GLIADIN STIMULATES HUMAN MONOCYTES TO PRODUCTION OF IL-8 AND TNF-α THROUGH A MECHANISM INVOLVING NF-κB

FEBS 28610 FEBS Letters 571 (2004) 81–85

Gliadin stimulates human monocytes to production of IL-8 and TNF-α through a mechanism involving NF-κB

Lenka Jelínková[a,*], Ludmila Tučková[a], Jana Cinová[a], Zuzana Flegelová[b], Helena Tlaskalová-Hogenová[a]

[a] *Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague, Czech Republic*
[b] *Research Institute of Biopharmacy and Veterinary Drugs, Jílové, Czech Republic*

Received 17 February 2004; revised 1 April 2004; accepted 15 June 2004

Available online 4 July 2004

Edited by Beat Imhof

Abstract Wheat gliadin is the triggering agent in coeliac disease. In this study, we documented that proteolytic fragments of gliadin, in contrast to other food antigens, induced interleukin (IL)-8 and tumour necrosis factor-α (TNF-α) production and significantly increased interferon (IFN)-γ-induced cytokine secretion in human monocytic line THP-1 cells. Stimulation with gliadin resulted in elevated phosphorylation of the IκBα molecule and increased NF-κB/DNA binding activity that was inhibited by sulfasalazine, L-1-tosylamido-2-phenylethyl chloromethyl ketone and pyrrolidine dithiocarbamate (PDTC). The activation pathway was shown to be independent of the CD14 molecule. Less mature U-937 monocytes responded to gliadin stimulation by low IL-8 secretion, TNF-α production was not detectable. We propose that gliadin-induced activation of monocytes/macrophages can participate in mechanisms leading to the impairment of intestinal mucosa in coeliac patients.

Keywords: Monocyte; Gliadin; Interleukin-8; Tumour necrosis factor-α; NF-κB; Innate immunity

1. Introduction

Coeliac disease, a chronic inflammation of small intestine, develops in genetically susceptible individuals because of intolerance to wheat gluten and related prolamins. Gluten, a complex mixture of gliadins and glutenins, is usually digested in the gastrointestinal tract by various enzymes of human and bacterial origin, mainly by pepsin in stomach.

Gliadin-peptide-specific CD4+ α/β T lymphocytes present in jejunal mucosa seem to be central in the immunopathology of the disease. After challenge, they produce increased levels of inflammatory cytokines of T-helper 1 profile (mainly interferon-γ, IFN-γ) and other mediators that can activate other cell types including cells of innate immunity. Macrophages and dendritic cells in intestinal mucosa play a role as antigen presenting cells and could be activated by various stimuli to the production of inflammatory cytokines (such as tumour necrosis factor-α (TNF-α), interleukin (IL)-1 and IL-6), chemokines (IL-8, monocyte chemotactic protein-1 [MCP-1]) and reactive oxygen and nitrogen intermediates [1–4].

Although the vast majority of bacterial and food components do not elicit intestinal inflammation, it is becoming increasingly recognised that pathogens that cause acute inflammation do activate the NF-κB pathway, resulting in a regulation of genes encoding proinflammatory cytokines, chemokines and adhesion molecules. The NF-κB family of proteins consists of homo- and heterodimeric subunits of the Rel family, including p50 and p65. The activity of NF-κB is regulated by a family of IκB inhibitor proteins (IκB α, β, γ and ε), which sequester NF-κB in the cytoplasm. Upon stimulation, IκB is phosphorylated, ubiquitinated, and subsequently degraded by the proteasome complex. Degradation of IκB allows NF-κB to translocate to the nucleus, bind to its specific promoter elements and activate gene transcription [5].

In recent years, the mechanism of activation of monocytes, intestinal epithelial cells or macrophages and the involvement of upregulated membrane expression and secretion of molecules such as HLA-DR, CD95/Fas, intercellular cell adhesion molecule-1 (ICAM-1), IL-15 or reactive nitric oxide (NO) radicals in coeliac disease immunopathogeny became a topic of intensive studies [6–10].

We have shown that wheat gliadin and its peptic fragments have the unique ability, in contrast to other food proteins, to activate the mouse peritoneal macrophages to the production of TNF-α, IL-10, RANTES and an inducible form of NO synthase. Gliadin fragments active in these processes were isolated and identified [3,4].

The aim of the present study was to investigate whether food proteins such as gliadin, soya protein or ovalbumin can also activate human monocytic cell lines (THP-1 and U-937 differing in surface marker expression) to cytokine and chemokine production and whether this activity could be modulated by IFN-γ, the main cytokine in coeliac disease. We also analysed the potential role of CD 14 receptor and NF-κB family members in the activation pathway.

2. Materials and methods

2.1. Food proteins

Ovalbumin and soya proteins (Sigma, St. Louis, MO) were diluted to 1 mg/ml concentration in incomplete RPMI-1640 medium (Endotoxin tested, Sigma), centrifuged (5000×*g*, 10 min) and supernatants were stored at −20 °C. The stock solution of crude gliadin 5.0 mg/ml in

* Corresponding author. Fax: +420-241-721-143.
E-mail address: lenicka@biomed.cas.cz (L. Jelínková).

0014-5793/$22.00
doi:10.1016/j.febslet.2004.06.057

50 mM HCl was prediluted in RPMI-1640 medium, centrifuged (10 000×*g*, 10 min), the sediment was dissolved in HCl solution, the concentration of soluble molecules was measured and the final dilution calculated.

Peptic fragments of proteins were prepared using the pepsin–agarose gel (ICN, Biomedicals, OH, USA). 7 ml of protein (10 mg/ml) in 0.1 M HCl, pH 1.8, was incubated with 5 ml of pepsin–agarose gel (45 min, 37 °C). Removing the gel by centrifugation (1500×*g*, 10 min) stopped enzymatic cleavage. The supernatants were then centrifuged (12 000×*g*, 10 min) and soluble protein fragments divided into aliquots and frozen at −20 °C.

The gliadin 33-amino acid (AA) peptide [11], gliadin peptide p31-43 [6], and B peptide [4] were synthesised using the Fmoc/tBu protection strategy on aminoethyl copoly (styrene-1% divinylbenzene) resin with Knorr linker. After cleavage from the resin, the peptides were purified using high-performance liquid chromatography and characterised by AA analysis and liquid chromatography/mass spectrometry (System Waters 2690 Separation Module and Waters 2487 Dual λ Absorbance Detector, connected to a Micromass Platform L.C.).

Potential presence of lipopolysaccharide (LPS) was tested using the E-toxate test (Sigma). The LPS level in all reagents used in the study was below the detection limit.

2.2. Cell lines and their activation

THP-1 and U-937 cells (GCMCC, Braunschweig, Germany) were cultured as described earlier [12]. Cells in concentration of 1×10^6/ml were exposed to gliadin or its peptic fragments (10–500 μg/ml) alone or together with human IFN-γ (150 U/ml, R&D System, Minneapolis) or to LPS (*Salmonella typhimurium*, 1–10 μg/ml, Sigma) for 24 h. THP-1 cells were also incubated with soya protein, ovalbumin or their peptic fragments (100–500 μg/ml) or synthetic gliadin peptides [33 AA gliadin peptide, B peptide and p31-43 peptide (200 μg/ml)] alone and/or with IFN-γ (150 U/ml). Alternatively, the cells were preincubated with IFN-γ for 2 or 24 h, washed twice with PBS and stimulated for additional 24 h with gliadin digest alone or together with IFN-γ. In some experiments, THP-1 cells were preincubated for 1 h at 37 °C with mouse anti-CD14 monoclonal antibody (mAb) MEM-18 or with isotype control IN-O5 mouse anti-insulin mAb (both mAb provided by Prof. V. Hořejší); the gliadin fragments or LPS were then added to cultured cells in the presence of anti-CD14 mAb or IN-O5 mAb for 24 h. Furthermore, NF-κB inhibitors sulfasalazine (0.1–2.0 mM), pyrrolidine dithocarbamate (PDTC) (0.1–10 μM) and L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) (1.0–25.0 μM) (Sigma) were added to cultured cells for 30 min, and then gliadin digest was added for 24 h.

2.3. Enzyme-linked immunosorbent assay

The level of IL-8 and TNF-α was determined in cell culture supernatants collected after 6, 24 and 48 h of cultivation by enzyme-linked immunosorbent assay DuoSet kit (R&D System, Minneapolis) according to the manufacturer's instructions.

2.4. Western blot analysis

THP-1 cells were stimulated for 30 min with gliadin digest (200 μg/ml), IFN-γ (150 U/ml) or LPS (1 μg/ml), rinsed in cold 1 μM Na_3VO_4 in Tris buffered saline, lysed in ice-cold lysis buffer (25 mM Tris–HCl, pH 7.4, 1 mM DTT, 1 mM Na_3VO_4 and 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 10 mM NaF and 1 mM PMSF) for 30 min at 4 °C and centrifuged (14 000×*g*, 15 min, 4 °C). Supernatants were assayed for protein by the BCA protein kit (Pierce, USA). The same amount of protein from each lysate (30 μg/well) was subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE) [13] and transferred onto nitrocellulose membranes. Phosphorylated IκB protein was detected after incubation with rabbit anti-phospho-IκBα Abs (1:1000 final dilution) (Cell Signaling, USA). The membrane was then incubated with goat anti-rabbit horseradish peroxidase-conjugated Abs (1:2500 final dilution) (Cell Signaling, USA). Immunoreactive bands were visualised by ECL detection kit (Amersham, UK) and quantified by densitometry using Scan Pack 3.0 Software (Biometra, Göttingen, Germany).

2.5. Preparation of cell nuclear extract and colorimetric NF-κB assay

Nuclear extracts were prepared from THP-1 cells stimulated for 90 min with gliadin digest (500 μg/ml) alone or together with IFN-γ (150 U/ml), sulfasalazine (2 mM), PDTC (0.5 μM) and TPCK (5 μM) using a Nuclear extract Kit (Active Motif, USA). NF-κB DNA binding activity was detected using a TransAM NF-κB family transcription factor assay kit (Active Motif, USA) according to the manufacturer's protocol. Briefly, microwells coated with a double-stranded oligonucleotide containing the NF-κB consensus sequence were incubated with the nuclear extract for 1 h at room temperature and washed three times with washing buffer. The captured active transcription factor was incubated for 1 h with Ab specific for p65 or p50 NF-κB subunit, then for 1 h with anti-rabbit IgG coupled-horseradish peroxidase and after washing exposed to developing solution for 10 min. The optical density was measured at 450 nm using a Titertec Multiscan MCC/340 (Flow Lab., Irvine, Scotland).

2.6. Statistical analysis

Data are presented as arithmetic means of at least three independent experiments + S.E.M. Statistical analysis was performed by Student–Newman–Keuls multiple range test and Student's *t*-test. *P* values smaller than 0.005 were considered to be significant.

3. Results and discussion

3.1. Cytokine production by human monocytic cell lines stimulated with gliadin

Since the phenotypic and functional characteristics of human jejunal macrophages have not been precisely determined in healthy and diseased conditions, the effect of food proteins was tested using human cell lines THP-1 and U-937. THP-1 and U-937 cells represent different stages of monocyte/macrophage maturation. Unlike the polyploid U-937 cells (CD 14-, CD 68-), THP-1 cells (CD 14+, CD 68+) posseses a normal complement of chromosomes and resemble primary monocyte-derived macrophages in terms of inducible functions [14].

The crude gliadin had a very low direct effect on the activation of THP-1 cells evaluated by IL-8 and TNF-α production, while gliadin proteolytic fragments (100–500 μg/ml) elicited a significant secretion of both cytokines that was enhanced on its joint administration with IFN-γ (150 U/ml). Soya protein and ovalbumin, treated similarly to gliadin, had no effect on IL-8 and TNF-α production either when applied alone or in combination with IFN-γ (Fig. 1).

Since joint administration of IFN-γ with gliadin fragments enhanced the gliadin-induced production of IL-8 and TNF-α, we investigated the effect of IFN-γ prestimulation (Fig. 2). Interestingly, prestimulation of THP-1 cells with IFN-γ (150 U/ml) for 2 or 24 h prior to the addition of gliadin fragments resulted also in a higher secretion of IL-8 and TNF-α with respect to non-prestimulated cells (Fig. 2A and B). The production of IL-8 and TNF-α was even higher when gliadin fragments were applied to prestimulated cells simultaneously with IFN-γ. In contrast to THP-1 cells, the response of U-937 cells to gliadin fragments was very low and these cells produced significantly increased amount of IL-8 only when gliadin was added to the cells prestimulated for 24 h with IFN-γ (Fig. 2C). The TNF-α production after gliadin challenge was not detectable (data not shown).

To complete the time schedule, the IL-8 and TNF-α secretion by THP-1 cells was measured also 6 and 48 h after triggering with gliadin fragments (200 μg/ml) alone or along with IFN-γ (150 U/ml). IL-8 production increased in time, reaching a maximum value after 48 h of cultivation. TNF-α secretion was elevated during the first 24 h. Extension of the cultivation to 48 h did not elevate cytokine secretion in response to gliadin fragments and increased spontaneous release of TNF-α by cells was observed (Fig. 3).

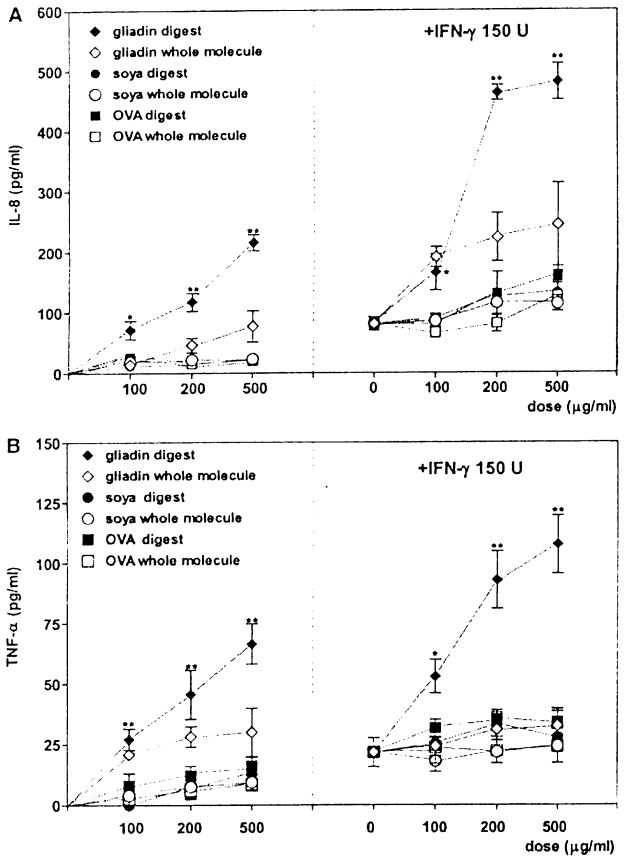

Fig. 1. Effect of gliadin, soya protein and ovalbumin and their proteolytic fragments on IL-8 (A) and TNF-α (B) secretion by THP-1 cells measured after 24 h of cell cultivation (left); the synergistic effect of IFN-γ applied together with food proteins (right). Data are expressed as means ± S.E.M. from at least three independent experiments. * $P < 0.05$, ** $P < 0.01$.

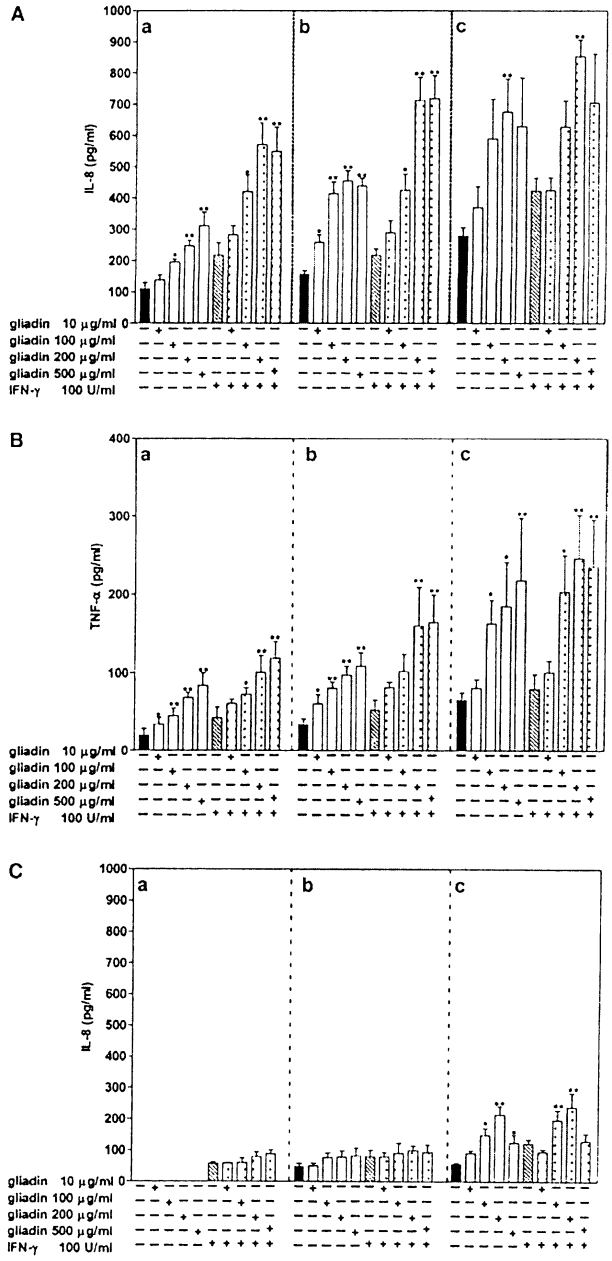

Fig. 2. Response of THP-1 (A, B) and U-937 cells (C) to gliadin fragments added directly to cell cultures (a) or after preincubation with IFN-γ for 2 (b) or 24 h (c) measured as IL-8 (A, C) and TNF-α (B) production. Data are expressed as means ± S.E.M. from at least three independent experiments. * $P < 0.05$, ** $P < 0.01$.

For comparison, we tested the stimulatory capacity of 33-AA peptide derived from α-gliadin (resistant to digestive enzymes), described as an inducer of gliadin-specific T cells [11], synthetic dodecapeptide B (FQQPQQQYPSSQ), a potent mouse macrophage-stimulating peptide [4] and the gliadin peptide (p31-43) that stimulates innate response in cultivated coeliac biopsies [6]. The IL-8 production by THP-1 cells in response to the 33-AA peptide reached only about 20% and to the B peptide about 60% of the response detected with the whole gliadin digest, while p31-43 gliadin peptide exerted the same stimulatory capacity as the gliadin digest. On evaluating TNF-α production, the B peptide reached about 10% and p31-43 about 40% of the response detected with the gliadin digest, while 33-AA peptide was unable to induce TNF-α secretion even in combination with IFN-γ. Similarity in the synergistic effect of IFN-γ and gliadin-derived peptide B, p31-43 and/or the whole gliadin digest on cytokine secretion was observed (data not shown). These results indicate that gliadin-derived peptides differ in their capacity to activate human monocytes and/or macrophages; however, the whole gliadin digest was found to be the most potent activator of these cells.

3.2. The CD14 molecule is not involved in the activation of THP-1 cells by gliadin

The response of THP-1 and U-937 cells to LPS (1 μg/ml), known to activate monocytes/macrophages, was compared with activation by gliadin and/or IFN-γ. When stimulated with LPS, THP-1 cells produced significantly higher levels of IL-8 (558.7 ± 23.7 pg/ml) and TNF-α (107.3 ± 13.0 pg/ml) than U-937 cells (IL-8 = 322.6 ± 16.1 pg/ml; TNF-α = 33.5 ± 5.2 pg/ml). The differences in response of THP-1 and U-937

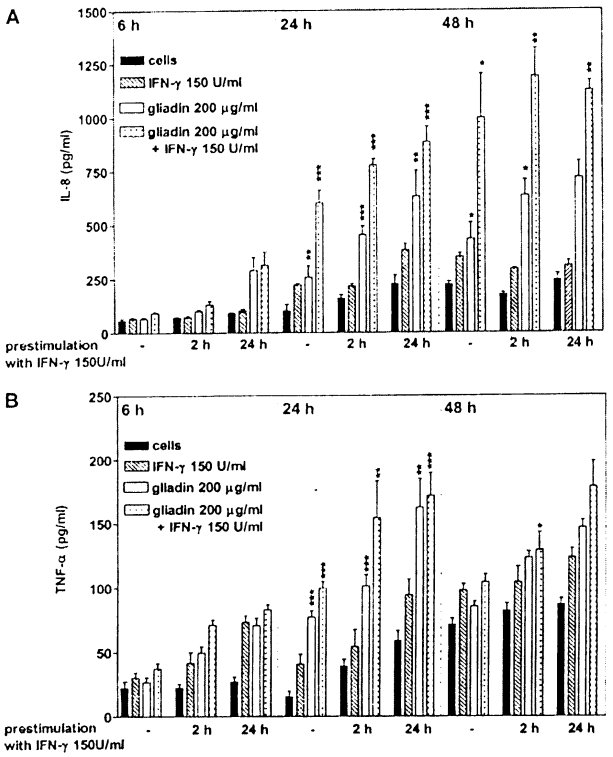

Fig. 3. The kinetics of IL-8 (A) and TNF-α (B) production by THP-1 cells. Cytokine production was determined 6, 24 and 48 h after addition of gliadin fragments and/or IFN-γ to cells directly or after IFN-γ-preincubation (for 2 or 24 h). Data obtained from at least three experiments are expressed as means ± S.E.M; differences between values at individual time intervals were calculated by Student's *t*-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

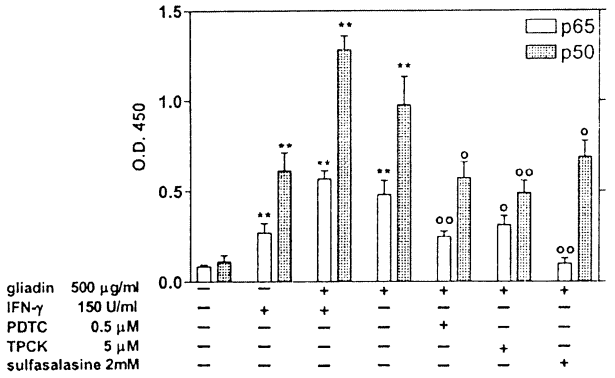

Fig. 4. The involvement of NF-κB subunits p50 and p65 in the activation pathway induced by gliadin fragments in THP-1 cells. The NF-κB DNA binding activity and the effect of inhibitors: sulfasalazine (2 mM), PDTC (0.5 μM) and TPCK (5 μM) was measured after a 90 min cultivation by a colorimetric NF-κB assay. Data are presented as means ± S.E.M. from three independent experiments. * $P < 0.05$, ** $P < 0.01$ was calculated vs. untreated cells and ° $P < 0.01$, °° $P < 0.01$ vs. gliadin-stimulated cells.

monocytes were also documented by other authors using *Escherichia coli* LPS and purified Shiga-like toxins [15,16].

Since the more pronounced response of THP-1 cells seemed to be in accordance with the higher expression of CD14 molecule, we examined the effect of anti-CD14 mAb on cytokine production. Addition of anti-CD14 mAb to THP-1 cells substantially reduced the IL-8 production induced by LPS, but had no effect on the activation by gliadin digest (Table 1). This observation throws doubt on the potential role of CD14 molecule in the activation of cells with gliadin.

3.3. Gliadin-induced IL-8 secretion is mediated via NF-κB pathway

Stimulation of THP-1 cells with gliadin digest (500 μg/ml) and/or with IFN-γ (150 U/ml) resulted in a marked increase of the binding activities of NF-κB subunits p50 and p65, more pronounced in the case of p50 subunit. To confirm the role of NF-κB in gliadin-induced activation of THP-1 cells, we examined the effects of NF-κB inhibitors: sulfasalazine, PDTC and TPCK. Sulfasalazine inhibits phosphorylation of Iκ-B [17], PDTC functions as a NF-κB inhibitor by blocking the dissociation of the NF-κB/IκB complex [18] and TPCK plays a role as proteasome inhibitor; it can therefore inactivate NF-κB by preventing the degradation of IκB [19]. Treatment with 0.5 μM PDTC and 2 mM sulfasalazine inhibited substantially p65 but only slightly p50 binding activity; 5 μM TPCK reduced the p50 binding capacity more efficiently than that of p65 (Fig. 4).

Moreover, sulfasalazine markedly suppressed, in a dose-dependent fashion, gliadin-stimulated IL-8 secretion by THP-1 cells, producing 15% inhibition at 0.1 mM and 90% inhibition at 2 mM concentration. The effect of PDTC was first evident at 0.1 μM concentration. TPCK produced 32% inhibition at 1 μM and completely abolished IL-8 production at 25 μM concentration (data not shown).

Since NF-κB activation involves the phosphorylation and subsequent degradation of IκBα, we assayed the phosphorylation of IκBα by Western blot analysis. Fig. 5 shows that the treatment of THP-1 cells with gliadin alone or together with IFN-γ increased phosphorylation of IκBα, when compared with unstimulated cells. Taken together, these results suggest that NF-κB molecule containing p50 and p65 subunits is involved in the activation pathway triggered by gliadin fragments in human monocytes.

In summary, our results document for the first time the direct effect of gliadin on human monocytes. In contrast to soya

Table 1
Anti-CD14 mAb (MEM-18) blockade of IL-8 production by LPS and gliadin-stimulated THP-1 cells

	IL-8 secretion (pg/ml)		
	mAb added	+ MEM-18 (10 μg) (% of blockade)	+ IN-05 (10 μg) (% of blockade)
LPS 10 μg	1030.3 ± 130.0	324.4 ± 76.8 (70)	1038.4 ± 56.6 (0)
Gliadin 500 μg	287.5 ± 21.2	273.1 ± 11.0 (5)	300.5 ± 20.2 (0)
Cells	98.0 ± 11.8	100.2 ± 10.5 (0)	98.2 ± 15.6 (0)

Note. Data are shown as means ± S.E.M. MEM-18, anti-CD14 monoclonal antibody IN-05, anti-insulin control monoclonal antibody.

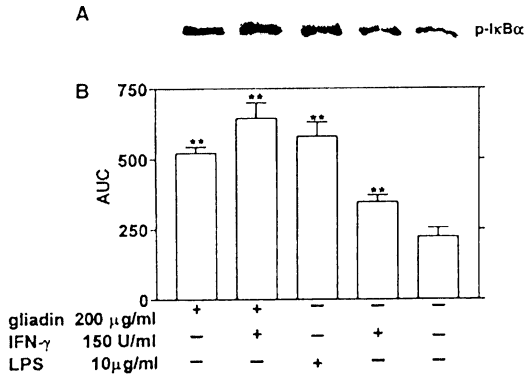

Fig. 5. Representative Western blot shows the effect of gliadin fragments, IFN-γ or LPS (positive control) on phosphorylation of IκBα in THP-1 cells (p-IκBα formation) (A) and its densitometric evaluation presented as mean AUC (arbitrary units) ± S.E.M. of three independent experiments, ** $P < 0.01$ (B).

protein and ovalbumin, gliadin and its fragments stimulated human monocytes to increased IL-8 and TNF-α production. IFN-γ, the main cytokine produced by gluten-specific T cells of coeliac patients, exerted a costimulatory effect on chemokine and cytokine production. Moreover, the data from our ongoing experiments show that gliadin peptides also activate human peripheral blood monocytes and that the production of IL-8 and TNF-α significantly differs in cells isolated from healthy donors and coeliac patients. The analysis of factors affecting the responsiveness is under study. Based on these findings, it could be suggested that increased secretion of IL-8 (a chemokine attracting immune cells to inflamed tissue) and TNF-α (cytokine involved in the activation of metalloproteinases digesting tissue components) produced by activated monocytes and/or macrophages could be involved in the impairment of coeliac intestinal mucosa.

Acknowledgements: This work was supported by Grants 310/02/1470, 310/03/H147 and 310/04/P242 from the Grant Agency of the Czech Republic, Grants A 5020210, A 5020205, S 5020203 and KJB 5020407 from the Grant Agency of the Academy of Sciences, Grant DAAD-CAS and Institutional Research Concept AV0Z5020903.

References

[1] Sollid, L. (2002) Nat. Rev. 2, 647–655.
[2] Nielsen, E.M., Jahnsen, F.L., Lundin, K.E., Johansen, F.E., Fausa, O., Sollid, L.M., Jahnsen, J., Scott, H. and Brandtzaeg, P. (1998) Gastroenterology 115, 551–563.
[3] Tučková, L., Flegelová, Z., Tlaskalová-Hogenová, H. and Zídek, Z. (2000) J. Leukoc. Biol. 67, 312–318.
[4] Tučková, L., Novotná, J., Novák, P., Flegelová, Z., Květoň, T., Jelínková, L., Zídek, Z., Man, P. and Tlaskalová-Hogenová, H. (2002) J. Leukoc. Biol. 71, 625–631.
[5] Ghosh, S., May, M.J. and Kopp, E.B. (1998) Annu. Rev. Immunol. 16, 225–260.
[6] Maiuri, L., Ciacci, C., Ricciardelli, I., Vacca, L., Raia, V., Auricchio, S., Picard, J., Osman, M., Quarantino, S. and Londei, M. (2003) Lancet 362, 30–37.
[7] Mention, J.J., Ben Ahmed, M., Begue, B., Barbe, U., Verkarre, V., Asnafi, V., Colombel, J.F., Cugnens, P.H., Ruemmele, F.M., McIntyre, E., Brousse, N., Cellier, C. and Cerf- Bensussan, N. (2003) Gastroenterology 125, 730–745.
[8] Maiuri, M.C., De Stefano, D., Mele, G., Iovine, B., Bevilaqua, M.A., Greco, L., Auricchio, S. and Carnuccio, R. (2003) Naunyn-Schmied. Arch. Pharmacol. 368, 63–71.
[9] Giovannini, C., Matarrese, P., Scazzocchio, B., Vari, R., D'Archivio, M., Straface, E., Masella, R., Malorni, W. and De Vincenzi, M. (2003) FEBS Lett. 540, 117–124.
[10] Schuppan, D., Esslinger, B. and Dieterich, W. (2003) Lancet 362, 3–4.
[11] Shan, L., Molberg, O., Parott, I., Hausch, F., Filiz, F., Gray, G.M., Sollid, L.M. and Khosla, C. (2002) Science 297, 2275–2279.
[12] Pugin, J., Kravchenko, V.V., Lee, J.D., Kline, L., Ulevitch, R.J. and Tobias, P.S. (1998) Infect. Immun. 66, 1174–1180.
[13] Laemmli, U.K. (1970) Nature 227, 680–685.
[14] Auwerx, J. (1991) Experientia 47, 22–31.
[15] Eperon, S. and Jungi, T.W. (1996) J. Immunol. Meth. 194, 121–129.
[16] Ramegowda, B. and Tesh, V.L. (1996) Infect. Immun. 64, 1173–1180.
[17] Wahl, C., Liptay, S., Adler, G. and Schmid, R.M. (1998) J. Clin. Invest. 101, 1163–1174.
[18] Snyder, J.G., Prewitt, R., Campsen, J. and Britt, L.D. (2002) Shock 17, 304–307.
[19] Baldwin, A.S. (1996) Annu. Rev. Immunol. 14, 649–683.

INVOLVEMENT OF INNATE IMMUNITY IN THE DEVELOPMENT OF INFLAMMATORY AND AUTOIMMUNE DISEASES

Involvement of Innate Immunity in the Development of Inflammatory and Autoimmune Diseases

HELENA TLASKALOVÁ-HOGENOVÁ,[a] LUDMILA TUCKOVÁ,[a] RENATA STEPÁNKOVÁ,[a] TOMÁS HUDCOVIC,[a] LENKA PALOVÁ-JELÍNKOVÁ,[a] HANA KOZÁKOVÁ,[a] PAVEL ROSSMANN,[a] DANIEL SANCHEZ,[a] JANA CINOVÁ,[a] TOMÁS HRNČÍŘ,[a] MILOSLAV KVERKA,[a] LENKA FROLOVÁ,[a] HOLM UHLIG,[b] FIONA POWRIE,[b] AND PAUL BLAND[c]

[a]*Department of Immunology and Gnotobiology, Institute of Microbiology, Academy of Sciences of the Czech Republic, 1st Faculty of Medicine, Charles University, Prague, Czech Republic*

[b]*University of Oxford, Oxford, United Kingdom*

[c]*University of Goteborg, Goteborg, Sweden*

ABSTRACT: **Initial events and effector mechanisms of most inflammatory and autoimmune diseases remain largely unknown. Dysfunction of the innate and adaptive immune systems associated with mucosae (the major interface between the organism and its environment, e.g., microbiota, food) can conceivably cause impairment of mucosal barrier function and development of localized or systemic inflammatory and autoimmune processes. Animal models help in elucidating the etiology and pathogenetic mechanisms of human diseases, such as the inflammatory bowel diseases, Crohn's disease and ulcerative colitis, severe chronic diseases affecting the gut. To study the role of innate immunity and gut microbiota in intestinal inflammation, colitis was induced by dextran sulfate sodium (DSS) in mice with severe combined immunodeficiency (SCID). Conventionally reared (microflora-colonized) SCID mice displayed severe inflammation like that seen in immunocompetent Balb/c mice, whereas only minor changes appeared in the intestinal mucosa of DSS-fed gnotobiotic germ-free SCID mice. The presence of microflora facilitates the inflammation in DSS-induced colitis that develops in immunodeficient SCID mice, that is, in the absence of T and B lymphocytes. Celiac disease, a chronic autoimmune small bowel disorder, afflicts genetically susceptible individuals with wheat gluten intolerance. We showed that, in contrast with any other food proteins, wheat gliadin and its peptic fragments activate mouse macrophages and human monocytes to produce proinflammatory cytokines through the nuclear factor-κB signaling pathway. Activation of innate immunity cells by food proteins or components from gut microbiota thus could participate in the impairment of intestinal mucosa and the development of intestinal and/or systemic inflammation.**

Address for correspondence: Prof. Helena Tlaskalová-Hogenová, M.D., Ph.D., Institute of Microbiology, Academy of Sciences of the Czech Republic, Department of Immunology and Gnotobiology, Víde-ská 1083, Prague 4, Czech Republic. Voice: +420-2-4106-2363; fax: +420-2-4172-1143.

tlaskalo@biomed.cas.cz

Ann. N.Y. Acad. Sci. 1051: 787–798 (2005).
doi: 10.1196/annals.1361.122

KEYWORDS: **innate immunity; mucosal immunity; mucosal barrier; inflammation; autoimmunity; animal models; germ-free animals; commensal bacteria; gut microbiota; inflammatory bowel diseases; celiac disease; gluten; gliadin**

INTRODUCTION

Body surfaces covered by epithelial cells come into contact with a number of microorganisms and foreign substances immediately after birth. Although the surface of the skin (~2 m^2) is protected mechanically by several epithelial layers, the surfaces of the gastrointestinal, respiratory, and urogenital tracts (~300 m^2) are mostly covered with a single-layered epithelium. To resist the invasion of microorganisms, they require extensive protection, as represented by a complex of mechanical and chemical mechanisms responsible for the degradation and removal of heterogeneous substances, and by highly effective innate and highly specific adaptive immune systems. However, the interface between the organism and the outside world is also the site of the exchange of nutrients, and the export of products and waste components. Mucosae must, therefore, be selectively permeable; at the same time, they must constitute a barrier equipped with local defense mechanisms against environmental threats (e.g., invading pathogens). The mucosal immune system has evolved mechanisms for discriminating between harmless antigens from food and microflora and dangerous antigens. Characteristic features of mucosal immunity distinguishing it from systemic immunity are (1) strongly developed mechanisms of innate defense; (2) the existence of characteristic populations of unique types of lymphocytes and their products; (3) colonization of the mucosal and exocrine glands by cells originating from the mucosal-organized tissues ("common mucosal system"); (4) transport of polymeric immunoglobulins through epithelial cells into secretions (sIgA); and (5) preferential induction of inhibitory mechanisms directed against mucosal nondangerous antigens ("oral/mucosal tolerance"). The innate mucosal immune system is represented by cells (epithelial cells, macrophages, dendritic cells, mast cells, and other cells) and their humoral products (e.g., antimicrobial proteins and peptides). Basic functions of the mucosal immune system are protection against pathogenic microorganisms and prevention of penetration of immunogenic components from mucosal surfaces into the internal environment of the organism (barrier and antiinfectious functions). Another important function is the induction of unresponsiveness of systemic immunity to antigens present on mucosal surfaces (oral/mucosal tolerance) and the maintenance of homeostasis on mucosal surfaces (immunoregulatory function).[1–4]

INNATE IMMUNE MECHANISMS IN INFLAMMATORY AND AUTOIMMUNE PROCESSES: THE ROLE OF MUCOSAL BARRIER

The basic mechanism of mucosal immunity is innate, natural immunity, as represented by processes that protect the host immediately, within the first minutes and hours of exposure to infection. It is of interest that these defense mechanisms of vertebrates are implemented by structurally related effector molecules present in plants

and insects, which do not possess higher, specialized forms of adaptive immunity. A characteristic, although not yet clearly defined, feature of innate immunity is an ability to distinguish between potentially pathogenic microbial components and harmless antigens by pattern recognition receptors (PRRs). Examples of these molecules are the so-called Toll-like receptors (TLRs) that enable mammalian cells to recognize conserved characteristic molecules present on microorganisms that represent so-called pathogen-associated molecular patterns (PAMPs).[5–7] Because these molecules (e.g., lipopolysaccharides, peptidoglycans) also are present on commensal bacteria, it seems more precise to call them microbe-associated molecular patterns (MAMPs). Toll receptors were originally described in *Drosophila*; their extracellular domain contains leucine-rich repeats, whereas the cytoplasmic domain is homologous to the IL-1 receptor (IL-1R). In insects, they were found to play an essential role in the immune response to fungal infection. In mammals, PRRs are present on macrophages, neutrophils, dendritic cells, epithelial cells, and other cells belonging to the innate immune system. It was demonstrated that recognition of microbes activates the NF-κB signaling pathway, triggering cytokine production and upregulation of costimulatory molecules on antigen-presenting cells that leads to activation of T cells. We have shown that human intestinal epithelial cells express an important lipopolysaccharide-binding molecule, CD14, which, together with Toll-like receptors, can participate in maintaining the intricate balance between self and the outer environment in the gut. Western blotting and reverse transcriptase polymerase chain reaction (RT-PCR) confirmed CD14 positivity. Furthermore, we found that these cells release a soluble form of CD14 that may have important implications in shaping the interaction between the mucosal immune system and gut bacteria.[8] Innate immunity is closely linked to adaptive, acquired immunity; the link is represented by dendritic cells.

In addition to well-known humoral components of innate immunity (humoral forms of PRRs) present on mucosal surfaces such as complement, lysozyme, lactoferrin, and mannan-binding protein, recently described factors have been the subject of intensive study. Important components of nonspecific mechanisms are antimicrobial peptides widely distributed throughout the plant and animal kingdoms. Various antibiotic peptides, defensins, were found in epithelial cells (e.g., in apical granules of Paneth epithelial cells). In addition to their wide antimicrobial activity, defensins display chemotactic activity toward T cells; in other words, they represent components of innate immunity interacting with adaptive immunity.[4,9]

The intestine harbors an enormously complex microflora of a large variety of indigenous bacteria. These bacteria are in close proximity to a large population of rapidly renewing epithelial cells and other components of the mucosal innate immune system. Components of normal microflora maintain a balanced, "physiological" inflammatory response that is missing in animals reared under germ-free conditions.[10,11] Starting from the first hours after delivery from the sterile uterine environment, microorganisms colonize most of the mucosal surfaces and skin. The number of autochthonous bacteria ($\sim 1 \times 10^{14}$) exceeds the number of cells forming the human body. The highest numbers of commensal bacteria, exhibiting enormous diversity, are found in distal parts of the gut; their identification and characterization is, however, hampered by the fact that many intestinal bacteria are not cultivable. Molecular biological methods help in analyzing the complexity of the microflora and in identifying its components.[12] The highly protective colonization of the mu-

cosal surfaces by commensals has an important stimulatory effect on innate and adaptive immunity, metabolic processes (e.g., nutrition), and other host activities.[13–15] Using gnotobiotic animal models (animals reared in germ-free conditions), we and others demonstrated that components of intestinal microflora play a crucial role during early postnatal development of the immune system.[10] However, unlimited immune activation in response to signals from commensal bacteria could pose the risk of pathological inflammation. The mucosal immune system has developed specialized regulatory, anti-inflammatory mechanisms for eliminating or tolerating nondangerous commensal microorganisms and food antigens (oral/mucosal tolerance).[16,17] Still, under specific conditions, commensal bacteria could participate in the development of intestinal or systemic inflammation.[11,18]

The epithelium of most mucosal surfaces consists of a layer of interconnected, polarized epithelial cells separated by a basal membrane from the connective and supporting tissue surrounding various types of cells present in the lamina propria. The epithelial layer is reinforced by tight junctions present in paracellular spaces of epithelial cells and forming an interconnected network. In addition to laterally situated tight junctions present in zonula occludens, there are intermediary junctions and desmosomes in the so-called zonula adherens. Tight junctions were found to act as a dynamic and strictly regulated port of entry that opens and closes in response to various signals (e.g., cytokines) originating in the lumen, the lamina propria, and the epithelium. Tight junctions participate in preserving cellular polarity and are regarded as key elements in intestinal diffusion mechanisms. Occludin, members of claudin family, and junctional adhesion molecule (JAM) were identified as the molecules forming transmembrane tight junction strands. The cytoplasmic plaque of tight junctions includes many proteins (e.g., zonula occludens–1 [ZO-1], ZO-2, ZO-3) that interact with each other and with cytoskeletal proteins. Tight junction proteins also appear to be direct targets and effectors of different signaling pathways. Although our knowledge of the ultrastructure of tight junctions has progressed during the last several years, the pathogenic mechanism of diseases where tight junctions are affected remained poorly understood, because of limited knowledge about their regulation.[19–21]

The intestinal epithelium represents the primary site for active transport of fluid and electrolytes from the gut lumen through the transcellular pathway; however, the predominant route for passive transepithelial solute flow is the paracellular pathway. Gut mucosa serves as the main barrier to the passage of macromolecules, that is, foreign antigens entering the host via the oral route, components of commensal flora such as toxins. The majority of luminal proteins cross the intestinal barrier through the transcellular pathway, followed by lysosomal degradation. Lysosomal degradation changes proteins into nonimmunogenic peptides. Small but immunologically significant amounts of antigens cross the barrier in intact form through the paracellular pathway. The paracellular pathway involves a subtle regulation of intercellular tight junctions that leads to antigen (mucosal) tolerance. When the integrity of tight junctions is compromised, for example, as a consequence of prematurity, exposure to toxins, drugs, or radiation, aberrant immune reactions to environmental antigens occur and could lead to inflammatory and autoimmune diseases.[19–21] Various types of epithelial cells participate in the mucosal barrier function; the main and most common cells are conventional enterocytes (colonocytes in colon). Also of importance are goblet cells producing both mucus and trefoil peptides required for epithelial growth and repair, enteroendocrine cells producing neuroendocrine molecules

having a paracrine effect, and Paneth's cells secreting antibiotic peptides-defensins. Epithelial cells are maintained on a network of interconnected myofibroblasts, which produce molecules necessary for the basal membrane, in addition to factors required for epithelial growth. Mucosal barrier function is greatly influenced by the products of the nervous system (neurotransmitters), increasing, for instance, the passage of macromolecules through tight junctions.[4]

Initial events leading to the development of chronic inflammatory and autoimmune diseases have not yet been elucidated. We propose that dysfunction of the immune system associated with the gut and other mucosal surfaces is a prerequisite for impairment of physiologically developing regulatory mechanisms. The balance in intestinal mucosa may be disturbed by pathogenic microorganisms and toxins attacking the mucosae by qualitative or quantitative changes in the composition of mucosal microbiota, or by inadequately functioning components of the innate or adaptive immune system occurring in cases of dysregulated mechanisms of mucosal immunity, or in immunodeficiencies. An expression of pathologically increased immunological activity may induce inflammatory processes of a different character, depending on the type and mediators of inflammation. Thus, numerous chronic diseases may occur as a result of disturbances of mucosal barrier function or of changes in mechanisms regulating mucosal immunity.[2,11,14] The main characteristics of chronic, "idiopathic," inflammatory, and autoimmune diseases are tissue destruction and functional impairment as a consequence of immunologically mediated mechanisms that are principally the same as those functioning against dangerous (pathogenic) infections. One of the most attractive explanations for inflammatory and autoimmune phenomena has centered on various infections as natural events capable of initiating the process in genetically predisposed individuals.[2,11,22–25] We propose that not only pathogenic microorganisms but also components of normal microflora could participate in the triggering and development of inflammatory and autoimmune processes.

THE ROLE OF COMMENSAL BACTERIA AND INNATE IMMUNE MECHANISMS IN INFLAMMATORY BOWEL DISEASES: STUDY IN EXPERIMENTAL MODELS

Both forms of inflammatory bowel disease (IBD), that is, Crohn's disease (CD) and ulcerative colitis (UC), are severe chronic disorders that affect approximately 0.2% of the human population. They represent an important medical problem because they have a devastating impact on quality of life and require long-standing medical care. Despite the long-lasting efforts, the etiology and pathogenesis of IBD remain unclear. The inflammatory bowel diseases seem to involve interactions among immune, environmental, and genetic factors; the combination of these factors results in induction of inflammation, subsequent mucosal lesions, and then repair.[26] Environmental factors seem to be responsible for the remarkably growing incidence of IBD, Crohn's disease, in particular, observed during the last half-century. It has been shown that northern geographic location and high social and economic status increase the risk of IBD. This finding is in agreement with "the hygiene hypothesis" for allergic and autoimmune diseases.

Recently, experimental models of intestinal inflammation, induced chemically or developing spontaneously, have been described that make it possible to examine early events during the induction of disease, to control all steps in disease progression, and to develop new preventive and therapeutic strategies.[27–29] Chemically induced intestinal inflammation is used to study the participation of immunological mechanisms in the pathogenesis of the diseases. Several murine and rat models with spontaneously developing colitis suggest that disruption of T lymphocyte regulatory functions or mucosal barrier defects could lead to intestinal inflammation. Mice with a null mutation in the interleukin-2 (IL-2), interleukin-10 (IL-10), transforming growth factor β1 (TGF-β1), MHC class II, T cell receptor (TCR)-α chain, and TCR-β chain, and mice lacking signaling protein G protein subunit Gαi2 chain were shown to develop chronic intestinal inflammation spontaneously. Interestingly, when some of these mice were reared in germ-free conditions, disease did not appear.[27,30]

Dysregulation of the intestinal immune response to bacterial flora was suggested to play a crucial role. Loss of physiologically normal regulatory mechanisms of the local immune system, perhaps a breakdown of oral tolerance to environmental antigens/commensal gut bacteria, could be involved in the pathogenic mechanism. Findings from experimental models of IBD indicate that T cells are responsible for the regulation of the intestinal immunological response to luminal antigens.[16] The finding that there is an abnormal T cell responsiveness against indigenous microflora in human inflammatory bowel disease, and its experimental model, awakened interest in the possibility that commensals may initiate and/or maintain IBD lesions.[30,31] Breakdown of oral tolerance to microfloral antigens was suggested to play a role in the development of intestinal inflammat ion. Under conditions of an immunoregulatory or mucosal barrier defect, the common intestinal flora obviously is capable of evoking stimulation leading to a chronic intestinal or systemic inflammation.[29,31] The solutions to the questions of which and how gut bacteria, and which cells, are involved in the induction and maintenance of chronic intestinal inflammation are of great importance because they could bring about new approaches to the therapy and/or prevention of this severe disease.

We used colitis induced by dextran sulfate sodium (DSS) feeding of mice to study the immunological factors involved in the pathogenetic mechanisms of chemically triggered intestinal inflammation. The role of adhesion molecules in intestinal inflammation was studied in our laboratory using mice with deletion of the gene for ICAM-1, in comparison with genetically corresponding wild-type controls.[32] Acute and chronic colitis was induced by oral administration of DSS in drinking water. Only minor changes were found in ICAM-1 knockout (KO) mice; in contrast, wild-type controls exhibited severe intestinal changes with ulcerations, suggesting direct involvement of ICAM-1 molecules in the development of the inflammatory response. The chronic colitis was accompanied by an increased level of anti-epithelial IgA autoantibodies detected in wild-type control mice.[32]

These experimental models were used also to analyze the role of commensal bacteria and innate immunity in the development of intestinal inflammation. Using the DSS-induced model of intestinal inflammation, we have shown that, as in conventionally reared, immunocompetent Balb/c mice, mice with severe combined immunodeficiency (SCID) developed profound inflammatory changes in colonic mucosa. Immunocompetent Balb/c and immunodeficient SCID mice were transferred into isolators for germ-free rearing by special gnotobiological techniques. Balb/c and

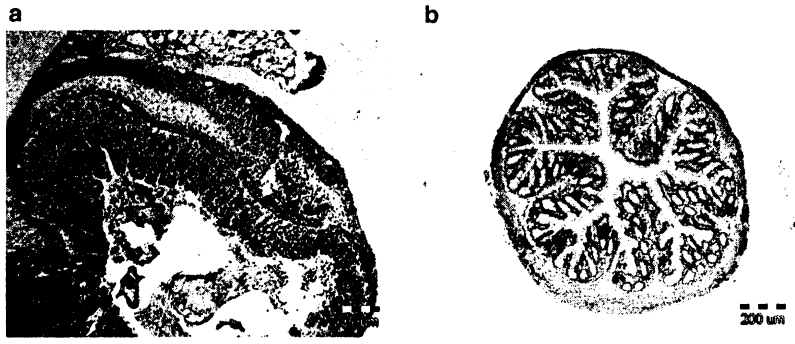

FIGURE 1. The colon after 1 week of ingestion of dextran sodium sulfate in (**a**) conventional SCID mice, showing ulcerative colitis with severe infiltration of inflammatory cells (grade 4 inflammation); and (**b**) germ-free SCID mice, in which no inflammation is observed.

SCID mice reared in germ-free conditions developed only minor signs of mucosal inflammation.[33] Interestingly, conventionally reared SCID mice, lacking T and B cells, developed intestinal inflammation similar to the inflammation that developed in immunocompetent Balb/c mice (FIG. 1). This finding suggests that under physiological conditions, innate immunity components are able to regulate (keep in balance) the interaction of the macroorganism with commensal bacteria, and, after chemically induced breakdown of mucosal barrier, commensal bacteria could induce severe forms of intestinal inflammation in the absence of components of adaptive immunity (T and B cells).

Another model of intestinal inflammation, developing spontaneously in the colons of conventional SCID mice restored with the CD45 RB^{high} subset of $CD4^+$ T cells isolated from the spleens of normal Balb/c mice, was used in further studies.[16,17] The $CD4^+$ $CD45RB^{high}$ subpopulation of T cells was purified from the spleens of conventional Balb/c mice and transferred to SCID mice. As recipients, germ-free SCID mice, conventional SCID mice, or SCID mice monoassociated with defined bacterial strains or colonized with bacterial mixtures were used. The clinical picture as well as morphological results revealed that severe colitis was present only in conventional SCID mice and in mice colonized with the cocktail of specific pathogen–free (SPF) microflora plus segmented filamentous bacteria (SFB). A monoassociation of SCID mice with SFB or colonization with the cocktail of SPF bacteria did not lead to intestinal inflammation.[34] Signs of impairment of tight junctions of the intestinal barrier in the terminal ileum of colitic mice was documented by anti–ZO-1 antibody (FIG. 2). Our studies performed in the model of reconstituted SCID mice suggest that noncultivable segmented filamentous bacteria, together with a defined mixture of cultivable bacteria from SPF mice, were effective in triggering intestinal inflammation.[34]

Experiments performed in gnotobiotic models suggest that the composition of gut microbiota plays a decisive role in the pathogenetic mechanism of intestinal inflammation. Exogenous application of commensal organisms (probiotics) exerting beneficial effects on host health has recently been shown to have protective and

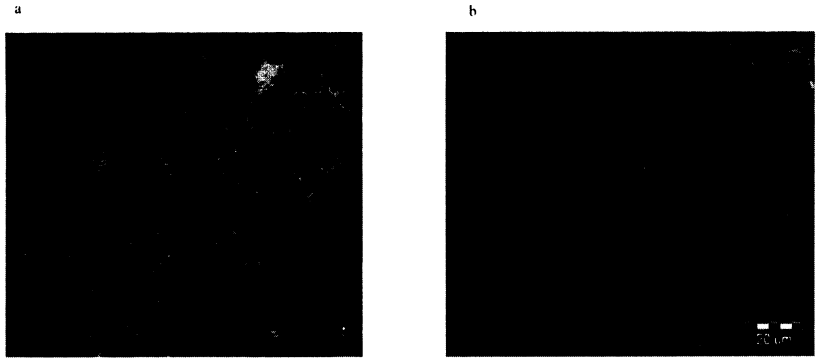

FIGURE 2. Tight junctions in terminal ileum of SCID mice colonized 12 weeks after transfer of $CD4^{+}CD45RB^{high}$ T cells with (**a**) specific pathogen–free (SPF) microflora, showing preserved integrity of tight junctions; and (**b**) mixture of SPF microflora and segmented filamentous bacteria, showing altered structure of tight junctions (staining for zonula occludens–1).

therapeutic effects on diarrheal diseases, including IBD, and to reduce the risk of infections and allergies.[35–38] Oral introduction of probiotic bacteria is associated with an alleviation of intestinal inflammation and normalization of increased intestinal permeability, together with promotion of intestinal barrier functions. Orally ingested probiotic microorganisms do not exert health effects entirely in the intestine. Investigations in germ-free mice have shown that after oral monoassociation of the mice with a *Bifidobacterium longum* strain, bifidobacteria translocated and were found in the mesenteric lymph nodes, liver, and kidneys 1 week later. Translocation of probiotic bacteria had no harmful effects but increased the barrier function of the intestinal mucosa and inhibited translocation of other bacteria. Recently, we showed that monoassociation with *B. bifidum* speeds up the biochemical maturation of enterocytes, resulting in a shift of specific activities of brush-border enzymes between the values found for germ-free and conventionally reared mice.[39] Regulation of microflora composition (e.g., by probiotics and prebiotics) offers the potential to influence the development of mucosal and systemic immunity but also can play a role in the prevention and treatment of human IBD.[30,31,40]

INNATE IMMUNITY MECHANISMS IN PATHOGENESIS OF CELIAC DISEASE

Celiac disease is a disorder characterized by gluten-dependent enteropathy. Small intestinal mucosal villous atrophy with hyperplasia of the crypts, abnormal surface epithelium, and increased inflammatory cell infiltration are regularly found in biopsy specimens taken from the jejunum or duodenum of patients with active disease. The features of the mucosal lesions suggest that gliadin and related prolamins of other cereals lead to an aberrant, pathologically increased immune response in genetically predisposed individuals. The pathogenetic concept of celiac disease is based on

TABLE 1. Effects of gliadin, soy protein, ovalbumin, and their proteolytic fragments on IL-8 and TNF-α secretion by THP-1 (monocytic cell line) cells, measured after 24 h of cell cultivation

	Food proteins only			+IFN-γ 150 U	
	0 μg	100 μg	500 μg	100 μg	500 μg
IL-8					
Gliadin crude	81.2 (10.9)	14.1 (6.0)	77.5 (25.6)	191.3 (17.5)	245.0 (68.6)
Soy crude	81.2 (10.9)	19.1 (8.7)	24.2 (2.3)	86.3 (11.3)	114.2 (12.8)
OVA crude	81.2 (10.9)	21.7 (2.3)	21.7 (3.1)	67.2 (8.4)	127.0 (25.1)
Gliadin digest	81.2 (10.9)	71.7 (15.2)^a	216.5 (13.6)^b	166.3 (30.2)^a	481.3 (30.0)^b
Soy digest	81.2 (10.9)	12.0 (1.2)	24.3 (5.8)	90.6 (10.4)	133.5 (19.6)
OVA digest	81.2 (10.9)	27.3 (2.2)	21.3 (3.1)	82.3 (12.6)	162.2 (14.4)
TNF-α					
Gliadin crude	21.6 (6.5)	20.6 (2.5)	29.8 (10.1)	24.3 (7.2)	32.4 (6.0)
Soy crude	21.6 (6.5)	4.7 (3.6)	9.2 (1.2)	17.9 (4.4)	24.1 (7.2)
OVA crude	21.6 (6.5)	2.5 (2.1)	8.5 (2.2)	23.5 (4.2)	23.9 (2.0)
Gliadin digest	21.6 (6.5)	27.2 (4.4)^b	66.4 (8.4)^b	52.9 (7.5)^a	107.6 (12.0)^b
Soy digest	21.6 (6.5)	0.0 (0.0)	12.8 (7.1)	25.3 (5.9)	27.7 (4.0)
OVA digest	21.6 (6.5)	8.0 (5.1)	16.1 (3.5)	31.5 (3.5)	34.5 (5.6)

Right: Synergistic effect of IFN-γ applied together with food proteins. Data are expressed as mean (± SEM).
$^aP < .05$, $^bP < .01$.[50]
OVA, ovalbumin.

the interaction of toxic gliadin peptides with HLA-DQ2 antigens, which occurs in antigen-presenting dendritic cells of the lamina propria. Intolerance to gluten seems to be caused by increased activity of gliadin-specific lamina propria $CD4^+$ T cells producing Th0/Th1 cytokines, and cytotoxic intraepithelial $CD8^+$ T cells expressing NK receptors.[41–44] The increased level of antibodies to gliadin in sera of patients is regularly accompanied by the presence of autoantibodies. The molecular target of antiendomysial autoantibodies recently was identified as tissue transglutaminase. We identified common epitopes on gliadin and another autoantigen, calreticulin, recognized by antibodies present in sera of patients with celiac disease.[45] Also, other features of the disease suggest that celiac disease fulfills the criteria for autoimmune diseases.[22,46]

Based on our previous findings, it seems that the unique structure of gliadin and its fragments could be responsible for the involvement of innate immunity mechanisms in the pathogenetic mechanism of this disease.[47] The question of how gliadin affects innate immunity was answered by experiments in which mouse macrophages were used. We have shown that in contrast with other dietary proteins tested, gliadin stimulated IFN-γ–treated mouse macrophages to produce nitric oxide and secrete cytokines (TNF-α, IL-10, RANTES).[48] Moreover, the active gliadin peptide was separated and identified.[49] Using the human monocytic cell line THP-1, we tried to see whether human monocytes could be activated similarly by a peptic digest of gliadin.

Cultivation of THP-1 cells with a digest of gliadin was found to lead to the production of IL-8 and TNF-α; the production was augmented by pretreatment of cells with IFN-γ or its addition to the culture. Ovalbumin and soy protein or their peptic digests had no effect on IL-8 and TNF-α production when applied alone or in combination with IFN-γ (TABLE 1). The participation of nuclear factor–κB (NF-κB) in the stimulatory effect of the gliadin digest on monocytes was documented by a marked increase of the DNA-binding activities of NF-κB subunits p50 and p65. Moreover, the inhibition of p65 and p50 subunit binding was detected using NF-κB inhibitors sulfasalazine, PDTC, and TPCK.[50] Because NF-κB activation involves phosphorylation and subsequent degradation of Iκ-Bα, the phosphorylation of Iκ-Bα was determined by immunoblotting. We found that stimulation of THP cells with gliadin and/or with IFN-γ leads to an increase of phosphorylation of Iκ-Bα, suggesting that NF-κB is involved in the activation pathway triggered by gliadin fragments. Similar findings were recently described by other authors.[43,44,51,52]

ACKNOWLEDGMENTS

This work was supported by grants A 5020205, A 5020210, B 5020407 (Grant Agency of the Academy of Sciences of the Czech Republic), 310/05/2245, 310/03/H147, 303/05/2249, 310/04/P242(Grant Agency of the Czech Republic), S 5020203 (Academy of Sciences of the Czech Republic), NR/8356-3 (Internal Grant Agency of Ministry of Health of the Czech Republic), and AVOZ 50200510 (Institute of Microbiology).

REFERENCES

1. MESTECKY, J., M.W. RUSSEL, S. JACKSON, *et al.* 1995. Advances in Mucosal Immunology. Plenum Press. New York, London.
2. TLASKALOVÁ-HOGENOVÁ, H., L. TUCKOVÁ, R. LODINOVÁ-ZÁDNÍKOVÁ, *et al.* 2002. Mucosal immunity: its role in defense and allergy. Int. Arch. Allergy Immunol. **128:** 77–89.
3. BRANDTZAEG, P. & R. PABST. 2004. Let's go mucosal: communication on slippery ground. Trends Immunol. **25:** 570–577.
4. MESTECKY, J., M.E. LAMM, W. STROBER, *et al.* 2005. Mucosal Immunology, 3rd ed. Elsevier Academic Press. Amsterdam.
5. MEDZHITOV, R. & C. JANEWAY, JR. 2000. Innate immune recognition: mechanisms and pathways. Immunol. Rev. **173:** 89–97.
6. ADEREM, A. & R.J. ULEVITCH. 2000. Toll-like receptors in the induction of the innate immune response. Nature **406:** 782–787.
7. AKIRA, S. & K. TAKEDA. 2004. Toll-like receptor signalling. Nat. Rev. Immunol. **4:** 499–511.
8. FUNDA, D.P., L. TUCKOVÁ, M. FARRÉ, *et al.* 2001. CD14 is expressed and released as soluble CD14 by human intestinal epithelial cells in vitro: lipopolysaccharide activation of epithelial cells revisited. Inf. Immun. **69:** 3772–3781.
9. BEVINS, C.L. 2004. The Paneth cell and the innate immune response. Curr. Opin. Gastroenterol. **20:** 572–580.
10. TLASKALOVÁ-HOGENOVÁ, H., J. STERZL, R. STEPÁNKOVÁ, *et al.* 1983. Development of immunological capacity under germfree and conventional conditions. Ann. N.Y. Acad. Sci. **409:** 96–113.
11. TLASKALOVÁ-HOGENOVÁ, H. 1997. Gnotobiology as a tool—an introduction. *In* Immunology Methods Manual. I. Lefkovits, Ed.: 1524–1559. Academic Press. London.

12. HARMSEN, H.J., G.C. RAANGS, T. HE, *et al.* 2002. Extensive set of 16S rRNA-bases probes for detection of bacteria in human feces. Appl. Environ. Microbiol. **68:** 2982–2990.
13. HOOPER, L.V., M.H. WONG, A. THELIN, *et al.* 2001. Molecular analysis of commensal host-microbial relationships in the intestine. Science **291:** 881–884.
14. TLASKALOVÁ-HOGENOVÁ, H., R. STEPÁNKOVÁ, T. HUDCOVIC, *et al.* 2004. Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases. Immun. Lett. **93:** 97–108.
15. CEBRA, J.J., H.Q. JIANG, N. BOIKO & H. TLASKALOVÁ-HOGENOVÁ. 2005. The role of mucosal microbiota in the development, maintenance, and pathologies of the mucosal immune system. *In* Mucosal Immunology, 3rd ed. J. Mestecky, M.E. Lamm, W. Strober, *et al.*, Eds.: 335–368. Elsevier Academic Press. Amsterdam.
16. SINGH, B., S. READ, C. ASSEMAN, *et al.* 2001. Control of intestinal inflammation by regulatory T cells. Immunol. Rev. **182:** 190–200.
17. POWRIE, F. & H. UHLIG. 2004. Animal models of intestinal inflammation: clues to the pathogenesis of inflammatory bowel disease. Novartis Found. Symp. **263:** 164–174.
18. RAKOFF-NAHOUM, S., J. PAGLINO, F. ESLAMI-VARZANEH, *et al.* 2004. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell **118:** 229–241.
19. FASANO, A., T. NOT, W. WANG, *et al.* 2000. Zonulin, a newly discovered modulator of intestinal permeability, and its expression in coeliac disease. Lancet **355:** 1518–1519.
20. FASANO, A. 2001. Intestinal zonulin: open sesame! Gut **49:** 159–162.
21. NEUTRA M.P. & J.P. KRAEHENBUHL. 2005. Cellular and molecular basis for antigen transport across epithelial barriers. *In* Mucosal Immunology, 3rd ed. J. Mestecky, M.E. Lamm, W. Strober, *et al.*, Eds.: 111–130. Elsevier Academic Press. Amsterdam.
22. SHOENFELD, Y. & N.R. ROSE. 2004. Infection and Autoimmunity. Elsevier. Amsterdam.
23. BACH, J.F. 2002. The effect of infections on susceptibility to autoimmune and allergic diseases. N. Engl. J. Med. **347:** 930–931.
24. KITA, H., X.S. HE & M.E. GERSHWIN. 2004. Autoimmunity and environmental factors in the pathogenesis of primary biliary cirrhosis. Ann. Med. **36:** 72–80.
25. TOUBI, E. & Y. SHOENFELD. 2004. Toll-like receptors and their role in the development of autoimmune diseases. Autoimmunity **73:** 183–188.
26. MACDONALD, T.T., G. MONTELEONE & S.L. PENDER. 2000. Recent developments in the immunology of inflammatory bowel disease. Scand. J. Immunol. **52:** 2–9.
27. STROBER, W. & R.O. EHRHARDT. 1993. Chronic intestinal inflammation: an unexpected outcome in cytokine or T cell receptor mutant mice. Cell **78:** 203–295.
28. STROBER, W., I.J. FUSS & R.S. BLUMBERG. 2002. The immunology of mucosal models of inflammation. Annu. Rev. Immunol. **20:** 495–549.
29. ELSON, C.O., A. KONRAD, Y. CONGH, *et al.* 2004. Gene disruption and immunity in experimental colitis. Inflamm. Bowel Dis. **10:** S25–S28.
30. SARTOR, R.B. 2005. Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. Gastroenterology **126:** 1620–1633.
31. SARTOR, R.B. 2003. Innate immunity in the pathogenesis and therapy of IBD. J. Gastroenterol. **38** (Suppl. 15): 43–47.
32. BENDJELLOUL, F., P. MALY, V. MANDYS, *et al.* 2000. Intercellular adhesion molecule-1 (ICAM-1) deficiency protects mice against severe forms of experimentally induced colitis. Clin. Exp. Immunol. **119:** 57–63.
33. HUDCOVIC, T., R. STEPÁNKOVÁ, J. CEBRA, *et al.* 2001. The role of microflora in the development of intestinal inflammation: acute and chronic colitis induced by dextran sulfate in germ-free and conventionally reared immunocompetent and immunodeficient mice. Folia Microbiol. (Praha) **46:** 565–572.
34. STEPÁNKOVÁ, R., H. TLASKALOVÁ, F. POWRIE, *et al.* 2004. The role of segmented filamentous bacteria (SFB) in experimental colitis induced in SCID mice reconstituted with CD4+CD45RB/hight T cells [abstract]. Gut **36** (Suppl. 1): A305.
35. CUKROWSKA, B., R. LODINOVÁ-ZÁDNÍKOVÁ, C. ENDERS, *et al.* 2002. Specific proliferative and antibody responses of premature infants to intestinal colonization with nonpathogenic probiotic *E. coli* strain Nissle 1917. Scand. J. Immunol. **55:** 204–209.

36. LODINOVÁ-ZÁDNÍKOVÁ, R., B. CUKROWSKA & H. TLASKALOVÁ-HOGENOVÁ. 2003. Oral administration of probiotic *Escherichia coli* after birth reduces frequency of allergies and repeated infections later in life. Int. Arch. Allergy Immunol. **131:** 209–211.
37. KALLIOMAKI, M.A. & E. ISOLAURI. 2004. Probiotics and down-regulation of the allergic response. Immunol. Allergy Clin. North Am. **24:** 739–752.
38. CUMMING, J.H., J.M. ANTOINE, E. AZPIROZ, *et al.* 2004. PASSCLAIM—gut health and immunity. Eur. J. Nutr. **43** (Suppl. 2): II118–II173.
39. KOZÁKOVÁ, H., Z. REHÁKOVÁ & J. KOLÍNSKÁ. 2001. Bifidobacterium bifidum monoassociation of gnotobiotic mice: effect on enterocyte brush-border enzymes. Folia Microbiol. (Praha) **46:** 573–576.
40. DANESE, S., M. SANS & C. FIOCCHI. 2004. Inflammatory bowel disease: the role of environmental factors. Autoimmun. Rev. **3:** 394–400.
41. STEPÁNKOVÁ, R., H. TLASKALOVÁ, J. SINKORA, *et al.* 1996. Changes in jejunal mucosa after long-term feeding of germfree rats with gluten. Scand. J. Gastroenterol. **31:** 551–557.
42. SOLLID, L.M. 2002. Coeliac disease: dissecting a complex inflammatory disorder. Nat. Rev. Immunol. **2:** 647–655.
43. SCHUPPAN, D., B. ESSLINGER & W. DIETRICH. 2003. Innate immunity and coeliac disease. Lancet **362:** 3–5.
44. CERF-BENSUSSAN, N., N. BROUSSE, C. CAILLAT-ZUCMAN, *et al.* 2003. Coeliac Disease. John Libbey Eurotext. Esber, UK.
45. KRUPICKOVÁ, S., L. TUCKOVÁ, Z. FLEGELOVÁ, *et al.* 1999. Identification of common epitopes on gliadin, enterocytes, and calreticulin recognised by antigliadin antibodies of patients with celiac disease. Gut **44:** 168–173.
46. ROSE, N.R. 2002. Mechanisms of autoimmunity. Semin. Liver Dis. **22:** 387–394.
47. NOVÁK, P., P. MAN, L. TUCKOVÁ, *et al.* 2002. Monitoring of in vitro deamidation of gliadin peptic fragments by mass spectrometry may reflect one of the molecular mechanisms taking place in celiac disease development. J. Mass Spectrom. **37:** 507–511.
48. TUCKOVÁ, L., H. TLASKALOVÁ-HOGENOVÁ, Z. FLEGELOVÁ, *et al.* 2000. Activation of macrophages by food antigens: enhancing effect of gluten on nitric oxide and cytokine production. J. Leukoc. Biol. **67:** 312–318.
49. TUCKOVÁ, L., J. NOVOTNÁ, P. NOVÁK, *et al.* 2002. Activation of macrophages by gliadin fragments: isolation and characterization of active peptide. J. Leukoc. Biol. **71:** 625–631.
50. JELÍNKOVÁ, L., L. TUCKOVÁ, J. CINOVÁ, *et al.* 2004. Gliadin stimulates human monocytes to production of IL-8 and TNF-γ through a mechanism involving NF-κB. FEBS Letters **571:** 81–85.
51. MAIURI, M.C., D. DE STEFANO, G. MELE, *et al.* 2003. Nuclear factor -B is activated in small intestinal mucosa of celiac patients. J. Mol. Med. **81:** 373–379.
52. NIKULINA, M., C. HABICH, S.B. FLOHE, *et al.* 2004. Wheat gluten causes dendritic cell maturation and chemokine secretion. J. Immunol. **173:** 1925–1933.

GLIADIN PEPTIDES ACTIVATE BLOOD MONOCYTES FROM PATIENTS WITH CELIAC DISEASE

Journal of Clinical Immunology, Vol. 27, No. 2, March 2007 (© 2006)
DOI: 10.1007/s10875-006-9061-z

Gliadin Peptides Activate Blood Monocytes from Patients with Celiac Disease

JANA CINOVA,[1] LENKA PALOVÁ-JELÍNKOVÁ,[1] LESLEY E. SMYTHIES,[2,6] MARIE ČERNÁ,[3] BARBARA PECHAROVÁ,[1] MILOS DVOŘÁK,[4] PAVEL FRUHAUF,[4] HELENA TLASKALOVÁ-HOGENOVÁ,[1] PHILLIP D. SMITH,[2,5] and LUDMILA TUČKOVÁ[1]

Received August 30, 2006; accepted December 1, 2006
Published online: 27 January 2007

To elucidate the role of innate immune responses in celiac disease, we investigated the effect of gliadin on blood monocytes from patients with celiac disease. Gliadin induced substantial TNF-α and IL-8 production by monocytes from patients with active celiac disease, lower levels by monocytes from patients with inactive celiac disease, and even lower levels by monocytes from healthy donors. In healthy donor monocytes gliadin induced IL-8 from monocytes expressing HLA-DQ2 and increased monocyte expression of the costimulatory molecules CD80 and CD86, the dendritic cell marker CD83, and the activation marker CD40. Gliadin also increased DNA binding activity of NF-κB p50 and p65 subunits in monocytes from celiac patients, and NF-κB inhibitors reduced both DNA binding activity and cytokine production. Thus, gliadin activation of HLA-DQ2$^+$ monocytes leading to chemokine and proinflammatory cytokine production may contribute to the host innate immune response in celiac disease.

KEY WORDS: Celiac disease; Innate immunity; Blood monocytes.

INTRODUCTION

Celiac sprue is a chronic inflammatory disorder of the small intestine induced by dietary gluten or related rye and barley proteins in genetically predisposed individuals. More than 90% of patients with celiac disease express the HLA class II molecule HLA-DQ2, encoded by the DQA1*05 and DQB1*02 alleles, compared to 20–30% in the general population in Europe and Northern America. Symptoms and pathological changes in the small intestine, including villous atrophy, crypt hyperplasia, infiltration of inflammatory cells, and activation of the infiltrating inflammatory cells, are effectively treated by the exclusion of gluten from the diet (1–6).

Lamina propria CD4$^+$ T cells that produce interferon (IFN)-γ in response to gliadin and intraepithelial CD8$^+$ lymphocytes cytotoxic for epithelial cells expressing MHC class I polypeptide-related sequence A (MICA) appear to play fundamental roles in the pathogenesis of celiac disease (7–14). The activation of local as well as systemic humoral responses is reflected in the presence of circulating IgG and IgA antibodies to gliadin, endomysium, tissue transglutaminase (tTG), and other autoantigens (15–22).

Recent evidence indicates that innate immune response cells, including monocytes/macrophages and dendritic cells (DCs), also contribute to celiac disease pathogenesis. Gliadin fragments formed after pepsin digestion induce mouse peritoneal macrophages to produce TNF-α, IL-10, and RANTES and to release increased amounts of nitric oxide in the presence of IFN-γ (23–25). In addition, gliadin triggers NF-κB activation, interferon regulatory factor (IRF)-1 release, and STAT-1-mediated signal transduction in mouse monocytes (26). Importantly, gliadin-induced activation of human monocytes appears to be dependent on the stage of cell differentiation (23–25, 27–29). Gliadin fragments also induce phenotypic and functional maturation of human monocyte-derived dendritic cells (30).

[1]Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic.
[2]Department of Medicine (Gastroenterology), University of Alabama at Birmingham, Birmingham, Alabama.
[3]3rd Medical Faculty, Charles University, Prague, Czech Republic.
[4]1st Medical Faculty, Charles University, Prague, Czech Republic.
[5]VA Medical Center, Birmingham, Alabama.
[6]To whom correspondence should be addressed to Department of Medicine (Gastroenterology), University of Alabama at Birmingham, 703 19th Street South, ZRB 633, Birmingham, Alabama, 35294; e-mail: lesmy@uab.edu.

To further understand the possible role of innate immune cells in celiac disease, we investigated whether peptic fragments of gliadin activate blood monocytes and the mechanism of that activation, and whether the gliadin-induced response of monocytes from celiac patients with active disease differs from the response of monocytes from patients with inactive disease on a gluten-free diet.

METHODS

Food Proteins

Peptic fragments of gliadin and soya protein were prepared using pepsin-agarose gel (ICN, Biomedicals, Inc., Ohio), as previously described (24, 30). Protein concentrations were measured by Bicinchoninic acid assay (BCA Protein assay, Pierce, Rockford, IL). All reagents were tested by the E-toxate test for lipopolysaccharide (LPS) (Sigma, St. Louis, MO) and shown to be below the limit of detection (2 pg/mL).

Study Subjects

The study population consisted of 54 patients with biopsy-proven celiac disease, including 14 symptomatic, untreated subjects [ESPGAN criteria; (31)] and 40 asymptomatic, treated (gluten-free diet) subjects. The control group consisted of 45 healthy blood donors. Patients were recruited after appropriate local ethics committee approval, and informed consent was obtained from all subjects.

Cells and Their Activation

Peripheral blood mononuclear cells were isolated by Ficoll paque (Amersham Biosciences, Piscataway, NJ) and incubated for 24 h in 24-well plates (Nunc, Roskilde, Denmark). Nonadherent cells were removed by washing, and the adherent monocytes were recultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 μg/mL streptomycin, and 100 U/mL penicillin (Sigma). The monocytes were removed by scraping and analyzed by flow cytometry (BD FACSVantage SE, San Jose, CA) after staining with the indicated monoclonal antibodies (mAbs). The monocytes (70–80% $CD14^+$) were cultured at a concentration of $1 \cdot 10^6$ cells/mL for 24 h in complete RPMI-1640 with gliadin (100–200 μg/mL) alone or with gliadin (100 μg/mL) plus IFN-γ (150 U/mL; R&D Biosciences, Minneapolis, MN), as described previously (7, 29, 32). In parallel monocyte cultures, the NF-κB inhibitors L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK, 1.0–25.0 μM) or pyrrolidine dithiocarbamate (PDTC, 1.0–10.0 μM) (both Sigma) were added to the cells for 30 min prior to the addition of gliadin. Cultures were maintained at 37° C, 5% CO_2 in a humidified incubator.

Flow Cytometric Analysis

To evaluate the effect of gliadin fragments on monocyte surface antigen expression, blood monocytes ($5 \cdot 10^5$) were incubated with optimal concentrations of FITC-labeled mouse mAbs to HLA-DR, CD40, CD80, CD83, CD86 (all from BD Biosciences Pharmingen), and CD14 and MEM-18 (kindly provided by V. Hořejší, Charles University, Prague). Cells also were incubated with FITC- or PE-conjugated irrelevant antibodies of the same concentration and isotype. After two washings, cells were resuspended in ice-cold PBS with 0.1% NaN_3 or fixed with 2% paraformaldehyde and analyzed by flow cytometry (FACSCalibur, BD Bioscience). Data were evaluated using CellQuest software (BD Biosciences). Staining with propidium iodide was performed to assess cell viability.

Preparation of Nuclear Extracts and Colorimetric NF-κB Assays

Nuclear extracts were prepared from purified monocytes stimulated for 90 min with gliadin digest (100 μg/mL) as mentioned earlier, with or without TPCK (1–25 μM) and PDTC (1–10 μM), using the Nuclear Extract Kit (Active Motif, Carlsbad, CA). NF-κB DNA binding activity was determined using the TransAM NF-κB family transcription factor assay (Active Motif), according to the manufacturer's protocol. Briefly, microwells coated with a double-stranded oligonucleotide containing the NF-κB consensus sequence were incubated with nuclear extracts for 1 h at room temperature and washed with washing buffer. The wells containing captured active transcription factor were incubated for 1 h with mAb specific for p50 or p65 NF-κB subunits, then for 1 h with anti-rabbit IgG coupled to horseradish peroxidase and, after washing, exposed to developing solution for 10 min. Optical density was measured at 450 nm using a Titertec Multiscan MCC/340 (Flow Lab., Irvine, Scotland).

Measurement of IL-8 and TNF-α Proteins

The amounts of IL-8 and TNF-α in supernatants of monocytes ($1 \cdot 10^6$ cells/mL) cultured for 24 h with or without gliadin (100 μg/mL) in the presence or absence of IFN-γ (150 U/mL) were determined by ELISA (R&D Systems, Europe, Germany) according to the manufacturer's instructions.

Preparation of Genomic DNA

QIAamp spin columns (QIAGEN, GmbH, Hilden, Germany) were used for rapid DNA purification according to the specifications of the manufacturer. Briefly, lysis buffer and proteinase K were added to the monocyte cultures, which were mixed by pulse-vortexing and incubated for 10 min at 56° C. After the addition of 100% ethanol, the mixture was applied to the QIAamp Spin Column, centrifuged, and the filtrate discarded. After the QIAamp Spin Column was washed with washing buffers, elution buffer was applied, and the column was incubated at 15–20° C for 1 min. The DNA filtrate was collected by centrifugation and stored at 4° C.

Analysis of the HLA Class II Molecule HLA-DQ2

Genes were typed by PCR with sequence-specific primers (33, 34). Oligonucleotide primers (Genovision, West Chester, PA) were designed to amplify the second exon of the class II genes. PCR amplification was carried out in a final volume of 10 μL containing: 6 ng/μL of genomic DNA, PCR Master Mix (200 μM of each dNTP, PCR buffer: 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.3, 0.001% w/v gelatin, 5% glycerol, 100 μg/mL cresol red) complete with 0.4 U/μL Taq polymerase and specific primer mix. Thermal cycling was accomplished by heating the sample at 94° C for 2 min and then running 10 cycles of 10 s at 94° C (denaturation), 60 s at annealing temperature 65° C, and 20 cycles of 10 s at 94° C (denaturation), 50 s at annealing temperature 61° C, 30 s at 72° C (extension). After 30 cycles, the extension was completed, and the samples were stored at 4° C.

Statistical Analysis

The differences among healthy donors and active and treated celiac patients groups were evaluted by the Kruskal–Wallis test and Mann–Whitney test for pairwise comparison. The levels of significance of the Mann–Whitney tests were adjusted according to Holm's method (35). P values < 0.05 were considered significant.

RESULTS

Cytokine Production by Gliadin-Stimulated Monocytes

Since monocytes are a major source of the chemokine IL-8 and the proinflammatory cytokine TNF-α, we evaluated blood monocytes from celiac patients and healthy donors for spontaneous and inducible IL-8 and TNF-α production. Monocytes from patients with active celiac disease and patients with inactive celiac disease on a gluten-free diet spontaneously released low levels of IL-8 (525 • 404 pg/mL and 965 • 682 pg/mL, respectively, $P > 0.05$), which were significantly greater than the amounts spontaneously released by monocytes from healthy control subjects (131 • 202 pg/mL) ($P < 0.001$) (Fig. 1A). The addition of IFN-γ (150 U/mL) to the cultures did not significantly enhance IL-8 production by monocytes from patients or healthy donors. In sharp contrast, the addition of gliadin fragments (100 μg/mL) to the cultures induced monocytes from all three groups to secrete markedly higher levels of IL-8; monocytes from patients with active celiac disease produced more IL-8 (3365 • 2451 pg/mL than monocytes from patients with inactive disease on a gluten-free diet (2645 • 1723 pg/mL), which in turn produced more IL-8 than monocytes from healthy donors (1046 • 783 pg/mL) ($P < 0.001$). The production of

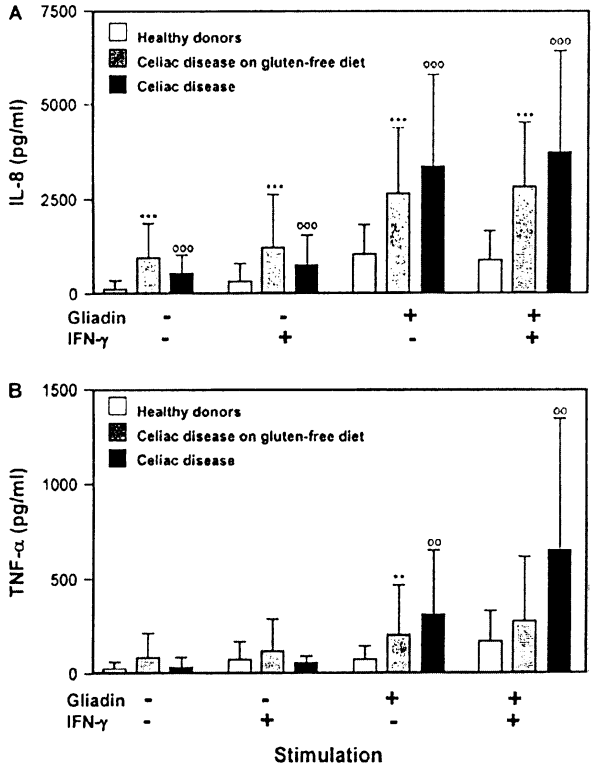

Fig. 1. IL-8 and TNF-α production by monocytes from patients with celiac disease. Monocytes isolated from patients with active celiac disease (n • 14), asymptomatic patients with celiac disease on a gluten-free diet (n • 40), and healthy donors (n • 45) were cultured for 24 h with gliadin (100 μg/mL) alone or with gliadin plus IFN-γ (150 U/mL). The amounts of (A) IL-8 and (B) TNF-α (mean • SD) released into the supernatants were determined by ELISA. The level of significance for patients versus healthy donors are indicated as follows: $^{**}P < 0.01$, $^{***}P < 0.001$, $^{oo}P < 0.01$, $^{ooo}P < 0.001$.

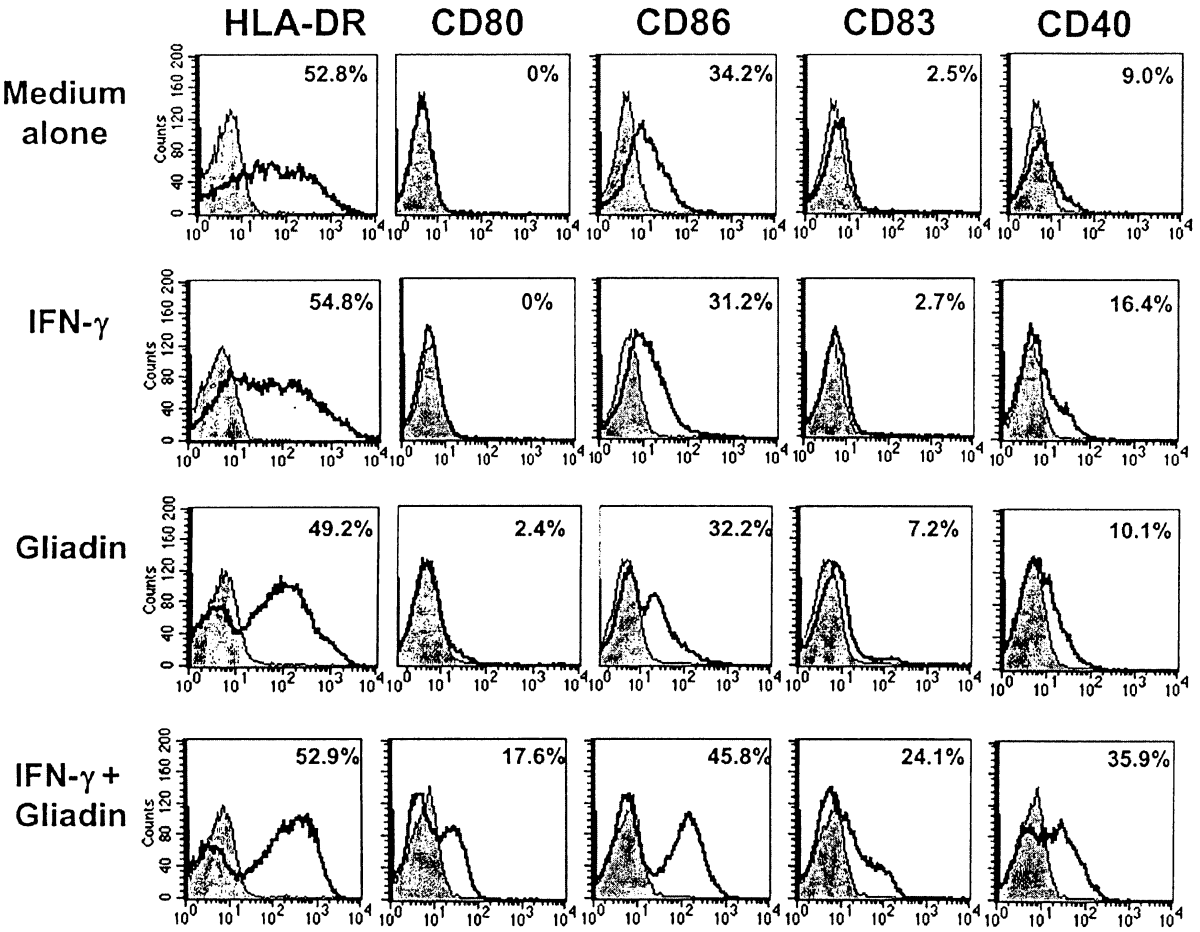

Fig. 2. Effect of gliadin on monocyte phenotype. Blood monocytes from healthy donors were incubated for 24 h in media or with IFN-γ alone, gliadin alone, or IFN-γ plus gliadin at the indicated concentrations and then analyzed by FACS for the expression of activation and differentiation markers. Staining after exposure to isotype-matched Ig is shown as a *shaded histogram*. Values are the percent monocytes that expressed the indicated marker from a representative donor (n • 3).

TNF-α by monocytes from the three groups paralleled that of IL-8 (Fig. 1B). The addition of IFN-γ did not further enhance gliadin-induced IL-8 production, but did enhance TNF-α production. Similar results were obtained for IL-8 and TNF-α production when the monocytes were preincubated with IFN-γ (data not shown). In contrast to gliadin, soya proteins (100 μg/mL) with or without IFN-γ did not induce production of IL-8 or TNF-α by monocytes from the three groups.

Gliadin Fragments Induce Differentiational Changes in Monocyte Surface Phenotype

Systemic immune activation is well documented in patients with celiac disease (36–39). In related studies (30), we have shown that gliadin fragments induce maturation of monocyte-derived dendritic cells. Therefore, we determined whether incubation of blood monocytes from healthy persons with gliadin fragments (100 μg/mL) induced changes in monocyte phenotype. Incubation of blood monocytes with gliadin, and to a lesser extent with IFN-γ (150 U/mL) alone, induced a slight increase in the percentage of cells expressing dendritic cell (DC) markers of maturation (CD83) and activation (CD80, CD40), compared to monocytes incubated in medium (Fig. 2). Importantly, incubation of monocytes with gliadin fragments plus IFN-γ induced a marked upregulation in the surface density and percentage of cells expressing CD80, CD86, CD83, and CD40 (Fig. 2). In contrast to the ability of gliadin to induce phenotypic changes, soya proteins (in the presence or absence of IFN-γ) did not alter

Table I. Interleukin (IL)-8 Production by HLA-DQ2-Positive and -Negative Monocytes from Healthy Donors

		Monocyte IL-8 Production (pg/mL)[a]					
HLA Type	Donors (n)	Medium	Gliadin 100 μg/mL	Gliadin 100 μg/mL • IFN-γ 150 U/mL	Gliadin 200 μg/mL	Gliadin 200 μg/mL • IFN-γ 150 U/mL	IFN-γ 150 U/mL
HLA-DQ2$^+$	11	228 • 116	1792 • 272	1463 • 358	1959 • 271	1801 • 394	566 • 222
HLA-DQ2$^-$	34	98 • 24	788 • 116	677 • 97	1073 • 121	809 • 96	273 • 68

[a]Values are expressed as mean • SEM of IL-8 protein.

monocyte surface protein expression (data not shown). This altered phenotype suggests a synergistic effect of gliadin and IFN-γ on monocyte activation.

Effect of HLA-DQ2 on Monocyte Cytokine Production

Since the majority of celiac patients are HLA-DQ2$^+$, we studied the relationship between HLA genotype and monocyte IL-8 production in healthy control subjects. Monocytes from HLA-DQ2$^+$ healthy donors secreted IL-8 spontaneously. However, in the presence of gliadin or IFN-γ, the monocytes released two- to three-fold more IL-8 than monocytes from HLA-DQ2$^-$ donors (Table I). These findings suggest that the HLA-DQ2$^+$ genotype predisposes monocytes to increased IL-8 secretion.

Gliadin Induces Monocyte Cytokine Production via NF-κB Activation

We have shown perviously that NF-κB activation is involved in gliadin-induced cytokine production by monocyte-derived DCs and THP-1 cells (30). Therefore, we next determined whether the NF-κB signal transduction pathway is also involved in gliadin-induced cytokine production by monocytes. Stimulation of monocytes from celiac patients with gliadin digest (200 μg/mL) resulted in a marked increase in the binding activity of the NF-κB p50 and p65 subunits ($P < 0.05$). Incubation of monocytes from celiac patients with the NF-κB inhibitor TPCK reduced p50 and p65 binding to 20% and 54%, respectively (Fig. 3). Supporting the role of NF-κB in gliadin-induced cytokine production by monocytes from patients with active celiac disease, NF-κB inhibitors also reduced gliadin-induced IL-8 and TNF-α production (Fig. 4). In contrast, stimulation of monocytes from healthy subjects with gliadin caused weak NF-κB binding, which was completely inhibited by TPCK (25 μM/mL), and reduced cytokine production to background levels.

DISCUSSION

We report for the first time that gliadin fragments stimulate cytokine production by blood monocytes from patients with celiac disease. The level of cytokine production varied with disease activity, as monocytes from patients with active celiac disease produced more gliadin-induced TNF-α and IL-8 than monocytes from patients with inactive disease, which in turn produced more inducible TNF-α and IL-8 than monocytes from healthy subjects. We also investigated whether IFN-γ further enhanced gliadin-induced TNF-α and IL-8 production by blood monocytes, since gliadin-induced T cells from patients with celiac disease produce increased levels of IFN-γ. IFN-γ plus gliadin upregulated TNF-α, but not IL-8,

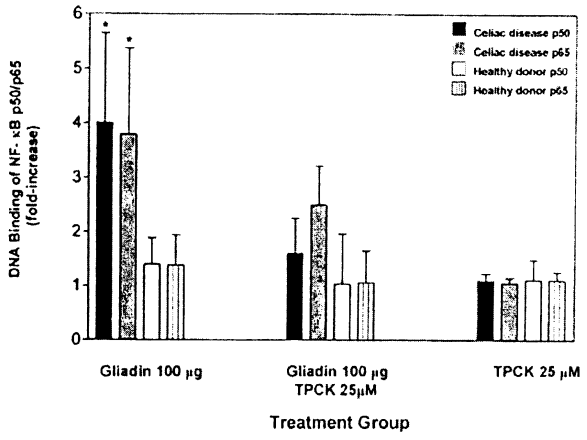

Fig. 3. Gliadin induction of monocyte NF-κB p50 and p65 subunit binding. Monocytes from patients with active celiac disease and healthy donors were analyzed for NF-κB DNA binding activity after a 90 min incubation with gliadin (in the presence or absence of the NF-κB inhibitor TPCK). Data are presented as the fold-increase in DNA binding activity by gliadin-stimulated versus nonstimulated monocytes (mean • SD for three separate experiments). $^*P < 0.05$ corresponds to the binding activity of monocytes from celiac patients versus that of monocytes from healthy donors.

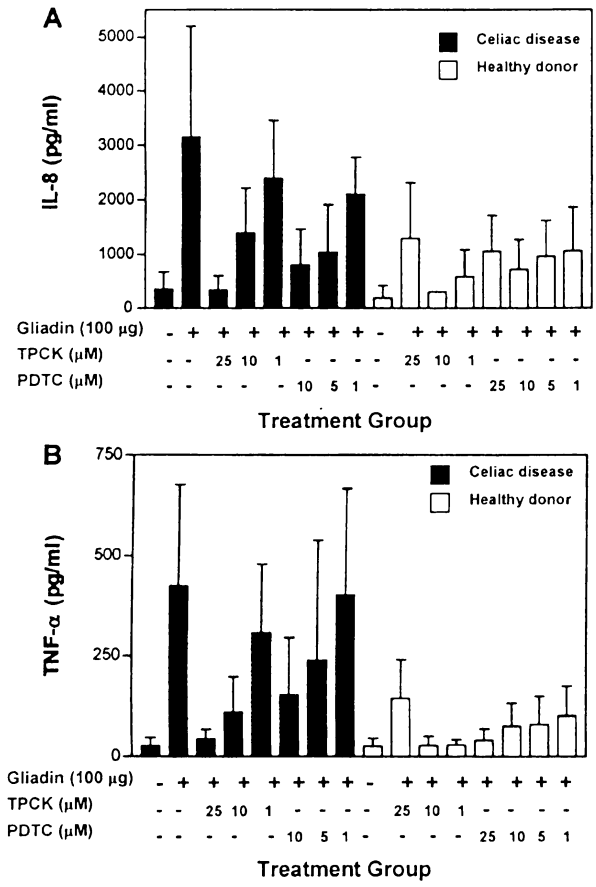

Fig. 4. NF-κB inhibitors block gliadin-induced monocyte IL-8 and TNF-α production. Monocytes from patients with celiac disease and healthy donors were cultured for 24 h with gliadin in the presence or absence of the NF-κB inhibitors TPCK (1–25 μM) or PDTC (1–10 μM), and the amount of IL-8 and TNF-α released into the culture supernatants were measured by ELISA. Results are expressed as mean • SD of cytokine production of monocytes from three patients with active celiac disease and six healthy donors.

production. These data suggest that monocytes from celiac patients are activated *in vivo*, consistent with the increased levels of other cytokines, i.e., IL-6, that have been detected in patients with celiac disease (38, 39). Coincident with gliadin-induced monocyte activation, gliadin also induced a DC phenotype in blood monocytes, consistent with our earlier observation that gliadin promoted maturation of monocyte-derived DCs (30). Moreover, coincubation of gliadin with IFN-γ further enhanced the upregulation in the surface density and percentage of cells expressing CD80, CD86, CD83, and CD40, suggesting a synergistic effect of gliadin and IFN-γ on monocyte activation. Since our data indicates that gliadin enhances monocyte IL-8 release, and the majority of celiac patients are HLA-DQ2$^+$ (1–6), we examined the ability of monocytes from HLA-DQ2$^+$ and HLA-DQ2$^-$ healthy subjects to release IL-8 following exposure to gliadin. Monocytes from HLA-DQ2$^+$, but not HLA-DQ2$^-$, healthy subjects spontaneously released IL-8, and monocytes from HLA-DQ2$^+$ persons released two- to threefold more IL-8 than monocytes from HLA-DQ2$^-$ persons. These findings suggest that the enhanced cytokine production by monocytes from patients with celiac disease is due, at least in part, to the activated HLA-DQ2$^+$ population of monocytes.

Celiac disease is characterized by increased infiltration of lymphocytes into the lamina propria and epithelium. Increased production of IL-8 and TNF-α by cells of the innate immune system (monocytes, macrophages, DCs) within the lamina propria could contribute to the pathogenesis of celiac disease through their ability to recruit lymphocytes to the mucosa. In this regard, IL-8 is reported chemotactic for T lymphocytes (40, 41) at concentrations equivalent to those reported in our study (40). Moreover, TNF-α induces the upregulation of vascular ICAM-1, a receptor for the adhesion molecule LFA-1 on memory T cells (42–44). Thus, the production of TNF-α by gliadin-stimulated monocytes in the lamina propria would increase adhesion properties on mucosal vessels and thereby promote T-lymphocyte infiltration into the adjacent mucosa (in celiac disease). In this regard, increased mucosal expression of ICAM-1 has been reported in the intestinal mucosa of patients with celiac disease (42, 43). In addition, IFN-γ, which is abundant in the mucosa of patients with celiac disease (44, 45) and has been implicated in T-lymphocyte recruitment (42–44) (via ICAM-1 upregulation) also has an additive effect on TNF-α-induced T-cell migration (43, 46). Thus, IFN-γ alone, and together with TNF-α, could contribute to T-lymphocyte recruitment in celiac disease. Our findings, therefore, suggest an important potential mechanism whereby gliadin-stimulated release of IL-8 and TNF-α from mucosal innate immune cells contribute to the recruitment of T cells to the mucosa.

While our report has focused on the potential inflammatory effects of gliadin on innate immune cells of the monocytic lineage, other cells within the mucosa, such as epithelial cells, also likely contribute to the innate mucosal response to gliadin in celiac disease. To our knowledge, there are no data available concerning gliadin-induced IL-8 production by primary intestinal epithelial cells, but gliadin is known to alter intercellular tight junction and barrier function in intestinal epithelial cell lines (47, 48), possibly through the induction of zonulin from gliadin-stimulated epithelial cells (47) and macrophages (49).

Members of the NF-κB/Rel family, including the p50 and p65 subunits, regulate inflammatory and immune

responses by inducing the expression of specific genes (50). We show here that exposure of monocytes to gliadin increased the binding activity of the p50 and p65 subunits. Increased p50 and p65 binding was more pronounced in monocytes from celiac patients with active disease than in monocytes from healthy subjects. This finding is consistent with our previous observations that gliadin activates the NF-κB p50/p65 complex in human monocyte-derived DCs (30) and THP-1 monocytes (29) and with observations by others that gliadin activation of macrophages is MyD88- and NF-κB-dependent (26, 49). In this regard, both NF-κB/DNA binding activity and p50/p65 nuclear levels are reported to be elevated in the inflamed mucosa of celiac patients (51). The involvement of the NF-κB/IκB complex in gliadin-induced activation of monocytes was confirmed using the NF-κB inhibitors TPCK, which prevents degradation of IκB inhibitor, and PDTC, which blocks dissociation of the NF-κB/IκB complex (52, 53). Both NF-κB inhibitors substantially reduced gliadin-induced NF-κB binding as well as TNF-α and IL-8 secretion by monocytes. Taken together, these findings underscore the likely involvement of monocyte/macrophage-derived innate immune reponses in celiac disease and suggest that NF-κB regulation should be considered in designing future therapeutic strategies.

In normal persons, wheat gluten, the major source of dietary gliadin, does not induce the T-cell activation or intestinal inflammation characteristic of celiac disease. In addition, intestinal macrophages isolated from healthy subjects do not respond to gliadin *in vitro* (Smythies, unpublished observation), which is consistent with the profound inflammation anergy characteristic of intestinal macrophages (54). Since intestinal macrophages are derived from blood monocytes (55), our findings suggest that local mucosal factors likely downregulate the ability of monocytes newly recruited to the mucosa to respond to gliadin. Since the "normal" downregulation of gliadin-induced monocyte activation and differentiation may be impaired in celiac disease, indentification of these mucosa-derived factors could have important therapeutic implications.

CONCLUSIONS

In summary, gliadin-derived fragments activate monocytes from celiac patients and HLA-DQ2$^+$ monocytes from healthy donors. The higher activity of monocytes in celiac patients could augment gliadin-specific immune responses and thus contribute to the pathogenesis of celiac disease.

ACKNOWLEDGMENTS

This work was supported by the Grant Agency of the Czech Republic (Grants 310/05/2245 and 310/03/H147); the Grant Agency of the Czech Academy of Sciences (Grants AA5020210, AA5020205, 1QS500200572, and B5020407); the Grant Agency of the Ministry of Education (MSM 0021620814, GA UK 30/2006), Mininstry of Agriculture 1B53002 and Institutional Research Concept (Grant AV0Z50200510); and the National Institutes of Health (U.S.) (DK-74033, DK-47322, DK-54495, HD-41361, and DK-064400); the Crohn's and Colitis Foundation of America; and the Research Service of the Veterans Administration

REFERENCES

1. Trier JS: Celiac sprue. N Engl J Med 325:1709–1719, 1991
2. Marsh MN: Gluten, major histocompatibility complex, and the small intestine. A molecular and immunbiologic approach to the spectrum of gluten sensitivity (celiac sprue). Gastroenterology 102:330–354, 1992
3. Maki M, Collin P: Coeliac disease. Lancet 349:1755–1759, 1997
4. Schuppan D: Current concepts of celiac disease pathogenesis. Gastroenterology 119:234–242, 2000
5. Sollid LM, Markussen G, Ek J, Gjerde H, Vartdal F, Thorsby E: Evidence for a primary association of celiac disease to a particular HLA DQ alpha/beta heterodimer. J Exp Med 169:345–350, 1989
6. Sollid LM: Coeliac disease: Dissecting a complex inflammatory disorder. Nat Rev Immunol 2:647–655, 2002
7. Nilsen EM, Jahnsen FL, Lundin KE, Johansen FE, Fausa O, Solid LM, Jahnsen J, Scott H, Brandtzaeg P: Gluten induced an intestinal cytokine response strongly dominated by interferon gamma in patients with celiac disease. Gastroenterology 115:551–563, 1998
8. Strober W, Fuss IJ: Gluten-sensitive enteropathy and other immunologically mediated enteropaties. *In* Mucosal Immunology, PL Ogra, J Mestecky, ME Lamm (eds). San Diego, CA, Academic, 1999, pp 1101–1128
9. Eiras P, Leon F, Camarero C, Lombardia M, Roldan E, Bootello A, Roy G: Intestinal intraepithelial lymphocytes contain a CD3-CD7$^+$ subset expressing natural killer markers and a singular pattern of adhesion molecules. Scand J Immunol 52:1–6, 2000
10. Hue S, Mention JJ, Monteiro RC, Zhang S, Cellier C, Schmitz J, Verkarre V, Fodil N, Bahram S, Cerf-Bensussan N, Caillat-Zucman S: A direct role for NKG2D/MICA interaction in villous atrophy during celiac disease. Immunity 21:367–377, 2004
11. Meresse B, Chen Z, Ciszewski C, Tretiakova M, Bhagat G, Krausz TN, Raulet DH, Laner LL, Groh V, Spies T, Ebert EC, Green PH, Jabri B: Coordinated introduction by IL-15 of TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. Immunity 21:357–366, 2004
12. Sollid LM: Intraepithelial lymphocytes in celiac disease: Licence to kill revealed. Immunity 21:303–304, 2004
13. Gianfrani C, Auricchio S, Troncone R: Adaptive and innate immune response in celiac disease. Immunol Lett 99:141–145, 2005

14. Ciccocioppo R, Di Sabatino A, Corazza GR: The immune recognition of gluten in celiac disease. Clin Exp Immunol 140:408–416, 2005
15. Valeski JE, Kumar V, Beutner EH, Lerner A, Chorzelski TP: Immunology of coeliac disease: Tissue and species specificity of endomysial and reticulin antibodies. Int Arch Allergy Appl Immunol 93:1–7, 1990
16. Karpati S, Burgin-Wolff A, Krieg T, Meurer M, Stolz W, Braun-Flaco O: Binding to human jejunum of serum IgA antibody from children with coeliac disease. Lancet 336:1335–1338, 1990
17. Volta U, Molinaro N, Fratangelo D, Bianchi FB: IgA antibodies to jejunum. Specific immunity directed against target organ of gluten-sensitive enteropathy. Dig Dis Sci 39:1924–1929, 1994
18. Ascher H, Hahn-Zoric M, Hanson LA, Kilander AF, Nilsson LA, Tlaskalova H: Value of serological markers for clinic diagnosis and population studies of coeliac disease. Scand J Gastroenterol 31:61–67, 1996
19. Karska K, Tuckova L, Steiner L, Tlaskalova-Hogenova H, Michalak M: Calreticulin—the potential autoantigen in celiac disease. Biochem Biophys Res Commun 209:597–605, 1995
20. Dieterich W, Laag E, Schopper H, Volta U, Ferguson A, Gillett H, Riecken EO, Schuppan D: Autoantibodies to tissue transglutaminase as predictor of coeliac disease. Gastroenterology 115:1317–1321, 1998
21. Stulik J, Hernychova L, Porkertova S, Pozler O, Tuckova L, Sanchez D, Bures J: Identification of new celiac disease autoantigens using proteomic analysis. Proteomics 3:951–956, 2003
22. Sollid LM, Jabri B: Is celiac disease an autoimmune disorder? Curr Opin Immunol 17:595–600, 2005
23. Tuckova L, Flegelova Z, Tlaskalova-Hogenova H, Zidek Z: Activation of macrophages by food antigens: Enhancing effect of gluten on nitric oxide and cytokine production. J Leukoc Biol 67:312–318, 2000
24. Tuckova L, Novotna J, Novak P, Flegelova Z, Kveton T, Jelinkova L, Zidek Z, Man P, Tlaskalova-Hogenova H, Bezouska K, Havlicek V: Activation of macrophages by gliadin fragments: Isolation and characterization of active peptide. J Leukoc Biol 71:625–631, 2002
25. Novak P, Man P, Tuckova L, Tlaskalova-Hogenova H, Bezouska K, Havlicek V: Monitoring of *in vitro* deamidation of gliadin peptic fragments by mass spectrometry may reflect one of the molecular mechanisms taking place in celiac disease development. J Mass Spectrom 37:507–511, 2002.
26. De Stefano D, Maiuri MC, Iovine B, Ialenti A, Bevilacqua MA, Carnuccio R: The role of NF-kappa B, IRF-1, and STAT-1 alpha transcription factors in the iNOS gene induction by gliadin and IFN-gamma in RAW 264.7 macrophages. J Mol Med 84:65–74, 2006
27. Maiuri L, Ciacci C, Ricciardelli I, Vacca L, Raia V, Auricchio S, Picard J, Osman M, Quaratinos S, Londei M: Association between innate response to gliadin and activation of pathogenic T cells in coeliac disease. Lancet 362:30–37, 2003
28. Maiuri MC, De Stefano D, Mele G, Iovine B, Bevilacqua MA, Greco L, Auricchio S, Carnuccio R: Gliadin increases iNOS gene expression in interferon-gamma-stimulated RAW 264.7 cells through a mechanism involving NF-kappa B. Naunyn Schmiedebergs Arch Pharmacol 368:63–71, 2003
29. Jelinkova L, Tuckova L, Cinova J, Flegelova Z, Tlaskalova-Hogenova H: Gliadin stimulates human monocytes to production of IL-8 and TNF-alpha through a mechanism involving NF-kappa B. FEBS Lett 571:81–85, 2004
30. Palová-Jelínková L, Roková D, Pecharová B, Bartova J, Sediva A, Tlaskalova-Hogenova H, Spisek R, Tuckova L: Gliadin fragments induce phenotypic and functional maturation of human dendritic cells. J Immunol 175:7038–7045, 2005
31. Walker-Smith JA, Guandalini S, Schmitz J, Working Group of European Society of Paediatric Gastroenterology and Nutrition: Revised criteria of diagnosis of coeliac disease. Arch Dis Child 65:909–911, 1990
32. Mazzarella G, Maglio M, Paparo F, Nardone G, Stefanile R, Greco L, van de Wal Y, Kooy Y, Koning F, Auricchio S: An immunodominant DQ-8 restricted gliadin peptide activates small intestinal immune response in *in vitro* cultured mucosa from HLA DQ-8 positive but not HLA-DQ8 negative coeliac patients. Gut 52:57–62, 2003
33. Olerup O, Zatterquist H: HLA DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 h: An alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. Tissue Antigens 39:225–235, 1992
34. Zetterquist H, Olerup O: Identification of HLA-DRB1*04, DRB1*07 and DRB1*09 alleles by PCR amplification with sequence-specific primers (PCR-SSP) in 2 h. Hum Immunol 34:64–74, 1992
35. Holm S: A simple sequentially rejective multiple test procedure. Scand J Stat 6:65–70, 1979
36. Jelinkova L, Tuckova L, Sanchez D, Krupickova S, Pozler O, Nevoral J, Kotalova R, Tlaskalova-Hogenova H: Increased levels of cirulating ICAM-1, E-selectin and IL-2 receptors in celiac disease. Dig Dis Sci 45:398–402, 2000
37. Merendino RA, Di Pasquale G, Sturniolo GC, Ruello A, Albanese V, Minciullo PL, Di Mauro S, Gangemi S: Relationship between IL-18 and sICAM-1 serum levels in patients affected by coeliac disease: Preliminary considerations. Immunol Lett 85:257–260, 2003
38. Romaldini CC, Barbieri D, Okay TS, Raiz R Jr, Cancado EL: Serum soluble interleukin-2 receptor, interleukin-6, and tumor necrosis factor-alpha levels in children with coeliac disease: Response to treatment. J Pediatr Gastroenterol Nutr 35:513–517, 2002
39. Cataldo F, Lio D, Marino V, Scola L, Crivello A, Corazza GR, Working Group of the SIGEP, Working Group of "Club del Tenue": Plasma cytokine profiles in patients with celiac disease and selective IgA deficiency. Pediatr Allergy Immunol 14:320–324, 2003
40. Pace E, Gjomarkaj M, Melis M, Profita M, Spatafora M, Vignola A, Bonsignore G, Mody CH: Interleukin-8 induces lymphocyte chemotaxis into the pleural space. Role of pleural macrophages. Am J Respir Crit Care Med 159:1592–1599, 1999
41. Roth SJ, Carr MW, Springer TA: C-C chemokines, but not the C-X-C chemokines interleukin-8 and interferon-g inducible protein-10, stimulate transendothelial chemotaxis of T lymphocytes. Eur J Immunol 25:3482–3488, 1995
42. Issekutz AC, Meager A, Otterness I, Issekutz TB: The role of tumour necrosis factor-a and IL-1 in polymorphonuclear leucocyte and T lymphocyte recruitment to joint inflammation in adjuvant arthritis. Clin Exp Immunol 97:26–32, 1994
43. Issekutz TB: Effects of six different cytokines on lymphocyte adherence to microvascular endothelium and *in vivo* lymphocyte migration in the rat. J Immunol 144:2140–2146, 1990
44. Ding Z, Xiong K, Issekutz TB: Chemokines stimulate human T lymphocyte transendothelial migration to utilize VLA-4 in addition to LFA-1. J Leukoc Biol 69:458–466, 2001
45. Sturgess RP, Macartney, JC, Makgoba MW, Hung CH, Haskard DO, Ciclitira PJ: Differential upregulation of intercellular

adhesion molecule-1 in coeliac disease. Clin Exp Immunol 82:489–492, 1990
46. Pober JS, Gimbrone MA Jr, Lapierre LA, Mendrick DL, Fiers W, Rothlein R, Springer TA: Overlapping patterns of activation of human endothelial cells by interleukin-1, tumor necrosis factor, and immune interferon. J Immunol 137:1893–1896, 1986
47. Drago S, El Asmar R, Di Pierro M, Grazia Clemente M, Tripathi A, Sapone A, Tacar M, Iacono G, Carroccio A, D'Agate C, Not T, Zampini L, Catáis C, Fasano A: Gliadin, zonulin and gut permeability: Effects on celiac and non-celiac intestinal mucosa and intestinal cell lines. Scand J Gastroenterol 41:408–419, 2006
48. Sander GR, Cummins AG, Henshall T, Powell BC: Rapid disruption of intestinal barrier function by gliadin involves altered expression of apical junctional proteins. FEBS Lett 579:4851–4855, 2005
49. Thomas KE, Sapone A, Fasano A, Vogel SN: Gliadin stimulation of murine macrophage inflammatory gene expression and intestinal permeability are MyD88-dependent: role of the innate immune response in celiac disease. J Immunol 176:2512–2521, 2006
50. Ghosh S, May MJ, Kopp EB: NF-kappa B and Rel proteins: Evolutionarily conserved mediators of immune responses. Ann Rev Immunol 16:225–260, 1998
51. Maiuri MC, De Stefano D, Mele G, Fecarotta S, Greco L, Troncone R, Carnuccio R: Nuclear factor kappa B is activated in small intestinal mucosa of celiac patients. J Mol Med 81:373–379, 2003
52. Baldwin AS: The NF-kappa B and I kappa B proteins: New discoveries and insights. Ann Rev Immunol 14:649–683, 1996
53. Snyder JG, Prewitt R, Campsen J, Britt LD: PDTC and Mg132, inhibitors of NF-kappa B, block endotoxin induced vasodilatation of isolated rat skeletal muscle arterioles. Shock 17:304–307, 2002
54. Smythies LE, Sellers M, Clements RH, Mosteller-Barnum M, Meng G, Benjamin WH, Orenstein JM, Smith PD: Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. J Clin Invest 115:66–75, 2005
55. Smythies LE, Maheshwari A, Clements R, Eckhoff D, Novak L, Vu HL, Mosteller-Barnum M, Sellers M, Smith PD: Mucosal IL-8 and TGF-b recruit blood monocytes: Evidence for cross-talk between the lamina propria stroma and myeloid cells. J Leuk Biol 80:492–499, 2006

CYTOKINE PROFILING IN HUMAN COLOSTRUM AND MILK BY PROTEIN ARRAY

Clinical Chemistry 53:5
955–962 (2007)

Clinical Immunology

Cytokine Profiling in Human Colostrum and Milk by Protein Array

MILOSLAV KVERKA,[1*] JAROSLAVA BURIANOVA,[1] RAJA LODINOVA-ZADNIKOVA,[2] INGRID KOCOURKOVA,[2] JANA CINOVA,[1] LUDMILA TUCKOVA,[1] and HELENA TLASKALOVA-HOGENOVA[1]

Background: **Human colostrum and milk contain components that influence development. Our aim was to use a protein array to determine the cytokine profile of human lacteal secretions and changes that occur during the early postpartum period.**

Methods: **We collected 17 samples of colostrum during the first 2 days postpartum and a 2nd group of 5 sets of 2 to 3 sequential colostrum or milk samples (at 20- to 30-h intervals). We analyzed the samples with array membranes consisting of 42 or 79 antibodies directed against cytokines.**

Results: **In most samples, we detected the previously described cytokines interleukin-8 (IL-8)/CXCL8, epidermal growth factor (EGF), growth-related oncoprotein (GRO)/CXCL1–3, angiogenin, transforming growth factor β-2, and monocyte chemotactic protein 1 (MCP-1/CCL2). In addition, we found 32 cytokines that have not been described before in colostrum. Cytokine concentrations differed among mothers, and the spectrum of cytokines changed with time after delivery. A significant decrease occurred in IL-12 and macrophage inflammatory protein-1δ/CCL15 and a significant increase in MCP-1/CCL2. The production of angiogenin, vascular endothelial growth factor, GRO/CXCL1–3, EGF, and IL-8/CXCL8 remained high throughout. The concentrations of 2 selected cytokines measured with the array technique and ELISA showed moderate to strong correlation (r = 0.63 for EGF and r = 0.84 for IL-8/CXCL8).**

Conclusion: **Despite the lack of precise quantification, the protein array might be suitable for cytokine screening. It allows simultaneous detection of a broad spectrum of cytokines (including those not described before) in lacteal secretions.**

Breastfeeding is an important factor helping the newborn adapt to the environment. Microbial colonization of epithelial surfaces after birth is a potential threat to newborns but is also the main contributor to the proper development of the immune system *(1, 2)*. The beneficial effect of breastfeeding may extend well beyond weaning and has been shown to prevent or mitigate several diseases later in life *(3)*. Many of these beneficial effects are the result, at least partially, of secretory IgA, which is the major immunoglobulin isotype in human lacteal secretions, and to other immunologically active components such as antimicrobial factors, cytokines, chemokines, and growth factors. Our knowledge about the variety of these factors is restricted, however, by limitations of the techniques used to study them.

In recent years, methodological approaches for detection of various biological factors have developed rapidly. Bioassays, in which the biological activity of a given component in the sample is tested on a target cell, were replaced by immunoassays (enzymatic immunoassay, RIA) affording generally higher specificity, sensitivity, and reproducibility. Recently, proteomics has been successfully used to detect multiple proteins at once using various multiplex methods available in commercial form *(4, 5)*. Antibody-based protein arrays are a valuable tool, especially in characterizing the spectra of biologically active components in body fluids and cells.

The main objective of our study was to screen human colostrum and milk for cytokines, chemokines, and growth factors and evaluate their changes early after delivery using a proteomic method based on protein array.

[1] Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic.
[2] Institute for the Care of Mother and Child, Prague, Czech Republic.
* Address correspondence to this author at: Fax 420-24172-1143; e-mail: kverka@biomed.cas.cz, immuno@biomed.cas.cz.
Received July 28, 2006; accepted February 8, 2007.
Previously published online at DOI: 10.1373/clinchem.2006.077107

Materials and Methods

CHARACTERISTICS OF THE COLOSTRUM OR MILK DONORS AND BREAST MILK SAMPLE COLLECTION

We collected 31 colostrum (within the first 72 h postpartum) or milk (after 72 h postpartum) samples from 22 mothers within 4 days postpartum. Median (SD) age of the colostrum and milk donors was 29 (4.7) years, and in 12 cases (54.5%) it was their first delivery. The specimens were collected into sterile plastic tubes, immediately frozen at −20 °C, transported to the laboratory, and stored at −20 °C until analysis, which was performed within 8 weeks. To address the question of cytokine degradation during storage, we measured a pool of samples that included both fresh samples and samples frozen at both −20 °C and −70 °C. Freezing the samples did not affect the results of the assay (data not shown). The breast milk fatty layer and cellular elements were removed by 2 centrifugations, at 680*g* for 10 min at 4 °C, after which the supernatants were removed and then at 10 000*g* for 30 min at 4 °C. The resulting translucent whey was used for analysis.

PROTEIN ARRAY

RayBio™ Human Cytokine Array (Raybiotech) was used for detection. Two groups of samples differed according to the protein array used. The 1st group of 17 samples was taken during the first 2 days postpartum and was tested using RayBio Human Cytokine Array V. This array is designed to detect 79 cytokines. The 2nd group, 14 colostrum samples comprising 5 sets of 2 to 3 sequential samples (obtained at 20- to 30-h intervals) from the same mother, was tested using RayBio Human Cytokine Array III, which can detect 42 cytokines.

We processed human cytokine array membranes according to the manufacturer's recommendation. Briefly, the membranes were blocked by incubation with the blocking buffer at room temperature for 30 min and incubated with the sample at room temperature for 90 min. Membranes were washed 3 times with Wash Buffer I and 2 times with Wash Buffer II at room temperature for 5 min per wash and incubated with biotin-conjugated antibodies at room temperature for 90 min. Finally, the membranes were washed and incubated with horseradish peroxidase–conjugated streptavidin at room temperature for 2 h and with detection buffer for 2 min.

We used a luminescence detector (LAS-1000, Fujifilm) for detection, and the data were digitized and subjected to image analysis (AIDA 3.28, Raytest). By subtracting the background staining and normalizing to the positive controls on the same membrane, we obtained relative protein concentrations. We then compared the mean values for each cytokine detected 30, 60, and 90 h postpartum. The manufacturer claims that the imprecision (CV) of the array is <10%. We tested a pool of colostrum samples on 5 arrays. Each of the 5 results was within 11% of the mean of the 5 results.

We measured total milk protein concentration by the bicinchoninic acid protein assay (Pierce).

EPIDERMAL GROWTH FACTOR AND INTERLEUKIN-8/CXCL8 ELISA

The results of the array were compared with data obtained by ELISA. The concentrations of epidermal growth factor (EGF)[3] and interleukin-8 (IL-8)/CXCL8 were analyzed in all 31 samples with commercial ELISA kits from R&D according to the manufacturer's protocol. All samples and calibrators were analyzed in duplicate, and the mean value was used. The absorbance of the controls was subtracted from the absorbance of the calibrators and samples.

The influence of breast milk components on the measured EGF and IL-8/CXCL8 concentrations was evaluated by adding known amounts of recombinant cytokines to colostrum samples, which were then analyzed. The recovery was tested with a concentration expected to be in the middle of the linear part of the standard curve. The recovery was 93% for EGF and 94% for IL-8/CXCL8. Our results are in agreement with recovery studies performed by other authors who tested broader spectra of cytokines in colostrum and maternal milk by ELISA *(6)*.

STATISTICS

We performed statistical analysis by use of the MedCalc package for Windows version 8.1.1.0 (MedCalc Software). We used Passing and Bablok regression analysis and Bland–Altman plots for method comparisons *(7, 8)* and the cusum test to evaluate linearity of the paired data. To analyze the association between the 2 methods, we used the Pearson correlation coefficient. The data obtained at various time points were compared by paired Student *t*-test with post hoc Bonferroni correction.

ETHICS

The Local Ethics Committee for Human Research at the Institute for Care of Mother and Child approved the study, and all participants gave informed consent.

Results

SPECTRUM OF CYTOKINES

Using an array designed to detect 79 proteins, we found altogether 68 proteins, 32 of which were detected in human colostrum or milk for the 1st time. Three cytokines [EGF, IL-8/CXCL8, and growth-related oncoprotein (GRO)/CXCL1–3] were present in all the tested samples, and 19 were found very often (≥50% samples) (Table 1). The median number of cytokines detected in samples was

[3] Nonstandard abbreviations: EGF, epidermal growth factor; IL-8, interleukin-8; GRO, growth-related oncoprotein; BLC, B lymphocyte chemoattractant; MIP, macrophage inflammatory protein; TIMP, tissue inhibitor of metalloproteinase; and MDC, macrophage-derived chemokine.

Table 1. Comprehensive array map (RayBio Human Cytokine Array V) of cytokines present in human colostrum.

	1	2	3	4	5	6	7	8
a	NT-4[b]	IGFBP-3	FGF-4[b]	Oncostatin M[b]	MIP-1δ[b]	IL-12	I-309	Pos
n	3/17	9/17	4/17	8/17	9/17	1/17	0/17	
Average amount (SE)	3.5 (1.48)	2.3 (0.51)	1.5 (0.62)	3.4 (0.73)	3.6 (1.20)	5.8	0.0	
Sensitivity, ng/L	2	1000	1000	100	100	1	1000	
b	OPG	IGFBP-4[b]	FGF-6[b]	Thrombopoietin	RANTES	IL-13	IL-1α	Pos
n	11/17	2/17	2/17	2/17	8/17	0/17	2/17	
Average amount (SE)	6.0 (0.94)	1.1, 3.3	1.5, 1.7	1.4, 1.9	3.7 (0.71)	0.0	2.5, 4.8	
Sensitivity, ng/L	100	1000	1000	100	2000	100	1000	
c	PARC[b]	IL-16	FGF-7[b]	VEGF	SCF[b]	IL-15[b]	IL-1β	Pos
n	12/17	2/17	2/17	11/17	1/17	1/17	1/17	
Average amount (SE)	8.1 (1.74)	1.8, 2.9	1.6, 2.2	4.3 (0.74)	2.5	7.9	27.7	
Sensitivity, ng/L	1000	1	1	100	10	100	100	
d	PlGF[b]	IP-10	FGF-9[b]	PDGF-BB[b]	SDF-1	IFN-γ	IL-2	Pos
n	5/17	12/17	2/17	1/17	2/17	3/17	0/17	
Average amount (SE)	2.3 (0.42)	3.6 (0.49)	1.4, 2.3	1.6	0.7, 2.9	4.4 (1.95)	0.0	
Sensitivity, ng/L	100	10	100	1000	2000	100	25	
e	TGF-β2	LIF[b]	Flt-3 ligand	Leptin	TARC[b]	MCP-1	IL-3[b]	Neg
n	13/17	7/17	0/17	0/17	1/17	13/17	1/17	
Average amount (SE)	4.3 (0.53)	2.1 (0.26)	0.0	0.0	1.3	6.1 (0.99)	10.6	
Sensitivity, ng/L	1000	1000	1	100	100	3	100	
f	TGF-β3[b]	LIGHT	Fractalkine[b]	BDNF[b]	TGF-β1	MCP-2[b]	IL-4	Neg
n	2/17	0/17	2/17	1/17	1/17	2/17	2/17	
Average amount (SE)	0.9, 1.4	0.0	1.4, 1.8	0.6	18.5	1.8, 18.4	1.6, 1.9	
Sensitivity, ng/L	100	1	1600	100	200	100	1	
g	TIMP-1[b]	MCP-4[b]	GCP-2	BLC[b]	TNF-α	MCP-3	IL-5	ENA-78
n	15/17	1/17	0/17	8/17	2/17	0/17	0/17	1/17
Average amount (SE)	7.4 (1.26)	13.6	0.0	2.0 (0.26)	1.0, 1.7	0.0	0.0	3.2
Sensitivity, ng/L	100	100	100	10	100	1000	1	1
h	TIMP-2[b]	MIF	GDNF[b]	Ck β 8–1[b]	TNF-β[b]	M-CSF	IL-6	G-CSF
n	15/17	6/17	2/17	2/17	1/17	2/17	3/17	1/17
Average amount (SE)	6.8 (1.76)	3.1 (0.76)	2.1, 2.4	1.1, 1.4	12.3	1.5, 2.8	7.3 (3.27)	61.5
Sensitivity, ng/L	1	100	100	1000	1000	1	1	2000
i	Neg	MIP-3α[b]	HGF	Eotaxin	EGF	MDC[b]	IL-7	GM-CSF
n		4/17	12/17	0/17	17/17	5/17	0/17	2/17
Average amount (SE)		2.7 (0.18)	9.5 (3.50)	0.0	42.5 (4.63)	2.3 (0.36)	0.0	2.4, 33.7
Sensitivity, ng/L		100	200	1	1	1000	100	100
j	Pos	NAP-2	IGFBP-1	Eotaxin-2[b]	IGF-1	MIG	IL-8	GRO
n		5/17	12/17	1/17	1/17	1/17	17/17	17/17
Average amount (SE)		3.4 (0.94)	5.0 (1.21)	1.8	0.2	15.7	80.4 (13.15)	40.7 (14.14)
Sensitivity, ng/L		100	1	1	10	1	1	1–1000
k	Pos	NT-3[b]	GFBP-2	Eotaxin-3[b]	Angiogenin	MIP-1β	IL-10	GRO-α
n		2/17	12/17	2/17	16/17	12/17	2/17	6/17
Average amount (SE)		1.0, 1.7	6.7 (1.20)	0.2, 1.3	9.0 (2.05)	2.4 (0.32)	1.6, 7.0	8.5 (6.43)
Sensitivity, ng/L		20	10	320	10	10	10	1000

n is the number of positive results for each cytokine of all 17 samples.

[a] Claimed by manufacturer.

[b] Newly discovered cytokines *(32)*.

Designations of columns and rows (numbers 1–8 and letters a-k) are used only for the purpose of easy orientation.

Pos, positive control; Neg, negative control; BDNF, brain-derived neurotrophic factor; Ck, chemokine; ENA, epithelial cell–derived neutrophil activating protein; FGF, fibroblast growth factor; Flt-3, *fms*-related tyrosine kinase-3; GCP-2, granulocyte chemotactic protein 2; G-CSF, granulocyte colony-stimulating factor; GDNF, glial cell line–derived neurotrophic factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HGF, hematopoietic growth factor; IFN, interferon; IGFBP–, insulin-like growth factor binding protein; IGF-1, insulin-like growth factor; IP-10, interferon-γ–inducible protein of 10 kDa; LIF, leukemia inhibitory factor; MCP–, monocyte chemoattractant protein; M-CSF, macrophage colony-stimulating factor; MIF, macrophage migration inhibitory factor; MIG–, monokine induced by interferon-γ; NAP-2, neutrophil activating peptide 2; NT, neurotrophin; PARC, pulmonary and activation-regulated chemokine; PDGF, platelet derived growth factor; PlGF, placenta growth factor; SCF, stem cell factor; SDF-1, stromal cell–derived factor 1; TARC, thymus and activation-regulated chemokine; TGF, transforming growth factor; TNF, tumor necrosis factor; and VEGF, vascular endothelial growth factor.

20 (range 4 to 38) (Fig. 1). The variability in cytokine spectrum of individual samples is shown in Fig. 1.

The cytokine concentration in colostrum or milk taken from an individual mother was quite variable, and changes of the same protein were not consistent over time (Fig. 2). The number of detected cytokines did not change significantly with time, although there was an overall decreasing tendency (Fig. 3). The mean number of cytokines (SD) we found 20 to 30 h after delivery was 22.80 (8.61), 50 to 60 h after delivery, 18.60 (5.32), and 80 to 90 h after delivery, 15.75 (5.32).

We found 32 proteins that have not been described in human colostrum or milk before. Some of these were present in more than 3 samples: growth factors (fibroblast growth factor 4, placental growth factor), chemotactic factors [B lymphocyte chemoattractant/CXCL13, macrophage inflammatory protein (MIP)-1β/CCL4, MIP-1δ/CCL15, MIP-3α/CCL20, pulmonary and activation-related chemokine (PARC)/CCL18, leukemia inhibitory factor, oncostatin M], and antiinflammatory factors [tissue inhibitor of metalloproteinase (TIMP)-1, TIMP-2, macrophage-derived chemokine (MDC)/CCL22].

The protein content in 14 samples was in the range of 23.4 to 203 g/L, with a mean (SE) of 70.67 (16.30) g/L. We did not find any statistically significant difference between the protein content in milk at 20 to 30 h [135.48 (27.10) g/L], 50 to 60 h [38.68 (6.28) g/L], and 80 to 90 h postpartum [29.70 (1.69) g/L].

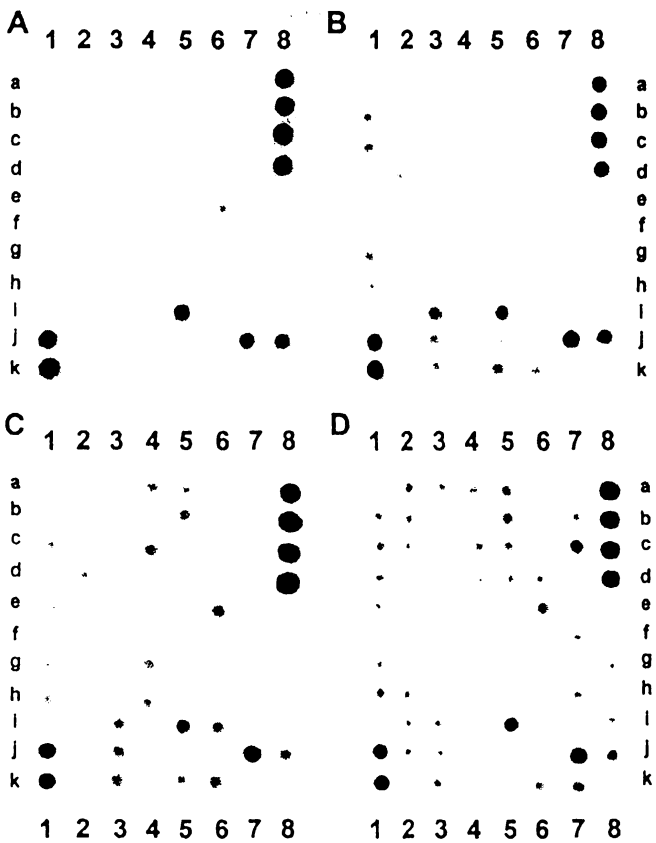

Fig. 1. Examples documenting the variability of the individual samples.

Sample *A* shows 4 (minimum), sample *B* 16, sample *C* 22, and sample *D* 38 (maximum) positive cytokines. These arrays are designed to detect 79 cytokines, chemokines, or growth factors. See Table 1 for cytokine identification.

COMPARISON OF ELISA WITH PROTEIN ARRAY

We statistically evaluated and compared the data obtained for IL-8/CXCL8 and EGF using ELISA and protein array. The correlation between the array and ELISA was moderate to strong ($r = 0.63$, $P < 0.001$ for EGF and $r = 0.84$, $P < 0.001$ for IL-8/CXCL8). The Passing and Bablok regression analysis (Fig. 4A and B) and Bland–Altman plot (Fig. 4C and D) did not suggest any constant or proportional difference between the 2 methods. The cusum test did not show a significant deviation from linearity for either of the cytokines ($P > 0.10$ for EGF and $P > 0.05$ for IL-8/CXCL8).

Discussion

One of the approaches to characterize the proteomic profile in biological samples is protein array. The cytokine array we used in our experiments belongs to a multiplex immunoassay that allows detection of many proteins simultaneously. Nowadays, more multiplex techniques based on antigen–antibody binding are available. Because it uses the largest number of cytokines, the protein array is most suitable to screen for biological factors and their changes.

The cytokine arrays give information about relative changes in cytokine concentrations, but they do not provide specific quantitative information as does an ELISA. The sensitivity for each protein of interest varies because of differences in antibody affinity. However, correlation with ELISA, at least for the 2 cytokines we tested (EGF and IL-8/CXCL8), is moderate to strong. Correlation between these 2 methods for IL-8/CXCL8 detection was also reported in human plasma *(9)*. Interestingly, the concentrations of EGF and IL-8/CXCL8 in colostrum are >1000 times higher than concentrations in plasma *(10)*.

The advantages of this approach are that the number of proteins detected on an array is high and steadily increasing (moreover, custom arrays are offered); the manufacturer (Raybiotech) tests the sensitivity and specificity for each protein of interest and claims that no cross-reactivities have been found for the antibodies on the array; and according to the user's manual, the detection range may be at least 100-fold greater (IL-2) with the protein array than with ELISA. We used this advantageous technique to measure the content of cytokines in biologically important body fluids—human colostrum and milk. The quality of measurement is influenced by many factors including preparation, processing, and standardization of samples. Moreover, interindividual, diurnal, and other variations may affect the evaluation of the biologically active components of mucosal and exocrine gland secretions *(11)*. The number of cytokines detected in individual samples of colostrum and milk shows considerable interindividual variability. The problem of normalization of the cytokine

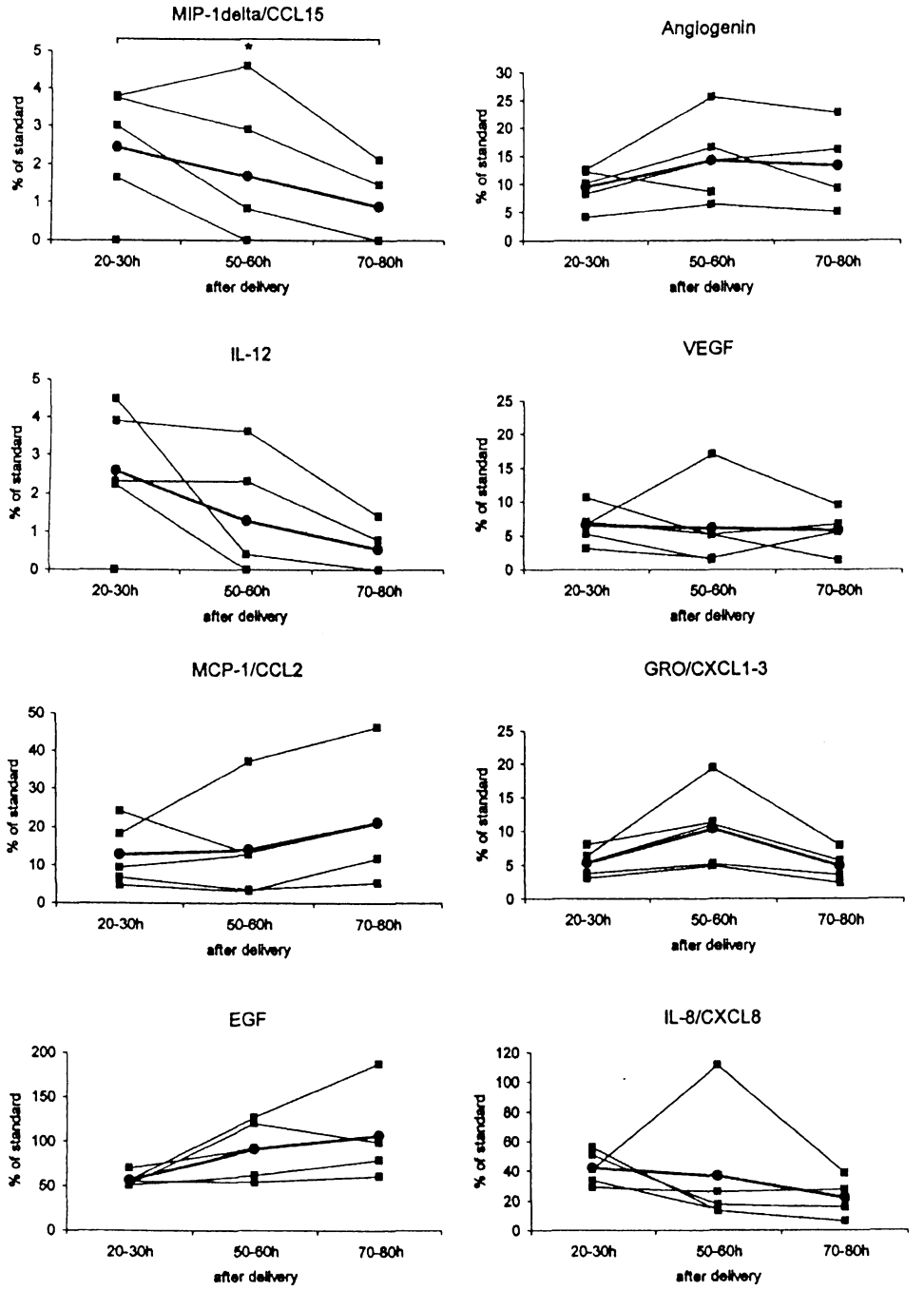

Fig. 2. Changes in cytokine relative concentration with time after delivery.

Thin lines represent the trends in relative protein concentration in individual mothers. The *thick line* represents the mean values. *Significant differences between the groups of samples taken at the same time.

content to some constant in mucosal fluids is not yet solved and standardized *(12)*.

It seems that cytokines are secreted in the mammary gland mostly by the epithelium and resident leukocytes, but a minor part derives from the serum *(13)*. The production of chemotactic factors and specific adhesion molecules in the mammary gland allows the cells of mucosal origin to enter the mammary gland from the circulation and increase the production of immunologically active proteins in situ. Human colostrum and milk contain high concentrations (10^6 to 10^7 for colostrum, 10^5 to 10^6 for milk) of numerous cells capable of producing various cytokines even in the infant's gut *(12)*. Besides nutritional components, antibodies, and major antibacterial proteins (e.g., lysozyme, lactoferrin, defensins), a wide array of immunomodulatory factors such as cytokines, chemokines, and growth factors are present *(3)*. These factors may influence not only the suckling's gut,

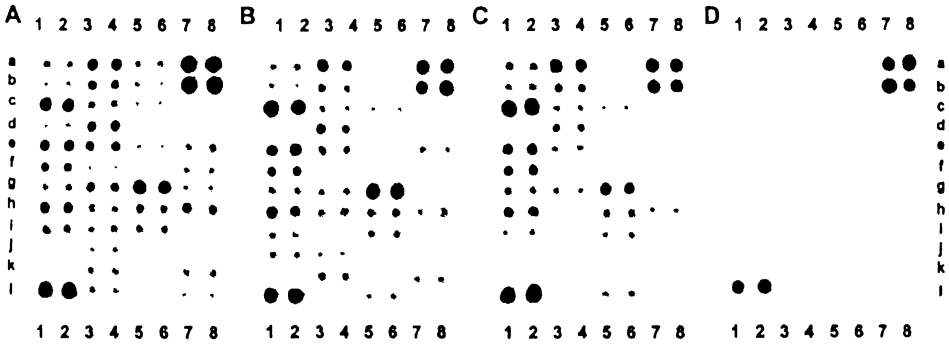

Fig. 3. Cytokine profile 30 h (*A*), 60 h (*B*), and 80 h (*C*) after delivery in one of the sample sets, demonstrating the changes in cytokine spectrum. The rightmost array (*D*) represents a negative control array. These arrays are designed to detect 42 cytokines, chemokines, or growth factors. See Table 2 for cytokine identification.

but the whole organism as well, as the complex proteins may be absorbed into the newborn's bloodstream through the permeable intestinal barrier and thus help to adapt the newborn's immune system to the environment *(14, 15)*. Moreover, all the above factors are similarly important for protecting the maternal mammary gland *(13)*.

Using multiplex analysis, this study confirmed the presence of various cytokines described previously; EGF, GRO/CXCL1–3, and IL-8/CXCL8 were detected in all samples of lacteal secretions. There is little evidence concerning the in vivo activities of human milk cytokines in sucklings; however, their immunomodulatory effects in vitro and in animal models have been proven repeatedly. In an animal model of human celiac disease, we have shown that EGF, in concentrations similar to those in milk, exerts a protective effect when orally administered to newborn rats *(16)*.

In connection with supposed biological activities, it would be interesting to speculate on the total intake of these factors. Based on the known daily volume of milk ingested by breast-fed infants, we can calculate the intake of those cytokines detected in human colostrum, by use of a method that gives quantitative results *(17)*. EGF concentration in colostrum is ~222 μg/L (7.3 μg/day) on the 1st day, 228 μg/L (9.8 μg/day) on the 2nd day, and 268 μg/L (28.7 μg/day) on the 3rd day. For other cytokines, we can use sensitivity values for the array given by the manufacturer: e.g., sensitivity for angiogenin is 10 ng/L; because it was detected in the sample, it could be expected that the suckling ingests >330 pg on the 1st day, >430 pg on the 2nd day, and >1070 pg on the 3rd day.

Interestingly, by use of the protein array, we found several immunologically active proteins not yet described in human colostrum or milk (search performed using PubMed database, http://www.ncbi.nlm.nih.gov/PubMed, accessed July 24, 2006). These proteins (cytokines) differ in their main biological activities; they exert chemoattractant, growth-promoting, and antiinflammatory activities, and some of them display multiple functional properties.

Cytokines with chemoattracting activity found in colostrum or milk for the 1st time included PARC/CCL18, MIP-3α/CCL20 (mainly for lymphocytes and immature dendritic cells), MIP-1β/CCL4 (mainly for NK cells and macrophages), and B lymphocyte chemoattractant/CXCL13 (mainly for naive B lymphocytes). Their activity, promoting the attraction of various types of cells, could be advantageous for the proper development and priming of the intestinal lymphocytes, which may protect the new-

Table 2. Comprehensive array map (RayBio Human Cytokine Array III) of cytokines for identification of individual cytokines in Fig. 3.

	1	2	3	4	5	6	7	8
a	TNF-α	TNF-α	MCP-1	MCP-1	IL-2	IL-2	Pos	Pos
b	TNF-β	TNF-β	MCP-2	MCP-2	IL-3	IL-3	Pos	Pos
c	EGF	EGF	MCP-3	MCP-3	IL-4	IL-4	Neg	Neg
d	IGF-I	IGF-I	M-CSF	M-CSF	IL-5	IL-5	Neg	Neg
e	Angiogenin	Angiogenin	MDC	MDC	IL-6	IL-6	ENA-78	ENA-78
f	Oncostatin M	Oncostatin M	MIG	MIG	IL-7	IL-7	G-CSF	G-CSF
g	Thrombopoietin	Thrombopoietin	MIP-1δ	MIP-1δ	IL-8	IL-8	GM-CSF	GM-CSF
h	VEGF	VEGF	RANTES	RANTES	IL-10	IL-10	GRO	GRO
i	PDGF-BB	PDGF-BB	SCF	SCF	IL-12p40p70	IL-12p40p70	GRO-α	GRO-α
j	Leptin	Leptin	SDF-1	SDF-1	IL-13	IL-13	I-309	I-309
k	Neg	Neg	TARC	TARC	IL-15	IL-15	IL-1α	IL-1α
l	Pos	Pos	TGF-β1	TGF-β1	IFN-γ	IFN-γ	IL-1β	IL-1β

Designations of columns and rows (numbers 1–8 and letters a–k) are used only for the purpose of easy orientation.

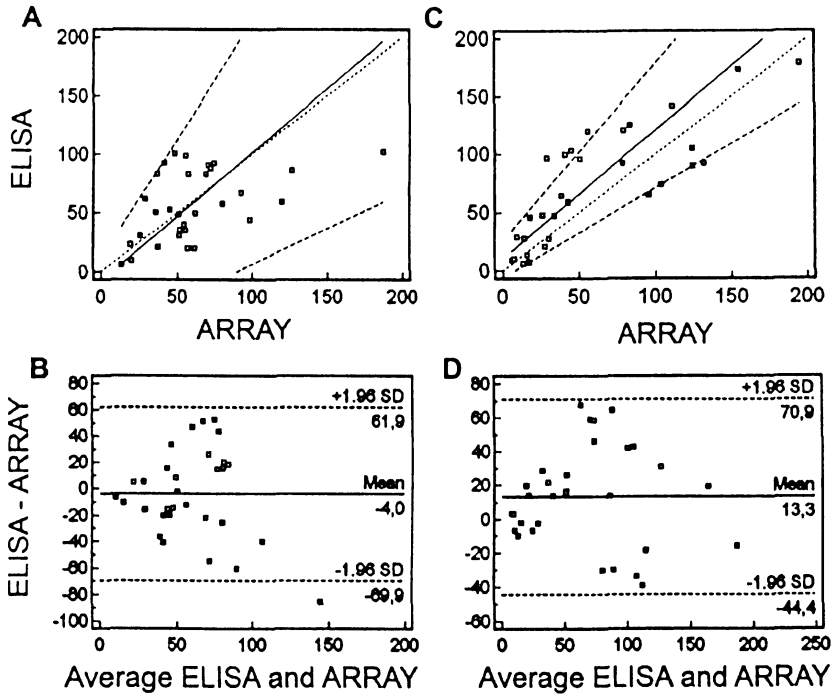

Fig. 4. Passing and Bablok regression scatter plot for EGF (*A*) and IL-8/CXCL8 (*C*).

Solid line, regression line ($y = -7.7398 + 1.0913x$ for EGF and $y = 10.1347 + 1.1076x$ for IL-8); *dashed lines*, 95% confidence interval for the regression line; *dotted line*, line of identity. Bland–Altman difference plot for EGF (*B*) and IL-8 (*D*).

born against several diseases immediately after birth and later in life *(18)*.

Some of the growth factors found in colostrum and milk for the 1st time, such as leukemia inhibitory factor and fibroblast growth factors, may contribute to developing gut structure and function including epithelial barrier maturation; others, such as placenta growth factor, may mainly influence angiogenesis. Interestingly, we found several neuronal growth factors (brain-derived neurotrophic factor, glial cell line–derived neurotrophic factor, neurotropin-3, and neurotropin-4), which may be important for development of the newborn's enteric nervous system and possibly also for the proper development of the central nervous system *(19)*.

Along with several cytokines with antiinflammatory activity described earlier, we found the following antiinflammatory factors for the first time: TIMP-1, TIMP-2, and MDC. These factors may help in establishing mucosal homeostasis in both the mammary gland and the suckling's gut *(13, 20, 21)*.

Because of the increasing interest in the detection of multiple biomarkers, there is a need to identify them in a time-saving and efficient way. The protein array offers parallel identification of individual protein biomarkers, which makes it very suitable for use in basic and clinical research as well as in clinical practice.

Grant/funding support: The study was supported by grants 310/03/H147 and 303/06/0974 of the Czech Science Foundation; S500200572 of the Academy of Sciences of Czech Republic; and Institutional Research Concept Grant AV0Z50200510.

Financial disclosures: None declared.

Acknowledgements: We are grateful to Dr. Brauner, Institute of Physiology, Academy of Sciences of the Czech Republic, who helped us with the use of the chemiluminescence detector LAS-1000.

References

1. Tlaskalova-Hogenova H, Tuckova L, Lodinova-Zadnikova R, Stepankova R, Cukrowska B, Funda DP, et al. Mucosal immunity: its role in defense and allergy. Int Arch Allergy Immunol 2002;128: 77–89.
2. Field CJ. The immunological components of human milk and their effect on immune development in infants. J Nutr 2005;135:1–4.
3. Hanson LA, Korotkova M, Telemo E. Human milk: its components and their immunobiologic functions. In: Mestecky J, Lamm ME, Strober W, Bienenstock J, McGhee JR, Mayer L, eds. Mucosal Immunology, 3rd ed. San Diego: Academic Press 2005:1795–827.
4. Saviranta P, Okon R, Brinker A, Warashina M, Eppinger J, Geierstanger BH. Evaluating sandwich immunoassays in microarray format in terms of the ambient analyte regime. Clin Chem 2004; 50:1907–20.
5. Joos TO, Stoll D, Templin MF. Miniaturized multiplexed immunoassays. Curr Opin Chem Biol 2002;6:76–80.
6. Bottcher MF, Jenmalm MC, Bjorksten B. Cytokine, chemokine and secretory IgA levels in human milk in relation to atopic disease and IgA production in infants. Pediatr Allergy Immunol 2003;14:35–41.
7. Passing H, Bablok W. A new biometrical procedure for testing the equality of measurements from two different analytical methods. Application of linear regression procedures for method comparison studies in clinical chemistry, Part I. J Clin Chem Clin Biochem 1983;21:709–20.
8. Bland JM, Altman DG. Measuring agreement in method comparison studies. Stat Methods Med Res 1999;8:135–60.

9. Copeland S, Siddiqui J, Remick D. Direct comparison of traditional ELISAs and membrane protein arrays for detection and quantification of human cytokines. J Immunol Methods 2004;284:99–106.
10. Berrahmoune H, Lamont JV, Herbeth B, FitzGerald PS, Visvikis-Siest S. Biological determinants of and reference values for plasma interleukin-8, monocyte chemoattractant protein-1, epidermal growth factor, and vascular endothelial growth factor: Results from the STANISLAS cohort. Clin Chem 2006;52:504–10.
11. Chaturvedi P, Warren CD, Altaye M, Morrow AL, Ruiz-Palacios G, Pickering LK, et al. Fucosylated human milk oligosaccharides vary between individuals and over the course of lactation. Glycobiology 2001;11:365–72.
12. Butler JE, Kehrli ME Jr, Immunoglobulins and immunocytes in the mammary gland and its secretions. In: Mestecky J, Lamm ME, Strober W, Bienenstock J, McGhee JR, Mayer L eds. Mucosal Immunology, 3rd ed. San Diego: Academic Press 2005:1763–94.
13. Garofalo RP, Goldman AS. Cytokines, chemokines, and colony-stimulating factors in human milk: the 1997 update. Biol Neonate 1998;74:134–42.
14. Van Elburg RM, Fetter WP, Bunkers CM, Heymans HS. Intestinal permeability in relation to birth weight and gestational and postnatal age. Arch Dis Child Fetal Neonatal Ed. 2003;88:F52–5.
15. Pacha J. Development of intestinal transport function in mammals. Physiol Rev 2000;80:1633–67.
16. Stepankova R, Kofronova O, Tuckova L, Kozakova H, Cebra JJ, Tlaskalova-Hogenova H. Experimentally induced gluten enteropathy and protective effect of epidermal growth factor in artificially fed neonatal rats. J Pediatr Gastroenterol Nutr 2003;36:96–104.
17. Dollberg S, Lahav S, Mimouni FB. A comparison of intakes of breast-fed and bottle-fed infants during the first two days of life. J Am Coll Nutr 2001;20:209–11.
18. Brandtzaeg P. Mucosal immunity: integration between mother and the breast-fed infant. Vaccine 2003;21:3382–8.
19. Chalazonitis A, Pham TD, Rothman TP, DiStefano PS, Bothwell M, Blair-Flynn J, et al. Neurotrophin-3 is required for the survival-differentiation of subsets of developing enteric neurons. J Neurosci 2001;21:5620–36.
20. Goldman AS, Chheda S, Garofalo R, Schmalstieg FC. Cytokines in human milk: properties and potential effects upon the mammary gland and the neonate. J Mammary Gland Biol Neoplasia 1996; 1:251–8.
21. Kelleher SL, Lonnerdal B. Immunological activities associated with milk. Adv Nutr Res 2001;10:39–65.