the other common bands

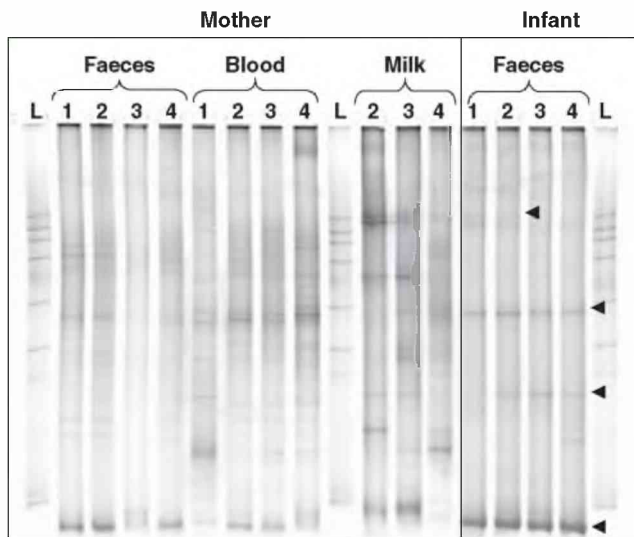


Fig. 1. Transmission of bacterial signatures from mother to child via maternal peripheral blood mononuclear cells and milk cells during lactation. Profiling of dominant bacterial signatures in maternal faeces, blood and milk cells and in infant faeces using temporal temperature gradient-gel electrophoresis amplified fragments of ribosomal DNA (rDNA). Each dominant bacterial species leads to the formation of one or a few bands in the pattern. Duplicate PCR were used for the maternal blood and milk cell fractions. A ladder (L) obtained by PCR amplification of cloned rDNA sequences was used for normalization of gels and image analysis. Some bacterial signatures (indicated by arrows) were common to the infant faeces and to samples of maternal origin.

identified DNA from *Streptococcus salivarius* and *Rhodococcus erythropolis*.

Next, PCR products were used to prepare rDNA libraries from the milk cells. Besides the species previously identified by plating and by sequencing of the excised TTGE gel bands, sequencing of milk cell clones revealed the presence of *Bacteroides*, Clostridia, *Eubacterium* and Enterobacteria among a total of 15 genera typical of ileal and colonic microbiota. The most represented bacterial genera in infant faeces were less diverse and essentially lactose degrading, lactic acid producing bacteria together with *Staphylococcus* species. Out of 23 sequences corresponding to bifidobacteria, 17 were related to *B. longum*, five to *Bifidobacterium bifidum* and 1 to *Bifidobacterium infantis*. Two identical rDNA sequences (99% identity out of 1117 base pairs), corresponding to *Streptococcus thermophilus* and *Staphylococcus epidermidis*, were identified in the milk cell clones and in the infant's faeces (data not shown).

Interestingly, PBMC of lactating mothers contained a restricted variety of bacterial rDNA sequences (Fig. 1). Bacterial signals were also present in blood cells of non-gravidus, non-lactating women, but the complexity of bacterial signatures was greater in PBMC from breastfeeding mothers (data not shown). Profiles for control women were similar, while those of lactating women were specific for each individual.

Acridine orange staining of milk and blood cytopreparations identified bacterial bodies in association with mononuclear cells (Fig. 2).

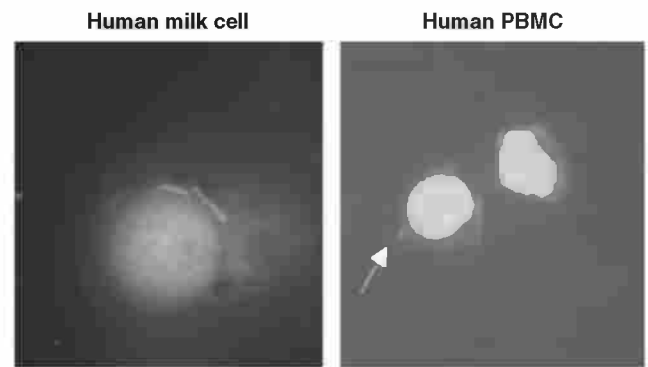


Fig. 2. Intact bacterial structures in milk cells and peripheral blood mononuclear cells (PBMC) from lactating mothers stained with acridine orange.

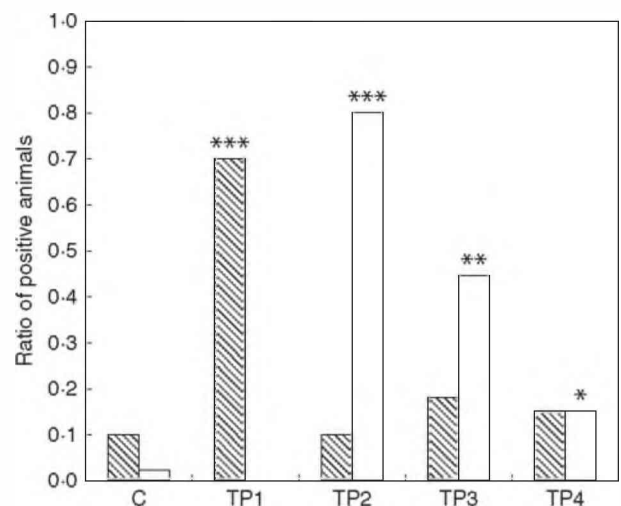


Fig. 3. Microbiological analysis of mouse tissues reveals increased bacterial translocation during pregnancy and lactation. Proportion of control virgin mice (C), pregnant mice (5–6 d before parturition, TP1) and lactating mice at 1–2 d (TP2), 3–4 d (TP3) and 14–15 d (TP4) postpartum with viable bacteria in mesenteric lymph nodes (MLN) (▨) and mammary gland tissue (MG) (□). *** $P < 0.00005$; ** $P < 0.005$; * $P < 0.05$ as compared to controls (Fisher's exact test).

Increased translocation of intestinal bacteria occurs in pregnant and lactating mice

To further define endogenous cellular transport of bacterial components, we next examined intestinal uptake of bacteria in conventional non-pregnant, pregnant and lactating mice.

In contrast to the confined bacterial translocation to MLN observed in control animals, heightened translocation to MLN in the perinatal period was followed by bacterial colonization of the breast in the immediate postpartum period (Fig. 3). While 10% control animals (C) had positive MLN cultures, 70% pregnant animals had detectable levels of bacteria in their MLN, 5–6 d before delivery (time point (TP) 1). Within 24 h postpartum, fewer animals had positive MLN cultures, but 80% of the mice had viable bacteria in the mammary tissue. Although

Table 1. Bacterial translocation to extra-intestinal tissues during pregnancy and lactation. The proportion of animals with viable bacteria in the spleen, liver and blood was examined after plating on blood agar. Positive samples had a low number of micro-organisms (blood: 10^1 – 10^2 cfu/ml; spleen and liver: 10^2 – 10^3 cfu/g). Bacteria isolates were characterized by macroscopic and microscopic morphology, Gram's staining, culture characteristics and using Fluorescence *in situ* hybridisation technology⁽⁴²⁾ and specific probes (Bif 1412, MWG Biotech). Time points TP1, TP2 and TP3 are the same as for Fig. 3.

Group	Proportion of animals with positive cultures		
	Blood	Spleen	Liver
Non-pregnant	0/40	1/30	0/30
Pregnant	3/10	3/10	3/10
TP1	0/10	1/10	0/10
TP2	1/10	0/10	1/10
TP3	0/10	0/10	3/10
	<i>Bifidobacterium</i> spp.	<i>Bifidobacterium</i> spp.	<i>Bifidobacterium</i> spp.
	<i>Staphylococcus/Micrococcus</i> spp.	<i>Staphylococcus/Micrococcus</i> spp.	<i>Staphylococcus/Micrococcus</i> spp.
	<i>Streptococcus</i> spp.		

this value decreased to 50% by 3–4 d postpartum, it was still significantly different from that of control mice ($P < 0.005$). From the present study, one cannot deduce whether subsequent waves of bacteria translocate at other time points later during lactation. Bacterial colonization of the MG coincided with an increased number of positive blood cultures and occasional translocation to the spleen and liver (Table 1). In contrast to pathological conditions where translocating micro-organisms are mainly Gram negative, penetrating organisms in pregnant and lactating mice included *Streptococcus*, *Lactobacillus* and *Bifidobacterium* species whose numbers gradually subsided over time (data not shown).

During lactation, bacteria were visualized histologically in the lamina propria of the small bowel, in the sub-epithelial dome and interfollicular regions of the Peyer's patch and associated with cells in the glandular tissue of the MG (data not shown). Double labelling using antibodies to CD11c and bacterial lipoteichoic acid showed co-localization of micro-organisms and CD11c⁺ cells suggesting that bacterial cells were inside the DC (data not shown).

The Peyer's patches of pregnant and lactating mice were enlarged compared to those of controls, had a more prominent sub-epithelial dome and more dilated lymphatic vessels (Fig. 4). Furthermore, CD11c⁺ cells were localized predominantly in the sub-epithelial dome and interfollicular regions of the Peyer's patches. In the patches of pregnant and lactating animals, mononuclear cells were observed within the draining lymphatics (Fig. 4).

Myeloid and lymphoid dendritic cells are diminished in the circulation of lactating women

Breast milk has a high proportion of phagocytes which readily ingest microbes but are ineffective at killing⁽¹⁷⁾. Recent work demonstrating that CD14⁺ milk mononuclear cells express HLA-DR, CD86 and CD83 as well as DC-specific intercellular adhesion molecule 3-grabbing non-integrin, a C-type lectin considered to be exclusively expressed on DC, suggests that these cells are partially differentiated DC⁽¹⁸⁾. Since milk DC-like cells may be

derived from the maternal circulation and are potential vehicles of intestinally derived microbial components, we next evaluated the distribution of DC phenotypes in the PBMC of lactating and non-lactating women using flow cytometry. The percentages of CD123⁺ lymphoid DC and CD11c⁺ myeloid DC were determined after gating on lin⁻ HLA-DR⁺ PBMC (data not shown). In addition, the numbers of CD14⁺CD11c⁺, potential DC precursors were studied. We found that the frequencies of differentiated lymphoid DC (lin⁻CD14⁻HLA-DR⁺CD11c⁻CD123⁺) and myeloid DC, (lin⁻CD14⁻HLA-DR⁺CD11c⁺CD123⁻) phenotypes and CD14⁺CD11c⁺ cells in the circulation of lactating women are lower than those of controls. Of note, both CD14^{hi}CD11c⁺ and CD14^{lo}CD11c⁺ cells are present in the circulation of control and lactating women and while there is a tendency towards a higher proportion of the CD14^{lo}CD11c⁺ intermediate DC-like phenotype during the first week of lactation, the results were not significantly different from controls.

Discussion

After discarding the foremilk, we found that aseptically collected human breast milk contained a low total concentration of up to 10^3 cfu/ml microbes comprising *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Peptostreptococcus*, *Staphylococcus* and/or *Corynebacterium*, with an occasional *Escherichia* species. In spite of every precaution, some of these isolates may still arise from contamination. One might argue that bacterial contaminants in milk may be biologically relevant to the breast-fed infant. Indeed, *Staphylococcus* species are common constituents of the early neonatal microbiota⁽²⁾. However, a bacterial presence in all samples suggests that a discrete microbiota may naturally exist in breast milk. Consistent with this, we made the observation that the bacteria were associated with breast milk cells examined microscopically (Fig. 2).

Human breast milk contains an appreciable leucocyte population, the lymphocytes of which originate from the gut-associated lymphoid tissue and bronchial lymphoid

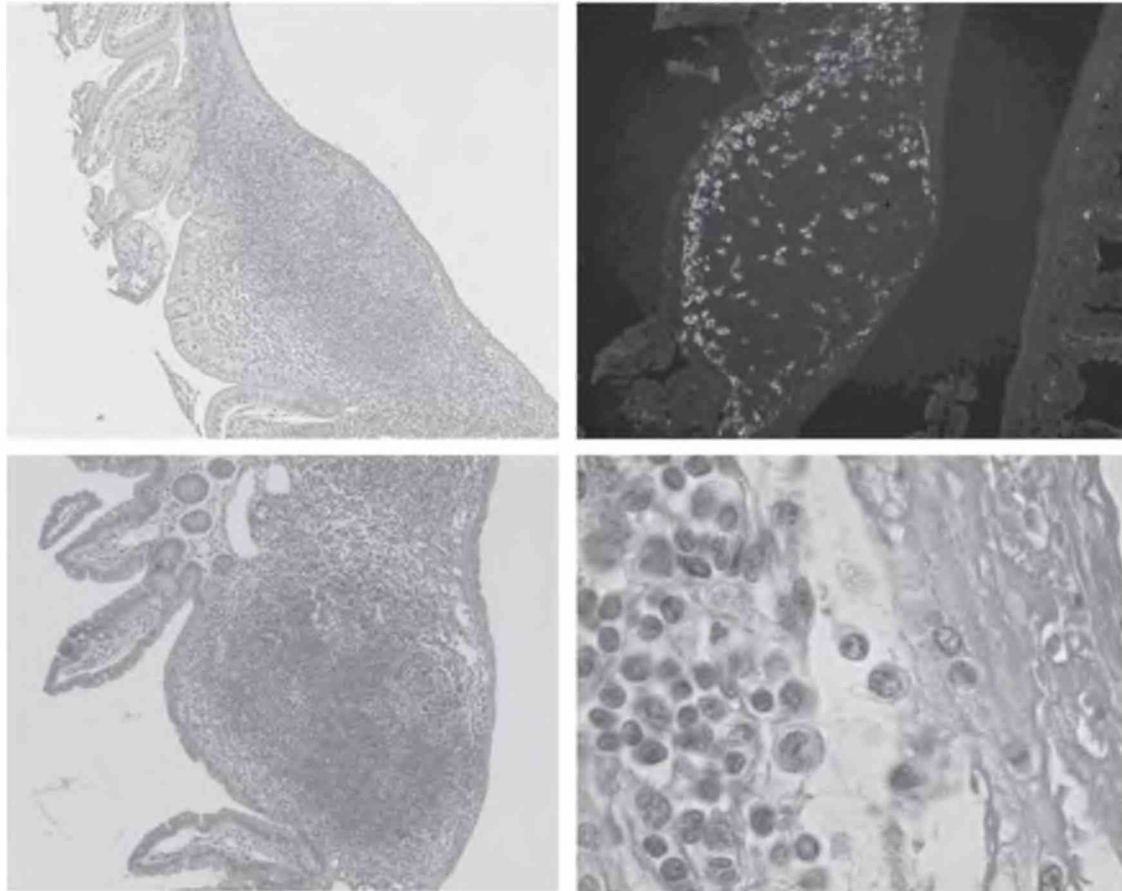


Fig. 4. Staining of Peyer's patch tissue from a pregnant mouse. Haematoxylin and eosin staining showed a reactive lymphoid tissue with dilated lymphatic vessels containing mononuclear cells (lower left, $\times 100$ and lower right, $\times 1000$) that are not present in virgin mice (upper left, $\times 100$). Fluorescently labelled CD11c⁺ cells were prominent in the sub-epithelial dome and interfollicular region (upper right).

tissue of the mother⁽¹⁹⁾ and the mononuclear phagocytes from peripheral blood⁽²⁰⁾. Here, we propose that the latter also arise in the gut-associated lymphoid tissue, capture components of the luminal microbiota prior to their departure and transfer them to the suckling infant via breast milk. Since it is generally accepted that tissue macrophages are resident, non-migrating cells, we considered DC-like cells as the most likely vehicles for trafficking within the common mucosal system.

In contrast to the two or three viable bacterial genera observed by plating of individual milk samples, TTGE analysis of human milk cells revealed a wide diversity of bacterial signatures, corresponding to the dominant autochthonous ileal and colonic commensal micro-organisms. This confirms the expected uptake of bacteria at these tissue sites and suggests that non-culturable bacteria or the DNA from dead bacteria may also be present intracellularly.

Migration of bacteria, or bacterial components, within intestinally derived leucocytes to the lactating breast is supported by the observation that some of the rDNA bands on TTGE gels are common to the faecal, blood and milk samples of each mother examined. Furthermore, some of these bands are also found in infant faeces (Fig. 1) and may

represent microbes transferred to the suckling infant via the milk.

To our surprise, PBMC contained a restricted variety of bacterial rDNA sequences which was more extensive in the blood of breast-feeding mothers. However, no viable bacteria were isolated from these cells. The reason for this discrepancy is not known. One could speculate that the few, bacterially laden cells may be diluted in the circulation. However, it is also possible that certain blood-borne bacteria may be quiescent or dead due to intracellular antimicrobial effects.

Awareness that some bacteria may nevertheless be environmental contaminants and that PCR amplification may have inadvertently led to false positives, we confirmed microscopically whether bacteria were associated with cells in the milk or maternal blood. Unlike sepsis where translocating bacteria are associated with polymorphonuclear cells⁽¹⁵⁾, intact bacterial bodies were observed in association with a limited number (<0.1%) of milk and blood mononuclear cells (Fig. 2). However, it is not excluded that the components from dead organisms may be associated with milk and blood polymorphonuclear cells.

During lactation, bacteria were visualized beneath the epithelium of both the Peyer's patch dome and the intestinal

lamina propria of mice. This suggests M-cell-mediated uptake towards DC in the Peyer's patches, direct sampling of luminal bacteria by dendrites of lamina propria DC or a low-level, physiological leakiness of the epithelium⁽²¹⁾. In any event, an extremely limited number of bacteria are thought to cross the intestinal epithelium, evade uptake and killing by intestinal macrophages, but remain viable after phagocytosis by DC⁽²²⁾. Bacterially loaded DC are then thought to migrate to the MLN where they initiate protective immune responses⁽²²⁾. These migrating DC phagocytose bacteria well but are relatively ineffective at killing internalized organisms⁽²³⁾. The more prominent Peyer's patches observed in pregnant and lactating animals, with localization of CD11c⁺ at the sites of noticeable mononuclear cell exit through lymphatic vessels (Fig. 4), provides indirect support of mononuclear cell-mediated transport of intestinally derived microbes to the lactating MG.

The presence of a modest population of enteric-like bacteria in association with a milk cell compartment suggests that this inoculum might serve an alternative purpose to that traditionally associated with bacterial implantation in the neonatal intestine. These organisms may indeed eventually colonize neonatal tissues, but we speculate that they educate the neonatal immune system in the recognition of commensal-associated molecular patterns of bacteria.

It has been previously shown that human milk contains soluble pattern recognition receptors for bacterial molecular motifs and that these may mediate a differential response to Gram-negative and Gram-positive organisms^(5,6). Uptake of maternal leucocytes into neonatal tissues occurs during gestation and lactation⁽²⁴⁾. Perhaps prolonged penetration of inconspicuous bacterial molecular patterns via maternal DC, induces tolerogenic responses that are analogous to those for self-antigens. The TNF-family molecule receptor activator of NF- κ B ligand (osteoprotegerin ligand, TNF-related activation-induced cytokin and osteoclast differentiation factor) and its receptor activator of NF- κ B are key regulators of bone remodelling⁽²⁵⁾ and regulate T-cell/DC differentiation communication^(26,27), lymph node formation⁽²⁸⁾ and the development of a lactating MG during pregnancy⁽²⁹⁾. It is noteworthy that osteoprotegerin, a soluble decoy receptor for receptor activator of NF- κ B ligand, is a DC survival factor⁽³⁰⁾ which is elevated in serum during pregnancy and lactation⁽³¹⁾ and is present in significant quantities in human breast milk⁽³²⁾. It has been shown that Peyer's patch DC express receptor activator of NF- κ B and that treatment with receptor activator of NF- κ B-Fc, which like osteoprotegerin, antagonizes receptor activator of NF- κ B ligand activity and enhances the induction of tolerogenic immune responses⁽³³⁾. It is feasible that the high levels of osteoprotegerin during pregnancy and lactation influence the generation of tolerogenic *v.* activating DC in the mother. It is also possible that milk osteoprotegerin has a similar effect in the suckling neonate.

Elevated translocation of bacteria or their components in the mother should certainly have some bearing on her immune status. The traditional paradigm that normal pregnancy is a T-helper 2-mediated phenomenon is challenged by reports of physiological activation of innate immunity with monocyte and granulocyte activation and increased

production of IL-12 and TNF- α ^(34–36). We consistently observed IL-12 in the serum of three out of eight lactating mothers (range 12.8–27.3 pg/ml, data not shown). An increased physiological translocation of viable and dead intracellular bacteria or their constituents could explain this activation. Interestingly, cytosine-phosphorothioate-guanine oligodeoxynucleotides stimulate innate immunity in pregnant mice, improve maternal survival and prevent pathogen transmission to the fetus⁽³⁷⁾. The association between specific intra-uterine infections or exaggerated production of T-helper 1 cytokines, and the risk for spontaneous premature birth or abortion may reflect dysregulation of the quantity or type of microbial components translocating.

We detected a decrease of DC subsets in the blood of lactating women. These findings are in agreement with previously reported results that showed reduced numbers of circulating DC subsets in the third trimester of pregnancy⁽³⁸⁾. These decreased DC numbers may reflect cellular trafficking towards the breast or intestine. Indeed, we detected fetal liver tyrosine kinase-3 ligand, a stimulator of DC differentiation and mobilization⁽³⁹⁾, in serum samples of three out of eight mothers (range 13.9–71.7 pg/ml, data not shown).

Our observations not only imply a novel form of mother–infant communication, but they also highlight a potentially new mechanism of immune regulation in healthy individuals. As shown previously^(40,41), the blood of normal healthy subjects contains bacterial components. Some DNA may arise from human or microbial contamination. However, the greater number of bacterial DNA signatures in the PBMC of healthy, lactating women suggests that certain bacterial species are inherent to circulating cells. It is tempting to speculate that this represents an evolutionary strategy of immune surveillance and that such bacterial imprinting maintains tolerance to specific bacterial species and alerts distant anatomical sites of changes in local lymphoid tissues. Such a mechanism may also provide a colonization advantage to bacteria of the mother's intestinal microbiota at a time when the low bacterial diversity in the neonatal intestine is permissive to colonization.

Acknowledgements

A. D.-H., J. B., P. S., I. S.-R. and E. J. S. are Nestlé employees. P. F. P., J. D., M. L. and F. L. declare no conflict of interest. P. F. P. performed animal studies, microbiology, analysis of bacteria-associated human cells and flow cytometry; J. D., M. L. and F. L. performed TTGE analysis and cloning of bacterial DNA; J. B. contributed to the animal studies and cytokine analysis in human blood; P. S. and I. S. R. prepared and analysed samples for flow cytometry and immunohistochemistry; E. J. S. and A. D. H. generated the original hypothesis, prepared and analysed human samples, contributed to the animal studies, histological and cell analysis. A. D. H. and E. J. S. wrote the manuscript. All the authors discussed the results and commented on the manuscript. Much of the work described in this paper has been published elsewhere⁽⁴²⁾.

References

1. Stagg AJ, Hart AL, Knight SC *et al.* (2003) The dendritic cell: its role in intestinal inflammation and relationship with gut bacteria. *Gut* **52**, 1522–1529.
2. Falk PG, Hooper LV, Midtvedt T *et al.* (1998) Creating and maintaining the gastrointestinal ecosystem: what we know and need to know from gnotobiology. *Microbiol Mol Biol Rev* **62**, 1157–1170.
3. Wright AL, Bauer M, Naylor A *et al.* (1998) Increasing breastfeeding rates to reduce infant illness at the community level. *Pediatrics* **101**, 837–844.
4. Lonnerdal B (2003) Nutritional and physiologic significance of human milk proteins. *Am J Clin Nutr* **77**, 1537S–1543S.
5. Labeta MO, Vidal K, Nores JE *et al.* (2000) Innate recognition of bacteria in human milk is mediated by a milk-derived highly expressed pattern recognition receptor, soluble CD14. *J Exp Med* **191**, 1807–1812.
6. Vidal K, Donnet-Hughes A & Granato D (2002) Lipoteichoic acids from *Lactobacillus johnsonii* strain La1 and *Lactobacillus acidophilus* strain La10 antagonize the responsiveness of human intestinal epithelial HT29 cells to lipopolysaccharide and gram-negative bacteria. Responsiveness of human intestinal epithelial HT29 cells to lipopolysaccharide and gram-negative bacteria. *Infect Immun* **70**, 2057–2064.
7. Moughan PJ, Birtles MJ, Cranwell PD *et al.* (1992) The piglet as a model animal for studying aspects of digestion and absorption in milk-fed human infants. Nutritional triggers for health and disease. *World Rev Nutr Diet* **67**, 40–113.
8. Gavin A & Ostovar K (1977) Microbiological characterization of human milk. *J Food Prot* **40**, 614–616.
9. Martín R, Langa S, Reviriego C *et al.* (2003) Human milk is a source of lactic acid bacteria for the infant gut. *J Pediatr* **143**, 754–758.
10. West PA, Hewitt JH & Murphy OM (1979) The influence of methods of collection and storage on the bacteriology of human milk. *J Appl Bacteriol* **46**, 269–277.
11. El Mohandes AE, Schatz V, Keiser JF *et al.* (1993) Bacterial contaminants of collected and frozen human milk used in an intensive care nursery. *Am J Infect Control* **21**, 226–230.
12. Lepage P, Seksik P, Sutren M *et al.* (2005) Biodiversity of the mucosa-associated microbiota is stable along the distal digestive tract in healthy individuals and patients with IBD. *Inflamm Bowel Dis* **11**, 473–480.
13. Mangin I, Bonnet R, Seksik P *et al.* (2004) Molecular inventory of faecal microflora in patients with Crohn's disease. *FEMS Microbiol Ecol* **50**, 25–36.
14. Ho PC & Lawton JW (1978) Human colostrum cells: phagocytosis and killing of *E. coli* and *C. albicans*. Human colostrum cells: phagocytosis and killing of *E. coli* and *C. albicans*. *J Pediatr* **93**, 910–915.
15. Kite P, Millar MR, Gorham P *et al.* (1988) Comparison of five tests used in diagnosis of neonatal bacteraemia. *Arch Dis Child* **63**, 639–643.
16. Granato D, Bergonzelli GE, Pridmore RD *et al.* (2004) Cell surface-associated elongation factor Tu mediates the attachment of *Lactobacillus johnsonii* NCC533 (La1) to human intestinal cells and mucins. *Infect Immun* **72**, 2160–2169.
17. Buescher ES & McIlheran SM (1993) Polymorphonuclear leukocytes and human colostrum: effects of *in vivo* and *in vitro* exposure. *J Pediatr Gastroenterol Nutr* **17**, 424–433.
18. Ichikawa M, Sugita M, Takahashi M *et al.* (2003) Breast milk macrophages spontaneously produce granulocyte-macrophage colony-stimulating factor and differentiate into dendritic cells in the presence of exogenous interleukin-4 alone. *Immunology* **108**, 189–195.
19. Roux ME, McWilliams M, Phillips-Quagliata JM *et al.* (1977) Origin of IgA secretory plasma cells in the mammary gland. *J Exp Med* **146**, 1311–1322.
20. Goldman AS & Goldblum RM (1997) Transfer of maternal leukocytes to the infant by human milk. *Curr Top Microbiol Immunol* **222**, 205–213.
21. Uhlig HH & Powrie F (2003) Dendritic cells and the intestinal bacteria flora: a role for localized mucosal immune responses. *J Clin Invest* **112**, 648–651.
22. MacPherson AJ & Uhr T (2004) Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science* **303**, 1662–1665.
23. Nagl M, Kacani L, Mullauer B *et al.* (2002) Phagocytosis and killing of bacteria by professional phagocytes and dendritic cells. *Clin Diagn Lab Immunol* **9**, 1165–1168.
24. Zhou L, Yoshimura Y, Huang Y *et al.* (2000) Two independent pathways of maternal cell transmission to offspring: through placenta during pregnancy and by breast-feeding after birth. *Immunology* **101**, 570–580.
25. Theill LE, Boyle WJ & Penninger JM (2002) RANK-L and RANK: T cells, bone loss, and mammalian evolution. *Annu Rev Immunol* **20**, 795–823.
26. Anderson DM, Maraskovsky E, Billingsley WL *et al.* (1997) A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature* **390**, 175–179.
27. Josien R, Wong BR, Li HL *et al.* (1999) TRANCE, a TNF family member, is differentially expressed on T cell subsets and induces cytokine production in dendritic cells. *J Immunol* **162**, 2562–2568.
28. Kong YY, Yoshida H, Sarosi I *et al.* (1999) OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* **397**, 315–323.
29. Fata JE, Kong YY, Li J *et al.* (2000) The osteoclast differentiation factor osteoprotegerin-ligand is essential for mammary gland development. *Cell* **103**, 41–50.
30. Wong R, Josien R, Lee SY *et al.* (1997) TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. *J Exp Med* **186**, 2075–2080.
31. Uemura H, Yasui T, Kiyokawa M *et al.* (2002) Serum osteoprotegerin/osteoclastogenesis-inhibitory factor during pregnancy and lactation and the relationship with calcium-regulating hormones and bone turnover markers. *J Endocrinol* **174**, 353–359.
32. Vidal K, van den Broek P, Lorget F *et al.* (2004) Osteoprotegerin in human milk: a potential role in the regulation of bone metabolism and immune development. *Pediatr Res* **55**, 1001–1008.
33. Williamson E, Bilsborough JM & Viney JL (2002) Regulation of mucosal dendritic cell function by receptor activator of NF-kappa B (RANK)/RANK ligand interactions: impact on tolerance induction. *J Immunol* **169**, 3606–3612.
34. Sacks GP, Redman CW & Sargent IL (2003) Monocytes are primed to produce the Th1 type cytokine IL-12 in normal human pregnancy: an intracellular flow cytometric analysis of peripheral blood mononuclear cells. *Clin Exp Immunol* **131**, 490–497.
35. Naccasha N, Gervasi MT, Chaiworapongsa T *et al.* (2001) Phenotypic and metabolic characteristics of monocytes and granulocytes in normal pregnancy and maternal infection. *Am J Obstet Gynecol* **185**, 1118–1123.
36. Kupfermanc MJ, Peaceman AM, Wigton TR *et al.* (1994) Immunoreactive tumor necrosis factor-alpha is elevated in maternal plasma but undetected in amniotic fluid in the second trimester. *Am J Obstet Gynecol* **171**, 976–979.

37. Ito S, Ishii KJ, Shirota H *et al.* (2004) CpG oligodeoxynucleotides improve the survival of pregnant and fetal mice following *Listeria monocytogenes* infection. *Infect Immun* **72**, 3543–3548.
38. Ueda Y, Hagihara M, Okamoto A *et al.* (2003) Frequencies of dendritic cells (myeloid DC and plasmacytoid DC) and their ratio reduced in pregnant women: comparison with umbilical cord blood and normal healthy adults. *Hum Immunol* **64**, 1144–1151.
39. Maraskovsky E, Brasel K, Teepe M *et al.* (1996) Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. *J Exp Med* **184**, 1953–1962.
40. Nikkari S, McLaughlin IJ, Bi W *et al.* (2001) Does blood of healthy subjects contain bacterial ribosomal DNA? *J Clin Microbiol* **39**, 1956–1959.
41. McLaughlin RW, Vali H, Lau PC *et al.* (2002) Are there naturally occurring pleomorphic bacteria in the blood of healthy humans? *J Clin Microbiol* **40**, 4771–4775.
42. Perez PF, Doré J, Leclerc M *et al.* (2007) Bacterial imprinting of the neonatal immune system: lessons from maternal cells? *Pediatrics* **119**, e724–e732.