⟨*Original Article*⟩

Unique Enhancement of Multinuclear Giant Cell Formation in AGS Cell Line Infected with *Helicobacter pylori*

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ABSTRACT

Helicobacter pylori (H. pylori) causes pathological changes of gastric epithelial cells induced by pathogenic factors such as CagA and VacA, namely hummingbird cells (HBC) formation and vacuolization, respectively, in cultured cell lines. Cytopathic effects of other pathogenic factors produced by *H. pylori* have not been reported. In this study, we examined whether *H. pylori* induces unique morphological changes other than HBC formation and vacuolization, and we established a new marker of the bacterial infection *in vitro*. The cytotoxicity of *H. pylori* was examined in the AGS cell line, and a new morphological change, namely multinuclear giant cells (MNGC) formation, was observed in this cell line. The enhancement of MNGC formation was observed following *H. pylori* infection but was not associated with CagA, which causes HBC formation. We characterized the factor causing MNGC formation enhancement as heat-stable and water-soluble, and finally considered the factor to be *H. pylori* lipopolysaccharide (LPS). We considered that *H. pylori* LPS enhances MNGC formation *in vitro*. The cytopathic effect may provide an important marker that may clarify the mechanism of *H. pylori* pathogenesis in human gastric epithelial cells.

Introduction

Helicobacter pylori (*H. pylori*) is a gram-negative, spiral and microaerobic bacterium. The bacterium, which was isolated from the gastric mucosa of a patient with chronic gastritis [1] and also from a peptic ulcer lesion [2], is considered to be an etiologic factor of gastric disorders, such as gastritis, peptic ulcer, nonulcerous dyspepsia and possibly gastric cancer [2,3]. Bacterial infection occurs in young individuals and persists throughout life [4]. The pathogenesis of gastric

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disorders is caused by bacterial urease, cytotoxin and the invasion of what causes pathological changes of gastric epithelial cells [5,6]. Recent studies on the pathogenesis of gastric disorders revealed a high production of urease [7], the expression of cytotoxins such as VacA [8], CagA [6] and endotoxin [9], the involvement of host-tissue-specific cell receptors for bacterial adherence, and pathological changes of gastric epithelial cells [10,11]. Considering the above phenomenon *in vitro*, the pathological changes and cytopathic mechanism need to be clarified.

H. pylori CagA induces a unique morphological change termed the hummingbird phenotype characterized by the elongation of human gastric adenocarcinoma epithelial cell line (AGS) [12~14]. Another morphological change *in vitro* is the vacuolization of host cells induced by *H. pylori* VacA [15,16]. The pathogenic mechanisms of CagA and VacA have been well studied using morphological markers, namely, hummingbird phenotype and vacuolization, respectively [12,15]. Because *H. pylori* produces various etiological factors, it is possible that unique morphological changes other than hummingbird phenotype and vacuolization may occur in the same cell line. In this study, we examined whether *H. pylori* induces other unique morphological changes in AGS cell line, and established a new marker of the bacterial infection *in vitro*.

Materials and Methods

Bacteria and cell culture:
The *H. pylori* strain ATCC 43504 and clinical strains that were isolated from biopsy samples were used in this study. The bacterial strains were cultured on Pylori agar plates (bioMéieux, Marcy-l'Étoile, France) in a jar containing an anaerobic gas pack (Becton Dickinson Microbiology Systems, Sparks, MD, USA) and Campypack Microaerophilic System Envelopes (Becton Dickinson) at 37 ˚C for 3 d. Colonies were collected, suspended, and treated in either McIlvaine buffer containing 100 mM citric acid monohydrate and 200 mM disodium hydrogen phosphate (pH 5 or pH 7) at 37 ˚C for 15 min. To obtain heat-stable factors from *H. pylori*, a bacterial suspension in phosphate-buffered saline (PBS) was heated at 80 ˚C for 10 min, and rapidly chilled on ice.

AGS cells of the CRL 1739 line (Dainippon Pharmaceuticals, Osaka, Japan), a human gastric adenocarcinoma epithelial cell line, were cultured in Ham's F12 medium (DaiNippon Pharmaceuticals) supplemented with 10 % fetal bovine serum at 37 ˚C in 5 % CO_2 atmosphere in a 25-cm^2 tissue culture flask and a 10-cm^2 one-well chamber glass slide for 2 d to obtain a monolayer of ~70 % confluence.

For microscopy, AGS cells were seeded on a one-well chamber glass slide for 2 d (1×10^6 AGS cells), and then inoculated with *H. pylori* at 1×10^8 bacterial cells at a multiplicity of infection (MOI) of 100, which were treated with McIlvaine buffer (pH 7 and pH 5) or PBS. In some experiments, supernatant of heat-treated *H. pylori* suspension or *H. pylori* lipopolysaccharide (LPS) was inoculated to the cell culture. The cells were then cultured in Ham's F12 medium, harvested 1 h to 24 h after the inoculation, washed with PBS twice and stained with Giemsa solution. The number of cells with HBC features and other morphological changes were observed under an Olympus BH-2 microscope (Tokyo, Japan).

Enzyme immunoassay (EIA):
Mouse monoclonal and rabbit polyclonal antibodies against *H. pylori* CagA (Austral Biologicals, CA, USA), and a horseradish-peroxidase-conjugated anti-mouse IgG antibody (STR, Tokyo, Japan) were used as previously described [17].

In brief, *H. pylori* cells were lysed by sonication for 30 min (W-208, Masuda, Osaka, Japan), and clarified by centrifugation at 10,000 g for 5 min. The supernatant was harvested as a sample. We used an immunoaffinity-purified rabbit polyclonal anti-*H. pylori* CagA antibody adsorbed to microwells. The microwells were incubated at 4 ˚C for 24 h. After washing with 0.05% Tween-20 in PBS (TPBS), the supernatant of the bacterial lysate was diluted and added to the wells, which were then incubated for 1 h and then washed 5 times with TPBS. A mouse monoclonal anti-*H pylori* CagA antibody was added to the wells, which were then incubated for 1 h at room temperature and then washed. A horseradish-peroxidase-conjugated anti-mouse IgG antibody was added to the wells, which were then incubated for 1 h at room temperature. The wells were washed with TPBS to remove any unbound materials, and 2, 2'-azino-di-(3-ethyl-benzthiazoline-6-sulfonate) (ABTS) (KRL, Gaithersburg MD, USA) was added before another 40 min incubation. A stop solution (10 % SDS:

sodium dodecyl sulfate) was added and absorbance was measured spectrophotometrically at 405 nm.

LPS extraction and measurement:

H. pylori LPS was extracted used LPS extraction kit (iNtRON Biotechnology, Kyungki-Do, Korea). The kit functions on the basis of the principle of hot phenol-water extraction. The amount of extracted samples was determined using an endotoxin test kit (BioWhittaker, MD, USA) which converted to LPS quantity unit (mg/ml). The kit is used in referenced standard endotoxin (RSE) / control standard endotoxin (CSE) potency determinations in conjunction with limulus amebocyte lysate products. The extraction and measurement of LPS were carried out according to the manufacturer's instructions.

Results

To clarify whether *H. pylori* induced unique morphological changes other than the HBC formation, *H. pylori*-inoculated AGS cells were observed under a light microscope. The hummingbird cells (HBC) formations were observed as previously reported by Segal et al. [13], and multinuclear giant cells (MNGC) were also observed in a culture inoculated with wild-type *H. pylori*. Representative light microscopic photographs of number of MNGC and HBC formed among AGS cells line are shown in **Fig. 1A**: The MNGC were 80-120 μm in diameter and contains 3-8 nuclei. Upon careful observation, AGS cells noninoculated with *H. pylori* showed a very small number of MNGC (**Fig. 1B**). Approximately 4000 cells of each culture were counted and the percentages of number of MNGC were compared between AGS cell cultures inoculated and those noninoculated with wild-type *H. pylori*. The percentage frequently observed in the cultures inoculated with wild-type *H. pylori* 24 h after the inoculation (**Fig. 1C**). The difference in percentage for MNGC between AGS cell cultures inoculated and those noninoculated with wild-type *H. pylori* was found to be statistically significant (**Fig.** 1C; $p<0.01$)

The above findings indicate that the increase in the number of MNGC in AGS cell line was associated with factors of *H. pylori*. To clarify whether the enhancement in the number of MNGC formation is associated with *H. pylori* CagA, the MNGC formation was compared with that of HBC associated with CagA under different experimental conditions. First, HBC and MNGC were counted at various incubation periods, after the inoculation of *H. pylori* (**Fig. 2A**). The
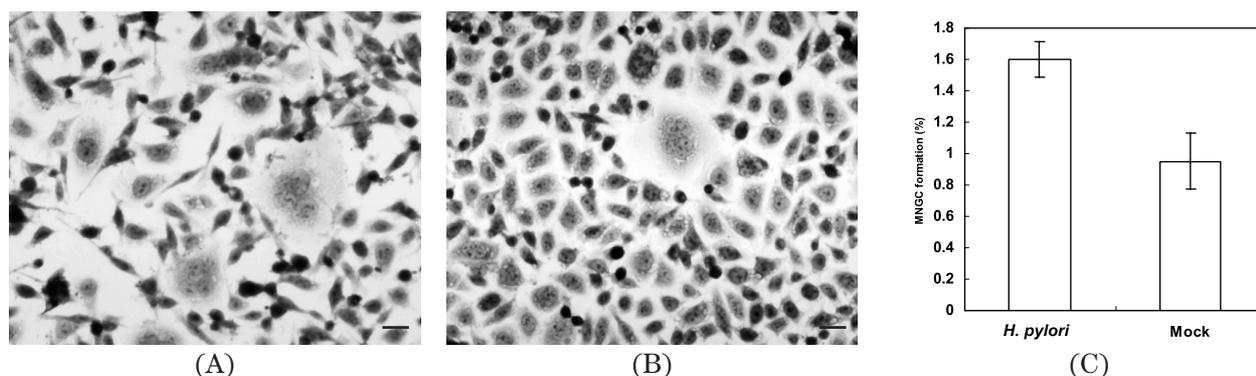

(A) (B) (C)

Fig. 1 *H. pylori* induced MNGC formation in AGS cell line after 24 h of incubation.
The morphology of AGS cells was examined under a light microscope 1 h after incubation with *H. pylori*. The AGS cells were stained with Giemsa. AGS cells incubated with wild-type *H. pylori* cells for 24 h showed MNGC and HBC formation (A) MNGC formation, which was observed as a unique morphological change in AGS cells, was frequently observed in a culture inoculated with wild-type *H. pylori*. As compared with AGS cells uninoculated with *H. pylori* as control (B), MNGC formation occurred less frequently. Bar: 10 μm. The percentage of number of MNGC formed in *H. pylor*-inoculated AGS cells is shown in (C). The percentage markedly increased in wild-type *H. pylori*-inoculated AGS cells. The difference in the percentage of MNGC formed was found to be statistically significant between wild-type *H. pylori*-inoculated and -noninoculated AGS cells ($p<0.01$).
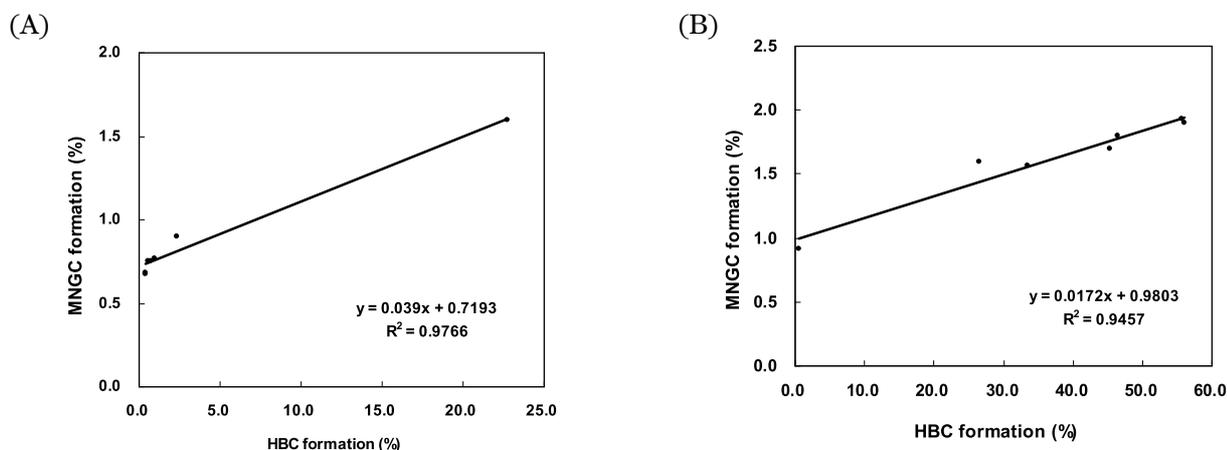
(A)

(B)

Fig. 2 Correlation diagram of HBC and MNGC formations in AGS cells induced by *H. pylori*.
(A) The correlation coefficient of HBC and MNGC formations markedly increased with time of incubation with *H. pylori*. (B) The correlation coefficient of HBC and MNGC formations markedly increased with the concentration of wild-type *H. pylori* after 24 h of incubation.

increased percentage for MNGC correlated to that of HBC with a high correlation coefficient (R^2 = 0.9766). Second, the formations of MNGC and HBC after the inoculation of *H. pylori* at various densities were compared (**Fig. 2B**). The increase in the number of both cells correlated well with each other (R^2 = 0.9457). The correlation suggests that the increase in the number of MNGC is induced by CagA, which also enhances HBC formation.

Wu et al. [17] reported that acid-treated *H. pylori* specifically accelerates HBC formation by the association with the intrabacterial rapid nanotransportation of CagA. To examine whether MNGC formation is associated with CagA, AGS cells inoculated with acid-treated *H. pylori* were observed for the acceleration of MNGC formation compared with that of HBC formation. Acid-treated *H. pylori* rapidly induced HBC formation compared with neutral-pH-treated *H. pylori* (**Fig. 3A**). In contrast, acid-treated *H. pylori* as well as neutral-pH-treated *H. pylori* did not accelerate MNGC formation (**Fig. 3B**). These findings suggest that MNGC formation in *H. pylori*-inoculated AGS cell line is not associated with CagA.

To confirm that CagA does not induce MNGC formation, 20 clinical isolates were examined for MNGC and HBC formations and compared in terms of CagA production. We obtained 15 clinical isolates with a high CagA production and 5 isolates with a low production and found that HBC

formation strongly correlated with CagA production (R^2=0.9098). In contrast, a strong correlation was not found between CagA production and the induction of MNGC formation (R^2=0.4544, **Fig. 4**). These findings indicate that MNGC formation is not induced by CagA, and MNGC formation is activated by factors other than CagA.

To determine the causative factor for MNGC formation, we examined whether the causative factor of *H. pylori* is heat-stable. Heat-treated *H. pylori* induced MNGC formation similarly to untreated *H. pylori* (**Fig. 5A**). On the other hand, HBC formation was not induced by heat-treated *H. pylori* (**Fig. 5B**). The results indicate that the causative factor for MNGC formation is a heat-stable factor.

To clarify whether the heat-stable factor is soluble in water, AGS cells were incubated with the supernatant of heat-treated *H. pylori* and the percentage for MNGC formed as determined after incubation. The percentage for MNGC increased for AGS cells incubated with the supernatant of heat-treated *H. pylori* and untreated *H. pylori* (**Fig. 6**). The difference in the percentage of MNGC formed was found to be statistically significant between AGS cells incubated and not incubated with the supernatant of heat-treated *H. pylori* (p<0.01), suggesting that the heat-stable factor is dissolved in water.
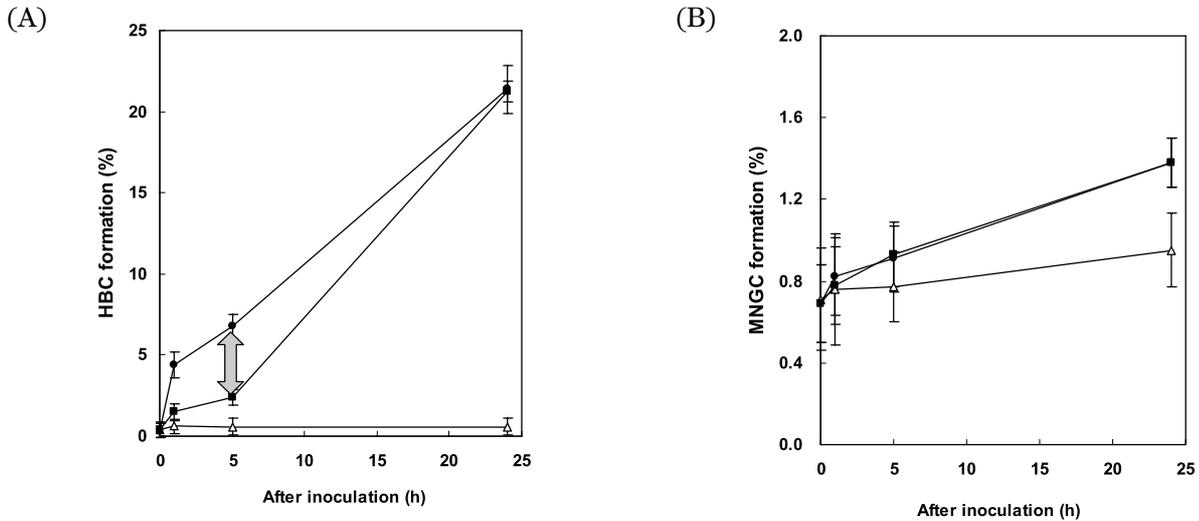
(A)

(B)

Fig. 3 MNGC and HBC formations in AGS cells induced by acidic-pH-treatment of *H. pylori.*
    (A) Acceleration of HBC formation in AGS cells induced by acidic-pH-treatment of *H. pylori.*
        CagA-specific cytotoxicity induced by *H. pylori* treated at different pHs. AGS cells showing
        the hummingbird phenotype were counted in five different fields in each of four chambers.
        About 4000 AGS cells were counted per sample in each experiment. The experiments were
        performed in triplicate. The number of cells showing the hummingbird phenotype with time
        was determined. The percentage of number of HBC was higher for those inoculated with *H.*
        pylori cells treated at acidic pH than for those inoculated with *H. pylori* cells treated at
        neutral pH immediately after inoculation.
    (B) MNGC formation in AGS cells induced by inoculation of *H. pylori* treated at different pHs.
        The difference in the percentage of numbers of MNGC was not found between AGS cell
        lines inoculated with acidic pH and neutral pH treated *H. pylori* statistically (α=0.01). Wild-
        type *H. pylori* cells were treated at pH 7 (■) and pH 5 (●); △ indicates uninfected.
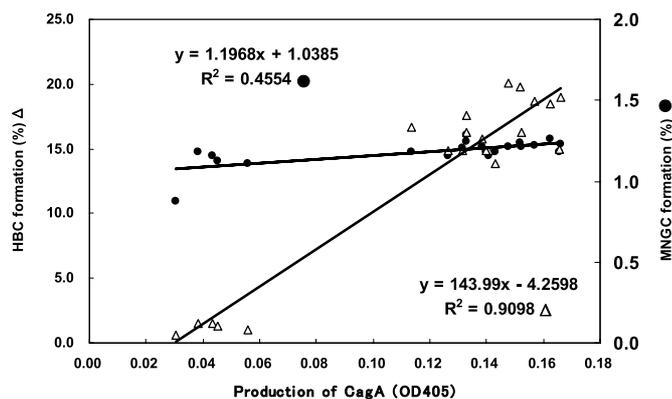

Fig. 4 Correlation diagram of HBC and MNGC formation in AGS cells after inoculation with *H. pylori*
    at different CagA expression levels.
    The correlation coefficient of HBC and CagA was markedly high after inoculation with *H. pylori*
    at different CagA expression levels. However, the correlation coefficient of MNGC and CagA was
    markedly low.
        ● indicates the percentage of MNGC formed; △ indicates the percentage of HBC formed.
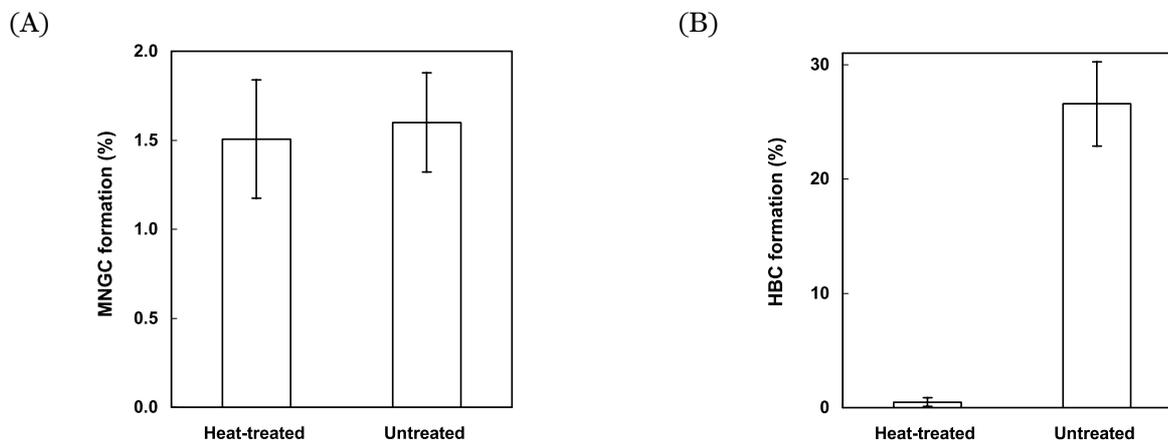
(A)

(B)

Fig. 5   Influence of percentage of number of MNGC and HBC formed in AGS cell line after heat-treated *H. pylori* incubation for 24 h.
(A) The percentage of MNGC formed in the AGS cells after heat-treated *H. pylori* inoculation was similar to that after untreated *H. pylori* inoculation. (B) The percentage of HBC formed was markedly higher after non-heat-treated *H. pylori* incubation than that after heat-treated *H. pylori* inoculation.
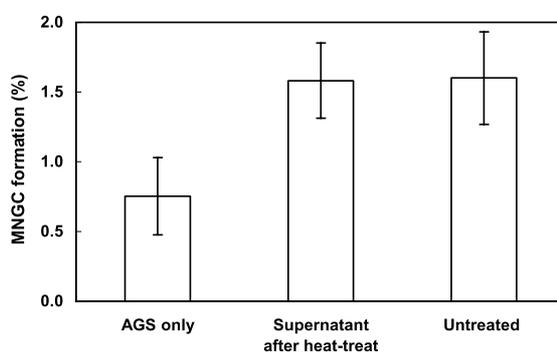

Fig. 6   MNGC formation in AGS cell line incubated by supernatant of heat-treated *H. pylori*.
The percentage of MNGC formed in AGS cell line induced by the supernatant of heat-treated *H. pylori* inoculation was similar to that of the untreated one. The difference in the percentage of MNGC formed was found to be statistically significant between AGS cells treated with the supernatant of heat-treated *H. pylori* and the untreated AGS cells (p<0.01)

One of the heat-stable and water-soluble toxins of *H. pylori* is LPS which is a component of the outer membrane of gram-negative bacteria. To clarify whether *H. pylori* LPS also induces MNGC formation, AGS cells were incubated with different concentrations of *H. pylori* LPS and the percentage of number of MNGC formed was measured. LPS induced MNGC formation in AGS cells in a concentration-dependent manner (**Fig. 7A**) and a time-dependent manner (**Fig. 7B**). These findings indicate that *H. pylori* LPS is the causative factor for MNGC formation.

Discussion

The *in vitro* cytopathogenicity of *H. pylori* CagA has been reported to cause a unique morphological change termed the hummingbird phenotype in the AGS cell line [12,14]. One of the other cytopathogenic factors of *H. pylori* is VacA [8,15,16], which induces the vacuolization of host cells *in vitro*. Another possible cytopathogenic factor is LPS of *H. pylori*; however, the morphological changes LPS in a epithelial cell line have not been reported. In this study, we found that *H. pylori* enhanced MNGC formation in the AGS cell line and HBC formation induced by
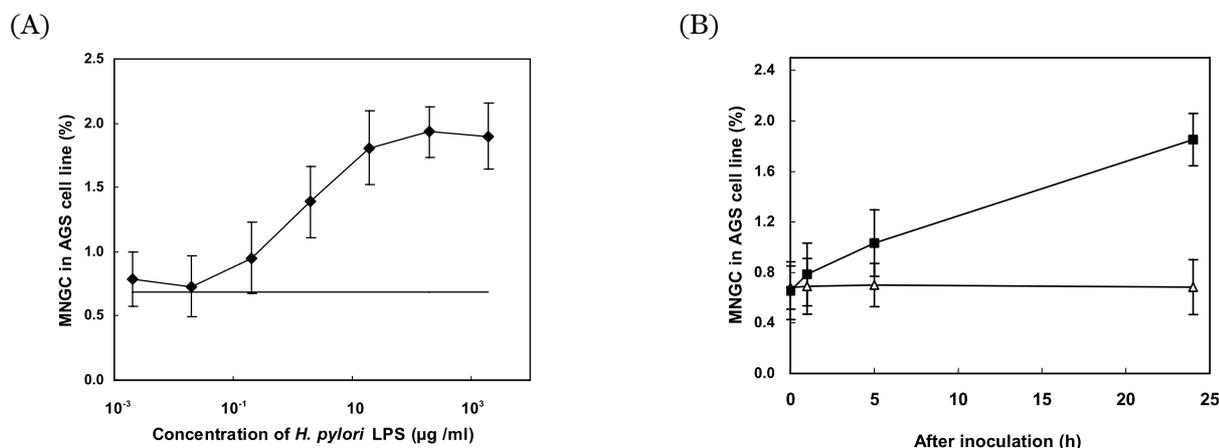
(A)

(B)

Fig. 7   Concentration and time course relationship between MNGC formation and *H. pylori* LPS in AGS cell line.
(A) The x-axis is shown as log LPS concentration. The number of MNGC in AGS cells incubated at different concentrations of *H. pylori* LPS was determined and showed concentration-dependence. (B) The number of MNGC during a time course was determined and showed time-dependence. *H. pylori* LPS (10^3 µg/ml) was incubated with AGS cell line (■); – indicates no *H. pylori* LPS as control.

CagA. The enhancement of MNGC formation, however, was not associated with CagA. We characterized the causative factor in *H. pylori* for MNGC formation as heat-stable and water-soluble, and finally considered that the causative factor is LPS. The enhancement of MNGC formation by *H. pylori* LPS, a new unique morphological change of AGS cell line, may provide an important marker that clarify the mechanism by which *H. pylori* pathogenesis in human gastric epithelial cells.

H. pylori LPS is capable of disrupting gastric mucosal integrity, has an inhibitory effect on gastric mucin synthesis through the laminin receptor [18], induces apoptosis of gastric mucosal cells [19], and induces gastric mucosal inflammation [9,20,21]. Slomiany BL and Slomiany A [9] reported that the *H. pylori* LPS inhibitory effect on gastric mucin synthesis is accompanied by marked increases in endothelin-1 generation, endothelin-convering-1 activity and severity of inflammatory involvement elicitation. It is not entirely clear, however, whether *H. pylori* LPS plays a main role in carciogenesis and inflammation of the stomach.

The enhancement of MNGC formation in AGS cells may be useful in the study on cytopathogenicity of *H. pylori* LPS *in vitro*.

Lepper et al. [22] reported that *H. pylori* LPS-induced cell activation is mediated through TLR2, in addition to antagonize TLR4, which is an advantage over the host and may be associated with the clinical outcome of *H. pylori* infection. Moreover, Ohmae et al. [23] reported that *H. pylori* activates the alternative NF-κB pathway in B lymphocytes through the release of cytokines resulting in gastric MALT lymphoma. These reports indicated that the inflammation of the gastric epithelium by *H. pylori* is induced through the signal transduction of host cells; therefore, MNGC formation in the AGS cell line may be enhanced by these modifications of signal transduction induced by *H. pylori* infection. Concerning MNGC in the AGS cell line, the morphological change was observed only in the AGS cell line, but not in other culture cell lines such as the Vero cell line and HeLa cell line (data not shown). It should be noted that uninfected AGS cell line showed a small number of MNGC. Therefore, MNGC formation may be associated with another factor in the AGS cell line. Although we could not identify the factor in this study, it may play an important role in the morphological changes in the AGS cell line.

In conclusion, we discovered MNGC formation in AGS cell line, consider that *H. pylori* LPS enhances the formation, and suggest that this enhancement may be useful in the study of *H. pylori* LPS.

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