Macrophages Are Mediators of Gastritis in Acute *Helicobacter pylori* Infection in C57BL/6 Mice

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*Helicobacter pylori* is the etiological agent of human chronic gastritis, a condition seen as a precursor to the development of gastrointestinal ulcers or gastric cancer. This study utilized the murine model of chronic *H. pylori* infection to characterize the role of macrophages in the induction of specific immune responses and gastritis and in the control of the bacterial burden following *H. pylori* infection and vaccination. Drug-loaded liposomes were injected intravenously to deplete macrophages from C57BL/6 mice, and effective removal of CD11b+ cells from the spleens and stomachs of mice was confirmed by immunofluorescence microscopy. Transient elimination of macrophages from C57BL/6 mice during the early period of infection reduced the gastric pathology induced by *H. pylori* SS1 but did not affect the bacterial load in the stomach. These data suggest that macrophages are important to the severity of gastric inflammation during *H. pylori* infection.

**MATERIALS AND METHODS**

**Mice.** Six-week-old female C57BL/6 mice were bred and housed under specific-pathogen-free conditions at the Department of Microbiology and Immunology Animal Facility, The University of Melbourne. Mice were fed sterile food and H2O ad libitum and euthanized by CO2 asphyxiation. All experiments were approved by The University of Melbourne Animal Ethics Committee.

**In vivo macrophage depletion.** Macrophages were depleted in vivo by using the well-characterized liposome-mediated macrophage “suicide” technique (21). Mice were intravenously (i.v.) injected with 200 μl of dichloromethylene diphosphonate (Cl2MDP)-loaded liposomes. Cl2MDP was a kind gift from Roche Diagnostics GmbH (Mannheim, Germany). Macrophage depletion was maintained, when required, by i.v. injecting 50 μl of Cl2MDP-loaded liposomes every 4 to 5 days for the indicated periods.

**H. pylori culture and infection of mice.** *H. pylori* SS1 was grown in brain heart infusion broth (Oxoid, England) supplemented with 5% (vol/vol) fetal calf serum (10). Mice were anesthetized with Penthane (Abbot Laboratories) and received approximately 106 bacteria in 200 μl of phosphate-buffered saline (PBS) by oral gavage. The animals were infected three times within 5 days for infection studies or challenged after prophylactic immunization with a single dose (108 CFU) of bacteria.

**H. pylori colonization and gastritis levels of infected mice.** Mouse stomachs were opened along the greater curvature, washed in PBS, and cut in half to include the greater curvature. One half was used to quantify *H. pylori* colonization by determining the number of CFU per gram of stomach tissue (10). The remaining half was embedded in Tissue-Tek O.C.T. compound (Sakura Finetech) and frozen over liquid nitrogen. Hematoxylin-and-eosin-stained cryosections (7 μm) were used to grade the inflammatory response as previously described (10). The following six-point scale was used to define mononuclear cell infiltration: 1, mild multifocal; 2, mild widespread or moderate multifocal; 3, mild widespread and moderate or severe multifocal; 4, moderate widespread; 5, moderate wide-
spread and severe multifocal; 6, severe widespread. All sections were graded blindly.

**H. pylori**-specific ELISA. Mouse serum H. pylori-specific antibody levels were determined by enzyme-linked immunosorbent assay (ELISA). In brief, MaxiSorp amine (Pierce) with H2O2 as the substrate. Absorbances were read by determining optical density at 492 nm with a TiterTec Multiskan (TiterTec, Finland). Serum endpoint titers are expressed as the reciprocal of the dilution of the specific antibody that gave an optical density at 492 nm of five times the value of horseradish peroxidase-conjugated antibody alone. Positive and negative control sera with known *Helicobacter* antibody titers were included in all ELISA experiments.

Immunohistochemistry. Acetone-fixed cryosections of spleen and stomach tissues were washed in PBS and blocked for 30 min with 20% (vol/vol) goat serum–2% mouse serum–FcRIII antibody (eBiosciences) in PBS. Sections were incubated with anti-CD11b clone M1/70 (BD Biosciences) and/or rabbit anti-H. pylori serum diluted in 5% (vol/vol) goat serum–PBS overnight at 4°C. Bound antibody was detected with fluorescein isothiocyanate-conjugated anti-rat antibody and/or phycoerythrin-conjugated anti-rabbit antibody (Jackson ImmunoResearch). Slides were mounted with Mowiol 4-88 (Merek, Australia) and examined with a UV epifluorescence microscope.

Statistics. The nonparametric two-tailed Mann-Whitney U test was used for statistical analysis of the results. One stomach section removed from along the greater curvature per animal was analyzed for pathology and colonization by *H. pylori* bacteria. Antibody levels below the detection limit of the assay were assigned the minimum level of detection of the assay to enable statistical analysis. Differences were considered statistically significant at *P* < 0.05.

### RESULTS

**Macrophage elimination by treatment with Cl2MDP-liposomes.** The role of macrophages in the control of *H. pylori* colonization and immunopathology were examined by transiently depleting mice of macrophages with Cl2MDP-liposomes. Mice were injected i.v. with Cl2MDP-liposomes, and macrophage depletion was maintained by i.v. injection of Cl2MDP-liposomes every 4 to 5 days, from 8 days prior to the first dose of infection to 10 days after the final dose of infection with *H. pylori* (a total of six injections). Control mice received PBS instead of Cl2MDP-liposomes. Three days after the final dose of bacteria, mice were euthanized and the spleens and stomachs of the mice were frozen and used for histological analysis. Cryostat sections were stained with anti-CD11b antibody to determine if macrophages were depleted from Cl2MDP-liposome-treated mice. Figure 1 shows that CD11b⁺ cells were easily distinguished in the spleens of control naive and *H. pylori*-infected animals. Cl2MDP-liposome treatment resulted in successful elimination of CD11b⁺ cells from the red pulp and marginal zone area of the spleens of naive and *H. pylori*-infected animals. There were very few detectable CD11b⁺ cells in the gastric tissue of PBS-treated mice and no detectable CD11b⁺ cells in Cl2MDP-liposome-treated naive mice. Analysis of the presence of CD11b⁺ cells (green) and *H. pylori* bacteria (red) detected in the stomachs of Cl2MDP-liposome-treated and control *H. pylori*-infected animals demonstrated that infection with *H. pylori* resulted in an influx of CD11b⁺ cells into the gastric tissue and that treatment with Cl2MDP-liposomes before and during administration of the bacteria resulted in a substantial reduction in the number of detectable CD11b⁺ cells in the stomach.

**Cl2MDP-liposome treatment during early *H. pylori* infection does not alter gastric colonization levels.** Cl2MDP-liposome-treated and PBS-treated control mice were euthanized 3 days, 10 days, or 3 months after the final dose of *H. pylori* infection, and gastric *H. pylori* colonization levels were determined by culture (Fig. 2A). *H. pylori* was isolated from the gastric tissue of all infected mice at all time points, and colonization levels of Cl2MDP-liposome-treated mice were comparable to those of PBS-treated control mice during the 3-month infection period (at 3 days [data not shown; *n* = 5]; *P* = 0.31 [ *n* = 5] and *P* = 0.10 [ *n* = 10] at both 10 days and 3 months, respectively).

The *H. pylori*-specific antibody response is not affected by Cl2MDP-liposome treatment. *H. pylori*-specific antibody re-
responses in the sera of all infected mice were measured by ELISA 3 months after infection (Fig. 2B). *H. pylori*-specific antibodies were detectable in the sera of all infected mice, and Cl2MDP-liposome treatment did not affect the levels of *H. pylori*-specific antibodies in serum. Reduced gastric pathology in *H. pylori*-infected mice as a result of treatment with Cl2MDP liposomes. Gastric mononuclear cellular infiltrate in Cl2MDP-liposome-treated and PBS-treated control *H. pylori*-infected mice was scored 3 months after infection. Figure 3A shows an immunohistological analysis of the inflammatory infiltrate of a representative sample from each stomach region from each group of mice. The average inflammatory score of each group of mice is shown in Fig. 3B. Overall, Cl2MDP-liposome-treated mice had lower inflammation scores, with significantly reduced levels of chronic inflammation in the lower regions of the stomach than the PBS-treated control mice \((P = 0.035 \ [n = 10])\) in the antrum and lower-body regions, respectively. The average inflammation in the mid- and upper-body regions of Cl2MDP-liposome-treated mice \((1.8 \pm 0.41 \ [mean \pm standard\ deviation; \ n = 10])\) and \(1.3 \pm 0.5 \ [n = 10]\) was reduced compared with that of PBS-treated control mice \((2.4 \pm 0.53 \ and \ 2.1 \pm 1.0,\ respectively), although the differences were not statistically significant \((P = 0.068\ in\ the\ mid-body\ region\ and\ P = 0.09\ in\ the\ upper-body\ region). Gastric tissue from all mice was stained with hematoxylin and eosin and examined for gastritis by light microscopy. Values for mononuclear cell infiltration, described as chronic inflammation, were estimated by using a six-point scale. Shown are representative images of PBS-treated (a to d) and Cl2MDP-liposome-treated (e to h) mice showing inflammatory infiltrate (arrows) in the upper-body (a [score = 2.5] and e [score = 1.0]), mid-body (b [score = 2.0] and f [score = 1.5]), lower-body (c [score = 4.0] and g [score = 2.5]), and antrum (d [score = 3.0] and h [score = 2.0]) regions of the stomach. Original magnification, \(\times 20.\) (B) Box plot showing the gastric inflammatory scores of Cl2MDP-liposome \((n = 10,\ hatched\ boxes)\) and PBS-treated \((n = 10,\ white\ boxes), H. pylori\)-infected animals determined by histology 3 months after infection. Data are representative of two independently performed experiments.

**FIG. 2.** Cl2MDP-liposome treatment during *H. pylori* infection does not affect colonization levels or *H. pylori*-specific antibody responses. C57BL/6 mice were treated with either PBS (white symbols) or Cl2MDP-liposomes (black symbols) from 8 days prior to the first dose of *H. pylori* until 10 days after the final dose of *H. pylori*. (A) At 10 days \((n = 5)\) and 3 months \((n = 10)\) after infection, mice were euthanized and stomach homogenates were plated onto selective media to determine the gastric colonization levels. (B) The *H. pylori*-specific serum endpoint titer was determined by ELISA 3 months after infection. The median levels of the groups in panels A and B are indicated by dashes, and each symbol represents an individual animal. Data are representative of two independently performed experiments.

**FIG. 3.** Cl2MDP-liposome treatment during *H. pylori* infection reduces gastric inflammation. C57BL/6 mice were treated with either PBS (white symbols) or Cl2MDP-liposomes (black symbols) from 8 days prior to the first dose of *H. pylori* until 10 days after the final dose of *H. pylori*. (A) Sections of gastric tissue from mice infected *H. pylori* for 3 months were stained with hematoxylin and eosin and examined for gastritis by light microscopy. Values for mononuclear cell infiltration, described as chronic inflammation, were estimated by using a six-point scale. Shown are representative images of PBS-treated (a to d) and Cl2MDP-liposome-treated (e to h) mice showing inflammatory infiltrate (arrows) in the upper-body (a [score = 2.5] and e [score = 1.0]), mid-body (b [score = 2.0] and f [score = 1.5]), lower-body (c [score = 4.0] and g [score = 2.5]), and antrum (d [score = 3.0] and h [score = 2.0]) regions of the stomach. Original magnification, \(\times 20.\) (B) Box plot showing the gastric inflammatory scores of Cl2MDP-liposome \((n = 10,\ hatched\ boxes)\) and PBS-treated \((n = 10,\ white\ boxes), H. pylori\)-infected animals determined by histology 3 months after infection. Data are representative of two independently performed experiments.
negative for acute gastric inflammation (influx of neutrophils) and atrophy.

**DISCUSSION**

Macrophages are bone marrow-derived mononuclear phagocytes that reside within tissues, act as scavengers of immunogenic debris, and eliminate foreign particles by phagocytosis (7). Macrophages are thought to have a central function in controlling and regulating the immune response; once activated, they produce proinflammatory cytokines and chemokines (7). Activated macrophages also express major histocompatibility complex class II and can therefore activate antigen-specific CD4+ T cells (4).

In this study, macrophages were transiently depleted in vivo by the well-characterized liposome-mediated macrophage suicide technique (21). Van Rooijen et al. demonstrated that Cl$_2$MDP affects cellular metabolism once intracellular concentrations of Cl$_2$MDP exceed a threshold and macrophages are depleted by apoptosis. It should be noted that elimination of macrophages (22). The depletion of macrophages from the spleens of Cl$_2$MDP-liposome-treated C57BL/6 mice was confirmed by immunohistochemistry and reproduced previously published results (21).

We also demonstrated the removal of macrophages from the stomachs of naïve animals and a substantial reduction in the number of CD11b$^+$ cells in the stomachs of Cl$_2$MDP-liposome-treated, H. pylori-infected animals.

This paper describes the effect of in vivo macrophage depletion during experimental infection of mice with H. pylori. Mice were injected with Cl$_2$MDP-liposomes prior to infection with H. pylori until 10 days postinfection. While the H. pylori colonization levels were unaltered by the liposome treatment over the 3-month period following infection, the transient elimination of macrophages from the spleen and stomach significantly reduced the H. pylori-induced chronic gastric inflammation in C57BL/6 mice.

There are several potential explanations for the observation that macrophages modulate H. pylori-mediated gastritis during the establishment of infection. The accumulation of phagocytic cells in the gastric tissue during H. pylori infection correlates with the development of gastritis (25). Macrophages accumulated in the gastric mucosa are stimulated by H. pylori proteins, which results in the production of IL-1, tumor necrosis factor alpha, IL-6, IL-8, MIP-1α, and GROα (5, 6, 13). These chemokines and inflammatory molecules produced during H. pylori infection may play a key role in the regulation, recruitment, and activation of inflammatory cells, such as macrophages themselves, in the gastric mucosa. For example, MIP-1α is implicated in the continuation of mucosal immune and inflammatory reactions of H. pylori-positive patients (13). Also, inducible nitric oxide synthase is important in the pathogenesis of H. pylori gastritis, as it is up-regulated in patients with chronic active gastritis or gastritis with intestinal metaplasia (23). Ihrig et al. reported that iNOS$^{-/-}$ mice infected with H. felis had decreased antibody levels and less gastritis than wild-type mice (9). Therefore, cytokine production by mucosal macrophages may be central to the genesis and severity of H. pylori-induced inflammation and malignancy.

Furthermore, macrophages express Toll-like receptors that recognize H. pylori antigens and induce MyD88 and NF-κB signaling pathways (15) that may aid in the development of H. pylori-induced gastritis. Moreover, products generated by macrophages can also have adverse effects on host tissue. Matrix metalloproteinase 9 and 2 levels are increased in the gastric mucosa of H. pylori-infected individuals, contributing to the destruction of gastric tissue during infection (2).

This study demonstrates that macrophages have a central role in Helicobacter infection-induced gastritis but do not affect H. pylori-specific antibody responses. This effect is most probably due to cytokine secretion and/or antigen presentation, and this hypothesis is the subject of ongoing studies. In identifying a role for macrophages in the initiation of gastritis during H. pylori infection, this study may assist in future studies targeting the inhibition of gastritis in the host and provide a stimulus to study the capacity of macrophage-modifying drugs to reduce the gastritis associated with Helicobacter disease.

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