In breast milk and paired serum from 70 lactating women and 40 of their term, infection-free neonates, on the 2nd and 5th day postpartum sICAM-1, sVCAM-1, sE- and sL-selectin were measured by ELISA and compared with those in 26 healthy adults (controls). Seven infant formulas and fresh milk from five cows were also analyzed. Human colostrum values of sICAM-1, sVCAM-1 (similar to those in maternal and control serum), sE-selectin and sL-selectin (~10 and ~100 times lower than in maternal and control serum) were significantly higher than those in milk, while they varied widely. None of the adhesion molecules was detected in fresh cow’s milk or infant formulas. Exclusively breast-fed infants showed significantly higher values of sICAM-1 and sL-selectin on the 2nd day of life than those supplemented also with formula. Only sICAM-1 values correlated positively between colostrum and time-matched maternal serum. These findings show in human milk important amounts of sICAM-1 and sVCAM-1 but minimal amounts of sE- and sL-selectin, which could affect the immune system of the neonate.

Key words: Adhesion molecules, Breast milk, Colostrum, Lactating women, Neonatal serum

Materials and methods

Subjects and sample collection

This study was approved by the Institutional Review Board for Human Research at our Teaching Hospital. Maternal informed consent was also obtained. All participants were non-allergic, healthy lactating women (n = 70) of mean age (range) 27.8 (19–40) years and had delivered vaginally (n = 53) or by elective cesarean section (n = 17) single, mature, healthy, appropriate for gestational age infants (29 females/41 males), after full term (38.8 weeks, 37.0–42.0), uncomplicated pregnancies. Thirty-seven of them were nulliparous and the remaining 33 multiparous.

A peripheral venous blood sample and a milk sample were collected on the 2nd and the 5th day postpartum. The milk samples were obtained by manual expression, just after venipuncture for the blood sample, one hour after last feeding in the morning. Furthermore, venous blood (~1 ml) from 40 of their 70 neonates was also obtained simultaneously with blood and milk sample collection from their mothers. Twelve neonates out of the 40 on the 2nd day of life were exclusively breast fed, whereas the remaining 28 were breast fed and supplemented with bovine milk-based infant formula. On the 5th day of life only 10 neonates were exclusively breast fed.

All neonates, lactating mothers and controls were infection free as judged by clinical examination and C-reactive protein (CRP) values (<3.5 mg/L), measured in the same blood samples as adhesion molecules.

Peripheral venous blood was also taken from 26 (15 females/11 males) healthy blood donors of mean age
28.3 years (22–38), who were neither allergic, nor taking any medication and without any signs of infection or inflammation for the last 3 months (controls).

Moreover, fresh milk samples from five cows and reconstituted, according to the manufacturing company instructions, samples of seven different powdered cow’s milk-based infant formulas, used in the Neonatal Unit of our Department (Similac, Abbott; Aptanyl, Milupa; Frisolac, Nounou; Almiron, Nutricia; Premia, Bebelac; S-26, Wyeth; and Nativa, Karamolegos) were also analysed.

Preparation of serum and milk samples
Peripheral venous blood samples were obtained in polypropylene pyrogen-free tubes and were kept at 5–8°C for clotting. Immediately after clotting, serum was separated by centrifugation in two steps in a refrigerated centrifuge at 2500 rpm for 10 min, aliquoted in five specimens and kept at −30°C until assayed.

Milk specimens were collected also in pyrogen-free tubes and they were refrigerated immediately at 2–5°C for two hours. Then they were centrifuged in two consecutive steps, 2000 rpm for 10 min and 2500 rpm for 15 min at 6°C, to remove the cells and lipid layer. The defatted and cell-free aqueous layer of colostrum and milk samples was aspirated through the needle of a sterilized syringe and was passed through a special filter with pore diameter 0.45 μm (Minisart, Sartorius, Goetingen, Germany), to remove any last membrane-associated molecules and lipid droplets. Then cell-free aqueous fractions were aliquoted in small pyrogen-free tubes, and kept at −30°C until assayed.

Adhesion molecule assays
Adhesion molecules sCAM-1, sVCAM-1, sE- and sL-selectin were measured by sandwich-type microELISAs, with commercially available reagents: Parameter Human soluble ICAM-1 Immunoassay, Parameter Human soluble VCAM-1 Immunoassay, Parameter Human soluble E-Selectin Immunoassay, and Parameter Human soluble L-Selectin Immunoassay (R&D Systems, Minneapolis, MN, USA). The typical sensitivities of the assays were 0.35 ng/ml for sCAM-1, 0.20 ng/ml for sVCAM-1, 0.106 ng/ml for sE-selectin and 0.30 ng/ml for sL-selectin. These correspond to a sample sensitivity equivalent to: 7.0 ng/ml for sCAM-1 in serum and milk samples; 10 ng/ml in serum and 5 ng/ml in milk samples for sVCAM-1; 2.12 ng/ml in serum and 1.06 ng/ml in milk samples for sE-selectin and 30 ng/ml in serum and 3.0 ng/ml in milk samples for sL-selectin, when the serum sample predilutions were respectively 1:20, 1:50, 1:20 and 1:100 and the milk sample predilutions were 1:20, 1:25, 1:10 and 1:10. The intra- and interassay coefficients of variation (CV%) of the assays used were respectively 3.90% and 6.40% for sCAM-1, 4.50% and 9.30% for sVCAM-1, 4.90% and 7.30% for sE-selectin and 3.75% and 10.35% for sL-selectin. The validity of the assays for quantifying sCAM-1, sVCAM-1, sE- and sL-selectin in the human milk was tested by the following recovery experiment. Five milk preparations (aqueous phase) were divided in two aliquots. Twenty-five, 50, 100, 200 and 400 ng/ml of recombinant human sCAM-1 and sVCAM-1, 3, 6, 12, 25 or 50 ng/ml sE-selectin and 5, 10, 20, 40, 80 ng/ml sL-selectin were added. Each of the four adhesion molecules was then quantified by the respective microELISA. In addition to the endogenous adhesion molecule measured in the preparation, over 95% of the exogenously added quantity of sCAM-1, sVCAM-1, sE- and sL-selectin were detected. A serum, colostrum and transitional milk sample from every lactating woman as well as age-matched serum samples of her neonate were analysed in the same run.

Statistical analysis
For the statistical analysis of the results, the statistical program Statgraphics, version 1.0 (Manugistics Inc, Rockville, MD, USA) was used. The frequency distribution of sCAM-1, sVCAM-1, sE- and sL-selectin values in neonatal, maternal and control serum was normal, while that in milk samples was not (Kolmogorov-Smyrnov test). For this reason, results in sera are given as mean values (±SD), whereas in milk as median values (upper and lower quartile). Data in serum were analysed using parametric methods (Student’s t-test or paired t-test, as appropriate, if natural pairing occurred in experimental series). Comparisons with and between milk samples were done using non-parametric methods: Wilcoxon test for unpaired measurements or paired Wilcoxon test (Wilcoxon signed-ranks test), as appropriate. Correlations of every adhesion molecule values between colostrum and milk samples or between them and the time-matched maternal or neonatal serum samples were examined by the non-parametric Spearman rank correlation coefficient (rS), while between serum samples by the parametric Pearson correlation coefficient (rP). The effect of the perinatal factors, mode of delivery, maternal age and parity or neonatal gender and birthweight on every adhesion molecule in breast milk, maternal or neonatal serum was examined by multiple regression analysis.

A probability of p < 0.05 was taken as statistically significant.

Results
Concentrations in box-plots of sCAM-1, sVCAM-1, sE- and sL-selectin on the 2nd and 5th day postpartum in
breast milk (median values, 25, 75 percentile and range) as well as in time-matched sera (mean ± SD, range) of lactating mothers and their neonates in comparison with the respective values in controls are depicted in Figs 1–4 respectively.

In colostrum (2nd day of life) and human transitional breast milk (5th day):

- Important amounts of sICAM-1 (520 ng/ml; 253, 892; 43–1600 and 206.5 ng/ml; 133, 300; 26–700, respectively) and sVCAM-1 (223 ng/ml; 158, 338; 48–1206, and 142 ng/ml; 111, 187; 60–732, respectively), while very low levels of sE-selectin (2.8 ng/ml: 1.9, 3.8; 0–11 and 1.2 ng/ml; 1, 2; 0–6, respectively) and sL-selectin (8.5 ng/ml; 6.8, 12.5;
0.3–2.1, and 5 ng/ml; 2, 7.5; 0–12.5, respectively) are found.

- A wide variation in concentrations of all four studied adhesion molecules is observed.

- Values of sCAM-1, sVCAM-1, sE- and sL-selectin are significantly higher on the 2nd than on the 5th day postpartum ($p<10^{-7}$, $p<2 \times 10^{-8}$, $p<1.5 \times 10^{-11}$, $p<2.5 \times 10^{-7}$, respectively).

- Colostrum values of sCAM-1 are significantly higher than those in time-matched maternal serum (256 ± 110 ng/ml, 79–570; $p<3 \times 10^{-7}$) and controls (243 ± 59 ng/ml, 128–349; $p<0.0003$), while human milk sCAM does not differ significantly from controls, but is significantly lower than that in time-matched maternal serum (287 ± 125 ng/ml, 86–756; $p<0.01$).

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**FIG. 3.** Values in box plots of soluble E-selectin (sE-selectin) on the 2nd and 5th day postpartum in human milk (●, median value; ξ, lower quartile; Γ, upper quartile; ↓, minimum; T, maximum), and in time-matched sera (●, mean value; ξ, −SD; Γ, +SD; ↓, minimum; T, maximum) of lactating mothers, and neonates in comparison with that in controls.

**FIG. 4.** Values in box plots of soluble L-selectin (sL-selectin) on the 2nd and 5th day postpartum in human milk (●, median value; ξ, lower quartile; Γ, upper quartile; ↓, minimum; T, maximum), and in time-matched sera (●, mean value; ξ, −SD; Γ, +SD; ↓, minimum; T, maximum) of lactating mothers, and neonates in comparison with that in controls.
Soluble adhesion molecules in human milk

- sVCAM-1 in both colostrum and transitional milk was significantly lower than that in controls (516 ± 99 ng/ml, 318–678; p<5×10⁻⁶) and time-matched maternal serum (596 ± 280 ng/ml, 263–1689; and 584 ± 242 ng/ml, 264–1657; respectively, p<2×10⁻¹¹).

- Colostrum and breast-milk sE-selectin is ~20 and ~40 times lower than that in controls (48 ± 13 ng/ml, 27–66) and ~10 and ~30 times lower than that in time-matched maternal serum (32 ± 13.3 ng/ml, 11–77 and 34 ± 14.8, 14–75, respectively).

- Colostrum and milk sL-selectin is ~100 times lower in comparison with that in controls (938 ± 181 ng/ml, 656–1266) and ~10 and ~30 times lower than that in expressed easily (362 ng/ml; 206, 460; 87–697) compared with those in samples expressed with difficulty (362 ng/ml; 124, 261; 26–573; p<0.02).

- In contrast with the presence of all four adhesion molecules in human milk, in the bovine milk-based formulas (n= 7) and in the fresh milk of five cows, none of the four adhesion molecules studied was detected.

In maternal serum:

- Values of sICAM-1 and sVCAM-1 on both the 2nd (256 ± 110 ng/ml and 596 ± 280 ng/ml, respectively) and the 5th day postpartum (287 ± 125 ng/ml and 584 ± 242 ng/ml respectively), showing no significant change from the 2nd to 5th day postpartum, do not differ significantly from the respective values in controls (243 ± 59 ng/ml and 516 ± 99 ng/ml).

- Values of sE- and sL-selectin on both the 2nd (256 ± 110 ng/ml and 596 ± 280 ng/ml, respectively) and the 5th day postpartum (34 ± 14.8 ng/ml and 668 ± 205 ng/ml, respectively) are significantly lower than the respective values in controls (48 ± 13 ng/ml, p<0.00002 and p<0.00005 and 938 ± 181 ng/ml, p<1×10⁻⁶ and p<1×10⁻⁶, respectively), showing a significant increase from the 2nd to the 5th day postpartum (p<0.01).

- A positive correlation is also noticed in sICAM-1 values between colostrum on the 2nd day postpartum and time-matched maternal serum samples (rₛ=0.306; p<0.02).

- Between maternal serum samples on the 2nd and 5th day postpartum values of sICAM-1, sVCAM-1, sE- or sL-selectin are correlated rather strongly (rₚ=0.607, rₚ=0.687, rₚ=0.856 or rₚ=0.507, respectively; p<0.00001).

- A negative correlation is noticed between sVCAM-1 and sE-selectin values in maternal serum samples on the 5th postpartum day (rₚ=−0.320, p<0.01).

- A positive correlation is observed in sE-selectin values between maternal and neonatal serum on the 5th postpartum day (rₚ=0.386, p<0.05).

In neonatal serum:

- sICAM-1 values on the 2nd day of life (178 ± 50 ng/ml, 106–401) are significantly lower than those in controls (243 ± 59 ng/ml, 128–349; p<0.00005), whereas on the 5th day of life they increase significantly (230 ± 72 ng/ml, 132–457; p<3×10⁻⁷), reaching control values.

- From the 2nd to the 5th day of life a significant rise was observed in sVCAM-1 values (p<0.005) but a significant fall in sE-selectin values (p<1×10⁻⁷).

- sL-selectin values on both the 2nd (674 ± 213 ng/ml) and the 5th day of life (684 ± 221 ng/ml) are lower than those in controls (938 ± 181 ng/ml; p<0.001 and p<0.003, respectively), showing no significant change from the 2nd to the 5th day of life.

The study of correlations of the four adhesion molecules in breast milk, maternal and neonatal age-matched serum samples revealed that:

- Between colostrum on the 2nd and transitional milk on the 5th day postpartum there is a positive correlation in sICAM or sL-selectin values (rₛ=0.316; p<0.02 or rₛ=0.430; p<0.00001 respectively) and a strong correlation in sVCAM-1 (rₛ=0.700; p<0.00001) or sE-selectin values (rₛ=0.862; p<0.00001).

- A positive correlation is also observed between sICAM-1 and sVCAM-1 values in human colostrum (rₛ=0.342; p<0.01) but in breast milk on the 5th day postpartum a negative correlation between sICAM-1 and sE-selectin values (rₛ=−0.270; p<0.03).

- A positive correlation is also noticed in sICAM-1 values between colostrum on the 2nd day postpartum and time-matched maternal serum samples (rₛ=0.306; p<0.02).

- Between maternal serum samples on the 2nd and 5th day postpartum values of sICAM-1, sVCAM-1, sE- or sL-selectin are correlated rather strongly (rₚ=0.607, rₚ=0.687, rₚ=0.856 or rₚ=0.507, respectively; p<0.00001).

- A negative correlation is noticed between sVCAM-1 and sE-selectin values in maternal serum samples on the 5th postpartum day (rₚ=−0.320, p<0.01).

- A positive correlation is observed in sE-selectin values between maternal and neonatal serum on the 5th postpartum day (rₚ=0.386, p<0.05).
Between neonatal serum samples on the 2nd and 5th day of life strong correlations were noticed in sICAM-1, sVCAM-1, sE- and sL-selectin values ($r_p=0.788$, $r_p=0.725$, $r_p=0.885$ and $r_p=0.813$, respectively; $p<0.00001$).

A positive correlation was also noticed between:
(a) sICAM-1 and sL-selectin values in neonatal serum samples on both the 2nd or the 5th day of life ($r_p=0.491; p<0.002$ or $r_p=0.476; p<0.008$ respectively) and (b) sVCAM-1 and sE-selectin or sL-selectin values in neonatal serum only on the 5th day of life ($r_p=0.359; p<0.045$ or $r_p=0.435; p<0.015$, respectively).

The study of the effect of several perinatal factors on the cell adhesion molecule values in breast milk, maternal serum and neonatal serum showed that:

- Values of sICAM-1, sVCAM-1, sE- and sL-selectin on either the 2nd or the 5th day postpartum are not dependent on the mode of delivery, maternal age or parity, neonatal gender or birthweight.

**Discussion**

In this study we measured the serum adhesion molecules sICAM-1, VCAM-1, sE- and sL-selectin in the defatted and acellular aqueous phase of humancolostrum and transitional milk during the first days of lactation. Our findings reveal that (a)colostrum and transitional milk contain important amounts of sICAM-1 and sVCAM-1 but minimal amounts of sE- and sL-selectin, (b) significantly higher concentrations of all four adhesion molecules are found in humancolostrum on the 2nd day compared with those in milk samples on the 5th day postpartum and (c) all four adhesion molecules studied present large variations in their concentrations in bothcolostrum and milk samples. Similar results have been reported for sICAM-1, sVCAM-1 and sE-selectin in a small number of humancolostrum ($n=10$) and milk ($n=13$) samples and for sICAM-1 only in a very recent report$^{13}$ and an abstract.$^{12}$ To the best of our knowledge sL-selectin in human milk that we detected in 90% of the humancolostrum samples and 80% of the transitional milk samples is reported here for the first time.

The significantly lower human milk content inadhesion molecules than that incolostrum led some investigators to suggest that sICAM-1, sVCAM-1 and sE-selectin were unlikely to contribute significantly in human milk’s anti-inflammatory effects.$^{13}$ We believe, however, that the important amounts of sICAM-1 and sVCAM-1 incolostrum from the 2nd day postpartum suggest that at least these compounds could affect the recipient infant, particularly in the first days of life. On the other hand, the significantly higher concentrations of all four adhesion molecules in humancolostrum (2nd day) compared with those in milk (5th day postpartum), as well as the higher concentrations of sICAM-1 incolostrum than incontrols and lactating mothers, seem to be in analogy with the protein content of humancolostrum and later breast milk, like albumin, protein hormones, and growth factors.$^{14,15}$

During the first 24 hr postpartum thecolostrum production is $\approx 120\text{ mL}$, whereas on the 5th day the total volume/day of transitional milk reaches $\approx 250-300\text{ mL}$. Consequently, the total amount of sICAM-1/day incolostrum should be $\approx 5000$ to $187500\text{ ng/day}$, considering that concentrations range from 40 to 1500 ng/ml, while on the 5th day postpartum the total amount in milk should be $\approx 6500-175000\text{ ng/day}$, considering that concentrations ranged from 26 to 700 ng/ml. Regarding even sL-selectin, the respective content ofcolostrum per day should be 850–1560 ng and that in milk 500–1875 ng/day, considering that the respective concentrations were 6.8–12 ng/ml and 2–7.5 ng/ml, respectively.

Hence, we consider that physiologically important amounts per day of all four adhesion molecules are taken by newborn infants through breast-feeding and could potentially act as immunomediators$^{8,10}$ orangiogenetic$^{17}$ and maturational agents. They might protect the newborn against infections of respiratory and intestinal tract and suppress certain diseases such as atopy, coeliac disease$^{18}$ and inflammatory diseases of the neonatal gut during the first days of life, when there is increased risk of overshooting immune reactions, leading maybe to chronic inflammation, as proposed for certain cytokines and their soluble receptors.$^{5,9,13}$ They could also promote the growth, differentiation and maturation of very important neonatal tissues like gut and lung, and induce developmental and maturational processes of the immune system in neonates, similarly to milk hormones, cytokines and growth factors.$^{3,6,7,19}$

sICAM-1 and sVCAM-1, found in human milk in considerable amounts, do not differ significantly inmaternal serum from those in controls. In contrast, sE- and sL-selectin, which show very low concentrations in human milk, present inmaternal serum significantly lower concentrations than those in controls. Matthiesen et al. have suggested that a state of systemic suppression of the maternal immune system seems to be present during pregnancy and the early postnatal period.$^{20}$

Values of soluble cell adhesion molecules in human milk were compared and correlated with those in time-matched maternal serum in order to get an indication of the origin of these biomolecules in human milk. However, for sICAM-1 values only, a significant positive trend betweenocolostrum and time-matched maternal serum was noticed, similarly to a previous study.$^{13}$ Thus, it appears that only sICAM-1 could be derived at least partially directly from maternal circulation. Possible sources of the
studied adhesion molecules in human milk could be cellular components such as leukocytes, macrophages, some T cells, vascular or epithelial cells found abundantly especially in early milk. This latter observation is probably an explanation of the higher values in colostrum than in transitional milk. They could also derive from the cellular activity of the mammary gland itself, where components such as IgA, complex proteins and carbohydrates are synthesized. Further studies are needed to explore this hypothesis.

Our finding of a significant positive correlation between sICAM-1 and sVCAM-1 in colostrum is not in agreement with the results of a previous report,11 this may be due to the very small number of the colostrum samples they analysed.

The low human milk content of selectins might be attributed to the transient expression of E-selectin, which peaks and begins to decline before the appearance of CD35+ cells.10

In contrast with our results showing that all four adhesion molecules were found in human milk in quantities similar to (sICAM-1 and sVCAM-1), or less than (sE-selectin, sL-selectin) those in control serum, none of the four adhesion molecules could be detected in the reconstituted seven different bovine milk-based formulas, or in the fresh milk samples of five cows, treated with exactly the same protocol as human milk samples. It is possible that cow’s milk contains adhesion molecules with different epitopes than those in human breast milk, and consequently they cannot be detected with the microELISAs used, with antibodies specific against recombinant human adhesion molecules. For this reason, it is apparent that the beneficial effects of breast-feeding to the recipient child are lacking, when the newborn infants are formula-fed.

Similarly to previous reports21–26 the results of the present study on sICAM-1, sVCAM-1 and sE- and sL-selectin in the serum of healthy term neonates show the following. First, shedding of sICAM-1, sVCAM-1, sE- and sL-selectin is an established component of the immune system of the newborn infant from birth. Second, the increase of postnatal age has a positive effect on the neonatal serum sICAM-1 and sVCAM-1, suggesting an expansion of the neonatal immune system and response. Breast-feeding seems to be beneficial to this expansion and maturation of the immune system considering the elevated concentrations in human milk of the latter two adhesion molecules. Third, the highly elevated neonatal sVCAM-1 and sE-selectin values, as also reported previously,24–26 might be attributed to their strong angiogenic function,17 considering that in the pre-natal period intense angiogenesis is noticed. Finally, in the early neonatal period, s-selectin values were significantly lower then in healthy adults, due possibly to the well-known functional immaturity of neonatal leucocytes, characterized by diminished expression of L-selectin and impaired shedding of sL-selectin.27,28

The finding in the present study of significantly higher sICAM-1 and sL-selectin values in neonates exclusively breast-fed than in neonates supplemented also with cow’s milk formula, suggests that breast-feeding, at least in the very early neonatal period, offers the recipient newborn infant what it is lacking, since these two molecules show lower neonatal values than those in healthy controls.

In conclusion, the findings of this study reconfirm the presence of sICAM-1, sVCAM-1 and sE-selectin, reveal that of sL-selectin in human milk in the early lactation period and indicate that all four adhesion molecules are physiologically important constituents of human breast milk in the very early postpartum period. This suggests potential immunomodulatory and anti-inflammatory effects of breast-feeding on the recipient newborn infant, as well as the promotion of maturation, development and expansion of the neonatal immune systems. Only sICAM-1 in human milk seems to derive, at least partially, from the maternal circulation, while for the origin of the remaining three adhesion molecules only putative suggestions may be proposed.

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