Evidence increasingly indicates that breast milk protects against gastrointestinal, respiratory tract, middle ear infections, asthma, and sudden infant death syndrome (SIDS) (Klonoff-Cohen et al. 1995; Kovar et al. 1984; Oddy et al. 2003). Several components of human milk have been postulated to confer this protective effect (Goldman et al. 1998). Breastfeeding might provide an immediate line of defense against infectious agents, which compensates directly for the immaturity of the newborn's immune system and lowered ability to resist infection. Protection might also be achieved through specific and nonspecific factors in milk, including bioactive enzymes, hormones, growth factors, and immunologic agents that augment and stimulate the development of immature host defense. Thus, it is not clear which of the many components of this complex, changing biologic fluid account for the protective effect.

Passive smoking in the same room as the infant increases the risk for respiratory disease and SIDS (Gordon et al. 2002), whereas breast-feeding is protective for SIDS among nonsmokers, but not smokers, when adjusted for potential confounders (Klonoff-Cohen et al. 1995), raising the possibility that there are also immunologic changes in the breast milk. However, a clear negative effect of nursing by a smoker mother has not been demonstrated (Mitchell et al. 1993), and very little is known of the mechanisms by which smoke might account for the reduced protective effect of breast milk (Blizzard et al. 2003; Elliot et al. 2003).

Cigarette smoke is composed of > 5,000 chemicals, including approximately 70 carcinogens (Stedman 1968), very few of which have actually been assayed for immunosuppressive activity (Ouyang et al. 2000). Conflicting reports exist regarding the effects of nicotine on cytokine production, and little is known about the nature of the immunosuppressive compounds in cigarette smoke (Ouyang et al. 2000). Nevertheless, the major phenolic components of cigarette tar, hydroquinone and catechol, have been reported to suppress the production of interleukin-1β, IL-2, and interferon-γ (Kalf et al. 1996; Ouyang et al. 2000). Breast-feeding substantially increases absorption of nicotine compared with only environmental tobacco smoke when the mother smokes (Dahlström et al. 1990; Luck and Nau 1985; Schultz-Hobein et al. 1992).

Smokers, but not IL-1α, is frequently
Our objective was to determine whether there is a relationship between IL-1-α levels in milk and smoking habits in nursing mothers, with the consequent implications on the development of the immune system. Other factors in milk considered for association with infection risk in infancy were the immunomodulators β-endorphin and leptin. β-Endorphin was assessed because it is produced by the epithelial cells (i.e., the primary cells in human milk) (Khachatryan et al. 1958), it exists at detectable levels in human milk (Zanardo et al. 2001), and it influences the behavior of other cells and tissues. Leptin, induced by lipopolysaccharide (LPS) and cytokines, participates in the host response to inflammation by modulating the host immune and cytokine responses after LPS (Faggioni et al. 1999). Thus, any effects of a given cytokine could be tested for specificity by comparison with the other immunologically active components.

**Materials and Methods**

Longitudinal and cross-sectional human milk samples were obtained from mothers hospitalized in regional tertiary maternity care at Padua University (Italy), from June to December 2002. Human milk was collected by mothers via commercial breast pumps, frozen immediately, and stored. Collection date and time were recorded, as were infants’ birth date, gestational age, birth weight, and sex and mothers’ demographic and anthropometrical characteristics [age, education, parity, body mass index (BMI)], diagnoses, pregnancy complications, and route of delivery (cesarean section vs. vaginal).

Before the infant’s birth, pregnant women were asked by experienced research midwives to participate in a prospective study. The women received verbal and written information about the aim and design of the study. Only healthy women ≥ 18 years of age, not taking anti-inflammatory medications at the time of enrollment, with otherwise uncomplicated gestation, delivery, and puerperium and who planned to obtain care for their newborns through rooming-in and exclusively breast-feeding their newborns were eligible. Allergy in mothers was not an exclusion criterion because of the findings that maternal atopy shows no relationship with cytokine level in milk (Bottcher et al. 2000). After the women accepted participation, their smoking history was recorded.

Of the 1,217 eligible participants, 25 of 26 self-identified as smokers (≥ 5 cigarettes per day through pregnancy until last trimester) were recruited for study participation. One was excluded from the final analysis because of maternal fever. Control participants included consecutive women without history of smoking and matched a smoking participant on the basis of overall inclusion criteria. A written informed consent was obtained from all participants.

**Milk specimen collection and processing.** Breast milk samples were collected during the third postpartum day (before discharge) and on the 10th postpartum day, close to expression of the colostrum and transitional milk production phases. Collection was standardized to reduce bias and potential diurnal variability in cytokine measurements. Specimens were obtained within 1 h of the first feeding in the morning, defined as 0800 hr to 0900 hr. Mothers were asked not to feed for 3 h before collection. All mothers were able to provide milk at each sampling point. An aliquot of 3 mL was taken using a manual breast pump. Samples were temporarily stored at –70°C in sterile plastic tubes and used for assays.

Samples were thawed and centrifuged for 10 min at 1800 rpm at 4°C, after which the lipid layer and cellular elements were removed. The aqueous fraction was filtered (0.45-µm Acrodisc; Gelman Sciences, Ann Arbor, MI, USA) and was used for cytokine, β-endorphin, and leptin determinations, with the lipid layer frozen for other studies.

**Milk IL-1-α, β-endorphin, and leptin assays.** Filtered aqueous milk fractions were assayed for IL-1-α (by CytElisa Human IL-1α ACCUCYTE; CytImmune Sciences Inc., Rockville, MD, USA), β-endorphin (beta-Endorphin 60 T kit RIA; Nichols Institute Diagnostic, San Juan Capistrano, CA, USA), and leptin (ACTIVE Human Leptin IRMA, DSL-23100; Diagnostic Systems Laboratories Inc., Webster, TX, USA) levels. All fractions were assayed undiluted. The coefficients of variation for intraassay and interassay, respectively, are the following: IL-1-α, 8.3 and 11.2%; β-endorphin, 4.1 and 7.1%; and leptin, 3.7 and 6.6%.

**Statistical analysis.** Unless otherwise specified, results are expressed as mean concentrations in nanograms per liter or micrograms per liter ± SE. The p = 0.05 significance level was used for the statistical analysis. We grouped human milk samples into third postpartum day smoker and nonsmoker mother groups, with 42 and 40 samples per group, respectively, and into 10th postpartum day smoker and nonsmoker mother groups, with 42 and 40 samples per group, respectively.

Because the concentrations of cytokine IL-1-α, β-endorphin, and leptin were not normally distributed, we performed a paired analysis with the Mann-Whitney U-test. We used the Student t-test for the analysis of the other data.

**Ethics.** The research protocol was approved by the Hospital Ethical Committee for Human Research of the University of Padua.

**Results**

We analyzed 164 milk samples, collected from 82 mothers, 42 smokers, and 40 nonsmokers. Measurable immunoreactivity levels were observed in most colostral (third day) and transitional (10th day) milk samples throughout the collection period (≥ 90%). The smoker mothers of healthy-term infants and control nonsmoker mothers of healthy-term infants who planned to breast-feed their infants were comparable in their anthropometric characteristics and those of the respective neonates. The birth weight of the newborn infants of smoker mothers was not significantly lower (Table 1).

IL-1-α concentrations were significantly reduced in the colostrum of smoker compared with the nonsmoker control mothers. β-Endorphin and leptin colostrum concentrations were comparable (Table 2).

Moreover, β-endorphin concentrations were significantly reduced in transitional milk samples compared with colostrum of both smoker and nonsmoker mothers. Also, leptin concentrations were significantly reduced in transitional milk samples compared with colostrum of both smoker and nonsmoker group. IL-1-α concentrations were lower in transitional milk than in colostrum samples of smoker and nonsmoker mothers, but without any significance (Table 2).

We found no significant differences between smoker and nonsmoker lactating mothers in transitional milk concentrations of IL-1-α, β-endorphin, and leptin (Table 2). And we found no correlations between reduced IL-1-α levels of the colostral milk samples and related immunomodulator β-endorphin and leptin levels.

**Discussion**

In this article, we present novel evidence suggesting that exposure to tobacco smoke during pregnancy may affect the development of the immunologic function of the mammary gland, significantly influencing colostral milk provision of the proinflammatory cytokine IL-1-α for breast-feeding infants. By contrast, the concentration of the immunomodulatory factors β-endorphin and leptin were unaffected. In this context, the possibility that delivery modalities, parity, or BMI affects the levels of cytokines and immunomediators in the breast milk is open to speculation.

The identification of reduced IL-1-α levels in the colostral milk of mothers exposed to tobacco smoke during pregnancy offers support to the hypothesis that some clinical consequences of smoking on infancy might be initiated and enhanced by altered levels of inflammatory cytokines (Froen et al. 2000; Sayers and Drucker 1999).

Early breast milk may be of particular importance in the development of the mammalian newborn innate and acquired immunity (Goldman et al. 1998). Colostrum has been reported to contain a large amount of...
cytokines, but little is known as to their concentrations and associations within breast milk (Laiho et al. 2003). IL-1 is probably the first cytokine that has been quantified in human milk (Garfalo and Goldman 1998; Munoz et al. 1990). The synthesis, processing, secretion, and activity of IL-1 are tightly regulated events. For instance, the presence of an IL-1 receptor antagonist is an important mechanism through which IL-1 signaling is down-regulated, and therefore its activity is reduced (Dinarello 1997). Of interest, IL-1β receptor antagonists have been measured in human breast milk (Buescher and Malinowska 1996). To what extent the biologic effect of the cytokine in milk is modulated by the presence of soluble receptors and other cytokine antagonist is open to speculation.

Smoking is a major risk factor for both infantile infections and SIDS. Such infections, both viral and bacterial, also increase the SIDS risk (Gordon et al. 2002; Guntheroth 1989). In experimental animals, nicotine has been shown to depress both the primary and secondary immune response of the lungs, lymph nodes, and spleen (Sayers and Drucker 1999). Interestingly, most studies have shown a decrease in proinflammatory cytokine product (IL-1α, IL-6, and tumor necrosis factor-α) by lung macrophages in smokers, accounting for the greater concentration of tobacco in the lungs (Froen et al. 2000; Sayers and Drucker 1999). These cells have proven very sensitive to both smoking history and the duration of the smoke-free period (Zeidel et al. 2002). Similarly, the greater concentration of tobacco components reported in the breast milk of smoker mothers could imply a different magnitude of immune impairment of the mammary gland macrophages (Klonoff-Cohen et al. 1995). The possibility that altered levels of cytokines and other biologic factors in breast milk modify the immune function in the nursed infants is a very attractive hypothesis (Jones and Warner 2000). Physiologic delays or imbalance in the production of immune factors can occur in mammals, and such delays increase the risk of infection and SIDS (Laiho et al. 2003).

Human milk is an important source of other bioactive substances, including hormones, growth factors, and immunologic factors such as cytokines, but the functional consequences of an overexpression or of a down-regulation of the most milk immunomodulatory constituents in neonates are unknown. In our study, the levels of β-endorphin and leptin in breast milk were not related to smoking habits of nursing mothers. The lack of additive effects and the dissimilarity in response to tobacco smoke might indicate that these immunomodulatory molecules are involved in different pathways. In this context, it is interesting that nicotine can cause a release of β-endorphin in various brain regions, and that cytokines and endotoxin can cause a release of β-endorphin in different tissues. Moreover, in mice, leptin, induced by LPS and cytokines, participates in the host response to inflammation by modulating the host immune and cytokine responses (Faggioni et al. 1999).

There are certain limitations in our work. First, only colostral and transitional milk samples were collected. It is well established that milk composition differs with delivery modalities, increasing parturition, throughout the day, at each feeding, and with time. Generally, milk proteins that are produced and secreted in the mammary gland are expected to constitute the largest proportion of protein in human milk and to have more postpartum variation than do serum proteins that are passively transferred into milk (Londerald and Atkinson 1995). Having multiple cytokine and immunomediators measures taken over time from breast-feeding mothers with a dose-response relationship between smoking levels and levels of agents would permit verification of the assumption underlying this analysis. In addition, the selection of IL-1, of leptin, and of β-endorphin, among the wide range of immunomodulatory factors present in breast milk and interconnecting the neuroendocrine and immune systems, has been made in the context of the present study mainly for convenience and not according to solid scientific reasons.

However, the study has several strengths. Our measure of cytokines incolostrum has been used in previous studies and is valid. Furthermore, most of the colostral and transitional milk samples (> 90%) in the study contained IL-1α, so this is a large enough group to be confident of the results and of measurable presence of IL-1α in the early milk. Nevertheless, it is premature to know how the findings will pertain to later infections, because most children of smoking mothers do not develop infections in the early perinatal time.

### Conclusion

Our data support the suggestion that some clinical consequences of smoking might be initiated and enhanced by the production of inflammatory cytokines from colostrum. Reduced colostral IL-1α concentrations provide additional data to the delineation of the pathophysiologic mechanism that includes mammary gland immunologic dysfunction in the cascade of events that can lead to infections and SIDS. To our knowledge, this is the first study on the effects of smoking on the content of cytokines and immunomediators in human milk. Prospective studies are required to thoroughly assess the differences in the composition of breast milk from mothers with different smoking histories and the immunologic consequences of this for the offspring.

### Table 1. Demographics, pregnancy, and birth outcomes characteristics of the study groups.

| Characteristic | Smokers (n = 42) | Nonsmokers (n = 40) |
|---------------|-----------------|---------------------|
| Maternal data |                 |                     |
| Maternal age (years) | 31.1 ± 0.9 | 31.2 ± 1.1 |
| Maternal smoking (cigarettes/day) | 3.2 ± 0.7 | 3.7 ± 1.2 |
| Maternal education (years) | 10.5 ± 2 | 10.6 ± 3 |
| Parity (primiparous (%)) | 35.1 | 41.4 |
| BMI (kg/m²) | 25.3 ± 4.0 | 25.7 ± 4.2 |
| Vaginosis (%) | 2.3 | 2 |
| Labor induction (%) | 7.1 | 7.2 |
| Duration of labor (hr) | 8.9 ± 4.2 | 8.6 ± 4.3 |
| Mode of delivery [vaginal (%)] | 88 | 92 |
| Birth data |                 |                     |
| Gestational age (weeks) | 39.0 ± 0.3 | 40.0 ± 0.3 |
| Birth weight (kg) | 3.23 ± 0.14 | 3.48 ± 0.09 |
| Sex (%male) | 44 | 42 |
| Apgar score |                 |                     |
| 1st min | 8.8 ± 0.4 | 9.2 ± 0.2 |
| 5th min | 9.5 ± 0.1 | 9.8 ± 0.1 |

Data are presented as mean ± SD unless otherwise indicated.

### Table 2. IL-1α, β-endorphin, and leptin concentrations in the colostrum and transitional milk of smoker compared with nonsmoker mothers.

| Cytokine | Smoker | Nonsmoker | p-Value |
|----------|--------|-----------|---------|
| IL-1α (ng/L) | 17.2 ± 4.0 | 38.4 ± 7.4 | a vs. b* |
| β-Endorphin (ng/L) | 353.5 ± 29.1 | 317.5 ± 27.6 | 152.8 ± 26.5 | 127.7 ± 2.3 | a vs. c** | b vs. d** |
| Leptin (μg/L) | 1.6 ± 0.3 | 1.5 ± 0.2 | 0.7 ± 0.1 | 0.5 ± 0.1 | a vs. c* | b vs. d* |

Data are presented as mean ± SE, or p-value from Mann-Whitney U-test.

*p < 0.05; **p < 0.01.
The name of co-author Angelo Barbato was added after publication online.

**REFERENCES**

Bentzen K. 1994. Cytokines and natural regulators of cytokines. Immunol Lett 43:111–123.

Blizzard L, Panzony AL, Dwyer T, Venn A, Cochrane JA. 2003. Parental smoking and infant respiratory infection: how important is not smoking in the same room with the baby? Am J Public Health 93:482–488.

Bottcher MF, Jenmalm MC, Garofalo RP, Biokstén B. 2000. Cytokines in breast milk from allergic and non-allergic mothers. Pediatr Res 47:157–162.

Buescher ES, Malinowska I. 1996. Soluble receptors and cytokine antagonists in human milk. Pediatr Res 40:839–844.

Dahlström A, Lundell B, Curvall M, Thapper L. 1999. Nicotine and cotinine concentrations in the nursing mother and her infant. Acta Paediatr Scand 79:142–147.

Dinarello CA. 1997. Interleukin-1. Cytokine Growth Factor Rev 8:235–365.

Dinarello CA. 1998. Interleukin-1 receptors and interleukin-1 receptor antagonist. Int Rev Immunol 16:407–499.

Elliot JC, Carroll NG, James AL, Robinson PJ. 2003. Airway alveolar attachment points and exposure to cigarette smoking in utero. Am J Resp Crit Care Med 167:45–49.

Faggioni R, Fantuzzi G, Gabay C, Moser A, Dinarello CA, Feingold KR, et al. 1999. Leptin deficiency enhances sensitivity to endotoxin-induced lethality. Am J Physiol 276:36–42.

Groen JJ, Akre H, Stray-Pedersen B, Saugstad OD. 2000. Evolution of immunologic functions of the mammary gland and the postnatal development of immunity. Pediatr Res 43:155–162.

Goldman AS, Chheda S, Garofalo R. 1998. Cytokines in human milk: properties and potential effects upon the mammary gland and the neonate. J Mammary Gland Biol Neoplasia 3:251–258.

Gordon AE, El Alhmr OR, Chan R, Al Madani OM, Braun JM, Weir DM, et al. 2002. Why is smoking a risk factor for sudden infant death syndrome? Child Care Health Dev 28:23–25.

Guntheroth WG. 1989. Interleukin-1 as intermediary causing prolonged sleep apnea and SIDS during respiratory infections. Med Hypotheses 28:121–123.

Jones CA, Wanner JO. 2000. Breast milk as an alternative source of cytokines for offspring. Clin Exp Allergy 30:599–601.

Kall GF, Renz JF, Niculescu R. 1996. β-Benzoinquinone, a reactive metabolite of benzene, prevents the processing of pre-interleukins-1α and -1β to active cytokines by inhibition of the processing enzymes, calpain, and interleukin-1β converting enzyme. Environ Health Perspect 104:1251–1256.

Khachaturian H, Lewis ME, Tsou K. 1958. β-Endorphin, β-MSH, and -1α-MSH, and related peptides. In: Proteins and Hormones (Björklund A, Hokfelt T, eds). New York:Elsevier Science, 216–272.

Klontz-Hoven HS, Edelstein SL, Lefkowitz ES, Srinivasan AP, Kaege D, Chang JC, et al. 1995. The effect of passive smoking and tobacco exposure on breast milk upon sudden infant death syndrome. JAMA 273:795–798.

Kovar MG, Serdula MK, Marks JS, Fraser DW. 1984. Review of the epidemiologic evidence for an association between infant feeding and infant health. Pediatrics 74:615–638.

Laio K, Lampi AM, Hamalainen M, Moilanen E, Pironen V, Arvola T, et al. 2002. Breast milk fatty acid, eicosanoids and cytokines in mothers with and without allergic disease. Pediatr Res 53(4):642–647.

Londerdal B, Atkinson S. 1995. Human milk proteins. In: Handbook of Milk Composition (Jensen R, ed). San Diego:Academic Press, 351–368.

Luck W, Nau H. 1985. Nicotine and cotinine concentrations in serum and urine of infants exposed via passive smoking or milk from smoking mothers. J Pediatr 107:816–820.

Mitchell EA, Ford RP, Stewart AW, Bercroit DM, Taylor BJ, Thompson JM, et al. 1993. Smoking and the SIDS. Pediatrics 91:893–896.

Munoz C, Enders S, Van Der Meer J, Schlesinger L, Arevalo M, Dinarello C. 1990. Interleukin-1β in human colostrum. Res Immunol 141:501–513.

Oddy WH, Halonen M, Martinez FD, Lohman IC, Stern DA, Kurzisz-Spencer M, et al. 2003. TGF-β in human milk is associated with wheeze in infancy. J Allergy Clin Immunol 112:723–728.

Ouyang Y, Virasch N, Hao P, Aubrey MT, Mukerjee N, Bierer BE, et al. 2000. Suppression of human IL-1β, IL-2, IFN-γ, and TNF-α production by cigarette smoke extracts. J Allergy Clin Immunol 106:280–287.

Sayers NM, Drucker DB. 1999. Animal models used to test the interactions between infectious agents and products of cigarette smoke implicated in sudden infant death syndrome. FEMS Immunol Med Microbiol 25:115–123.

Schultz-Hobein B, Schwartz-Bickenbach D, Abt S, Plum C, Nau H. 1992 Cigarette smoke exposure and development of infants throughout the first year of life: influence of passive smoking and nursing on cotinine levels in breast milk and infant’s urine. Acta Paediatr 81:550–557.

Sopori ML, Kozak W, Savage SM, Geng Y, Soszynski D, Kluger MJ, et al. 1998. Effect of nicotine on the immune system: possible regulation of immune response by central and peripheral mechanism. Psychoendocrinology 23:189–204.

Stedman RL. 1968. The chemical composition of tobacco and tobacco smoke. Chem Rev 68:153–207.

Zanardo V, Nicolussi S, Giacomini C, Faggian D, Favaro F, Piebani M. 2001. Labor pain effects on colostral milk β-endorphin concentration of lactating mothers. Biol Neonate 79:87–90.

Zeidel A, Belin B, Yardeni I, Mayburd E, Smirnov G, Bessler H. 2002. Immune response in asymptomatic smokers. Acta Anaesthesiol Scand 46:959–964.