

Symposium: Innate Immunity and Human Milk

Human Milk Inactivates Pathogens Individually, Additively, and Synergistically^{1,2}

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ABSTRACT Breast-feeding can reduce the incidence and the severity of gastrointestinal and respiratory infections in the suckling neonate by providing additional protective factors to the infant's mucosal surfaces. Human milk provides protection against a broad array of infectious agents through redundancy. Protective factors in milk can target multiple early steps in pathogen replication and target each step with more than one antimicrobial compound. The antimicrobial activity in human milk results from protective factors working not only individually but also additively and synergistically. Lipid-dependent antimicrobial activity in milk results from the additive activity of all antimicrobial lipids and not necessarily the concentration of one particular lipid. Antimicrobial milk lipids and peptides can work synergistically to decrease both the concentrations of individual compounds required for protection and, as importantly, greatly reduce the time needed for pathogen inactivation. The more rapidly pathogens are inactivated the less likely they are to establish an infection. The total antimicrobial protection provided by human milk appears to be far more than can be elucidated by examining protective factors individually. *J. Nutr.* 135: 1286–1288, 2005.

KEY WORDS: • human milk • antimicrobial lipids • antimicrobial peptides • synergy

Breast-feeding can reduce the incidence and the severity of respiratory and gastrointestinal infections in the suckling neonate by providing additional protective factors at mucosal surfaces (1,2). A number of common infectious disorders that are prevented or lessened by breast-feeding, including those caused by viruses, bacteria, and protozoa are shown in **Table 1** (3).

Protection against infection is provided by a multiplicity of protective factors, including secretory antibodies, and by innate immune factors, including lipids, carbohydrates, oligosaccharides, lysozyme, and lactoferrin (4–9). The immune system

in milk uses a combination of direct-acting antimicrobial factors, anti-inflammatory factors, and immunomodulators (3).

Direct-acting antimicrobial compounds in milk attack pathogens at multiple points in their life cycles, and multiple protective factors attack each point in the pathogen's replication cycle. Thus the immune system in milk provides redundancy at multiple levels. Pathogens can be inactivated directly by antimicrobial lipids, antimicrobial peptides, antibodies, and lysozymes, and can be prevented from binding to cellular receptors and coreceptors by fucosylated oligosaccharides and milk glycoproteins, such as lactoferrin and lactadherin (10–12). Binding inhibitors also protect against bacterial toxins by preventing receptor binding.

Protective lipids in milk

Milk lipids provide an example of how an integral milk component can serve both a nutritional and a protective function. The lipids in human milk do not initially have antimicrobial activity but become antiviral, antibacterial, and antiprotozoal *in vivo* after digestion in the gastrointestinal tract (13). Microbial killing by milk lipids is due primarily to FFAs and monoglycerides released from milk triglycerides by both milk-derived bile-salt-stimulated lipase and lipolytic activity in the infant's gut (14,15). The primary antimicrobial fatty acid released from human milk triglycerides is oleic acid (16).

Milk lipids are not unique in possessing antimicrobial activity. Human epidermis-derived skin lipids, especially fatty acids, inactivate *Staphylococcus aureus* (17). Lung surfactant from humans, dogs, rats, and guinea pigs contain fatty acids

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TABLE 1

Antimicrobial activity of human milk

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|------------------------------------|
| I. Enteric infections |
| 1. Poliovirus |
| 2. Coxsackie virus |
| 3. Echo virus |
| 4. <i>Escherichia coli</i> |
| 5. Shigella |
| 6. Salmonella |
| 7. Rotavirus |
| 8. <i>Giardia lamblia</i> |
| 9. Cryptosporidium |
| II. Respiratory infections |
| 1. Respiratory syncytial virus |
| 2. <i>Haemophilus influenzae</i> |
| 3. <i>Streptococcus pneumoniae</i> |
| 4. Enteroviruses |

that inactivate pneumococci (18). Because surfactant-like particles, which are biochemically similar to lung surfactant, have been isolated from intestine (19), fatty acids may also have an antimicrobial function in the intestinal tract, which is supplemented by milk lipids in the suckling neonate. Interestingly, fatty acids found in algae also have antimicrobial activity against both gram-positive and gram-negative bacteria (20).

Lipid-dependent antiviral activity is found in the stomach contents of milk-fed infants within 1 h of feeding (13,21). This lipid-dependent antimicrobial activity can persist for as long as 3 h after ingestion, which is the usual time between infant feedings (21). Differences in the persistence of lipid activity between milk-fed infants probably reflect variability in the gastrointestinal transit rate.

The concentration of antimicrobial lipids in the infant's stomach contents 1 h after feeding can in most cases be diluted 10-fold, with minimal decrease in activity against enveloped viruses (21). In fact, even after a 40-fold dilution, 10 to 15% of lipid-dependent activity remains.

Purified fatty acids can be used to duplicate the antimicrobial activity of milk lipids (14). Antimicrobial fatty acids and monoglycerides primarily fall into 2 groups, which are long-chain unsaturated fatty acids and medium-chain saturated fatty acids. The antimicrobial activity of each antimicrobial lipid, whether medium chain or long chain, is additive, such that lipid mixtures can be made in which the concentration of individual components is below an antimicrobial range, but the mixture is antimicrobial, because the total lipid concentration is in the antimicrobial range. Previous studies in our laboratory have shown that milk samples treated with lipase, which had FFAs concentrations of 2 g/L or below, did not inactivate enveloped viruses, whereas samples with ≥ 7 g/L FFAs were strongly antiviral (14). Enveloped viruses are effectively inactivated by both long-chain and medium-chain fatty acids, whereas gram-positive and gram-negative bacteria show varying susceptibilities to different chain lengths of antimicrobial fatty acids (22,23). For example, *Escherichia coli* is inactivated by mixtures of medium-chain fatty acids but is resistant to long-chain unsaturated fatty acids (24). However, it is possible that the presence of multiple different membrane destabilizing compounds, e.g., lipids and antimicrobial peptides, each at suboptimal levels may effectively inactivate pathogens. Therefore, there may be additive and synergistic effects not only between lipids but also between lipids and other protective factors.

Inactivation of herpes simplex virus by the synergistic activity of antimicrobial lipids and peptides

To examine the possibility of additive or synergistic interactions between antimicrobial compounds in human milk, we set up an in vitro system using an antimicrobial lipid ether [1-0-octyl-sn-glycerol (OG)] and a synthetic antimicrobial peptide (D2A21; Demegen). Studies in our laboratory have shown that OG inactivates herpes simplex virus (HSV) and chlamydia but that in the presence of interfering substances, e.g., serum, viral inactivation took at least 1 h and required lipid concentrations of 10 to 15 mmol/L (25). When D2A21 is used by itself at 30 and 60 $\mu\text{mol/L}$, it only inactivates HSV-2 titers by 25- and 200-fold, respectively, in 3 h. This study was designed not only to determine whether combining 2 antimicrobial compounds reduced the concentrations of each active compound required to inactivate pathogens but also to determine whether the time required for pathogen inactivation is reduced by combining antimicrobial lipids and peptides (26).

When OG was used alone, at a concentration of 3 mmol/L, it had a minimal effect against HSV-1 after 10 min but reduced the titer of HSV-1 by 100-fold in 20 min and by 1000-fold in 60 min. By itself, 9 $\mu\text{mol/L}$ D2A21 does not inactivate HSV-1 after 60 min. However, when 3 mmol/L OG is combined with 9 $\mu\text{mol/L}$ D2A21, the HSV titer is reduced by 1000–10,000-fold within 1 min and can be reduced below detectable levels within 10 min. These studies show that combining antiviral lipids and peptides has a synergistic effect not only on the concentrations of active lipid and peptide required for viral inactivation but also on the time required for HSV inactivation. By reducing the time required for pathogen inactivation, the possibility of establishing an initial infection is reduced. The results in this simplified system demonstrate that combining an antiviral lipid with an antiviral peptide, thus targeting the HSV envelope simultaneously by 2 separate mechanisms, synergistically produces antimicrobial activity that is greater than when each of the active compounds is used individually. It is likely that even greater additive and synergistic protective effects are produced when binding inhibitors and other protective factors in human milk simultaneously attack a pathogen at multiple points in its replication cycle.

What is the total antimicrobial activity present in human milk?

Immune factors provided to the suckling infant in the milk will also interact with those present in the gastrointestinal tract, providing an additional opportunity for additive and synergistic effects. This suggests that looking only at undigested milk may underestimate the total protective benefit provided by breast-feeding. As our studies with milk lipids have shown, antimicrobial activity is not present in milk but is released from milk triglycerides in the gastrointestinal tract. Studies with lactoferrin, which is a major milk protein, also show that some of its antiviral activity is due to peptides released by proteolysis (7).

Studies that examine the antimicrobial potential of human milk also may not always give a picture of its full protective activity, because the issue of co-infection is not addressed. It has been shown with sexually transmitted infections, e.g., HIV, that eliminating prior infection with another viral, bacterial, or protozoal pathogen reduces the incidence of HIV by 40% (27–29). One pathogen may disrupt mucosal barriers, suppress the immune system, or provide molecular cofactors,

thereby facilitating the spread of a second infectious agent that would not establish, or as frequently establish, an infection by itself. Human milk may indirectly reduce transmission of one pathogen by preventing another pathogen from establishing a successful infection. It should also be remembered that while laboratory-adapted strains of viruses and bacteria are commonly used to study the antimicrobial potential of human milk, clinical isolates often show a different susceptibility to antimicrobial agents than laboratory strains. Therefore, to obtain a more accurate picture of the protection from infection that the suckling neonate derives from milk, future studies should use, where possible, panels of clinical isolates of infectious agents.

In summary, our present picture of the mechanisms by which human milk protects the suckling infant likely underestimates its antimicrobial potential. This is illustrated by Svanborg and co-workers (30) in this symposium whose studies indicate that α -lactalbumin, in addition to its recognized role in lactose synthesis, also inactivates neoplastic cells. Future studies elucidating the protective mechanisms present in milk will have to explore the potential multifunctional roles of individual compounds, as well as the additive and synergistic interactions between protective factors in the milk and the suckling neonate's gastrointestinal tract.

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