Human Monoclonal Antibodies from Stomach Carcinoma Patients React with Helicobacter pylori and Stimulate Stomach Cancer Cells In Vitro

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Background. In recent studies, an increased incidence of gastric adenocarcinomas was observed in patients with Helicobacter pylori infection. However, the extent to which this coincidence is caused by immunologic mechanisms is unknown.

Methods. Two human monoclonal antibodies (MoAbs) from patients with stomach carcinoma and H. pylori-associated gastritis were isolated and established by fusion of spleen cells with the heteromyeloma HAB-1. The reactivity of these human MoAbs was investigated in functional adhesion assays and on Western blots of tissue, tumor cell, and bacterial extracts. Their stimulation and proliferation were tested by the MTT test and 3-H-thymidine incorporation tests.

Results. The two monoclonal immunoglobulin-M antibodies, 103/51 and 105/79, inhibited adhesion of tumor cells. On bacterial extracts antibody 103/51 identified protein bands of 55 kilodaltons (kd) and 80 kd, and in tumor cell extracts, a specific protein of approximately about 110 kd and 140 kd. Antibody 105/79 identified a 55 kd protein in bacterial extracts and a 110 kd protein in tumor extracts. In addition, in the 3-H-thymidine incorporation and MTT assay the antibodies showed a stimulatory and growth-enhancing effect on tumor cells in vitro. A similar activity was observed in sera from patients with gastric carcinoma, indicating a physiologic role of such antibodies in vivo.

Conclusion. The human monoclonal antibodies described here react with H. pylori and cross-react with and stimulate gastric carcinoma cells. It is possible that the production of these antibodies is primarily stimulated by bacterial antigens which cause chronic gastritis and that they might be indirectly responsible for the recently described higher incidence of gastric cancer because of the simultaneous reaction and stimulation of tumor cells they cause. Cancer 1994; 74:1525-32.

Key words: Helicobacter pylori, gastric cancer, human monoclonal immunoglobulin M antibodies, tumor cell stimulation.

Gastric carcinoma is estimated to be one of the most frequently occurring cancers worldwide.¹ Studies on environmental risk factors for gastric cancer have concentrated in the recent years on Helicobacter pylori, a gram-negative microaerophilic spiral bacterium whose colonization of the gastric mucosa is strongly associated with chronic gastritis.²-⁶ According to the Correa model, a commonly accepted etiopathogenic hypothesis for intestinal-type gastric carcinoma, chronic gastritis is followed by successive stepwise changes through atrophic gastritis, intestinal metaplasia of Type I–III, dysplasia, and carcinoma.⁷

In addition, recent investigations showed an increased risk of gastric adenocarcinoma in patients with H. pylori infections.³,⁴ The ability to isolate human monoclonal antibodies from patients with cancer by in vitro immortalization offers an ideal tool for identifying tumor associated antigens and studying tumor associated immunological mechanisms of the patients' immune system.
Materials and Methods

Source of Tissue and Light Microscopic Methods

From surgically removed gastrectomy specimens, native tissue sections with stomach carcinoma and with tumor-free antrum and corpus mucosa were snap frozen in liquid nitrogen and stored at −80°C until use. The gastrectomy specimens were fixed in buffered 3% formaldehyde solution. Five-micron thick tumor sections of paraffin embedded tissue were stained with hematoxylin and eosin, periodic acid–Schiff reaction, and Alcian blue. The tumor classification and grading were performed according to World Health Organization guidelines, and the tumor type was determined according to Lauren. Tissue sections of tumor-free antrum and corpus mucosa were stained with hematoxylin and eosin, periodic acid–Schiff reaction, Giemsa, and silver impregnation according to Whartin-Starry as described by Price to determine the grade of gastritis and the presence of H. pylori.

Cell Culture

Hybridomas were prepared and cultured as previously described. Briefly, spleen cells from patients with stomach carcinoma were prepared by mechanical disruption of the tissue, and the single cell suspension was fused at a 1:1 ratio with the heteromyeloma HAB-1 using 40% polyethylene glycol. Hybridomas were cultured in RPMI-1640 containing 10% fetal calf serum (FCS) and hypoxanthine-aminopterin-thymidine supplement; after 4–6 weeks, supernatants were screened for antibody production in an enzyme linked immunosorbent assay (ELISA). Positive clones were tested in attachment inhibition and binding assays and cloned by limiting dilution using irradiated nude mouse lymphocytes as feeder cells. The stomach carcinoma cell lines 23132 and 2975 used in this study have been described.

Antibody ELISA

Plastic plates were precoated overnight with rabbit anti-human immunoglobulin (Ig) antibodies and incubated with supernatants from hybridoma cells for 2 hours at 20°C. Nonspecific binding sites on the plastic were blocked by treatment with 0.1 M borate buffer (pH 8.2) containing 1% FCS for 15 minutes at 20°C. Plates were washed twice with borate buffer and incubated with peroxidase-conjugated rabbit immunoglobulin to human immunoglobulin for 30 minutes at 37°C. Plates were washed twice with borate buffer and incubated with substrate (0.03% orthophenyl-diamine and 0.02% hydrogen peroxide in citrate phosphate buffer). After 10–15 minutes, the reaction was stopped with 3 M sulfuric acid, and the absorbance was recorded at 492 nm in an ELISA reader (Biorad, Munich, Germany).

Adhesion and Growth Inhibiting Assay

The adhesion assay was performed as described previously. Briefly, tumor cells were removed from culture flasks with trypsin/edetic acid, washed in RPMI-1640, and plated in hybridoma supernatants, normal medium, or medium from HAB-1 cultures. Plates were incubated for 1 hour at 37°C and washed with phosphate buffered saline (PBS), and the remaining cells were fixed with 3% formaldehyde. With the aid of an inverted microscope, the attached cells were counted, and the inhibition of attachment was evaluated by comparison to the control specimens. For the growth inhibiting assay, 10,000 stomach carcinoma cells were plated in triplicate in 96-well plates with or without antibodies for 1 week. Every second day, one well was trypsinized, and the cells were counted.

Antibody Purification

IgM producing hybridomas were cultured in roller flasks in RPMI-1640 with 10% basal medium supplement. Supernatant was concentrated by a factor of 20 in an ultrafiltration system, and the IgM was purified on a Superose-12-column (Pharmacia, Freiburg, Germany) using an FPLC system. The antibody was eluted in PBS and stored at −70°C until use. Purity was determined by sodium dodecyl sulfate/polyacrylamide (SDS) gel electrophoresis.

3-H-thymidine Incorporation Assay and Mitochondrial Hydroxylase Enzymatic Activity Test (MTT test)

Both assays were performed in flat-bottomed 96-well plates. Ten thousand cells were grown in 200 μl medium to subconfluency. Medium was replaced by antibody containing media and control media. After incubation for 1 day, 10 μl (methyl-3-H)-thymidine with a specific activity of 5 μCi/mmol diluted to a concentration of 50 μCi/ml was added to each well. After 24 hours, cells were harvested and thymidine incorporation was measured. The MTT test for stimulation of tumor cells was performed as described by Mosmann and Carmichael et al. with minor modifications. Cells
Figure 1. (A) Morphologic aspects of chronic atrophic gastritis with intestinal metaplasia (H & E, original magnification ×100, Patient 1) and (B) with numerous H. pylori in the mucus layered on the surface epithelia (Whartin-Starry, original magnification ×550, Patient 1). (C) Morphologic aspects of a mixed type gastric adenocarcinoma in areas of diffuse type (H & E, original magnification ×220, Patient 1) and (D) of intestinal type with ulceration (H & E, original magnification ×100, Patient 1).

were grown for 2 days, and medium was replaced by 100 μl phenol red free RPMI-1640 medium containing 1 mg/ml 3(4,5 dimethylthiazo1)-2,5 diphenyltetrazolium bromide. After 2 hours, incubation plates were centrifuged at 800 g for 5 minutes, MTT medium was removed, and the blue dye was dissolved in warm dimethyl sulfoxide and measured at 540-690 nm wave length.

Electrophoretic Procedures and Western Blot Analysis

SDS-polyacrylamide (10%) gel electrophoresis was performed as described by Laemmli. Western blots were done as described by Pfaff et al. Briefly, cultured cells were washed with cold PBS, harvested in PBS containing 1% Triton X-100 and 1 mM phenylmethylsulphonyl fluoride, and incubated for 30 minutes on ice. To obtain extracts from frozen tissues, cryostat sections (10 μm) were performed, harvested in PBS, and extracted in a similar way. The extracts were cleared of nuclei and cell debris by centrifugation at 12,000 g for 5 minutes, diluted with Laemmli sampling buffer for SDS-gel electrophoresis, and heated to 95°C. Electrophoretic transfer of separated components from polyacrylamide gels to 0.45 μm nitrocellulose membranes was performed in a semidry blotting apparatus. The dried membrane was treated with PBS containing 10% FCS and 0.5% Tween 20 for 1 hour at room temperature, followed by a 2-hour incubation with human IgM antibodies. After five washings with PBS and 0.05% Tween 20, peroxidase coupled rabbit anti-human IgM antibody was added. After a 2-hour incubation, the nitrocellulose membranes were washed four times with Tris buffered saline, and the color was developed in a freshly prepared mixture of 20 ml Tris buffered saline, 4 ml 4-cloro 1-naphtol (3 mg/ml in methanol) and 5 μl hydrogen peroxide. Extraction, electrophoretic separation and Western blotting of bacterial antigens were performed as described by von Wulffen et al. Immuno- peroxidase staining of nitrocellulose filters was done as described here.

Results

Morphology

The two human monoclonal antibodies described in this study were isolated from 2 male patients with gastric cancer (58 and 59 years old). Histologically in both patients moderately or low differentiated tubular adenocarcinoma occurred in conjunction with areas of signet ring cell carcinoma in nearly equal proportions (mixed, i.e., intestinal and diffuse type according to Lauren; Fig. 1, C and D). In one patient, an early cancer was seen in the antrum with infiltration only of the mucosa and submucosa and without lymph node metastases, whereas in the other patient, there was an advanced tumor in the antrum/corpus region with regional lymph node metastases (Table 1). Chronic
atrophic gastritis with focal intestinal metaplasia was observed, and *H. pylori* were demonstrable in each patient (Fig. 1, A and B).

**Monoclonal Antibodies**

To isolate human monoclonal antibodies with tumor reactivity, spleen cells from patients with stomach cancer were immortalized in fusion experiments with the heteromyeloma HAB-1. The lymphoid source was not preselected in terms of age or sex of patients. The antibodies were tested primarily for tumor reactivity in functional adhesion tests, such as cell-substrate adhesion assay with established tumor cell lines, to select for antibodies with cell surface reactivity. In addition, functionally active antibodies were routinely tested in vitro growth assays with stomach carcinoma activity. It was found that two antibodies, 103/51 and 105/79, both IgM, had a growth enhancing, but not inhibiting, effect (Table 2).

**Western Blot Analysis on Cell and Tissue Extracts**

The characterization of the antibodies was performed on established gastric adenocarcinoma cell lines and extracts from cryostat sections of normal and tumor tissues. On Western blots of protein extracts from cryostat sections of the autologous tumors, the antibodies identify proteins with 140 kilodalton (kd) (103/51) and 110 kd (105/79, Table 3). On extracts of the tumor cell line 23132, the antibodies bind to similar molecules (Fig. 2, A–D). To rule out unspecific binding of IgM antibodies on tissue and cell extracts, which often is observed with IgM antibodies, unrelated human control IgM, also isolated from patients with stomach cancer, were used routinely as controls. Figure 2 shows that a nonspecific IgM binding can be observed at approximately 60 kd.

When tested on tissue extracts from different diffuse and intestinal type tumors, only antibody 107/79 reacts with 4/10 intestinal type stomach cancers (Table 3). When tested on normal tissue, reactivity was observed only with antibody 105/79 on colon (Table 4).

**Western Blot Analysis on *H. pylori* Extracts**

The two patients from whom the antibodies originally were isolated showed positive infection of the gastric mucosa with *H. pylori* (Table 1) and a chronic gastritis type B with atrophy and intestinal metaplasia (Fig. 1). Western blot analysis on extracts from *H. pylori* revealed that the human monoclonal antibodies cross-react with bacterial antigens (Fig. 2, E–H). They identify specific molecules with 55 and 80 kd (103/51, 55 and 80 kd; 105/79, 55 kd). Similar proteins have been described as major proteins from *H. pylori* on SDS-polyacrylamide gel electrophoresis of whole cell lysates. As in the case of blots from tumor extracts, a nonspecific binding of IgM antibodies can be seen in the range of

**Table 1. Characteristics of Patients and Tumors**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Tumor type</th>
<th><em>H. pylori</em></th>
<th>Gastritis type</th>
<th>Stage</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>58</td>
<td>M</td>
<td>Mixed</td>
<td>++</td>
<td>CAG/IM</td>
<td>T1N0</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>59</td>
<td>M</td>
<td>Mixed</td>
<td>++</td>
<td>CAG/IM</td>
<td>T3N2</td>
<td>3</td>
</tr>
</tbody>
</table>

Mixed: mixed type of gastric cancer with nearly equal proportions of intestinal and diffuse type according to Lauren; CAG/IM: chronic atrophic gastritis with intestinal metaplasia.

*H. pylori* infection was morphologically demonstrated by the Wartini–Starr reaction.

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**Table 2. Characterization of Human Monoclonal Antibodies**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Antibody</th>
<th>Source of lymphocytes</th>
<th>Ig class</th>
<th>Fusion partner</th>
<th>Blot*</th>
<th>Adhesion†</th>
<th>Stimulation‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>103/51</td>
<td>Spleen</td>
<td>IgM</td>
<td>HAB-1</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>105/79</td>
<td>Spleen</td>
<td>IgM</td>
<td>HAB-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Western blot with specific bands on extracts from tumor cell line 23132 and extracts from *H. pylori.

† Adhesion assay with tumor cells 23132; ++: 40–60% inhibition; +: 60–80% inhibition.

‡ Stimulation of tumor cells was performed as described in Materials and Methods.
Stomach Carcinoma Cells and *Helicobacter pylori* MoAb/\textit{Vollmers et al.} 1529

### Table 3. Western Blot Analysis on Stomach Cancer Tissue

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Antibody (103/51)</th>
<th>Antibody (105/79)</th>
<th>Antibody (105/9^*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autologous (kd)</td>
<td>140</td>
<td>110(\dagger)</td>
<td>—</td>
</tr>
<tr>
<td>Intestinal</td>
<td>0/10</td>
<td>4/10</td>
<td>0/10</td>
</tr>
<tr>
<td>Diffuse</td>
<td>0/7</td>
<td>0/7</td>
<td>0/7</td>
</tr>
</tbody>
</table>

One percent Triton extracts from cryostat sections were separated by electrophoretic procedures and blotted and stained as described in Materials and Methods.  
* Control IgM.  
\(\dagger\) Molecular weight in positive cases: 110 kd.

60–65 kd. In addition, Western blots with purified heat shock proteins gave negative results (data not shown).

### Stimulation and Proliferation Assays

To investigate the stimulating effect of the two human antibodies on stomach carcinoma cell lines in more detail, 3-H-thymidine incorporation and mitochondrial hydroxylase enzymatic activity was tested with stomach carcinoma cell lines 23132 and 2975. A significant stimulation can be seen with both antibodies on both cell lines in MTT assay (Fig. 3, A and B) compared with the controls. Similar effects can be seen in 3-H-thymidine incorporation. Figure 3 (C and D) shows that both antibodies enhance growth of cell lines 23132 and 2975. The antibody concentration used was approximately 2 \(\mu\)g, but dilution experiments have shown that there is a stimulatory effect on the stimulation with approximately 100 ng antibody (data not shown).

### Investigation of Patient Sera

In a supplementary study, sera from different patients with stomach carcinoma were collected and investigated for antibodies against *H. pylori* in Western blots and for stimulation of tumor cell 23132 in the MTT assay. Table 5 shows that in five of seven patients, IgG antibodies could be detected that cross-react with *H. pylori*, and in four of seven patients, MTT activity could be determined. Three sera show a positive reaction against *H. pylori* but are negative in the stimulation assay, and two sera stimulate but do not bind *H. pylori* extracts. One explanation for this discrepancy is that not all *H. pylori* positive antibodies stimulate tumor cells, and MTT positive/*H. pylori* negative antibodies might be of the IgM class, which is not detected in the IgG blot. Two sera from patients with carcinoma were positive in both assays, indicating a physiologic role of such antibodies in vivo.

### Discussion

In this article, we report two monoclonal IgM antibodies isolated from patients with gastric adenocarcinomas of the mixed type and *H. pylori* associated chronic gastritis. The antibodies 103/51 and 105/79 inhibit the adhesion of tumor cells, and they identify antigens with 140 and 110 kd on Western blots of tumor cells and tumor tis-
sues. Tested on normal tissue, only colon shows positive reactivity (antibody 105/79). In addition, the antibodies stimulate stomach carcinoma cells and induce their proliferation. They cross-react with *H. pylori*, indicating common structures on tumor cells and bacteria.

Human gastric carcinogenesis is a multistep, multifactorial process. Incriminated environmental influences include irritants, antibodies, gastrectomy, nutritional deficits, intake of nitrogen compounds, and *H. pylori*. In 84–94% of the patients, stomach carcinoma of the intestinal and diffuse type is associated with *H. pylori* infection, compared with 61–76% of matched tumor-free control subjects, and a strong association of *H. pylori* infection with an increased risk of gastric carcinoma is reported. In addition, in crude etiologic-fraction calculations, 60% of gastric adenocarcinomas are supposed to be attributable to infection with *H. pylori*. Several investigations demonstrate that stomach carcinoma has a higher incidence in regions in which *H. pylori* associated gastritis occurs relatively frequently. However, the precise role of *H. pylori* remains unclear. It is likely that it plays an important role in the induction of chronic gastritis, which is followed by atrophy, intestinal metaplasia, dysplasia, and carcinoma.

Several factors are responsible for the pathogenicity of *H. pylori*, motility of the bacteria, hemagglutinin production, adhesion factors, proteases, cytokins, ammonia produced by ureases, phospholipases, katalases, and platelet-aggregating factors.

Because patients with *H. pylori* associated chronic gastritis have antibodies developed against the bacteria, it is not surprising that monoclonal antibodies can be isolated and established from such patients. The antibodies described here cross-react with stomach carcinoma cells and have a growth enhancing effect on tumor cells in vitro.

The search for antibody mediated proliferation of tumor cells in vitro is a new aspect in the study of immunological reaction on tumor cells because until now

| Table 5. Antibody Reactivity in Sera of Stomach Carcinoma Patients |
|------------------------|-----------------|------------------------|------------------------|
| Patient no. | Tumor type | *H. pylori* IgG blot | MTT test |
| 3 | Mixed | + | + |
| 4 | Mixed | – | + |
| 5 | Intestinal | + | – |
| 6 | Intestinal | + | – |
| 7 | Intestinal | + | – |
| 8 | Intestinal | – | + |
| 9 | Intestinal | + | + |

Mixed: mixture of intestinal and diffuse type of stomach cancer; Intestinal: intestinal type of stomach cancer according to Lauren. *H. pylori* Western blots and MTT test were done as described in Materials and Methods.

Figure 3. Stimulation of stomach carcinoma cell lines 23132 and 2975 by human monoclonal antibodies. (Top left and right) 3-H-thymidine uptake with cell lines 23132 and 2975. (Bottom left and right) MTT test with cell line 23132 and 2975; 105/9 control antibody (IgM) at a similar concentration (all antibody concentrations, 2 µg/ml). Assays were done in triplicate; differences are statistically significant.
the inhibitory effects of antibodies on tumor cells were the focus of investigations. In an earlier study, we screened human monoclonal antibodies in the same adhesion assays as used here and found antibodies against tumor specific (signet ring cell carcinoma) differentiation antigens\textsuperscript{12} and modified secreted keratin structures (TPA structures).\textsuperscript{13} In addition, functional antiadhesive murine antibodies have been shown to be of significant value in the study of tumor growth and metastasis of murine and human malignant melanoma cells.\textsuperscript{28-33} They all have in common the inhibition of tumor growth in vitro or in vivo by interfering with unknown adhesion molecules\textsuperscript{28-30} or known differentiation antigens, such as proteoglycans or gangliosides.\textsuperscript{31-33} The two different human monoclonal antibodies described here do not inhibit, but enhance, growth of tumor cells in vitro by stimulating mitochondrial activity and increasing DNA synthesis in a concentration dependent manner. They are functionally active at a concentration of 0.1 $\mu$g/ml purified antibody.

It has been known for a long time that tumor cells can be triggered by antibodies against particular surface receptors. For instance, the effect of epidermal growth factor on A431 carcinoma cells can be simulated by a functional monoclonal antibody directed against the epidermal growth factor receptor,\textsuperscript{34} and antibodies against the insulin receptor can induce insulin action in certain cells.\textsuperscript{35,36}

How the monoclonal antibodies 103/51 and 105/79 induce their enhancing effect is not known. They might mimic the binding of a growth factor to tumor cells (e.g., epidermal growth factor) or bind to an unknown cell surface receptor and produce a direct stimulating effect. The antibodies bind to different antigens on carcinoma cells, indicating different receptors and possibly different mechanisms of stimulation.

The reactivity of the two antibodies on human tissues is restricted to tumor cells and tumor tissue, with one exception: antibody 105/79 binds on Western blots of extracts from colon tissue, but the reason is not known. With few exceptions, most of the earlier described monoclonal antibodies isolated from patients with tumor show a broad cross-reactivity with cellular components and normal tissue.\textsuperscript{37-39} One reason for a restricted reactivity might be the method of selecting the antibodies, e.g., in binding assays on living cells or assays with tissue sections and tissue extracts, for which the reactivity against cytoplasmic or nuclear components dominates the reactivity against membrane bound structures.

Antibodies 103/51 and 105/79 cross-react with \textit{H. pylori} and on Western blots define bacterial antigens with molecular weights of 55 and 80 kd, which might belong to the major proteins of the bacteria because their molecular weights are similar.\textsuperscript{19} Several groups have reported monoclonal antibodies that react with DNA or antigens such as thyroglobulin, myosin, actin, collagen and cross-react with different mycobacteria.\textsuperscript{40,41} In most patients, these are naturally occurring autoantibodies, cross-reacting over epitopes, which are shared by bacteria. With regard to the antibodies described here, we do not know whether they initially were induced by the bacterial infection or by an immune response to the tumor cells.

If these antibodies are primarily induced by and directed against \textit{H. pylori} as a cause of chronic gastritis, they could be directly or indirectly responsible for the higher incidence of gastric cancer in \textit{H. pylori} associated gastritis because of the simultaneous reaction and stimulation of tumor cells by the same antibodies. Like growth factors, they could play a role in tumor progression and possibly also in the initiation of tumors from precancerous stages, e.g., from intestinal metaplasia or dysplastic changes, ideas that must be investigated.

Tumor stimulating and \textit{H. pylori} cross-reacting antibody activity also has been found in sera from patients with mixed and intestinal type gastric cancer. We have tested only a limited number of sera, but we found \textit{H. pylori} positive IgG antibodies in five specimens of sera, stimulating activity on stomach carcinoma cells in MTT tests in four of seven, and both in two specimens of sera. These results might indicate a physiological role of such antibodies in vivo.

References