BACTERIAL PATHOGENICITY

Establishment and characterisation of a monoclonal antibody to inhibit adhesion of *Helicobacter pylori* to gastric epithelial cells

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Monoclonal antibodies (MAbs) that inhibit adhesion of *Helicobacter pylori* to human gastric cancer (MKN45) cells were established to clarify the mechanism of adhesion of *H. pylori*. Of 53 hybridoma clones screened by the primary inhibition assay for adhesion, MAb A20 of IgM class was selected on the basis of both its reactivity to whole cells of *H. pylori* by ELISA and its inhibitory effect on adhesion of *H. pylori*. The adhesion of *H. pylori* strain TK1029 to MKN45 cells was inhibited by MAb A20, depending on the concentration of the MAb. The MAb recognised the surface antigen, lipopolysaccharide (LPS) of *H. pylori*, suggesting that LPS is associated with adhesion of *H. pylori* to human gastric epithelial cells.

Introduction

Helicobacter pylori is a causative agent of active chronic gastritis and gastro-duodenal ulcer, and may be associated with gastric cancer and lymphoma [1–4]. The micro-organism has been shown to colonise gastric mucus and to attach to mucosal epithelial cells by histological technique [5] and by electron microscopy. Adhesion of bacteria to mucosal surfaces is essential for colonisation and subsequent pathogenesis. The bacterial adhesion factors, which include flagella, capsules, glycocalyxes and lipopolysaccharides (LPS), are also important as virulence factors. *H. pylori* has a strong ability to adhere in various in-vitro test systems [6–10]. Various binding specificities and putative adhesins of *H. pylori* have been reported: an N-acetylneuraminylactose (NAML)-binding fibrillar haemagglutinin [11], haemagglutination activities with strain specificity [12, 13], adhesin binding to GM₃ ganglioside and sulphatides [14, 15] and laminin-binding protein [16, 17]. Similarly, various types of receptor for *H. pylori* have been reported, e.g., phosphatidyl-ethanolamine [18, 19] and Lewis^b blood group antigen [20]. NAML-binding fibrillar haemagglutinin is now known to be an intracellular lipoprotein and the adhesin recognising phosphatidyl-ethanolamine is the catalase

of *H. pylori*. However, it is not known whether a *H. pylori*-specific adhesin is involved in adhesion to epithelial cells. These adhesion properties alone may not explain tissue adhesion of *H. pylori*.

In the present study, to clarify the mechanism by which *H. pylori* adheres to gastric epithelial cells, establishment of a monoclonal antibody (MAb) to inhibit adhesion of *H. pylori* to human gastric epithelial cells was attempted.

Materials and methods

Bacterial strains and culture condition

H. pylori strain TK1029 used for immunisation of mice was isolated from gastric biopsy material from a patient with gastric ulcer. Other *H. pylori* strains were isolated from gastric biopsy materials. *H. pylori* NCTC11638 and *H. mustelae* NCTC12032 strains were kindly provided by Dr T. Ito (Tokyo Metropolitan Research Laboratory of Public Health, Japan). *Helicobacter* strains were cultured in Brain Heart Infusion (BHI) Agar (Difco) with horse blood 7% in an atmosphere of O₂ 5%, CO₂ 10%, N₂ 85% for 4 days at 37°C. *Campylobacter jejuni*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Shigella sonnei* and *Vibrio cholerae* were laboratory strains from the Department of Microbiology, Kyorin University School

of Medicine. These bacteria were cultured in BHI agar at 37°C under aerobic or micro-aerobic conditions.

Cell line

Human gastric carcinoma cells, MKN45 and Kato III, were obtained from the Japanese Cancer Research Resources Bank. The human intestinal cell line, Intestine (Int)-407 was also used. For the adhesion assay, cells were grown at 37°C in RPMI-1640 (Gibco BRL, Gaithersburg, USA) containing fetal calf serum (FCS) 10% and harvested from a flask by scraping with a sterile cell scraper.

H. pylori inhibition of adhesion assay

The inhibition of adhesion of *H. pylori* to epithelial cells by MAb was tested by the following two methods. As a screening test, a qualitative method based upon urease activity of *H. pylori* was used. MKN 45 cells (5×10^4 cells) were cultured in a 96-well plate coated with poly-L-Lysin (Sumitomo Bakelite Co. Ltd, Tokyo, Japan) 1% for 2 days. The monolayer MKN45 cells were washed once with RPMI-1640 containing FCS 10%, and the medium was aspirated completely. Culture supernates (50 µl) of hybridoma cells (MAb) and 5×10^6 cfu of *H. pylori* in 50 µl of RPMI-1640 were added to each well. After incubation for 30 min, the culture medium was aspirated from each well. The cells in the plate were washed twice with RPMI-1640, and 100 µl of urea broth medium (Difco) were added. After 1 or 2 h, the plates were examined for a change in colour of the medium. Red colour showed the presence of adherent *H. pylori*, whereas no change in the colour of the medium showed the inhibition of adhesion of *H. pylori* to MKN45 cells by the MAb.

To confirm the inhibitory effect of MAb, the adhesion of *H. pylori* was assessed by flow cytometry [21–24]. *H. pylori* (5×10^8 cfu), labelled at room temperature for 15 min with 4 µM lipophilic dye, PKH-2 (Zynaxis Cell Sciences, Phoenixville, PA, USA), were incubated with MAb A20 for 20 min at 4°C and washed twice with Hanks's Balanced Salts Solution containing gelatin 0.1% (HGS). MKN45, Kato III and Int-407 cells ($c. 1 \times 10^6$ cells) were washed once with HGS and resuspended in 500 µl of HGS. *H. pylori* were also resuspended in 500 µl of HGS and co-incubated with the cells for 1 h at 25°C. After incubation, the cells were washed once with PBS containing sucrose 15% and then washed twice with HGS. Fluorescence intensity of the cells was analysed by flow cytometry with a 530-nm filter (FACS Vantage, Beckton Dickinson Immunocytometry Systems, San Jose, CA, USA). Fluorescence data were obtained in a logarithmic mode on a 1024 channel scale.

MAb production

MAbs were prepared by the method described by

Yamaguchi *et al.* [25]. BALB/c mice were immunised with sonicated whole-cell antigen of *H. pylori* strain TK1029 and boosted three times. Hybridoma cells secreting a MAb were tested by ELISA with whole-cell sonicate and cell surface antigen of *H. pylori*, and the inhibition of adhesion assay, by assessment of urease activity of *H. pylori*, was performed. BALB/c mice, pretreated with pristane 4 days before inoculation, were inoculated intraperitoneally with cloned hybridoma cells secreting a MAb. The immunoglobulin of the ascites fluid obtained from the mice was purified with an Immunoglobulin-E-Z-Separation kit (Pharmacia Biotech Co., Tokyo, Japan).

ELISA

Microtitration plates (Greiner Labortechnik Japan, Tokyo, Japan) were coated at 4°C for 18 h with sonicated antigen (100 µg/ml) of *H. pylori*. After washing with PBS, the plate was incubated with PBS containing skim milk (Yukijirushi Nyugyo Co., Tokyo, Japan) 1% (PBS-S) for 1 h at room temperature. After washing with PBS, the plate was incubated with MAbs for 1 h at room temperature. The bound MAbs were detected with an affinity-purified goat anti-mouse IgG and IgM horseradish peroxidase conjugate (Biosource International, Camarillo, CA, USA) diluted 1 in 5000 with PBS-S. Then, the plates were developed with OPD buffer (0.1 M citric acid, 0.07 M sodium phosphate dibasic, H₂O₂ 0.015%) containing *o*-phenylenediamine 0.1%. After reaction at room temperature for 5 min, the developed colour was measured at 490 nm.

The whole-cell ELISA method was used [26] for the assay of reactivity to surface antigen of *H. pylori*. Microtitration plates were coated with 100 µl of a suspension of bacterial cells (8×10^7 cfu/ml) in Na₂CO₃ 0.38% solution containing methylglyoxal (Sigma) 0.3% v/v and incubated overnight at 4°C. The incubation steps and development were performed by the method described above.

Immuno-electron microscopy

The post-embedding labelling on ultra-thin sections were performed by the method described by Taguchi *et al.* [27]. *H. pylori* strain TK1029 was cultured on BHI agar containing horse blood 7% under micro-aerobic conditions for 3 days. The micro-organisms were washed with PBS and fixed with glutaraldehyde 2% in PBS for 2 h at room temperature. The cell pellet was dehydrated in a graded series of dimethylformamide at 4°C and embedded in Lowicryl K4M (Chemische Werke Lowi GmbH, Waldkaraiburg, Germany). After infiltration with Lowicryl K4M, UV polymerisation was performed for 24 h at 4°C. Ultra-thin sections were cut with glass knives and mounted on 400-mesh nickel grids. The grid was floated on a drop of PBS containing bovine serum albumin (BSA) 1% for 10 min and incubated with one drop of PBS containing

goat serum 5% for 10 min. The grid was washed with PBS and then incubated with one drop of PBS containing the diluted MAb A20 (10 μ g/ml) in BSA-PBS 0.1% for 1 h at room temperature. Then the grid was washed and floated on a drop of PBS containing a 1 in 50 dilution of 12 nm diameter colloidal gold particles-affinipure goat anti-mouse IgG + IgM (Jackson Immuno Research Laboratories, Inc. West Grove, PA, USA). After incubation at room temperature for 1 h, the unbound colloidal gold particles were removed by floating the grid on three drops of PBS. The sections were stained with a saturated aqueous solution of uranyl acetate for 30 s and with lead citrate solution for 1 min. The specimens were observed with a JEM-100 electron microscope (JEOL, Tokyo, Japan).

SDS-PAGE and immunoblotting

SDS-PAGE with acrylamide 10 or 15% w/v was performed by the method of Laemmli [28]. Protein or LPS was stained with a silver stain kit (BioRad Laboratories, Hercules, CA, USA). Separated proteins were transferred from the polyacrylamide gel to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). Electroblothing was performed in a semi-dry transblot apparatus (Millipore Corporation, Bedford, MA, USA) at 10 V for 45 min. After blocking with PBS-S, the membranes were incubated at room temperature for 30 min with MAb (10 μ g/ml) in PBS-S and then at room temperature for 30 min with goat anti-mouse IgG and IgM conjugated with horseradish peroxidase (Biosource International) diluted 1 in 1000 with PBS-S. Immunoblots were developed with 50 mM Tris saline containing H_2O_2 0.12% and 1 mM *o*-diaminobenzidine.

Detection of glycochain after Western blotting

SDS-PAGE and transblotting were performed as described above. The membrane was treated with G-P-sensor kit (Honen Corporation, Tokyo, Japan). After incubation with avidine conjugated with horseradish peroxidase at room temperature for 20 min, blots were developed as described above.

LPS preparation

LPS was prepared from whole cells by the hot-phenol-water technique [29]. The LPS preparations were purified by treatment with RNAase (Sigma) 50 μ g/ml and DNAase I (Sigma) 16 μ g/ml, followed by dialysis against 50 mM Tris buffer and centrifugation at 20 000 *g* for 30 min. The resulting suspension was freeze-dried.

Results

Selection of MAbs against *H. pylori* antigen

Culture supernates of hybridomas (862 clones) were screened for the production of antibodies to whole-cell sonicate by ELISA, and to surface antigen of *H. pylori* strain TK1029 by whole-cell ELISA. In addition, the inhibitory activity of MAbs for adhesion of *H. pylori* strain TK1029 to MKN45 cells was evaluated by qualitative adhesion assay based upon the urease activity of *H. pylori* (Fig. 1). The numbers of positive clones for ELISA, whole-cell ELISA and inhibition of adhesion assay were 251, 356 and 82, respectively. A total of 53 clones was selected by these criteria.

Fig. 1. Inhibition of adhesion of *H. pylori* to MKN45 cells by culture supernate of hybridoma cells according to urease activity of *H. pylori*. C8, E6 and F8 Wells exhibit no change in the colour of the medium indicating the inhibition of adhesion of *H. pylori* to MKN45 cells by MAb.

Establishment of MAb A20 to inhibit adhesion of *H. pylori* to MKN45 cells

These 53 clones were investigated for their inhibitory effect on adhesion of *H. pylori* to MKN45 cells by flow cytometry. Only one MAb A20 was selected according to these criteria. MAb A20 was confirmed to be of the IgM class. The adhesion of *H. pylori* to MKN45 cells was inhibited after co-cultivation with MAb A20 (Fig. 2). After co-cultivation with MAb A20 500 µg/ml, the inhibition rate estimated by comparison with positive control cells was 50.6%. None of the other 52 clones showed any inhibition of adhesion in flow cytometry.

A pretreatment experiment was performed to determine whether MAb A20 reacted with either *H. pylori* or MKN45 cells. Pretreatment of *H. pylori* with MAb A20 significantly inhibited its adhesion to MKN45 cells (Table 1). After pretreatment of *H. pylori* with MAb A20 500 µg/ml, the inhibition rate was estimated to be 74.4%. On the other hand, pretreatment of MKN45 cells with MAb A20 did not inhibit adhesion of *H. pylori* to these MKN45 cells at all (data not shown).

Kato III and Int-407 cells were used to examine the inhibition of adhesion of *H. pylori* to other cells by MAb A20 (Table 2). After treatment of *H. pylori* with

Table 1. Inhibition of adhesion of *H. pylori* to MKN45 cells by MAb A20 in flow cytometry

Concentration of MAb (µg/ml)	Fluorescence intensity*			Inhibition rate†
	Mean	SD	Peak channel	
500	89.5	66.6	119	74.4
250	110.1	80.7	143	68.3
125	146.5	106.9	164	58.1
0	347.9	219.5	284	—

* *H. pylori* (5×10^8 cfu, PKH-2 treated) was pretreated with each concentration of MAb A20 and then incubated with MKN45 cells (1×10^6 cells).

† $100 - (\text{mean fluorescence of the sample with MAb} / \text{mean fluorescence of the sample without MAb}) \times 100$.

Table 2. Inhibition of adhesion of *H. pylori* to epithelial cells by MAb A20*

Cell line	Concentration of MAb A20 (µg/ml)	Mean fluorescence intensity	Inhibition rate (%)†
Kato III	500	477.6	27.5
	0	658.4	—
Int-407	500	264.8	0
	0	266.7	—

* *H. pylori* (5×10^8 cfu, PKH-2 treated) was pretreated with MAb A20 and then incubated with each cell line (1×10^6 cells).

† $100 - (\text{mean fluorescence of the sample with MAb} / \text{mean fluorescence of the sample without MAb}) \times 100$.

Fig. 2. Inhibition of adhesion of *H. pylori* to MKN45 cells by MAb A20 in flow cytometry: ·····, negative control MKN45 cells; —, positive control, MKN45 cells were co-incubated with *H. pylori* strain TK1029; — —, MKN45 cells were co-incubated with *H. pylori* strain TK1029 in the presence of MAb A20 (500 µg/ml).

MAB A20 500 µg/ml, the inhibition of adhesion of *H. pylori* to Kato III cells was also demonstrated and the inhibition rate was 27.5%. In contrast, MAB A20 did not inhibit the adhesion of *H. pylori* to Int-407 cells.

Surface exposure of MAb-reactive antigens

To confirm that MAB A20 recognises surface antigen of *H. pylori*, immuno-electron microscopy was performed. The MAB A20 bound to the cell surface of *H. pylori* strain TK1029, but not to cytoplasm (Fig. 3). The mean numbers of gold particles counted inside and outside of *H. pylori* were 101.7 SD 32.9 and 876.7 SD 62.3, respectively (data not shown).

Enzyme treatment and detection of glycochain

To characterise the MAB A20-recognising antigen, immunoblot analysis after SDS-PAGE of whole *H. pylori* antigen with or without treatment with proteinase K was performed. MAB A20 reacted with a 35-kDa antigen of the whole-cell lysate of *H. pylori* strain TK1029 (Fig. 4A). Treatment with proteinase K did not inhibit the reaction of MAB A20, although the same treatment inhibited completely the reaction of MAB H20, which recognises heat-shock protein 60 of *H. pylori* (data not shown). Glycochain was detected by the G-P sensor kit in the same size molecule recognised by MAB A20 (Fig. 4B).

Reactivity of LPS of H. pylori with MAB A20

Immunoblot analysis and ELISA of purified LPS of *H. pylori* strains TK1029 and TK1028 were performed with MAB A20. The purified LPS of strains TK1029 and TK1028 were reactive with MAB A20 in immunoblot analysis (Fig. 5). In ELISA, it was also shown that the purified LPS reacted with MAB A20 (data not shown).

Specificity in the reaction of LPS with MAB A20

A total of 42 strains of *H. pylori* was tested by ELISA with MAB A20 (Table 3). MAB A20 reacted with 31 strains of *H. pylori* examined. The strains of *H. pylori* were separated into three groups according to their reactivity in ELISA. The adhesion of highly reactive strains ($OD_{490} > 1.0$) of *H. pylori* was inhibited after treatment with MAB A20. Correlation between the reactivity with MAB A20 in ELISA and inhibitory effect of MAB A20 on adhesion estimated by flow cytometry among *H. pylori* strains was statistically significant ($r = 0.7436$).

MAB A20 had no reactivity in ELISA with other bacteria such as *C. jejuni*, *E. coli*, *H. mustelae*, *K. pneumoniae*, *P. mirabilis*, *Ps. aeruginosa*, *S. marcescens*, *Sh. sonnei* and *V. cholerae* (data not shown).

Fig. 3. Electron micrograph of *H. pylori* strain TK1029 incubated with MAB A20 with immunogold labelling ($\times 58\,000$). Gold particles were mainly observed on the surface of *H. pylori*, but not in the cytoplasm.

Fig. 4. Immunoblot analysis of *H. pylori* strain TK1029 with MAb A20 (A) and detection of glycochain (B) after SDS-PAGE of *H. pylori* strains.

Fig. 5. SDS-PAGE and immunoblot analysis of purified LPS of *H. pylori* strains with MAb A20 (A) SDS-PAGE of LPS of strains TK1029 (lane 1) and TK1028 (2) followed by staining with silver reagent kit; (B) immunoblot analysis after SDS-PAGE of LPS of strains TK1029 (lane 1) and TK1028 (2) with MAb A20.

Table 3. Reactivities of *H. pylori* strains with MAb A20 and inhibition of adhesion of *H. pylori* to MKN45 cells by MAb A20

ELISA value* (OD ₄₉₀)	Number of strains examined	Number of inhibition- positive strains†
> 1.0	16	16
0.1–1.0	15	2
< 0.1	11	0

*Microtitration plate was coated with 2500 ng of whole-cell lysate of *H. pylori*/well.

†*H. pylori* (5×10^8 cfu) were incubated with 1×10^6 MKN45 cells at 37°C for 1 h with MAb A20 (250 µg/ml). Positive control was incubated without MAb A20.

Discussion

The study established MAb A20, as an inhibitor of adhesion of *H. pylori* to MKN45 cells (Figs. 1–3). Pretreatment of *H. pylori* with MAb A20 inhibited the adhesion of *H. pylori* to MKN45 cells, depending on the concentration of the MAb (Table 1), but pretreatment of MKN45 cells with the MAb did not inhibit adhesion of *H. pylori* at all. These data show that the

MAb blocked bacterial substances involved in the adhesion system. After pretreatment with MAb A20 500 µg/ml, the inhibition effect was incomplete and the inhibition rate estimated by comparison with positive control cells was 74.4%. Even when lower numbers of *H. pylori* ($c. 5 \times 10^6$) were used for the adhesion assay, the inhibition of adhesion by MAb A20 was still only partial (data not shown). It is possible that several receptor–ligand interactions are involved in *H. pylori* adhesion to gastric epithelial cells.

A previous study compared the adhesion of *H. pylori* to MKN45, Kato III, Int-407 and MKN28 cells [23]. It was shown that cell line MKN45 is the most sensitive for adhesion of *H. pylori* and Int-407 is the least sensitive. In the present study, it was observed that adhesion of *H. pylori* to Int-407 cells was not inhibited by MAb A20. These results suggest that Int-407 cells have a different type of adhesion receptor to MKN45 and Kato III cells.

It was shown that the adhesion of *H. pylori* to MKN45 cells could be inhibited by MAb A20 recognising the bacterial surface antigen, LPS.

Furthermore, purified LPS of *H. pylori* strain TK1029 bound to MKN45 cells. This result indicates that LPS is also an important factor for adhesion of *H. pylori* to MKN45 cells. The role of LPS in adherence to host cells has been reported recently. Valkonen *et al.* [30] showed that initial recognition and binding of laminin by *H. pylori* may occur through LPS. A similar adhesion mechanism involving LPS and protein receptors on epithelial cells has been described for *C. jejuni* [31], *Sh. flexneri* [32], *Actinobacillus pleuropneumoniae* [33], *E. coli* [34], *Pasteurella multocida* [35], and *Ps. aeruginosa* [36]. These LPSs show inhibition of adhesion of these bacteria to various host cells. LPS of *H. pylori* can inhibit the interaction between laminin and its receptor on epithelial cells [37], but LPS has received only limited attention as a potential tissue adhesin [38]. However, LPS is a surface antigen of *H. pylori*, and anti-LPS MAb A20 may be able to agglutinate bacteria. This is likely to result in decreased adhesion. It is also possible that MAb A20 masks another surface adhesin and blocks adhesion of *H. pylori*.

MAb A20 had different reactivity to *H. pylori* strains tested in ELISA, suggesting antigenic variety of *H. pylori* in terms of LPS expression. Moran *et al.* [38] found structural or substitutional differences, or both, in the core composition of the rough LPS of various strains. Doig and Trust [39] also reported that two MAbs, recognising the core region of LPS and the flagella sheath of *H. pylori*, were strain-specifically reactable.

The biological activities of LPS of *H. pylori* have been compared with activities of LPS of Enterobacteriaceae. Mitogenicity in mouse spleen cells, pyrogenicity in rabbits, lethal toxicity in galactosamine-sensitized mice and macrophage activation of LPS of *H. pylori* were demonstrated, but its activity was lower than that of Enterobacteriaceae [40, 41]. Generally, LPS is an immunodominant substance and anti-LPS MAb-producing hybridoma is found with high frequency. In the present study, almost 900 hybridoma cells were assayed and only one, MAb A20, was selected.

It has recently been reported that MAbs specific for Lewis blood group antigen (Le^X and Le^Y) reacted with LPS of *H. pylori* strains in immuno-electron microscopy and ELISA, and that the MAbs specific for *H. pylori* LPS reacted with purified Lewis antigens [42, 43]. These data show that *H. pylori* expresses a complex surface carbohydrate corresponding to Lewis blood group antigens in LPS. In the preliminary study, the MAb specific for Le^X reacted with the antigen which MAb A20 recognised from whole-cell lysate of strain TK1029 in immunoblot analysis (data not shown). Whether the MAb A20 blocks the core O-antigenic chain or another component of LPS as terminal Lewis antigen remains to be demonstrated.

This research was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture.

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