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Helicobacter pylori antigen in the glomeruli of patients with membranous nephropathy

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Abstract Renal biopsy specimens from patients with membranous nephropathy (MN) were studied using immunohistochemical labelling to clarify the aetiological significance of *Helicobacter pylori* antigen in this disease. Sixteen specimens were examined, from 7 male and 9 female MN patients. Renal specimens from patients with diabetic nephropathy and IgA nephropathy, and from autopsied patients without renal diseases were obtained as controls. Immunohistochemical labelling was performed using one polyclonal antibody and three monoclonal antibodies against *H. pylori*. Specimens from 11 of the MN patients revealed granular deposits along the glomerular capillary walls, which reacted positively with polyclonal antibody after trypsin pretreatment. None of the control specimens revealed positive labelling. The MN specimens showed no positive reaction with the primary antibody, which had been treated for immunoabsorption testing using sonicated *H. pylori*.We also determined *H. pylori* status in these MN patients histologically and/or serologically. Of the 11 patients whose glomeruli were positive for anti-*H. pylori* antibody, 7 were suitable for analysis, and all were regarded as positive for *H. pylori* infection. These results suggest that the presence of a specific antigen in the glomeruli of patients with MN and *H. pylori* infection may be involved in the pathogenesis of MN.

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Introduction

Helicobacter pylori, a causal agent of active chronic gastritis in humans, is associated with the development of duodenal ulcers and may also be associated with adenocarcinoma or low-grade lymphoma of mucosa-associated lymphoid tissue (MALT) in the stomach [1, 4, 7, 17]. Depending on the infection, a systemic chronic inflammatory state may be induced. However, few papers have addressed the causal relationship between *H. pylori* and extragastric diseases [14, 19].

Membranous nephropathy (MN) is the most common cause of idiopathic nephrotic syndrome in adults, and it is well known that this disease appears as a complication of various infectious, neoplastic, toxic and autoimmune conditions [2, 5, 6, 9]. Some reports have described the resolution of proteinuria after therapy for complicating neoplasms or infected virus in patients with MN [13, 18, 23], and a causal relationship between these diseases and renal abnormality is strongly suggested. However, in most patients with MN there are no proven underlying conditions and the disease is diagnosed as idiopathic.

In this study, the presence of *H. pylori* antigen was investigated in renal tissue from needle biopsy samples, using immunohistochemistry to elucidate the relationship between *H. pylori* infection and MN.

Materials and methods

Sixteen renal specimens were obtained from patients with MN by needle biopsy at Yamagata City Hospital Saiseikan and Yamagata Prefectural Medical Center for Adults between 1992 and 1994. The diagnosis of MN was established on the basis of the characteristic findings of light microscopy and immunohistochemistry. We used all the specimens from MN patients that were appropriate for the analysis. All the material showed granular deposits positive for IgG, IgM, IgA and complement components such as C1q, C3c or C3d, along the glomerular capillary walls. The characteristics of these pa-

Table 1 Patient characteristics at renal biopsy

No.	Sex	Age		Clinical characteristics
1	Male	55	Idiopathic MN	Nephrotic syndrome
2	Male	57	Idiopathic MN	Nephrotic syndrome
3	Male	57	Idiopathic MN	Proteinuria
4	Male	65	Idiopathic MN	Nephrotic syndrome
5	Male	67	Idiopathic MN	Nephrotic syndrome
6	Male	68	Idiopathic MN	Nephrotic syndrome
7	Male	81	Idiopathic MN	Nephrotic syndrome
8	Female	28	Systemic lupus erythematosus	Oedema in both legs
9	Female	42	Systemic lupus erythematosus	Proteinuria
10	Female	42	Idiopathic MN	Nephrotic syndrome
11	Female	50	Idiopathic MN	Oedema in both legs
12	Female	56	Idiopathic MN	Oedema in both legs
13	Female	64	Idiopathic MN	Nephrotic syndrome
14	Female	68	Early gastric cancer	Nephrotic syndrome
15	Female	69	Idiopathic MN	Oedema in both legs
16	Female	76	Idiopathic MN	Oedema in both legs

MN: membranous nephropathy

Table 2 List of primary antibodies employed in the present study (*W.D.* working dilution)

Antibody	Subclass	W.D.	Source
Anti-*Helicobacter pylori*	Polyclonal rabbit serum	1:100	Dakopatts, Glostrup, Denmark
CP15	Mouse IgMk	1:50	Bioline Diagnostici, Giaveno, Italy
1G6	Mouse IgG1κ	1:50	Bioline Diagnostici, Giaveno, Italy
51–13	Mouse IgG1	1:50	Monosan, Uden, Netherlands

tients are listed in Table 1. There were 7 male and 9 female patients, with a mean age of 59.1 (28–81) years. Some had secondary MN complicating systemic lupus erythematosus (SLE) or early gastric cancer (established using the Japanese classification of gastric carcinoma [12]). The patients complained of oedema or proteinuria, and many of them were diagnosed as having nephrotic syndrome. Negative control material was obtained from needle biopsy or autopsy samples. They included samples affected by diabetic nephropathy (n = 3) and IgA nephropathy (n = 13); none of the autopsy specimens was affected by any renal disease (n = 5). All the material was fixed in 95% ethanol and processed routinely to obtain conventional paraffin sections. Moreover, gastric mucosa from *H. pylori*-infected patients (n = 4) obtained by endoscopic biopsy was used as positive control material for immunohistochemical labelling or immunoabsorption testing. Biopsy specimens were fixed soon after biopsy, and the material obtained at autopsy, within 3 h after death.

The monoclonal and polyclonal antibodies employed as primary antibodies are listed in Table 2. Isotype-matched antibodies of inappropriate specificity or normal serum were used as controls.

Immunohistochemical labelling was achieved using the avidin-biotin-peroxidase method as follows. All specimens were divided into two groups, trypsin-pretreated and trypsin-non-treated. The sections were dewaxed in xylene, rehydrated in a graded ethanol series, and immersed in methanol containing 0.5% H_2O_2 to block their endogenous peroxidase activity. In the trypsin-pretreated group, after washing in PBS the sections were pretreated with 0.1% trypsin (trypsin 1:250, Difco, Detroit, Mich.) in PBS containing 0.1% $CaCl_2$ for 10 min at 37°C to unmask their antigenicity. This process was omitted in the trypsin-non-treated group. Thereafter, all the specimens were incubated with 2% skim milk/PBS for 15 min at ambient temperature to block any nonspecific binding of the antibodies. After washing in PBS, the sections were incubated with each primary antibody in moist boxes at 4°C overnight. Next day, the sections were rinsed in PBS at least 3 times, and further processed for immunohistochemistry by the avidin-biotin-peroxidase method [3] using a SAB-Po kit (Nichirei, Tokyo, Japan). Finally, the peroxidase activity was developed in 0.003% diaminobenzidine (DAB)/Tris-HCl (pH 7.6) solution, as described by Graham and Karnovsky [10]. The sections were counterstained with haematoxylin, dehydrated in a graded series of ethanol dilutions, cleared in xylene and mounted with Permount (SP15-100, Fisher Scientific, Fair Lawn, N.J.) for light microscopic observations.

To absorb the *H. pylori*-specific antibody from the primary polyclonal antibody, we used sonicated cells of *H. pylori* ATCC43504 (NCTC11637), generously supplied by Drs. N. Mukai and M. Kikuchi, Otsuka Pharmaceutical Co., Tokushima, Japan. Anti-*H. pylori* polyclonal antibodies diluted 1:100 were incubated in a gradient series of sonicated bacterial cells suspended in PBS (10 mg/ml–0.0001 mg/ml) at 4°C overnight after shaking for 1 h at 37°C. As a control, the primary antibody was incubated in 5% bovine serum albumin/PBS in the same manner. On the following day, all samples were centrifuged at 1500 *g* for 60 min and the supernatants were obtained as primary antibodies. Using these antibodies, the immunohistochemical reaction was performed after trypsin pretreatment as described above.

Endoscopically biopsied gastric mucosa from the patients with MN were fixed in 10% formalin and processed routinely to obtain conventional paraffin sections. At least two gastric biopsies were performed, one fundic and one antral. The material was stained with haematoxylin and eosin (HE) for conventional microscopic observation, and with Giemsa to detect *H. pylori*. In addition, serum samples from MN patients were subjected to enzyme-linked immunosorbent assay (ELISA) for evaluation of IgG antibodies against *H. pylori* protein performed using a HEL-p TEST II kit (AMRAD Operations, Melbourne, Australia). A sample was considered seropositive for *H. pylori* IgG antibodies when the titre exceeded 50 U/ml and negative when below 30 U/ml. Furthermore, serum *H. pylori* antibodies were evaluated by latex agglutination using a PYLORISET Dry Kit (ORION Diagnostics, Espoo, Finland). All these materials were obtained from MN patients less than 2 years after diagnosis.

Results

Many MN specimens revealed granular deposits giving a positive reaction with polyclonal anti-*H. pylori* antibody along the glomerular capillary walls (Fig. 1). This reactivity was observed only in trypsin-pretreated materials,

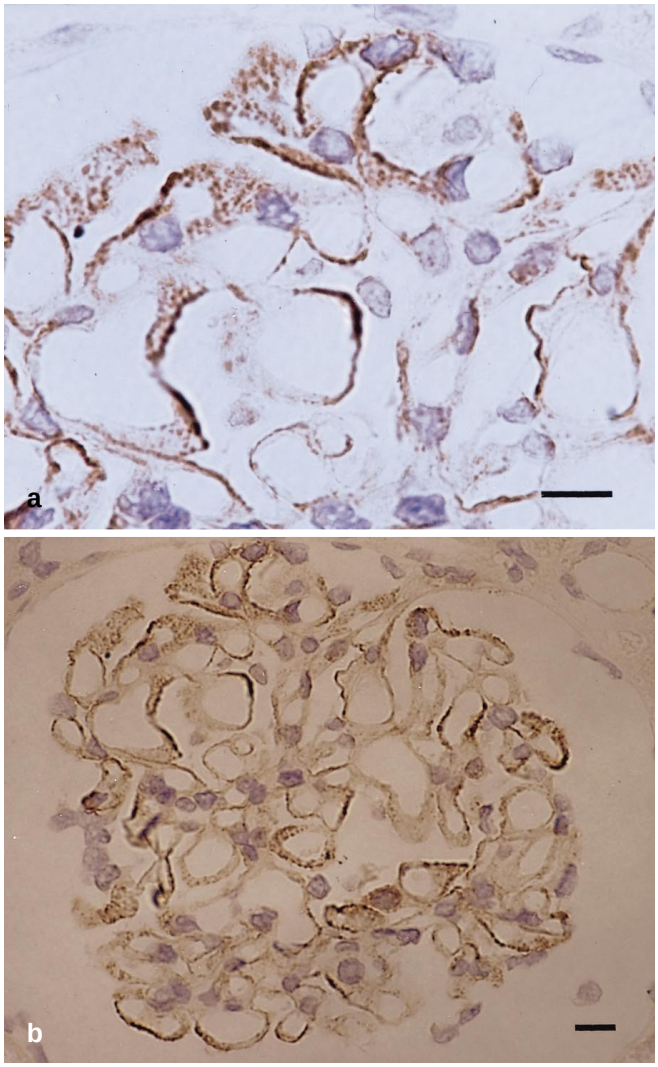

Fig. 1a, b Photomicrograph showing immunoreactivity with anti-*Helicobacter pylori* antibody in glomerulus of a patient with membranous nephropathy. Note the prominent granular positive deposits along the glomerular capillary walls. Avidin-biotin-peroxidase immunolabelling with trypsin pretreatment, **a** ×2000, **b** ×800; bars 10 μm

and not in specimens without trypsin pretreatment. No positive labelling was observed using monoclonal antibodies. The results of immunohistochemical examination are summarized in Table 3. Eleven of 16 specimens had glomerular deposits that were reactive with anti-*H. pylori* antibody. Positive control material obtained from *H. pylori*-infected patients revealed a positive reaction using polyclonal anti-*H. pylori* antibody with trypsin pretreatment and using monoclonal antibodies with or without trypsin pretreatment. None of the negative control specimens (including histopathologically unremarkable kidney) revealed a reaction.

Bacterial cells in specimens obtained from gastric mucosa reacted positively with antibody incubated as a control and incubated with 0.01, 0.001 or 0.0001 mg/ml *H. pylori* antigen. However, we were unable to detect any positive reaction using the antibodies incubated with 10 or 1 mg/ml antigen. The immunostaining obtained using the supernatant incubated with 0.1 mg/ml antigen was regarded as equivocal. Immunohistochemical investigation of MN specimens using the antibodies absorbed in 10 or 1 mg/ml *H. pylori* antigen did not produce a positive reaction in a single case.

The 11 MN patients whose glomeruli were positively stained with anti-*H. pylori* antibody included 7 (cases 1, 2, 6, 7, 10, 12, 14) who were appropriate for analysis of specimens from the stomach and/or serum samples. All these 7 patients were regarded as positive for *H. pylori* infection according to histological and/or serological tests (Table 4).

Discussion

Intense reactivity with polyclonal anti-*H. pylori* antibody was evident along the glomerular capillary walls in many MN specimens, but there was no positive labelling with three monoclonal antibodies, two of which are specific for the 20-kDa (CP15) and 80-kDa (1G6) polysaccharidic epitope components. Since specimens demonstrated

Table 3 Results of immunohistochemical labelling (+ revealing granular positive deposits along glomerular capillary walls, – no reaction in glomerulus)

No.	Trypsin-pretreated materials				Trypsin-non-treated materials			
Antibody	Polyclone	CP15	1G6	51—13	Polyclone	CP15	1G6	51–13
1	+	–	–	–	–	–	–	–
2	+	–	–	–	–	–	–	–
3	–	–	–	–	–	–	–	–
4	–	–	–	–	–	–	–	–
5	–	–	–	–	–	–	–	–
6	+	–	–	–	–	–	–	–
7	+	–	–	–	–	–	–	–
8	+	–	–	–	–	–	–	–
9	+	–	–	–	–	–	–	–
10	+	–	–	–	–	–	–	–
11	–	–	–	–	–	–	–	–
12	+	–	–	–	–	–	–	–
13	–	–	–	–	–	–	–	–
14	+	–	–	–	–	–	–	–
15	+	–	–	–	–	–	–	–
16	+	–	–	–	–	–	–	–
Positive control	Positive	Positive	Positive	Positive	Negative	Positive	Positive	Positive

Table 4 Results of determination of *H. pylori* infection (+ seropositive, ± equivocal, –seronegative)

No.	ELISA (U/ml)	Latex agglutination	Gastric biopsy specimen
1	120 +	+	Positive
2	545 +	+	Positive
6	43 ±	+	Positive
7	Not done	Not done	Positive
10	13 –	+	Positive
12	Not done	Not done	Positive
14	240 +	+	Positive

a positive reaction only after trypsin pretreatment, the labelled antigen was thought to be unmasked by trypsin. We were able to rule out the possibility of nonspecific reaction because no reaction was noted in control material treated using the same procedures. Only the glomerular capillary walls revealed distinct reactivity, and we were unable to detect any positive reaction after immunoabsorption using appropriately diluted sonicated bacterial cells. We consider that our observations represent specific findings in some MN patients, that is the presence of a specific antigen reactive with anti-*H. pylori* antibody in the glomerulus.

As this was a retrospective study, we were unable to obtain serum or gastric material from all the MN patients. However, all 7 patients analysed for *H. pylori* infection among the 11 whose glomeruli were positively stained with anti-*H. pylori* antibody proved to be infected with *H. pylori*. This result supports the possibility that *H. pylori* infection involves the presence of a specific antigen in the glomeruli of some patients with MN.

The present findings suggest that acquisition of *H. pylori* may be involved in the occurrence of MN. However, neither the origin nor the aetiological significance of this antigen along the capillary walls is yet clear. We suggest three possibilities. First, the antigen is from *H. pylori* itself and forms by deposition of immune complexes circulating in serum after *H. pylori* infection. Secondly, antigen from the bacterial cells is deposited directly on the basement membrane of the glomerular capillary walls, and immune complexes may form in situ. A few reports have indicated that some *H. pylori* antigens have a strong affinity for laminin or type IV collagen [20, 21], and it has also been shown that laminin and type IV collagen are effectively increased in the basement membrane of MN patients [11]. However, if this is the case, the deposition of *H. pylori* antigen may not necessarily be related to the initial pathogenesis, since there is a possibility that *H. pylori* antigens having high affinity for the basement membrane are deposited after the occurrence of MN. Finally, the antigen is an *H. pylori*-like antigen that exists specifically in the glomerulus of MN.

Our current understanding of the pathogenesis of MN in humans is based largely on animal models that closely resemble the human disease [8, 15]. Autoantibodies against glomerular basement membrane components may play a significant part, and immune complexes may form in situ [22]. Moreover, a relationship between production of autoantibodies and *H. pylori* infection has been reported [14, 16]. MN may occur after the production of autoantibody induced by *H. pylori* infection. However, human MN differs from that in experimental models, and its pathogenesis is still unknown.

Interestingly, granular deposits in glomeruli reactive with anti-*H. pylori* antibody have been observed in patients with the complications of systemic lupus erythematosus and early gastric cancer. This interesting finding supports the concept that *H. pylori* may not take part in the initiation of MN. The deposition of the *H. pylori* antigen may be a secondary phenomenon. However, we do not yet know, how secondary MN occurs and the most important factor contributing to the disease is still obscure.

It was surprising that these specific findings were observed in so many MN patients. It will be necessary to study *H. pylori* in relation to the occurrence of MN. If this bacillus behaves as a pathogen, its elimination may be beneficial for patients with MN. Moreover, analysis of the antigen along the glomerular capillary walls should contribute further to elucidation of the aetiological mechanism of MN.

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