Identification of neutral and acidic sphingomyelinases in *Helicobacter pylori*

Yuh-Ling Lin[b], Jai-Shin Liu[b], Kuei-Tian Chen[b], Chien-Tsu Chen[a], Err-Cheng Chan[b,*]

[a]*Department of Biochemistry, Taipei Medical College, Taipei, Taiwan*
[b]*School of Medical Technology, Chang Gung University, 259 Wen-Hua 1st Road, Taoyuan, Taiwan*

Received 8 January 1998

Abstract We demonstrated for the first time the presence of sphingomyelinase (SMase) in *Helicobacter pylori*. Activation of SMase has been implicated as the cause of elevation of cellular ceramide levels and consequently of apoptosis. The data indicate that there are two classes of SMase, defined by their optimal pHs and cellular locations, existing in *H. pylori*. One is an Mg^{2+}-dependent membrane-bound enzyme with an optimal activity at pH 7, and the other is an Mg^{2+}-independent cytosolic enzyme with an optimal activity at pH 5. Bisalumin, a bismuth salt, was found to inhibit the activities of both forms of SMase regardless of the presence of Mg^{2+}. By Western blot analysis, the membrane-bound SMases of *H. pylori* and *Bacillus cereus* were shown to be antigenically related and to have a similar denatured molecular mass of 28 kDa.

Key words: Sphingomyelinase; Phospholipase; *N*-ω-Trinitrophenylaminolauryl-sphingomyelin; *Bacillus cereus*; *Helicobacter pylori*

1. Introduction

Helicobacter pylori is a bacterial pathogen which is strongly associated with human gastritis, peptic ulcer and gastric cancer [1–7]. Infection of *H. pylori* begins with gastric mucus colonization, followed by attachment of the bacteria to the specific portions of epithelial cells [8]. Human gastric epithelium protects itself against various noxious factors attacks by the lipid hydrophobic barrier [9–12]. A recent report on the ultrastructure analysis has documented that *H. pylori* infection damages the phospholipid-rich epithelial layer of gastric mucosa [13], and the gastric mucosal hydrophobicity of *H. pylori*-infected patients can return to normal values after eradication of *H. pylori* [14]. Among the enzymes of *H. pylori*, phospholipases have reactive targets on gastric epithelial cell membrane as well as on the lipid hydrophobic layer on the surface of the mucus gel. In previous studies, two types of phospholipases, phospholipases A and C, have been identified in *H. pylori*. They were regarded as the pathogenic factors owing to their actions on epithelial cell membrane [15,16]. In the present study, we attempt to determine whether or not sphingomyelinase (SMase), an important phospholipase, exists in the common pathogenic bacteria, *H. pylori*. SMase catalyzes the hydrolytic reaction of sphingomyelin (SM) to form the lipid moiety ceramide and water soluble cholinephosphate derivative. These sphingolipids are regarded as active participants in the regulation of cell growth, differentiation, and apoptosis [17]. Therefore, should SMase exist in *H. pylori*, it may play a potential role in pathogenesis of gastric ulcer diseases.

2. Materials and methods

2.1. Materials

N-ω-Trinitrophenylaminolauryl-sphingomyelin (TNPAL-SM), crude sphingomyelin (SM) (bovine brain extract, type viii), phenylmethylsulfonyl fluoride (PMSF), bisalumin, bovine serum albumin, ethylenediamine tetraacetic acid (EDTA, trisodium salt) and all other organic compounds were purchased from Sigma (St. Louis, MO). Chocolate-agar plates were purchased from Difco (Detroit, MI).

2.2. Bacterial isolates and cultivation

The *Helicobacter pylori* strains from ulcer patients were kindly given by Dr. N. Lee of Chang Gung memorial hospital, Taiwan. Twelve strains were screened quantitatively for SMase activity. The bacteria were cultured on chocolate agar plates at 37°C in a microaerophilic atmosphere of 10% CO_2 in air and 99% humidity for three days. *H. pylori* strains were identified by Gram stain morphology, urease test, and histology.

2.3. Preparation of H. pylori proteins

The *H. pylori* cells grown on chocolate agar plates were washed out with phosphate buffer saline (PBS, pH 7.2). The harvested bacterial cells were washed twice in PBS and then resuspended in 0.2 M glycine-HCl buffer (pH 2.2). The suspension was stirred at 25°C for 15 min and centrifuged at 4°C for 15 min. The supernatant was neutralized with NaOH and dialyzed against distilled water at 4°C overnight, and used as a membrane fraction for SMase assay. The intact *H. pylori* pellet was then resuspended in 50 mM PBS containing 1 mM PMSF and 2 mM EDTA and was disrupted by sonication (Heat-System Model XL 200, NY) in an ice bath at 35% pulse with 30-s intervals for 5 min. The solution was centrifuged at $15\,000 \times g$ for 10 min at 4°C. The supernatant was used as the cytosolic portion for SMase assay. Protein concentration was determined by the Bradford dye binding assay using bovine serum albumin as a standard.

2.4. SMase assay

The activity of both neutral SMase (N-SMase) and acidic SMase (A-SMase) were determined by the ability of the enzyme to hydrolyze the substrate analog, TNPAL-SM, as described previously [20,21]. For determining N-SMase activity, enzyme preparation (100 μl, 0.3–0.6 mg of protein) diluted to 200 μl with 50 mM PBS, pH 7.4, was mixed with 20 nmol of TNPAL-SM dissolved in 0.05% Triton X-100 in 50 μl of 100 mM PBS, pH 7.4, containing 5 mM magnesium chloride. The reaction processed for 2 h at 37°C and was then terminated by the addition of 0.75 ml of isopropanol/heptane/5 M H_2SO_4 (40:10:1, v/v) followed by 0.4 ml of H_2O and 0.45 ml of heptane. After phase separation, a portion of the upper phase was removed and the reaction product, trinitrophenylamino (TNPAL) residue was measured at 330 nm spectrophotometrically, and 1 nmol gave 0.023 absorbance units. A-SMase activity was determined using TNPAL-SM resuspended in 50 μl of 100 mM sodium acetate, pH 5.0. One unit of activity was defined as the amount of SMase which catalyzed the hydrolysis of 1 nmol of TNPAL-SM per hour at 37°C. Specific activity was given as units of enzyme activity per milligram of protein. The effects of different pH, magnesium ion, and bisalumin concentrations on the SMase activity were examined.

*Corresponding author. Fax: (886) (3) 3288741.

PII S0014-5793(98)00087-8

2.5. Immunoblotting

Purified *Bacillus cereus* SMase and *H. pylori* lysate were subjected to SDS-PAGE (10% polyacrylamide) and were transferred by Western blot to nitrocellulose membrane (Millipore, Bedford, MA) in 10 mM phosphate buffer, pH 7.2, and immunostaining was performed as described previously [18,22]. Antiserum to the purified SMase of *B. cereus* (Sigma Co.) was raised in female New Zealand White rabbits as previously described [19]. Two milliliters of enzyme solution were mixed with an equal volume of Freund complete adjuvant and injected subcutaneously into eight sites on the back and hind legs of each rabbit. Booster doses were given in a similar manner in Freund incomplete adjuvant 14 and 28 days after initial immunization. On day 42, the rabbits were bled, and the serum was collected and stored at −20°C in 100-μl samples. Preimmune serum was taken and used as a negative control serum. For the immunoblotting experiments, the rabbit antisera were diluted 1:200.

3. Results

Twelve strains of *H. pylori* isolated from humans were screened quantitatively for SMase activity. SMase activity was measured as the ability to hydrolyze an SM analog TNPAL-SM. *H. pylori* cells were harvested from chocolate agar plates and lysing them by sonication. Lysates were then assayed for SMase activity. We determined the specific activities of SMase which varied from 2.12 to 15.62 nmol of TNPAL-SM hydrolyzed per h/mg of protein from 12 strains (Table 1). Data show that strain K8 has an SMase-specific activity of 15.6 nmol/h/mg protein, which is much higher than that of others. Interestingly, the strain K8 was from a patient with a severe duodenal ulcer as well as gastric ulcer, and the other isolates were from patients with either duodenal ulcer, gastric ulcer, or dyspepsia. Strain K8 was used exclusively in the characterization of SMase because it grew well under laboratory conditions and showed no significant change in SMase-specific activity on repeated subculture.

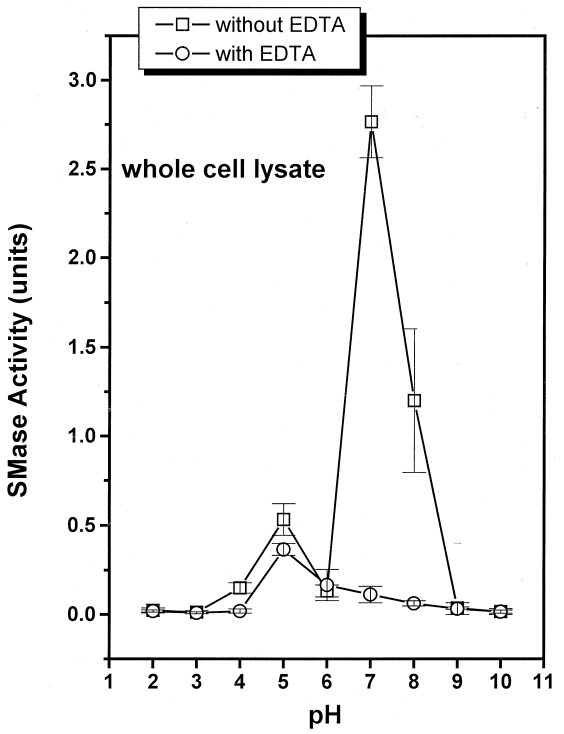

Fig. 1. Effect of pH on the SMase activity in *H. pylori* lysate. Activity measurements of 100 μl whole cell lysate (400 μg protein) were carried out after 20 min incubation at 37°C with buffers ranging from pH 2 to 10 (pH 2–5.6, acetate; pH 5.8–8.5, $Na_2B_4O_7$/HCl; pH 9–10, $Na_2B_4O_7$/NaOH). Symbol □ indicates lysate mixing with 20 mM EDTA; symbol ○ indicates lysate not mixing with EDTA. The results are the means ± S.E.M. of four separate experiments, each performed in at least triplicate.

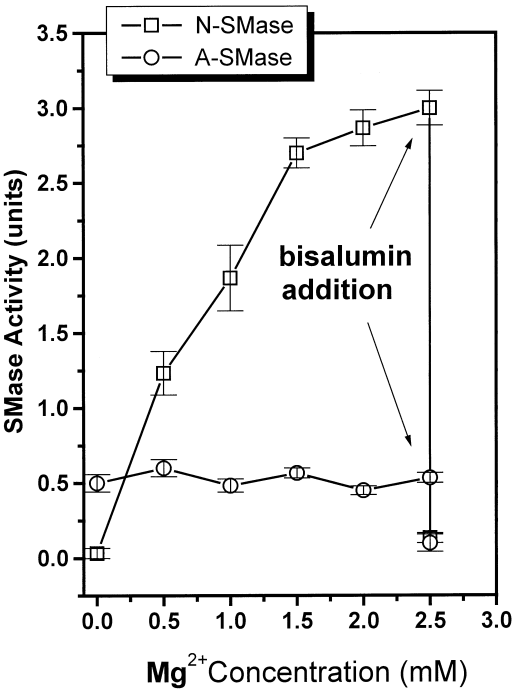

Fig. 2. Effect of the concentration of Mg^{2+} on the SMase activity. *H. pylori* lysate (100 μl, 400 μg protein) was mixed with 100 μl of 5 mM EDTA in 0.05 M PBS (pH 7.2) and in 0.05 M acetate buffer (pH 5), and was kept at 5°C for 2 h. Then 0.1 ml of magnesium chloride solution at various concentrations was added to each mixture. After being allowed to stand for 1 h at 5°C, the sample was subjected to SMase assay.

As part of the biochemical characterization of SMase, we initially examined the effect of pH on the in vitro activity of the enzyme. SMase activity of *H. pylori* lysate was measured at 37°C with buffers ranging from pH 2 to 10, and two optimal pH values for activity were determined at pH 5 and pH 7, respectively (Fig. 1). This result supports the existence of different forms of SMase in *H. pylori*; one is neutral isoform and the other one is acidic isoform of SMase. In *H. pylori*, N-SMase contributes approximately 85% of the total activity,

Table 1
SMase activities from twelve clinical *H. pylori* isolates

Clinical strains	Endoscope diagnosis	SMase activity[a] (nmol/h/mg protein)
K7	GU[b]	3.50
K8	GU, DU[b]	15.62
K34	DU	2.83
K85	None[b]	2.93
K86	DU	3.13
K89	DU	2.12
K93	GU	3.10
34	Dyspepsia	2.85
36	DU	2.45
37	GU	2.55
41	DU	2.88
46	Dyspepsia	2.93

[a]The whole cell lysate buffered in pH 7 was used for SMase assay.
[b]GU: gastric ulcer; DU: duodenal ulcer; None: no symptom.

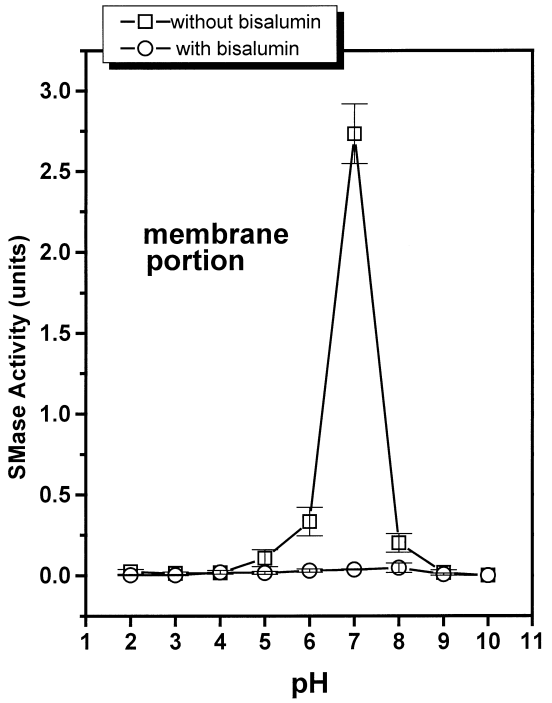

Fig. 3. Effect of pH on the SMase activity in *H. pylori* membrane portion. The experimental conditions were described in Fig. 1. Symbol □ indicates lysate mixing with 1 mM bisalumin; symbol ○ indicates lysate not mixing with bisalumin. The results are the means ± S.E.M. of four separate experiments, each run performed in at least triplicate.

and A-SMase contributes the rest. We next examined the effect of Mg^{2+} concentrations on SMase activities. As shown in Fig. 2, incubation of *H. pylori* lysate with concentrations of magnesium chloride ranging from 0 to 2.5 mM resulted in a linear activation of the SMase. In contrast to N-SMase, magnesium chloride at concentrations ranging from 0 to 2.5 mM was completely ineffective on the activity of A-SMase (Fig. 2). By adding EDTA to the *H. pylori* lysate, the activity of N-SMase was completely inhibited, and the activity of A-SMase was not affected (Fig. 1). Nevertheless, adding the excess amount of Mg^{2+} ion could restore the EDTA-inhibited N-SMase activity (data not shown). These results indicate that N-SMase is Mg^{2+}-dependent, but A-SMase is not. Data also showed that adding bisalumin, a bismuth-containing compound used in the treatment of gastric disorders, at a concentration of 1 mM, could inhibit the activities of both N-SMase and A-SMase in the presence of Mg^{2+} (Fig. 2).

We next investigated cellular locations of SMases to determine whether it was in a membrane fraction or in *H. pylori* cytosol. To this end, we used the glycine-extractable proteins from *H. pylori* as a membrane fraction for SMase assay. As shown in Fig. 3, the majority of N-SMase activity was found in a membrane fraction with an insignificant amount of A-SMase activity, and incubation of the membrane fraction at 37°C with 1 mM bisalumin resulted in a 100% inhibition of N-SMase activity regardless the presence of Mg^{2+}. The intact cells without any treatment also appeared to have an N-SMase activity and to be free of A-SMase activity (Fig. 4). This result implicates that N-SMase may be located on the outer membrane of *H. pylori*. On the other hand, the N-SMase activity was found to be nearly absent from *H. pylori* cytosolic fraction which was prepared from cells previously extracted by 0.2 M glycine-HCl buffer, and A-SMase was found to be a major form in *H. pylori* cytosol (Fig. 5).

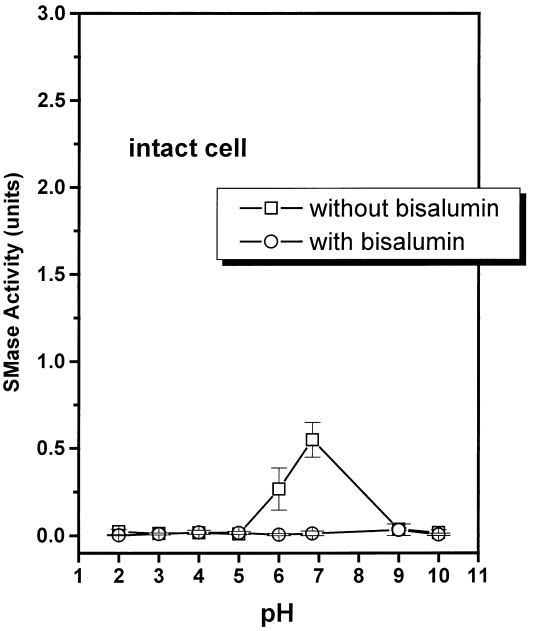

Fig. 4. Effect of pH on the SMase activity of intact *H. pylori* cells. The experimental conditions were described in Fig. 1, and 10^6 cells were used for SMase assay. Symbol □ indicates lysate mixing with 1 mM bisalumin; symbol ○ indicates lysate not mixing with bisalumin. The results are the means ± S.E.M. of four separate experiments, each run performed in at least triplicate.

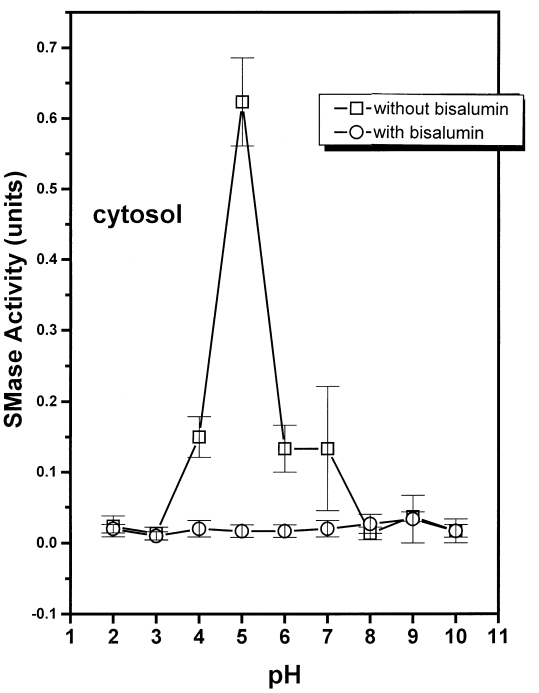

Fig. 5. Effect of pH on the SMase activity in *H. pylori* cytosol. The experimental conditions were described in Fig. 1. Symbol □ indicates lysate mixing with 1 mM bisalumin; symbol ○ indicates lysate not mixing with bisalumin. The results are the means ± S.E.M. of four separate experiments, each run performed in at least triplicate.

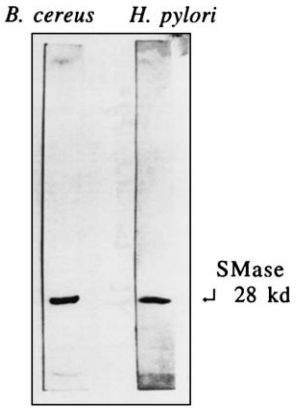

Fig. 6. Western blot recognition of the M_r 28 kDa membrane-bound protein of *H. pylori* as well as that of *B. cereus* by antiserum raised against the purified *B. cereus* N-SMase.

By Western blot analysis, antiserum raised against the purified *B. cereus* N-SMase recognized the 28-kDa protein from *H. pylori* membrane and no other protein constituents (Fig. 6). This result suggests that N-SMase produced by *H. pylori* and *B. cereus* are antigenically related. The antiserum did not recognize any protein from *H. pylori* cytosol (data not shown). There are two implications to this result. First, *H. pylori* A-SMase is not antigenically related to *B. cereus* N-SMase. Second, the amount of A-SMase is insufficient to be detected. The data of Fig. 6 also show that the denatured N-SMases from both bacteria have a similar molecular mass of approximately 28 kDa.

4. Discussion

In the present study, SMase activity of *H. pylori* is observed which is generally expressed by the clinical isolates. We identified two isoforms of SMase existing in *H. pylori*. One is a neutral Mg^{2+}-dependent membrane-bound enzyme with an optimal activity at pH 7, and the other is an acidic Mg^{2+}-independent cytosolic enzyme with an optimal activity at pH 5. Among the twelve *H. pylori* isolates, a strain K8 isolated from a patient with duodenal and gastric ulcers was found to have a much higher total activity of SMase than that of others. Because of the insufficient number of *H. pylori* isolates tested at this stage, it was impossible to draw a conclusion on SMase activity appearing to correlate with pathogenesis. However, in epidemiology it is interesting to know the correlation between them.

Ikezawa et al. showed that SMase of *Bacillus cereus* was a neutral enzyme with a molecular mass of 24 kDa [23]. In the present study, results show that the properties of *H. pylori* membrane-bound N-SMase resemble those in *B. cereus* with respect to Mg^{2+} dependence and optimal pH value. In addition to the Mg^{2+}-dependent form of membrane-bound N-SMase, we also found an Mg^{2+}-independent A-SMase form in *H. pylori* cytosol. In mammalian cells, although the A-SMase has been primarily found to reside in the lysosomes, it has also been detected as a soluble form in cytosol [24]. Both N-SMase and A-SMase have been suggested to play a role in induction of cellular apoptosis [25]. In this study we demonstrated that both *H. pylori* N-SMase and A-SMase were inactivated at pH 2 (Fig. 2). This evidence suggests that *H. pylori* SMases are inactivated in the stomach. However, *H. pylori* membrane-bound urease gives this bacterium the ability to survive in acid, by production of ammonia from urea substrate, to penetrate the mucus surface and colonize a more alkaline environment. Therefore, in the local alkaline environment created by *H. pylori*, activities of SMases may be restored in the stomach and subsequently involved in the induction of apoptosis. Study results also showed that bismuth salt, an agent that was found to inhibit phospholipases A and C [26], inhibited the activity of *H. pylori* SMase. Although the inhibitory mechanism of bismuth salt is not clear and remains to be studied, a possible model for inactivation due to an ionic interaction between bismuth and enzyme has been proposed [27].

In Western blot analysis, unfortunately, the amount of the purified *H. pylori* SMase was not enough to perform the immunization to raise the antiserum. Instead, a purified *B. cereus* N-SMase from commercial source was used to raise antiserum. Results showed that antiserum recognized the denatured 28-kDa protein from membrane portion of *H. pylori*. This indicates that the N-SMases produced by both *H. pylori* and *B. cereus* possess common antigenic epitopes, although we have not yet identified those regions.

It is now well known that approximately 90% of duodenal ulcer patients and 60% of gastric ulcer patients are *H. pylori* infected, and the patients are at high risk to develop gastric cancer [2,28,29]. Pathogenic mechanisms behind the effects of *H. pylori* on the gastroduodenal mucosa are not fully understood. Enzymes of *H. pylori* involving gastric diseases such as urease, catalase, lipase, phospholipase and protease have been claimed [30]. In ongoing studies searching *H. pylori* enzymes involved in pathogenesis, we discovered the presence of SMase activity in *H. pylori*. SMase is present in some pathogenic microorganisms, and it is for the first time identified in *H. pylori*. Among the bacterial pathogenic factors, investigations have been insufficient in relation to phospholipases of *H. pylori* and pathology. Until detailed studies are performed, it is not possible to interpret correctly the pathology associated with *H. pylori* SMases.

SM hydrolysis through the activation of SMases and generation of ceramide have been implicated as important signaling pathways in the regulation of cell growth, differentiation, apoptosis, and inflammatory responses [31,32]. A number of extracellular agents such as tumor necrosis factor, interleukin-1β, nerve growth factor, chemotherapeutic agents, and serum deprivation, which are known to induce apoptosis, activate SMase and cause elevation of intracellular ceramide levels [33–37]. Thus, an interesting question arises whether the bacterial membrane-bound SMase from *H. pylori* acts as an apoptosis inducer. Further investigation is required to determine whether *H. pylori* SMase plays an important role in apoptosis of gastric cells.

Acknowledgements: This work was supported by Grant NSC-85-2331-B-182-072 from the National Science Council of ROC.

References

[1] Price, A.B., Levi, J., Dolby, J.M., Dunscombe, P.L., Smith, A., Clark, J. and Stephenson, M.L. (1985) Gut 26, 1183–1188.
[2] Buck, G.E., Gourely, E.G., Lee, W.K., Subramanyam, K., Latimer, L.M. and Dinuzzo, A.R. (1986) J. Infect. Dis. 153, 664–669.

[3] Anderson, L.P., Holck, S., Poulsen, C.C., Elsborg, L. and Justensen, T. (1987) Scand. J. Gastroenterol. 22, 219–224.
[4] Leunk, R.D., Johnson, P.T., David, B.C., Kraft, W.G. and Morgan, R.D. (1987) J. Med. Microbiol. 26, 93–99.
[5] Marshall, B.J. (1988) Scand. J. Gastroenterol. (Suppl.) 23, 58–66.
[6] Forman, D., Newell, D.G., Fullerton, F., Yarnell, J.W.G., Stacey, A.R., Wald, N. and Sitas, F. (1991) Br. Med. J. 302, 1302–1305.
[7] Genta, R.M. and Hamner, H.W. (1994) Arch. Pathol. Lab. Med. 118, 740–743.
[8] Blaser, M.L. (1993) Trends Microbiol. 1, 255–260.
[9] Bulter, B.D., Lichtenberger, M. and Hill, B.A. (1983) Am. J. Physiol. 244, G645–G651.
[10] Hill, B.A., Bulter, B.D. and Lichtenberger, L.M. (1983) Am. J. Physiol. 244, G561–G568.
[11] Hill, B.A. (1985) Am. J. Physiol. 249, G342–G349.
[12] Slomiany, B.L., Kasinathan, C. and Slomiany, A. (1989) Am. J. Gastroenterol. 84, 1273–1277.
[13] Mauch, F.G., Ditschuneit, H. and Malfertheiner, P. (1993) Gastroenterology 105, 1698–1704.
[14] Goggin, P.M., Marrero, J.M., Spychal, R.T., Jackson, P.A. and Corbishley, C.M. (1992) Gastroenterology 103, 1486–1490.
[15] Langton, S.R. and Cesareo, S.D. (1992) J. Clin. Pathol. 45, 221–224.
[16] Weltkamp, J.H., Perez-Perez, G.I., Bode, G.I., Bode, G., Malfertheiner, P. and Blaser, M.J. (1993) Int. J. Med. Microbiol. 280, 11–27.
[17] Hannun, Y.A. and Linardic, C.M. (1993) Biochim. Biophys. Acta 1154, 223–236.
[18] Cover, T.L. and Blaser, M.J. (1992) J. Biol. Chem. 267, 10570–10575.
[19] Dunn, B.E., Campbell, G.P., Perez-Perez, G.I. and Blaser, M.J. (1990) J. Biol. Chem. 265, 9464–9469.
[20] Gatt, S., Dinur, T. and Barenholz, Y. (1978) Biochim. Biophys. Acta 530, 503–507.
[21] Gillmore, M.S., Cruz-Rodz, A.L., Leimeister-Wachter, M., Kreft, J. and Goebel, W. (1989) J. Bacteriol. 171, 744–753.
[22] Laemmli, U.K. (1970) Nature 227, 680–685.
[23] Ikezawa, H., Mori, M., Ohyabu, T. and Taguchi, R. (1978) Biochim. Biophys. Acta 528, 247–256.
[24] Quintern, L.E., Weitz, G., Nehrkorn, H., Tager, J.M., Schram, A.W. and Sandhoff, K. (1987) Biochim. Biophys. Acta 922, 323–336.
[25] Haimovitz-Friedman, A., Kan, C.C., Ehleiter, D., Perssaud, R.S., McLoughlin, M., Fuks, Z. and Kolesnick, R.N. (1994) J. Exp. Med. 180, 525–535.
[26] Otlecz, A., Romero, J.J., Hazell, S.L., Graham, D.Y. and Lichtenberger, L.M. (1993) Digest. Dis. Sci. 38, 2071–2080.
[27] Beil, W., Bierbaum, S. and Sewing, S.F. (1993) Pharmacology 47, 141–144.
[28] Graham, D.Y. (1989) Gastroenterology 96, 615–625.
[29] Forman, D., Webb, P., Newell, D., Coleman, M., Palli, D., Moller, H., Hengels, K., Elder, J. and DeBacker, G. (1993) Lancet 341, 1359–1362.
[30] Hazell, S.L. and Mendz, G.L. (1993) in: *Helicobacter pylori*: Biology and Clinical Practice (Goodwin, C.S. and Worsely, B.W., Eds.) pp. 115–141, CRC Press, London.
[31] Okazaki, T., Bell, R.M. and Hannun, Y.A. (1989) J. Biol. Chem. 264, 19076–19080.
[32] Hannun, Y.A. (1994) J. Biol. Chem. 269, 3125–3128.
[33] Wiegmann, K., Schutze, S., Machleidt, T., Witte, D. and Kronke, M. (1994) Cell 78, 1105-1015.
[34] Mathias, S., Younes, A., Kan, C.C., Orlow, I., Joseph, C. and Kolesnick, R.N. (1993) Science 250, 519–522.
[35] Dobrowsky, R.T., Werner, M.H., Castellino, A.M., Chao, M.V. and Hannun, Y.A. (1994) Science 265, 1596–1599.
[36] Zhang, J., Alter, N., Rood, J.G., Barner, C., Obeid, I.M. and Hannun, Y.A. (1996) Proc. Natl. Acad. Sci. USA 93, 5325–5328.
[37] Jayadev, S., Liu, B., Bielawska, A.E., Lee, J.Y., Nazairo, F., Pushkareva, M.Y., Obeid, I.M. and Hannun, Y.A. (1995) J. Biol. Chem. 270, 2047–2052.