

UNIVERZITA KARLOVA V PRAHE

Prírodovedecká fakulta



Dizertačná práca

**Úloha monocytov v patogenetických
mechanizmoch celiakie**

Mgr. Jana Cinová



Vedúci dizertačnej práce: Doc. RNDr. Ludmila Tučková, DrSc.

Oddelenie Imunológie a gnotobiológie

Mikrobiologický ústav

Akadémia vied Českej republiky

Praha, Júl 2007

Týmto by som chcela vyjadriť svoje poďakovanie predovšetkým mojej školiteľke Doc. RNDr. Ludmile Tučkovej, DrSc. za jej odborné vedenie mojej dizertačnej práce, za cenné rady a pripomienky.

Moje poďakovanie patrí všetkým pracovníkom oddelenia imunológie a gnotobiológie Mikrobiologického ústavu AV ČR, osobne by som chcela poďakovať kolegom z nášho laboratória, RNDr. Lenke Palovej-Jelínkovej, PhD., Ing. Danielovi Sánchezovi, PhD., Ing. Barbare Pecharovej, MUDr. Anree Kitanovičovej a pani Pavle Kašparovej za ich rady, neoceniteľnú pomoc a vytvorenie príjemného pracovného zázemia.

Vrelé poďakovanie patrí mojej rodine, blízkym a priateľom za ich podporu.

Praha, Júl 2007

Jana Cinová

Dizertačná práca bola vypracovaná na základe nasledujúcich publikácií:

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

Jelínková L, Tučková L, Cinová J, Flegelová Z, Tlaskalová-Hogenová H.

FEBS Letters; 571: 81-85, 2004

Involvement of innate immunity in the development of inflammatory and autoimmune diseases.

Tlaskalová-Hogenová H, Tučková L, Štěpánková R, Hudcovič T, Palová-Jelínková L, Kozáková H, Rossmann P, Sanchez D, Cinová J, Hrnčíř T, Kverka M, Froňová L, Uhlig H, Powrie F, Bland P.

Ann NY Acad Sci; 1051: 1-12, 2005

Gliadin peptides activate blood monocytes from patients with celiac disease.

Cinová J., Palová-Jelínková L, Smythies LE, Černá M, Pecharová B, Dvořák M, Fruhauf P, Tlaskalová-Hogenová H, Smith PD, Tučková L.

J Clin Immunol; 27:201-209, 2007

Cytokine profiling in human colostrum and milk by protein array.

Kverka M, Burianová J., Lodinová-Žadniková R, Kocourková I, Cinová J, Tučková L, Tlaskalová-Hogenová H.

Clin Chem; 53:955-962, 2007

Zoznam použitých skratiek:

AGA	antigliadinové protilátky
AJA	antijejunálne protilátky
APC	antigén prezentujúce bunky
ARA	antiretikulinové protilátky
CD	aktívni celiaci
CTLA-4	cytotoxický leukocytárny antigén
CXCL13	B lymfocytárny chemoatraktant
DCs	dendritické bunky
EGF	rastový epidermový faktor
EMA	antiendomomyziálne protilátky
FGF-4	rastový faktor fibroblastov
GFD	bezlepková diéta
GM-CSF	faktor stimulujúci rast kolóni granulocytov a makrofágov
GRO	s proliferáciou súvisiaci onkoproteín
HD	zdraví darci
ICAM-1	intracelulárna adhezívna molekula
IELs	intraepitelové lymfocyty
IFN- γ	interferón gamma
LP	lamina propria
LPS	lipopolysacharid
MALT	lymfoidné tkanivo asociované s mukózou
MCP	proteín atrahujúci monocyty
MDC	chemokín odvodený od makrofágov
MHC	hlavný hitokompatibilný komplex
MIP-1 α	zápalový proteín makrofágov
NK	prirodzeni zabijáči
PAMPs	s patogénom asociovaná molekula
PDTC	pyrrolidine dithiocarbamate
PIGF	rastový faktor placenty
PRRs	pattern recognition receptors

RS	refraktórna sprue
TGF- β	transformujúci rastový faktor
TIMP	tkanivový inhibítor metaloproteináz
TLRs	toll-like receptors
TNF- α	faktor nekrotizujúci nádory
TPCK	L-1-tosyloamido-2-phenyl chloromethyl ketone
tTG2	tkanivová transglutamináza
VCAM-1	adhezívna molekúla ciev

OBSAH:

1. CELIAKIA	2
1.1 Klinické príznaky	2
1.2 Glutén	3
1.3. Genetické pozadie celiakie	4
1.4 Celiakia ako autoimunitné ochorenie	4
1.4.1 Tkanivová transglutamináza	5
1.4.2. Kalretikulin	6
1.5. Diagnostika	7
1.5.1 Sérologické vyšetrenie	7
1.5.2. Biopsia - histologický obraz celiakie	8
1.5.3. Liečba a obnova črevnej sliznice	9
1.5.3.1. Formy celiakie	9
2. SLIZNIČNÝ IMUNITNÝ SYSTÉM	11
2.1 Adaptívna imunita	11
2.1.1 T- lymfocyty	11
2.1.1.1. Intraepitelové lymfocyty	12
2.1.1.2. CD4 ⁺ T- lymfocyty v lamina propria (LP)	13
2.1.2. B lymfocyty	16
2.2. Zložky prirodzenej imunity	17
2.2.1. Epitelové bunky	17
2.2.2. Antigén prezentujúce bunky	19
2.2.2.1. Mononukleárno-fagocytový systém	19
2.2.2.2. Dendritické bunky	20
2.2.3. NF-κB systém	21
3. CIEĽ PRÁCE	22
4. VÝSLEDKY	23
5. DISKUSIA	30
6. ZÁVER	35
7. ZOZNAM LITERATÚRY	36

1. CELIAKIA

Celiakia je imunologicky mediované ochorenie tenkého čreva, ktoré je vyvolané u geneticky predisponovaných jedincov po požití pšeničného gluténu/lepku a jemu príbuzných prolamínov, ako sú hordeíny v jačmeni a sekalíny v žite. Skrinigové štúdie v Európe a v USA poukazujú na to, že prevalencia celiakie v populácii je 1:100/200, čo sú oveľa vyššie hodnoty než sa vo všeobecnosti predpokladalo (Catassi et al., 1994; Csizmadia et al., 1999; Hill et al., 2000).

V počiatočnej fáze ochorenia dochádza k infiltrácii tenkého čreva T a B lymfocytmi, makrofágmi, dendritickými bunkami a k ich aktivácii. Chronický zápal tenkého čreva vedie k atrofii klkov a k splošteniu črevnej sliznice. Celiakia je úspešne liečená nasadením bezlepkovej diéty (Trier, 1991; Marsh, 1992; Mäki a Collin, 1997; Sollid, 2002; Fasano a Catassi, 2001).

1.1 Klinické príznaky

Na základe prítomnosti klinických symptómov sa celiakia môže klasifikovať na symptomatickú a asymptomatickú. Symptomatická, aktívna forma je prezentovaná klasickými gastrointestinálnymi príznakmi, ako je diarhoea, abdominálna distenzia, zatiaľ čo u asymptomatickej, tichej formy, tieto príznaky chýbajú (Green a Jabri, 2003).

Celiakia je často diagnostikovaná v rannom detstve. V dôsledku sploštenia črevnej sliznice je znížená absorpcia živín, u detí sú prítomné gastrointestinálne problémy, poruchy rastu, anémia, a celkové neprospievanie (deficit kys. listovej, vápnika, železa, vitamínu D a ďalších vitamínov a minerálov) (Wahab et al., 2002 a). V dospelosti sa môže celiakia prejaviť širším spektrom príznakov. V poslednej dobe je až 60% novo diagnostikovaných pacientov dospelých, z toho 15-20% je nad 60 rokov (Mulder a Cellier, 2005; Jansen et al., 1993). Môže byť pozorovaná zvýšená únava, úbytok na hmotnosti, osteoporóza, neplodnosť, môže byť postihnutý aj endokrinný, nervový a pohybový systém. U pacientov sa môžu nešpecificky

vyvinúť neurologické symptómy ako je neuropatia, ataxia, alebo epilepsia. U niektorých pacientov sú pozorované afty a defekty zubnej skloviny (Lahteenoja et al., 1998; Lepore et al., 1996; Aine et al., 1990 Wierink et al., 2007). Celiakia môže byť asociovaná s ďalšími autoimunitnými ochoreniami. Ak je postihnutá len proximálna časť tenkého čreva, pacienti sa nezvyknú sťažovať na diarrhoeu, pretože absorpčné straty tukov a karbohydrátov sú kompenzované distálnou časťou tenkého čreva (Green et al., 2003), diarrhoea je pozorovaná u pacientov u ktorých choroba zasiahla aj distálnu časť tenkého čreva (Rubin, 1960).

1.2 Glutén

Vyvolávací agens - glutén/lepok predstavuje zložitú zmes bielkovín. Podľa rozpustnosti vo vodnom roztoku alkoholu sa glutén delí na 2 frakcie: nerozpustné gluteníny a rozpustné gliadiny. Gliadiny sú monoméry, zatiaľ čo gluteníny tvoria polymérne frakcie tvorené z podjednotiek navzájom viazaných S-S väzbou. Podľa pohyblivosti v elektrickom poli sa gliadiny delia na α/β , γ a ω typy (~250-300 aminokyselinových zvyškov, molekulová hmotnosť 20-75 kDa). Gluteníny sa podľa molekulovej hmotnosti delia na nízkomolekulové (~270-330 aminokyselinových zvyškov) a vysokomolekulové (~650-800 aminokyselinových zvyškov). Glutén patrí do skupiny prolaminov, je bohatý na prolín a glutamín (Sollid et al., 2002). V gastrointestinálnom trakte sú gliadiny a gluteníny pôsobením tráviacich enzýmov (pepsín, trypsín, chymotrypsín) degradované. Alfa-gliadiny s vysokým zastúpením prolínu sú viac odolné proti enzymatickému pôsobeniu pankreatických a žalúdočných proteáz. Všetky uvedené zložky gluténu a gliadinové peptidy prispievajú k poškodeniu črevnej sliznice. Shan a spolupracovníci opísali α -gliadinový peptid 33-mér o sekvencii LQLQFPQPQLPYPQPQLPYPQPQLPYPQPQPF, ktorý indukoval T-bunkové klony izolované z čreva celiakálnych pacientov. Tento peptid ostáva stabilný aj po pôsobení proteáz tráviaceho traktu. Nachádzajú sa v ňom 3 gliadinové epitopy, ktoré boli rozpoznávané T-bunkami pacientov (PFPQPQLPY, PQPQLPYPQ, PYPQPQLPY) (Shan et al., 2002). Boli opísané bakteriálne a rastlinné enzýmy, ktoré štiepia tieto odolné peptidy na menšie fragmenty, strácajú väzobnú aktivitu na HLA-

DQ2 a HLA-DQ8 a tým aj schopnosť stimulovať T-bunky. Vhodnou kombináciou týchto bakteriálnych a rastlinných enzýmov by mohli byť zavedené nové terapeutické postupy v celiakii (Stepniak et al., 2006; Shan et al., 2002; 2004; Siegel et al., 2006).

1.3. Genetické pozadie celiakie

Asociácia celiakie s HLA komplexom bola po prvý krát opísaná v roku 1972 (Falchuk et al., 1972). Väčšina celiakov má DR3-DQ2 haplotyp alebo sú DR5-DQ7/DR7-DQ2 heterozygoti. Približne 95% celiakov exprimuje HLA-DQ2 heterodimér (DQA1*0501/DQB1*0201), zvyšných 5% pripadá na DQ8 heterodimér (DQA1*0301/DQB1*0302) (Green a Jabri, 2003; Sollid et al., 1989; Mazzarella et al., 2003; Karell et al., 2003). Až 30% kaukaziánskej populácie je nositeľom HLA-DQ2 alel (Sollid et al., 1989). V prípade heterodimérov sa alely môžu vyskytovať v *cis* alebo vzácnejšie v *trans* konfigurácii. *Cis* a *trans* forma sa líšia jednou aminokyselinou v každej podjednotke (van Heel et al., 2005). U nositeľov homodimérov kódujúcich alelami DQA1*05 alebo DQB1*02 v *cis* pozícii je vyššie riziko vzniku ochorenia (Vader et al., 2003).

Polanco a spoluprac. (1984) popisujú 70% výskyt celiakie u jednovaječných dvojčiat. Štúdie u monozygotných a dizygotných dvojčiat, ktoré majú 100% a 50% genetickú variabilitu a rovnaké okolité podmienky poukazujú na to, že prítomnosť DQ2 je nevyhnutný, ale nie postačujúci faktor pre vznik celiakie. Určitý podiel na rozvoji celiakie majú aj gény pre neklasické-HLA molekuly (Greco et al., 2002; van Heel et al., 2005). Niektoré práce opisujú vplyv génov kódujúcich kostimulačné molekuly cytotoxických T-lymfocytov CTLA4/CD28/ICOS na chromozóme 2q33, ale ich všeobecný efekt je slabý (Djilali-Saiah et al., 1998; King et al., 2002; Haimila et al., 2004). Typizácia DQ2/8 by mohla byť nápomocná pri stanovení diagnózy, v prípade negativity sa diagnóza celiakie môže vylúčiť.

1.4 Celiakia ako autoimunitné ochorenie

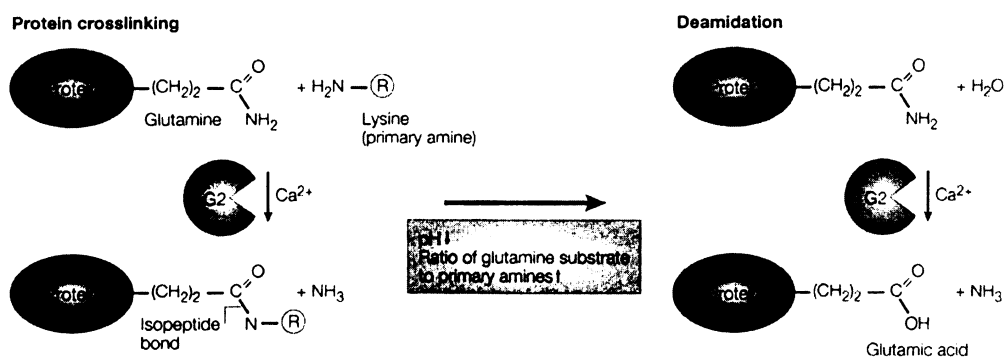
Celiakia spĺňa kritéria autoimunitných ochorení (Rose, 1998):

- v sére je zvýšená hladina IgG, IgA protilátok proti gliadinu, autoprotilátok proti endomyziálnemu autoantigénu tkanivovej transglutamináze a proti kalretikulínu (Karská et al., 1995; Dietrich et al., 1997)
- je asociovaná s ďalšími autoimunitnými ochoreniami ako je napr. autoimunitná tyreoidída, diabetes mellitus I typu, reumatoidná artritída, systémový lupus erytematodes, sarkoidóza, Sjögrenov syndróm, primárna biliárna cirhóza. Vysoko riziková sú aj pacienti s neurologickými symptómami, epilepsiou, pacienti s Downovým a Turnerovým syndrómom (Chin et al., 2003; Bonamico et al., 1998, 2001; Ventura et al., 1999; Cronin et al., 1997; Luft et al., 2003; Dickey et al., 1997; Collin a Mäki, 1994).
- ochorenie bolo prenesené intraepitelovými lymfocytmi u kryšieho modelu celiakie a neúmyselne bunkami pri transplantácii kostnej drene medzi jednovaječnými dvojčatami (Štěpánková et al., 1989; Bargetzi et al., 1997)
- polygénny spôsob dedičnosti celiakie je viazaný na MHC komplex (Sollid 1997; Troncone et al., 1996)
- častejší je výskyt u žien než u mužov (Marsh, 1992)

1.4.1 Tkanivová transglutamináza

Dietrich a spoluprac. (1997) identifikovali endomysialny autoantigén - tkanivovú transglutaminázu (tTG2), ktorá sa exprimuje vo všetkých orgánoch, včítne tenkého čreva, intracelulárne alebo extracelulárne. Z buniek sa uvoľňuje počas mechanického poškodenia, stresu, pri infekcii, zápale alebo apoptózou. Je to kalcium dependentný enzým (76-85 kDa) s transaminačnou a GTPázovou aktivitou. Transamináciou alebo deamidáciou glutamínu zodpovedá za posttranslačné modifikácie proteínov. U celiakálnych pacientov je zvýšená expresia subepitelovej tTG2 (Molberg et al., 1998; Greenberg et al., 1991). Spôsobuje prekríženie proteínov, vznikom ϵ -(γ -glutamyl)-lysín kovalentných väzieb medzi vedľajším reťazcom glutamínu na jednom proteíne s aminoskupinou na lysíne druhého proteínu. Za neprítomnosti lysínu a pri nízkom pH (v tenkom čreve), glutamín interaguje s vodou a je deamidovaný na kyselinu glutámovú (Obr. č.1) (Sollid et al., 2002). V procese

demidácie významnú úlohu zohráva vzdialenosť glutamínu a prolínou v gluténových peptidoch (Vader et al., 2002).



Obrázok č.1 (Sollid et al., 2002)

Aktívna forma tTG2 sa exprimuje na povrchu aktivovaných makrofágov, monocytov a DCs (Murtaugh et al., 1984; Akimov a Belkin; 2001; Le Noaur et al.; 2001). Nedávno bola preukázaná GTP-ázová aktivita tTG2 a jej ovplyvnenie multifunkčnou molekulou kalretikulinu (Mhaouty-Kodja, 2004).

1.4.2. Kalretikulin

Kalretikulin je všadeprítomný, kalcium viažúci proteín a molekulárny chaperón o molekulovej hmotnosti 46 kDa (pI=4.2), migrujúci v SDS-PAGE ako molekula o hmotnosti 60-63 kDa. Je lokalizovaný v endoplazmatickom retikule, ale bol opísaný aj v jadre, v lýtických granulách T-lymfocytov, na povrchu buniek a bol detekovaný aj v krvnom sére (extracelulárne) (Zhu et al, 1997; Andrin et al., 1998; Kishore et al., 1997).

Na základe krížovej reaktivity anti gliadinových protilátok izolovaných zo séra celiakálnych pacientov i myších monoklonálnych protilátok bol v našom laboratóriu identifikovaný kalretikulin ako autoantigén u celiakie (Karská et al., 1995; Tučková et al., 1997). Po prvý krát boli opísané antikalretikulinové protilátky izotypu IgA (Tučková et al., 1997). Až 93% pacientov s celiakiou má signifikantne zvýšené hladiny IgA a IgG antikalretikulinových protilátok v sére (Sánchez et al., 2000).

Antikalretikulínové protilátky izotypu IgA boli zvýšené aj v sére pacientov s primárnou biliárnou cirhózou, autoimunitnou hepatítidou a alkoholickou cirhózou pečene (Sánchez et al., 2003). Autoprotilátky (IgG a IgM) proti kalretikulínu boli nájdené tiež u niektorých pacientov so systémovým lupus erytematodes, lupus neonatalis a cutaneus, Sjögrenovým syndrómom, reumatoidnou artritídou, hepatocelulárnym karcinómom, halotanovou hepatítidou a u pacientov infikovaných parazitmi (Gut et al., 2005; Rokeach et al., 1991; Routsias et al., 1993; Le Naour et al., 2002; McKhalife et al., 1993).

1.5. Diagnostika

Kritéria pre diagnózu celiakie boli schválené v r. 1974, a korigované v r. 1979 a 1990 Európskou spoločnosťou pediatrickej gastroenterológie a výživy (ESPGAN) (McNeish et al., 1979; Walker-Smith et al., 1990).

1.5.1 Sérologické vyšetrenie

Pri podozrení na celiakiu sa testuje prítomnosť IgA antigliadinových (AGA) a antiendomyziálnych (EMA) protilátok, protilátky proti tTG2, prípadne antijejunálnych (AJA) a antiretikulinových (ARA) protilátok a celková hladina IgA v sére (Karpati et al., 1990; Troncone a Ferguson, 1991).

Pre klinické využitie sa začali prednostne stanovovať EMA protilátky, ktoré majú vyše 90% sensitivitu (percento pacientov, ktorí boli v teste pozitívni) a až 100% špecifickosť (percento zdravých kontrol, ktorí boli v teste negatívni) (Fernandez et al., 2005). Viaceré štúdie poukazujú na fakt, že u neliečených celiakov hladina EMA protilátok koreluje s mierou atrofie klkov (Rossi et al., 1988; Dickey et al., 2000). Stanovujú sa metódou nepriamej imunofluorescencie na tkanivových rezoch opičieho ezofágu a ľudskeho pupočníka (Ladinsler et al., 1994).

Protilátky proti tTG2 sa stanovujú ELISA testom, použitím tTG z pečene morčiat alebo rekombinantnej ľudskej tTG (Wong et al., 2002). U pediatrických

pacientov hladina IgA protilátok proti tTG2 a EMA korelovala s histopatologickým nálezom (Donaldson et al., 2007).

U pacientov s normálnym histologickým nálezom alebo s miernym poškodením sa citlivosť detekcie tTG2 protilátok znižuje. U pacientov s čiastočnou atrofiou klkov, nemusia byť tieto protilátky detekované, ale zvyčajne sú pozitívni na AGA protilátky (Tursi et al., 2003). Ak sú sérologické testy negatívne, ale všetky klinické symptómy silne poukazujú na celiakiu, môže ísť o imunoglobulinové deficiencie. U pacientov so selektívnym deficitom IgA až 1,7-2,6 % postihnutých má celiakiu. V takom prípade sa odporúča merať tTG2 protilátky triedy IgG (Cataldo et al., 1998).

1.5.2. Biopsia - histologický obraz celiakie

Pozitívne sérologické testy poukazujú na celiakiu, ich detekcia je významná pri záchyťte potenciálnych pacientov, avšak nie sú konečnou diagnostikou. Histologické vyšetrenie biopsie z jejuna či duodena čreva sa pokladá za konečnú metódu diagnostiky celiakie (Green a Jabri, 2003; Murray a Green, 1999).

Podľa rozsahu poškodenia črevnej sliznice Marsh (1992) klasifikoval štyri stupne celiakie. U typu I (Marsh I) sa opisuje normálna architektúra klkov, v sliznici je zvýšená infiltrácia intraepitelových lymfocytov (IELs). Za fyziologickú hodnotu sa pokladá, ak na 100 enterocytov pripadá 25 lymfocytov (Hayat et al., 2002). Keď je počet vyšší než 30 IELs/100 enterocytov, považuje sa tento stav za abnormálny. Zvýšený počet IELs sa považuje za citlivý, aj keď nešpecifický marker celiakie. Zvýšená infiltrácia IELs môže byť zaznamenaná aj u ďalších autoimunitných ochorení, u tropickej sprue, u Crohnovej choroby, pri giardióze, akútnej infekčnej enteropatii (Marsh 1992; Lebowhl et al. 2003; Shah et al., 2000; Kakar et al., 2003; Ferguson a Murray, 1971). Marsh II je definovaný, ak je v histologickom náleze zvýšená infiltrácia lymfocytmi sprevádzaná hyperpláziou krypt. Marsh III bol podrobnejšie klasifikovaný do troch podskupín A až C, podľa miery atrofie klkov (Wahab, 2002). Väčšina diagnostikovaných pacientov (50-60%) spadá do tejto kategórie (Green et al., 2005). Marsh IIIA - čiastočná atrofia klkov, kĺky sú tupé a skrátané, pomer klkov a krypt je pod 1:1. Marsh IIIB - kĺky sú silne atrofické, avšak stále rozpoznateľné, pretrváva lymfocytóza, krypty sú rozšírené s patrnou mitotickou

aktivitou. Typ IIIC - totálna atrofia kĺkov, hyperplastické krypty s ťažkou lymfocytózou, mukóza tenkého čreva je podobná rektálnej mukóze. Marsh charakterizoval aj stupeň IV- celková atrofia sliznice s hyperpláziou krypt (Marsh, 1992).

1.5.3. Liečba a obnova črevnej sliznice

K regenerácii architektúry črevnej sliznice dochádza pri dôslednom dodržiavaní bezlepkovej diéty. Minimálne 6-12 mesiacov po jej nasadení vymiznú sprievodné symptómy a znižuje sa hladina všetkých protilátok. Obnova sliznice môže trvať aj niekoľko rokov, závisí to od stupňa a rozsahu poškodenia. U detí je uzdravenie rýchlejšie než u dospelých (Uil et al., 1996; Grefte et al., 1988). U 65% pacientov došlo k zlepšeniu počas 2 rokov dodržiavania bezlepkovej diéty, u 85,3% do 5 rokov a u 89,9% u dlhodobejšie liečených. U pacientov so silným poškodením (Marsh IIIC) je obnova pomalšia a neúplná, i naďalej môžu pretrvávajú abnormality črevnej sliznice (Wahab, 2002a).

Stanovenie protilátok v sére je vhodnou skriningovou metódou v populácii rizikových skupín pre celiakiu. Do tejto skupiny spadajú jednostupňoví príbuzní pacientov s celiakiou, s diabetes mellitus 1. typu, s Downovým syndrómom a primárnou biliárnou cirhózou. Napomáhajú tiež monitorovať dodržiavanie bezlepkovej diéty.

1.5.3.1. Formy celiakie

Dermatitis herpetiformis (Duhringova choroba) sa pokladá za kožnú formu celiakie. Prejavuje sa svrbivými pluzgierovitými vyrážkami na vonkajšej strane kolena, laktí, na zápästí a na temene hlavy. Pacienti majú zvýšenú hladinu protilátok proti gliadinu a tTG2. Histologický nález z tenkého čreva poukazuje na rovnaké funkčné a morfológické zmeny ako u celiakie. Nasadením bezlepkovej diéty dochádza po niekoľkých mesiacoch k zlepšeniu slizničných aj kožných prejavov (Rodrigo, 2006).

Asi u 5% pacientov nedochádza k obnove črevnej sliznice. Ak pri dlhodobom dodržiavaní bezlepkovej diéty (vyše 12 mesiacov) naďalej pretrváva atrofia klkov, hyperplázia krypt a infiltrácia IELs, ide o *refraktórnu sprue (RS)*. Podľa fenotypu IELs sa rozlišujú 2 typy RS: typ I. s IELs $CD3^+CD8^+ TCR\beta^+$, u typu II. je 50% IELs aberantných s $CD3^+CD8^-TCR\beta^+$ fenotypom. Diagnóza sa musí potvrdiť histologickým nálezom (Cellier et al., 1998; 2000).

2. SLIZNIČNÝ IMUNITNÝ SYSTÉM

Povrch gastrointestinálneho, respiračného a urogenitálneho traktu, očnej spojivky a vývodov exokrinných žliaz tvorí asi 300m². Sliznice majú vytvorený vysoko účinný obranný systém, ktorého úlohou je rozlíšiť škodlivé antigény od neškodných.

Celiakia sa vyvíja v prostredí slizničného systému, ktorého integrálnou súčasťou sú rezidentné cirkulujúce zložky imunitného systému. Sliznica čreva je anatomicky tvorená jednou vrstvou epitelových buniek a v nej prítomných imunitných bunkách. Hlavnou úlohou slizničného imunitného systému je zabrániť vniknutiu patogénnych mikroorganizmov a patogénnych zložiek z povrchu sliznice do vnútorného prostredia organizmu. Slizničný imunitný systém má vyvinuté regulačné mechanizmy, ktoré eliminujú nebezpečné antigény (mechanizmom antigénnej exklúzie), alebo navodzujú orálnu toleranciu proti komenzálnym mikroorganizmom a potravinovým antigénom (Singh et al., 2001). Pri celiakii nastáva porušenie orálnej tolerancie proti gluténu.

Do patogenetických mechanizmov celiakie sú zapojené obe zložky imunitného systému, jak adaptívna (T a B-bunkami mediovaná), tak aj prirodzená imunita (Maiuri et al., 2003; Hüe et al., 2004; Meresse et al., 2004).

2.1 Adaptívna imunita

MALT (lymfoidné tkanivo asociované s mukózou) je tvorený T a B lymfocytmi, ktoré môžu byť zoskupené v lymfoidných folikuloch (Payerove plaky v čreve), alebo sú rozptýlené v epiteli (IELs) a v lamina propria (Ogra et al., 1999).

2.1.1 T- lymfocyty

T-lymfocyty sa vyvíjajú v týmuse, do krvi prestupujú ako naivné T-lymfocyty, migrujú do lymfoidných tkanív, kde sú aktivované cudzorodými antigénmi v komplexe s MHC predkladanými na povrchu antigén prezentujúcich buniek. Vzniká subpopulácia efektorových a pamäťových buniek.

T-lymfocyty recirkulujúce v krvi aj v tkanive sa rozdeľujú na pomocné T_H ($CD3^+CD4^+$) a cytotoxické T_C ($CD3^+CD8^+$) bunky. Podľa typu nesúceho TCR receptoru sa rozdeľujú na $\alpha\beta$ alebo $\gamma\delta$. Až 95% lymfocytov je $\alpha\beta$ typu, zvyšných 5% nesie $\gamma\delta$ reťazec. Minoritná subpopulácia $\gamma\delta$ T-lymfocytov je zväčša fenotypu $CD3^+CD4^-$, $CD8^-$ a rozpoznáva antigény nepeptidovej povahy bez účasti antigén prezentujúcich buniek (APC) a HLA molekúl. Na základe rozdielnej distribúcie a funkcie sa môžu T-bunky deliť do 2 skupín: jedna skupina sa nachádza v lymfatickom tkanive a druhá medzi epitelovými bunkami kože a čreva a zrejme sú súčasťou imunologického dohľadu (Buc, 2001). Ich zvýšený počet je pozorovaný pri autoimunitných chorobách.

2.1.1.1. Intraepitelové lymfocyty

IELs sa nachádzajú v epiteli kože a slizníc. Sú fenotypovo heterogénne. Rozlišujú sa 2 základné populácie, jedna nesie $\alpha\beta^+$ T bunkový receptor, druhá $\gamma\delta^+$ T-receptor. Počet $\alpha\beta^+$ T-buniek sa zvyšuje pri reakcii na antigénny podnet alebo pri strese. Úloha $\gamma\delta^+$ buniek nie je úplne objasnená, mohli by zohrávať určitú regulačnú úlohu pri imunitných reakciách. (Collin et al., 2005).

Väčšina IELs je $CD3^+CD8^+$, nesú $\alpha\beta^+$ T-receptor, s $CD45R0^+$ fenotypom, s adhezívnymi molekulami typu integrínov ($\alpha4\beta7$) a cytoplazmatickými granulami s perforínom, ktoré sa podieľajú na bunkami mediovanej cytotoxicite. U myší bola opísaná subpopulácia nesúca homodimér $CD8\alpha\alpha$, ktorá sa bežne nevyskytuje v cirkulácii (Ogra et al., 1999; McDonald, 1998; Štěpánková et al., 1998).

Intraepitelové T-lymfocyty spolu s epitelovými bunkami sa podieľajú na zápalových reakciách, ako sú nešpecifické črevné zápaly, Crohnova choroba a celiakia (Tlaskalová-Hogenová et al., 1995). Charakteristickým znakom celiakie je zvýšený počet intraepitelových TCR $\alpha\beta^+$ a $\gamma\delta^+$ T-buniek. Zvýšený počet $\gamma\delta^+$ T-buniek nie je typický fenomén len u celiakie, bol pozorovaný aj pri alergii na mlieko, post-enteritíde ale aj u zdravých jedincov (Spencer et al., 1991; Brandtzaeg et al., 1998; Collin et al., 2005). Napriek tomu, v prípade chýbajúcej atrofie, je vyšetrenie ich počtu nápomocné pri stanovení diagnózy celiakie (Mäki et al., 1991).

Atrofia klkov súvisí s apoptózou enterocytov vedúcou k deštrukcii epitelu (Ciccocioppo et al., 2001). Jedným z možných mechanizmov je zabíjanie epitelových buniek cez systém perforín/granzým (Di Sabatino et al., 2001). Apoptóza môže byť spustená interakciou NKG2D receptora a CD94 na povrchu NK buniek a CD8⁺ cytotoxických T-lymfocytov a jeho ligandu MICA (neklasická-HLA molekula) a HLA-E na povrchu epitelových buniek. U zdravých jedincov len veľmi malé percento enterocytov exprimuje na svojom povrchu MICA, jeho nízka expresia chráni epitelové bunky pred IELs. MICA a HLA-E môžu byť indukované stresom a interferénom (IFN)- γ (Jabri et al., 2000; Maiuri et al., 2001). H \ddot{u} e a spoluprac. (2004) potvrdili, že MICA je po *in vitro* stimulácií gliadinom a jeho peptidom (p31-49) silne exprimovaný na povrchu epitelových buniek u celiakálnych pacientov a je rozpoznávaná NKG2D receptorom na povrchu cytotoxických IELs. Až 87% epitelových buniek u pacientov s RS je MICA pozitívnych, interakcia NKG2D/MICA je zrejme hlavným mechanizmom masívneho poškodenia črevného epitelu (H \ddot{u} e et al., 2004). IELs izolované z pacientov s RS (aberantné CD3⁻CD7⁺NKG2D⁺) zabíjali MICA-pozitívne HT-29 a HeLa epitelové línie (Di Sabatino et al., 2001). U refraktórnej sprue pretrváva aktivácia IELs aj po nasadení bezlepkovej diéty, u týchto pacientov je vysoké riziko vzniku T- bunkových lymfómov (Meijer et al., 2004).

2.1.1.2. CD4⁺ T- lymfocyty v lamina propria (LP)

CD4⁺ T-lymfocyty sú prekurzorom pomocných T-buniek, ktoré sa podľa produkovaných cytokínov rozdeľujú na T_H1 (IL-2, IFN- γ , lymfotoxín), T_H2 (IL-4, IL-5, IL-6, IL-9, IL-13), T_H3 (IL-10, TGF- β). Subpopulácie sa polarizujú z T_H0 klonov (produkujú zmes cytokínov oboch typov).

Hlavnú úlohu v imunopatogenetickom mechanizme celiakie zohrávajú mukózne CD4⁺ α/β T-lymfocyty v LP, ktoré produkujú zápalové cytokíny T-bunkového typu s T_H1/T_H0 profilom (Sollid et al., 2002; Schuppan et al., 2003; Cerf-Bensussan et al., 2003). Zvyšuje sa počet gluténom aktivovaných CD4⁺ T-buniek v lamina propria a CD8⁺ T-buniek v epiteli (Shan et al., 2002; Sollid et al., 2002). V tkanive tenkého čreva sa zvyšuje hladina cytokínov, najmä IFN- γ , ktorý aktivuje ďalšie typy buniek zahrňujúcich i bunky prirodzenej imunity (Nilsen et al., 1995). IFN- γ spolu s tumor

nekrotizujúcim faktorom (TNF)- α výrazne prispieva k zvýšeniu epitelovej permeability (Madara a Stafford, 1989). Glutén-špecifické T-bunky boli izolované z periférnej krvi celiakálnych pacientov (Ráki et al., 2007).

Väčšina glutén-špecifických T-buniek rozpoznáva v alkohole rozpustné frakcie gliadinu. Na základe štúdií na T-bunkových klonoch izolovaných z črevnej mukózy celiakálnych pacientov boli opísané imunogénne gliadinové epitopy (Sjöström et al., 1998; Molberg et al., 1998; Arentz-Hansen et al., 2000; 2002; Sollid et al., 2000). Dva z nich sú HLA-DQ2 restričné peptidy α -9(QLQPFQLPY) a α -2(PQPQLPYPQPQLPY), zdieľajú spoločný motív 7 aminokyselín (PQPQLPY). T-lymfocyty z celiakálnych pacientov rozpoznávajú prevažne peptidy na N-terminálnom konci α -gliadinu (prezentované HLA-DQ2 alebo DR7 molekulami) (Franco et al., 1994; Johansen et al., 1996).

Prehľad známych peptidov, ktoré sú schopné stimulovať T-bunky je uvedený v tab. č. 1 (Koning, 2005; Stepniak a Koning, 2005).

Gliadinové peptidy stimulujúce T-bunky

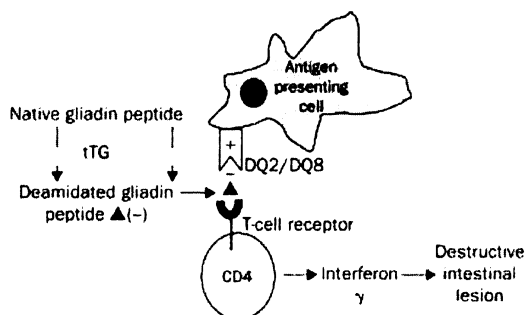
Peptid	Sekvencia	Zdroj proteínu	HLA-DQ	tTG závislosť
Glia- α 2	PQPQLPYPQ	α -gliadin	DQ2	+++
Glia- α 9	PFPQPQLPY	α -gliadin	DQ2	+++
Glia- α 20	FRPQQPYPQ	α -gliadin	DQ2	+++
Glia- α	QGSFQPSQQ	α -gliadin	DQ8	+
Glia- γ 2	FPQQPQQPF	γ -gliadin	DQ2	+++
Glia- γ 1	PQQSFPPQQ	γ -gliadin	DQ2	+++
Glia- γ 30	IIQPQQPAQ	γ -gliadin	DQ2	+
Glt-156	FSQQQQSPF	LMW-glutenin	DQ2	+++
Glt-17	FSQQQQQPL	LMW-glutenin	DQ2	+++
Glt	QGYYPSTSPQ	HMW-glutenin	DQ8	-
Glu-5	QLPQQPQQF	Unknown	DQ2	+++

Tabuľka č.1: Deamidované aminokyseliny sú zvýraznené. +++ peptidy sú rozpoznávané T-bunkami po demidácii tTG2; + deamidácia tTG2 zvyšuje T-stimulačnú kapacitu, - peptid je rozpoznávaný T-bunkami nezávisle na demidácii tTG2 (Koning, 2005; Stepniak a Koning, 2005)

Peptid p31-43 pochádzajúci z α -gliadinu nie je rozpoznávaný CD4⁺ intestinálnymi T-lymfocytmi, ale vyvoláva poškodenie tenkého čreva u celiakálnych

pacientv a u pacientov v remisii (Maiuri et al., 2003; Fraser et al., 2003; Sturgess et al., 1994). Stimuluje makrofágy k produkcii cytokínov a aktivuje dendritické bunky v lamina propria, zvyšuje u nich expresiu IL-15, ktorý je podstatný pre aktiváciu a zachovanie pamäťových T- buniek (Maiuri et al., 1996; 2000; 2003; Jelínková et al., 2004).

Peptidy prestupujú do lamina propria tenkého čreva pravdepodobne cez porušené medzibunkové spoje. Glutamín týchto peptidov je pôsobením tTG2 v čreve deamidovaný na kyselinu glutámovú. Deamidáciou glutamínových zvyškov získavajú gliadinové peptidy negatívny náboj a zvyšuje sa ich väzobná aktivita na HLA-DQ2 alebo HLA-DQ8 a tým sa zvyšuje aj ich T-stimulačná kapacita (Obr. č 2) (Green a Jabri, 2003; van de Wal et al., 1998; Sollid, 1998; Sjöstrom et al., 1998; Molberg et al., 1998; Shan et al., 2002; Stepniak a Koning, 2005).



Obrázok č. 2 (Green a Jabri, 2003)

Nedávno bola opísaná ďalšia efektorová subpopulácia lymfocytv $CD4^+ T_H17$, ktorá sa diferencuje pod vplyvom IL-23, IL-6 a transformujúceho rastového faktora (TGF)- β 1. Tieto bunky produkujú IL-6, TNF- α , IL-17A a IL-17F cytokíny a sú pravdepodobne zodpovedné za zápalovú imunitnú odpoveď u mnohých autoimunitných ochorení (Kikly et al., 2006). IL-17 produkovaný hlavne aktivovanými T-bunkami stimuluje fibroblasty, endotelové bunky, makrofágy a epitelové bunky k produkcii prozápalových mediátorov (IL-1, IL-6, TNF- α , metaloproteínáz a chemokínov), ktoré indukujú zápal (Kolls a Linden, 2004; Nakae et al., 2003).

Zvláštnu skupinu tvoria regulačné T bunky - Treg ($CD4^+CD25^+$), T_H3 a Tr1, ktoré majú tlmiaci účinok na vznik autoimunitných reakcií, regulujú autoreaktívne lymfocyty, ktoré unikli negatívnej selekcii v týmuse (Thornton a Shevach, 2000).

Produkujú prevažne IL-10, TGF- β a prostaglandíny. Inhibujú hlavne T_H1 imunitnú odpoveď. Regulujú cez neznámy mechanizmus, vyžadujú priamy kontakt bunka-bunka, alebo cez účinok produkovaných cytokínov (Sakaguchi et al., 1995; Akbari et al., 2003; Kretschmer et al., 2005). Gianfrani a spoluprac. izolovali z črevnej mukózy celiakálnych pacientov v remisii gliadin-špecifické T-bunky, ktoré kultivovali v prostredí s gliadinom a s IL-10. Získali klon T-buniek s T_H0 cytokínovým profilom. Z biopsií sa im podarilo izolovať aj T-regulačné bunky Tr1, ktoré boli anergické, po stimulácii gliadinom produkovali IL-10 a IFN- γ , ale len málo IL-2 a IL-4 a potláčali proliferáciu izolovaných patogénnych T_H0 bunkových klonov, zistenie prítomnosti Tr1 buniek v čreve by mohlo viesť k novým terapeutickým prístupom v celiakii (Gianfrani et al., 2006).

2.1.2. B lymfocyty

Diferencujúce sa B-lymfocyty sú prítomné v germinálnych centrách lymfoidných folikulov. V lamina propria sú prezentované hlavne plazmatickými bunkami produkujúcimi prevažne IgA, v menšej miere aj IgM a IgG. B lymfocyty môžu fungovať aj ako APC, komplexy tTG-gliadin sú endocytované a prezentované s HLA-DQ príbuzným T-lymfocytom (Sollid et al, 1997; Tlaskalová-Hogenová et al., 2002).

Boli charakterizované imunodominantné B bunkové epitopy α/β a γ -gliadinu rozpoznávané IgA a IgG protilátkami pacientov s celiakiou (Kasarda et al., 1984; Okita et al., 1985; Krupičková et al, 1999, Osman et al., 2000). Osman a spoluprac. použitím syntetických peptidov identifikovali niekoľko epitopov α -gliadinu charakterizovaných prítomnosťou spoločného motívu QQQFPF a jeden repetitívny epitop v γ -gliadine (Osman et al, 2000).

V našom laboratóriu boli študované B-bunkové epitopy gliadinu a autoantigénu kalretikulínu, ktoré boli rozpoznávané IgA autoprotiátkami v sére celiakálnych pacientov a pacientov s asociovanými autoimunitnými a črevnými ochoreniami (Karská et al., 1995, Krupičková et al., 1999, Sánchez et al., 2000). Sánchez a spoluprac. (2003) ďalej metódou PEPSCAN, použitím dodekapeptidov kalretikulínu,

mapovali epitopy B-lymfocytov pacientov s celiakiou a s autoimunitnými ochoreniami pečene a preukázali radu spoločných a pre nemoc špecifických imunodominantných epitopov kalretikulínu.

2.2. Zložky prirodzenej imunity

Prirodzená zložka imunitného systému je v mukóze zastúpená epitelovými bunkami, makrofágmi, dendritickými bunkami, NK bunkami, mastocytmi, fibroblastami, neutrofilmi, eozinofilmi a ich humorálnymi produktmi (cytokíny, chemokíny, zložky komplementu, lyzozým, laktoferín), charakteristická je aj produkcia antimikrobiálnych proteínov a peptidov (Mestecký et al., 2005; Tlaskalová-Hogenová, 2002).

2.2.1. Epitelové bunky

Epitelové bunky tenkého čreva sú vysoko polarizované bunky s apikálnym a bazolaterálnym koncom. Sú to krátko žijúce bunky, ich obnova sa uskutočňuje každých 3-5 dní. Základným predpokladom pre ich správnu funkciu je celistvosť epitelovej vrstvy (Tlaskalová-Hogenová, 1995). Úzko kooperujú s intraepitelovými T-lymfocytmi a spoločne tvoria prvú obrannú líniu pred vstupom cudzorodých antigénov do organizmu. Reagujú navzájom prostredníctvom E-kadherínových adhezívnych molekúl epitelových buniek a integrínov z podrodiny β_7 ($\alpha_E\beta_7$) lymfocytov. Zabezpečujú interakciu organizmu s vonkajším prostredím a extracelulárnou matrix, konštitutívne exprimujú MHC molekuly I. aj II. triedy, ich expresia sa zvyšuje po stimulácii napr. IFN- γ . Produkujú široké spektrum cytokínov a chemokínov (IL-1, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, faktor stimulujúci rast kolónií granulocytov a makrofágov (GM-CSF), TGF- β), ktoré napomáhajú pri regulácii slizničnej imunity. Majú určitú úlohu pri edukácii IELs nezávislých na týmuse (Eckmann, 1993; Stadnyk, 2004; Sartor, 1994). Intestinálne epitelové bunky exprimujú aj neklasické molekuly MHC I, ktoré napomáhajú prenosu signálov z lúmenu čreva k lymfocytom v lamina propria a robia z nich tak unikátne APC

v čreve. Spoločne s molekulami CD1d, MICA/B, HLA-E, FcRn, MR1 a UL16-viažúcim proteínom sa podieľajú na aktivácii lamina propria T-lymfocytov a na regulácii zápalu (Dahan et al., 2007).

Enterocyty viažu polymérne imunoglobulíny (IgA, IgM) produkované plazmatickými bunkami v lamina propria a transportujú ich do lúmenu čreva ako sekrečné imunoglobulíny (Brandtzaeg, 1995).

Zvláštnu úlohu medzi epitelovými bunkami majú Panethove bunky a membránové epitelové bunky (M-bunky). Panethové bunky sú sekrečné epitelové bunky produkujúce látky s antimikróbnymi účinkami (lyzozým, fosfolipáza A2, defenzíny) (Huttner a Bevins, 1999). M-bunky, pohlcujú antigén z lúmenu čreva a transportujú ho do lymfatických folikulov, kde aktivujú T-lymfocyty a tým indukujú slizničnú imunitu.

Aktivované T-lymfocyty v epiteli tenkého čreva prostredníctvom produkovaných cytokínov priamo pôsobia na epitelové bunky. V poslednej dobe sa pozornosť zameriava na úlohu IL-15, ktorý zrejme zohráva jednu z významných úloh v patogenéze celiakie. IL-15 je pleiotropný cytokín, ktorý spoločne zdieľa s IL-2 dva receptorové reťazce (IL-2R β a γ reťazec) (Giri et al., 1994). Exprimujú sa však na rozdielnych bunkách, zatiaľ čo IL-2 je exprimovaný na povrchu aktivovaných T-lymfocytov, IL-15 je exprimovaný viacerými typmi buniek, vrátane monocytov, makrofágov a epitelových buniek (Waldmann a Tagaya, 1999; Perera et al., 2000). IL-15 stimuluje produkciu IFN- γ intraepitelovými lymfocytmi, zvyšuje ich cytotoxicitu a prispieva k poškodeniu epitelu (Meresse et al., 2004). Súčasne oba cytokíny (IL-2 a IL-15) stimulujú T-bunkovú proliferáciu (Li et al., 2001).

Mention a spoluprac. (2003) detekovali zvýšenú expresiu IL-15 v čreve pacientov s aktívnou formou celiakie, ale nebola zaznamenaná u Crohnovej choroby. IL-15 indukuje expresiu MICA v biopsiách pacientov aj zdravých kontrol, a tým neustále aktivuje adaptívnu imunitu (Maiuri et al., 2000).

Intestinálne epitelové bunky sú rozpoznávané IgA i IgG autoprotiátkami v sére pacientov a v kultivačnom médiu z jejunálnych biopsií (Tlaskalová et al., 1990; Krupičková et al., 1999; Sanchez et al. 2000).

2.2.2. Antigén prezentujúce bunky

Profesionálne antigén prezentujúce bunky, medzi ktoré patria monocyty, makrofágy a dendritické bunky, zohrávajú v lamina propria významnú úlohu pri udržiavaní orálnej tolerancie. Za fyziologických podmienok exprimujú nízku hladinu povrchových molekúl B7 (CD80) a B7.2 (CD86), ktoré sú kostimulačnými molekulami T-buniek (Qiao et al., 1996).

2.2.2.1. Mononukleárno-fagocytový systém

Zástupcami mononukleárno-fagocytového systému sú makrofágy. Za nezrelú formu makrofágov sa označujú monocyty, ktoré sú prítomné v krvnom obeh. Na základe povrchovej expície diferenciačných znakov sa monocyty rozdeľujú na „klasické“ (CD14^{hi}CD16⁻), ktoré tvoria 90-95% z celkového množstva monocytov u zdravých jedincov a „neklasické“, alebo tiež označované ako „prozápalové“ (CD14⁺CD16⁺(FcγIII pozitívne)), ktoré produkujú menej IL-10 a viac TNF-α. V porovnaní s klasickými monocytmi majú vyššiu expresiu HLA-DR. U zdravých jedincov táto subpopulácia predstavuje 5-10% z celkového počtu krvných monocytov a sú akýmsi prekursorom migrujúcich DCs (Frankenberger et al., 1996; Belge et al., 2002; Randolph et al., 2002). Jednotlivé subpopulácie sa líšia aj expresiou povrchových molekúl. Receptory pre CCR5 a CX3CR1 sú silne exprimované u CD14⁺CD16⁺ buniek, expresia CCR2 a nízka expresia CX3CR1 sú typické pre CD14^{hi}CD16⁻ bunky. Expícia rozdielnych chemokínových receptorov a adhezívnych molekúl v jednotlivých subpopuláciách môže mať významnú úlohu v ich odlišnej migrácii a citlivosti na jednotlivé infekcie (Weber et al, 2000; Geissmann et al., 2003).

Makrofágy sa nachádzajú v celom organizme, v rôznych tkanivách a orgánoch, majú spoločný pôvod a rovnakú funkciu. Tvoria asi 5% z celkovej populácie leukocytov. Makrofágy sa vyvíjajú z prekursorovej bunky z kostnej drene (monoblast). Pod vplyvom rastových faktorov sa diferencujú na promonocyty a ďalej

na monocyty. Po 3 dňoch v cirkulácii sa monocyty usídľujú v tkanivách kde diferencujú na makrofágy. Sú to dlho žijúce bunky schopné lokálnej proliferácie.

Makrofágy patria medzi prvé bunky, ktoré sa infiltrujú na miesto zápalu. V odpovedi na mikrobiálne stimuly a IFN- γ produkujú prozápalové cytokíny a chemokíny (IL-12, TNF- α , IL-1, IL-6, IL-8, zápalový proteín makrofágov (MIP)-1 α). Zohrávajú dôležitú imunoregulačnú úlohu jak v prirodzených, tak aj v adaptívnych imunitných obranných mechanizmoch. Zúčastňujú sa fagocytózy, syntetizujú imunoregulačné látky (monokíny), zložky komplementu a fungujú ako APC (Buc, 2001).

2.2.2.2. Dendritické bunky

Dendritické bunky sa vyvíjajú z progenitorovej bunky CD34⁺ v kostnej dreni, ktoré diferencujú na krvné prekurzory DCs (CD11⁺CD14⁺). Pod vplyvom IL-4 a GM-CSF alebo TNF- α sa diferencujú na nezrelé DCs (CD11⁻,CD11⁺CD14⁻). Nezrelé tkanivové DCs majú vysokú fagocytárnu schopnosť a po aktivácii migrujú do lymfatických uzlín, kde aktivujú T-lymfocyty. Prostredníctvom špecifických receptorov reagujú na široké spektrum chemokínov. Po stimulácii antigénom podliehajú funkčným a fenotypovým zmenám. V priebehu vyzrievania zvyšujú expresiu molekúl CD80, CD86, CD40, HLA-DR, CCR7, znižuje sa ich fagocytárna schopnosť. V lymfatických folikuloch slizníc sa nachádzajú dva typy DCs: subpopulácia nezrelých DCs tesne pod epitelom folikulov a subpopulácia diferencovaných DCs v interfolikulárnej oblasti, kde sú v kontakte s T-bunkami. Dendritické bunky prispievajú k polarizácii naivných T-lymfocytov. Štúdie v poslednej dobe ukazujú, že výsledná imunitná odpoveď na antigén (indukcia tolerancie alebo stimulácia) závisí od zúčastnených subpopulácii DCs (Banchereau et al., 2000). Neinfekčné antigény sú prezentované nezrelými DCs, ktoré indukujú T_H2 alebo T_H3 polarizáciu a navodzujú orálnu toleranciu. Patogénne antigény sú prezentované diferencovanými DCs, indukujúcimi T_H1 imunitnú odpoveď, vedúcu k zápalu alebo k rezistencii proti intracelulárnym infekciám (Tlaskalová-Hogenová et al. 2007).

2.2.3. NF- κ B systém

Signálne dráhy predstavujú sieť kaskád, ktoré zabezpečujú odpoveď bunky na rozličné stimuly, ako sú napr. cytokíny, rastové faktory alebo stres. Jedna z významných signálnych dráh vedie cez NF- κ B transkripčný faktor, ktorý indukuje expresiu mnohých génov. Reguluje apoptózu, migráciu buniek, produkciu zápalových cytokínov (IL-1 β , TNF- α , GM-CSF, IL-2, IL-11, IL-17), chemokínov (IL-8, RANTES, MIP-1 α , proteín atrahujúci monocyty (MCP)-2), enzýmov (indukovateľnú NO syntázu, cyklooxygenázu) a adhezívne molekuly (VCAM-1, ICAM-1, E-slektin) a receptory (receptor pre IL-2). Pozostáva z 5 podjednotiek: NF- κ B1 (p105/p50), NF- κ B2 (p100/p52), RelA (p65), RelB a c-Rel. Najčastejšie sú do signálnych dráh zapájené diméry z podjednotiek p65 a p50 alebo s p52 (Siebenlist et al., 1994; Baeuerle a Baltimore, 1996). V cytoplazme buniek sa diméry nachádzajú v neaktívnom komplexe spoločne s inhibítorom I κ B. Po stimulácii je I κ B podjednotka fosforylovaná, ubikvitinovaná a následne degradovaná v proteázovom komplexe. Uvoľnený dimér translokuje do jadra a viaže sa na DNA špecifický promótor, čím spúšťa transkripciu génov (Barnes, 1997).

3. CIEĽ PRÁCE

1. Analyzovať a porovnať stimulačný účinok gliadinu a jeho peptidov a iných potravinových antigénov (ako je sója a ovalbumín) na bunky ľudských monocytárných línií, ktoré sa od seba líšia stupňom diferenciácie. Sledovať produkciu cytokínov a chemokínov a charakterizovať signálnu dráhu indukovanú gliadinom.
2. Študovať odpoveď monocytov izolovaných z periférnej krvi pacientov s aktívnou formou celiakie (CD), pacientov na bezlepkovej diéte (GFD) a zdravých darcov (HD) na peptické fragmenty gliadinu. Porovnať produkciu cytokínov a chemokínov medzi jednotlivými skupinami monocytov a analyzovať vplyv pridaného IFN- γ . Určiť vplyv genetickej predispozície na kvantitu produkovaných cytokínov u zdravých jedincov a expresiu aktivačných a maturačných znakov buniek. U pacientov liečených bezlepkovou diétou charakterizovať vzťah výšky hladiny produkovaných protilátok proti gliadinu a tTG2 na hladiny gliadinom indukovaných cytokínov.
3. Analyzovať ochranný účinok materského mlieka na vznik celiakie. Charakterizovať a monitorovať zmeny cytokínového profilu materského mlieka odoberaného od matiek v rôznych časových intervaloch po pôrode.

4.VÝSLEDKY

Zložky prirodzenej a adaptívnej imunity spolu úzko kooperujú. Antigén prezentujúce bunky na základe povahy antigénov rozhodujú, ktorým smerom bude imunitná odpoveď indukovaná. V našom laboratóriu sme zistili, že peptické štepy gliadinu a jeho peptidy majú výnimočnú schopnosť stimulovať myšie peritoneálne makrofágy k produkcii chemokínov, cytokínov a indukovateľnej NO syntázy. Bol identifikovaný dodekapeptid B (FQQPQQQYPSSQ), ktorý výrazne indukoval makrofágy k produkcii TNF- α , IL-10, a RANTES (Tučková et al., 2002).

Zaujímalo nás, či má gliadin rovnaký vplyv aj na ľudské monocyty. V našej práci sme sledovali stimulačný účinok potravinových proteínov (sója, ovalbumínu, gliadinu a jeho peptidov) na produkciu cytokínov a chemokínov (IL-8 a TNF- α) ľudskými monocytárnymi líniami THP-1 a U937 a ovplynenie tejto aktivity IFN- γ , ktorý je hlavným cytokínom pri celiakii. IL-8 a TNF- α boli stanovené v kultivačnom médiu metódou ELISA testu. Aby sme sa čo najviac priblížili podmienkam, aké sú v gastrointestinálnom trakte, použité proteíny boli opracované pepsínom (naviazaným na agarózou) v kyslom prostredí (pH 1.8). Monocytové/makrofágové línie THP-1 (CD14⁺CD68⁺) a U937 (CD14⁻CD68⁻) sa líšia od seba stupňom diferenciacie. Neštiepený gliadin má len slabý vplyv na produkciu IL-8 a TNF- α línou THP-1. Jeho peptické štepy vyvolávali signifikantne vyššiu produkciu oboch cytokínov (100-500 μ g/ml), po stimulácii spoločne s IFN- γ (150 U/ml) sa produkcia ešte viac zvýšila. Naštiepená sója a ovalbumín nemali žiaden účinok na produkciu IL-8 a TNF- α ani v kombinácii s IFN- γ . Sledovali sme aj vplyv prestimulácie IFN- γ . Produkcia IL-8 a TNF- α bola najvyššia po stimulácii gliadinovými fragmentami v kombinácii s IFN- γ , po 2h a 24h prestimulácii IFN- γ v porovnaní s neprestimulovanými bunkami sa ešte viac zvýšila.

Menej diferencované U937 bunky produkovali po stimulácii gliadinovými fragmentami len veľmi nízku hladinu IL-8, ktorá sa zvýšila po 24h prestimulácii IFN- γ . Produkcia TNF- α nebola detekovateľná.

Súčasne s celou zmesou gliadinových peptidov sme sledovali produkciu IL-8 THP-1 bunkami po stimulácii syntetickým peptidom 33-mérom, peptidom p31-43, a B-peptidom. Produkcia IL-8 stimulovaná 33-mérom dosahovala len 20% a B

peptidom 60% odpoveď zo stimulačnej kapacity celkovej zmesi gliadinu a p31-43. TNF- α produkcia po stimulácii B peptidom dosiahla len 10% a u p31-43 len 40% účinnosť v porovnaní s gliadinovými fragmentami. 33-mér neindukoval produkciu TNF- α ani po stimulácii spoločne s IFN- γ . Jednotlivé gliadinové peptidy sa líšia svojou stimulačnou kapacitou, najsilnejším aktivátorom ľudskej monocytárnej línie THP-1 je celá zmes gliadinu.

Ako pozitívnu kontrolu sme použili LPS (1 μ g/ml). Odpoveď na LPS (obdobne ako na gliadin) bola u línie THP-1 a U937 odlišná. THP-1 produkovali signifikantne vyššiu hladinu IL-8 (558.7 \pm 23.7 pg/ml) a TNF- α (107.3 \pm 13.0 pg/ml) než U937 línia (IL-8: 322.6 \pm 16.1 pg/ml; TNF- α : 33.5 \pm 5.2 pg/ml). Zaujímalo nás, či expresia CD14 molekuly, ktorá je vysoko exprimovaná u THP-1, ale nie u U937 línie, má vplyv na produkciu cytokínov. Monoklonálna protilátka anti-CD14 znižovala produkciu IL-8 u THP-1 buniek po stimulácii LPS, ale nemala žiaden efekt na aktiváciu buniek gliadinovými fragmentami.

Po stimulácii THP-1 buniek gliadinovými fragmentami (500 μ g/ml) sa zvyšovala väzobná aktivita podjednotiek (p50 a p65) NF- κ B signálnej dráhy. Pre potvrdenie zapojenia NF- κ B do signalizačných mechanizmov po stimulácii gliadinom (500 μ g/ml) sme sledovali efekt inhibítorov NF- κ B: sulfasalazinu, PDTC a TPCK. Sulfasalazin inhibuje fosforyláciu I κ -B (Wahl et al., 1998), PDTC (pyrrolidine dithiocarbamate) blokuje disociáciu NF- κ B/I κ -B komplexu (Snyder et al., 2002), TPCK (L-1-tosyloamido-2-phenyl chloromethyl ketone) bráni degradácii I κ -B (Baldwin, 1996). PDTC (0.5 μ M) a sulfasalazin (2 mM) inhibovali väzobnú aktivitu p65 podjednotky, ale len slabo p50; TPCK (5 μ M) inhiboval väzobnú aktivitu podjednotky p50 výraznejšie než p65 podjednotky.

Sulfasalazin znižoval produkciu IL-8 THP1-bunkami, jeho efekt bol dávkovo závislý, v koncentrácii 0,1 mM bola pozorovaná 15% inhibícia a 2 mM mal až 90% účinnosť. PDTC pôsobil už pri koncentrácii 0,1 μ M. TPCK mal pri koncentrácii 1 μ M 32% efekt a 25 μ M úplne inhiboval produkciu IL-8.

Pri aktivácii NF- κ B sa I κ B α fosforyluje a degraduje. Pomocou Western Blotu sme sledovali fosforyláciu I κ B α u THP1-buniek po stimulácii štiepeným gliadinom samotným a v kombinácii s IFN- γ , v porovnaní s nestimulovanými bunkami sa

fosforylácia I κ B α zvyšovala. Tieto výsledky potvrdzujú zapojenie NF- κ B do signalizačnej dráhy ľudských monocytov po stimulácii gliadinom.

Naše výsledky ukázali, že gliadin na rozdiel od iných potravinových antigénov, ako je sója a ovalbumín, je schopný priamo stimulovať ľudské monocyty k produkcii IL-8 a TNF- α . T-bunkový cytokín IFN- γ , ktorý je hlavný cytokín u celiakálnych pacientov, má synergistický účinok na produkciu cytokínov a chemokínov. Zvýšená produkcia IL-8 (chemoatraktant imunitných buniek) a TNF- α (vyvoláva produkciu metaloproteináz) aktivovanými monocytmi/makrofágmi prispievajú k poškodeniu črevnej sliznice.

Gliadin stimulates human monocytes to production of IL-8 and TNF- α through a mechanism involving NF- κ B. Jelínková L, Tučková L, Cinová J, Flegelová Z, Tlaskalová-Hogenová H. *FEBS Letters*; 571: 81-85, 2004

Aby sme lepšie pochopili úlohu prirodzenej imunity v celiakii, porovnávali sme odpoveď na gliadin medzi ľudskými monocytmi izolovanými z periférnej krvi zdravých darcov, aktívnych celiakov a pacientov na bezlepkovej diéte.

V jednotlivých skupinách monocytov sme použitím ELISA metódy v kultivačnom médiu merali spontánnu a gliadinom indukovanú produkciu IL-8 a TNF- α . Spontánnu produkciu IL-8 bola v skupine aktívnych celiakov a pacientov liečených bezlepkovou diétou vyššia (525 ± 404 pg/ml a 965 ± 682 pg/ml, $P > 0.05$) než u monocytov zdravých darcov (131 ± 202 pg/ml, $P < 0.001$). Po stimulácii IFN- γ (150U/ml) samotným nedošlo k výraznejšiemu zvýšeniu produkcie, v kombinácii spoločne s gliadinom (100 μ g/ml) sa zvýšila produkcia IL-8 u všetkých troch skupín. Monocyty celiakálnych pacientov produkujú viac IL-8 než monocyty zdravých darcov. Najvyššia produkcia bola v skupine aktívnych celiakov (3365 ± 2451 pg/ml). U pacientov na bezlepkovej diéte poklesla produkcia IL-8 (2645 ± 1723 pg/ml), avšak v porovnaní s produkciou monocytov u zdravých darcov (1046 ± 783 pg/ml) ostala štatisticky významne zvýšená ($P < 0.001$). Obdobné rozdiely medzi skupinami boli nájdené i v prípade produkcie TNF- α . Zaujímavé je, že pridaný IFN- γ nemal vplyv na produkciu IL-8, ale výrazne zvýšil produkciu TNF- α .

U krvných monocytov zdravých jedincov sme sledovali vplyv gliadinových fragmentov na fenotypové zmeny. Po kultivácii monocytov s gliadinovými fragmentami (100µg/ml) alebo s IFN-γ (150 U/ml), v porovnaní s nestimulovanými monocytmi, sa len mierne zvýšila expresia maturačných (CD83) a aktivačných (CD80, CD40) molekúl, ktoré predstavujú znaky dendritických buniek. Po stimulácii gliadinom v kombinácii s IFN-γ sa u monocytov výrazne zvýšila povrchová expresia molekúl CD80, CD86, CD40, a hlavne CD83. Inkubácia buniek v prítomnosti sóje a jej proteolytických fragmentov, na rozdiel od gliadinu, nevyvolala fenotypové zmeny na bunkovom povrchu, ani v kombinácii s IFN-γ.

Väčšina celiakálnych pacientov (95%) exprimuje antigén HLA-DQ2⁺. Zaujímalo nás vplyv genotypu na produkciu IL-8 monocytmi zdravých darcov. Po stimulácii gliadinom a IFN-γ monocyty HLA-DQ2⁺ zdravých darcov produkovali 2 až 3-krát viac IL-8 než monocyty HLA-DQ2⁻ jedincov. Výsledky poukazujú na to, že monocyty s HLA-DQ2⁺ genotypom sú predisponovaní k vyššej produkcii IL-8.

Podobne ako u THP-1 línie, zaujímalo nás, či gliadinom indukovaná produkcia cytokínov monocytmi z periférnej krvi vedie tiež cez NF-κB signálnu dráhu. U monocytov aktívnych celiakov sa po stimulácii gliadinom zvýšila väzobná aktivita podjednotiek p50 a p65 ($P < 0.05$). Po inkubácii s inhibitorom TPCK sa väzobná aktivita redukovala u p50 podjednotky na 20% a u podjednotky p65 na 54%. NF-κB inhibitory TPCK (1-25 µM) a PDTC (1-10 µM) znižovali produkciu IL-8 a TNF-α. U monocytov zdravých darcov bola po stimulácii gliadinom zaznamenaná len slabá väzobná aktivita oboch podjednotiek, ktorú TPCK (25 µM/ml) úplne redukoval. Produkciu IL-8 a TNF-α inhiboval až na úroveň pozadia.

V tejto práci sme ukázali, že gliadinové fragmenty aktivujú monocyty celiakálnych pacientov a monocyty HLA-DQ2⁺ zdravých jedincov k zvýšenej produkcii prozápalových cytokínov a chemokínov. Zvýšené hladiny cytokínov aktivujú bunky prirodzenej imunity a tým ich zapájajú do patogenetických mechanizmov celiakie.

Gliadin peptides activate blood monocytes from patients with celiac disease.

Cinová J., Palová-Jelínková L, Smythies LE, Černá M, Pecharová B, Dvořák M, Fruhauf P, Tlaskalová-Hogenová H, Smith PD, Tučková L. *J Clin Immunol*; 27:201-209, 2007

Materské mlieko obsahuje veľa imunologických špecifických aj nešpecifických faktorov, vrátane imunitných buniek. U kojencov, ktorí ešte nemajú plne vyvinutý imunitný systém zabezpečujú ochranu pred infekčnými agens (Ogra, 1999; Tlaskalová-Hogenová et al., 1982). V materskom mlieku sa nachádzajú imunologicky aktívne zložky, ako je sekrečný IgA, antimikróbne faktory, cytokíny a rastové faktory. Jedným z nich je aj rastový epidermový faktor (EGF), ktorý sa podieľa na proliferácii a diferenciácii epitelových buniek. EGF bol detekovaný v rôznych telových tekutinách, vrátane kolostra a materského mlieka (Goodland et al, 1996; Xu, 1996). Štěpánková a spoluprac. vypracovali experimentálny model celiakie na krysách, ktoré boli kŕmené včasne po narodení gliadinom spolu s IFN- γ . Na tomto modeli demonštrovali ochranný vplyv materského mlieka pred rozvojom celiakie a ukázali, že jeden z faktorov, ktorý je v tomto mechanizme významný je EGF (Štěpánková et al., 2003). Zdá sa, že kojenie má vplyv na nástup celiakie a ukazuje sa, že u detí, ktoré sú prikrmované a zároveň aj kojené je nižšie riziko vzniku celiakie. Pre nás bolo zaujímavé vedieť, aké je zloženie kolostra a materského mlieka, či sa ich zloženie postupom času mení a či by jednotlivé zložky mohli mať ochranný účinok pred rozvojom celiakie.

Podieľali sme sa na pokusoch, v ktorých bolo odobraných 31 vzoriek kolostra (prvých 72 h po pôrode), alebo 22 vzoriek mlieka (72 h po pôrode) odobraných počas prvých 4 dní po pôrode. Použitím testov na stanovenie cytokínov (RayBio Human Cytokine Array V) sme sledovali zastúpenie jednotlivých proteínov v kolostre a v mlieku, ich koncentráciu a zmeny v časovo postupne odoberaných vzorkách. Jedna skupina vzoriek (17) bola odobraná počas prvých 2 dní po pôrode a boli použité array (na ktorých je možné detekovať až 79 rôznych proteínov v jednej vzorke. V druhej skupine bolo testovaných 42 proteínov (RayBio Human Cytokine Array III), pozostávala zo 14 vzoriek kolostra, kde od jednej matky bolo niekoľko odberov v rôznych časových intervaloch. Použitím cytokínových testov pre 79 proteínov, sme detekovali 68 rôznych proteínov, z toho 32 z nich boli opísané v ľudskom kolostre alebo v mlieku po prvý krát. Tri cytokíny (EGF, IL-8, a GRO-s proliferáciou súvisiaci onkoproteín) boli prítomné vo všetkých testovaných vzorkách, 19 proteínov malo veľmi častý výskyt (u vyše 50 % vzoriek). Priemerný počet cytokínov detekovaný

v jednej vzorke je 20. Zastúpenie jednotlivých cytokínov v kolostre a v mlieku matiek vykazuje individuálne rozdiely a zmena koncentrácie v čase nie je jednotná, ale väčšinou sa znižuje, preto sme súčasne so stanovením cytokínov prevádzali aj meranie celkovej hladiny bielkovín v týchto vzorkách.

V našej štúdii bola po prvý krát v kolostre a v mlieku preukázaná prítomnosť rastových faktorov (rastový faktor fibroblastov (FGF)-4, rastový faktor placenty (PIGF)), chemotaktických faktorov (B lymfocytárny chemoatraktant/CXCL13, zápalový proteín makrofágov (MIP)-1 δ -1 β /CCL4, MIP-1 δ /CCL15, MIP-3 α /CCL20, (PARC)/CCL18, faktor inhibujúci leukémiu, oncostatin M) a protizápalové faktory (tkanivový inhibítor metaloproteináz (TIMP)-1, TIMP-2, chemokín odvodený od makrofágov (MDC)/CCL22). Všeobecnou črtou bol pokles koncentrácie IL-12 a MIP-1 δ /CCL15 a nárast koncentrácie MCP-1/CCL2 v materskom mlieku.

Cytokine profiling in human colostrum and milk by protein array. Kverka M, Burianová J., Lodinová-Žadníková R, Kocourková I, Cinová J, Tučková L, Tlaskalová-Hogenová H. *Clin Chem*; 53:955-962, 2007

Časť výsledkov z našej práce je uvedených v prehľadnom článku, ktorý opisuje úlohu slizničného imunitného systému a jeho vplyv na celkový vývoj imunitného systému. Hneď po narodení, keď ešte nie je dokonale vyvinutý imunitný systém, je organizmus chránený pred infekciou prirodzenou zložkou imunitného systému. Významným znakom prirodzenej imunity je schopnosť rozlíšiť patogénne mikroorganizmy pomocou pattern recognition receptors (PRRs). Tieto molekuly sú tiež známe aj ako Toll-like receptors (TLRs), ktoré rozpoznávajú charakteristické, s patogénom asociované molekuly na povrchu mikroorganizmov (pathogen-associated molecular patterns-PAMPs) (Medzhitov a Janeway, 2000; Aderem a Ulevitch, 2000; Akira a Takeda, 2004), ich interakcia vedie k signalizácii a k aktivácii transkripčného faktoru NF- κ B, ktorý zodpovedá za aktiváciu génov kódujúcich produkciu cytokínov, chemokínov a ďalších mediátorov zápalu. Baktérie črevnej mikroflóry sú v úzkom kontakte s epitelovými bunkami. Po narodení sa osídľuje črevná sliznica mikroorganizmami ($\sim 1 \times 10^4$). Je otáznou, akými mechanizmami je zabezpečené, že tieto komenzálne baktérie u zdravých jedincov nevyvolávajú na rozdiel od patogénnych baktérií zápal sliznice. Väčšina týchto komenzálnych baktérií je nekultivovateľná,

preto presné zloženie črevnej mikroflóry je neustále predmetom mnohých štúdií. Použitím gnotobiologických zvieracích modelov sa potvrdilo, že jednotlivé zložky črevnej mikroflóry majú rozhodujúcu úlohu pri postnatálnom vývoji imunitného systému.

Zistenie, ktoré baktérie a bunky, a akým spôsobom sa podieľajú na indukci a udržiavaní chronického zápalu čreva, by mohlo priniesť nové prístupy v terapii a v prevenci nešpecifických črevných zápalov medzi ktoré patrí Crohnova choroba, ulcerózna kolitída a celiakia.

Involvement of innate immunity in the development of inflammatory and autoimmune diseases. Tlaskalová-Hogenová H, Tučková L, Štěpánková R, Hudcovič T, Palová-Jelínková L, Kozáková H, Rossmann P, Sanchez D, Cinová J, Hrnčír T, Kverka M, Frolová L, Uhlig H, Powrie F, Bland P. *Ann NY Acad Sci*; 1051: 1-12, 2005

5. DISKUSIA

Celiakia je multifaktoriálne ochorenie sliznice tenkého čreva, ktoré je u geneticky vnímavých jedincov vyvolané pšeničným lepkom - gluténom a jeho hlavnými zložkami. Z ďalších faktorov, ktoré môžu mať vplyv na nástup celiakie, je vplyv vonkajšieho prostredia, prekonaných infekčných ochorení a výživy. Aj keď je známy vyvolávajúci agens celiakie, patogenetický mechanizmus tohoto ochorenia nie je doposiaľ dostatočne objasnený. Veľká pozornosť bola venovaná úlohe CD4⁺ T-buniek, ktoré rozpoznávajú gliadinové peptidy prezentované APC. Aktivované špecifické T bunky stimulujú jak B-bunkovú produkciu špecifických protilátok, tak aj odpoveď T_H1 buniek, sú zapojené do produkcie celej rady cytokínov a chemokínov, ktoré ovplyvňujú ďalšie bunky adaptívnej aj prirodzenej imunity. V poslednej dobe sa predmetom intenzívnych štúdií stáva úloha buniek prirodzenej imunity v patogenéze ochorení. Je študovaná aktivácia monocytov, črevných epitelových buniek a makrofágov, expresia povrchových molekúl ako sú HLA-DR, CD95/Fas, intracelulárnych adhezívnych molekúl (ICAM-1), produkcia cytokínov a ich podiel v mechanizmoch zapojených do morfológických a funkčných zmien črevnej sliznice (Maiuri et al., 2003; 2003a; Mention et al., 2003; Giovannini et al., 2003; Shuppan et al., 2003).

V našom laboratóriu sme sa zapojili do tejto štúdie a ukázali sme, že peptické štepy gliadinu, na rozdiel od intaktného gliadinu a obdobne spracovaných ostatných testovaných potravinových proteínov (sója, ovalbumín, kasein) stimuloval v prítomnosti IFN- γ (hlavného cytokínu celiakie) myšie peritoneálne makrofágy (*in vitro*) k produkcii cytokínov a NO (Tučková et al., 2000).

Zaujímalo nás, či podobný efekt budú mať gliadinové štepy aj na ľudské monocyty. Použili sme ľudské monocytárne línie U937 (CD14⁻CD68⁻) a THP-1 (CD14⁺CD68⁺), ktoré predstavujú odlišné stupne diferenciácie monocytov/makrofágov. Ukázali sme, že viac diferencované bunky ľudskej monocytárnej línie THP-1 odpovedali na gliadinové fragmenty zvýšenou produkciou IL-8 a TNF- α a ich produkcia je významne zvyšovaná v prítomnosti exogenného IFN- γ .

V tkanive jejuna aktívnych celiakov bola detekovaná vysoká hladina mRNA pre IFN- γ (Nilsen et al., 1998). Jeho hlavným producentom sú gliadin-senzitívne T-lymfocyty (Nilsen et al., 1995). IFN- γ spolu s TNF- α prispievajú k zvýšenej permeabilite črevného epitelu (Deem et al, 1991; Madara a Stafford, 1989). Odpoveď THP-1 buniek na gliadin bola signifikantne vyššia, jak v prípade, keď bunky boli inkubované spoločne s gliadinom a IFN- γ , tak aj u buniek prestimulovaných IFN- γ a následne stimulovaných gliadinovými fragmentami samotnými alebo v kombinácii s IFN- γ . Neštiepený gliadin, sója a ovalbumín mali len veľmi slabý vplyv na aktiváciu monocytov, ktorý sa nezvyšoval ani po pridaní INF- γ . Odpoveď U-937 monocytov bola veľmi nízka, známky produkcie IL-8 boli pozorované po stimulácii gliadinom a IFN- γ a to len v prípade buniek prestimulovaných IFN- γ (24h), zatiaľ čo TNF- α produkcia nebola detekovaná. THP-1 monocytárna línia reagovala intenzívnejšie nielen na stimuláciu gliadinom, ale tiež na bakteriálny LPS signifikantne vyššou produkciou IL-8 a TNF- α než línia U-937. Rozdielna odpoveď THP-1 a U-937 monocytov bola dokumentovaná aj inými autormi po stimulácii *E. Coli* LPS a *Shiga*-toxínmi (Eperon a Jungi, 1996; Ramegowda a Tesh, 1996). Imunohistochemické štúdie poukazujú na zvýšený počet CD14 pozitívnych makrofágov a vyššiu expresiu iNOS v biopsiách z aktívnych celiakov, na rozdiel od zdravých jedincov (Steege et al., 1997). THP-1 bunky sú charakterizované vysokou expresiou CD14, použitím anti-CD14 monoklonálnej protilátky bola LPS-indukovaná produkcia IL-8 redukovaná, avšak na gliadinom indukovanú odpoveď to nemalo žiaden efekt. Účasť CD14 molekuly na aktivácii buniek gliadinom sa v týchto experimentoch nepotvrdila.

Analýzou povrchového receptora pre gliadin sa zaoberal aj Fasano a spoluprac., na myších bunkách sledovali možnú úlohu povrchových TLR2 a TLR4 pri aktivácii gliadinom. Autori ukázali, že u myší s defektom v expresii týchto molekúl nie je ovplyvnená produkcia cytokínov po aktivácii gliadinovými peptidmi a s najväčšou pravdepodobnosťou nepredstavujú receptorové molekuly pre gliadin na povrchu týchto buniek (Thomas et al., 2006).

Súbežne s pokusmi na monocytoch prebiehali v našom laboratóriu v spolupráci s oddelením Imonológie 2.LF v Motole pokusy testujúce účinok gliadinových fragmentov na dendritické bunky pripravené z ľudskej periférnej krvi. Pokusy boli inšpirované výsledkami Nikulina a kol, ktorí na dendritických bunkách odvodených

z kostnej drene myši ukázali, že gliadinové fragmenty indukujú expresiu maturačných znakov MHC II, CD40, CD54 a CD86 (Nikulina et al., 2004). V našej pracovnej skupine boli dendritické bunky pripravené v prostredí IL-4 a GM-CSF. Bolo ukázané, že gliadinové fragmenty majú nielen stimulačnú schopnosť, opísanú v predošlých pokusoch, ale navodzujú aj funkčné a fenotypové vyzrievanie DCs. Gliadinové fragmenty zvyšovali povrchovú expresiu maturačných znakov CD83, CD80, CD86, CD40 a HLA-DR a znižovali fagocytárnu kapacitu DCs. Podobne ako aj u monocytov, proteolytické fragmenty sóje a ovalumínu nemali žiaden zreteľný efekt na maturáciu DCs. Maturácia DCs súvisí s produkciou širokého spektra cytokínov a chemokínov (McColl, 2002). U gliadinom stimulovaných DCs buniek bola nameraná vysoká produkcia IL-6, IL-8, TNF- α . Produkcia regulačných cytokínov (IL-4, IL-10) bola nízka, gliadin dokonca znížil produkciu TGF- β v porovnaní s nestimulovanými DCs. Zaujímavé bolo zistenie, že LPS-stimulované a gliadinom-stimulované DCs sa líšia spektrom produkovaných cytokínov, hlavne v produkcii IL-12, ktorá nebola zaznamenaná u DCs stimulovaných gliadinom. Neprítomnosť IL-12 je charakteristickým znakom u celiakie, IL-12 nebol detekovaný ani na úrovni mRNA izolovanej z tkaniva celiakálnych pacientov (Nilsen et al., 1998). Domnievame sa, že úloha IL-12 pri T_H1 polarizácii by mohla byť zastúpena IL-23 alebo IL-18 molekulami.

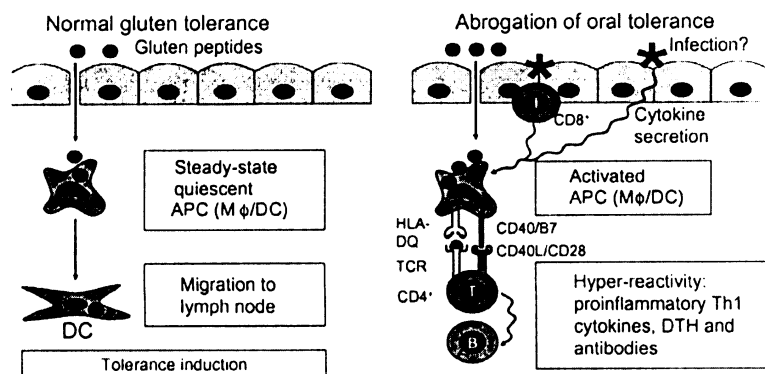
Výsledky našich, už spomínaných experimentov zameraných na analýzu signálnej dráhy aktivovanej gliadinovými peptidmi u ľudských monocytárných línií THP-1 a U937 a ľudských monocytov izolovaných z periférnej krvi, tak aj pozorovanie ďalších autorov (De Stefano et al., 2006; Palová-Jelínková et al., 2005) poukazujú na významnú účasť molekúl NF- κ B komplexu. U všetkých týchto buniek bolo potvrdené zapojenie dvoch hlavných väzobných podjednotiek (p50 a p65) NF- κ B signálnej dráhy. Gauss a spoluprac. zistili, že ľudské monocyty po kultivácii s TNF- α zvyšujú aktivitu NADPH oxidázy, to vedie k zvýšenej produkcii kyslíkových radikálov a k aktivácii NF- κ B, čím TNF- α eventulálne podporuje zápal (Gauss et al., 2007)

Subepitelové ľudské (Rugtveit et al., 1997) aj myšie (Chirido et al., 2005) črevné makrofágy a DCs exprimujú za fyziologických podmienok len nízke hladiny kostimulačných molekúl CD80 a CD86 a nie sú schopné navodiť stimuláciu T-

lymfocytov (Qiao, 1996). Keďže črevné makrofágy sú odvodené od krvných monocytov (Smythies et al., 2006), mikroprostredie čreva pravdepodobne znižuje schopnosť novo osídlených monocytov odpovedať na gliadin.

Monocyty zdravých jedincov po stimulácii gliadinom samotným nezvyšovali povrchovú expresiu znakov CD80, CD86, CD83 a CD40, avšak po pridaní gliadinu spoločne s IFN- γ sa prejavil synergistický účinok IFN- γ na aktiváciu monocytov. V rámci skupiny monocytov zdravých jedincov sme v produkcii IL-8 zaznamenali široký rozptyl hodnôt, preto sme na základe HLA-DQ2 typizácie monocyty rozdelili na HLA-DQ2⁺ a HLA-DQ2⁻. Monocyty HLA-DQ2⁺ jedincov spontánne uvoľňovali viac IL-8, než monocyty HLA-DQ2⁻. Výsledky naznačili, že zvýšená produkcia cytokínov u celiakálnych pacientov je asociovaná s expresiou HLA-DQ2, avšak toto spojenie môže byť súčasťou doposiaľ neobjasnených genetických faktorov a komplexných mechanizmov pri vzniku tohoto ochorenia.

Naše výsledky zapadajú do hypotézy navrhovanej Brantzaegom (2006), podľa ktorej väčšina APC, ktoré sú prítomné v čreve za fyziologických podmienok v nezrelej forme, vedie skôr k indukcii tolerancie než k aktivácii T-buniek. Jedným z možných mechanizmov vzniku celiakie je porušenie orálnej tolerancie za spoluúčasti ďalších, doteraz neznámych činiteľov, ako je napr. infekcia a mikroprostredie v tkanive. Gliadinové peptidy prestupujúce črevnou sliznicou stimulujú epitelové bunky a CD8⁺ IELs k produkcii cytokínov. Kombinácia týchto cytokínov s gliadinovými peptidmi stimuluje makrofágy a vedie k maturácii dendritických buniek. Na DCs sa zvyšuje expresia kostimulačných molekúl a dochádza k vzniku gluten-špecifických CD4⁺ T_H1 lymfocytov (Obr. č. 3). Gliadin navodzuje aktiváciu a maturáciu dendritických buniek a tak zvyšuje celkový počet buniek prezentujúcich jeho peptidy. Predstavu o vplyve mikroprostredia v tkanive na reaktivitu buniek podporujú aj nálezy L. Smythies, ktorá ukázala, že makrofágy izolované z črevného tkaniva zdravých jedincov nenesú znaky umožňujúce ich aktiváciu bakteriálnymi antigénmi. (Smythies et al., 2005; Smith et al, 1991; 2005). V rámci spolupráce s touto skupinou bol testovaný aj efekt gliadinu, ktorý nenavodil v *in vitro* podmienkach aktiváciu makrofágov izolovaných z čreva zdravých jedincov, na rozdiel od ich krvných monocytov, ktoré boli gliadinom aktivované k produkcii širokého spektra cytokínov a chemokínov (viď naša spoločná publikácia).



Obrázok č. 3 (Brandtzaeg et al., 2006)

Keďže súhrn činiteľov podieľajúcich sa na vzniku celiakie je stále otáznny, do istej miery by mohli určitú úlohu zohrávať aj baktérie mikrobiálnej flóry. V súčasnej dobe niekoľko pracovných skupín sa zaoberá porovnaním bakteriálnej flóry u zdravých jedincov a u detí s celiakiou (Tjellström et al., 2005, Sanz et al., 2007). Do tejto štúdie sme sa zapojili spoločne s pracovnou skupinou Y. Sanz z Valencie, kde v rámci spoločného grantového projektu sme zahájili štúdium vplyvu vybraných bakteriálnych kmeňov, (ktorými sa líšili zdraví a celiakálni pacienti) na bunky prirodzenej imunity. Bude testovaný ich samotný účinok na bunky prirodzenej imunity a ich vplyv spoločne s gliadinom na monocyty a makrofágy izolované zo zdravých jedincov a pacientov s celiakiou.

6. ZÁVER

Záverom by som zhrnula, že našou štúdiou sa nám podarilo prispieť k objasneniu účasti buniek prirodzenej imunity pri vzniku a vývoji celiakie. Ukázali sme, že gliadin, na rozdiel od ostatných potravinových antigénov má schopnosť aktivovať jak ľudské monocytárne línie, tak aj monocyty izolované z periférnej krvi celiakálnych pacientov a zdravých jedincov k produkcii cytokínov a chemokínov, pričom odpoveď monocytov z celiakálnych pacientov je vyššia v porovnaní s monocytmi zdravých jedincov. Podarilo sa nám čiastočne analyzovať gliadinom aktivované signálne dráhy stimulované gliadinovými fragmentami. Stále ostáva kľúčovou otázkou v patogenéze celiakie, čím je glutén tak výnimočný, že navodzuje rozsiahlu bunkami mediovanú imunitnú odpoveď, aké receptorové molekuly viažu gliadinové fragmenty na povrch buniek a ktoré z týchto fragmentov sú patogenetické pre celiakiu.

Podieľali sme sa na štúdiu, ktorá v náväznosti na preukázaný protektívny vplyv kojenia na rozvoj celiakie, charakterizovala cytokínové zloženie kolostra a materského mlieka. V našej štúdiu bola po prvý krát opísaná celá rada cytokínov a ďalších faktorov a ich zmeny v čase, ktoré by mohli byť zodpovedné za ochranu črevnej sliznice u kojencov.

Tieto výsledky sú len malou súčasťou zložitého mechanizmu vzniku celiakie. Vedú k celej rade ďalších otázok, ktoré je potrebné ďalej analyzovať. Patrí sem aj identifikácia gliadinových peptidov, ktoré stimulujú bunky prirodzenej imunity, identifikácia receptora pre tieto imunogénne peptidy, podrobnejšia analýza signálnych dráh a potenciálna účasť baktérií pri tomto ochorení.

7. ZOZNAM LITERATÚRY

Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. *Nature*; 406: 782–787, 2000

Aine L, Maki M, Collin P, Keyrilainen O. Dental enamel defects in celiac disease. *J Oral Pathol Med*; 19: 241-245, 1990

Akimov SS, Belkin AM. Cell-surface tissue transglutaminase is involved in adhesion and migration of monocytic cells in fibronectin. *Blood*; 98: 1567-1576, 2001

Akira S, Takeda K. Toll-like receptor signalling. *Na. Rev Immunol* 4: 499–511. 2004

Akbari O, Stock P, DeKruyff RH, Umetsu DT. Role of regulatory T cells in allergy and asthma. *Curr Opin Immunol*; 15:627-633, 2003

Andrin C, Pinkoski MJ, Burns K, Atkinson EA, Krahenbuhl O, Hudig D, Fraser SA, Winkler U, Tschopp J, Opas M, Bleackley RC, Michalak M. Interaction between a Ca²⁺-binding protein calreticulin and perforin, a component of the cytotoxic T-cell granules. *Biochemistry*; 37: 10386-10394, 1998

Arentz-Hansen H, Korner R, Molberg Ø, Quarsten H, Kristiansen C, Madsen L, Fugger L, Scott H, Noren O, Roepstorff P, Lundin KE, Sjostrom H, Sollid LM. The intestinal T cell response to alpha-gliadin in adult celiac disease is focused on a single deamidated glutamine targeted by tissue transglutaminase. *J EXP Med*; 191:603-612, 2000

Arentz-Hansen H, McAdam SN, Molberg Ø, Fleckenstein B, Lundin KE, Jorgenson Tj, Jung G, Roepstorff, Sollid LM. Celiac lesion T cells recognize epitopes that cluster in regions of gliadins rich in proline residues. *Gastroenterology*; 123: 803-809, 2002

Askling J, Linet M, Gridley G, Halstensen T, Ekstrom K, Ekborn A. Cancer incidence in a population-based cohort of individuals hospitalized with coeliac disease and dermatitis herpetiformis in Sweden. *Gastroenterology*; 123: 1428-1452, 2002

Baeuerle PA, Baltimore D. NF-κB: ten years after. *Cell*; 87: 13-20, 1996

Baggiolini M, Dewald B, Moser B. Interleukin-8 and related chemotactic cytokines-CXC and CC chemokines. *Adv Immun*; 55: 97-179, 1994

Baldwin AS. The NF-kappa B and I kappa B Proteins: new discoveries and insights. *Annu Rev Immunol*; 14: 649-683, 1996

Banchereau J, Briere Caux Ch, Davoust J, Lebecque S, Liu Ym Pulendran B, Palucka K. Immunobiology of dendritic cells. *Annu Rev Immunol*, 18: 767-811, 2000

Bargetzi M, Schönenberger A, Tichelli A, Fried R, Cathoma SG, Grawohl A. Celiac disease transmitted by allogeneic non-T cell-dependent bone marrow transplantation. *Bone Marrow Transplant*; 20:607-609, 1997

Barnes PJ. Nuclear factor-kappa B. *Int J Biochem Cell Biol*; 29: 867-70, 1997

Belge KU, Dayyani F, Horelt A, Siedlar M, Frankenberger M, Frankenberger B, Espevik T, Ziegler-Heitbrock L. The proinflammatory CD14+CD16+DR++ monocytes are a major source of TNF. *J Immunol*; 168: 3536-3542, 2002

Bonamico M, Bottaro G, Pasquino AM, Caruso-Nicoletti M, Mariani P, Gemme G, Paradiso E, Ragusa MC, Spina M. Celiac disease and Turner syndrome. *J Pediatr Gastroenterol Nutr*; 26: 496-499, 1998

Bonamico M, Mariani P, Danesi HM, Crisogianni M, Failla P, Gemme G, Quartino AR, Giannotti A, Castro M, Balli F, Lecora M, Andria G, Guariso G, Gabrielli O, Catassi C, Lazzari R, Balocco NA, De Virgiliis S, Culasso F, Romano C, SIGEP (Italian Society of Pediatric Gastroenterology and Hepatology) and Medical Genetic Group. Prevalence and clinical picture of celiac disease in Italian Down syndrome patients: a multicenter study. *J Pediatr Gastroenterol Nutr*; 33: 139-143, 2001

Brandtzaeg P, Farstad IN, Helgeland L. Phenotypes of T cells in the gut. In: MacDonald TT, editor. Mucosal T cells. *Chem Immunol Basel: Karger*, 71: 1-26, 1998

Brandtzaeg P. Molecular and cellular aspects of the secretory immunoglobulin system. *APMIS*; 103: 1-19, 1995

Brandtzaeg P. The changing immunological paradigm in coeliac disease. *Immunol Letters*; 105: 127-139, 2006

Buc M. Imunológia. Slovenská akadémia vied; vyd. VEDA, 2001

Cataldo F, Marino V, Ventura A, Bottaro G, Corazza GR. Prevalence and clinical features of selective immunoglobulin A deficiency in coeliac disease: an Italian multicentre study. Italian Society of Pediatric Gastroenterology and Hepatology (SIGEP) and "Club del Tenue" Working Groups on Coeliac Disease. *Gut*; 42: 362-365, 1998

Catassi C, Fabiani E, Corrao G, Barbato M, De Renzo A, Carella AM, Gabrielli A, Leoni P, Carroccio A, Baldassarre M, Bertolani P, Caramaschi P, Sozzi M, Guariso G, Volta U, Corazza GR; Italian Working Group on Coeliac Disease and Non-Hodgkin's-Lymphoma. Risk of non-Hodgkin lymphoma in coeliac disease. *Jama*; 287: 1413-1419, 2002

Catassi C, Ratsch IM, Fabiani E, Rossini M, Bordicchia F, Candela GV, Giorgi PL. Coeliac disease in the year 2000: exploring the iceberg. *Lancet*; 343: 200-203, 1994

- Cellier C, Delabesse E, Helmer C, Patey N, Matuchansky C, Jabri B, Macintyre E, Cerf-Bensussan N, Brousse N. Refractory sprue, coeliac disease, and enteropathy-associated T-cell lymphoma. *Lancet*; 256: 203-208, 2000
- Cellier C, Patey N, Mauvieux L, Jabri B, Delabesse E, Cervoni JP, Burtin ML, Guy-Grand D, Bouhnik Y, Modigliani R, Barbier JP, Macintyre E, Brousse N, Cerf-Bensussan N. Abnormal intestinal intraepithelial lymphocytes in refractory sprue. *Gastroenterology*; 114: 471-481, 1998
- Cerf-Bensussan N, Brousse N, Caillat-Zucman C...Coeliac Disease. *John Libbey Eurotext. Esber UK*, 2003
- Ciccocioppo R, Di Sabatino A, Parroni R, Muzi P, D'Alo S, Ventura T, Pistoia MA, Cifone MG, Corazza GR. Increased enterocyte apoptosis and Fas-Fas ligand system in celiac disease. *Am J Clin Pathol*; 115: 494-503, 2001
- Collin P, Mäki M. Associated disorders in coeliac disease: clinical aspects. *Scand J Gastroenterol*; 29:769-775, 1994
- Collin P, Wahab PJ, Murray JA. Intraepithelial lymphocytes and coeliac disease. *Best Practice and Research*; 19: 341-350, 2005
- Cronin CC, Feighery A, Ferriss JB, Liddy C, Shanahan F, Feighery C. High prevalence of celiac disease among patients with insulin-dependent (type I) diabetes mellitus. *Am J Gastroenterol*; 92: 2210-2212, 1997
- Csizmadia CG, Mearin ML, von Blomberg BM, Brand R, Verloove-Vanhorick SP. An iceberg of childhood coeliac disease in the Netherlands. *Lancet*; 353: 813-814, 1999
- Dahan S, Roth-Walter F, Arnaboldi P, Agarwal S, Mayer L. Epithelia: lymphocyte interactions in the gut. *Immuno Rev*; 215: 243-253, 2007
- De Stefano D, Maiuri MC, Iovine B, Ialenti A, Bevilacqua MA, Carnuccio R. The role of NF-kappaB, IRF-1, and STAT-1alpha transcription factors in the iNOS gene induction by gliadin and IFN-gamma in RAW 264.7 macrophages. *J Mol Med*; 84: 65-74, 2006
- Deem RL, Shanahan F, Targan SROV. Triggered human mucosal T cells release tumor necrosis factor- α and interferon- γ which kill human colonic epithelial cells. *Clin Exp Immunol*; 83: 79-84, 1991
- Di Sabatino A, Ciccocioppo R, D'Alo S, Parroni R, Millimaggi D, Cifone MG, Corazza GR. Intraepithelial and lamina propria lymphocytes show distinct patterns of apoptosis whereas both populations are active in Fas based cytotoxicity in coeliac disease. *Gut*; 49:380-386, 2001

Dickey W, Hughes DF, McMillan SA. Reliance on serum endomysial antibody testing underestimates the true prevalence of coeliac disease by one fifth. *Scand J Gastroenterol*; 35: 181-183, 2000

Dickey W, McMillan SA, Callender ME. High prevalence of celiac sprue among patients with primary biliary cirrhosis. *J Clin Gastroenterol*; 25: 328-329, 1997

Dietrich W, Ehnis T, Bauer M, Donner P, Volta U, Riecken EO, Schuppan D. Identification of tissue transglutaminase as the autoantigen of celiac disease. *Nature Med*; 3: 797- 801, 1997

Djilali-Saiah I, Schmitz J, Harfouch-Hammoud E, Mougnot JF, Bach JF, Caillat-Zucman S. CTLA-4 gene polymorphism is associated with predisposition to coeliac disease. *Gut*; 43: 187-189, 1998

Donaldson MR, Firth SD, Wimpee H, Leiferman KM, Zone JJ, Horsley W, O'gorman MA, Jackson WD, Neuhausen SL, Hull CM, Book LS. Correlation of duodenal histology with tissue transglutaminase and endomysial antibody levels in pediatric celiac disease. *Clin Gastroenterol Hepatol*, 5:567-73, 2007

Eckman L, Kagnoff MF, Fierer J. Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry. *Infect Immun*; 63:4569-4574, 1993

Eperon S, Jungi TW. The use of human monocytoid lines as indicators of endotoxin. *J Immunol Methods*; 194: 121-129, 1996

Falchuk ZM, Rogentine GN, Strober W. Predominance of histocompatibility antigen HL-A8 in patients with gluten-sensitive enteropathy. *J Clin Invest*; 51: 1602-1605, 1972

Fasano A, Catassi C. Current approaches to diagnosis and treatment of celiac disease: an avolving spectrum. *Gastroenterology*; 120: 636-651, 2001

Ferguson A, Murray D. Quantitation of intraepithelial lymphocytes in human jejunum. *Gut*; 12: 988-994, 1971

Fernandez E, Riestra S, Rodrigo L, Blanco C, Lopez Vazquez A, Fuentes D, Moreno M, Lopez-Larrea C. Comparison of six human anti-transglutaminase ELISA-tests in the diagnosis of celiac disease in the Saharawi population. *World J Gastroenterol*; 11: 3762-3766, 2005

Franco A, Appella E, Kagnoff MF, Chowes Y, Sakaguchi K, Grey HM, Sette A. Peripheral T-cell response to A-gliadin in celiac disease: differential processing and presentation capacities of Epstein-Barr-transformed B cells and fibroblasts. *Clin Immunol Immunopathol* 71:75-81, 1994

Frankenberger M, Sternsdorf T, Pechumer H, Pforte A, Ziegler-Heitbrock HW. Differential cytokine expression in human blood monocyte subpopulations: a polymerase chain reaction analysis. *Blood*; 87: 373-377, 1996

Fraser JS, Engel W, Ellis HJ, Moodie SJ, Pollock EL, Wieser H, Ciclitira PJ. Coeliac disease: in vivo toxicity of the putative immunodominant epitope. *Gut*; 52: 57-62, 2003

Gauss KA, Nelson-Overton LK, Siemsen DW, Gao Y, Deleo FR, Quinn MT. Role of NF- κ B in transcriptional regulation of the phagocyte NADPH oxidase by tumor necrosis factor- α . *J Leukoc Biol*; 30: 2007 [Epub ahead of print]

Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity*; 19: 71-82, 2003

Gianfrani C, Levings MK, Sartirana C, Mazzarella G, Barba G, Zanzi D, Camarca A, Iaquinto G, Giardullo N, Auricchio S, Troncone R, Roncarolo MG. Gliadinspecific type-1 regulatory T cells from the intestinal mucosa of treated celiac patients inhibit pathogenic T cells. *J Immunol*; 177: 4178-4186, 2006

Giovannini C, Matarrese P, Scazzocchio B, Vari R, D'Archivio M, Straface E, Masella R, Malorni W, De Vincenzi M. Wheat gliadin induces apoptosis of intestinal cells via an autocrine mechanism involving Fas-Fas ligand pathway. *FEBS Lett*; 540: 117-124, 2003

Giri JG, Ahdieh M, Eisenman J, Shanebeck K, Grabstein K, Kumaki S, Namen, Park LS, Cosman D, Anderson D. Utilization of the beta and gamma chains of the IL-2 receptor by the novel cytokine IL-15. *EMBO J*; 264: 965-968, 1994

Goodland RA, Wright NA. Epidermal growth factor (EGF). *Baillieres Clin Gastroenterol*; 10:33-47, 1996

Greco L, Romino R, Coto I, Di Cosmo N, Percopo S, Maglio M, Paparo F, Gasperi V, Limongelli MG, Cotichini R, D'Agate C, Tinto N, Sacchetti L, Tosi R, Stazi MA. The first large population based twin study of coeliac disease. *Gut*; 50: 624-628, 2002

Green PHR, Jabri B. Coeliac disease. *Lancet*; 362: 383-391, 2003

Green PHR, Rostami K, Marsh MN. Diagnosis of coeliac disease. *Best Pract Res Clin Gastroenterol*; 19: 389-400, 2005

Greenberg CS, Birckbichler PJ, Rice RH. Transglutaminase: Multifunctional cross-linking enzymes that stabilize tissues. *FASEB J*; 5:3071-3077, 1991

Grefte JM, Bouman JG, Grond J, Jansen W, Kleibeuker JH. Slow and incomplete histological and functional recovery in adult gluten sensitive enteropathy. *J Clin Pathol*; 41: 886-891, 1988

Gut J, Christen U, Fey N, Koch V, Stoffer D. Molecular mimicry in halothane hepatitis. Biochemical and structural characterization of lipoylated autoantigens. *Toxicol*; 97: 199-224, 1995

Haimila K, Smedberg T, Mustalahti K, Mäki M, Partanen J, Holopainen P. Genetic association of coeliac disease susceptibility to polymorphisms in the ICOS gene on chromosome 2q33. *Genes Immun*; 5: 85-92, 2004

Hayat M, Cairns A, Dixon MF, O'Mahony S. Quantitation of intraepithelial lymphocytes in human duodenum: what is normal? *J Clin Pathol*; 55: 393-394, 2002

Hill I, Fasano A, Schwartz R, Counts D, Glock M, Horvath K. The prevalence of celiac disease in at-risk groups of children in the United States. *J Pediatr*; 136: 86-90, 2000

Hüe S, Mention JJ, Monteiro RC, Zhang S, Cellier C, Schmitz J, Verkarre V, Fodil N, Bahram S, Cerf-Bensussan N. A direct role for NKG2D/MICA interaction in villous atrophy during celiac disease. *Immunity*; 21: 367-377, 2004

Huttner S, Bevins C. Antimicrobial peptides as mediator of epithelial host defense. *Pediatr Res*; 45:785-794, 1999

Chin RL, Sander HW, Brannagan TH, Green PH, Hays AP, Alaedini A, Latov N. Celiac neuropathy. *Neurology*; 60:1581-1585, 2003

Jabri NP, deSerre, Cellier, Evans, Gache C, Carvalho C, Mougenot JF, Allez M, Jian R, Desreumaux P, Colombel JF, Matuchansky C, Cugnenc H, Lopez-Botet M, Vivier E, Moretta A, Roberts AI, Ebert EC, Guy-Grand D, Brousse N, Schmitz J, Cerf-Bensussan N. Selective expansion of intraepithelial lymphocytes expressing the HLA-E specific natural killer receptor CD94 in coeliac disease. *Gastroenterology*, 118:867-79, 2000

Jansen TL, Mulder CJJ, Karssen PHZ, Wagenaar CGJ. Epidemiological survey of the Dutch Coeliac Disease Society: an update 1992. *Eur J Gastroenterol Hepatol*; 5: 73-78, 1993

Jelínková L, Tučková L, Cinová J, Flegelová Z, Tlaskalová-Hogenová H. Gliadin stimulates human monocytes to production of IL-8 and TNF- α through a mechanism involving NF- κ B. *FEBS Lett*; 571:81-85, 2004

Jelínková L, Tučková L, Sánchez D, Krupičková S, Pozler O, Nevorál J, Kotalová R, Tlaskalová-Hogenová H. Increased levels of circulating ICAM-1, E-selectin, and IL-2 receptors in celiac disease. *Dig Dis Sci*; 45: 398-402, 2000

Johansen BH, Gjertsen HA, Vartdal F, Buus S, Thorsby E, Lundin KE, Sollid LM. Binding of peptides from the N-terminal region of α -gliadin to the celiac disease associated HLA-DQ2 molecule assessed in biochemical and T cell assays. *Clin Immunol Immunopathol*; 79: 288-293, 1996

Kakar S, Nehra V, Murray JA, Dayharsh GA, Burgart LJ. Significance of intraepithelial lymphocytosis in small bowel biopsy samples with normal mucosal architecture. *Am J Gastroenterol*; 98: 2027-2033, 2003

Karell K, Louka AS, Moodie SJ, Ascher H, Clot F, Greco L, Ciclitira PJ, Sollid LM, Partanen J, members of European genetics cluster on celiac disease. European genetics cluster on celiac disease. HLA types in celiac disease patients not carrying the DQA1*05-DQB1*02(DQ2) heterodimer: results from the european genetics cluster on celiac disease. *Hum Immunol*; 64: 469-477, 2003

Karpati S, Burgin-Wolf A, Krieg T, Meuer M, Stolz W, Braun-Falco O. Binding to human jejunum of serum IgA antibody from children with coeliac disease. *Lancet*; 36: 1335-1338, 1990

Karská K, Tučková L, Steiner L, Tlaskalová-Hogenová H, Michalak M. Calreticulin the potencial autoantigen in celiac disease. *Biochem Biophys Res Commun*; 209:597-605, 1995

Kasarda DD, Okita TW, Bernardin JE, Baecker PA, Nimmo CC, Lew EJ, Dietler MD, Greene FC. Nucleic acid (cDNA) and amino acid sequences of alpha-type gliadins from wheat (*Triticum aestivum*). *Proc Natl Acad Sci USA*; 81: 4712-4716, 1984

Kikly K, Liu L, Na S, Sedgewick JD. The IL-23/Th17 axis: therapeutic targets for autoimmune inflammation. *Curr Opin Immunol*; 18: 670-675, 2006

King AL, Moodie SJ, Fraser JS, Curtis D, Reid E, Dearlove AM, Ellis HJ, Ciclitira PJ. CTLA4/CD28 gene region is associated with genetic susceptibility to coeliac disease in UK families. *J Med Genet*; 39: 51-54, 2002

Kishore U, Sontheimer RD, Sastry KN, Zappi EG, Hughes GRV, Khamashta MA, Reid KB, Eggleton P. The systemic lupus erythematosus (SLE) disease autoantigen-calreticulin can inhibit C1q association with immune complexes. *Clin Exp Immunol*; 108: 181-190, 1997

Kolls JK, Linden A. Interleukin-17 family members and inflammation. *Immunity*; 21: 467-476, 2004

Koning F. Disease: Caught between a rock and a hard place. *Gastroenterol*; 129: 1294-1301, 2005

Kretschmer K, Apostolou I, Hawiger D, Khazie K, Nussenzweig MC, von Boehmer H. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol*; 6: 1219-1227, 2005

Krupičková S, Tučková L, Flegelová Z, Michalak m, Walters JR, Whelan A, Harries J, Vencovský J, Tlaskalová-Hogenová H. Identification of common epitopes on

gliadin enterocytes, and calreticulin recognised by antigliadin antibodies of patients with celiac disease. *Gut*; 44: 168-173, 1999

Ladinser B, Rossipal E, Pittschieler K. Endomysium antibodies in coeliac disease: an improved method. *Gut*; 35: 776-778, 1994

Lahteenoja H, Toivanen A, Viander M, Maki M, Irjala K, Raiha I, Syrjanen S. Oral mucosal changes in coeliac patients on a gluten-free diet. *Eur J Oral Sci*; 106: 899-906, 1998

Le Naour F, Brichory F, Misek DE, Bréchet C, Hanash SM, Beretta L. A distinct repertoire of autoantibodies in hepatocellular carcinoma identified by proteomic analysis. *Molecular & Cellular Proteomics*; 1: 197-203, 2002

Le Naour F, Hohenkirk L, Grolleau A, Misek DE, Lescure P, Geiger JD, Hanash S, Beretta L. Profiling changes in gene expression during differentiation and maturation of monocyte-derived dendritic cells using both oligonucleotide microarrays and proteomics. *J Biol Chem*; 276: 17920-17931, 2001

Lebwohl B, Deckelbaum RJ, Green PH. Giardiasis. *Gastrointest Endosc*; 57: 906-913, 2003

Lepore L, Martellosi S, Pennesi M, Falcini F, Ermini ML, Ferrari R, Perticarari S, Presani G, Lucchesi A, Lapini M, Ventura A. Prevalence of celiac disease in patients with juvenile chronic arthritis. *J Pediatr*; 129: 311-313, 1996

Li XC, Demirci G, Ferrari-Lacraz S, Groves C, Coyle A, Malek TR, Strom TB. IL-15 and IL-2: a matter of life and death for T cells in vivo. *Nat Med*; 7: 114-118, 2001

Luft LM, Barr SG, Martin LO, Chan EK, Fritzler MJ. Autoantibodies to tissue transglutaminase in Sjogren's syndrome and related rheumatic disease. *J Rheumatol*; 30: 2613-2619, 2003

Madara JL, Stafford J. Interferon- γ directly affects barrier function of cultured intestinal epithelial monolayers. *J Clin Invest*; 83: 724-727, 1989

Maiuri L, Troncone R, Mayer M, Coletta S, Picarelli A, De Vincenzi M, Pavone V, Auricchio S. In vitro activities of A-gliadin related synthetic peptides: damaging effect on the atrophic coeliac mucosa and activation of mucosal immune response in the treated coeliac mucosa. *Scand J Gastroenterol*; 31:247-253, 1996

Maiuri L, Ciacci C, Auricchio S, Brown V, Quarantino S, Londei M. Interleukin 15 mediates epithelial changes in celiac disease. *Gastroenterology*; 119:996-1006, 2000

Maiuri L, Ciacci C, Raia V, Vacca L, Ricciardelli I, Raimondi F, Auricchio S, Quarantino S, Londei M. FAS engagement drives apoptosis of enterocytes of coeliac patients. *Gut*; 48: 418-424, 2001

Maiuri L, Ciacci C, Ricciardelli I, Vacca L, Raia V, Auricchio S, Picard J, Osman M, Quarantino S, Londei M. Association between innate response to gliadin and activation of pathogenic T-cells in coeliac disease. *Lancet*; 362: 30-37, 2003

Maiuri L, Picarelli A, Boirivant M, Coletta S, Mazzilli MC, De Vincenzi M, Londei M, Auricchio S. Definition of the initial immunologic modifications upon in vitro gliadin challenge in the small intestine of celiac patients. *Gastroenterology*; 110: 1368-1378, 1996

^aMaiuri MC, De Stefano D, Mele G, Iovine B, Bevilaqua 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 *Nuanyin-Schmied Arch Pharmacol*; 368: 63-71, 2003

Mäki M, Collin P. Coeliac disease. *Lancet*; 349: 1755-1759, 1997

Mäki M, Holm K, Collin P, Hallstrom O, Viander M, Collin P, Savilahti E, Koskimies S. Increase in gamma/delta T cell receptor bearing lymphocytes in normal small mucosa in latent coeliac disease. *Gut*; 32: 1412-1414, 1991

Marsh MN. Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity (celiac sprue). *Gastroenterology*; 102: 330-354, 1992

Mazzarella G, Maglio M, Paparo F, Nardone G, Stefanile R, Greco L, van de Wal Y, Kooy Y, Koning F, Auricchio S, Troncone R. An immunodominant DQ8 restricted gliadin peptide activates small intestinal immune response in in vitro cultured mucosa from HLA-DQ8 positive but not HLA-DQ8 negative coeliac patients. *Gut*; 52: 57-62, 2003

McColl. Chemokines and dendritic cells: a crucial alliance. *Immunol Cell Biol*, 80: 489-496, 2002

McDonald TT. Mucosal T cells. *Basel*, Karger, 1998

McKhalife J, Trottei F, Schacht AM, Godin C, Pierce RJ, Capron A. Cloning of the gene encoding a *Schistosoma mansoni* antigen homologous to human Ro/SS-A autoantigen. *Mol Biochem Parasitol*; 57: 193-202, 1993

McNeish AS, Harms JK, Rey J. The diagnosis of coeliac disease. A commentary on the current practise of members of the European Society for Pediatric Gastroenterology and Nutrition (ESPGAN). *Arch Dis Child*; 54: 629-637, 1979

Medzhitov R a Janeway JR. Innate immune recognition: mechanisms and pathways. *Immunol. Rev*; 173: 89-97, 2000

Meijer JW, Mulder CJ, Goerres MG, Boot H, Schweizer JJ. Coeliac disease and (extra) intestinal T-cell lymphomas: definition, diagnosis and treatment. *Scand J Gastroenterol Suppl*; 78: 2004

Mention JJ, Ben AM, Begue B, Barbe U, Verkarre V, Asnafi V, Colombel JF, Cugnenc PH, Ruemmele FM, McIntyre E, Brousse N, Cellier C, Cerf-Bensussan N. Interleukin 15: a key to disrupted intraepithelial lymphocyte homeostases and lymphomagenesis in celiac disease. *Gastroenterology*; 125: 730-745, 2003

Meresse B, Chen Z, Ciszewski C, Tretiakova M, Bhagat G, Krausz TN, Raulet DH, Lanier LL, Groh V, Spies T, Ebert EC, Green PH, Jabri B. Coordinated induction by IL-15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. *Immunity*; 21:357-366, 2004

Městecký J, Lamm ME, Strober W at al. *Mucosal Immunology* 3rd ed. Elsevier Academic Press Amsterdam, 2005

Mhaouty-Kodja S. Galpha/tissue transglutaminase 2: an emerging G protein in signal transduction. *Biol Cell*; 96: 363-367, 2004

Molberg Ø, McAdam SN, Korner R, Quarsten H, Kristiansen C, Madsen L, Fugger L, Scott H, Noren O, Roepstorff P, Lundin KE, Sjostrom H, Sollid LM. Tissue transglutaminase selectively modifies gliadin peptides that are recognised by gut-derived T-cells in celiac disease. *Nat Med*; 4: 713-717, 1998

Mulder CJJ, Cellier C. Coeliac disease: changing views. *Best Pract Res Clin Gastroenterol*; 19: 313-321, 2005

Murray JA, Green PH. Biopsy is the gold standard of diagnosis of coeliac sprue. *Gastroenterology*; 116: 1273-1274, 1999

Murtaugh MP, Arend WP, Davies PJ. Induction of tissue transglutaminase in human peripheral-blood monocytes. *J Exp Med*; 159: 114-125, 1984

Nakae S, Saijo S, Horai R, Sudo K, Mori S, Iwakura Y. IL-17 production from activated T cells is required for the spontaneous development of destructive arthritis in mice deficient in IL-1 receptor antagonist. *Proc Natl Acad Sci U.S.A*; 100: 5986-5990, 2003

Nikulina M, Habich C, Flohe SB, Scott FW, Kolb H. Wheat gluten causes dendritic cell maturation and chemokine secretion. *J Immunol*; 173:1925-1933, 2004

Nilsen EM, Jahnsen FL, Lundin KE, Johansen FE, Fausa O, Sollid LM, Jahnsen J, Scott H, Brandtzaeg P. Gluten induces an intestinal cytokine response strongly dominated by interferon gamma in patients with celiac disease. *Gastroenterology*; 115: 551-63, 1998

Nilsen EM, Lundin KEA, Krajci P, Scott H, Sollid LM, Brandtzaeg P. Gluten specific, HLA-DQ restricted T cells from coeliac mucosa produce cytokines with Th1 or Th0 profile dominated by interferon γ . *Gut*; 37: 766-776, 1995

Ogra PL, Městecký J, Lamm ME, Strober W, Bienenstock J, McGhee J. *Mucosal Immunology*. New York, Academic Press, 1999

Okita TW, Cheesbrough V, Reeves CD. Evolution and heterogeneity of the alpha/beta type gliadin DNA sequences. *J Biol Chem*; 260: 8203-8213, 1985

Osman AA, Gunnel T, Dietl A, Uhlig HH, Amin M, Fleckenstein B, Richter T, Mothes T. B cell epitopes of gliadin. *Clin Exp Immunol*; 121: 284-254, 2000

Palová-Jelínková L, Rožková D, Pecharová B, Bartová J, Šedivá A, Tlaskalová-Hogenová H, Spišek R, Tučková L. Gliadin induce phenotypic and functional maturation of human dendritic cells. *J Immunol*; 175: 7038-7045, 2005

Perera LP. Interleukin 15: its role in inflammation and immunity. *Arch Immunol Ther Exp*; 48:457-464, 2000

Polanco I, Biemond I, van Leeuwen A et al. Gluten sensitive enteropathy in Spain: genetic and environmental factors. In McConnell R: *The genetics of Coeliac Disease*. Lancaster, MTP Press; 211-231, 1984

Powrie F. Immune regulation in the intestine: a balancing act between effector and regulatory T cell responses. *Ann NY Acad Sci*; 1029: 132-141, 2004

Qiao L, Braunstein J, Golling M, Schurmann G, Autschbach F, Moller P, Meuer S. Differential regulation of human T cell responsiveness by mucosal versus blood monocytes. *Eur J Immunol*; 26: 922-927, 1996

Ráki M, Fallang LE, Brottvet M, Bergseng E, Quarsten H, Lundin KEA. Tetramer visualization of gut-homing gluten-specific T cells in the peripheral blood of celiac disease patients. *PNAS*; 104: 2831-2836, 2007

Ramegowda B, Tesh VL. Differentiation-associated toxin receptor modulation, cytokine production, and sensitivity to Shiga-like toxins in human monocytes and monocytic cell lines. *Infect Immun*; 64: 1173-1180, 1996

Randolph GJ, Sanchez-Schmitz G, Liebman RM, Schakel KT. The CD16(+) (Fc γ RIII(+)) subset of human monocytes preferentially becomes migratory dendritic cells in a model tissue setting. *J Exp Med*; 196:517-27, 2002. Erratum in: *J Exp Med*; 196:869, 2002

Rodrigo L. Celiac disease. *World J Of Gastroenterol*; 12: 6585-6593, 2006

Rokeach LA, Haselby JA, Meilof JF, Smeek RJT, Unnasch TR, Greene BM, Hoch SO. Characterization of the autoantigen calreticulin. *J Immunol*; 147: 3031-3039, 1991

Rose NR. Fundamental concepts of autoimmunity and autoimmune disease. V: Krawit EL, Wiesner RH, Nishioka M, editori. *Autoimmune liver diseases*. 2.vydanie New York, Elsevier, s. 1-120, 1998

Rossi TM, Kumar V, Lerner A, Heitlinger LA, Tucker N, Fisher J. Relationship of endomysial antibodies to jejunal mucosal pathology: Specificity towards both symptomatic and asymptomatic coeliacs. *J Pediatr Gastroenterol Nutr*; 7: 858-863, 1988

Routsias JG, Tzioufas AG, Sakarellos-Daitsiotis M, Sakarellos C, Moutsopoulos HM. Calreticulin synthetic peptide analogues: anti-peptide antibodies in autoimmune rheumatic diseases. *Clin Exp Immunol*; 91: 437-441, 1993

Rubin CE. Some reflections on reversibility, gluten and the intestine. *Gastroenterology*; 39: 260-261, 1960

Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol*; 155:1151-1164, 1995

Sánchez D, Tučková L, Mothes T, Kreisel W, Beneš Z, Tlaskalová-Hogenová H. Epitopes of calreticulin recognized by IgA autoantibodies from patients with hepatic and coeliac disease. *J Autoimmun*; 21: 383-392, 2003

Sánchez D, Tučková L, Šebo P, Michalak M, Whelan A, Šterzl I, Jelínková L, Havrdová E, Imramovská M, Beneš Z, Krupičková S, Tlaskalová-Hogenová H. Occurrence of IgA and IgG autoantibodies to calreticulin in coeliac disease and various autoimmune diseases. *J Autoimmune*; 15:441-449, 2000

Sartor RB. Cytokines in intestinal inflammation: Pathophysiological and clinical considerations. *Gastroenterology*; 106:523-539, 1994

Shah VH, Rotterdam H, Kotler DP, Fasano A, Green PH. All that scallops is not celiac disease. *Gastrointest Endosc*; 51: 717-720, 2000

Shan L, Marti T, Sollid LM, Gray GM, Khosla C. Comparative biochemical analysis of three bacterial prolyl endopeptidases: implications for coeliac sprue. *Biochem J*; 383: 311-318, 2004

Shan L, Molberg Ø, Parrot I, Hausch F, Filiz F, Gray GM, Sollid LM, Khosla C. Structural basis for gluten intolerance in celiac sprue. *Science*; 297: 2275-2279, 2002

- Schuppan D, Esslinger B, Dietrich W. Innate immunity and coeliac disease. *Lancet*; 362: 3-5, 2003
- Siebenlist U, Franzoso G, Brown K. Structure, regulation and function of NF-kB. *Ann Rev Cell Biol*; 10: 405-455, 1994
- Siegel M, Bethune MT, Gass J, Ehren J, Xia J, Johannsen A, Stuge TB, Gray GM, Lee PP, Khosla C. Rational design of combination enzyme therapy for celiac sprue. *Chem Biol*; 13: 649-658, 2006
- Singh B, Read S, Asseman C, Malmstrom V, Mottet C, Stephens LA, Štěpánková R, Tlaskalová-Hogenová H, Powrie F. Control of intestinal inflammation by regulatory T cells. *Immunol Rev*; 182:190-200, 2001
- Sjöstrom H, Lundin KEA, Molberg Ø, Korner R, McAdam SN, Anthonsen D, Quarsten H, Noren O, Roepstorff P, Thorsby E, Sollid LM. Identification of a gliadin T-cell epitope in coeliac disease: generated importance of gliadin deamidation for intestinal T-cell recognition. *Scand j Immunol*; 48: 111-115, 1998
- Smith PD, Ochsenbauer-Jambor C, Smythies LE. Intestinal macrophages: unique effector cells of the innate immune system. *Immunol Rev*; 206:149-59, 2005
- Smith PD, Smythies LE, Mosteller-Barnum M, Sibley DA, Russell MW, Merger M, Sellers MT, Orenstein JM, Shimada T, Graham MF, Kubagawa H. Intestinal macrophages lack CD14 and CD89 and consequently are down-regulated for LPS- and IgA-mediated activities. *J Immunol*; 167:2651-6, 2001
- Smythies LE, Maheshwari A, Clements R, Eckhoff D, Novak L, Vu HL, Mosteller-Barnum LM, Sellers M, Smith PD. Mucosal IL-8 and TGF-beta recruit blood monocytes: evidence for cross-talk between the lamina propria stroma and myeloid cells. *Leukoc Biol*; 80: 492-9, 2006
- 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
- Snyder JG, Prewit R, Campsen J, Britt LD. PDTC and Mgl32, inhibitors of NF-kappaB, block endotoxin induced vasodilation of isolated rat skeletal muscle arterioles. *Shock*; 17: 304-307, 2002
- Sollid LM, Markussen G, Ek J, Gjerde H, Vartdal F, Thorsby E. Evidence for primary association of celiac disease to a particular HLA-DQ α/β heterodimer. *J Exp Med*; 169: 345-350, 1989
- Sollid LM, Molberg Ø, McAdam S, Lundin KE. Autoantibodies in coeliac disease: tissue transglutaminase-guilt by association? *Gut*; 41: 851-852, 1997

Sollid LM. Coeliac disease: dissecting a complex inflammatory disorder. *Nat Rev Immunol*; 2:647-655, 2002

Sollid LM. Molecular basis of coeliac disease. *Ann Rev Immunol*; 18: 53-81, 2000

Spencer J, Isaacson PG, MacDonald TT, Thomas AJ, Walker-Smith JA. Gamma/delta T cells and the diagnosis of coeliac disease. *Clin Exp Immunol*; 85: 109-113, 1991

Stadnyk AW. Cytokine production by epithelial cells. *FASEB J*; 8: 1041-1047, 1994

Steege J, Buurman W, Areds JW, Forget P. Presence of inducible nitric oxid synthase, CD68 and CD14 in the small intestine in celiac disease. *Lab Invest*; 77: 29-36, 1997

Stepniak D, Koning F. Celiac disease- sandwiches between innate and adaptive immunity. *Human Immunol*; 67: 460-468, 2005

Stepniak D, Spaenij-Dekking L., Mitea C, Moester M, de Ru A, Baak-Pablo R, van Veelen P, Edens L, Koning F. Highly efficient gluten degradation with a newly identified prolyl endoprotease: implications for celiac disease. *Am J Physiol Gastrointest Liver Physiol*; 291:621-629, 2006

Sturgess RP, Day P, Ellis HJ, Lundin KE, Gjertsen HA, Kontakou M, Ciclitira PJ, Wheat peptide challenge in coeliac disease. *Lancet*; 343: 758-761, 1994

Štěpánková R, Kofroňová O, Tučková L, Kozáková H, Cebra JJ, Tlaskalová-Hogenová H. Experimentally induced gluten enteropathy and protective effect of epidermal growth factor in artificially fed neonatal rats. *J Pediatr Gastroenterol Nutr*; 36: 96-104, 2003

Štěpánková R, Šinkora J, Hudcovič T, Kozáková H, Tlaskalová-Hogenová H. Differences in development of lymphocyte subpopulations from gut-associated lymphatic tissue (GALT) of germfree and conventional rats: Effect of aging. *Folia Microbiol*; 43:531-534, 1998

Štěpánková R, Tlaskalová-Hogenová H, Frič P, Trebichavský I. Enteropathy induced in young rats by feeding with gliadin: similarity with coeliac disease. *Folia Biol*; 35:19-26, 1989

Šterzl J. The immune system and its physiological functions. *Czech Society for Immunology*, Prague, 1993

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*; 176:2512-21, 2006

Thornton A, Shevach EM. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. *J Immunol*; 164: 183-190, 2000

Tjellström B, Stenhammar L, Högberg L, Fälth-Magnusson K, Magnusson KE, Midtvedt T, Sundqvist T, Norin E. Gut microflora associated characteristics in children with celiac disease. *Am J Gastroenterol*; 100: 2784-2788, 2005

Tlaskalová-Hogenová H, Farre-Castany MA, Štěpánková R, Kozáková H, Tučková L, Funda DP, Barot R, Cukrowska B, Šinkora J, Mandel L, Karská K, Kolínská J. The gut as a lymphoepithelial organ: the role of intestinal epithelial cells in mucosal immunity. *Folia Microbiol*; 40: 385-391, 1995

Tlaskalová-Hogneová H, Holáň V, Bilej M, a kol. Buněčné a molekulární základy imunologie. *Česká imunologická společnost*, 2007

Tlaskalova-Hogenova H, Vetvicka V, Sterzl J, Stepankova R. Development of immune potential and migration pattern of cells from germfree (GF) and conventionally(CONV) reared rats. *Adv Exp Med Biol*;149:515-20, 1982

Tlaskalová-Hogneová H, Štěpánková R, Frič P, Kolínská J, Vančíková Z, Jílek M, Kocna P, Lipská L, Slabý J, Dvořák M. Lymphocyte activation by gliadin in coeliac disease and in experimentally induced enteropathy of germ-free rats, 781-784 in *MacDonald TT et. Al. Advances in Mucosal Immunology*. Kluwer, Dordrecht, 1990

Tlaskalová-Hogneová H, Tučková L, Lodinová-Žadniková R, Štěpánková R, Cukrowska B, Funda DP, Stríž I, Kozáková H, Třebichavský I, Sokol D, Řeháková Z, Šinkora J, Fundová P, Horáková D, Jelínková L, Sanchez D. Mucosal immunity: its role in defense and allergy. *Int Arch Allergy Immunol*; 128:77-89, 2002

Trier JS. Celiac sprue. *N Engl J Med*; 325: 1709-1719. 1991

Troncone R, Ferguson A. Anti-gliadin antibodies. *J Pediatr Gastroenterol*; 12: 150-158, 1991

Troncone R, Greco L, Auricchio S. Gluten-sensitive enteropathy. *Pediatr Clin North Am*; 43: 355-373, 1996

Tučková L, Flegelová Z, Tlaskalová-Hogenová H, Zidek Z. Activation of macrophages by food antigens: enhancing effect of gluten on nitric oxide and cytokine production. *J Leukoc Biol*; 71: 625-631, 2000

Tučková L, Karská K, Walters JR, Michalak M, Rossmann P, Krupičková S, Verdu EF, Saalman R, Hanson LA, Tlaskalová-Hogenová H. Anti-gliadin antibodies in patients with celiac disease cross-react with enterocytes and human calreticulin. *Clin Immunol Immunopathol*; 85: 289-96, 1997

Tučková L, Novotná J, Novak P, Flegelová Z, Kveton T, Jelinková L, Zidek Z, Man P, Tlaskalová-Hogenová H. Activation of macrophages by gliadin fragments: isolation and characterization of active peptide. *J Leukoc Biol*; 71:625-631, 2002

Tučková L, Tlaskalová-Hogenová H, Farré MA, Karská K, Rossmann P, Kolínska J, Kocna P. Molecular mimicry as a possible cause of autoimmune reactions in celiac disease? Antibodies to gliadin cross-react with epitopes on enterocytes. *Clin Immun Immunopathol*; 74: 170-176, 1995

Tursi A, Brandimarte G, Giorgetti GM. Prevalence of antitissue transglutaminase antibodies in different degrees of intestinal damage in celiac disease. *J Clin Gastroenterol*; 36: 319-221, 2003

Uil JJ, van Elburg RM, van Overbeek FM, Meyer JW, Mulder CJ, Heymans HS. Follow-up of treated coeliac patients: sugar absorption test and intestinal biopsies compared. *Eur J Gastroenterol Hepatol*; 8: 219-223, 1996

Vader W, de Ru A, Wal Y, van de Kooy, Benckhuijsen W, Mearin L, Drijfhout JW, van Veelen P, Koning F. Specificity of tissue transglutaminase explains cereal toxicity in celiac disease. *J Exp Med*; 195: 643-649, 2002

Vader W, Stepniak D, Kooy Y, Mearin L, Thompson A, van Rood JJ, Spaenij L, Koning F. The HLA-DQ2 gene dose effect in celiac disease is directly related to the magnitude and breadth of gluten-specific t cell responses. *PNAC*; 100: 12390-12395, 2003

Van Belzen MJ, Meijer JW, Sandkuijl LA, Bardoel AFJ, Mulder CHJJ, Pearson PL, Roderick H. J. Houwen RHJ, Wijmenga C. A major non-HLA locus in celiac disease maps to chromosome 19. *Gastroenterology*; 125: 1032-1041, 2003

Van de Wal Y, Kooy YMC, Veelen van P, Peña AS, Mearin ML, Papadopoulos GK, Koning F. Selective deamidation by tissue transglutaminase strongly enhances gliadin-specific T cell reactivity. *J Immunol*; 161: 1585-1588, 1998

Van Heel DA, Hunt K, Greco L, Wijmenga C. Genetics in celiac disease. *Best Pract Res Clin Gastroenterol*; 19: 323-339, 2005

Ventura A, Magazzu G, Greco L. Duration of exposure to gluten and risk for autoimmune disorders in patients with celiac disease. SIGEP Study Group for Autoimmune Disorders in Celiac Disease. *Gastroenterology*; 117: 297-303, 1999

Wahab PJ, Meijer JW, Goerres MS, Mulder CJ. Coeliac disease: changing views on gluten-sensitive enteropathy. *Scand J Gastroenterol Suppl*; 236: 60-65, 2002

^aWahab PJ, Meijer JW, Mulder CJ. Histologic follow-up of people with celiac disease on a gluten-free diet: slow and incomplete recovery. *Am J Clin Pathol*; 118: 459-463, 2002

Wahl C, Liptay S, Adler G, Schmid RM. Sulfasalazine: a potent and specific inhibitor of nuclear factor kappa B. *J Clin Invest*; 101: 1163-1174, 1998

Waldmann TA, Tagaya Y. The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host response to intracellular pathogens. *Annu Rev Immunol*; 17:19-49, 1999

Walker-Smith JA, Guandalini S, Smith J, Shmerling DH, Visakorpi JK. Revised criteria for diagnosis of coeliac disease. *Arch Dis Child*; 65: 909-911, 1990

Weber C, Belge KU, von Hundelshausen P, Draude G, Steppich B, Mack M, Frankenberger M, Weber KS, Ziegler-Heitbrock HW. Differential chemokine receptor expression and function in human monocyte subpopulations. *J Leukoc Biol*; 67: 699-704, 2000

Wierink CD, van Diermen DE, Aartman IH, Heymans HS. Dental enamel defects in children with coeliac disease. *Int J Paediatr Dent*; 17:163-168, 2007

Wong RC, Wilson RJ, Steele RH, Radford-Smith G, Adelstein S. A comparison of 13 guinea pig and human anti-tissue transglutaminase antibody ELISA kits. *J Clin Pathol*; 55: 488-494, 2002

Xu RJ. Development of the newborn GI tract and its relation to colostrum/milk intake: a review. *Reprod Fertil Dev*; 8: 35-48, 1996

Zhu Q, Zelinka P, White T, Tanzer ML. Calreticulin-integrin bidirectional signaling complex. *Biochem Biophys Res Commun*; 232: 354-358, 1997

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

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

Lenka Jelínková^{a,*}, Ludmila Tučková^a, Jana Cinová^a, Zuzana Flegelová^b,
Helena Tlaskalová-Hogenová^a

^aInstitute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague, Czech Republic

^bResearch Institute of Biopharmacy and Veterinary Drugs, Jilové, 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 I κ B α molecule and increased NF- κ B/DNA binding activity that was inhibited by sulfasalazine, L-1-tosylamido-2-phenylethyl chloromethyl ketone and pyrrolidine dithiocarbamate (PDTTC). 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.

© 2004 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Monocyte; Gliadin; Interleukin-8; Tumour necrosis factor- α ; NF- κ B; 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- κ B pathway, resulting in a regulation of genes encoding proinflammatory cytokines, chemokines and adhesion molecules. The NF- κ B family of proteins consists of homo- and heterodimeric subunits of the Rel family, including p50 and p65. The activity of NF- κ B is regulated by a family of I κ B inhibitor proteins (I κ B α , β , γ and ϵ), which sequester NF- κ B in the cytoplasm. Upon stimulation, I κ B is phosphorylated, ubiquitinated, and subsequently degraded by the proteasome complex. Degradation of I κ B allows NF- κ B 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- κ B 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 \times 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).

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 μ g/ml) alone or together with human IFN- γ (150 U/ml, R&D System, Minneapolis) or to LPS (*Salmonella typhimurium*, 1–10 μ g/ml, Sigma) for 24 h. THP-1 cells were also incubated with soya protein, ovalbumin or their peptic fragments (100–500 μ g/ml) or synthetic gliadin peptides [33 AA gliadin peptide, B peptide and p31-43 peptide (200 μ g/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- κ B inhibitors sulfasalazine (0.1–2.0 mM), pyrrolidine dithiocarbamate (PDTC) (0.1–10 μ M) and L-1-tosylamidod-2-phenylethyl chloromethyl ketone (TPCK) (1.0–25.0 μ M) (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 μ g/ml), IFN- γ (150 U/ml) or LPS (1 μ g/ml), rinsed in cold 1 μ M Na₃VO₄ in Tris buffered saline, lysed in ice-cold lysis buffer (25 mM Tris-HCl, pH 7.4, 1 mM DTT, 1 mM Na₃VO₄ 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 μ g/well) was subjected to 10% SDS–polyacrylamide gel electrophoresis (PAGE) [13] and transferred onto nitrocellulose membranes. Phosphorylated I κ B protein was detected after incubation with rabbit anti-phospho-I κ B α 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- κ B assay

Nuclear extracts were prepared from THP-1 cells stimulated for 90 min with gliadin digest (500 μ g/ml) alone or together with IFN- γ (150 U/ml), sulfasalazine (2 mM), PDTC (0.5 μ M) and TPCK (5 μ M)

using a Nuclear extract Kit (Active Motif, USA). NF- κ B DNA binding activity was detected using a TransAM NF- κ B 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- κ B 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- κ B 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+) possesses 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 μ g/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 μ g/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).

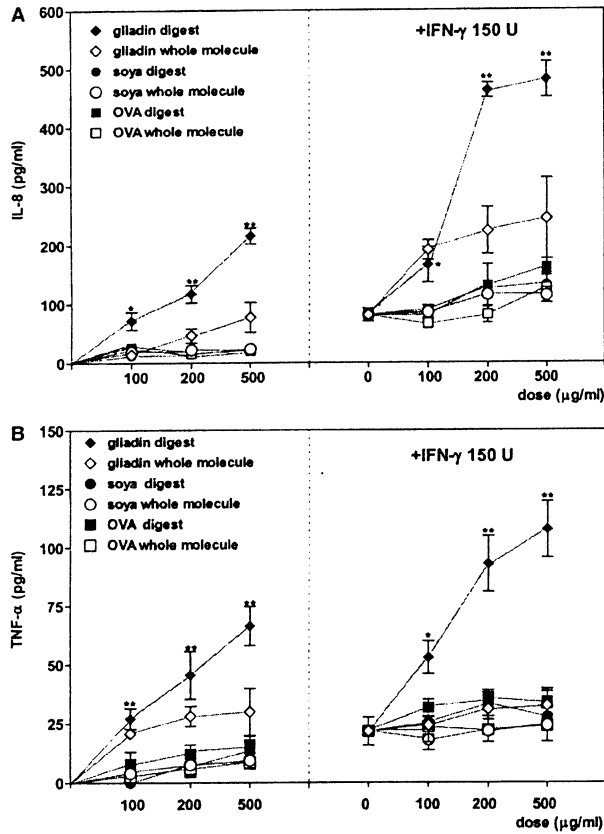


Fig. 1. Effect of gliadin, soya protein and ovalbumin and their proteolytic fragments on IL-8 (A) and TNF- α (B) secretion by THP-1 cells measured after 24 h of cell cultivation (left); the synergistic effect of IFN- γ applied together with food proteins (right). Data are expressed as means \pm S.E.M. 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 (FQQPQQYPSQ), 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.

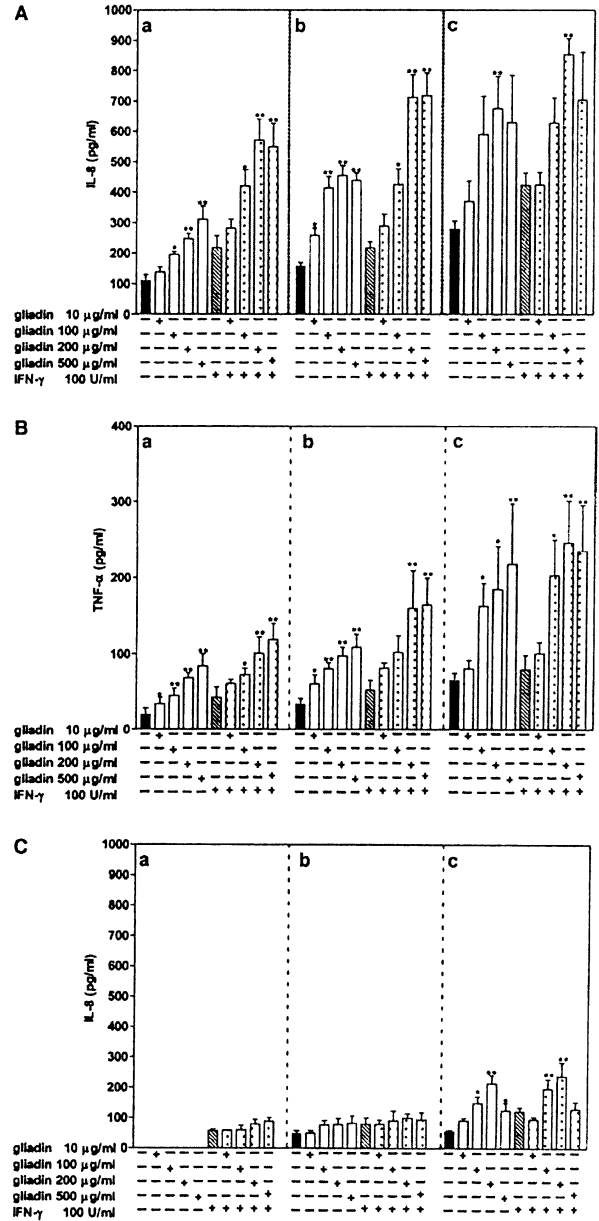


Fig. 2. Response of THP-1 (A, B) and U-937 cells (C) to gliadin fragments added directly to cell cultures (a) or after preincubation with IFN- γ for 2 (b) or 24 h (c) measured as IL-8 (A, C) and TNF- α (B) production. Data are expressed as means \pm S.E.M. from at least three independent experiments.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

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 μ g/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

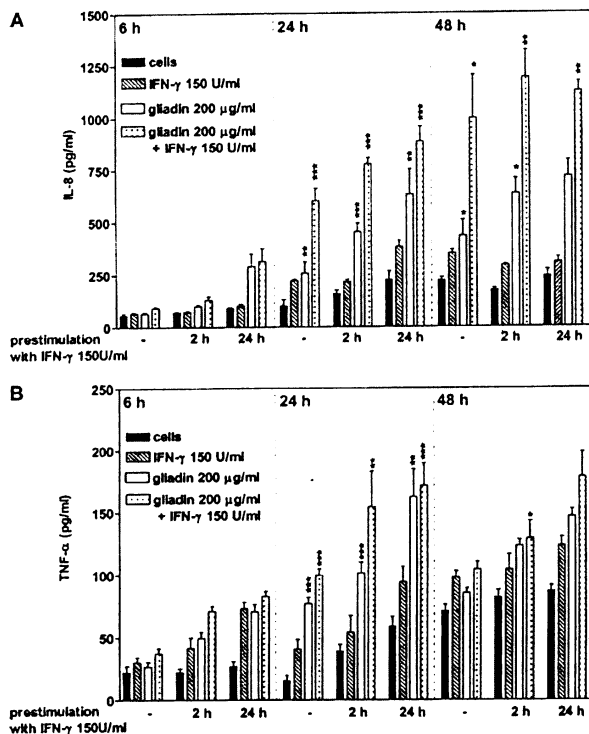


Fig. 3. The kinetics of IL-8 (A) and TNF- α (B) production by THP-1 cells. Cytokine production was determined 6, 24 and 48 h after addition of gliadin fragments and/or IFN- γ to cells directly or after IFN- γ preincubation (for 2 or 24 h). Data obtained from at least three experiments are expressed as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

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- κ B pathway

Stimulation of THP-1 cells with gliadin digest (500 μ g/ml) and/or with IFN- γ (150 U/ml) resulted in a marked increase of the binding activities of NF- κ B subunits p50 and p65, more

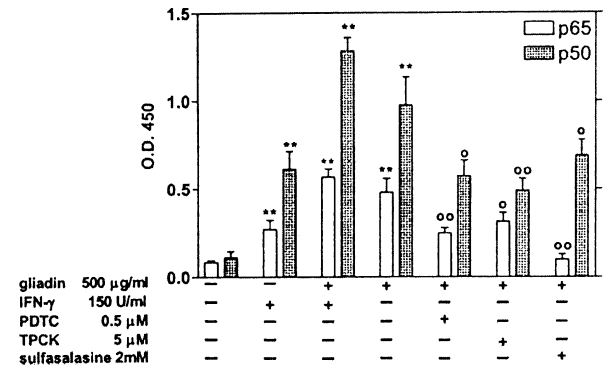


Fig. 4. The involvement of NF- κ B subunits p50 and p65 in the activation pathway induced by gliadin fragments in THP-1 cells. The NF- κ B DNA binding activity and the effect of inhibitors: sulfasalazine (2 mM), PDTC (0.5 μ M) and TPCK (5 μ M) was measured after a 90 min cultivation by a colorimetric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

pronounced in the case of p50 subunit. To confirm the role of NF- κ B in gliadin-induced activation of THP-1 cells, we examined the effects of NF- κ B inhibitors: sulfasalazine, PDTC and TPCK. Sulfasalazine inhibits phosphorylation of I κ B [17], PDTC functions as a NF- κ B inhibitor by blocking the dissociation of the NF- κ B/I κ B complex [18] and TPCK plays a role as proteasome inhibitor; it can therefore inactivate NF- κ B by preventing the degradation of I κ B [19]. Treatment with 0.5 μ M PDTC and 2 mM sulfasalazine inhibited substantially p65 but only slightly p50 binding activity; 5 μ M 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 μ M concentration. TPCK produced 32% inhibition at 1 μ M and completely abolished IL-8 production at 25 μ M concentration (data not shown).

Since NF- κ B activation involves the phosphorylation and subsequent degradation of I κ B α , we assayed the phosphorylation of I κ B α 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 I κ B α , when compared with unstimulated cells. Taken together, these results suggest that NF- κ B 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 μ g) (% of blockade)	+ IN-05 (10 μ g) (% of blockade)
LPS 10 μ g	1030.3 \pm 130.0	324.4 \pm 76.8 (70)	1038.4 \pm 56.6 (0)
Gliadin 500 μ g	287.5 \pm 21.2	273.1 \pm 11.0 (5)	300.5 \pm 20.2 (0)
Cells	98.0 \pm 11.8	100.2 \pm 10.5 (0)	98.2 \pm 15.6 (0)

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

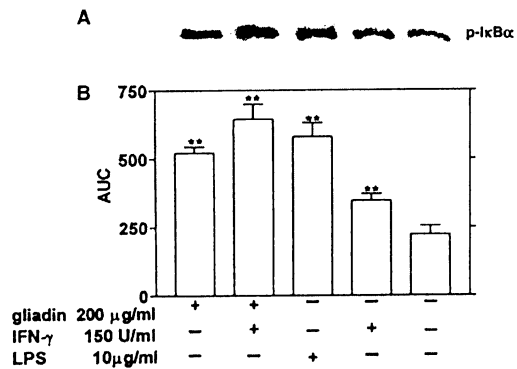


Fig. 5. Representative Western blot shows the effect of gliadin fragments, IFN- γ or LPS (positive control) on phosphorylation of I κ B α in THP-1 cells (p-I κ B α formation) (A) and its densitometric evaluation presented as mean AUC (arbitrary units) \pm S.E.M. of three independent experiments, ** $P < 0.01$ (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 Zidek, Z. (2000) *J. Leukoc. Biol.* 67, 312–318.
- [4] Tučková, L., Novotná, J., Novák, P., Flegelová, Z., Květoň, T., Jelínková, L., Zidek, 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., Bevilacqua, 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., Parrott, 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- κ B 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). © 2005 New York Academy of Sciences.
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 ($\sim 2 \text{ m}^2$) is protected mechanically by several epithelial layers, the surfaces of the gastrointestinal, respiratory, and urogenital tracts ($\sim 300 \text{ m}^2$) 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 non-dangerous 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 anti-infectious 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- κ B 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 non-dangerous 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 β 1 (TGF- β 1), MHC class II, T cell receptor (TCR)- α chain, and TCR- β chain, and mice lacking signaling protein G protein subunit G α i2 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 inflammation. 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

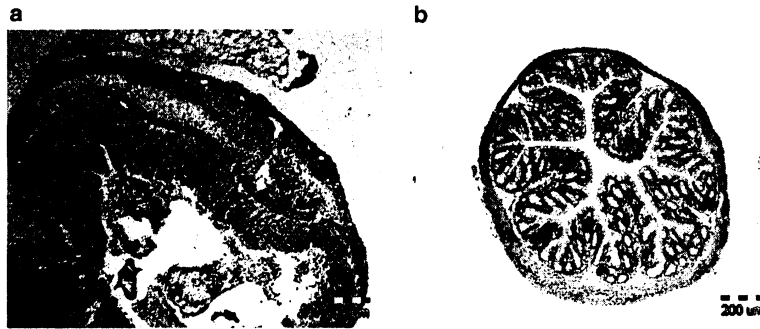


FIGURE 1. The colon after 1 week of ingestion of dextran sodium sulfate in (a) conventional SCID mice, showing ulcerative colitis with severe infiltration of inflammatory cells (grade 4 inflammation); and (b) germ-free SCID mice, in which no inflammation is observed.

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

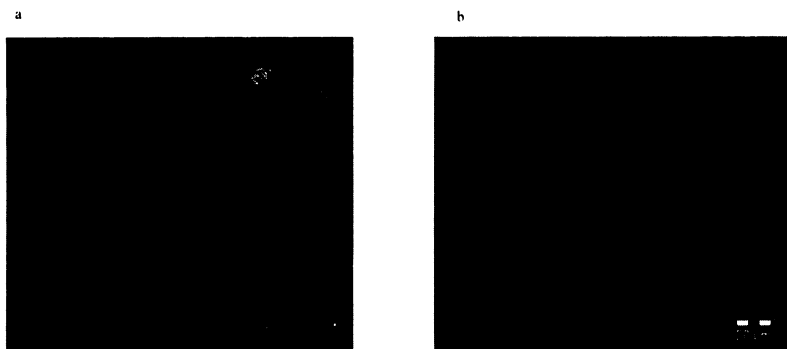


FIGURE 2. Tight junctions in terminal ileum of SCID mice colonized 12 weeks after transfer of CD4⁺CD45RB^{high} T cells with (a) specific pathogen-free (SPF) microflora, showing preserved integrity of tight junctions; and (b) mixture of SPF microflora and segmented filamentous bacteria, showing altered 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 μ g	100 μ g	500 μ g	100 μ g	500 μ g
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 (\pm SEM).

^a $P < .05$, ^b $P < .01$.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- κ B (NF- κ B) in the stimulatory effect of the gliadin digest on monocytes was documented by a marked increase of the DNA-binding activities of NF- κ B subunits p50 and p65. Moreover, the inhibition of p65 and p50 subunit binding was detected using NF- κ B inhibitors sulphasalazine, PDTTC, and TPCK.⁵⁰ Because NF- κ B activation involves phosphorylation and subsequent degradation of I κ -B α , the phosphorylation of I κ -B α 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 I κ -B α , suggesting that NF- κ B 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. Lefkowitz, Ed.: 1524–1559. Academic Press. London.

12. HARMSSEN, 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. KRAEHEBUHL. 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/high 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 non-pathogenic 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. ŠTEPÁNKOVÁ, 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 anti-gliadin 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. CÍNOVÁ, *et al.* 2004. Gliadin stimulates human monocytes to production of IL-8 and TNF- γ through a mechanism involving NF- κ B. *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**

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 MILOŠ DVOŘÁK,⁴ PAVEL FRUHAUF,⁴ HELENA TLASKALOVÁ-HOGENOVÁ,¹ PHILLIP D. SMITH,^{2,5} and LUDMILA TUČKOVÁ¹

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- κ B p50 and p65 subunits in monocytes from celiac patients, and NF- κ B 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- κ B 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).

¹Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic.

²Department of Medicine (Gastroenterology), University of Alabama at Birmingham, Birmingham, Alabama.

³3rd Medical Faculty, Charles University, Prague, Czech Republic.

⁴1st Medical Faculty, Charles University, Prague, Czech Republic.

⁵VA Medical Center, Birmingham, Alabama.

⁶To 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 μ g/mL streptomycin, and 100 U/mL penicillin (Sigma). The monocytes were removed by scraping and analyzed by flow cytometry (BD FACS Vantage 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 μ g/mL) alone or with gliadin (100 μ g/mL) plus IFN- γ (150 U/mL; R&D Biosciences, Minneapolis, MN), as described previously (7, 29, 32). In parallel monocyte cultures, the NF- κ B inhibitors L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK, 1.0–25.0 μ M) or pyrroli-

dine dithiocarbamate (PDTC, 1.0–10.0 μ M) (both Sigma) were added to the cells for 30 min prior to the addition of gliadin. Cultures were maintained at 37°C, 5% CO₂ 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% NaN₃ 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- κ B Assays

Nuclear extracts were prepared from purified monocytes stimulated for 90 min with gliadin digest (100 μ g/mL) as mentioned earlier, with or without TPCK (1–25 μ M) and PDTC (1–10 μ M), using the Nuclear Extract Kit (Active Motif, Carlsbad, CA). NF- κ B DNA binding activity was determined using the TransAM NF- κ B family transcription factor assay (Active Motif), according to the manufacturer's protocol. Briefly, microwells coated with a double-stranded oligonucleotide containing the NF- κ B 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- κ B 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 μ g/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 μ L containing: 6 ng/ μ L of genomic DNA, PCR Master Mix (200 μ M of each dNTP, PCR buffer: 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl pH 8.3, 0.001% w/v gelatin, 5% glycerol, 100 μ g/mL cresol red) complete with 0.4 U/ μ L 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 evaluated 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

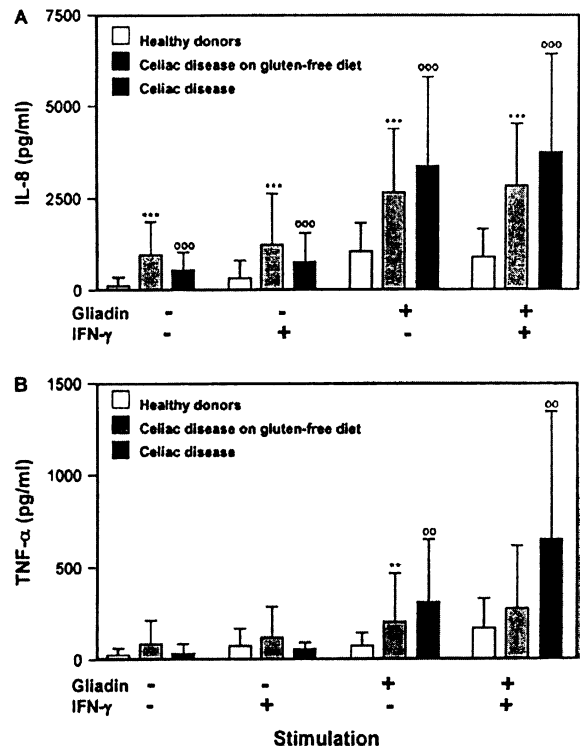


Fig. 1. IL-8 and TNF- α production by monocytes from patients with celiac disease. Monocytes isolated from patients with active celiac disease ($n = 14$), asymptomatic patients with celiac disease on a gluten-free diet ($n = 40$), and healthy donors ($n = 45$) were cultured for 24 h with gliadin (100 μ g/mL) alone or with gliadin plus IFN- γ (150 U/mL). The amounts of (A) IL-8 and (B) TNF- α (mean \pm 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, $^{\circ\circ}$ *P* < 0.01, $^{\circ\circ\circ}$ *P* < 0.001.

gluten-free diet spontaneously released low levels of IL-8 (525 \pm 404 pg/mL and 965 \pm 682 pg/mL, respectively, *P* > 0.05), which were significantly greater than the amounts spontaneously released by monocytes from healthy control subjects (131 \pm 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 μ g/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 \pm 2451 pg/mL) than monocytes from patients with inactive disease on a gluten-free diet (2645 \pm 1723 pg/mL), which in turn produced more IL-8 than monocytes from healthy donors (1046 \pm 783 pg/mL) (*P* < 0.001). The production of

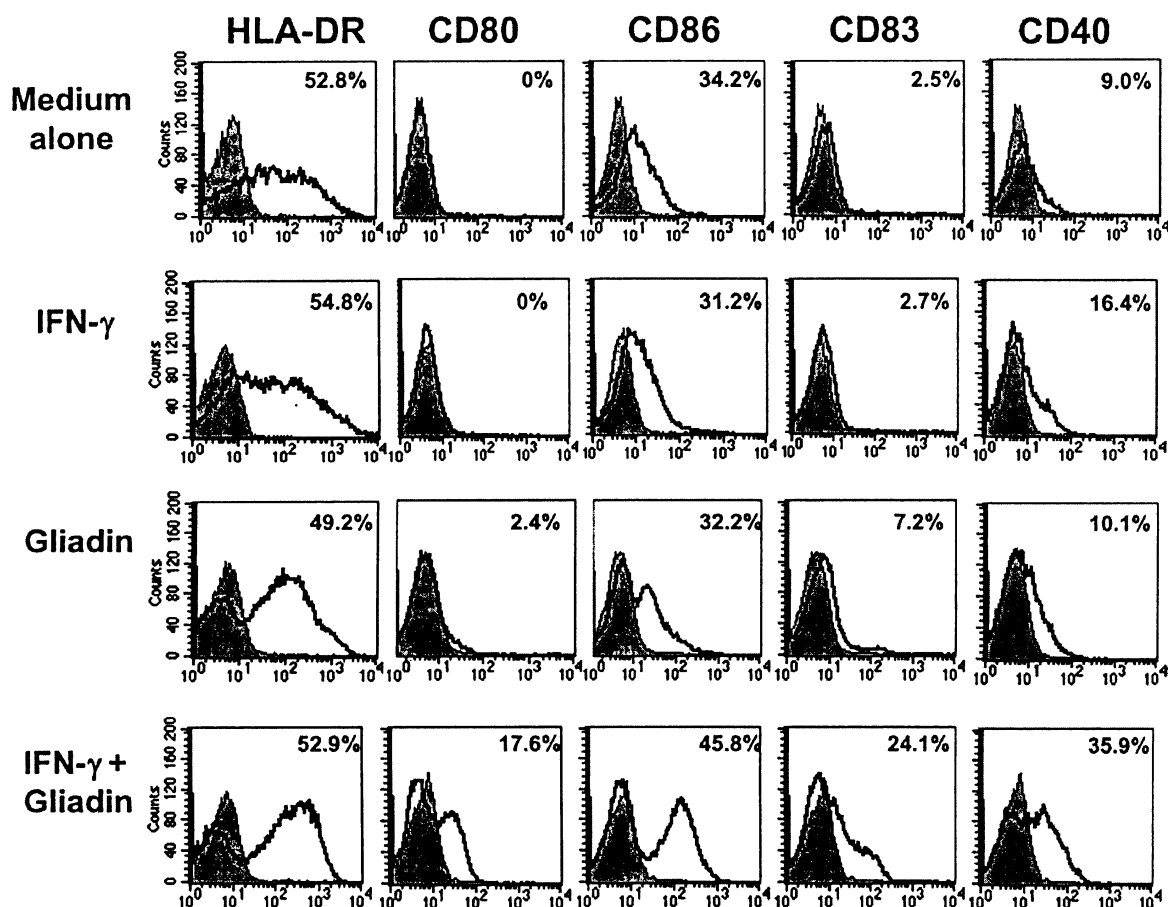


Fig. 2. Effect of gliadin on monocyte phenotype. Blood monocytes from healthy donors were incubated for 24 h in media or with IFN- γ alone, gliadin alone, or IFN- γ plus gliadin at the indicated concentrations and then analyzed by FACS for the expression of activation and differentiation markers. Staining 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 μ g/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 Differentiation 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 mat-

uration of monocyte-derived dendritic cells. Therefore, we determined whether incubation of blood monocytes from healthy persons with gliadin fragments (100 μ g/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

HLA Type	Donors (n)	Medium	Monocyte IL-8 Production (pg/mL) ^a				
			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

^aValues 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- κ B Activation

We have shown previously that NF- κ B activation is involved in gliadin-induced cytokine production by monocyte-derived DCs and THP-1 cells (30). Therefore, we next determined whether the NF- κ B 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- κ B p50 and p65 subunits ($P < 0.05$). Incubation of monocytes from celiac patients with the NF- κ B inhibitor TPCK reduced p50 and p65 binding to 20% and 54%, respectively (Fig. 3). Supporting the role of NF- κ B in gliadin-induced cytokine production by monocytes from patients with active celiac disease, NF- κ B 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- κ B 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,

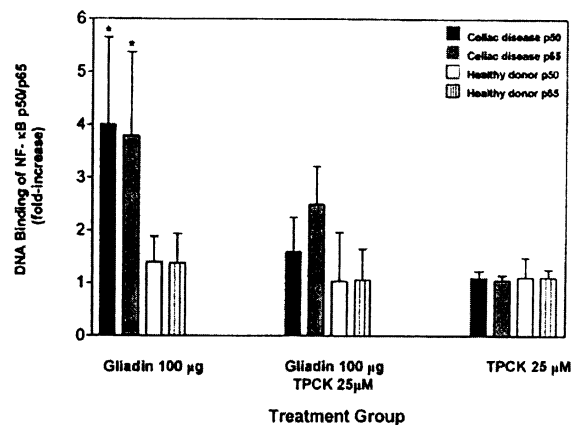


Fig. 3. Gliadin induction of monocyte NF- κ B p50 and p65 subunit binding. Monocytes from patients with active celiac disease and healthy donors were analyzed for NF- κ B DNA binding activity after a 90 min incubation with gliadin (in the presence or absence of the NF- κ B inhibitor TPCK). Data are presented 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.

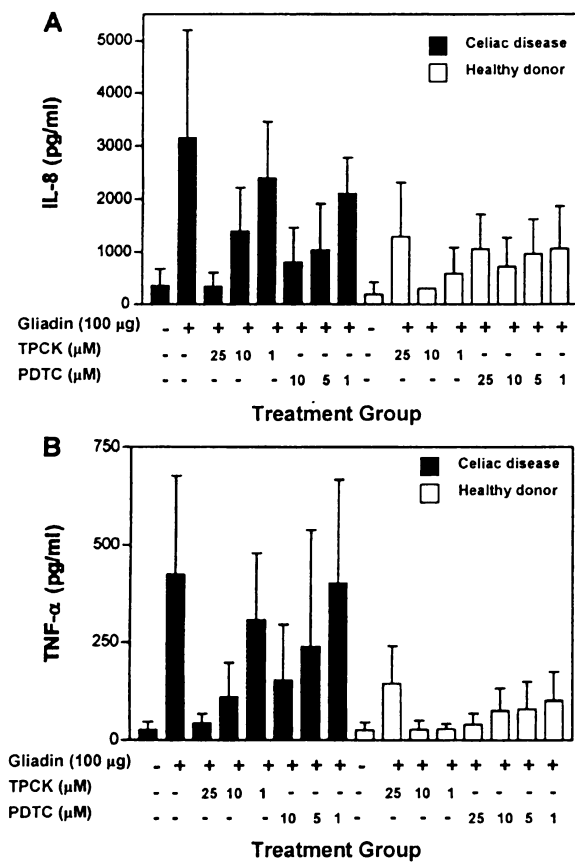


Fig. 4. NF- κ B inhibitors block gliadin-induced monocyte IL-8 and TNF- α production. Monocytes from patients with celiac disease and healthy donors were cultured for 24 h with gliadin in the presence or absence of the NF- κ B inhibitors TPCK (1–25 μ M) or PDTC (1–10 μ M), and the amount of IL-8 and TNF- α released into the culture supernatants were measured by ELISA. Results are expressed as mean \pm 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, cocubation 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- κ B/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- κ B 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- κ B-dependent (26, 49). In this regard, both NF- κ B/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- κ B/I κ B complex in gliadin-induced activation of monocytes was confirmed using the NF- κ B inhibitors TPCK, which prevents degradation of I κ B inhibitor, and PDTC, which blocks dissociation of the NF- κ B/I κ B complex (52, 53). Both NF- κ B inhibitors substantially reduced gliadin-induced NF- κ B 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 responses in celiac disease and suggest that NF- κ B 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 energy 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, identification 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), Ministry 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 immunobiologic 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, Sollid 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 enteropathies. *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, Quarantino 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, Bartová 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, Nevoval J, Kotalova R, Tlaskalova-Hogenova H: Increased levels of circulating 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 Tenuè”: 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-gamma 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-alpha 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**



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-HOGENOVA¹

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 oncprotein (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-1 δ /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 spec-

trum of cytokines (including those not described before) in lacteal secretions.

© 2007 American Association for Clinical Chemistry

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 680g for 10 min at 4°C , after which the supernatants were removed and then at 10 000g 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 ($\geq 50\%$ 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-18 ^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-1 α	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-1 β	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	PIGF ^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- β 2	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- β 3 ^b	LIGHT	Fractalkine ^b	BDNF ^b	TGF- β 1	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-3 α ^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-1 β	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 neurophil 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; PIGF, 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)-1 β /CCL4, MIP-1 δ /CCL15, MIP-3 α /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 be-

tween 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].

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 cumsum 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

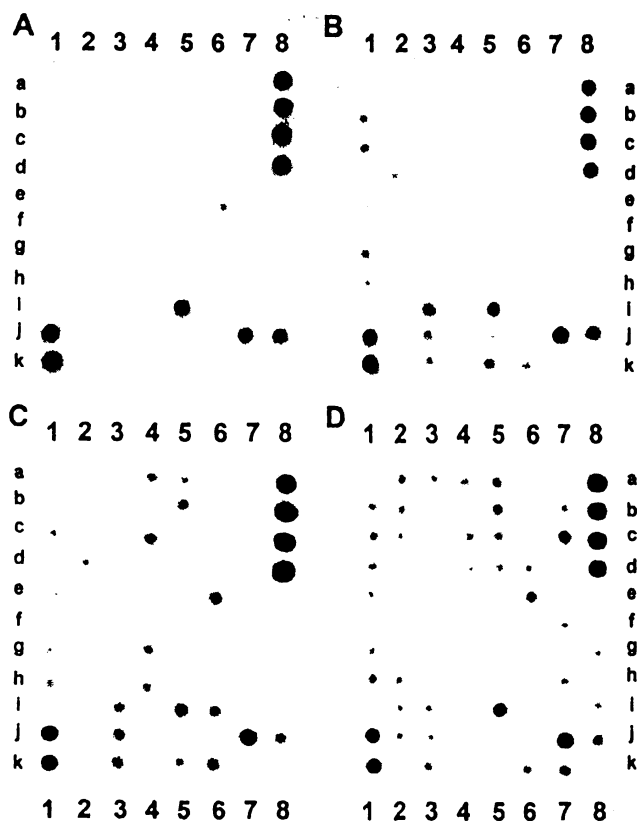


Fig. 1. Examples documenting the variability of the individual samples. Sample A shows 4 (minimum), sample B 16, sample C 22, and sample D 38 (maximum) positive cytokines. These arrays are designed to detect 79 cytokines, chemokines, or growth factors. See Table 1 for cytokine identification.

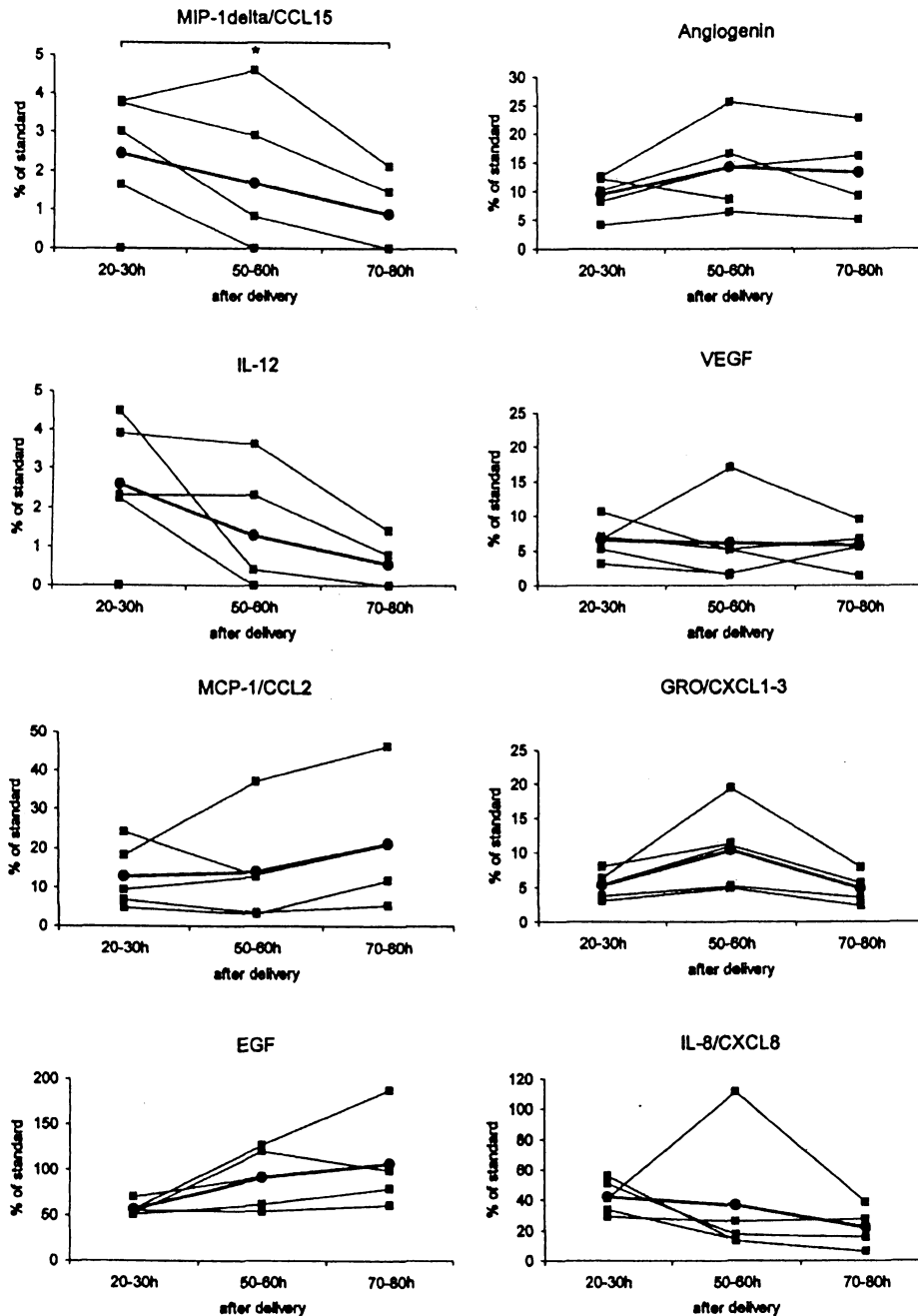


Fig. 2. Changes in cytokine relative concentration with time after delivery.

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 immunologi-

cally 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,

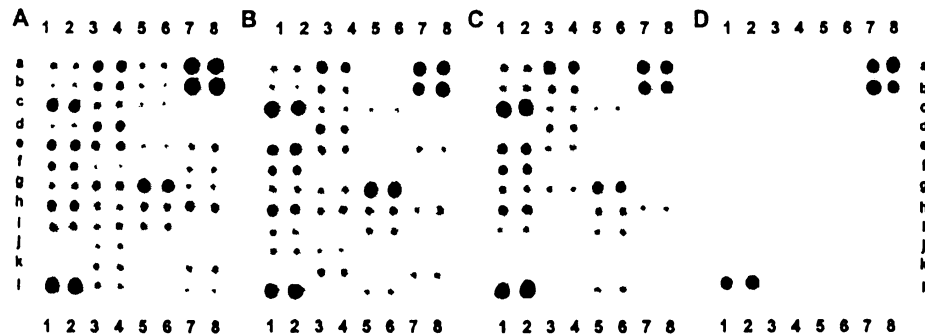


Fig. 3. Cytokine profile 30 h (A), 60 h (B), and 80 h (C) after delivery in one of the sample sets, demonstrating the changes in cytokine spectrum. The rightmost array (D) represents a negative control array. These arrays are designed to detect 42 cytokines, chemokines, or growth factors. See Table 2 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 $\sim 222 \mu\text{g/L}$ ($7.3 \mu\text{g/day}$) on the 1st

day, $228 \mu\text{g/L}$ ($9.8 \mu\text{g/day}$) on the 2nd day, and $268 \mu\text{g/L}$ ($28.7 \mu\text{g/day}$) 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 $>330 \text{ pg}$ on the 1st day, $>430 \text{ pg}$ on the 2nd day, and $>1070 \text{ pg}$ 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-3 α /CCL20 (mainly for lymphocytes and immature dendritic cells), MIP-1 β /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-1 δ	MIP-1 δ	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-1 α	IL-1 α
l	Pos	Pos	TGF- β 1	TGF- β 1	IFN- γ	IFN- γ	IL-1 β	IL-1 β

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

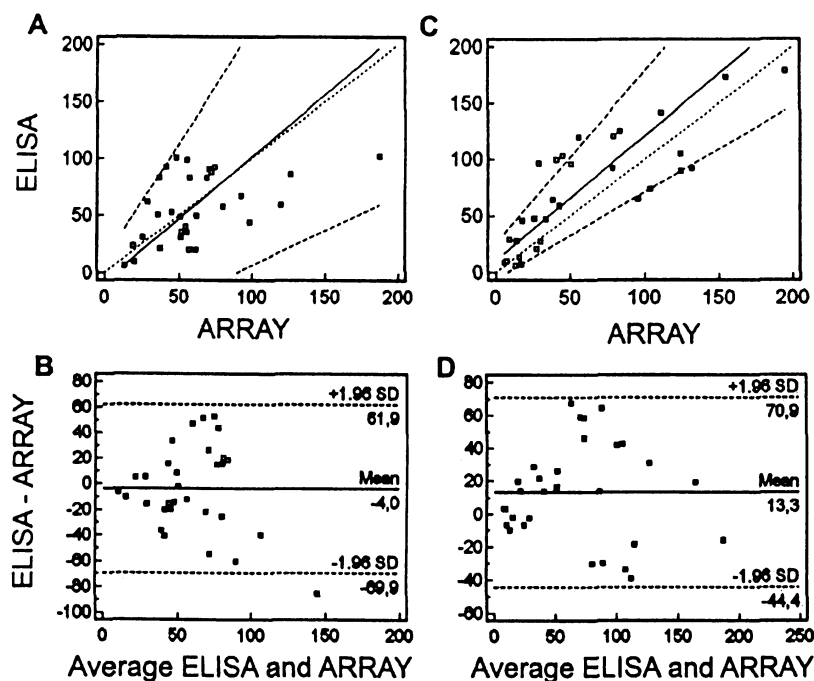


Fig. 4. Passing and Bablok regression scatter plot for EGF (A) and IL-8/CXCL8 (C).

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, neurotrophin-3, and neurotrophin-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, Ladinova-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, Telleo E. Human milk: its components and their immunobiologic functions. In: Mestecky J, Lamm ME, Strober W, Blenestock 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, TempIn 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, Kehrl 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 post-natal 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.