

Enhanced Fe Ion-Uptake Activity in *Helicobacter pylori* Strains Isolated from Patients with Iron-Deficiency Anemia

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Recent studies have suggested a link between iron-deficiency anemia and *Helicobacter pylori* infection. In the current study, strains of *H. pylori* derived from patients with iron-deficiency anemia showed enhanced Fe ion uptake and Fe ion-dependent rapid growth compared with those from patients with non-iron-deficiency anemia. *H. pylori* with enhanced Fe ion-uptake ability may be a causative factor for iron-deficiency anemia.

Helicobacter pylori is recognized as a causative agent of long-term gastritis, peptic ulcer, and stomach cancer. Recent studies suggest that diseases other than those of the gastroduodenal tract are also associated with *H. pylori* infection. In particular, epidemiological and clinical evidence suggests a relationship between iron-deficiency anemia (IDA) and *H. pylori* infection [1–6]. In summary of the evidence, IDA, which is suspected to relate to *H. pylori* infection, has some common features, as follows: (1) most of patients do not report gastrointestinal symptoms, (2) no gastrointestinal bleeding is observed, (3) no shortage of iron uptake is observed, (4) *H. pylori*-associated gastritis is evident on histopathological examination after endoscopy and biopsy, (5) anemia is unresponsive to treatment with iron or is responsive but exacerbated when treatment with iron is stopped, and (6) anemia is improved by eradication of *H. pylori* without treatment with an iron. However, the relationship between *H. pylori* and the etiology of IDA remains obscure. In the present study, we compared the Fe ion-uptake ability of *H. pylori* strains recovered from patients with IDA

and patients with gastroduodenal diseases without IDA (non-IDA).

Methods. The criteria for puberty IDA were as follows: hemoglobin concentration, <12 g/dL (for males) or 11.5 g/dL (for females); mean corpuscular volume, <78 fL; mean corpuscular hemoglobin concentration, <26 pg; Fe level, <50 µg/dL; serum ferritin level, <10 µg/mL; and transferrin saturation degree, <16%. *H. pylori* infection status was based on the presence of anti-*H. pylori* serum IgG, as measured by ELISA (HM-CAP; Enteric Products) and the *H. pylori* stool antigen test (Premier Platinum HpSA; Meridian Diagnostics). We isolated *H. pylori* from gastric biopsy specimens of *H. pylori*-infected children. Informed consent was obtained from parents of each patient. The isolation and identification of *H. pylori* were described elsewhere [7]. We selected 10 *H. pylori* isolates recovered from patients with IDA whose hemoglobin levels were <10 g/dL, in consideration of differences between male and female. We also selected 10 *H. pylori* isolates derived from patients without symptoms of anemia (i.e., non-IDA). The ratio of male to female subjects and age distribution of the patient group with non-IDA were comparable to those of the patient group with IDA. The ages (\pm SE) of the groups with IDA and with non-IDA were 13.4 ± 0.8 and 13.9 ± 3.4 years, respectively ($P =$ not significant). Both groups comprised 6 males and 4 females. RBC counts of IDA and non-IDA groups were 401.6 ± 60.6 and $481.1 \pm 35.6 \times 10^4/\mu\text{L}$, respectively ($P < .05$). Hemoglobin levels were 7.17 ± 1.62 and 13.77 ± 1.53 g/dL, respectively ($P < .05$). Fe levels were 12.8 ± 4.6 and 101.7 ± 23.8 µg/dL, respectively ($P < .01$). Pepsinogen I levels were 67.7 ± 18.4 and 86.1 ± 72.2 ng/mL, respectively ($P =$ not significant). Pepsinogen II levels were 17.7 ± 7.5 and 23.0 ± 17.6 ng/mL, respectively ($P =$ not significant). The pepsinogens were determined as a marker for gastric inflammation [8]. Statistical significances were evaluated by the unpaired t test.

H. pylori isolates were precultured on a *Helicobacter*-selective agar plate (Nissui Pharmaceuticals). The cells were collected from the agar and were inoculated into brain-heart infusion broth supplemented with 5% fetal bovine serum (BHI-FCS). Fe-restricted cell culture was performed with BHI-FCS supplemented with 20 µmol/L deferoxamine mesylate (Sigma-Aldrich). Fe-sufficient cell culture was performed with BHI-FCS. Cell number was determined by turbidity (measured by absorbance at 600 nm).

Fe ion-uptake ability was determined basically according to the method of Velayudhan et al. [9]. In brief, cells grown in BHI-FCS to the late exponential phase were collected and

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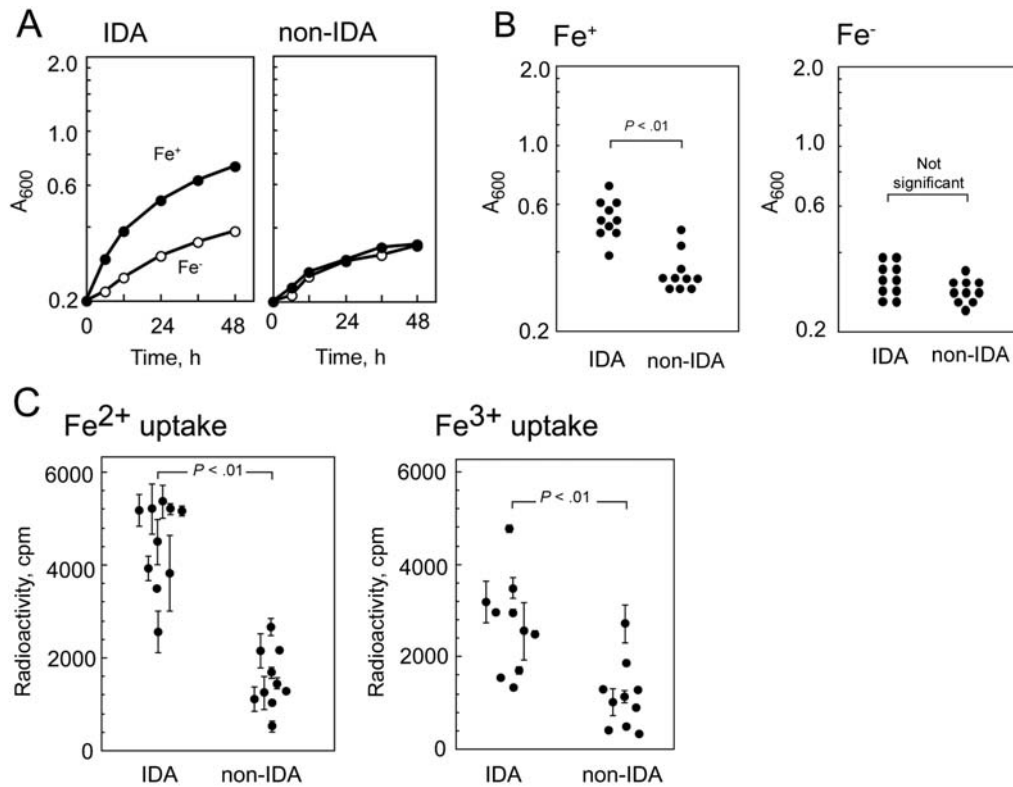


Figure 1. Cell growth under Fe ion–restricted and –sufficient conditions and Fe ion uptake in *Helicobacter pylori* strains recovered from patients with iron-deficiency anemia or without iron-deficiency anemia. *A*, Cell growth curves of representative strains. ●, Fe ion–sufficient culture conditions; ○, Fe ion–restricted culture conditions. *B*, Comparison of cell growth rates. Data represent absorbance at 600 nm at 48 h. *C*, Fe²⁺ (left graph) or Fe³⁺ (right graph) ion uptake of *H. pylori* cells. Data represent the mean ± SE of 4 independent experiments. Statistical significances were evaluated by the unpaired *t* test.

washed twice in Dulbecco's PBS. The cells were resuspended in PBS at a concentration of 20–30 mg total cell protein per milliliter and then were maintained under microaerobic conditions at 37°C for 1 h. The cell suspension (100 μL) was added to 2 mL of Chelex-100 (Sigma-Aldrich)–treated BHI-FCS, which contained 0.4% glucose and 100 μmol/L nitorilotriacetic acid. ⁵⁵FeCl₃ (Perkin-Elmer) was used for the Fe ion–uptake assay. To examine uptake of Fe²⁺, ⁵⁵FeCl₃ was diluted 10-fold in 1 mol/L of ascorbic acid. To examine uptake of Fe³⁺, ⁵⁵FeCl₃ was diluted 10-fold in 1 mol/L of sodium citrate before the assay. Each solution of ⁵⁵FeCl₃ (20 kBq per tube) was then added to the cell suspension. After 5 min, the suspension was layered onto 0.3 mL silicon oil (Sigma-Aldrich) and was centrifuged at 12,000 *g* for 1 min, to stop uptake by pelleting the cells through the oil. The aqueous layer was removed, and the surface of the oil was washed twice with PBS. The oil layer was removed, and the cell pellet was suspended in 100 μL of 1% (v/v) Triton X-100 to solubilize the cells. The solubilized cell suspension was then placed in Aqueous Counting Scintillant II (GE Healthcare Bioscience), and the radioactivity in the suspension was counted using ³H channel of a liquid scintillation counter and

was used as Fe ion–uptake activity. The experiments were performed in quadruplicate.

Results. We examined 10 *H. pylori* strains recovered from patients with IDA and 10 strains recovered from patients with non-IDA. First, we compared the growth rate of the 2 groups of strains under Fe-restricted or Fe-sufficient conditions (figure 1A and 1B). Under Fe-sufficient conditions, strains recovered from patients with IDA showed significantly higher growth rates than did those recovered from patients with non-IDA. Under Fe-restricted conditions, the growth rate of strains recovered from patients with IDA was markedly decreased compared with tests done under Fe-sufficient conditions. Most of the strains recovered from patients with non-IDA showed a similar growth rate under Fe-restricted and Fe-sufficient conditions. Under Fe-restricted conditions, the cell growth rates were not significantly different between strains recovered from patients with IDA and from patients with non-IDA.

Next, we examined Fe³⁺ or Fe²⁺ uptake by the strains (figure 1C). The uptake of both Fe²⁺ and Fe³⁺ was greater for strains recovered from patients with IDA than those recovered from patients with non-IDA. The differences in Fe ion uptake be-

tween IDA strains and non-IDA strains were more apparent for the Fe²⁺ ion than for the Fe³⁺ ion.

Discussion. Although epidemiological and clinical evidence of the relationship between *H. pylori* infection and the incidence of IDA has been accumulating, the etiology of *H. pylori*-associated IDA is not clear. The results of the current study showed that strains of *H. pylori* recovered from patients with IDA displayed a rapid cell growth rate in an Fe ion-dependent manner and an enhanced Fe ion uptake, particularly Fe²⁺, compared with strains recovered from patients with non-IDA. On the basis of these results, we propose that one of the risk factors for IDA is infection with certain strains of *H. pylori*. Fe is an essential factor for *H. pylori*, as for all bacteria. Strains of *H. pylori* that have an enhanced Fe ion-uptake efficiency consume Fe ions in the stomach and grow rapidly in the presence of Fe ions. Furthermore, *H. pylori* cells in which large amounts of Fe ions accumulate are excreted in stool. Enhanced Fe consumption and excretion due to certain strains of *H. pylori* may contribute to Fe deficiency in the host and the development of IDA. Thus, in addition to host and environmental factors, the particular strains of colonizing *H. pylori* may be involved in the development of IDA.

We have not identified the molecule(s) that contribute to the enhanced Fe ion uptake of IDA-associated *H. pylori*. Several candidates, however, can be explored. *H. pylori* in the human stomach acquires Fe via human lactoferrin [10]. Dhaenens et al. [11] identified a lactoferrin-binding protein of *H. pylori* that resides in the outer membrane. Recently, hepcidin, which is an antimicrobial peptide recovered from liver, was identified and characterized. The link among hepcidin induction, *H. pylori*-induced inflammation, and anemia, both in humans and in animal models, suggests its key role as a mediator of anemia [12]. However, we have not determined the levels of hepcidin in serum. FeoB is a major component of the Fe²⁺ ion-acquisition machinery in *H. pylori* [9]. Jeon et al. [13] reported several polymorphisms of *feoB* that appear to be associated with IDA; however, the relationship has not yet been fully elucidated because of the small number of strains studied. We determined the DNA sequence of *feoB* in the strains used in the present study and did not observe a significant relationship between *feoB* polymorphisms and IDA (data not shown). Park et al. [14] performed a comparative proteomic analysis of *H. pylori* strains recovered from patients with IDA and with non-IDA.

They showed that IDA- and non-IDA-associated strains could be distinguished by their protein-expression patterns and that a number of proteins had higher levels of expression in IDA strains. However, the identities of the proteins that contribute to IDA are unknown. Additional studies are required to elucidate the molecular mechanism of enhanced Fe ion uptake in *H. pylori* strains recovered from patients with IDA.

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