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Antigastric Autoantibodies in Ferrets Naturally Infected with *Helicobacter mustelae*

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Infection with *Helicobacter pylori* has been associated with induction of autoantibodies that cross-react with the gastric mucosa. There have been discordant reports as to whether or not these autoantibodies arise due to molecular mimicry between *H. pylori* and host cell antigens on parietal cells. In this study, we investigated whether molecular mimicry by *H. mustelae* causes autoantibodies in infected ferrets. Serum from *H. mustelae*-infected ferrets reacted with parietal cells in the ferret gastric mucosa but not with duodenal or colonic mucosa. These sera did not react with the blood group A epitope on erythrocytes or *H. mustelae* lipopolysaccharide, and absorption with *H. mustelae* whole cells or red blood cells did not remove autoantibodies. In conclusion, ferrets naturally infected with *H. mustelae* generate antibodies that react with parietal cells, but these autoantibodies are not due to molecular mimicry.

Helicobacter pylori is a gram-negative, spiral-shaped organism which colonizes the gastric mucosa of humans (18). *H. pylori* has been associated with gastritis (18, 30), duodenal ulcer disease (22, 26), and gastric cancer (23, 25, 29). Patients infected with *H. pylori* have been shown to have autoantibodies that react with antigens expressed on the gastric mucosa (20). The gastric H^+ K^+ATPase found in the canaliculi of parietal cells has been identified as a possible target of this autoimmune response (2, 6, 10, 11, 17). The presence of gastric autoantibodies, in particular those directed to parietal cells, was found to correlate with an increased corpus atrophy. It has thus been suggested that *H. pylori*-associated autoimmunity may play a crucial role in the pathogenesis of chronic atrophic gastritis, a risk factor for gastric cancer (10, 21).

Studies have shown that the O-antigen regions of lipopolysaccharide (LPS) from some *H. pylori* strains are structurally similar to the blood group antigens Lewis x and Lewis y (3, 4, 5). These antigens are expressed in more than 85% of strains obtained from various parts of the world (27). *H. pylori*-associated antigens Lewis x and Lewis y have been implicated in the induction of autoantibodies in humans. One group (21) suggested that molecular mimicry between *H. pylori* antigens and the gastric mucosa causes production of gastric autoantibodies, as they found that absorption of serum from *H. pylori*-infected patients with *H. pylori* resulted in reduced reactivity with the gastric mucosa. However, this has been the only report suggesting that gastric autoantibodies in humans are due to molecular mimicry between *H. pylori* and the gastric mucosa. Faller at al. (9) also absorbed serum from *H. pylori*-infected individuals with *H. pylori* organisms. They removed the reactivity of the serum with *H. pylori* but not with the gastric mucosa, suggesting that molecular mimicry between *H. pylori* and the gastric mucosa is not the cause of gastric autoantibodies. Similarly Ma et al. (17) did not succeed in removing anti-H^+ K^+ATPase autoantibodies by preabsorption with *H. pylori*. Claeys et al. (6) and Amano et al. (1) have recently shown that there is no increase in anti-Lewis antibodies in *H. pylori*-infected individuals compared with noninfected individuals, and they also concluded that molecular mimicry was not involved in gastric autoantibody production (6). Furthermore autoantibodies were shown to react with the peptide part of the H^+ K^+ATPase and not with carbohydrate structures (6). In contrast Guruge et al. (16) have recently described a transgenic mouse model for *H. pylori*-induced gastric autoantibodies in which the autoantibodies were directed against Lewis x antigen and were thought to be due to molecular mimicry between the organism and the mouse gastric mucosa. These autoantibodies were removed by absorption of the serum with *H. pylori*.

A naturally infected-animal model of *Helicobacter* infection may be more closely related to *H. pylori* infection in humans. *Helicobacter mustelae* infects ferrets naturally, colonizing the gastric mucosa (15). *H. mustelae* shares many virulence factors with *H. pylori*, including intimate adherence to gastric epithelial cells (13), sheathed flagella (28), and a potent urease enzyme (7). *H. mustelae* has also been associated with gastritis and duodenal ulcer disease (12, 15). More recently *H. mustelae*-positive ferrets have been shown to develop adenocarcinoma of the stomach (14) and a gastric mucosa-associated lymphoid tissue lymphoma (8). We and others have previously reported that *H. mustelae* expresses blood group antigen A (19, 24), which is also expressed on ferret gastric epithelial cells (24), indicating that *H. mustelae* like *H. pylori* displays molecular mimicry of a host blood group antigen. We have also demonstrated that *H. mustelae*-specific antibodies raised in a rabbit cross-react with blood group antigen A on ferret epithelial tissue (24). However, the antibody response obtained by

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injecting a rabbit with *H. mustelae* may be very different from that seen with natural infection of ferrets.

The aims of this study were, therefore, to investigate whether ferrets naturally infected with *H. mustelae* developed autoantibodies to epitopes in the ferret gastric mucosa. If any autoantibodies were present, we wanted to determine whether they were due to molecular mimicry, as is the case with animal models of *H. pylori* infection, or if there was no association with molecular mimicry of *Helicobacter* structures, as appears to be the case in natural *H. pylori* infection.

Serum samples were taken from a group of 10 ferrets including four adults (F1, F2, F9, and F10) and six younger ferrets ranging from 10 to 12 weeks old (F3 through F8). Blood was taken by cardiopuncture and allowed to clot before serum was removed. Ferrets were then euthanatized, and tissue samples were taken from the antrum fundus and duodenum for diagnosis of *H. mustelae* infection. Tissue was minced and plated onto blood agar plates at 37°C for 3 days in an atmosphere of 10% CO_2 and 5% O_2. Tissue was also tested for urease activity by incubation in 100 μl of urea solution containing 2% (wt/vol) urea and 0.001% (wt/vol) phenol red in 0.01 M phosphate buffer (pH 6.8). A positive reaction was indicated by a change in color from orange to pink within 30 min.

H. mustelae 12198 was obtained from the National Collection of Type Cultures (Public Health Laboratory Service, London, England). Strain 12198 and strains isolated from ferrets were cultured on Columbia blood agar plates (Oxoid, Columbia, Md.) containing 7% defibrinated horse blood for 3 days at 37°C in an atmosphere of 10% CO_2 and 5% O_2.

Serum was tested for anti-*H. mustelae* antibodies by enzyme-linked immunosorbent assay as previously described (2) *H. mustelae* whole cells (7×10^6) were suspended in 100 μl of phosphate-buffered saline (PBS), added to wells of microtiter plates, and incubated overnight at room temperature. Plates were washed with PBS containing 0.05% Tween 20 (PBST). Subsequently, ferret sera serially diluted in PBST were added and incubated for 2 h at room temperature. Plates were then washed three times in PBST and goat anti-ferret immunoglobulin G (Kirkegaard and Perry) conjugated to horseradish peroxidase was added, diluted 1/1,000 in PBST with 0.5% goat serum, and incubated for 2 h at 37°C. Plates were washed and developed using H_2O_2 and orthophenylene diamine in citrate phosphate buffer (pH 5.5) for 30 min at room temperature, and the optical density was read at 492 nm after stopping the reaction with 50 μl of sulfuric acid.

Rabbit-raised *H. mustelae* antiserum to strain NCTC 12198 (24), rabbit preimmune serum, and ferret sera were tested for the presence of anti-A and anti-B antibodies by titration against blood group A, B, and O red blood cells. Serum was diluted serially in microtiter plates, and red blood cells of blood group A, B, or O were added. The suspension was mixed, spun, and resuspended, and agglutination patterns were recorded. Serum was diluted 1:50 in PBS, and *H. mustelae* (10^{12} CFU/ml) or human red blood cells (3% packed cell volume) expressing blood group antigen A were added. Serum was absorbed with the *H. mustelae* strain isolated from each ferret and *H. mustelae* strain 12198, apart from serum from ferret 2, which was only absorbed with *H. mustelae* strain 12198, as no *H. mustelae* was cultured from this animal. Aliquots (1 ml) of suspensions were mixed well and left at 4°C overnight with shaking. The cells were removed by centrifugation at 10,000 × *g* for 5 min, and the supernatants were aliquoted and stored at −20°C.

Gastric, duodenal, and colonic biopsy specimens were taken from the gastrointestinal tracts of ferrets not infected with *H. mustelae*. Gastric biopsies were taken at endoscopy using a paediatric bronchoscope, whereas duodenal and colonic samples were taken postmortem. Samples were fixed in 10% formaldehyde and paraffin embedded. Sections were cut using a microtome and mounted on glass slides. Slides were deparaffinated in xylene and rehydrated in graded ethanol solutions (two changes of absolute ethanol, 3 min each, followed by two changes of 80% ethanol, 3 min each). Internal peroxidase was inactivated by incubation in 0.3% hydrogen peroxidase in methanol for 30 min. Normal goat serum diluted 1/10 in PBS was used to block nonspecific binding of the secondary antibody. Slides were stained with rabbit-raised anti-*H. mustelae* antibodies (1/100), ferret sera (1/50), or monoclonal antibodies raised against the β chain of the H^+ K^+ATPase (monoclonal antibody 2G11) (1/50) for 30 min at room temperature. The secondary antibodies used were either goat anti-mouse (Sigma), goat anti-rabbit (Sigma), or goat anti-ferret immunoglobulin G (Kirkegaard and Perry) conjugated to peroxidase. All secondary antibodies were used in accordance with the manufacturers' recommendations. Slides were developed for 5 min with 3,3′-diaminobenzidine tetrahydrochloride medium and counterstained with Weigerts hematoxylin (Sigma). After dehydration slides were mounted and analyzed using a light microscope.

H. mustelae cells were incubated with proteinase K (50 μg/200 μg of protein in the cell suspension) for 1 h at 60°C or with 50 mmol of sodium acetate (pH 4.5) per liter alone or containing 10 mmol of sodium metaperiodate per liter for 1 h at room temperature in the dark. Cells were lysed by boiling in sample buffer (Tris-mercapthoethanol) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% acrylamide), and proteins were transferred to nitrocellulose. Membranes were probed with serum from *H. mustelae*-infected ferrets (1/200 dilution) and monoclonal antibody raised against blood group antigen A (1/1,000 dilution; Dako). Antigen antibody complexes were detected using either goat anti-ferret IgG (Kirkegaard and Perry) (1/500 dilution) or goat anti-mouse IgG (Sigma) (1/1,000 dilution) conjugated to peroxidase. Blots were developed using enhanced chemiluminesence (Amersham).

The four adult ferrets tested positive for *H. mustelae* infection by urease activity and serology. *H. mustelae* strains were cultured from three of these ferrets (F1, F9, and F10), but in the fourth (F2) no strain was isolated due to contamination of the plates. The six younger ferrets (F3 through F8) tested negative for *H. mustelae* infection by serology culture and urease activity, although one did give a weakly positive urease test after 48 h. Thus, the adult ferrets (F1, F2, F3, and F10) were deemed to be infected with *H. mustelae* whereas the younger ferrets (F3 through F8) were deemed noninfected.

Immunohistochemistry was used to check for the presence of autoantibodies in the serum of each ferret. Reactivity was then compared with staining of the tissue with monoclonal antibodies to H^+ K^+ATPase and rabbit-raised *H. mustelae*-

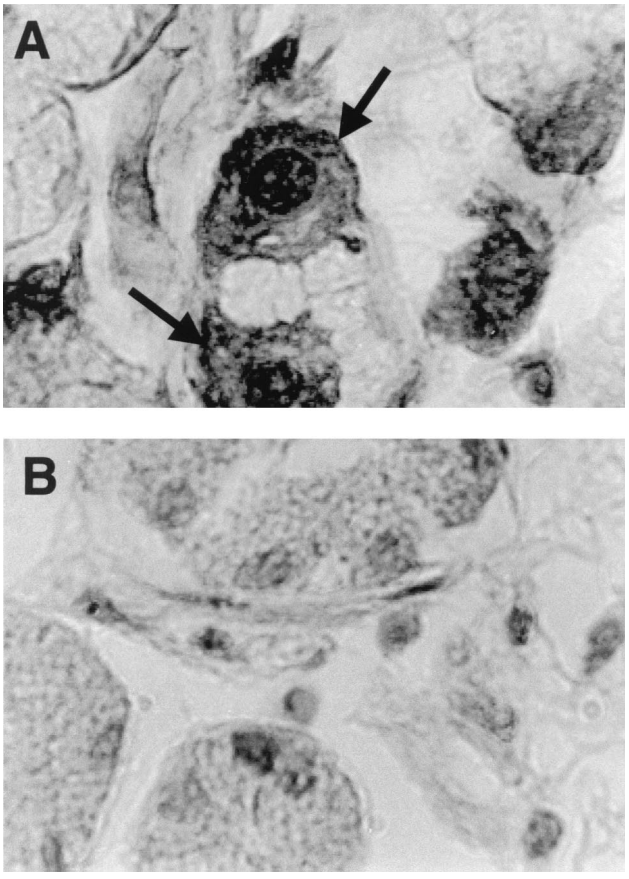

FIG. 1. Immunohistochemical staining of ferret gastric mucosa following incubation with serum from an *H. mustelae*-positive ferret, showing staining of the parietal cells (indicated by arrows), identified by their round nucleii (A), and serum from an *H. mustelae*-negative ferret, showing no specific staining (B). Magnification, ×810.

specific antibodies. Of the 10 sera 2 (F2 and F9) showed a strong staining of ferret gastric tissue, with 2 more sera (F1 and F10) showing a weaker staining, while the 6 sera from uninfected ferrets showed no significant staining of ferret gastric tissue (Fig. 1). The staining with sera from ferrets infected with *H. mustelae* was specifically of cells found in the gastric glands. These cells appeared morphologically to be parietal cells by their pyramidal shape and round nucleus. Furthermore, a similar pattern of staining was shown using monoclonal antibodies to the H^+ K^+ATPase found in the canaliculi of parietal cells (data not shown). With serum from *H. mustelae* infected ferrets, no staining was observed on duodenal tissue and only parietal cells were stained on the gastric mucosa. This pattern of staining was different from that observed using *H. mustelae*-specific antibodies raised in a rabbit, where all gastric and duodenal epithelial cells were stained by the antiserum (Fig. 2).

H. mustelae-specific antiserum raised in a rabbit and serum from ferrets naturally infected with *H. mustelae* who had autoantibodies were absorbed with *H. mustelae* or red blood cells expressing blood group antigen A. Subsequently these absorbed sera were tested for reaction with ferret gastric tissue by immunohistochemistry. In the case of the *H. mustelae* antiserum raised in a rabbit, the reactivity with ferret gastric epithelial cells was removed both by absorption with the red blood cells and *H. mustelae* strain NCTC 12198 (data not shown). However, the autoantibodies in the sera from naturally infected ferrets could not be absorbed out using red blood cells expressing blood group antigen A or by using *H. mustelae* whole cells from either NCTC 12198 or the infecting strain (Fig. 3).

Rabbit-raised *H. mustelae* antiserum and serum from *H. mustelae* infected and uninfected ferrets were tested for the presence of antibodies to blood groups A and B. *H. mustelae* antiserum raised in a rabbit reacted with blood groups A and B at a dilution of 1/16 and 1/2, respectively. However, none of the sera from *H. mustelae*-infected or uninfected ferrets showed any reaction with blood groups A or B.

Western immunoblotting showed that serum from *H. mustelae*-infected ferrets reacts with a range of different antigens on *H. mustelae* whole cells, and this reactivity is completely removed by treatment of *H. mustelae* with proteinase K. Sodium metaperiodate treatment, however, had no effect. In contrast sodium metaperiodate treatment completely abolished the reaction of anti-blood group A antibodies with *H. mustelae*, whereas proteinase K treatment has no effect. This suggests that ferrets naturally infected with *H. mustelae* do not have an antibody response against the blood group antigen A epitope on the LPS of the bacteria (Fig. 4).

Lewis x and Lewis y antigens have been shown to be expressed as part of the O-antigen region of LPS of *H. pylori* (3, 4, 5). It had been suggested that during *H. pylori* infection antibodies are raised against bacterial Lewis x and Lewis y antigens and cross-react with these antigens found on the gastric mucosa (2, 21). Claeys et al. (6) have demonstrated autoantibodies binding to canalicular structures within the parietal cells of humans (6). However, *H. pylori*-infected individuals with these autoantibodies did not have increased titers of antibodies against Lewis x or Lewis y antigens compared with noninfected individuals (6). The authors concluded that gastric autoantibodies in *H. pylori*-infected individuals are not caused by molecular mimicry between the bacteria and the gastric mucosa (6). These results contrast markedly with experimental models of *H. pylori* infection where titers of anti-Lewis x and Lewis y antigens are increased in infected or immunized animals (2, 16, 21).

We previously reported that *H. mustelae*-specific antibodies raised in a rabbit cross-react with blood group antigen A on the gastric mucosa of ferrets. *H. mustelae* has been shown to express blood group antigen A as part of its LPS (19, 24), and we have shown that blood group antigen A is also found on ferret gastric epithelial cells (24). This is analogous to the expression of Lewis x and Lewis y antigen on *H. pylori* LPS and the human gastric mucosa. Rabbit-raised *H. mustelae*-specific antibodies which reacted with blood group antigen A on the gastric mucosa could be removed by absorption with *H. mustelae* expressing blood group antigen A, proving that these antibodies had been induced by molecular mimicry.

In the present study we have identified for the first time anti-parietal cell autoantibodies in ferrets naturally infected with *H. mustelae*. However, these autoantibodies are not removed by absorption of sera with *H. mustelae* or red blood cells expressing blood group A. Thus, these autoantibodies are not

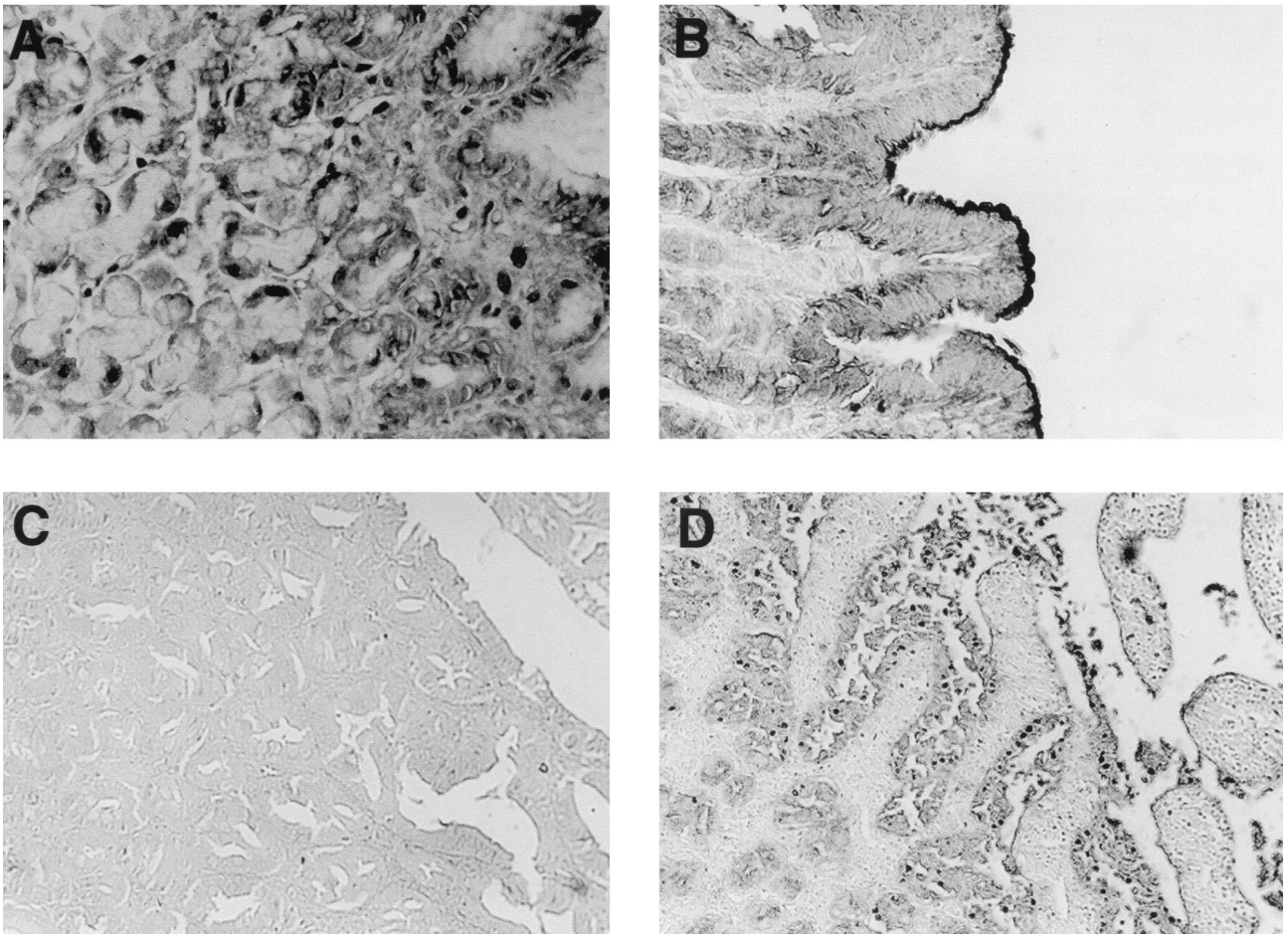

FIG. 2. Immunohistochemical staining of ferret gastric and duodenal tissue. (A) Ferret gastric epithelium stained with serum from ferret 9 (which was naturally infected with *H. mustelae*), showing staining of parietal cells only. (B) Ferret gastric epithelium stained with *H. mustelae* antibodies which were raised in a rabbit, showing staining of gastric epithelial cells. (C) Ferret duodenal epithelium stained with serum from ferret 9, showing no staining. (D) Ferret duodenal epithelium stained with *H. mustelae* antibodies raised in a rabbit, showing staining of duodenal epithelial cells. Magnification, ×340.

due to molecular mimicry between the bacteria and the gastric mucosa. The response is therefore different from that seen with immunized animals such as rabbits, where autoantibodies are directed against host antigens expressed by the bacteria. Our results suggest that the autoantibody response in *H. mustelae*-infected ferrets is similar or identical to that reported by most groups who have studied autoantibodies in humans with *H. pylori* gastritis (6, 9, 17).

Colonization of transgenic mice expressing the Lewis b antigen with *H. pylori* has been reported to be associated with the development of autoantibodies to parietal cells (16). However, these autoantibodies were shown to recognize the Lewis x antigen and thus were induced by molecular mimicry. This finding is in marked contrast to what has been reported in most studies of *H. pylori* infection in humans and our findings with ferrets naturally infected with *H. mustelae*. These findings emphasize the difference in the immune response seen with natural models of infection compared to the response that occurs in experimental animal models.

If gastric autoantibodies in *H. pylori* or *H. mustelae* infection were directed against blood group antigens it would be expected that the antibodies would react with the gastrointestinal tract as a whole rather than specifically with the gastric mucosa. We have shown that the *H. mustelae*-specific antibodies raised in a rabbit, which are directed against blood group antigen A, react with duodenal and colonic tissue as well as gastric tissue (24). In contrast in our present study the autoantibodies found with natural infection of the ferret only reacted with the gastric mucosa. This is again identical to the situation in *H. pylori* infection of humans, where autoantibodies react with the gastric mucosa but not with duodenal or colonic tissue (21). Our findings suggest that these anti-parietal cell antibodies occurring in association with natural *Helicobacter* infection are due to another process, such as gastric inflammation, rather than molecular mimicry.

H. pylori-induced autoantibodies have been suggested to play a role in the pathogenesis of chronic atrophic gastritis, which is thought to be a risk factor for gastric cancer. An animal model is required to further our understanding of the role of *Helicobacter*-induced autoantibodies in the pathogenesis of chronic atrophic gastritis. *H. mustelae*-infected ferrets have been shown to develop multifocal atrophic gastritis (13),

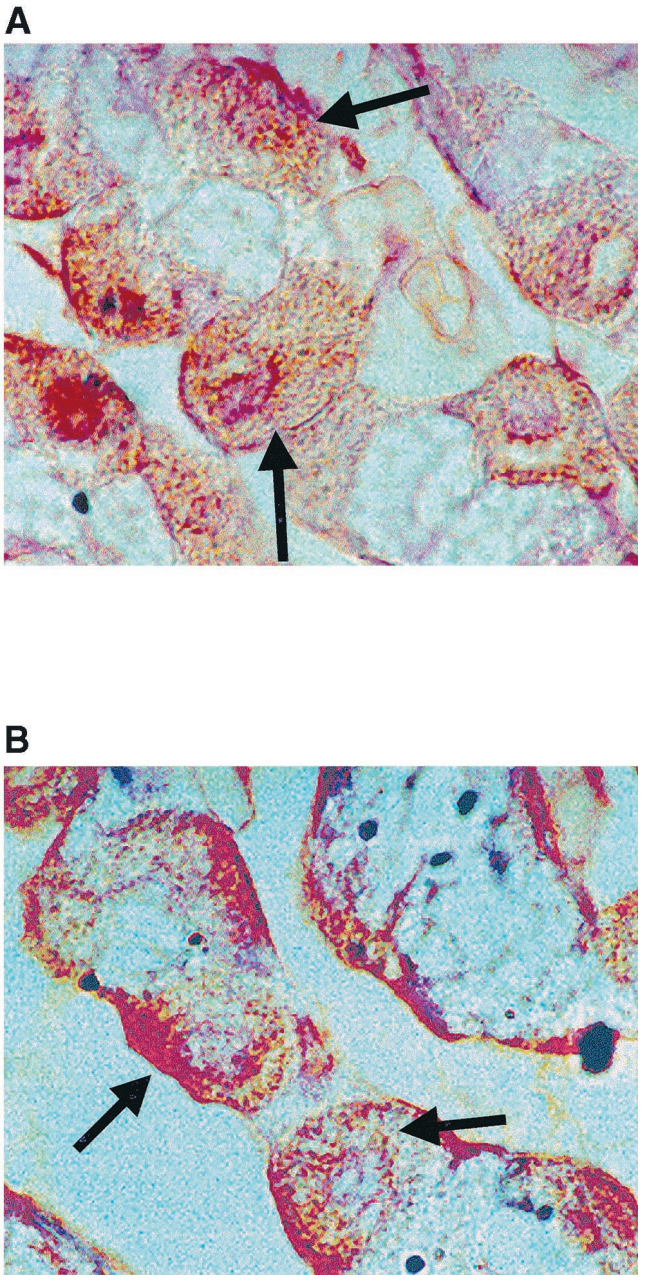

FIG. 3. Absorption of ferret serum from an *H. mustelae*-infected ferret with *H. mustelae* whole cells. (A) Ferret gastric epithelium stained with serum from an *H. mustelae*-infected ferret, showing staining of parietal cells. (B) Ferret gastric epithelium stained with serum from an *H. mustelae*-infected ferret after absorption with *H. mustelae* whole cells, showing no removal of reactivity. Parietal cells are indicated using arrows. Magnification, ×930.

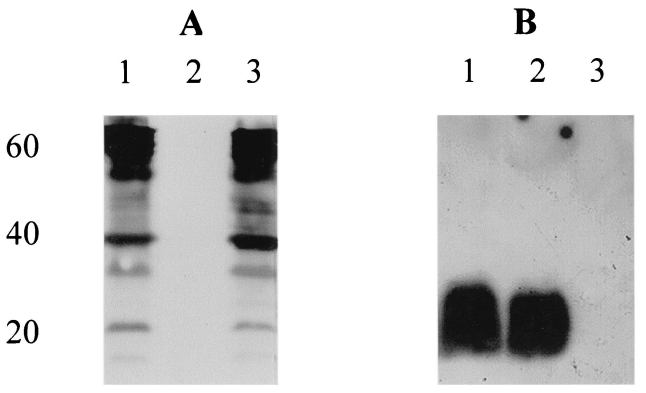

FIG. 4. Western immunoblot showing reaction of serum from a ferret naturally infected with *H. mustelae* and a monoclonal antibody against blood group antigen A with *H. mustleae*. Lanes 1, untreated *H. mustelae* whole cells; lanes 2, *H. mustelae* whole cells treated with proteinase K; lanes 3, *H. mustelae* whole cells treated with sodium acetate (50 mmol/liter) containing sodium metaperiodate (10 mmol/liter). (A) Blot is probed with serum from a *H. mustelae* infected ferret; (B) blot is probed with a monoclonal antibody against blood group antigen A. Molecular weights are indicated at left in kilodaltons.

gastric adenocarcinoma (14) and mucosa-associated lymphoid tissue lymphoma (8). These findings along with our report of the presence of autoantibodies which are not due to molecular mimicry suggest that the ferret model of naturally occurring *Helicobacter* infection may be useful for investigating possible associations between autoimmunity, atrophic gastritis, and gastric cancer. Further studies will focus on the association between *H. mustelae*-associated autoantibodies and atrophic gastritis in ferrets.

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