

RAPID COMMUNICATION

Cross-reactivity of anti-*H pylori* antibodies with membrane antigens of human erythrocytes

Feng-Hua Guo, Xiao-Mei Yan, Chun-Xiang Fan, Fei Zhao, Yuan Hu, Di Xiao, Xun Zeng, Mao-Jun Zhang, Li-Hua He, Fan-Ling Meng, Jian-Zhong Zhang

Feng-Hua Guo, Xiao-Mei Yan, Chun-Xiang Fan, Fei Zhao, Yuan Hu, Di Xiao, Xun Zeng, Mao-Jun Zhang, Li-Hua He, Fan-Ling Meng, Jian-Zhong Zhang, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, PO Box 5, Changping, Beijing 102206, China

Supported by the National Natural Science Foundation of China, No. 30370078

Correspondence to: Jian-Zhong Zhang, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, PO Box 5, Changping, Beijing 102206, China. helico99@sina.com

Telephone: +86-10-61739456 Fax: +86-10-61739456

Received: 2007-03-10 Accepted: 2007-04-18

Abstract

AIM: To investigate whether anti-*H pylori* antibodies have cross-reaction with antigens of erythrocyte membrane.

METHODS: Blood samples were collected from 14 volunteers (8 positive and 6 negative for *H pylori* detected by ¹³C-urea breath test) of the general population. Erythrocyte membrane proteins of the subjects were examined by Western blot using anti-*H pylori* serum. The proteins related to the positive bands were identified by mass spectrum analysis.

RESULTS: Anti-*H pylori* antibodies had cross-reaction with the proteins of about 50 kDa of erythrocyte membranes in all samples independent of *H pylori* infection. One protein in the positive band was identified as Chain S, the crystal structure of the cytoplasmic domain of human erythrocyte Band-3 protein.

CONCLUSION: Anti-*H pylori* antibodies cross-react with some antigens of human erythrocyte membrane, which may provide a clue for the relationship between *H pylori* infection and vascular disorders.

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Key words: *H pylori*; Antibodies; Erythrocyte; Cross-reactivity

Guo FH, Yan XM, Fan CX, Zhao F, Hu Y, Xiao D, Zeng X, Zhang MJ, He LH, Meng FL, Zhang JZ. Cross-reactivity of anti-*H pylori* antibodies with membrane antigens of human

erythrocytes. *World J Gastroenterol* 2007; 13(27): 3742-3746

<http://www.wjgnet.com/1007-9327/13/3742.asp>

INTRODUCTION

H pylori, first isolated by Marshall and Warren^[1], a gram-negative spiral bacterium, colonizing in gastric mucosa, is notorious for causing chronic infections and has been linked to various gastric diseases such as chronic gastritis, peptic ulcer, gastric mucosa-associated lymphoid tissue lymphoma and gastric cancer^[2-4]. In recent years, infection by *H pylori* has been linked to extradigestive pathologies including ischemic cardiac and cerebral diseases. Many seroepidemiological studies revealed the relationship between *H pylori* and vascular disorders^[5,6] even though the prevalence of positive findings varied widely between studies and not all studies reported positive results^[7-9]. However, the exact nature of the association is not completely elucidated.

Several investigations revealed that heat shock proteins (HSPs) of *H pylori* are extremely homologous with HSPs of humans^[10], the O-side chain of the lipopolysaccharide (LPS) of a number of *H pylori* strains is structurally similar to the Lewis histo-blood group antigens^[11], anti-CagA antibodies cross-reacted with antigens of blood vessels^[12]. All these imply that autoimmunity might take part in pathomechanisms of *H pylori*.

The changes of erythrocytes affect the whole blood viscosity, which contributes importantly to thrombosis and atherosclerosis (AS). Our previous studies found that anti-*H pylori* serum reacted with parts of erythrocytes and endothelial cells of heart valves using immunohistochemical method^[13,14]. But it remains unknown which antigen resulted in these positive reactions. The present study was aimed to investigate whether the proteins of erythrocyte membrane cross-react with anti-*H pylori* by Western blot assay and to identify the special proteins by mass-spectrum assay in an effort to provide a clue for pathogenic link between *H pylori* infection and vascular disorders.

MATERIALS AND METHODS

Blood samples

Fresh blood samples were collected from 14 subjects from the general population whose results of ¹³C-urea

Table 1 General data about the subjects

	Subject No.													
	01	02	03	04	05	06	07	08	09	10	11	12	13	14
Gender	F	F	M	M	F	M	M	F	F	M	M	M	F	F
Age (yr)	27	45	44	44	31	49	32	42	47	42	34	45	28	30
¹³ C-UBT (DOB)	40.93	-	8.48	-	-	8.81	11.23	-	10.33	23.08	-	-	25.10	38.71

F: female; M: male; -: UBT negative.

breath test (¹³C-UBT) were supplied by Chinese People's Liberation Army General Hospital. The kit for ¹³C-UBT was provided by Altachem Pharma Ltd. Current infection of *H pylori* was confirmed by a value of ¹³C-UBT greater than 4. General data about the subjects are shown in Table 1. Informed consents were obtained from all the volunteers before ¹³C-UBT and blood sampling.

Extraction of erythrocyte membrane proteins

Fresh blood collected from the subjects were mixed with heparin as anti-coagulant. The erythrocytes were separated by centrifugation at $1230 \times g$ and were lysed with deionized water and then centrifuged at $12000 \times g$ for 20 min at 4°C. The pellets were washed in three volumes of cold phosphate buffer at 5 mmol/L, pH 8.0, containing 1 mmol/L EDTA and 1 mmol/L PMSF (Sigma) 6 times until the membranes were white and then were resuspended in the same buffer and centrifuged at $30000 \times g$ for 1 h at 4°C. The pellets were frozen at -80°C and dried at -56°C in cold vacuum. The membranes were resuspended in the 2-DE lysis buffer cocktail consisting of 7 mol/L urea, 2 mol/L thiourea, 10 g/L DTT, and 40 g/L CHAPS at 4°C for 2 h, then ultrasonicated on ice. The concentration of proteins in each sample was 6-12 g/L determined by Bradford protein assay^[15]. The whole proteins of *H pylori* NCTC11637 were extracted as positive. All reagents in 2-DE lysis buffer were bought from Amersham.

Reactivity of anti-*H pylori* serum with erythrocyte membrane proteins by Western blot

SDS-PAGE was performed using a Bio-Rad Mini-Protean 3 electrophoresis cell. Approximately 120 µg of membrane proteins were parallelly loaded into two wells of 10% SDS-polyacrylamide minigel, 60 µg per well. Thirty µg of whole proteins of *H pylori* NCTC11637 as positive control and 5 µL prestained molecular weight standards marker (Fermantas) were also respectively loaded in two wells per gel.

Proteins were transferred to a PVDF membrane (Amersham) using Bio-Rad Semi-Dry transfer unit. Blocking was performed overnight at 4°C in blocking buffer (TBS containing 50 g/L BSA). The membrane was bisected and one part was incubated with the primary antibody, rabbit anti-*H pylori* NCTC11637 serum (from immunized rabbits with *H pylori* NCTC11637, the animals were provided by Vital River Laboratories Co. Ltd. and raised by the Department of Laboratory Animal Science, Peking University Health Science Center) for 2 h at room temperate (RT). To exclude the color reaction resulting from the direct conjugation of the second antibody and

the normal serum with the proteins on PVDF membranes, the normal serum (pre-immunization serum) of the same rabbits was used as control for another part of membranes with the same samples. Other steps were performed according to the Western blot assay. The second antibody, goat anti-rabbit IgG AP conjugate and AP substrates were from Vector.

Excision of protein bands and in-gel reduction, alkylation and trypsin digestion of proteins

The blots incubated in anti-*H pylori* serum were compared with the others of the same sample incubated in normal serum to find out the different reacted bands. The samples were chosen according to different bands and SDS-PAGE was performed and the gel was stained with Coomassie blue-R250 dye. The bands in the SDS-PAGE gel in accordance with different reacted ones in Western blot were excised, and in-gel reduction, alkylation and trypsin digestion was performed according to EMBL protocol (<http://www.proteomics.com.cn/paper/InGel.html>). Briefly, after a washing step, gel particles were reduced with DTT and alkylated with iodoacetamide. A second washing was performed before overnight digestion with 3 µL (40 mg/L) trypsin (Sigma). The resulting peptides were extracted with 500 mL/L ACN and 50 mL/L TFA and dried in a cold vacuum.

Mass spectrometric (MS) analyses of tryptic peptides and identification of proteins

The digested samples were mixed with a saturated matrix solution (1:1) (α-cyano-4-hydroxycinnamic acid prepared in 500 mL/L acetonitrile and 1 mL/L formic acid). All mass spectra were obtained on a 4700 Proteomics analyzer with TOF/TOF optics (Applied Biosystems, Foster City, CA, USA) in the positive ion reflector mode with a mass accuracy of about 50 ppm. The MALDI tandem mass spectrometer used a 200 Hz frequency-tripled Nd:YAG laser operating at a wavelength of 355 nm. MS spectra were obtained between *Mr* 800 and 4000 with ca. 1000 laser shots. MS/MS spectra were acquired with 2000 laser shots using air as the collision gas. The singly charged peaks were analyzed using an interpretation method present in instrument software, where the five most intense peaks were selected and MS/MS spectra were generated automatically, excluding those from the matrix, due to trypsin autolysis peaks. Spectra were processed and analyzed by the Global Protein Server Workstation (Applied Biosystems, Foster City, CA, USA), which uses internal Mascot v2.0 software (Matrix Science, UK) for searching the peptide mass fingerprints and MS/MS data. Searches

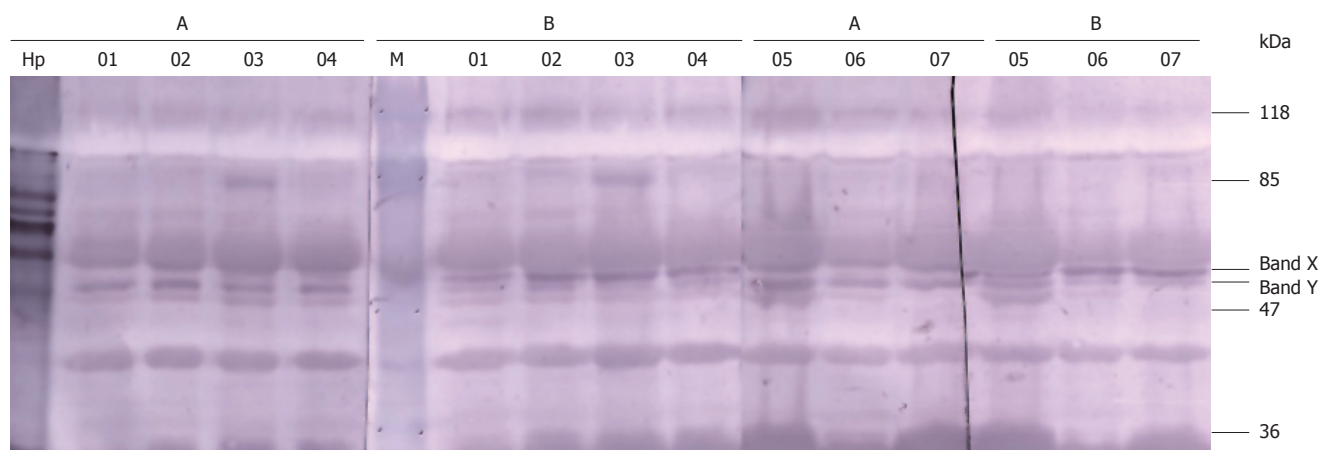


Figure 1 Anti-*H pylori* serum immunoreacted with erythrocyte membrane proteins in Western blot. **A:** incubated with anti-*H pylori* serum of rabbit; **B:** incubated with normal serum of rabbit. 01-07: the number of erythrocyte membrane protein samples. Hp: *H pylori* NCTC11637; M: prestained molecular weight standards marker.

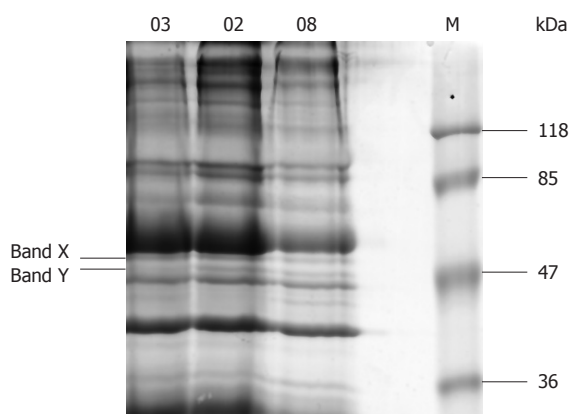


Figure 2 SDS-PAGE of erythrocyte membrane proteins. 03,02,08: the code of erythrocyte membrane protein samples. M: prestained molecular weight standards marker.

were performed against the NCBI non-redundant protein database (updated 18 November 2005). Identifications with a GPS confidence interval of greater than 95% were accepted.

RESULTS

Reactivities of anti-*H pylori* serum with erythrocyte membrane proteins

Both normal rabbit serum and anti-*H pylori* serum showed immunoreactivities with the membrane proteins of about 110 kDa, 55 kDa, 51 kDa, 50 kDa, 40 kDa and 27 kDa of all erythrocytes. However, anti-*H pylori* serum specially recognized antigens of about 50 kDa (marked as band Y in Figure 1) from erythrocytes compared with the normal serum. Remarkably, this feature existed not only in *H pylori*⁺ subjects (No. 01, 03, 06, 07, 09, 10, 13, 14) but also in *H pylori*⁻ subjects (No. 02, 04, 05, 08, 11, 12). The immunoreactivity of another band (marked as band X in Figure 1) with anti-*H pylori* serum was weaker than that with normal serum.

Identification of specific proteins

There were 17-18 bands in the SDS-PAGE 10% gel of

erythrocyte membrane protein sample (Figure 2). The special band of about 50 kDa and another one closely above it (respectively marked as band Y and band X in Figure 2) corresponding to the specially reacted bands in Western blot were faintly stained. Five proteins were identified in the two bands, 4 in band X and 1 in band Y (Table 2).

DISCUSSION

The pathogenesis of ischemic vascular diseases is multifactorial. AS and thrombosis, the principle basis of ischemic vascular disease, determine the occurrence of ischemic events. However, many AS patients lack traditional risk factors, suggesting that other mechanisms may be involved in the AS development^[16,17]. In recent years, more attention has been paid to the relationship between infection and ischemic diseases^[16,18,19].

Several studies indicated the association between *H pylori* infection and ischemic vascular disease especially when the CagA⁺ strain was involved^[5,6], although the results are currently being debated^[7-9]. By now, most studies have been based on seroepidemiology and nonspecific systemic inflammation. The exact mechanisms by which *H pylori* infection contributes to the progression of vascular disorders have not been elucidated.

The molecular mimicry between elements of *H pylori* and those of host cells^[10,11] provides clues for autoimmunity as one of the candidate pathogenesis. Franceschi and his colleagues^[12] reported that anti-CagA antibodies cross-reacted with antigens of both normal and atherosclerotic blood vessels by immunohistochemistry and anti-CagA antibodies also specifically immunoprecipitated two antigens of 160 and 180 kDa from both normal and atherosclerotic artery lysates. The authors speculated that the immunoprecipitated proteins were not CagA of *H pylori* but vascular elements because the two antigens were different from CagA (about 116-140 kDa) in molecular weight. The reactivity detected in vessels with anti-CagA antibodies was caused by the mimicking vascular antigens. We think this speculation reasonable. However, the two antigens were not identified. Moreover,

Table 2 List of proteins identified from the special bands in Figure 2

Band code	Protein name (source)	Accession No.	Protein score	Protein score CI%	Protein MW	Protein PI
X	Flotillin 1 (Sus scrofa)	gi 41529176	88	99.560	47 325.6	7.66
	Flotillin 1 (Macaca mulatta)	gi 55700801	87	99.433	47 383.6	6.71
	Flotillin 1 variant (Homo sapiens)	gi 62896619	87	99.378	47 324.6	8.18
	Predicted: similar to flotillin-1 isoform 5 (Canis familiaris)	gi 73972134	84	98.640	34 070.7	8.52
Y	Chain S, crystal structure of the cytoplasmic domain of human erythrocyte Band-3 protein (Homo sapiens)	gi 14277742	93	99.861	42 509.3	4.49

the difficulty in obtaining vascular tissue makes the investigation in the relationship between vascular endothelium and *H pylori* infection unfruitful.

Erythrocyte is one of most important factors affecting hemodynamics. Its membranes can be easily isolated in large quantities and many blood group antigens are expressed not only on the surface of blood cells but also on vascular endothelial cells. Thus, we chose erythrocyte to investigate the cross-reaction of human plasma membrane and anti-*H pylori* antibodies. Our previous study showed that anti-*H pylori* serum reacted with erythrocytes by immunohistochemical method^[13]. But we did not know which elements resulted in the immunoreaction and whether the elements belong to erythrocytes or to *H pylori*. In the present investigation, antigens of about 50 kDa from erythrocyte membrane strongly immunoreacted with anti-*H pylori* serum rather than normal serum in all 14 samples (Figure 1). This feature did not depend on current infection of *H pylori*. Therefore, we speculate the reacted antigens are not elements of *H pylori* but the mimicking erythrocyte antigens. The results of mass spectrum assay confirmed our speculation. One protein was identified as Chain S, the crystal structure of the cytoplasmic domain of human erythrocyte Band-3 protein (*Mr* 42.5 kDa) in the special band (band Y in Figure 2).

Band 3 protein is the most abundant transmembrane protein to maintain the normal metabolism and function of human erythrocyte. This protein of about 95-100 kDa has two domains. The N-terminal domain of about 40 kDa is located within the cytoplasm and participates in signal transmission across membranes and other functions such as growth, differentiation and interaction of cellules, while the C-terminal of 55 kDa domain is membrane-associated and mediates the exchange transportation of anions $\text{Cl}^-/\text{HCO}_3^-$ across the erythrocyte membrane^[20,21]. In this study, the two antigens of 160 and 180 kDa mimicking with CagA were not found possibly because of the diversity of erythrocytes and vascular cells.

We consider that antibodies against *H pylori* may not contact with cytoplasmic domain of Band 3 of normal erythrocyte. However, oxygen free radicals and systemic inflammation caused by acute or chronic infection could damage erythrocyte membrane leading to the decrease of erythrocyte deformability, increase of erythrocyte fragility and elevation of erythrocyte aggregation index. Some authors reported these changes in several ischemic cardiac disease patients with *H pylori* infection^[22]. The impaired erythrocytes might be easier to be disrupted, inducing internal antigens (including the cytoplasmic domain of Band 3 protein) to be exposed to circulating antibodies.

Then anti-*H pylori* antibodies could bind the exposed antigens and cause inflammatory cell activation, which might be associated with the changes of hemorheology and hemodynamics, plaque aggregation, thrombus formation and atherogenesis leading to ischemic events.

In band X (Figure 2), 4 proteins were identified, which were considered to be flotillin 1 variants according to their resource and molecular weight. The reason why the reaction of the band X incubated with normal serum was stronger than with anti-*H pylori* serum is being investigated.

The protein that cross-reacted with anti-*H pylori* antibodies probably is another one that we could not identify due to its trace quantity and the limit of separation ability of SDS-PAGE. Nevertheless, our study provides an experimental evidence of molecular mimicry between *H pylori* antigens and erythrocyte membrane proteins. The results support the hypothesis that autoimmunity induced by *H pylori* infection plays an important role not only in vascular disorders but also in various extragastric diseases.

COMMENTS

Background

The pathogenesis of ischemic vascular diseases is multifactorial. The conventional risk factors do not fully account for the risk of these diseases. In recent years, more attention has been paid to the relationship between infection and ischemic diseases. Several studies indicated the association between *H pylori* infection and vascular disorders. However, the exact nature of the association is not completely elucidated.

Research frontiers

The molecular mimicry between elements of *H pylori* and those of host cells provides clues for autoimmunity as one of candidate pathopoiesis. Autoimmunity has become one of the hot spots of studies in recent years. Some studies have found that anti-*H pylori* antibodies reacted with endothelial cells and erythrocytes.

Innovations and breakthroughs

This study choose erythrocyte, which is easily to be isolated in large quantities, to investigate the cross-reaction of human plasma membrane and anti-*H pylori* antibodies and found anti-*H pylori* antibodies cross-reacted with the proteins of about 50 kDa of erythrocyte membranes in Western blot. The protein was identified by mass spectroscopy.

Applications

Erythrocyte is one of most important factors affecting hemodynamics. Many blood group antigens are expressed not only on the surface of blood cells but also on vascular endothelial cells. The materials selecting and the results of this study provide a new clue and experimental evidence for autoimmunity as one of the potential pathopoiesis of *H pylori* infection in vascular disorders.

Peer review

This study looks at the cross-reaction of human plasma membrane and anti-*H pylori* antibodies. Although the contribution of the cross-reaction to the relationship

between *H pylori* infection and vascular disorders is not clear, this study provides some interesting observations and a new clue for autoimmunity as one of the potential pathopoiesis of *H pylori* infection.

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S- Editor Liu Y L- Editor Ma JY E- Editor Ma WH