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DEPARTMENT OF MEDICAL MICROBIOLOGY AND IMMUNOBIOLOGY

THE ROLE OF ALARMINS - HUMAN DEFENSINS, HMGB1 - IN GASTROINTESTINAL DISEASES

Ph.D. Thesis

by

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Publications with results incorporated in the thesis:

I. **Kocsis AK, Ocsovszky I, Tiszlavicz L, Tiszlavicz Z, Mándi Y.:**

Helicobacter pylori induces the release of alpha-defensin by human granulocytes. *Inflamm Res*. 2009 May;58(5):241-7.

IF:1,457

II. Kocsis AK, Kiss ZF, Tiszlavicz L, Tiszlavicz Z, Mándi Y.:

Potential role of human beta-defensin 1 in Helicobacter pylori-induced gastritis. *Scand J Gastroenterol.* 2009;44(3):289-95.

IF:1,98

III. Kocsis AK, Lakatos PL, Somogyvári F, Fuszek P, Papp J, Fischer S, Szamosi T, Lakatos L, Kovacs A, Hofner P, Mándi Y.:

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Scand J Gastroenterol. 2008 Mar;43(3):299-307.

IF:1,98

IV. Kocsis AK, Szabolcs A, Hofner P, Takács T, Farkas G, Boda K, Mándi Y.

Plasma concentrations of high-mobility group box protein 1, soluble receptor for advanced glycation end-products and circulating DNA in patients with acute pancreatitis.

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IF:3,061

ABBREVIATIONS

α-defensin: alpha-defensin

AGS: human adenocarcinoma cell

β-defensin: beta-defensin

CagA+: cytotoxin associated gene A

C. albicans: Candida albicans

cDNA: complementary DNA

CI: confidence interval

COPD: Chronic obstructive pulmonary disease

CRP: C-reactive protein

DAMP: damage-associated molecular pattern

DEFA: alpha-defensin

DEFB: beta-defensin

DNA: deoxyribonucleic acid

EDTA: ethylenediaminetetraacetic acid

EIMs: extraintestinal manifestations

ELISA: enzyme-linked immunosorbent assay

ERK: extracellular signal-regulated kinase

E.coli: Escherichia coli

FAM: Carboxyfluorescein

FCS: Fluorescence correlation spectroscopy

FITC: fluorescein isothiocyanate

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

HAPMap: haplotype map

hBD: human beta-defensin

HD5: human α-defensin 5

HMGB1: high mobility group box protein 1

HNP: human neutrophil protein

IBD: inflammatory bowel disease

IL-1β: interleukin-1 beta

IL-8: interleukin-8

LDH: lactate dehydrogenase

LPS: lipopolysaccharide

MOI: multiplicity of infection

NF κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NK cell: natural killer cell

NOD2: nucleotide-binding oligomerization domain containing 2

OR: odds ratio

PAI: cag pathogenicity island

PAMP: pathogen-associated molecular pattern

PBEC: primary bronchial epithelial cells

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PSC: primary sclerosing cholangitis

P. aeruginosa : Pseudomonas aeruginosa

RAGE: Receptor for Advanced Glycation Endproducts

RNA: ribonucleic acid

ROC: receiver-operating characteristic

RT-PCR: reverse transcription polymerase chain reaction

SDS: Sodium Dodecyl Sulphate

SNP: single-nucleotide polymorphism

S. aureus: Staphylococcus aureus

sRAGE: soluble Receptor for Advanced Glycation Endproducts

Th2: T helper cell

TNF α: tumor necrosis factor-alpha

TSC: The SNP Consortium

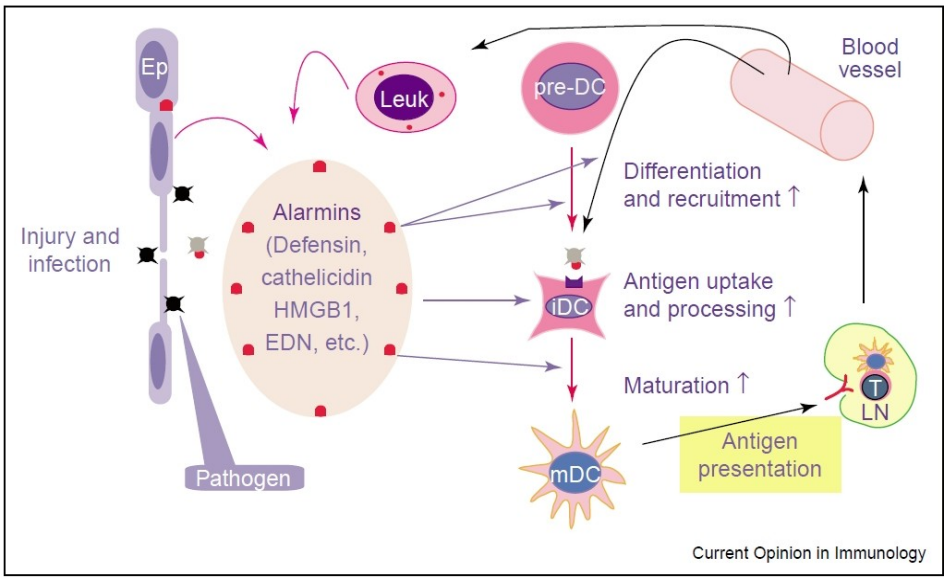
UBT: Urea Breath Test

VacA+: vacuolating cytotoxin A

1. INTRODUCTION

1.1. Alarmins

Multicellular organisms must distinguish whether their cells are alive or dead and detect when microorganisms intrude, and had evolved surveillance defense repair mechanisms to this [1.]. A complete system for the detection of damage caused to cells in the organism requires warning signals, cells to respond to them via receptors and signalling pathways. Danger signals include exogenous microorganisms, endogenous tissue injury, and intercellular inflammatory mediators. Alarmins are endogenous mediators of innate immunity. They are rapidly released in response to infection or tissue injury. In addition, they are rapidly released from nonprogrammed cell death, but are not released from apoptotic cells. Cells of the immune system also can be induced to produce alarmins by specialized secretion system [2.]. Alarmins recruit immune system and directly or indirectly promote adaptive immune responses. On the basis of their unique activities, they have been called „alarmin" molecules[2.3.]. Innate immune mediators possessing alarmin activity include defensins, cathelicidin, eosinophil-derived neurotoxin (EDN) and High Mobility group Box Protein 1 (HMGB1) (1.Fig.).



1.Fig. Schematic illustation of the contributions of alarmins to the initiation of host adaptive immune response. (EP-epitelial cells, Leuk-leukocytes, DC-dendritic cell, pre-DC-DC precursors, LN-lymph nodes, EDN-eosinophil derived neurotoxin)

1.2. Defensins

Defensins are antimicrobial peptide components of the innate host defense system.[4.5.6.] They exhibit a great spectrum of activity against Gram-negative and Gram-positive bacteria, fungi and enveloped viruses.[7.] The human defensins are a family of six cysteine 3–4 kD cationic peptides with a characteristic fold that is common to the two subfamilies, α- and β-defensins, despite a difference in the connectivity of their disulfide bonds.[8.]

1.2.1. α-defensins:

Human α-defensins are arginine-rich peptides, containing 29–35 amino acids. Their three disulfide bridges connect cysteines 1–6, 2–4 and 3–5. Human α-defensins are synthesized as 93–100 amino acid prepropeptides with a 19-amino acid signal peptide and a 41–51 amino acid anionic pro segment. The current view is that they are instrumental for the killing of phagocytosed microorganisms during intracellular delivery to phagolysosomes. [9.10.11.] In humans, six human α-defensins have been identified. The gene cluster, coding for α-defensins (DEFA1, DEFA3, DEFA4, DEFA5, DEFA6), is located within chromosomal region 8p21-p23. Four of them, human neutrophil proteins – HNP 1-4 -, are mainly produced by granulocytes.[4.] HNP 1-3 share identical amino acid sequences except for the first N-terminal residue. HNP 1-4 are localized in the azurophilic granules of neutrophil granulocytes. Neutrophils are the main source of human α-defensins (therefore also named human neutrophil peptides HNP1–4), although they have been found in other leukocyte subsets such NK cells, monocytes to a lesser extent, and in immature dendritic cells. The HNP1-3 induce interleukin (IL)-8 expression in lung epithelial cell line and in human primary bronchial epithelial cells (PBEC)[12.13.] Human α-defensin 5 (HD5) and human α-defensin 6 (HD6) are present in intestinal Paneth cells. In the small intestine, Paneth cells produce high quantities of α-defensins. Thus, they are called enteric defensins. HD-5 and -6 are stored in secretory vesicles as propeptides and cleaved by Paneth cell trypsin which acts a prodefensin convertase[14.15.] and by the metalloproteinase matrilysin.[16.] Inflammatory functions of the α-defensins could be improved by the ability to attract neutrophils, monocytes, macrophage, and T-cells to inflammation sites and production of inflammatory cytokines. These leukocytes are commonly found at the inflammation sites as part of innate immune response to the presence of pathogens.[3.17.18.19.20.] At concentrations higher than 100 μg/ml, HNP-1 and HNP-2 kill Gram-negative and Gram-positive bacteria, including both intracellular and extracellular organisms,[21.] as well as enveloped viruses such as members of the *Herpes* family.[22.23.]

1.2.2. β-defensins:

Human β-defensins also contain around 35 amino acid residues, including six cysteine residues with a distinct spacing pattern forming a disulfide array (1–5, 2–4, 3–6) [24.] that differs from that of the α-defensins. They are expressed predominantly in epithelial tissues, which provide the first line of defense between an organism and the environment.[25.] The first gene cluster, coding for β-defensins DEFB1, DEFB4 and DEFB103, is located within chromosomal region 8p21-p23, which also includes all the α-defensin genes. Subsequently, three other DEFB gene clusters were identified within chromosomes 6p12, 20q11.1 and 20p13.[5.27.28.6.29.] Although more than 90 human-defensins have been identified by gene-based searches, the function of six human β-defensins (hBD1-6) are to known, they are expressed mainly by epithelial cells. Whereas hBD1 is constitutively expressed by epithelial cells, expression of hBD2 and hBD3 can be induced by viruses, bacteria, microbial products and pro-inflammatory cytokines. The most prevalent expression of β-defensins is observed in the gastrointestinal and respiratory tracts; however, they are present throughout the entire epithelia.[30.27.]

Expression of hBD-1 has been demonstrated in keratinocytes associated with inflammatory skin lesions such as psoriasis vulgaris as well as in keratinocytes from normal skin and in sweat gland ducts within the dermis.[31.32.] hBD-1 is produced constitutively in cultured human epithelial cells derived from the trachea, bronchi, small airways and the mammary gland,[33.34.] as well as the parotid gland, buccal mucosa, tongue and gingiva. [35.36.37.] Moreover, hBD-1 is expressed in the epithelia of the small intestine, pancreas, kidney, prostate, testis, vagina, ectocervix, endocervix, uterus, fallopian tubes, the placenta, and the thymus.[8.38.]

hBD-2 is expressed in keratinocytes, the gingival mucosa and the tracheal epithelium. [39.40.41.42.] Transcription of the hBD-2 gene is induced by IL-1β, TNF-α and bacterial lipopolysaccharide, and by contact with Gram-negative (*E. coli*, *P. aeruginosa*) and Gram-positive (*S. aureus*) bacteria, and the yeast *C. albicans*.[35.36.8.40.43.] The expression of hBD-*2* is regulated by several intracellular signalling pathways, such as the NF-κB pathway and the G-protein-coupled protease-activated receptors that mediate cellular responses to extracellular proteinases. [27.] At concentration than near 10 mg/ml, hBD2 kill Gram negative bacteria, thus hBD-2 is predominantly active against Gram-negative bacteria and yeasts.[44.45.]

Human beta-defensin-3 (hBD-3) expression was demonstrated in keratinocytes and in tonsil tissue, whereas low hBD-3 expression was found in the epithelia of the respiratory,

gastrointestinal, and genitourinary tracts. In keratinocytes, transcription of the hBD-3 gene is induced by TNF-α and contact with heat-inactivated bacteria, including the Gram-negative organism *P. aeruginosa* and the Gram-positive *S. aureus*.[45.] Synthetic hBD-3 shows antimicrobial activity against the Gram-positive bacteria *Staphylococcus carnosus*, *Streptococcus pneumoniae* and *S. aureus*, the Gram-negative bacteria *E. coli* and *P. aeruginosa*, and the yeast *Saccharomyces cerevisiae*.[46.]

Human β-defensin-4 (hBD-4) was detected using bioinformatic and functional genomic analysis.[47.] In human tissues, the highest level of hBD-4 expression was found in the testis and in the gastric antrum.

The genes of human β-defensin 5 (hBD5) and human β-defensin 6 (hBD6) were discovered and cloned in 2002. hBD5 and hBD6 genes were found to be specifically expressed in the human epididymis, which suggests their important host defense role against microbial invasion in the epididymis.[48.]

The role of defensins in infection is not limited to causative agent (bacteria, virus, fungi) killing; in fact, the immunomodulatory repertoire of defensin is likely as important as their killing capabilities. It has been demonstrated that physiological concentration of β-defensin can increase the expression of pro-inflammatory cytokines and chemokines by purified human peripheral blood mononuclear cells. The anti-inflammatory functions of the defensins could be improved by the ability to attract neutrophils, monocytes, machrophage, and T-cells to inflammation sites.

Immune function of defensins may be induced by various physiological stimuli to mobilize pre-formed α-defensins or to upregulate β-defensin expression in various tissues. Released peptides interact with many target cells and tissues to promote secondary responses that may be critical for regulating acute inflammation, the recruitment of adaptive immune cells, angiogenesis and wound healing.[49.50.]

1.3. Defensins and *Helicobacter pylori* infections

Helicobacter pylori was first discovered in the stomachs of patients with gastritis and stomach ulcers nearly 25 years ago by Dr Barry J. Marshall and Dr J. Robin Warren of Perth, Western Australia. At the time (1982/83) the conventional thinking was that no bacterium can live in the human stomach as the stomach produced extensive amounts of acid which was similar in strength to the acid found in a car-battery.[51.] In recognition of their very important discovery, they were Awarded the 2005 Nobel Prize for Medicine & Physiology. *Helicobacter pylori* is a Gram-negative, microaerophilic, neutrophilic, spiral-shaped,

flagellated bacterium.[52.53.] *Helicobacter pylori* infection in the stomach can be found in more than half of the world's population. The bacterium induces gastric inflammation, and the diseases that can follow this infection include chronic gastritis, peptic ulcers and gastric cancer. There is marked diversity in the clinical outcome of *H. pylori* infection, which is able to persist for decades in infected individuals, the majority of them remaining asymptomatic. [54.] There is increasing evidence to show that both bacterial factors and subsequent host defence responses contribute to the inflammatory processes that determine the clinical manifestations of the disease.[55.] Attention has recently been focused on antimicrobial peptides as a component of the innate immune system against microorganisms. Defensins are endogenous antibiotic peptides that form a chemical barrier at the epithelial surface and their relative deficiency may lead to bacterial adherence to the mucosa and secondary mucosal inflammation.[4.] In the stomach, gastric epithelial cells constitutively express hBD-1, whereas hBD-2 is induced in response to pro-inflammatory cytokines or microbial infection. Recent reports have demonstrated that *H. pylori* infection induces hBD-2, whereas hBD-1 is thought to be expressed constitutively, and its role in *H. pylori* infection is controversial. *H. pylori* infection induces hBD-2 in gastric epithelium and hBD-2 inhibits the growth of *H. pylori*, in vitro.[56.] Bajaj-Elliott *et al.* reported that the constitutive expression of hBD-1 can be further modulated during infection and inflammation, which implies a greater role for hBD-1 in the innate host defence against *H. pylori* infection than previously thought.[30.]

1.4. Defensins and Crohn's disease

Crohn's disease, a chronic, inflammatory disease of the intestinal mucosa, is a complex multifactorial disease, the pathogenesis of which is still not fully understood. The commensal flora itself may cause inflammation in the absence of an adequate epithelial barrier function. Any part of the gastrointestinal tract can be affected, but most commonly, the terminal ileum, cecum, and colon. It is characterized by the presence of segments of normal bowel between affected regions. In the histology, a transmural, dense infiltration of lymphocytes and macrophages can be observed. The disease's course varies widely, with periods of remission and exacerbation including abdominal pain, diarrhea and fatigue. A major advance towards a better understanding of the disease was the discovery of mutations in the NOD2 gene in approximately one-third of Crohn's disease patients.[57.58.] NOD2 is a putative receptor of bacterial peptidoglycan, which is also expressed by Paneth cells and cryptic epithelial cells. [59.] The next step in the elucidation of the pathomechanism was the recognition that NOD may be involved in signalling cascade-mediated defensin expression,[60.] and therefore an

impaired defensin synthesis has been linked to the occurrence of Crohn's disease. Thus, decreased defensin levels lead to a weakened intestinal barrier function to intestinal microbes and might be crucial in the pathophysiology of Crohn's disease.[61.] Wehkamp reported a link in mutation in NOD2 to reduced α-defensin expression in Crohn's disease[62.]. Crohn's disease patients with ileal involvement have diminished expression of ileal Paneth cell defensins.[63.] This decrease is even more pronounced in Crohn's disease patients displaying a NOD2 mutation. It is therefore plausible that mutation in the NOD2 gene is associated with ileal involvement of Crohn's disease.[64.] Human neutrophil peptides 1-3 as well as lysozyme are expressed in surface enterocytes of mucosa with Crohn's disease but not in controls. HD-5 is stored in precursor form in normal Paneth cells. Notably, both α-defensins HD-5 and HD-6 are induced in the colonic mucosa of patients with Crohn's disease. In contrast, Crohn's disease of the colon is characterized by impaired induction of β-defensins in enterocytes. Human β-defensin 1 (hBD1) is constitutively expressed in the intestinal epithelium, but a decrease in hBD1 has been found in inflamed mucosa in Crohn's disease. Inducible β-defensin hBD2 is also expressed in the colon during inflammation and is diminished in Crohn's disease.[65.] In conclusion, the regional localizations of Crohn's disease, ileal or colonic disease can be linked to different defensin profiles.[66.]

1.5. SNP

DNA sequence polymorphisms are usually defined as variation present at greater than 1% frequency in the population. The most common are single nucleotide polymorphisms (SNPs) in which one of the four possible nucleotides in the DNA sequence is substituted by another, occurring on average every 800 nucleotides across the genome. SNPs can occur anywhere in the genom: both in the coding and noncoding regions or genes or in intergenic spacers.

SNPs within a coding sequence will not necessarily change the amino acid sequence of the protein that is produced, due to degeneracy of the genetic code. A SNP in which both forms lead to the same polypeptide sequence is termed synonymous or silent mutation. If a different polypeptide sequence is produced they are nonsynonymous. A nonsynonymous change may either be missense or nonsense, where a missense change results in a different amino acid, while a nonsense change results in a premature stop codon. SNPs that are not in protein-coding regions may still have consequences for gene splicing, transcription factor binding, or the sequence of non-coding RNA.[67.68.] Insertion-deletion polymorphisms (indels) as genetic markers in natural populations. Variations in the DNA sequences of humans can

affect how humans develop diseases and respond to pathogens, chemicals, drugs, vaccines, and other agents. SNPs are also thought to be key enablers in realizing the concept of personalized medicine.[69.] Current estimates are that SNPs occur as frequently as every 100-300 bases. This implies in an entire human genome there are approximately 10 to 30 million potential SNPs. More than 4 million SNPs have been identified and the information has been made publicly available through the efforts of TSC and others.[70.] The HapMap (short for "haplotype map") is a catalog of common genetic variants. The International HapMap Project is an international scientific effort to identify common genetic variations among people. The HapMap will make carrying out large-scale studies of SNPs and human disease (called genome-wide association studies) cheaper, faster, and less complicated.

SNPs are associated with diversity in the population, individuality, susceptibility to diseases and individual response to medicine. Recently, it has been suggested that SNPs can be used for homogeneity testing and pharmacogenetic studies and to identify and map complex, common diseases such as diabetes. Therefore, it is likely that sequence variation alone is not sufficient to predict the risk of disease susceptibility in humans.[71.] With the help of SNPs, we can also gain insight into the steps of evolution.

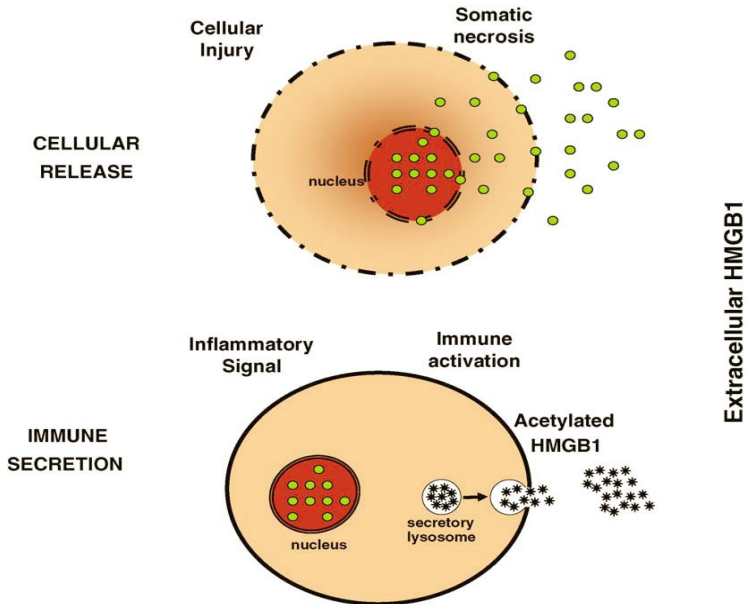
1.5.1. Defensin-β SNPs

Several polymorphisms of beta-defensin-1 have been reported.[72.73.74.75.] The frequency of polymorphisms in the coding region is low compared with polymorphisms in the promoter and untranslated regions. Of the reported polymorphisms, three polymorphisms in the 5' untranslated region at positions -20, -44, and -52 of the hBD1 gene have minor allele frequencies greater than twenty percent in a white population.[72.] The SNP at position -20 (G-20A) results in the formation of an nuclear factor-kappaB transcription factor-binding sequence; however, as hBD1 is constitutively expressed, the functional impact is unclear.[74.] It has been demonstrated that hBD1 function is compromised in cystic fibrosis.[76.] The SNP at position -44 (C-44G) is associated with *Candida* carriage status. Here both groups the SNP at site 668 (-44) in allele 2 is associated with a protective effect in type I diabetic and nondiabetic populations. This protective effect means that individuals carrying either one or two copies of the SNP allele 2 (G) are 25 times more likely (or 8.5 times more likely for the nondiabetic group) of not having elevated levels of *Candida* carriage compared to the likelihood for those individuals with only SNP allele 1 (C) at this position. The location of the SNP is in the 5' untranslated region of hBD-1, a region that is highly conserved in other primate species. The resultant change does not confer a change in the amino acid composition

of the peptide but may be associated with a variation in translation or transcription of hBD-1 or another linked gene.[77.] Variation in the hBD1 gene is associated with the pathogenesis of asthma,[78.] which is another atopic disease with a similar Th2 response,[79.] but different tissue is affected and a condition that shares some underlying characteristics with COPD. It is known that the frequencies of hBD1 SNPs differ between racial/ethnic groups.[77.]
The SNPs in the hBD1 gene have been associated with the pathogenesis of asthma[80.], and also with chronic obstructive pulmonary disease[81.] and infectious diseases.[82.]

1.6. HMGB-1

High mobility group box 1 protein (HMGB-1) is a member of the nonhistone chromatin-associated proteins. HMGB-1 is translated as a 214–amino acid protein, and extensively modified posttranslationally, by glycosylation, acylation, methylation, and phosphorylation.[83.84.] The primary structure is evolutionarily conserved, with 100% amino acid sequence homology between rat and mouse, and 99% homology between rodents and humans.[85.86.] Intracellular HMGB-1 has been studied previously for its roles in binding DNA; stabilizing nucleosome formation; as a general transcription factor for nucleolar and mitochondrial RNA polymerases; and as a gene- and tissue-specific transcriptional regulator that can enhance transcription and/or replication.[87.88.89.90.91.] Extracellular HMGB-1 was recently implicated as a “late” mediator of delayed endotoxin lethality.[92.] HMGB-1 mediates lethal toxicity in the absence of LPS signal transduction. HMGB-1 levels were increased significantly in critically ill patients with sepsis:[93.] the highest serum HMGB-1 levels were observed in patients that succumbed. HMGB-1 levels were also significantly increased in the serum of an uninfected patient with hemorrhagic shock, suggesting that extracellular HMGB-1 might play a mediator role in the setting of inadequate tissue perfusion. Toxic doses of HMGB-1 were associated with the development of fever, weight loss, piloerection, shivering, and microthrombi formation in the lungs and liver.[94.95.96.] Other proinflammatory mediators of endotoxemia that mediate similar pathological effects (e.g., TNF and IL-1) also function as potent stimulators of monocyte cytokine release in order to amplify and extend the “cytokine cascade”. Accordingly, it plausible that HMGB-1 might also function to stimulate monocytes to release TNF and other proinflammatory cytokines (2.Fig.).[97.98.]



2.Fig.''Passive release'' of HMGB1 isoforms from necrotic cells vs. ''active secretion'' of hyper-acetylated HMGB1 isoforms from activated immune cells.

The receptor for HMGB-1 is reported to be TLR and RAGE, a trans-membrane protein belonging to the immunoglobulin superfamily, which is expressed primarily by cells of the central nervous system, endothelial cells, smooth muscle cells, and mononuclear phagocytes. RAGE is the major functional receptor, responsible for the proinflammatory effects of HMGB-1.[98.99.100.] RAGE has secretory isoforms reffected to as soluble RAGE (sRAGE). Soluble RAGE has the same ligand-binding specificity, therefore competes with cell-bound RAGE.[101.]

1.7. Aims

The aim of our study was to investigate the role of defensin and HMGB1 as alarmins in multifactorial diseases, where the natural defense system and genetic factors may influence the consequences of infection and/or injury. Therefore we investigated

The effect of *H.pylori* on defensin-α (HNP1-3) production by human granulocytes

The role of hBD1 SNP in *H. pylori* induced gastritis

The role of hBD1 SNPs in the pathomechanism of Crohn's disease

The relevance of HMGB1 in the severity of acute pancreatitis

2.PATIENTS AND METHODS

2.1. Patients and controls

2.1.1. Patient with chronic acute gastritis and control group

150 *H. pylori*-positive patients with chronic active gastritis included the study. Biopsy specimens were taken during upper gastrointestinal endoscopy from adjacent sites of the gastric antrum and corpus for histology. In addition, the ^{13}C-Urea Breath Test (UBT) was carried out. Patients with *H. pylori* infection documented at histology and with positive results for the ^{13}C-UBT were considered eligible for the study. The presence of *H. pylori* and the severity of gastritis were graded with the Sydney Classification system. The 100 members of the control population for mutation analysis were age- and gender-matched serologically *H. pylori*-positive healthy blood donors without gastric or duodenal symptoms. All cases and controls were of Hungarian ethnic origin and resided in Hungary. Informed consent was obtained from all patients and controls, and the local Ethics Committee gave prior approval to the study.

2.1.2. Patient groups with Crohn's disease and control group

190 unrelated patients with Crohn's disease were investigated. The diagnosis was based on the Lennard-Jones criteria. Age, age at onset, the presence of extraintestinal manifestations (EIMs) (arthritis: peripheral and axial; ocular manifestations: conjunctivitis, uveitis, iridocyclitis; skin lesions:erythema nodosum, pyoderma gangrenosum; and hepatic manifestations: primary sclerosing cholangitis (PSC), frequency of flare-ups (frequent flare-up: therapeutic effectiveness , need for surgery , the presence of familial inflammatory bowel disease (IBD) and smoking habits and perianal involvement were investigated by reviewing the medical charts by the physician and by completing a questionnaire. The disease phenotype (age at onset, duration, location and behaviour) was determined according to the Vienna Classification. The control group for mutation analysis consisted of 95 age- and gender-matched healthy blood donors. Control subjects did not have any gastrointestinal and/or liver diseases and were selected from consecutive blood donors in Szeged.

2.1.3. Patient with acute pancreatitis and control group

Blood samples were obtained from 62 patients with acute pancreatitis. The criteria for the diagnosis of acute pancreatitis were a clinical history consistent with the disease, radiological evidence and serum amylase level greater than 660 U/L. All patients were classified as having mild or severe pancreatitis according to the original criteria of Ranson.

Patients with fewer than three positive prognostic signs (n=32) were considered to have mild pancreatitis; while those with three or more positive prognostic signs (n=30) were classified into the severe pancreatitis group. Patients were enrolled in this prospective study at the Departments of Surgery and Internal Medicine, Albert Szent-Györgyi Medical Center, Szeged University, between September 2006 and January 2008. For comparison, 20 patients with sepsis of different origin, in the surgical intensive care unit were enrolled in the study. The control cohort considered of a random, unrelated population of 20 healthy blood donors. All cases and controls were of Hungarian ethnic origin and resident in Hungary.

2.2. Genotyping procedures

2.2.1. DNA extraction

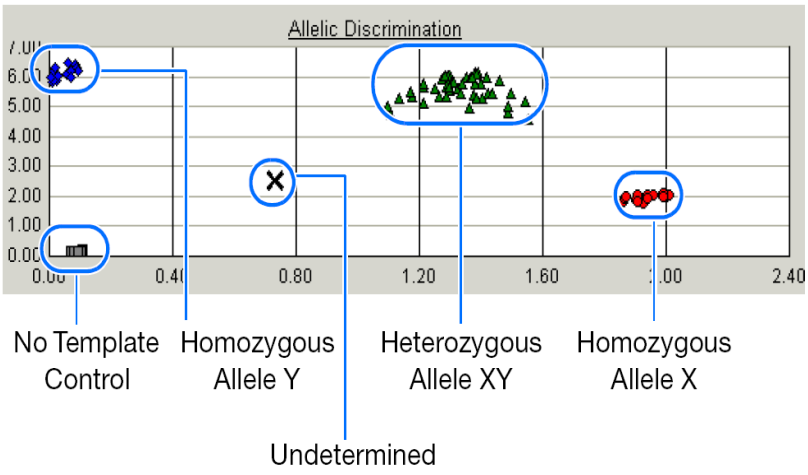
For the examination of human beta-defensin polymorphisms, leukocyte DNA purified from peripheral blood was isolated using the High Pure PCR Template Preparation Kit in accordance with the manufacturers' instructions (Roche Diagnostic GmbH, Mannheim, Germany). DNA concentration was measured by Qubit fluorometer (Invitrogen, Carlsbad, Calif., USA), again following the manufacturer's instructions. Genomic DNA was stored at -20 °C until further use.

2.2.2. Determination of hBD1 G-20A , C-44G and G-52A polymorphism

2.2.2.1. Custom TaqMan SNP Genotyping Assays

Genotyping was done using Custom TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, Calif., USA). Fluorogenic minor groove binder probes were used for each case using the dyes 6-carboxyfluorescein (FAM; excitation, 494 nm) and VIC (excitation, 538 nm): β-defensin 1 polymorphisms (hBD1) G-20A (rs11362) Applied Biosystems code c_11636793_ 20, hBD1 C-44G (rs1800972) c_11636794_10 and hBD1 G-52A (rs1799946) c_11636795_20. Thermal cycling was performed on GeneAmp 9700 PCR systems. The amplification mix contained the following ingredients: 7.5 µl TaqMan universal PCR master mix (Applied Biosystems), 0.375 µl primerprobe mix, 6.375 µl RNase- and DNase-free water (Sigma Chemical Co., St. Louis, Mo., USA), and 0.8 µl of sample DNA, in a total volume of 15 µl per single tube reaction. Assay conditions were 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each 96-well plate contained 90 samples of an unknown genotype and six reactions with reagents but no DNA. DNase free water was used as a non-template control. Initial and post-assay analyses were done using the Sequence Detection System (SDS) version 2.1 software (Applied Biosystems) as outlined in

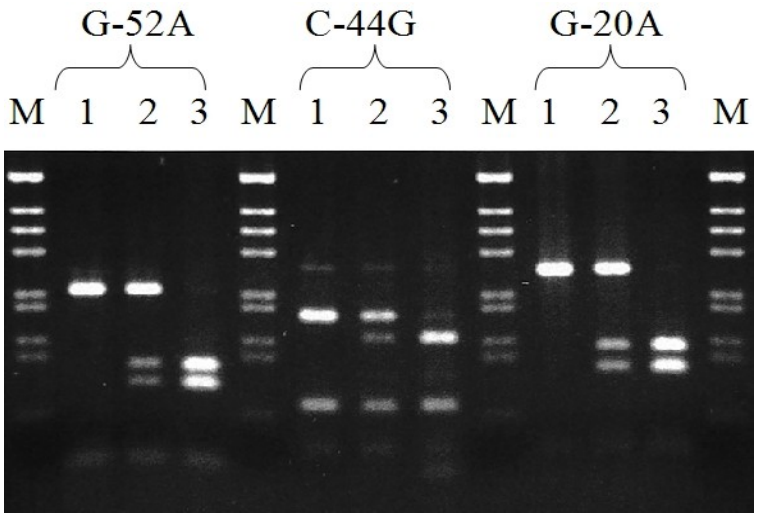
the Taq- Man Allelic Discrimination Guide (3.Fig).



3.Fig The Taq- Man Allelic Discrimination Guide

2.2.2.2. Restriction fragment length polymorphisms (RFLP)

Genotyping of the hBD1 polymorphisms at positions -20, -44, and -52 in the 5' untranslated region were performed by restriction fragment length polymorphism analysis. An amplicon of 260 bp was generated by 35 cycles of PCR using the sense primer 5'GTGGCACCTCCCTTCAGTTCCG and the antisense primer 5'CAGCCCTGGGGATGGGAAACTC. The PCR products were digested with 2 U Scr FI restriction endonuclease (New England BioLabs Inc.) to detect variation at position -20. The reaction mixtures were digested with 4 U Hga I restriction endonuclease (New England BioLabs Inc.) to detect variation at position -44. The reaction mixtures were digested with 2 U Nla IV restriction endonuclease (New England BioLabs Inc.) to detect variation at position -52. The digest mixtures were resolved on a 3% agarose gel stained with ethidium bromide (4.Fig.).



4.Fig. Detection of human β-defensin-1 gene variations by restriction enzyme digestion, followed by 3% agarose gel electrophoresis. M, Marker. Lanes 1, homozygotes for allele 1; lanes 2, heterozygotes for alleles 1

and 2; lanes 3, homozygotes for allele 2.

2.3. Bacterial strains and culture

The *H. pylori* 26695 CagA+ Vac A+ strain was used, which was generously donated by D.E. Berg (Department of Molecular Biology and Genetics, Washington University Medical School, St. Louis, USA). Bacteria were maintained on Brain Heart agar containing 10% sheep blood, and incubated in a microaerophilic atmosphere. Inocula for co-culturing were diluted from suspensions that had been prepared from 72-h subcultures and adjusted by comparison of the absorbance with standards.

The cag pathogenicity island (PAI)-negative Tx30a strain of ATCC 51932 was cultivated similarly. For co-culture experiments, both strains were used at a multiplicity of infection (MOI) of 100.

2.4. Expression experiments

2.4.1. Cells

The human gastric cell line AGS was maintained in RPMI 1640 culture medium supplemented with 10% heat inactivated foetal calf serum (FCS), (GIBCO), 2mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin. Cells (10^6) were seeded into 6-well tissue culture plates (COSTAR) and maintained at 37°C in 5% CO_2. Confluent cells were left uninfected, or were infected with *H. pylori* at a MOI of 100.

2.4.2. Inhibitor studies

AGS cells were pretreated with ERK activation inhibitor peptide (Calbiochem 328005) at 50 mg/ml for 30 min prior to and during bacterial stimulation.

2.4.3. RNA isolation and PCR amplification

Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, Calif., USA.) according to the manufacturer's instructions. RNA concentration was determined by the A260 value of the sample. Complementary DNA (cDNA) was generated from 1 μg total RNA using high-capacity cDNA reverse transcription kits (Applied Biosystems) in a final volume of 20 μl. Real-time reverse transcription PCR (RT-PCR) analyses were performed in a fluorescence temperature cycler (LightCycler; Roche Diagnostics GmbH) in accordance with the manufacturer's instructions. After reverse transcription, amplification was carried out using LightCycler FastStart DNA Master PLUS SYBR Green I mix (Roche Diagnostics). Samples were loaded into capillary tubes and placed in the fluorescence thermocycler (LightCycler).

Initial denaturation at 95°C for 10 min was followed by 45 cycles of 95°C for 15 s, the primer-specific annealing temperature for 5 s, and elongation at 72°C for 15 s. For hBD-1 (sense, 5'-TTG TCT GAG ATG GCC TCA GGT GGT AAC -3'; antisense, 5'- ATA CTT CAA AAG CAA TTT TCC TTTAT-3') the annealing temperature was set at 58°C. For the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase); sense, 5'-AAG GTC GGA GTC AAC GGA TTT-3'; antisense, 5'-TGG AAG ATG GTG ATG GGA TTT-3') primers were used. At the end of each run, melting-curve profiles were arrived at by cooling the sample to 40°C for 15 s and then heating the sample slowly at 0.20°C/s up to 95°C with continuous measurement of the fluorescence to confirm the amplification of specific transcripts. Cycle-to-cycle fluorescence emission readings were monitored and analysed using LightCycler software (Roche Diagnostics GmbH). Melting curves were generated after each run, to confirm amplification of specific transcripts. The specificity of the amplification products was verified by subjecting the amplification products to electrophoresis on a 2% agarose gel. The fragments obtained were visualized by ethidium bromide staining. All quantifications were normalized to the housekeeping GAPDH gene. Relative expression is given as a ratio between target gene and GAPDH gene expression.

2.5. Isolation of granulocytes and mononuclear cells

Peripheral white blood cells were prepared from EDTA-anticoagulated venous blood samples from healthy volunteers by density gradient purification. Peripheral blood mononuclear cells were separated by Ficoll Hypaque (Sigma Chemicals) density gradient centrifugation. Human polymorphonuclear granulocytes were isolated by the same density gradient centrifugation. Purified neutrophils were washed with phosphate buffered saline (PBS) and contaminating red blood cells were removed by brief hypotonic lysis followed by centrifugation. After washing, cells were resuspended in RPMI 1640 medium supplemented with 10 % FCS (fetal calf serum) to achieve final concentration of 1×10^6 cells/ml. Experimental cultures (1×10^6/ml) were incubated without or with 10^8/ml *Helicobacter pylori* 26695 for 1–4 h, following which the cells were centrifuged and processed for flow cytometry. Cell supernatants were tested for HNP1-3 content by ELISA.

2.6. Whole blood incubation method

Venous blood samples from healthy blood donors (whose granulocyte count varied from 3.8×10^6/ml to 4.7×10^6/ml) were incubated in the presence or absence of *H. pylori* for 4 h. Following the incubation period the cells were centrifuged and the supernatants were

tested for HNP1-3 content by ELISA.

2.7. Flow cytometric analysis

Granulocytes and mononuclear cells were fixed and permeabilized by Cytofix/Cytoperm solution (Becton-Dickinson) after washing in PBS. Cells were then labeled with monoclonal mouse anti human defensin (clone DEF3; Serotec Ltd. UK). As a secondary antibody FITC conjugated goat anti–mouse IgG (SIGMA) was applied. For controls, (background fluorescence) the cells were stained only with the secondary antibody. Flow cytometric analysis was performed thereafter with a FACStar plus fluorescence-activated cell sorter (Becton-Dickinson) at 488 nm excitation to estimate intacellular α-defensin in peripheral granulocytes and mononuclear cells.

2.8. HNP1-3 ELISA

The HNP 1–3 ELISA kit was obtained from HyCult Biotechnology, The Netherlands. Plasma samples of whole blood cultures and supernatants of granulocytes and mononuclear cells were analysed with standard measurements, according to the manufacturer's instructions. ELISA plates were measured on an Anthos 2010 Reader at 450 nm. Concentration of HNP1–3 in plasma and supernatants were calculated according to a standard curve.

2.9. LDH assay

To determine whether the tested granulocytes has been damaged during prolonged cultivation with or without *H. pylori,* which could lead to α-defensin release, 200 µl samples of the supernatants were assayed for lactate dehydrogenase activity using LDH kit Cyto Tax PROMEGA.

2.10. HMGB ELISA

Plasma HMGB1 concentration were determined with an established ELISA kit (Shino-Test-Corp., Japan) according to the instructions of the manufacturer.

2.11. sRAGE ELISA

The sRAGE antigen in the plasma was determined by ELISA (R&D Systems, Wiesbaden, Germany) according to the instructions of the manufacturer.

2.12. Measurement of Plasma DNA

Plasma DNA levels were estimated by the quantitative PCR method using primers for the β-globin gene, a housekeeping gene.

Blood samples were centrifuged at 3000 rpm, and plasma were carefully removed from EDTA-containing and plain tubes and were transferred into a 1,5 ml clear polypropylene tube taking care not disturb the buffy coat layer. The newly separated aliquot was centrifuged for a further 10 min at 13 000 rpm, after which the upper portion of plasma was removed, placed in a further clear tube and frozen at -20°C prior to extraction.

Plasma DNA was detected by quantitative PCR (Roch Lichtcycler; Roche GmbH), using primers (forward 5'-GTG CAC CTG ACT CCT GAG GAG A-3', reverse 5'-CCT TGA TAC CAA CCT GCC CAG-3') for the β-globin gene and Lightcycler-FastStart DNA Master SYBR gren 1 mix (Roche Diagnostics GmbH). The identity of the product was controlled by melting-point analysis. The generation of a plasma DNA standard curve was accomplished using human genomic DNA. Absolute quantification of plasma DNA was achieved using the Lightcycler software (version 3.5.2; Roche Diagnostics GmbH).

2.13. Statistical analyses

The α-defensin levels in the supernatants of *H. pylori* infected and control cell cultures were compared by using the Student's t-test, and statistical significance was defined as $p< 0.05$. Data are presented as means ± S.E.M.

Statistical analyses for comparison of genotype frequencies between groups were performed using the x^2 test, and the Fisher exact test if one cell had n=5. The probability level of $p<0.05$ indicated statistical significance. The relationship between genotypes and disease severity is presented as the odds ratio (OR), with a 95% confidence interval (95% CI). The genotype frequencies for each polymorphism were tested for deviation from the Hardy-Weinberg equilibrium by means of the x^2 test, with one degree of freedom used.

The levels of HMGB1, sRAGE and circulating DNA in the plasma were compared by means of one-way ANOVA. The Bonferroni test was used for post hoc pair-wise multiple comparisons. The relation between the plasma HMGB1 and plasma sRAGE, CT score and plasma DNA, and PCT and plasma HMGB1 were evaluated by regression analysis. The optimum cutoff levels of HMGB1, sRAGE and DNA were determined by analyses of receiver-operating characteristic (ROC) curves. Various cutoff values were selected, and the relationship between the sensitivity and specificity in ROC curves was plotted.

Statistical calculations were done with the Graph Pad Prism 5 (Graph Pad Software

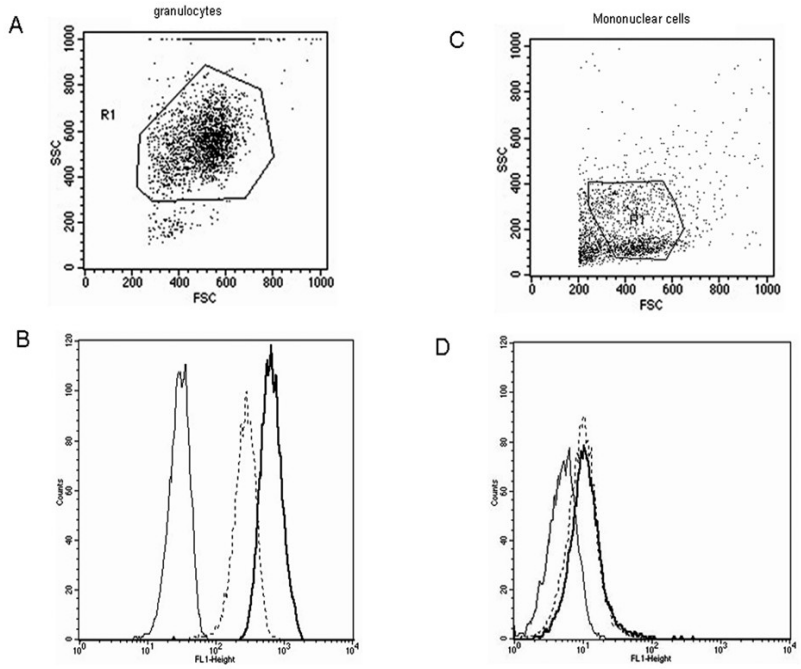
Inc., San Diego, Calif., USA) statistical program. Statistical power was calculated by application of the statistical power calculator program available through http:// statpages.org/Power, through the dssresearch.com/toolkit page. Linkage disequilibrium estimation was performed using Arlequin version 3.1. The multiple linear regression analysis was performed with SPSS.

3. RESULTS

3.1. *Helicobacter pylori* induces the release of α-defensin by human granulocytes

3.1.1. Effect of *H. pylori* on the intracellular expression and relase of α-defensin in granulocytes

A considerable intracellular α-defensin staining was observed in granulocytes mean fluorescence intensity (m. f. i.) 650 vs 29 of background fluorescense. Stimulation of granulocytes with *H. pylori* for 4 h resulted in a decrease in intracellular staining (m. f. i. 250). This decrease was due to the extracellular release of α-defensin; it was proved by measuring the α-defensin levels in the cell supernatants (5.Fig).



5.Fig. Flow cytometric analysis of the intracellular content of α-defensin in human granulocytes and mononuclear cells. A. gated granulocytes, B. α-defensin expression is detected by staining with MAb against HNP 1–3 following permeabilisation of cells, C. gated mononuclear cells, D. α-defensin expression is detected by staining with MAb against HNP 1–3 following permeabilisation of cells.

The HNP1–3 (α-defensin) concentrations in supernatants of granulocytes were determined by ELISA. Following a 4 h incubation with *H. pylori* (10^8), granulocytes produced 75713 ± 8376 pg/ml α-defensin which was significantly higher than the amount released during the 4-h incubation from the non-stimulated cells (5200 ± 2521 pg/ml). The α-defensin levels in the supernatants increased parallel with the decrease in the intracellular α-defensin content determined by flow cytometry. LDH activity analyses revealed that the release of α-defensin

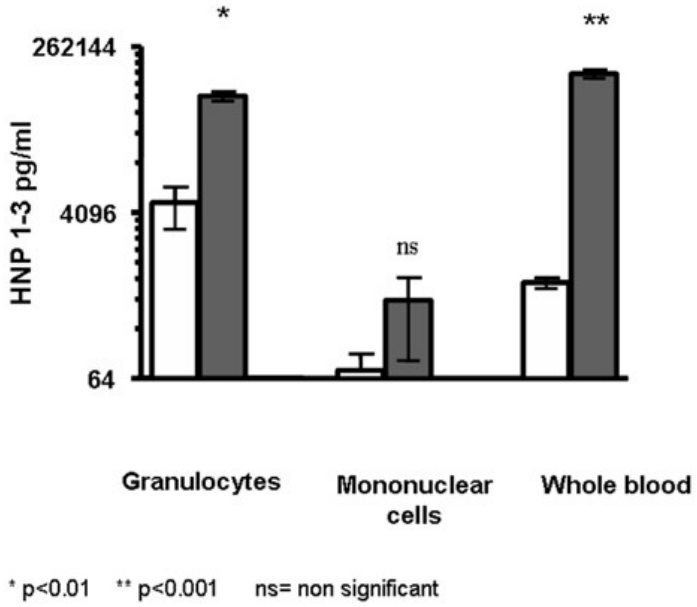
was not caused by cell damage in the 4 h culturing period.

3.1.2 Induction of α-defensin release from whole blood samples by *H. pylori*

The α-defensin concentration was significantly higher (p = 0.0007) in the supernatants of *H. pylori* induced blood, (131623 ± 13986 pg/ml) than in the controls (639.3 ± 90 pg/ml).

3.1.3. Intracellular expression of α–defensin in mononuclear cells; the effect of *H. pylori* infection on α-defensin release from mononuclear cells

To answer the question whether the α-defensin content of whole blood cultures mainly arose from granulocytes, mononuclear cells were subjected to intracellular α-defensin staining and the effect of *H. pylori* on the defensin release was assessed. Induction of mononuclear cells (1×10^6/ml) with *H. pylori* (10^8/ml) did not result in a significant change in the intracellular staining, i.e. α-defensin content of the cells (m. f. i.= 34.5). Similarly, the α-defensin concentration in the supernatants of the mononuclear cells increased only moderately relative to the basic α-defensin concentration of the control cells (455 ± 352 pg/ml vs. 78 ± 40.7 pg/ml, p = 0.350, non significant). These levels in the control and the induced samples were much less than that in the granulocytes at all (6.Fig).



6.Fig. Effect of *H. pylori* on α-defensin release.

3.2. Potential role of human β-defensin 1 in *Helicobacter pylori*-induced gastritis

3.2.1. hBD1 G-52A, C-20 G and C-44 G polymorphisms in patients with gastritis

The distribution of the hBD1 genotypes was in accordance with the Hardy- Weinberg

equilibrium in both the control population, and the patients. There was a significant difference in genotypic distribution between the patients overall and the healthy controls (p=0.001). To elucidate the reason for this difference, we analysed the difference in the numbers of AA homozygotes and GA heterozygotes among the patients and the healthy controls. The frequency of AA homozygotes was significantly higher in the group of patients (25%) than in the controls (11%) (p=0.005; OR2.745, 95% CI=1.3275.677). When the patients were stratified according to the density of *H. pylori* in the biopsy samples (i.e. mild colonization or severe colonization) a significant difference in the frequency of GA heterozygotes was observed between the controls and the patients with severe *H. pylori* colonization (p=0.005; OR2.250, 95% CI=1.2863.936). The frequency of AA heterozygotes among patients with severe *H. pylori* colonization was 26%, with an OR of 2.868. It is noteworthy, that the frequency of GA and AA genotypes in groups of patients with only mild *H. pylori* colonization was higher than that in the controls, but the difference was not statistically significant (1.Table).

	GG	GA	AA	
+	12/43 (27%)	21/43 (48%)	10/43 (23%)	
++	16/107 (15%)	63/107 (58%)[d]	28/107 (26%)[c]	
Gastritis	28/150 (18%)[b]	84/150 (56%)	38/150 (25%)[a]	$p<0.001$*
Control	49/100 (49%)	40/100 (40%)	11/100 (11%)	

*χ^2 test versus control; [a]Fisher test $p=0.005$, OR =2.745; [b]Fisher test $p=0.001$, OR=4.186; [c]Fisher test $p=0.007$ OR=2.868; [d]Fisher test $p=0.005$ OR=2.250.
+ =mild colonization; ++ =severe colonization.

1.Table hBD1 G-52A genotypes in patients with *H. pylori*-induced chronic active gastritis

The genotypic distribution of the hBD1 C-44G and hBD1 C-20 G polymorphisms was no significant difference in genotype distribution between the patients overall and the healthy controls in any of these two SNPs (2.Table).

	GG	GA	AA	
DEFB1 G-20A				
Gastritis	49/150 (33%)	74/150 (49%)	27/150(18%)	$p=0.913$* NS
Control	31/100 (31%)	49/100 (49%)	20/100(20%)	
DEFB1 C-44G				
Gastritis	92/150 (61%)	45/150 (30%)	13/150(9%)	$p=0.682$* NS
Control	56/100 (56%)	35/100 (35%)	9/100(9%)	

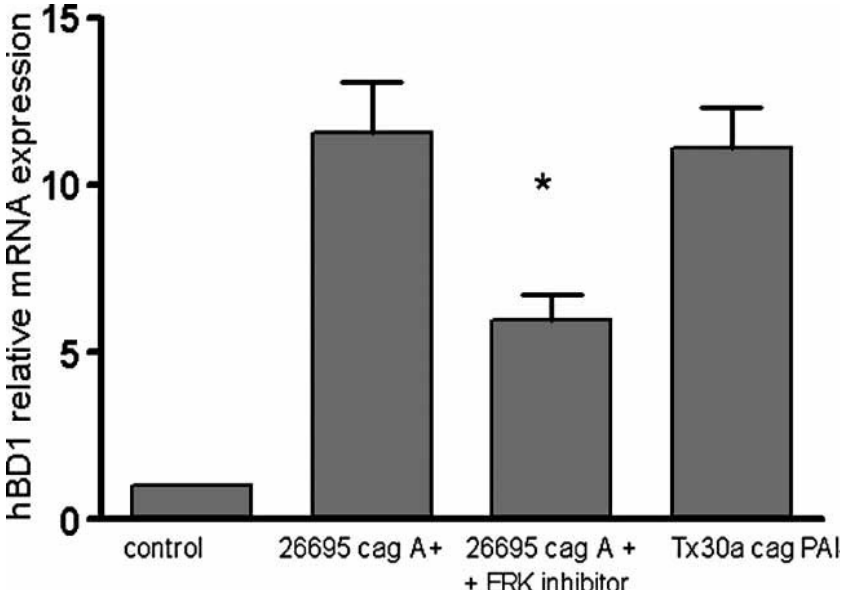
Abbreviation: NS =non-significant.
*χ^2 test versus controls.

2.Table hBD1 C-20 G and hBD1 C-44 G genotypes in patients with *H. pylori*-induced chronic active gastritis.

3.2.2. Human β–defensin 1 mRNA expression by *H. pylori*

A time-dependent increase in hBD-1 mRNA expression was observed in AGS cells over a 48-h incubation period. A maximum increase in hBD-1 mRNA expression was observed at 24 h. An MOI of 100 was chosen, because in pilot experiments a dose-dependent

increase in hBD1 mRNA was observed in AGS cells following infection with increasing numbers of *H. pylori.* To elucidate the role of the bacterial secretory system in hBD-1 expression, *H. pylori* strain Tx30a which lacks the cag PAI was employed. Infection by the cag PAI-negative strain showed expression of hBD-1 to a similar extent as observed for the cag A-positive *H. pylori* 26695 strain. As the ERK (extracellular signal-regulated kinase) pathway which also participates in β-defensin expression can be activated by cag PAI-negative strains [102.], we supplemented the experiments by applying the ERK activating inhibitor. There was a significant (p=0.029) reduction in the expression of hBD-1 following pretreatment of AGS cells with the ERK activating inhibitor (7.Fig).



7.Fig Effects of *H. pylori* strains on human β defensin-1 (hBD-1) mRNA expression in AGS cells.

3.3. Human β-defensin polymorphisms in patient with Crohn's disease

3.3.1. hBD1 G-20A, C-44GG and G-52A polymorphism in patients with Crohn's disease

The distribution of the hBD1 G-20A genotypes was in accordance with the Hardy-Weinberg equilibrium in the control population and in the patients with Crohn's disease. There was no significant difference in genotypic distribution between the Crohn's disease patients overall and the healthy controls. When the patients were stratified according to localization of the disease i.e. ileal, ileocolonic, or colonic a significant difference was observed between the controls and the patients with colonic localization (x^2 test, p=0.043) but not between the controls and the patients with ileal or even ileocolonic localization of the disease. The frequency of GA heterozygotes was significantly higher in this group of patients (60%) than in the controls (39%) (Fisher test, p=0.02; OR=2.393, 95% CI=1.176-4.869, statistical power 80.7%). No further significant differences were observed regarding the G-

20A SNP when the patients were stratified according to extraintestinal localization or behaviour of the disease.

The distribution of hBD1 C-44 G genotypes was in accordance with the Hardy-Weinberg equilibrium among the patients with Crohn's disease but not in the control population. Interestingly, there was a lower frequency of the GG genotype among the patients with Crohn's disease as compared with the controls. The difference concerning this genotype between patients with ileal localization was not significant, whereas it was marginally significant among the patients with colonic localization ($p=0.06$), and statistically significant among patients with ileocolonic localization. No further significant differences were observed for the hBD1 C44G SNP when the patients were stratified according to extraintestinal localization or behaviour of the disease (3.Table).

The distribution of the hBD1 G-52A genotypes was in accordance with the Hardy-Weinberg equilibrium in the control population but not in the patient group. There were no significant differences in the hBD1 G-52A genotypic distributions between the patients with Crohn's disease and healthy controls.

3.3.2. Smoking habit and SNPs

To investigate the connection between smoking habits and the hBD1 SNPs in Crohn's disease, the patients were stratified as smokers or nonsmokers. Eighty-two smokers (including the 21 who had already stopped) were included in the study. It is interesting that the correlation between the genotype frequencies of the SNPs of G-20A and those of C-44G was striking only among nonsmokers. However, a significant difference between the smokers and the controls was not observed regarding the genotypes (3.Table).

3.Table Allele and genotype frequencies of the polymorphisms of DEFB1 genes.

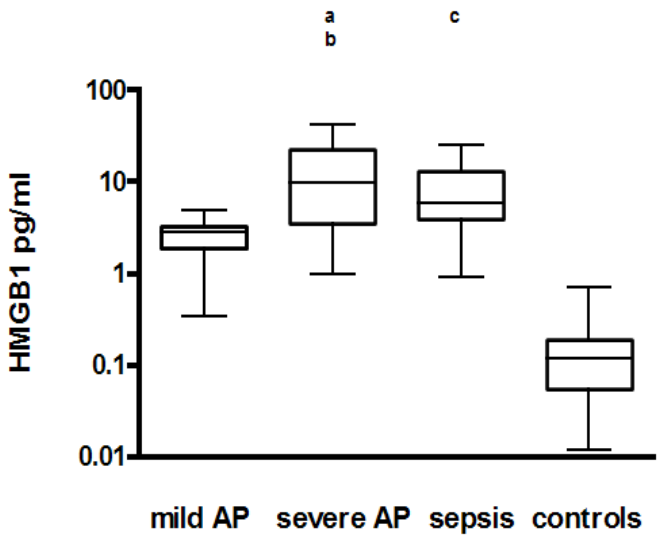
			Localization of CD						Smoking habits					
	Patients with CD ($n=190$)		Ileal		Ileocolonic		Colonic		Smokers		Non-smokers		Controls ($n=95$)	
Gene polymorphisms	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
DEFB1 (G-20A)														
Allele														
G	217	58	62	66	100	55	53	55	95	58	125	58	125	66
A	157	42	32	34	82	45	43	45	69	42	91	42	65	34
Genotype														
GG	61	33	21	45	27	30	12	25b	28	34	32	30	44	46
GA	95	51	20	42	46	50	29	60a	39	48	61	56f	37	39
AA	31	16	6	13	18	20	7	15	15	18	15	14	14	15
DEFB1 (C-44G)														
Allele														
C	300	80	69	73	151	83	80	82	127	77	181	84	140	74
G	74	20	25	27	31	17	18	18	37	23	35	16	50	26
Genotype														
CC	120	64	25	53	63	69	32	65	51	62	74	69	56	59
CG	60	32	19	40	25	28	16	33	25	31	33	30	28	29
GG	7c	4	3	7	3	3e	1	2d	6	7	1	1g	11	12
DEFB1 (G-52A)														
Allele														
G	226	60	56	60	111	61	59	60	109	66	129	58	118	62
A	148	40	38	40	71	39	39	40	55	34	95	42	72	38
Genotype														
GG	69	37	18	38	34	37	17	35	39	48	35	31	37	39
GA	88	47	20	43	43	47	25	51	31	38	59	53	44	46
AA	30	16	9	19	14	16	7	14	12	14	18	16	14	15

Abbreviations: CD = Crohn's disease; DEFB1 = β-defensin 1 gene.
[a]Fisher test, $p=0.02$ versus control, OR = 2.393; [b]Fisher test, $p=0.01$ versus control, OR = 2.588; [c]Fisher test, $p=0.01$ versus control, OR = 3.367; [d]Fisher test, $p=0.06$ versus control; [e]Fisher test, $p=0.04$ versus control, OR = 3.841; [f]Fisher test, $p=0.01$ versus control, OR = 2.035; [g]Fisher test, $p=0.001$ versus control, OR = 14.

3.4. Plasma Concentrations of High Mobility Group Box Protein 1 (HMGB1), sRAGE and Circulating DNA in Patients with Acute Pancreatitis

3.4.1. Plasma HMGB1 levels

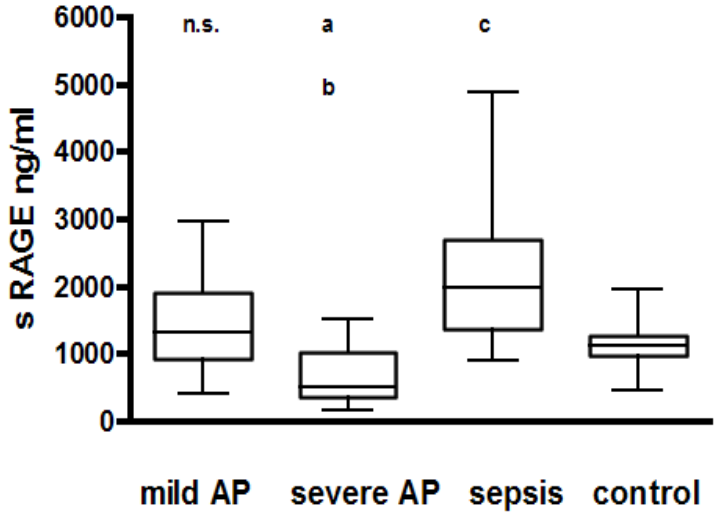
The plasma HMGB1 concentration was significantly higher in the patients with severe acute pancreatitis than in the healthy controls (p<0,001) or in patients with mild pancreatitis (p=<0,001). The HMGB1 plasma levels were comparable, and even higher in patients with severe acute pancreatitis than in the group of septic patients (p<0,01 vs controls). The highest HMGB1 concentration were detected in the plasma of patients infected necrosis (17,5-42 ng/ml, n=8) (8.Fig).



8.Fig Plasma HMGB1 concentrations in patients with mild and severe acut pancreatitis (AP), sepsis and healthy controls. (a. p<0,001 vs controls; b. p<0,001 vs patients with mild pancreatitis; c. p<0,01 vs controls)

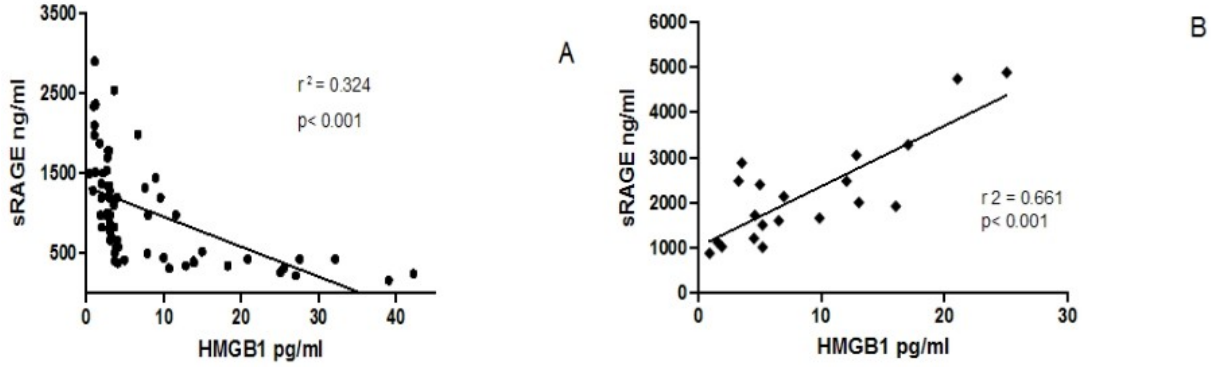
3.4.2. Plasma sRAGE levels

The circulating sRAGE levels were compared in the patients with mild and severe acute pancreatits, in the patients with sepsis, and in the healthy controls**.** The highest sRAGE level was detected in patients with sepsis. However, the sRAGE concentrations in patients with severe acute pancreatitis was significantly lower than in the septic patients (p<0,001), or even lower than the control level also in patients with severe acute pancreatitis (p<0,05) (9.Fig).



9.Fig Plasma concentrations of sRAGE in patients with mild and severe acut pancreatitis (AP), sepsis and healthy controls. (a. p<0,05 vs controls; b. p<0,001 vs patients with mild pancreatitis; c. p<0,001 vs patients with severe pancratitis; n.s.= nonsignificant vs controls)

The relation between the plasma sRAGE and plasma HMGB1 levels was separately investigated in the pancreatitis group, and the sepsis group. The plasma sRAGE level proved to be inversely associated with plasma HMGB1 level in patients with acute pancreatitis. In contrast, for the septic patients a positive correlation was observed between the plasma sRAGE and HMGB1 concentrations (10.Fig).

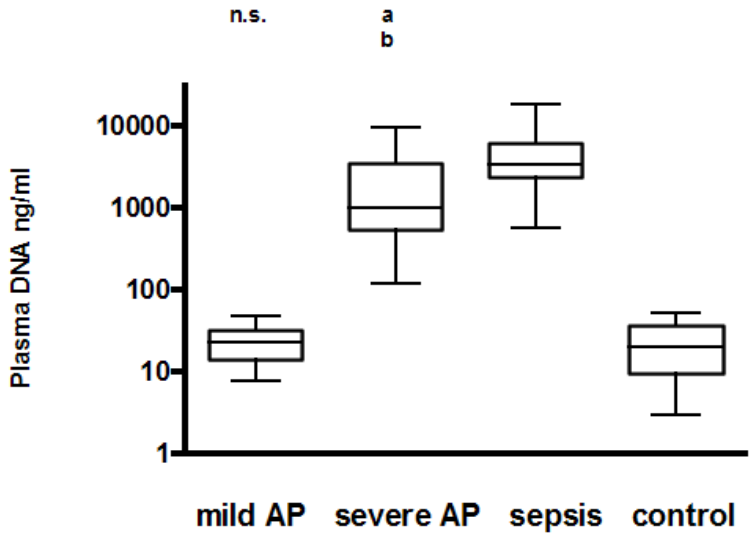


10.Fig Correlation between plasma concentrations of HMGB1 and sRAGE in patients with acute pancratitis (A) and in patients with sepsis (B)

3.4.3. Plasma DNA Concentrations

The highest plasma DNA concentration was measured in the patients with sepsis. The plasma DNA concentration was significantly higher than the controls level also in the case of the patients with severe acute pancreatitis. No significant difference was observed in plasma

DNA concentrations between the patients with mild pancreatitis and healthy controls. (11.Fig).



11.Fig Plasma DNA concentrations in patients with acute pancreatitis and sepsis, and in heathy controls. (a. $p<0,05$ vs. controls; b. $p<0,01$ vs. patients with mild pancreatitis; n.s.= nonsignificant vs. controls)

4. DISCUSSION

4.1. *Helicobacter pylori* induces the release of α-defensin by human granulocytes

From earlier studies we know that human beta defensin (hBD) inducing ability of *H. pylori,* and indicated an enhancement of hBD2 expression upon infection in patients with chronic gastritis[103.104.105.]. The hBD1 has been postulated to play an important role in the pathogenesis of *H. pylori* induced gastritis [30.]. However little information is available on the potential role of α-defensin derived from neutrophils during *H. pylori* infection, an intense α-defensin staining of granulocytes in biopsy samples of *H. pylori* positive patients with gastritis led to the assumption, that it was a secondary consequence of the inflammation involving neutrophil infiltration. Our experiments demonstrated the α-defensin inducing capacity of *H. pylori* not only in the case of purified granulocytes, but also when a whole blood incubation method was applied. In the peripheral blood the neutrophil granulocytes are the main source of α-defensin, and we therefore suggest that the elevation of the level of α–defensin following *H. pylori* infection is mainly due to the activity of the granulocytes. Monocytes, immature dendritic cells, lymphocytes also have been reported to contain α-defensin [13.106.].

Our flow cytometric analysis revealed intracellular α-defensin staining in mononuclear cells but with much lower intensity was much less than for the granulocytes, and only 23% of the mononuclear cells were stained positively intracellularly. A low basal level of α-defensin was measured in the supernatants of these cells, but here was no significant increase in the level following the incubation of mononuclear cells with *H. pylori.* Accordingly, we conclude that the α-defensin in the supernatants of whole blood cultures can be regarded as products of the granulocytes. This is all the more likely, if it is borne in mind, that the neutrophils account for the highest number of leukocytes in the peripheral blood. Whole blood culturing method is a reliable method with which to investigate the effects of activating agents on granulocytes [107.]. To reduce the artificial effects we used a whole blood incubation method. The advantages of this method include: prevention of neutrophil activation or even damage during separation, reduction in size of blood sample, and retention of a full components of blood mimicking natural conditions. Activation or priming of neutrophils during preparation procedures has been reported to affect neutrophil functions. This might be the explanation, why the baseline α-defensin release was higher in the supernatants of the control, non-stimulated granulocytes than the baseline values for the whole blood cultures. The exact mechanism of the induction of α–defensin release from granulocytes by *H. pylori* has not yet

been elucidated. The α-defensin release from the granulocytes was not increased when LPS or paraformaldehyde-fixed *H. pylori* cells were used as potential inducers (data not shown).

4.2. Potential role of human β-defensin 1 in *Helicobacter pylori*-induced gastritis

4.2.1. hBD1 polymorphisms in patients with gastritis

H. pylori-induced gastric inflammation is dependent on the persistence of the microorganism in the gastric epithelium. The modulation of host epithelial antimicrobial responses may be a critical determinant of bacterial adherence and subsequent pathology in *H. pylori*-induced gastritis. hBDs, epithelial-derived antimicrobial peptides are important components of the host defence at mucosal surfaces. Genetic variation in the hBD-1 gene is associated with cystic fibrosis, atopic dermatitis, asthma, and *Candida* infection in diabetic patients [75.76.77.] has been reported. We investigated the relavation of three hBD1 SNPs in patinets with *H. pylori* induced gastritis. In our study the analysis of hBD1 G-52 A genotypes revealed that the AA and GA genotype comprises a higher risk of chronic active gastritis among *H. pylori*-infected subjects, with a higher rate of colonization with the bacteria. Although the functional impact is unclear, it is tempting to speculate that this mutation at the -52 untranslated region might cause a lower hBD-1 expression in gastric epithelial cells with deficient function of human defensin. This could lead to increased colonization of *H. pylori* in the stomach, with ineffective clearance, and consequent inflammation. Additional functional studies will be necessary to establish the exact biological role of this SNP. No further significant differences were observed for the SNPs of hBD1 C-44 G and hBD1 C-20 G.

4.2.2. Human β–defensin 1 mRNA expression by *H. pylori*

The role of hBD-1 in *H. pylori* infections is still controversial. An increase in hBD-1 expression was reported during *H. pylori* infection both *in vitro* and *in vivo* [30.], but was not confirmed by others . In our co-culture experiments, the multiplicity of infection was similar to that applied by Bajaj-Elliott *et al.* [30.], and also resulted in an increase in hBD-1 expression. Increased hBD-1 expression was likewise observed following infection of AGS cells with the cag PAI strain. The bacterial effector injected by the cag PAI IV secretion system is peptidoglycan. This is recognized by the intracellular NOD1 (nucleotide binding oligomerization domain) receptor molecule, which directly activates NFκB. NOD1 activation results in activation of defensin-β [60.]. In an elegant experiment Viala *et al.* [108.] demonstrated that the type IV secretion system is necessary for activation of the NOD1 system. In our experiments, however, not only the CagA-positive, but also the cag PAI-

negative strain resulted in hBD-1 expression. Therefore it is tempting to speculate that, in contrast with hBD-2, the hBD-1 activation is possible in a NOD1-independent way. Boughan *et al.* [109.] found that hBD-2 expression was exclusively dependent on the presence of the bacterial cag PAI, with NOD1 as a critical host sensor. In contrast to hBD-2, hBD-3 expression was NOD1-independent, but EGFR and ERK pathway dependent. *H. pylori* infection activates ERK and MEK kinases in AGS gastric epithelial cells, in both a cag PAI-independent and cag PAI-dependent manner [102.]. In this way, ERK and MAP kinases are regulated during *H. pylori* infection through the use of secreted factors, and also in an epithelial contact dependent manner in which cag PAI-positive strains exert the ability to induce higher levels of ERK and MEK phosphorylation. We suggest a potential role for this pathway in hBD-1 expression. In contrast to hBD-2, it seems that hBD-1 expression could also be NOD1 independent, at least independent on cag PAI, but ERK pathway dependent. This might be the reason for a 50% reduction in the expression of hBD-1 following the pretreatment of AGS cells with the ERK activation inhibitor prior to infection with *H. pylori* 26695. As the ERK pathway can be activated by the cag PAI-independent strain, we suggest a potential role of this pathway in hBD1 expression in the presence of the Tx30a strain. Other signalling pathways that might be important in hBD-1 expression cannot be excluded.

4.3. Human β-defensin polymorphisms in patient with Crohn's disease

Ileal and colonic Crohn's disease can be linked with a lowered defensin profile [62.]. The colonic Crohn's disease is associated with the lack of β-defensin [64.65.]. A chromosome 8, gene-cluster polymorphism with a low human β-defensin 2 gene copy number has been stated [110.] as predisposing to colonic Crohn's disease. It was stated that a lower hBD2 gene copy number in a β-defensin locus predisposes to colonic disease, most likely through diminished β-defensin expression. The constitutive expression of hBD1 by various mucosal surface epithelia suggests a surveillance-like role of this peptide in maintaining tissue homeostasis in the absence of infection [33.]. It has recently been reported [30.] that the constitutive expression of hBD1 can be modulated during infection and inflammation. In our study, we analysed the genetic variation in the hBD1 gene in Crohn's disease. The analysis of hBD1 G-20A genotypes among patients with Crohn's disease revealed that the GA genotype comprises a higher risk of colonic Crohn's disease (OR2.393, 95% CI: 1.176 4.869). There was no correlation between this SNP and the ileal or ileocolonic form of the disease. The functional significance of this SNP resides in the formation of a nuclear factor-κB transcription factor-binding sequence, but the functional impact is unclear. As the colonic

localization of the disease is connected with impaired hBD1 expression [65.], it is tempting to speculate that this mutation might cause a lower hBD1 expression in colonic epithelial cells. Additional functional studies will be necessary to establish the exact biological role of this SNP. The present study has demonstrated that the distributions of C-44G (according to the initiation codon-based nomenclature), also known as the hBD1 668C/G genotype, were different in patients with Crohn's disease and healthy controls, while the frequency of the GG genotype was significantly higher in the controls. This result indicates that the C-G mutation probably leads to strengthened hBD1 antimicrobial activity, which is less frequent among patients with Crohn's disease. The SNP at C-44G generated a putative binding site for nuclear factor-κB, and induced overexpression [111.]. The proposed effect of this SNP could partly explain why the GG genotype was considered to be a protective genotype in atopic dermatitis [111.] and also in the susceptibility to *Candida* colonization [77.]. This is consistent with our observation, that the GG phenotype could also be protective in Crohn's disease. It is also possible that the SNP is in linkage disequilibrium with another gene, perhaps another β-defensin that has an effect on the barrier function of the colonic mucosa.

No further significant differences were observed for the SNPs when the patients were stratified according to behaviour of the disease or the extraintestinal manifestations. The only difference in genotypes was observed when patients were stratified as smokers and non-smokers. It is interesting that the correlation between genotype frequencies of SNPs of G-20A and C-44G was striking only among non-smokers. However, a significant difference between the smokers and the controls was not observed for the genotypes. We speculate that defensin SNP might basically influence the susceptibility to Crohn's disease, but regarding smoking habits, other factors might superimpose to worsen the genetically determined situation. This means that smoking increases the susceptibility to and severity of Crohn's disease; moreover, smoking habit and disease location are contributory factors in this process [112.]. Smoking, through increased carbon monoxide concentration, may amplify the impairment in vasodilatation capacity in the chronically inflamed microvessel, resulting in ischaemia, and perpetuating ulceration and fibrosis. A defect in bacterial clearance or macrophage deficiency may also have a detrimental role in the disease [113.].

For C-44G SNP, the distribution of genotypes was not in accordance with the Hardy-Weinberg equilibrium in the control group. Similar deviation from the Hardy-Weinberg equilibrium was observed by Prado-Montes *et al.* [111.]. There are several reasons for deviations; if the SNP sites are under selection forces independent of disease, it could be explained by the important role of hBD-1 in innate protection. Deviation from Hardy-

Weinberg equilibrium has been observed in a population without disease as a result of duplications [114.]; nevertheless, hBD1 has been defined as a unique copy number [115.]. It was suggested that the G allele might be a ‘‘protective’’ allele, and it functions as a selective factor in the control population. This also might explain the deviation from the Hardy-Weinberg equilibrium. Jurevic *et al.* [74.] reported a significant difference in the frequency of hBD1 polymorphism between different racial groups. Our study group comprised a white Caucasian population from Central-Eastern Europe.

4.4. Plasma Concentrations of High Mobility Group Box Protein 1 (HMGB1), sRAGE and circulating DNA in patients with Acute Pancreatitis

4.4.1. Plasma Concentrations of HMGB1

In acute pancreatitis, HMGB-1 produced by activated monocytes/macrophages can amplify the inflammation and may contribute to the tissue injury and organ failure. In the severe, necrotizing form of pancreatitis, HMGB-1 may also be produced by the injured pancreas and other damaged organs. As the level of circulating DNA in the plasma correlates with cell death, it appeared reasonable to supplement the study with measurment of the plasma DNA level. Yasuda *et al.*[116.] reported that the mean serum HMGB-1 level was significantly higher in patients with severe acute pancreatitis than in healthy volunteers, and correlated significantly positively with the Japanese severity score and Glasgow score. Corroborating the importance of HMGB-1 in severe acute pancreatitis, Sawa *et al.* [118.] reported that anti-HMGB-1 neutralizing antibodies significantly reduced the elevation of serum amylase, serum alanine aminotransferase and creatinine levels and improved the histological alterations in the pancreas and lung in experimental severe acute pancreatitis in mice. We found a significant elevation of the HMGB-1 concentration in the plasma of patients with severe acute pancreatitis. We compared the results with the data on septic patients, as the elevation of HMGB1 level in sepsis is well documented[95.96.]. The HMGB1 level in the patients with severe acute pancreatitis was comparable with the levels in septic patients, and even more higher (however no statistically significantly). In acute pancreatitis HMGB1 secretion can be induced by inflammatory cytokines, which play a definitive role in the pathomechanism of acute pancreatitis. In severe, necrotizing pancreatitis the passive release of HMGB1 from necrotic cells may be an additional source of HMGB1. This may be the reason for a significant difference in HMGB1 levels between patients with mild and severe form of disease. Infected pancreatic necrosis was diagnosed in 8 of the patients with severe acute pancreatitis. It is noteworthy that the highest levels were measured in the plasma

of these patients (17.5 – 42 pg/ml) very probably in consequence of the additive effects of inflammation, necrosis and infection.

4.4.2. Plasma Concentrations of sRAGE

Extracellular HMGB1 regulates cells through RAGE and Toll like receptors [101.116.].Bopp *et al.* [117.] reported an elevation in the level of sRAGE in septic patients. We likewise observed this in our septic patients, but there was an inverse correlation between the levels of sRAGE and HMGB1 in the patients with severe acute pancreatitis (Fig.3 A). Relatively low sRAGE concentrations were found in the patients with severe acute pancreatitis, where the highest HMGB1 levels were observed (Fig.2). Yamagishi *et al.* [124.] suggest that circulating sRAGE levels may reflect tissue RAGE expression and may be elevated in parallel with the levels of the ligands. Bopp *et al.* therefore supposed, that the level of sRAGE as the splice variant of RAGE or split off variants of cell surface RAGE, was elevated in sepsis. On the other hand, it must be considered, that sRAGE could be inversely correlated with HMGB1, because sRAGE can capture and eliminate circulating HMGB1 in a feedback loop of the process, in this way acting in competition with membrane-bound RAGE, binding, and inhibiting HMGB1. This was the situation when another ligand of RAGE (S100A12) was investigated [119.]. We found, that the level of released sRAGE relates inversely to the HMGB1 level, but only in the case of acute pancreatitis.

Thus it may be speculated, that the more HMGB1 there is in the circulation, the lower will be the level of sRAGE in severe acute pancreatitis. The question arises whether the low level of sRAGE is simply due to its consumption to bind HMGB1, or whether there are any other components which could decrease the level of sRAGE. The elevation of other (as yet undefined) components in the circulation, that can suppress the amount of soluble RAGE receptor can not be excluded. Whatever the reason, the relatively high HMGB1 levels relative to the low sRAGE levels make the feedback mechanism less effective, and further enhance the inflammatory loop in acute pancreatitis.

4.4.3. Plasma Concentrations of circulating DNA

In various pathologic conditions, an increase in circulating DNA have been demonstrated. Only low amount of serum or plasma DNA level has been observed in healthy individuals, whereas high concentrations have been described in patients with sepsis, trauma, stroke, or autoimmune diseases [120.]. Since most of these disorders are associated with increased rates of cell death events, from either apoptosis or necrosis, these mechanisms

should be considered to be the main sources for circulating DNA. As tissue damage and necrosis are a key feautures in acute pancreatitis, we considered it logical to measure the levels of circulating DNA in patients with mild and severe pancreatitis, and with sepsis. The accumulation of DNA in the circulation can result from an excessive release of DNA caused by massive cell death, inefficient removal of the dead cells or a combination of these two [120.]. Our data revealed, that the circulating DNA significantly elevated in patients with severe pancreatitis, or in sepsis. A significant elevation was not observed in those with mild pancreatitis. Our data are in contrast with those of Bagul *et al.* [121.], who found a decreased rather than an increased level of plasma DNA in severe acute pancreatitis. There may well be some methodological differences between the two sudies. First, our study involved the use of a real time quantitative PCR method for the β-globin gene, which is a widely accepted method [120.122.123.]. Bagul *et al.* used the RNase P transcription assay. Secondly, it is known, that contamination of a sample with nucleated cells can affect the concentrations of plasma DNA. The sample preparation is therefore crucial [120.123.]. We applied the two-step procedure of sample centrifugation, while Bagul *et al.* separated the plasma only with one centrifugation step at 2000 rpm. This could result in a high variation in DNA concentration, originating not only from cell the free DNA, but also from contaminating leukocytes. From the aspect that both inflammation and necrosis are involved in the pathogenesis in acute pancreatitis, a complex study of the levels of HMGB1, sRAGE, and plasma DNA might be informative in an evaluation of the effects of different levels of severity of acute pancreatitis.

4.5. SUMMARY: CONCLUSIONS AND POTENTIAL SIGNIFICANCES

The major new findings of our experiments are as follows:

1. *H. pylori* induce α-defensin release from granulocytes which may well be important in local host response to *H. pylori* infection in gastroduodenal diseases.
2. The association of the higher frequency of the G-52 A SNP of hBD1 with chronic active gastritis, and the elevated level of hBD-1 mRNA following *H. pylori* infection draw attention to the importance of hBD-1 in *H. pylori* infection. This indicates that not only the inducible, but also the constitutive form of hBD-1 plays a role in the development of chronic active gastritis following *H. pylori* infection.
3. The colonic Crohn's disease could be due to defective β-defensin 1 production. This means that not only the inducible, but also the constitutive form of hBD1 plays a definitive role in the colonic localization of Crohn's disease.
4. The circulating HMGB1 level was significantly higher in the patients with severe

acute pancreatitis than in the healthy controls or the patients with mild pancreatitis, and the level of HMGB1 correlated inversely with that of sRAGE. The plasma DNA level was increased in severe acute pancreatitis. These observations underline the role of HMGB1 and its receptor in the pathogenesis of acute pancreatitis, and in the severity of the disease. Blockade of HMGB1 should be considered as possible future therapy in sepsis and severe acute pancreatits.

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