# Human β-defensin-2 induction in *Helicobacter pylori*-infected gastric mucosal tissues: antimicrobial effect of overexpression

N. Uehara,[1] A. Yagihashi,[2] K. Kondoh,[1] N. Tsuji,[2] T. Fujita,[3] H. Hamada[4] and N. Watanabe[2]

Correspondence
N. Watanabe
watanabn@sapmed.ac.jp

Division of Laboratory Diagnosis[1] and Departments of Clinical Laboratory Medicine[2], Plastic and Reconstructive Surgery[3] and Molecular Medicine[4], Sapporo Medical University School of Medicine, South-1, West-16, Chuo-ko, Sapporo 060-8543, Japan

Received 6 June 2002
Accepted 20 September 2002

The objective of this study was to understand more of the innate immune response to *Helicobacter pylori* by determining the expression of human β-defensin-2 (hBD-2) in various gastric mucosal tissues and MKN45 gastric cancer cells with or without *H. pylori*. Semi-quantitative TaqMan RT-PCR and immunohistochemistry were carried out. The antimicrobial effects of a transfected hBD-2 gene against *H. pylori* were also evaluated. The results showed that hBD-2 was expressed in inflamed gastric mucosal tissues with *H. pylori* infection, but not in the absence of *H. pylori* infection. Expression was also detected in gastric cancers in patients with *H. pylori* infection. Expression was induced in the MKN45 gastric cancer cell line by *H. pylori* in a manner dependent on the abundance of bacteria. hBD-2-transfected 3T3J2-1 cells secreted hBD-2 protein into the culture medium and this protein inhibited growth of *H. pylori* completely. The results suggest that hBD-2 may be involved in the pathophysiology of *H. pylori*-induced gastritis.

## INTRODUCTION

Defensins are antimicrobial peptide components of the innate immune system against micro-organisms. Two subfamilies, α- and β-defensins, distinguished according to structural features at the gene and protein levels, have been identified in vertebrates (Diamond & Bevins, 1998). Three β-defensins were recently characterized in various human epithelial cells. Human β-defensin (hBD)-1 mRNA is expressed constitutively in various epithelial tissues (Harder *et al.*, 1997). hBD-2 mRNA expression is detected in epithelial cells of the skin, lung, trachea and urogenital tract and can be induced by treatment with tumour necrosis factor (TNF)-α or interleukin (IL)-1 or by exposure to micro-organisms (Wada *et al.*, 1999, 2001; Hamanaka *et al.*, 2001; O'Neil *et al.*, 2000). Both hBD-1 and hBD-2 show antimicrobial activity, predominantly against Gram-negative bacteria. hBD-3 mRNA, expressed in the skin, tonsil and trachea, can be induced in epithelial cells by treatment with TNF-α or contact with *Pseudomonas aeruginosa* and shows antimicrobial activity against Gram-negative and Gram-positive bacteria (Harder *et al.*, 2001).

Recently, induction of hBD-2 mRNA expression by *Helicobacter pylori* has been shown in human gastric cancer cell lines (MKN45 and AGS) (Wada *et al.*, 1999, 2001; Hamanaka *et al.*, 2001; O'Neil *et al.*, 2000). Gastric colonization by *H. pylori*, which is Gram-negative, is pathogenetically important in gastritis, peptic ulcer, gastric cancer and gastric mucosa-associated lymphoid tissue lymphoma. However, hBD-2 mRNA expression in gastric mucosal tissues has not been fully characterized.

To understand the innate immune response to *H. pylori*, we determined hBD-2 expression in various gastric mucosal tissues with or without *H. pylori* infection using a semi-quantitative TaqMan RT-PCR assay as well as immunohistochemistry. Additionally, the antimicrobial effect of hBD-2 against *H. pylori* was evaluated in transfection experiments.

Abbreviation: hBD-2, human β-defensin-2.

## METHODS

**Bacterial strains.** *H. pylori* ATCC 43504$^T$, *Salmonella typhimurium* ATCC 14028, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 33186 were used for hBD-2 mRNA induction.

**hBD-2 mRNA induction in MKN45 gastric cancer cells.** MKN45 gastric cancer cells were cultured in RPMI 1640 medium (BioWhittaker) supplemented with heat-inactivated fetal bovine serum (FBS) (JRH Biosciences) at 37 °C in an atmosphere including 5 % $CO_2$. Induction of hBD-2 mRNA was carried out as described by Wada *et al.* (1999). Briefly, $10^6$ MKN45 cells were seeded into dishes 60 mm in diameter and incubated for 12 h. Culture medium was replaced with 2 ml fresh RPMI 1640 medium without FBS. Bacterial suspensions

(100 μl; 0–$10^8$ c.f.u. $ml^{-1}$ in RPMI 1640 medium) were added to the dishes and incubation was continued for various periods of time.

**Tissue samples.** Samples of primary tumour, mucosa showing intestinal metaplasia, mucosa with chronic gastritis and normal mucosa were obtained from seven patients with previously untreated gastric cancer following surgery at Sapporo Medical University Hospital. Informed consent was obtained from all patients. After tissue removal, all samples were frozen immediately and fixed in 10 % formalin. All histological factors were evaluated according to the criteria of the Japanese Research Society for Gastric Cancer (1995).

**Determination of *H. pylori* infection.** Sections were Giemsa-stained and the rapid urease test (CLO test; Tri-Med Specialties Inc.) was performed with fresh samples from the prepyloric antrum, greater curvature of the corpus and fundus (Marshall *et al.*, 1987). *H. pylori* infection was defined as positive when *H. pylori* was detected and/or the CLO test was positive.

**Quantitative RT-PCR assay for hBD-2 mRNA.** ISOGEN (Nippon Gene) was used to extract total RNA from cells or tissues and this extract was assayed for RNA with the Gene Quant DNA/RNA calculator (Amersham Pharmacia Biotech). For quantitative RT-PCR, fluorescent hybridization probes and the TaqMan PCR core reagents kit with AmpliTaq Gold (Perkin-Elmer Applied Biosystems) were used with the ABI Prism 7700 sequence detection system (Perkin-Elmer Applied Biosystems). Expression of hBD-2 mRNA was quantified by methods reported previously (Yajima *et al.*, 1998). Primers and the TaqMan probe for hBD-2 mRNA were as follows: forward primer, 5′-ATC CAGTCTTTTGCCCTAGAAGGTA-3′; reverse primer, 5′-GGCTTT TTGCAGCATTTTGTTC-3′; TaqMan probe, 5′-AAACAAATTGGC ACCTGTGGTCTCCCT-3′. Aliquots of 25 ng total RNA from samples were used for one-step RT-PCR. Conditions of one-step RT-PCR were as follows: 30 min at 48 °C (stage 1, reverse transcription), 10 min at 95 °C (stage 2, RT inactivation and AmpliTaq Gold activation) and then 40 cycles of amplification for 15 s at 95 °C and 1 min at 60 °C (stage 3, PCR). Data for hBD-2 mRNA were normalized to data for glyceraldehyde-3-phosphate dehydrogenase.

**Immunostaining.** Formalin-fixed, paraffin-embedded tissue sections were stained with polyclonal goat antibody against hBD-2 (Santa Cruz Biotechnology) or non-immune goat serum using an indirect immunoperoxidase technique.

**Vector construction and generation of hBD-2-producing cells.** The full-length hBD-2 gene was amplified by RT-PCR using hBD-2-specific primers (forward primer, 5′-CGGGATCCATGAGGGTCTTG TATCTCCTCTTCTC-3′; reverse primer, 5′-CGGAATTCGGATCCT CATGGCTTTTTGCAGCATTTT GTTC-3′). Total cellular RNA was extracted from the human oral cancer cell line HSC2 as a template. The amplified product was digested with *Bam*HI and inserted at the *Bgl*II site of vector pCAcc (Yoshida & Hamada, 1997). The sequence and orientation of the hBD-2 gene in the vector were confirmed by sequencing. This construct was designated pCAhBD-2. Briefly, 3T3J2 cells were co-transfected with pCAhBD-2 and a plasmid containing a neomycin-resistance gene using LipofectAmine (Gibco-BRL) according to the manufacturer's instructions. After selection with G418 (500 μg $ml^{-1}$; Gibco-BRL), hBD-2 gene-transfected cells were cloned by a limiting-dilution method.

**Antimicrobial assay.** To evaluate the antimicrobial effect of hBD-2 on *H. pylori*, 25 μl of a suspension of $4 \times 10^6$ c.f.u. *H. pylori* strain ATCC $43504^T$ $ml^{-1}$ was cultured on HP agar (Eikenkagaku) after a 1–4 h pre-incubation at 37 °C in the presence or absence of culture supernatant obtained from hBD-2-3T3J2-1 cells. To determine the number of c.f.u., the pre-incubation mixture was diluted 100-fold immediately with culture medium and samples were cultured in triplicate. Viable cells (c.f.u. $ml^{-1}$) were counted after 3 days in culture at 37 °C.

## RESULTS AND DISCUSSION

### Induction of hBD-2 mRNA expression in MKN45 cells and expression of hBD-2 in various gastric mucosal tissues

To clarify the effect of *H. pylori* on hBD-2 mRNA expression by using TaqMan RT-PCR, MKN45 cells were first incubated for 1–20 h with *H. pylori*. hBD-2 mRNA expression was detected in MKN45 cells 1 h after starting incubation with *H. pylori* and reached a maximum at 10 h (Fig. 1).

To determine a suitable number of *H. pylori* bacteria for induction of hBD-2 mRNA expression, 100 μl aliquots of suspensions containing 0–$10^8$ c.f.u. *H. pylori* $ml^{-1}$ were incubated with MKN45 cells for 7·5 h. hBD-2 mRNA expression was up-regulated in a manner dependent on numbers of bacteria (Fig. 2), being first detectable at $10^4$ c.f.u. $ml^{-1}$.

To determine whether other species of bacteria could induce hBD-2 mRNA expression in MKN45 cells, the cells were exposed to *Salmonella typhimurium*, *Escherichia coli*, *Staphylococcus aureus* or *Enterococcus faecalis* for 7·5 h. hBD-2 mRNA expression in MKN45 cells was induced by all species of bacteria assessed in this study (Fig. 3). Gram-negative bacteria were more effective than Gram-positive bacteria in inducing hBD-2 mRNA expression in MKN45 cells (Fig. 3).

To evaluate the effect of *H. pylori* colonization in gastric tissues on hBD-2 expression, gastric cancer and paired adjacent mucosa showing gastritis from four *H. pylori*-positive and three *H. pylori*-negative patients were assessed by TaqMan RT-PCR analysis and immunostaining. In *H. pylori*-positive specimens, the mean expression of hBD-2

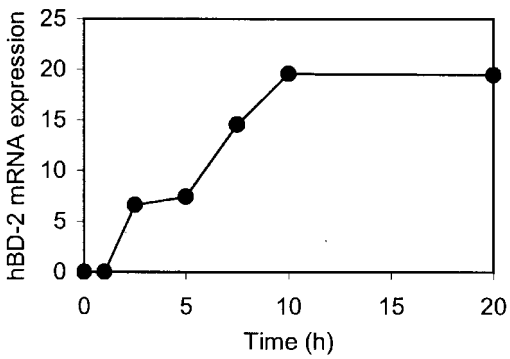


**Fig. 1.** Time-course of hBD-2 mRNA expression induced by *H. pylori* in a gastric cancer cell line. MKN45 cells ($10^6$) were seeded into dishes 60 mm in diameter and incubated for 12 h. Culture medium was replaced with 2 ml fresh RPMI 1640 medium without FBS. The cells were incubated for 1–20 h with 100 μl of a suspension of $10^8$ c.f.u. *H. pylori* $ml^{-1}$. hBD-2 mRNA expression was measured by TaqMan RT-PCR.

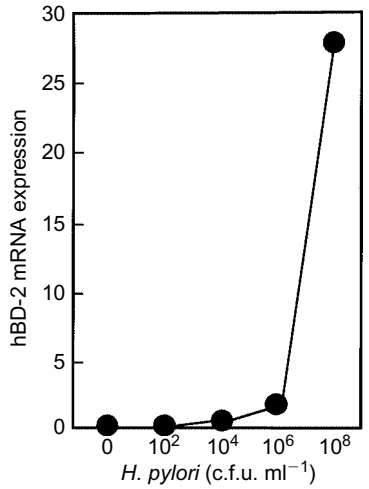


**Fig. 2.** Induction of hBD-2 mRNA expression by various numbers of *H. pylori* cells. MKN45 cells ($10^6$) were seeded into dishes 60 mm in diameter and incubated for 12 h. Culture medium was replaced with 2 ml fresh RPMI 1640 medium without FBS. Bacterial suspensions (100 μl; $0–10^8$ c.f.u. $ml^{-1}$ in RPMI 1640 medium) were added to the dishes and incubation was continued for 7·5 h. hBD-2 mRNA expression was measured by TaqMan RT-PCR.

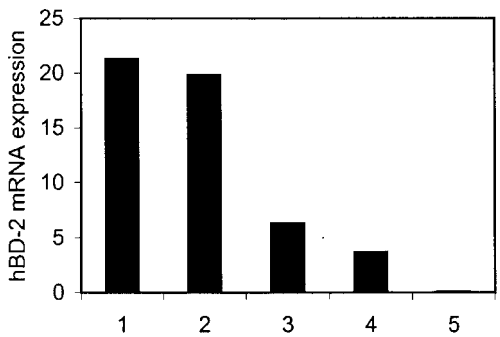


**Fig. 3.** Induction of hBD-2 mRNA expression by various bacterial species. MKN45 cells ($10^6$) were seeded into dishes 60 mm in diameter and incubated for 12 h. Culture medium was replaced with 2 ml fresh RPMI 1640 medium without FBS. The cells were exposed for 7·5 h to 100 μl of a suspension containing $10^8$ c.f.u. $ml^{-1}$ of *H. pylori* (1), *Salmonella typhimurium* (2), *Escherichia coli* (3), *Staphylococcus aureus* (4) or *Enterococcus faecalis* (5). hBD-2 mRNA expression was measured by TaqMan RT-PCR.

mRNA was 26·5 (Fig. 4). In contrast, the mean expression of hBD-2 mRNA in *H. pylori*-negative specimens was 0·27 (Fig. 4). The difference was significant ($P = 0·028$; Mann–Whitney U test).

hBD-2 protein was detected in gastric cancers and paired adjacent non-neoplastic tissue showing gastritis from *H. pylori*-positive patients, but not in specimens from two of three *H. pylori*-negative patients (Fig. 5).

Using TaqMan RT-PCR for hBD-2 mRNA and immunostaining for hBD-2 protein, we demonstrated that hBD-2 is expressed in gastric mucosa with *H. pylori* infection showing gastritis, but not in inflamed mucosa without *H. pylori* infection. In addition, hBD-2 mRNA expression was detected in gastric cancers from patients with *H. pylori* infection and hBD-2 mRNA expression was induced in the MKN45 gastric cancer cell line according to the intensity of *H. pylori* exposure. However, the level of expression of hBD-2 mRNA was variable in inflamed gastric mucosa and in cancers. A recent report has indicated that IL-1 and TNF-α can induce hBD-2 mRNA expression and that *H. pylori*, but not culture filtrate, increased hBD-2 mRNA expression in MKN45 cells (Wada *et al.*, 1999). These results imply that contact of gastric epithelial cells with *H. pylori* and the amounts of proinflammatory cytokines are important in induction of hBD mRNA expression. In addition, the magnitude of gastritis was variable in our cases. Our results might reflect the number of *H. pylori* cells in the gastric mucosa or influences of other factors such as proinflammatory cytokines.

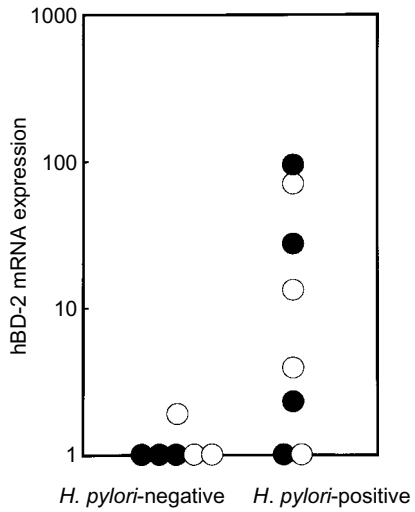


**Fig. 4.** hBD-2 mRNA expression in gastric cancers (○) and paired specimens of adjacent mucosa showing gastritis (●) from four *H. pylori*-positive and three *H. pylori*-negative patients.

Isomoto *et al.* (2000) detected activated NF-κB in epithelial cells in gastric mucosa of patients with *H. pylori*-associated gastritis. Recent reports suggest that only *H. pylori* strains (type I) that carry a *cag* pathogenicity island (PAI) induce activation at the NF-κB site of the hBD-2 promoter (Wada *et al.*, 1999, 2001). In the present study, all clinical *H. pylori* isolates from four patients with *H. pylori* infection had a *cag* A (data not shown), and as did *H. pylori* ATCC $43504^T$. Moreover, exposure of MKN45 cells to *Salmonella typhimurium* ATCC 14028, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 33186 resulted in induction of hBD-2 mRNA. These results suggested that *H. pylori* (*cag* PAI) and other pathogens may induce hBD-2 mRNA expression via direct or indirect activation of NF-κB. *Salmonella* species have pathogenicity

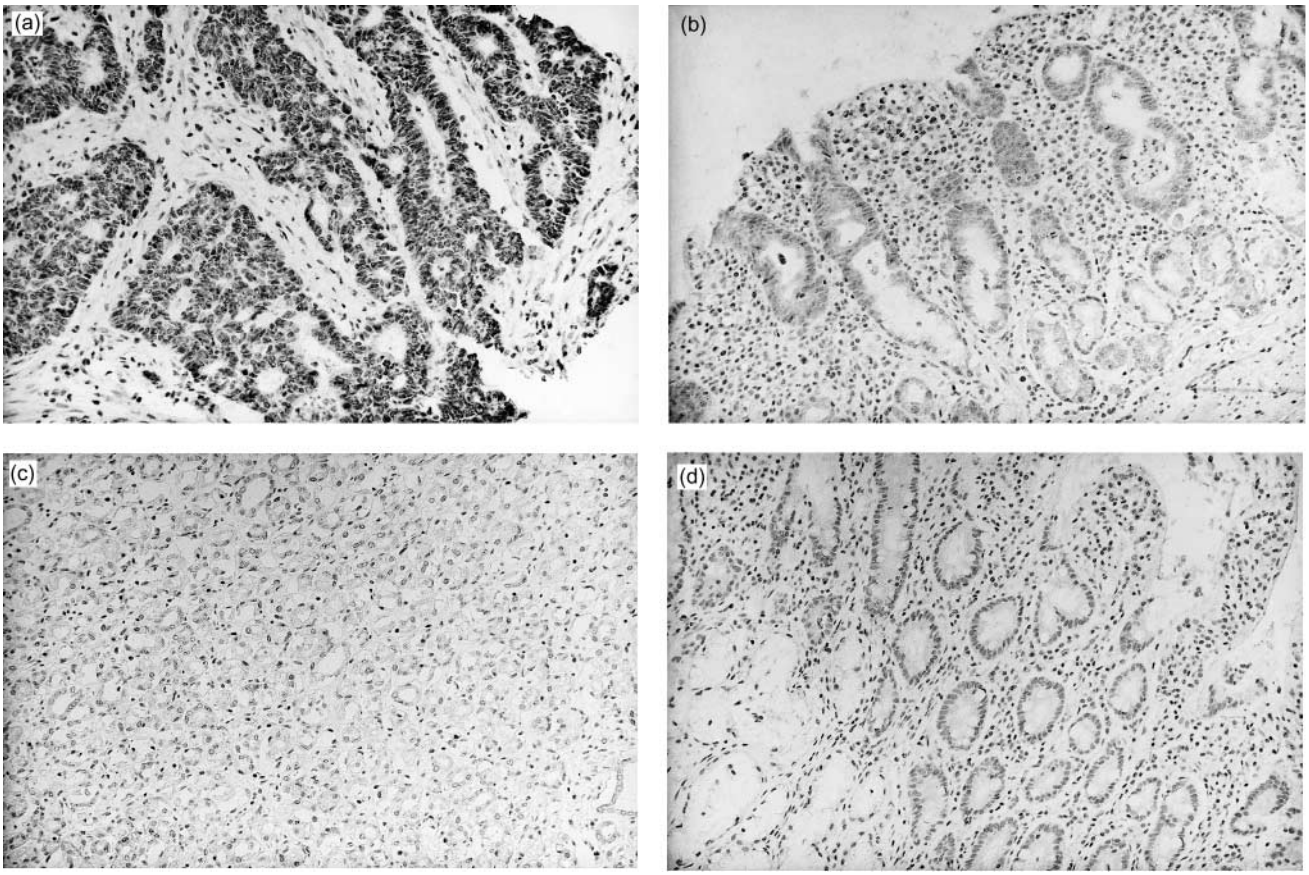


**Fig. 5.** hBD-2 protein expression in gastric cancer and paired adjacent tissue showing gastritis with or without *H. pylori* infection. Tissues were stained with anti-hBD-2 antibody. (a) and (b) Case 1: gastric cancer (moderately differentiated adenocarcinoma) and mucosa with gastritis with *H. pylori* infection. Positive staining was observed in gastric cancer cells in (a) and gastric epithelial cells in (b). (c) and (d) Case 2: gastric cancer (poorly differentiated adenocarcinoma) and mucosa with gastritis but without *H. pylori* infection. No positive staining was observed in (c) or (d). Magnification, × 120.

islands (SPI 1 and 2) that may be important in induction of hBD mRNA expression. Pathogen-associated molecular patterns in these bacterial species and pattern-recognition receptors in MKN45 cells should be studied.

### Assessment of expression of hBD-2 in hBD-2-gene-transfected cells and antimicrobial effect of medium from transfected cells

hBD-2 mRNA expression and secretion of hBD-2 protein into the culture medium were confirmed by the TaqMan RT-PCR for hBD-2 described above and by immunoblot analysis using anti-hBD-2 polyclonal antibody (Fig. 6). A mouse embryonic fibroblast clone showing high production of hBD-2 protein, hBD-2-3T3J2-1, was selected for further study.

Culture supernatants from hBD-2-3T3J2-1 cells were used to evaluate the antimicrobial effect of overexpressed hBD-2 against *H. pylori*. Aliquots of 25 μl ($4 \times 10^6$ c.f.u. $ml^{-1}$) of *H. pylori* ATCC 43504$^T$ were cultured on HP agar for 3 days. The mean numbers of c.f.u. of *H. pylori* after 0, 1, 2 and 4 h of pre-incubation with the culture supernatant (or with control medium) were respectively approximately $10^5$ ($10^5$), 0 ($10^5$), 0 ($10^5$) and 0 (82). Thus, growth of *H. pylori* was inhibited completely after 1 h of incubation with the culture supernatant.

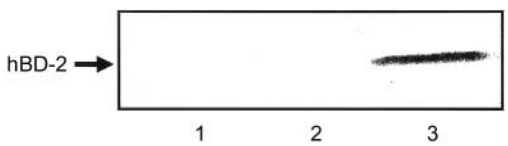


**Fig. 6.** Detection of hBD-2 protein in culture medium of hBD-2 gene-transfected 3T3J2 cells (lane 3), designated hBD-2-3T3J2-1, and parent 3T3J2 cells (lane 2) by immunoblot analysis using anti-hBD-2 antibody. Lane 1, RPMI 1640 medium control.

It has been reported that, at $10^{-5}$ M, chemically synthesized hBD-2 inhibits growth of *H. pylori* completely (Hamanaka *et al.*, 2001). Schröder & Harder (1999) reported that the $LD_{90}$

values of natural hBD-2 preparations against *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* were respectively 10, 10 and 25 μg $ml^{-1}$.

In the present study, we demonstrated that hBD-2-3T3J2-1 cells could secrete hBD-2 protein into the culture medium and that this protein inhibited growth of *H. pylori* completely. In conclusion, hBD-2 originating from the epithelium clearly can be bactericidal for *H. pylori*, yet is elevated in infection. This suggests a role for hBD-2 in the pathophysiology of *H. pylori* infection that has yet to be defined.

## REFERENCES

**Diamond, G. & Bevins, C. L. (1998).** β-Defensins: endogenous antibiotics of the innate host defense response. *Clin Immun Immunopathol* **88**, 221–225.

**Hamanaka, Y., Nakashima, M., Wada, A. & 7 other authors (2001).** Expression of human β-defensin 2 (hBD-2) in *Helicobacter pylori* induced gastritis: antibacterial effect of hBD-2 against *Helicobacter pylori*. *Gut* **49**, 481–487.

**Harder, J., Bartels, J., Christophers, E. & Schröder, J. M. (1997).** A peptide antibiotic from human skin. *Nature* **387**, 861.

**Harder, J., Bartels, J., Christophers, E. & Schröder, J. M. (2001).** Isolation and characterization of human β-defensin-3, a novel human inducible peptide antibiotic. *J Biol Chem* **276**, 5707–5713.

**Isomoto, H., Mizuta, Y., Miyazaki, M. & 7 other authors (2000).** Implication of NF-κB in *Helicobacter pylori*-associated gastritis. *Am J Gastroenterol* **95**, 2768–2776.

**Japanese Research Society for Gastric Cancer (1995).** *Japanese Classification of Gastric Carcinoma*, English edn. Tokyo: Kanehara.

**Marshall, B. J., Warren, J. R., Francis, G. J., Langton, S. R., Goodwin, C. S. & Blincow, E. D. (1987).** Rapid urease test in the management of *Campylobacter pyloridis*-associated gastritis. *Am J Gastroenterol* **82**, 200–210.

**O'Neil, D. A., Cole, S. P., Martin-Porter, E., Housley, M. P., Liu, L., Ganz, T. & Kagnoff, M. F. (2000).** Regulation of human β-defensins by gastric epithelial cells in response to infection with *Helicobacter pylori* or stimulation with interleukin-1. *Infect Immun* **68**, 5412–5415.

**Schröder, J. M. & Harder, J. (1999).** Human β-defensin-2. *Int J Biochem Cell Biol* **31**, 645–651.

**Wada, A., Mori, N., Oishi, K. & 10 other authors (1999).** Induction of human β-defensin-2 mRNA expression by *Helicobacter pylori* in human gastric cell line MKN45 cells on cag pathogenicity island. *Biochem Biophys Res Commun* **263**, 770–774.

**Wada, A., Ogushi, K., Kimura, T. & 9 other authors (2001).** *Helicobacter pylori*-mediated transcriptional regulation of the human β-defensin 2 gene requires NF-κB. *Cell Microbiol* **3**, 115–123.

**Yajima, T., Yagihashi, A., Kameshima, H., Kobayashi, D., Furuya, D., Hirata, K. & Watanabe, N. (1998).** Quantitative reverse transcription-PCR assay of the RNA component of human telomerase using the TaqMan fluorogenic detection system. *Clin Chem* **44**, 2441–2445.

**Yoshida, Y. & Hamada, H. (1997).** Adenovirus-mediated inducible gene expression through tetracycline-controllable transactivator with nuclear localization signal. *Biochem Biophys Res Commun* **230**, 426–430.