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Role of Sulfatides in Adhesion of *Helicobacter pylori* to Gastric Cancer Cells

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We have demonstrated that clinical isolates of *Helicobacter pylori* preferentially bind to sulfatides (I^3SO_3-GalCer) and GM_3 gangliosides (II^3NeuAcLacCer), two predominant acidic glycosphingolipids in the human gastric mucosa, on thin-layer chromatography plates. However, it has not yet been clarified that these glycosphingolipids truly serve as adhesion receptors for *H. pylori* in live cells. In this study, we used a gastric cancer cell line, KATO III, as a cellular model of *H. pylori* adhesion and examined the role of sulfatides in attachment. The adhesion of *H. pylori* (i.e., a standard strain of *H. pylori*, NCTC 11637) to KATO III cells and the effects of various substances on this adhesion were monitored and semiquantitated by flow cytometric analysis. Sulfated glycoconjugates, such as heparin and gastric mucin, significantly inhibited *H. pylori* adhesion to KATO III cells. Membrane preparations from KATO III cells strongly inhibited this adhesion. In the membrane preparations, sulfatides were present as a major acidic glycosphingolipid. With the exception of sulfatides, no distinct adhesion of *H. pylori* to glycosphingolipids from KATO III cells was observed. Moreover, *H. pylori* did not bind to any membrane proteins of KATO III cells. Finally, a monoclonal anti-sulfatide antibody markedly reduced *H. pylori* adhesion to KATO III cells. These results suggest that sulfatides, and possibly related sulfated compounds, serve as a major receptor for cell adhesion by *H. pylori*.

Helicobacter pylori has been recognized as a principal cause of chronic gastritis. Moreover, a number of investigators have demonstrated the association of *H. pylori* infection with peptic ulcers (2, 7). It has been demonstrated that *H. pylori* colonizes the epithelial surface of the gastric mucosa and in many cases forms a specific adhesion apparatus (8). However, the precise mechanisms underlying *H. pylori* adhesion have yet to be clarified.

Several candidate molecules have been proposed as receptors for *H. pylori* adhesion: *N*-acetylneuraminyllactose (5, 6), GM_3 ganglioside (17), sulfated lactosylceramide (13), phosphatidylethanolamine (13), and Lewis^b (Le^b) antigens (3). In a previous study, we found that clinical isolates of *H. pylori* bound specifically to sulfatides and GM_3 gangliosides, both of which are major acidic glycolipids in the human gastric mucosa (16), on thin-layer chromatography (TLC) plates. Whether these glycosphingolipids truly serve as cellular receptors for *H. pylori* adhesion remains, however, to be determined. The present study was undertaken to examine the role of glycosphingolipids in the adhesion of *H. pylori* to viable cells.

The abbreviated nomenclature for glycolipids used in this paper is based on the recommendation of the IUPAC-IUB commission (8a).

MATERIALS AND METHODS

Bacterial strain and bacterial culture. A standard strain of *H. pylori*, NCTC 11637, was cultured in Belo-Horizonte medium with Skirrow's selective supplement (Nikken Biomedical Laboratory, Kyoto, Japan) on agar plates in a microaerobic atmosphere at 37°C for 4 to 5 days. Subsequently, the bacteria were transferred to a liquid medium of brucella broth (Difco Laboratories, Detroit, Mich.) containing 10% fetal bovine serum and cultured in a microaerobic atmosphere for 24 h.

Cell culture. KATO III cells, a gastric cancer cell line, were cultured in a 1:1 (vol/vol) mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-10 medium supplemented with 10% fetal bovine serum in polystyrene tissue culture flasks at 37°C. The cells grew essentially in suspension, and some of the cells loosely attached to the base of the flask. For bacterial adhesion experiments, the cells were gently harvested with a rubber policeman (Coster, Cambridge, Mass.) without trypsin digestion or EDTA treatment.

Adhesion experiments. The assay of adhesion of *H. pylori* to KATO III cells conformed essentially to the methods of Dunn et al. (4). In short, KATO III cells at a concentration of 10^6 cells per ml were pelleted by centrifugation at $200 \times g$ for 5 min, and 1 ml of *H. pylori* at desired concentrations suspended in phosphate-buffered saline (PBS) was mixed with KATO III cells. After incubation, nonadherent bacteria were removed by centrifugation at $200 \times g$ through 15 ml of a 15% (vol/vol) sucrose layer. KATO III cells were washed once in PBS and then incubated for 20 min at 4°C in 100 μl of rabbit anti-*H. pylori* antibody diluted to 1/20 (16). After being washed twice in PBS, the cells were incubated for an additional 20 min at 4°C in 100 μl of a fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (Cappel Laboratories, Cochranville, Pa.) diluted to 1/20. The cells were then washed twice in PBS and fixed in 1.5 ml of 1% *p*-formaldehyde in PBS.

Analysis of bacterial adhesion to KATO III cells. The measurement of bacterial adhesion to KATO III cells was performed with a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.). The data were analyzed with a Lysis software program from Becton Dickinson. This program produced histograms of fluorescence intensity of each cell sample in proportion to the surface density of fluorescently labelled *H. pylori* adhering to the cell and calculated the mean fluorescence intensity (MFI) of each cell sample. Fluorescent microscopic observation of bacterium-cell interactions was also done.

Inhibition of adhesion. The following materials were tested to assess any possible influence on adhesion: mucin from the porcine stomach, heparin, fetuin, asialofetuin, *N*-acetylneuramic acid, L-fucose, D-galactose, and D-mannose (Sigma Chemical Co., St. Louis, Mo.). All of these materials except heparin were used at final concentrations of 0.001 to 1.0 mg/ml. Final concentrations of 10 to 1,000 U/ml were used for heparin. After preincubation of *H. pylori* with each material for 30 min, the assay procedure described above was carried out. The effects of membrane fractions from KATO III cells, a monoclonal anti-sulfatide antibody, a monoclonal anti-GM_3 ganglioside antibody, and a monoclonal anti-Le^b antibody on adhesion were also examined. The specificities of these antibodies have been described previously (9, 12, 19). Culture supernatants of each hybridoma were used as a source of antibody to sulfatides or GM_3 gangliosides. Ascites fluid from hybridoma-bearing mice was used as a source of the anti-Le^b antibody. Globulin fractions from these culture supernatants and ascites were

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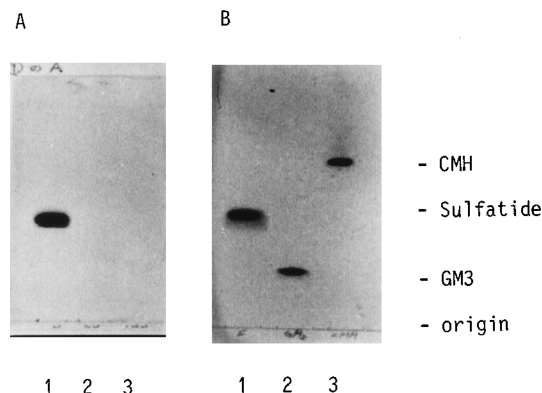

FIG. 1. Recognition of glycosphingolipids by *H. pylori* NCTC 11637. Glycosphingolipids were chromatographed in a solvent system with chloroform-methanol-water (65:35:8, vol/vol). (A) The plate was incubated with *H. pylori* and then stained by TLC-immunostaining as described in the text. (B) The spots were located with orcinol-H_2SO_4 reagent. Lanes: 1, standard sulfatides from bovine brain; 2, standard GM_3 gangliosides from bovine brain; 3, standard ceramide monohexosides from bovine brain.

obtained by salting out with 50% saturation of ammonium sulfate. Subsequently, precipitates were dissolved in 10 mM phosphate buffer (pH 7.4) and dialyzed against 10 mM phosphate buffer (pH 7.4) for 2 days. These purified antibodies were used in this study. The protein concentrations of these antibody preparations as measured with bicinchoninic acid protein assay reagent (Pierce, Rockford, Ill.) (18) using bovine serum albumin as a standard were as follows: anti-sulfatide antibody, 0.66 mg/ml; anti-GM_3 ganglioside antibody, 1.25 mg/ml; and anti-Le^b antibody, 92 mg/ml.

Adhesion of *H. pylori* to glycosphingolipids purified from KATO III cells on TLC plates. KATO III cells were harvested by a rubber policeman and washed in PBS. Subsequently, the total lipid contents were extracted from cells with chloroform-methanol (1:1, vol/vol). The total lipid extracts were then fractionated into neutral and acidic glycolipids by DEAE-Sephadex (A-25; acetate form) column chromatography. The neutral and acidic glycosphingolipids from each fraction were purified by a previously reported method (10).

Glycosphingolipids purified from KATO III cells were developed with chloroform-methanol-water (65:35:8, vol/vol) on silica-gel-coated plates (Polygram, SilG; Macherey-Nagel, Düren, Germany). The plates were blocked with 1% bovine serum albumin at 4°C overnight. The *H. pylori* overlay method was then carried out on a TLC plate as described previously (16).

To examine the role of Le^b antigens in *H. pylori* adhesion, we performed TLC-immunostaining on glycolipids extracted from KATO III cells with a monoclonal anti-Le^b antibody. Furthermore, bacterial attachment to a standard Le^b glycolipid ($III^4IV^2Fuc_2$-Lc_4Cer) applied to a TLC plate was also examined.

Adhesion of *H. pylori* to KATO III cell membrane proteins. After the membrane proteins from KATO III cells had been separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the separated proteins were transferred electrically to a polyvinylidene fluoride membrane (Millipore, Bedford, Mass.). The membrane was blocked with 5% bovine serum albumin at 4°C overnight. After being washed with PBS, the membrane was incubated with *H. pylori* in Tris-buffered saline (pH 7.9) at 37°C for 1 h. After the membrane was washed with a washing buffer (0.1% Tween 20 in Tris-buffered saline), bound bacteria on the blotted membrane were detected by a bacterial overlay method as described previously (16).

Localization of glycosphingolipid antigens on KATO III cells. Localization of sulfatides, GM_3 gangliosides, and Le^b antigens on KATO III cells was investigated by immunofluorescence staining. First, KATO III cells were washed twice with PBS and resuspended in PBS. After incubation for 20 min with a monoclonal antibody to sulfatides, GM_3 gangliosides, or Le^b antigens, the cells were washed twice in PBS. A fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Cappel Laboratories) diluted to 1/10 was added, and the incubation was continued for 30 min at 4°C. The samples were then suspended in PBS containing 6% bovine serum albumin. After centrifugation at 200 × *g* for 5 min, the supernatant was removed. Immunofluorescence of KATO III cells was observed by fluorescence microscopy (Nikon, Tokyo, Japan).

Statistical analysis. The statistical significance of the differences between MFI values for various treatments was determined by the Student *t* test. *P* values exceeding 0.05 were considered not significant.

RESULTS

Recognition of glycosphingolipids by a standard strain of *H. pylori*, NCTC 11637. When the *H. pylori* overlay method was carried out on TLC plates as described previously (16), NCTC 11637 cells adhered to sulfatides but did not bind to GM_3 gangliosides (Fig. 1).

Adhesion of *H. pylori* to KATO III cells. As described by Dunn et al. (4), separation of nonadherent *H. pylori* from KATO III cells could be achieved by centrifugation through a 15% (vol/vol) sucrose solution. Fluorescence microscopic examination revealed KATO III cells with adhered *H. pylori*; the contamination of unadherent *H. pylori* was negligible. Flow cytometric analysis revealed that the majority of KATO III cells exposed to *H. pylori* had higher fluorescence intensity than cells unexposed to *H. pylori* did (Fig. 2). The adhesion occurred rapidly and was saturated by 10 min, as reported by Dunn et al. (4). Additional incubation, for up to 30 min, did not increase the MFI. The incubation of bacteria and KATO III cells at 4°C reduced the MFI. In proportion to the increase in the ratio of bacteria to KATO III cells, up to 100:1, the MFI rose, but any further increase in this ratio reduced the adhesion (data not shown).

Inhibition of adhesion. At a bacterium/KATO III cell ratio of 100:1, and with a 30-min incubation at 37°C, the effects of various substances were assessed by the changes in MFI as compared with that of the control. Since the total membrane protein from 10^6 KATO III cells was 0.023 mg, the maximum concentration of 1.0 mg/ml was considered to be sufficient for inhibition study. Preincubation of KATO III cells with heparin at a concentration over 100 U/ml or porcine gastric mucin at 1.0 mg/ml significantly reduced adhesion (Fig. 3a and b). Preincubation with fetuin at a final concentration of 1.0 mg/ml also reduced adhesion but not to a statistically significant degree. Asialofetuin at 1 mg/ml did not influence the adhesion. Monosaccharides such as *N*-acetylneuramic acid, L-fucose, D-galactose, and D-mannose did not show any effect at a final concentration of 1.0 mg/ml (data not shown). On the other hand, membrane preparations from KATO III cells inhibited adhesion in a dose-dependent manner (Fig. 3c). Furthermore, pretreatment with a monoclonal anti-sulfatide antibody markedly inhibited the adhesion of *H. pylori* to KATO III cells. Preincubation with the monoclonal anti-GM_3 ganglioside antibody or the monoclonal anti-Le^b antibody also reduced adhesion, although not to a degree significantly different from that of the control (Fig. 3d).

Adhesion of *H. pylori* to glycosphingolipids purified from KATO III cells on TLC plates. Sulfatides were present as a major acidic glycospingolipid in KATO III cells (Fig. 4A). The

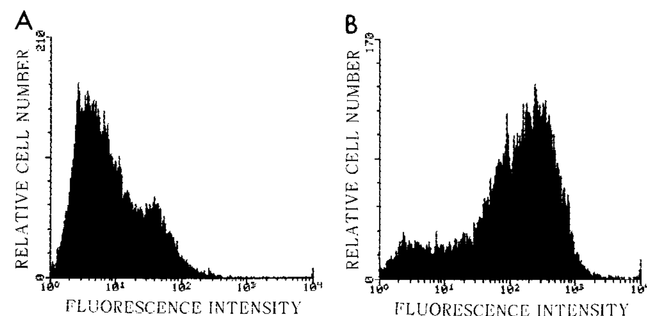

FIG. 2. Flow cytometric analysis of *H. pylori* adhesion to KATO III cells. The fluorescence intensities of control KATO III cells unexposed to *H. pylori* (A) and of those exposed to the bacteria (B) were analyzed by FACScan flow cytometry. The *x* axis represents the fluorescence intensity of KATO III cells, and the *y* axis represents the relative cell number. The distribution of fluorescence intensity of KATO III cells exposed to *H. pylori* markedly increased and shifted to the right. In this example, the MFIs as calculated with a Lysis software program from Becton Dickinson were 27.4 (A) and 249.6 (B).

adhesion of *H. pylori* to sulfatides was confirmed by TLC-immunostaining with an anti-*H. pylori* antibody. *H. pylori* did not, however, adhere to any of the neutral glycolipids or other acidic glycolipids (Fig. 4B).

Le^b antigens in neutral glycolipids from KATO III cells were not detectable on TLC plates by immunostaining with a monoclonal anti-Le^b antibody. These negative results were not due to weak reactivity of our antibody, since it clearly detected Le^b glycolipid extracted from SNG-II cells, a human uterine endometrial adenocarcinoma cell line (data not shown). Furthermore, *H. pylori* did not adhere to a standard Le^b glycolipid on TLC plates (Fig. 5).

Adhesion of *H. pylori* to KATO III cell membrane proteins. No distinct adhesion of *H. pylori* to any of the KATO III membrane proteins was observed (data not shown).

Immunostaining with a monoclonal anti-Le^b antibody revealed no Le^b antigens in glycoproteins from KATO III cells (data not shown).

Localization of glycosphingolipid antigens on KATO III cells. Fluorescence labelling of KATO III cells with a monoclonal antisulfatide antibody revealed a specific fluorescence on the cell surface (Fig. 6), but no such staining was observed with a monoclonal anti-GM_3 ganglioside antibody or Le^b antibody.

DISCUSSION

The attachment of microorganisms to host cells is considered to be the initial event in the infectious process. It has been well established that several bacteria, viruses, and bacterial toxins recognize lipid-linked oligosaccharides present in the plasma membrane (11).

Many putative adhesion receptors of *H. pylori* have been proposed. Evans et al. have shown that the hemagglutinin from *H. pylori* recognizes *N*-acetylneuraminyllactose, which is the oligosaccharide moiety of GM_3 gangliosides (5, 6). Slomiany et al. demonstrated that sulfated lactosylceramide and GM_3 gangliosides have a strong inhibitory activity towards *H. pylori* hemagglutinin (17). With a TLC-immunostaining method, Lingwood et al. found that *H. pylori* specifically bound to phosphatidylethanolamine from human erythrocytes (13). Recently, Boren et al. reported that soluble glycoproteins presenting the Le^b antigen or antibodies to the Le^b antigen inhibited bacterial adhesion in situ (3). On the other hand, our previous study showed that *H. pylori* bound specifically to sulfatides and GM_3 gangliosides from human gastric mucosa (16). These differences in results may be attributable, in part, to a difference in the *H. pylori* strains or the detection methods used in the experiments. To minimize these problems, we have used a type strain of *H. pylori*, NCTC 11637, in this study. This type strain, when cultured in the liquid medium, lacked binding to GM_3 gangliosides but retained the ability to bind to sulfatides on TLC plates. This type strain also bound to KATO III cells, as reported for other *H. pylori* strains by Dunn et al., who found that this adhesion was inhibited by fetuin, a glycoprotein containing *N*-acetylneuraminyllactose (4). In the present study,

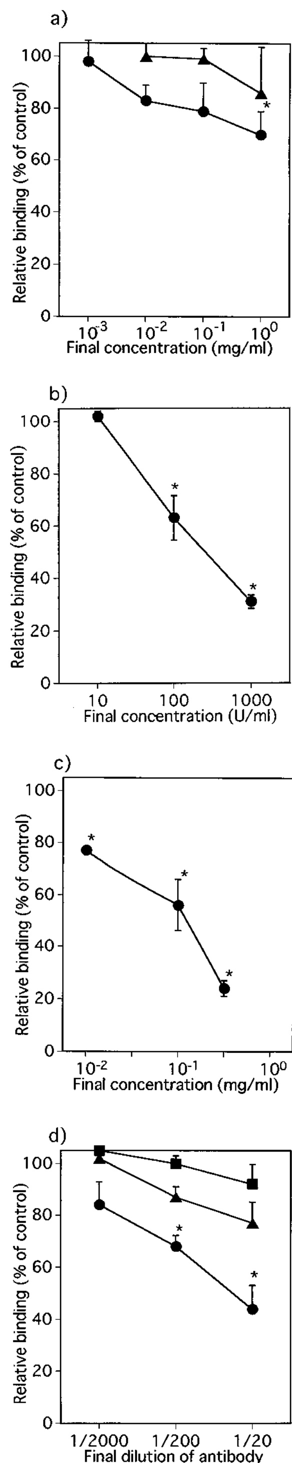

FIG. 3. Effects of various substances on *H. pylori* adhesion to KATO III cells. After preincubation of *H. pylori* with each material, the assay was carried out as described in the text. Each value, as a percentage of the control, was calculated as follows: $X = (A - C)/(A - B)$, where X is the percentage of control, A is the MFI of KATO III cells exposed to *H. pylori* and without preincubation, B is the MFI of KATO III cells unexposed to *H. pylori* and without preincubation, and C is the MFI of KATO III cells exposed to *H. pylori* and with preincubation. Each value represents the mean of three to seven determinations ($\pm$ standard error), and bars represent standard errors. *, <0.05 versus control. (a) Effect of mucin (●) and fetuin (▲) on adhesion. The final concentration of each material was expressed as dry weight (milligrams) per milliliter. (b) Effect of heparin on adhesion. The final concentration of heparin was expressed as USP units per milliliter. (c) Effect of KATO III cell membranes on adhesion. The final concentrations of KATO III cell membranes were expressed as milligrams of protein per milliliter. (d) Effect of antisulfatide antibody (●), anti-GM_3 ganglioside antibody (▲), and anti-Le^b antibody (■) on adhesion. For a monoclonal antisulfatide antibody and an anti-GM_3 antibody, the culture supernatant of each hybridoma was used as a source of the antibody. For a monoclonal anti-Le^b antibody, the ascites fluid from hybridoma-bearing mice was used as a source of the antibody. Protein concentrations of the antibody solution after salting out and dialysis were as follows: antisulfatide antibody, 0.66 mg/ml; anti-GM_3 ganglioside antibody, 1.25 mg/ml; anti-Le^b antibody, 92 mg/ml.

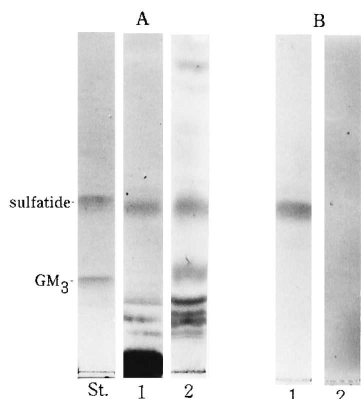

FIG. 4. *H. pylori* adhesion to glycosphingolipids purified from KATO III cells. (A) Sulfatides were present as a major acidic glycosphingolipid in the membrane preparations of KATO III cells. The spots were located with orcinol-H_2SO_4 reagent. (B) A TLC overlay assay demonstrates that *H. pylori* adhesion occurred only on sulfatides. Glycosphingolipids were chromatographed in the solvent system with chloroform-methanol-water (65:35:8, vol/vol). The plate was incubated with *H. pylori* and then stained by TLC-immunostaining as described in the text. Lanes: St, all standard glycosphingolipids prepared from bovine brain; 1, acidic glycosphingolipids from KATO III cells; 2: neutral glycosphingolipids from KATO III cells.

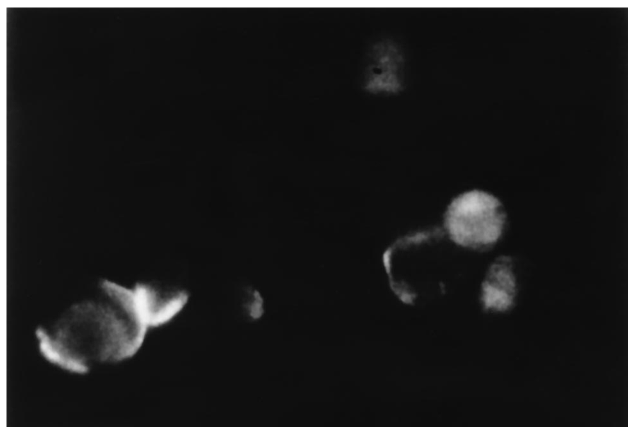

FIG. 6. Indirect immunofluorescence staining of KATO III cells. Fluorescence labelling of KATO III cells with a monoclonal antisulfatide antibody revealed a specific fluorescence on the cell surface. Original magnification, ×400.

however, the inhibitory effect of fetuin was not statistically significant. As reported by Evans et al. (5), sialyllactose-binding hemagglutinin may have been lost during the liquid culture in our study, which may explain the different results. On the other hand, our results showed that this adhesion was significantly inhibited by heparin and mucin from porcine stomach. Furthermore, this adhesion was also inhibited by membranes from KATO III cells in which sulfatides were the major acidic glycosphingolipids. Of the glycosphingolipids extracted from KATO III cells, selective adhesion of *H. pylori* to sulfatides was demonstrated. As for Le^b antigens, *H. pylori* did not bind to a standard Le^b glycolipid on TLC plates. The presence of Le^b antigen, either as a neutral glycosphingolipid or a glycoprotein from KATO III cells, could not be confirmed by TLC-immunostaining or by immunoblotting. Moreover, immunofluorescence labelling of intact KATO III cells with a monoclonal anti-Le^b antibody failed to show specific staining, indicating the absence of Le^b antigens on KATO III cells. In contrast, sulfatides were clearly labelled with the monoclonal antibody. GM_3 gangliosides were not labelled with the monoclonal antibody. This was not an artifact due to the cell preparation procedure or weak reactivity of these antibodies, because, in other cell lines, both Le^b glycolipids and GM_3 gangliosides could be detected easily by anti-Le^b and anti-GM_3 antibodies, respectively (data not shown). Therefore, the adhesion of *H. pylori* to KATO III cells appears to be mediated by binding to sulfatides rather than Le^b antigen or GM_3 gangliosides.

FIG. 5. Absence of involvement of Le^b glycolipids in the adhesion of *H. pylori* to KATO III cells. Glycosphingolipids were chromatographed in the solvent system with chloroform-methanol-water (65:35:8, vol/vol). (A) The plate was incubated with *H. pylori* and then stained by TLC-immunostaining as described in the text. (B) The spots were located by TLC-immunostaining with a monoclonal anti-Le^b antibody diluted to 1/500 (184 μg/ml). Lanes: 1, neutral glycosphingolipids from KATO III cells; 2, standard Le^b glycolipids.

The observation that this adhesion was inhibited by heparin and gastric mucin, both of which are sulfated glycoconjugates, suggests the importance of the sulfated moiety on saccharide chains. In this context, it is possible that glycosaminoglycans, having long repeating sulfated glycoconjugates, may also serve as a receptor for *H. pylori* adhesion (1). The specific localization and quantitative analysis of glycosaminoglycans in gastric mucosa remain, however, to be elucidated. In our previous study, we showed that sulfatides are highly enriched in human gastric mucosa (14, 15). Moreover, immunofluorescence staining using a monoclonal antisulfatide antibody revealed that sulfatides were specifically localized in the epithelial lining of human gastric mucosa (16). This specific localization and enrichment of sulfatides in human gastric mucosa indicate that sulfatides can serve as a major receptor for cell adhesion by *H. pylori* in the gastric mucosa.

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