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IgA Antibodies Impair Resistance against Helicobacter pylori Infection: Studies on Immune Evasion in IL-10-Deficient Mice

Ali A. Akhiani, Anneli Stensson, Karin Schön, and Nils Y. Lycke

We recently reported that Helicobacter pylori-specific Abs impair the development of gastritis and down-regulate resistance against H. pylori infection. In this study, we asked whether IgA Abs specifically can have an impact on H. pylori colonization and gastric inflammation. To obtain a sensitive model for the study of inflammation we crossed IgA- and IL-10-deficient mice. We found that IL-10−/−/IgA−/− mice were significantly less colonized than IL-10−/−/IgA+/+ mice, which in turn were less colonized than wild-type (WT) mice. The IL-10−/−/IgA−/− mice exhibited a 1.2-log reduction in bacterial counts compared with that in IL-10−/−/IgA+/+ mice, suggesting that IgA Abs rather promoted than prevented infection. The reduced colonization in IL-10−/−/IgA−/− mice was associated with the most severe gastritis observed, albeit all IL-10−/− mice demonstrated more severe gastric inflammation than wild-type mice. The gastritis score and the infiltration of CD4+ T cells into the gastric mucosa were significantly higher in IL-10−/−/IgA−/− mice than in IL-10−/−/IgA+/+ mice, arguing that IgA Abs counteracted inflammation. Moreover, following oral immunization, IL-10−/−/IgA−/− mice were significantly better protected against colonization than IL-10−/−/IgA+/+ mice. However, the stronger protection was associated with more severe postimmunization gastritis and gastric infiltration of CD4+ T cells. There was also a clear increase in complement receptor-expressing cells in IL-10−/−/IgA−/− mice, though C3b-fragment deposition in the gastric mucosa was comparable between the two. Finally, specific T cell responses to recall Ag demonstrated higher levels of IFN-γ production in IL-10−/−/IgA−/− as compared with IL-10−/−/IgA+/+ mice. Thus, it appears that IgA and IL-10 help H. pylori bacteria evade host resistance against infection.

Infection with Helicobacter pylori bacteria results in strong specific local and systemic Ab production, cell-mediated immunity as well as an inflammatory infiltrate of neutrophils, lymphocytes, plasma cells, macrophages, and eosinophils in the gastric mucosa (1–3). Despite this massive immune response, H. pylori infection in untreated people is usually lifelong, and an ongoing chronic infection could eventually result in the development of gastric ulcers, atrophic changes, and carcinoma (4). H. pylori bacteria reside in the mucus layer covering the gastric epithelium and therefore the type of protective measures which could confer resistance appear to be limited. Because polymeric IgA and IgM are actively transported across the epithelium, specific Abs could play a major role in protection against H. pylori infection (5, 6).

However, even though H. pylori infection stimulates strong local and systemic specific IgA and IgG Ab production, it is still controversial as to what influence Abs may have on the bacterial colonization (7–10). Whereas specific IgA mAbs administered directly into the gastric lumen of mice mediated protection against Helicobacter felis infection, passive transfer of serum from immunized protected mice to naive recipient animals failed to protect against infection (11, 12). Most importantly though, B cell-deficient μMT mice, as well as IgA- and IgM-deficient mice, following mucosal immunization with bacterial lysate plus adjuvant, were protected to the same extent as wild-type (WT) mice (12–15). Thus, the role of specific Abs for host resistance against H. pylori is not essential, rather most studies indicate that protection is conveyed by T cells and CD4+ Th1 cells in particular (12, 16–18).

We recently demonstrated that Abs were not only dispensable for protection, but also impaired elimination of bacteria and the development of gastric inflammation (14). We found that μMT mice infected with H. pylori were less colonized, developed more inflammation, and exhibited equivalent or stronger postimmunization gastritis compared with WT mice (14). Our data suggested that Abs promoted rather than prevented bacterial colonization and dampened gastric inflammation. Whether the higher bacterial load in WT mice was a direct effect of the presence of Abs or an indirect effect of a milder inflammation is unknown. Hence, the role of specific IgA Abs, in particular, on bacterial colonization and the inflammatory response is poorly understood. Although IgA-deficient mice were fully protected against an H. felis infection following oral immunization, the bacterial load was increased in IgA-deficient mice as compared with WT mice (15). However, whereas IgG Abs were unaltered, specific IgM Abs were significantly raised in gastric secretions in these mice, which could have compensated for the lack of IgA or may indicate different functions of IgA and IgM Abs (15). No observations of the gastric inflammation were reported (15). Therefore, it remains to be clarified whether IgA Abs act by promoting or preventing bacterial colonization and whether they might be influencing the development of gastric inflammation.

The postimmunization gastritis phenomenon, which occurs after challenge with live bacteria, may reflect the protective potential of induced immunity as it is consistently associated with protection in most mouse strains and dissipates over time when the bacterial

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3 Abbreviations used in this paper: WT, wild type; CT, cholera toxin; AP, alkaline phosphatase.
counts drop (14, 19). Also, a robust gastritis has been reported in mouse strains that spontaneously appear to clear the infection, such as the IL-10- and phagocyte oxidative-deficient (cybb−/−) mice (20, 21). However, the degree of gastric inflammation in response to a Helicobacter infection is related to the genetic background of the mouse strain and C57BL/6 mice were shown to have a severe form of gastritis (22–24). Mice on this background are usually also the mice that develop the strongest protective immune responses (14, 19, 23). As the genetic susceptibility for development of gastritis varies between different strains of mice and the fact that some immunodeficient mice, such as the IL-10−/− mice, are more prone to react with inflammation, we took advantage of this information to design a highly sensitive experimental model in which we could assess whether IgA Abs impact on bacterial colonization and gastritis in H. pylori-infected individuals (20, 22). With this intention, we crossed IgA− onto IL-10-deficient C57BL/6 mice and obtained double and single gene knockout mice, IL-10−/−/IgA−/− and IL-10−/−/IgA+/+. Our choice of the IL-10−/− mice was based on previous studies showing that these mice develop severe gastritis and that IL-10 is strongly produced in the gastric mucosa of patients as well as in mice infected with H. pylori (20, 25–32). Also, H. pylori-infected IL-10−/− mice demonstrated a 100-fold lower bacterial colonization compared with WT mice (20, 30). Of note, the IL-10−/− mice have successfully been used to show the involvement of complement and neutrophilic leukocytes in host resistance against Helicobacter infection (30, 31). Moreover, recent investigations have included IL-10 to explain the chronicity of the infection as IL-10-producing regulatory T cells, both in humans and in mice, have been found in infected individuals (21, 33). By promoting the presence of regulatory T cells in the gastric tissue and limiting the severity of the inflammation, the bacteria may evade the massive immune response of the infected host (21). By contrast, the lack of IL-10 and regulatory T cells would result in up-regulated IL-12 production and a stronger Th1 and IFN-γ response, which are critical components in host resistance against H. pylori infections (17, 18, 20, 30, 34, 35).

Materials and Methods

Mice

IL-10-deficient (IL-10−/−; Ref. 36) mice on a C57BL/6 background, IgA-deficient (IgA−/−; Ref. 37) mice on a mixed (C57BL/6 × 129Sv) background, and C3-deficient mice on a 129Ola/C57BL/6 background (38) were bred in ventilated cages under pathogen-free conditions at the Laboratory for Experimental Biomedicine, University of Gothenburg, (Gothenburg, Sweden). To obtain single and double gene knockout mice we crossed IL-10−/− (C57BL/6) mice with IgA−/− (C57BL/6 × 129Sv) mice and the F1 offspring was subsequently crossed to obtain F2 mice that were typed for IL-10 and IgA. F2 mice were typed by PCR using genomic DNA from tail tips to detect IL-10 and ELISA to detect IgA in sera (14, 36). This way we generated double (IL-10−/−/IgA−/−) or single (IL-10−/−/IgA+/+) gene knockout mice that were derived from the same founders. All mice were tested for IL-10 and IgA before experimentation, primarily to exclude potential IgA-negative mice in the breeding of IL-10-negative/IgA-positive F2 mice. C57BL/6 WT mice were obtained from M&B. All mice were seronegative for Helicobacter spp. Ags before infection or immunization. Age- and sex-matched animals were used throughout the study.

Immunization

Groups of 8–10 mice were immunized orally with a blunt feeding needle (Popper & Sons) four times at weekly intervals with 500 μg of H. pylori whole cell lysate Ags and 10 μg of cholera toxin (CT) adjuvant (List Biological Laboratories) in 3% (w/v) NaHCO3 in PBS in a total volume of 0.5 ml. Control mice received 10 μl of CT.

H. pylori growth conditions and challenge

H. pylori Sydney strain (SS1) cells were cultured on selective agar plates containing 5% sheep blood and antibiotics in a microaerophilic condition as previously described (17). The bacteria were then harvested and inoculated into brucella broth (BD Biosciences) supplemented with 5% heat-inactivated FCS (Biochrom) as described elsewhere (17). To establish a primary H. pylori infection, mice were inoculated intragastrically via a 20-gauge feeding needle with a 500-μl suspension of H. pylori containing 3 × 108 CFU of the bacteria on 2 consecutive days and sacrificed 7 wk postinoculation. To assess protection after immunization, the mice were challenged with 5 × 107 CFU of H. pylori 2 wk after the last immunization. At 2 or 9 wk after challenge, mice were sacrificed and gastric tissue processed for urease activity, H. pylori culture, histology, and immunohistochemistry as described below.

Preparation of H. pylori whole cell lysate Ags

H. pylori (SS1) was grown on selective blood agar plates as previously described (17). The bacteria were then washed three times in PBS by centrifugation at 6000 rpm for 10 min at 4°C before being disrupted by freeze-pressing with X-press (39) as described previously (17). After centrifugation (6000 rpm) to remove cell fragments, the preparation was filtered through a 0.2-μm pre size membrane filter (Schleicher & Schuell). The protein content was determined by the Bio-Rad protein assay, and aliquots were frozen at −85°C until used.

Gastric tissue analyses

The stomach was dissected along the greater curvature and divided into four longitudinal strips for assessment of urease activity, H. pylori culture, histopathology, or immunohistochemical analyses. For histopathology, longitudinal segments including the antrum and corpus plus a piece of attached intestine were fixed in 4% neutral buffered formaldehyde, embedded in paraffin, and sectioned at 3 μm by Histolab Products. For immunohistochemistry, gastric segments were placed into Histocon (Histolab Products) at 4°C. The tissues were then placed in plastic forms (Cryomold; Miles) filled with OCT compound (Miles) and subsequently snap-frozen in isopentane in liquid nitrogen (N2) for −60 s. Frozen cross-sections (7 μm) were prepared on microslides using a Cryostat-1720 (Leitz) and frozen at −85°C.

Assessment of bacterial colonization

The presence of H. pylori in gastric tissue was assessed by urease activity measured spectrophotometrically at 550 nm using a colorimetric assay (16). For a quantitative measurement of H. pylori bacteria longitudinal segments of gastric tissue were homogenized in 0.5 ml of Brucella broth supplemented with 5% FCS, and replicate serial 10-fold dilutions were plated on Helicobacter-selective blood agar plates (16). The plates were incubated at 57°C under microaerobic conditions (10% CO2, 5% O2, and 85% N2), and the quantitation of the CFU was performed 7 days later. In the present study, protection against H. pylori infection was defined as a significant reduction in the colonizing bacteria in the stomach.

Histopathology

For evaluation of gastritis, H&E-stained sections were scored based on the degree of infiltrating lymphocytes, plasma cells, and neutrophils (40). The scoring grades were defined as follows: 0, none; 1, a few leukocytes scattered in the deep mucosa; 2, moderate numbers of leukocytes in the deep mucosa and occasional neutrophils in gastric glands (microabscesses); 3, dense infiltrates in the deep to midmucosa, a few microabscesses, and one or two lymphoid aggregates; and 4, dense, diffuse infiltrates throughout the lamina propria and into the submucosa, frequent microabscesses, and prominent lymphoid aggregates.

Epithelial changes

For evaluation of epithelial changes, H&E-stained sections were scored based on the degree of epithelial destruction in the corpus including parietal cell loss and hyperplasia of the surface epithelium as described by Ermak et al. (41). Briefly, epithelial scores were defined as follows: 0, none; 1, small, focal areas of parietal cell loss in the corpus and/or hyperplasia of the surface epithelium; 2, epithelial changes throughout 75% of the mucosa; 3, epithelial changes throughout the mucosa plus one to three microabscesses or cystic glands; or 4, epithelial changes throughout the mucosa plus four or more microabscesses or cystic glands.

Immunohistochemistry of gastric tissue

Frozen sections from gastric tissue were stained for CD4 and CD8 (BD Pharmingen) as described previously (35). Control sections were incubated without specific mAb. Stained sections were scored from 0 to 4 based on the extent of infiltrating T cells (35). Scores were defined as follows: 0, none; 1, a few T cells scattered in the mucosa; 2, moderate numbers of T cells...
cells in the gastric mucosa; 3, dense infiltrates in the deep to mid mucosa; 4, dense, diffuse infiltrates throughout the lamina propria and into the submucosa. A quantitative description of each score level was defined as follows: 1, 100 ± 20; 2, 200 ± 40; 3, 400 ± 80; and 4, 800 ± 160 cells/mm². For detection of complement receptors or complement components (C3b) in the gastric tissue, frozen sections were incubated with rat anti-mouse CD21/CD35 (complement receptors) mAb (BD Pharmingen) or rat anti-mouse C3b/C3b/C3c (HyCult Biotechnology) mAb, respectively, followed by biotinylated rabbit anti-rat IgG (H + L; Vector Laboratories). Control sections were incubated with rat IgG1 isotype control Ab (BD Pharmingen), the secondary Ab (biotinylated rabbit anti-rat IgG) alone or with only the primary Ab. Sections were then incubated with HRP conjugated to an avidin-biotin complex (ABC-Elite kit; DAKO). Antibodies were detected by sequential incubation with a polyclonal rabbit anti-IFN-γ Ab (BD Pharmingen), the secondary Ab (biotinylated rabbit anti-rat IgG) alone or with only the primary Ab. Sections were then incubated with HRP conjugated to an avidin-biotin complex (ABC-Elite kit; DAKO). Antigen detection was scored from 0 to 4 based on the intensity of staining as described for CD4+ T cells (above), while C3b deposition was scored as follows: 0, none; 1, positive staining (C3b deposition) in a few areas including the surface and deep down in the mucosa; 2, moderate staining; 3, intense staining on the surface and in the deep to mid mucosa; 4, very intense staining on the surface and throughout the lamina propria and into the submucosa.

Serum and gastric samples

Blood was obtained from the axillary plexus of the mouse at sacrifice. Gastric secretions were collected with absorbent wicks positioned longitudinally in the gastric lumen (42) after extensive rinsing with PBS containing 0.2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (Calbiochem), 1 μg of aprotinin/ml, 10 mM leupeptin (Sigma-Aldrich), and 3.25 μM Bestatin (Boehringer Mannheim Biochemicals) protease inhibitors. For extraction of gastric secretions from the wick, 0.5 ml of protease inhibitor containing 5% nonfat dry milk was added to each sample tube containing two wicks, vortexed extensively, and then frozen at −85°C until analyzed.

Serum and gastric anti-H. pylori Ab determinations by ELISA

Flat-bottom 96-well microtiter plates (Nunc) were incubated with 10 μg/ml H. pylori lysate or 2.5 μg/ml CTA subunit (List Biological Laboratories) in PBS and incubated at 4°C overnight. After washing and blocking with PBS containing 0.1% BSA, the wells were incubated with serial dilutions of sera or gastric secretions. The wells were then incubated with the appropriate dilutions of alkaline phosphatase (AP)-conjugated goat anti-mouse IgG1, anti-mouse IgG2a, or anti-mouse IgA (Southern Biotechnology Associates), followed by the phosphatase substrate p-nitrophenyl phosphate (Sigma-Aldrich) in ethanalamine buffer (pH 9.8) and the enzymatic reactions were read at 405 nm. Ab titers were defined on the linear portion of the curve as the interpolated dilution of a sample giving rise to an absorbance of 0.4 U above background.

In vitro stimulation of spleen cells

Spleen cells were obtained by mechanical dissociation and filtering through a nylon mesh. RBC were subjected to lysis by osmotic shock using hypo-osmotic NaCl. Spleen cells were collected with absorbent wicks positioned longitudinally in the gastric lumen, rinsed with PBS, and incubated at 4°C overnight. After washing and blocking with PBS containing 0.1% BSA, the wells were incubated with serial dilutions of sera or gastric secretions. The wells were then incubated with the appropriate dilutions of alkaline phosphatase (AP)-conjugated goat anti-mouse IgG1, anti-mouse IgG2a, or anti-mouse IgA (Southern Biotechnology Associates), followed by the phosphatase substrate p-nitrophenyl phosphate (Sigma-Aldrich) in ethanalamine buffer (pH 9.8) and the enzymatic reactions were read at 405 nm. Ab titers were defined on the linear portion of the curve as the interpolated dilution of a sample giving rise to an absorbance of 0.4 U above background.

In vitro stimulation of spleen cells

Spleen cells were obtained by mechanical dissociation and filtering through a nylon mesh. RBC were subjected to lysis by osmotic shock using hypotonic Tris-ammonium chloride. After washing in HBSS (Invitrogen Life Technologies), the cells were resuspended in Iscove’s medium (Biochrom) supplemented with 10% heat-inactivated FCS (Biochrom), 50 μM 2-ME (Sigma-Aldrich), 1 mM l-glutamine (Biochrom), and 50 μg/ml gentamicin. Spleen cells (10⁴ cells/well) were then cultured (8% CO₂, 37°C) in round-bottom 96-well microtiter plates (Nunc) in the presence or absence of varying concentrations of H. pylori lysate Ags or anti-CD3 by adding 10% supernatant from the 145-2C11 cell line (43). Cell-free supernatants were collected 96 h after incubation, and stored at −85°C until assayed for IFN-γ concentration.

IFN-γ assay

The concentration of IFN-γ in the supernatants was assessed by ELISA. Briefly, 96-well round-bottom microtiter plates (Dynatech Laboratories) were incubated with 2.5 μg/ml rat anti-mouse IFN-γ (BD Pharmingen). The sample supernatant, or recombinant mouse IFN-γ standard, was then added to the appropriate wells. Bound IFN-γ was detected by sequential incubations with a polyclonal rabbit anti-IFN-γ antiserum followed by AP-conjugated goat anti-rabbit Ig (Southern Biotechnology Associates). Finally, the AP substrate p-nitrophenyl phosphate (Sigma-Aldrich) in ethanalamine buffer (pH 9.8) was added to each well and the extent of the reaction was read at 405 nm using a Titer-Tek multiscan spectrophotometer.

The IFN-γ concentration in stimulated culture supernatants was estimated from the standard curve generated with recombinant mouse IFN-γ. The sensitivity of detection of IFN-γ was 23 pg/ml.

Statistical analysis

The Wilcoxon rank sum test was used for independent samples for analysis of significance in all experimental groups, except for the IFN-γ values which were compared by the one-tailed Student t test.

Results

The influence of IgA Abs on primary H. pylori infection in IL-10-deficient mice

Our recent study suggested that H. pylori-specific Abs impair the development of gastric inflammation and promote bacterial colonization, resulting in decreased resistance against H. pylori infection (14). Here, we investigated further whether the lack of IgA Abs can affect the level of bacterial colonization and the development of inflammation. To this end we used a more sensitive model for assessment of changes in gastric inflammation, the IL-10-deficient mouse (20). By cross-breeding IgA- and IL-10 gene knockout mice we obtained double and single gene knockout mice that were genetically derived from the same founders. Mice of the IL-10−/−IgA−/− and IL-10−/−/IgA−/+ strains as well as the original IgA−/−, IgA−/+ (37), or C57BL/6 WT strains were inoculated intragastrically with 3 × 10⁸ CFU of H. pylori SS1 on 2 consecutive days, and the level of colonization and immunopathology was compared 7 wk later. Whereas we observed no difference in bacterial colonization between WT C57BL/6 or IgA−/− and IgA−/+ mice on a mixed (C57BL/6 × 129Sv) genetic background, we found significantly (p < 0.001) lower bacterial counts in IL-10-deficient mice compared with WT mice as assessed by urease activity (not shown) and by quantitative culture (Fig. 1). At least a 100-fold reduction in colonizing bacteria with a mean log₁₀ value of 3.68 ± 0.4 in IL-10−/−/IgA−/− mice vs 5.8 ± 0.1 in IgA−/+ and WT mice was observed (data pooled from three experiments). Most importantly, though, the absence of IgA in the IL-10−/− mice further reduced the bacterial colonization by 1.2 log (p < 0.05), clearly indicating that the presence of IgA augmented bacterial colonization of the gastric mucosa (Fig. 1). In fact, most bacteria were cleared from the gastric tissue of IL-10−/−/IgA−/− as opposed to what was seen in IL-10−/−/IgA−/+ mice. Thus, only in the absence of IL-10 could we clearly observe the negative influence of IgA Abs on host resistance against H. pylori infection. The IgA gene defect in both IL-10−/− and WT mice was confirmed by an H. pylori Ab-specific ELISA on sera and gastric secretions.

FIGURE 1. H. pylori colonization in IL-10−/−/IgA−/−, IL-10−/−/IgA−/+ and WT mice 7 wk after inoculation with live bacteria. Groups of 10 mutant and WT mice were infected with 3 × 10⁶ CFU of H. pylori SS1 on 2 consecutive days. The presence of H. pylori in gastric tissue was assessed by quantitative culture as described in Materials and Methods. The bars represent mean ± SEM of pooled data from three experiments. * p < 0.001 and ** p < 0.05 by Wilcoxon rank sum test.
from infected mice. High levels of serum anti-\textit{H. pylori} IgG1 and IgG2a Abs were detected in all infected mice while uninfected controls had no specific Abs and no IgA Abs were detected in the IL-10/−/IgA/−/ or IgA/−/+ mice, as expected. Specific IgM Ab levels in serum were raised in IgA-deficient IL-10/−/ mice.

**Gastric inflammation and immunopathology in the presence or absence of IgA**

Next we determined whether the lower bacterial colonization in double gene knockout mice was associated with increased gastric inflammation. Gastritis was scored in H&E-stained tissue sections as described in Materials and Methods. As shown in Fig. 2A, C57BL/6 WT or IgA/−/ and IgA/−/+ mice exhibited only a mild gastric inflammation 7 wk after inoculation with \textit{H. pylori} bacteria, whereas IL-10-deficient mice demonstrated more severe gastric inflammation and the pathology score in the IL-10/−/IgA/−/+ mice was significantly (\( p < 0.05 \)) higher than that in the IL-10/−/IgA/−/+ mice. Therefore, an even more severe gastritis developed in IL-10-deficient mice lacking IgA Abs as compared with IL-10 single gene knockout mice. Because previous studies have demonstrated that there is an inverse correlation between the gastritis score and the number of colonizing bacteria, our data indicated that the presence of IgA Abs in IL-10-deficient mice significantly had impaired host resistance against \textit{H. pylori} infection (14). The IL-10/−/IgA/−/+ mice also demonstrated the most dramatic alterations in epithelial cell morphology with hyperplasia in the corpus and parietal cell loss compared with single gene knockout mice or WT mice (Fig. 2B).

The lack of IgA also had a significant impact on the presence of CD4\(^+\) T cells in the gastric mucosa (Fig. 2C). The IL-10-deficient mice clearly had more gastritis and more CD4\(^+\) T cells in their inflamed tissues compared with WT mice, but the IgA-deficient double gene knockout mice exhibited the strongest infiltration of CD4\(^+\) T cells in the gastric mucosa (\( p < 0.01 \)). This may be important because \textit{H. pylori}-specific T cells are known to regulate gastric inflammation and are critical for clearance of the bacteria from the stomach (12, 16–18). Cytokine production by splenic T cells isolated from infected double gene knockout mice revealed elevated levels of IFN-\( \gamma \) (11 ± 2.9 \( \mu \)g/ml; mean ± SD), whereas T cells from IgA/−/ or WT mice, produced only very low levels of IFN-\( \gamma \) (0.1 ± 0.1 \( \mu \)g/ml) in response to recall Ag in vitro.

**Reduced protective immunity in the presence of IgA in IL-10-deficient mice**

Recent studies have demonstrated that IL-10/−/ mice develop severe gastritis and can partly clear an infection with \textit{H. pylori}, but whether IL-10/−/ mice develop protective immunity following immunization has been incompletely investigated (20, 30–32). We asked whether IgA would influence the development of protective immunity and whether it would change postimmunization gastritis in IL-10/−/ mice. WT, single, or double gene knockout mice were immunized orally with bacterial lysate plus CT adjuvant and subsequently challenged with live \textit{H. pylori} bacteria. We found that protection was substantially better in immunized IL-10/−/ mice (\( p < 0.001 \)) at 2 wk after challenge as compared with that seen in immunized WT mice (Fig. 3A). In fact, IL-10/−/ mice exhibited at least a 1000-fold reduction in bacterial load in the gastric tissue, indicating that elicitation of local IL-10 production in WT mice provides the bacteria with a better chance to survive in the gastric tissue (20). In addition, we observed a striking effect of IgA Abs on bacterial survival in IL-10/−/ mice. Indeed, protection in IL-10/−/ mice was significantly (\( p < 0.05 \)) weaker in the presence than in the absence of IgA Abs (Fig. 3B). Following 9 wk after challenge, both immunized single and double gene knockout mice were well-protected, but protection was significantly (\( p < 0.05 \)) greater in IL-10/−/IgA/−/+ mice as compared with IL-10/−/IgA/−/+ mice (data not shown). Of note, these experiments also suggested a genetic difference between IL-10/−/ mice on a C57BL/6 background and the IL-10/−/IgA/−/+ mice on a mixed C57BL/6 × 129.5v background, in that an 18-fold difference in bacterial clearance was observed between the two (Fig. 3, A and B). Notwithstanding this, we conclude that IgA Abs appeared to
counteract host resistance against *H. pylori* infection (Fig. 3B). Hence, both IL-10 and the presence of IgA Abs may promote bacterial evasion of host immunity against *H. pylori* infection in WT mice.

Given that CD4+ T cell infiltration in the gastric tissue is a reflection of host protection, this conclusion was further supported by the increased infiltration of CD4+ T cells in IgA-deficient as opposed to single gene knockout mice (Fig. 3D). There was a good correlation between the level of CD4+ T cell infiltration in the gastric mucosa and protective immunity in both WT and IL-10−/− mice (Fig. 3, C and D). Recall responses to *H. pylori* Ags by splenic T cells isolated from immunized well-protected mice revealed increased IFN-γ (15.8 ± 4.3 μg/ml in lysate + CT vs 1.7 ± 0.4 μg/ml in CT-only control group, mean ± SD) production in double gene knockout mice, whereas IL-10−/−/IgA−/− mice produced significantly (p < 0.001) less (3.3 ± 0.7 μg/ml in lysate + CT vs 0.4 ± 0.1 μg/ml in the CT-only control group).

Postimmunization gastritis in immunized and protected mice was substantial when compared with control CT-only immunized mice in WT as well as IL-10−/− mice (Fig. 4, A and B). Double gene knockout mice lacking both IL-10 and IgA exhibited more gastritis than IL-10-deficient mice and the latter mice demonstrated more inflammation than WT mice (Fig. 4, A and B). Following 9 wk after challenge, both single and double gene knockout mice exhibited severe postimmunization gastritis, but the latter mice lacking both IL-10 and IgA had significantly more gastritis than the single IL-10-deficient mice (Fig. 4B). The immunized well-protected mice hosting the most severe gastritis also exhibited the most marked epithelial changes in the corpus region, characterized by parietal cell loss and hyperplasia of the epithelium (Fig. 4, C and D).

**FIGURE 3.** *H. pylori* colonization (A and B) and CD4+ T cell infiltration (C and D) in IL-10−/−, C57BL/6 WT, IL-10−/−/IgA−/−, and IL-10−/−/IgA−/− mice after mucosal immunization. Groups of 8–10 mutant and WT mice were immunized orally with *H. pylori* lysate together with CT alone (black bar) or CT plus IgA Abs (red bar). The presence of *H. pylori* bacteria in the gastric tissue was assessed by quantitative culture at 2 wk after challenge as described in Materials and Methods. The bars represent the mean ± SEM of pooled data from two experiments. C and D, CD4+ T cells were detected in the gastric tissue by immunohistochemistry 2 wk after challenge. Sections were scored from 0 to 4 as described in Materials and Methods. The bars represent the mean ± SEM of pooled data from two experiments. A–D, *p < 0.05; †, p < 0.01; **, p < 0.001; and ‡, p < 0.0001 by Wilcoxon rank sum test.

**Increased IgM responses to oral immunizations in IL-10−/−/IgA−/− mice**

Unimmunized infected IL-10−/−/IgA−/− mice exhibited slightly increased specific IgM production in sera as compared with infected IL-10−/−/IgA−/− mice (Fig. 5A). Following oral immunization with lysate plus CT adjuvant in double gene knockout mice, the enhanced specific IgM responses in sera as well as in gastric secretions were even more pronounced when compared with those seen in IL-10−/−/IgA−/− mice (Fig. 5, B and C). Supporting the fact that increased specific IgM Abs were a compensation for lack of IgA-production, we noted no difference in specific serum IgM Abs between IL-10−/− or WT mice on the C57BL/6 genetic background (Fig. 5B). By contrast, IL-10−/− mice had generally higher specific IgG1, IgG2a, and IgA Ab levels as compared with those found in WT mice (Fig. 5, B and C). As predicted, no IgA Ab production was detected from IgA−/− mice, whereas IL-10−/−/IgA−/− mice demonstrated *H. pylori*-specific local and serum IgA responses (Fig. 5). Thus, in agreement with previous studies in IgA-deficient mice we found IgM Abs to be enhanced in the IL-10−/−/IgA−/− mice, suggesting that local IgM Abs could functionally substitute for the lack of IgA Abs (15).

**H. pylori-induced complement deposition in the gastric mucosa appears unaffected by IgA**

Earlier studies have indicated that complement activation may be involved in *Helicobacter*-induced gastric inflammation and coupled with the notion that IgA Abs poorly activate complement, or even may inhibit IgG-mediated complement activation, we investigated the degree of complement deposition in our IL-10-deficient
mice (31, 44, 45). Because complement activation and tissue deposition could be linked to an increased level of complement receptor expression (CD21/CD35), we analyzed frozen sections of gastric mucosa from infected mice for C3b fragments as well as CD21/CD35 expression (46). We found that C3b deposition was comparable in the gastric tissues of IL-10−/−/IgA−/− (2.60 ± 0.16, mean ± SEM) and IL-10−/−/IgA++/+ mice (2.45 ± 0.19, mean ± SEM) (Figs. 6 and 7). This observation was also confirmed when comparing C3b deposition in the well-immunized and protected IL-10−/−/IgA−/− (2.83 ± 0.17, mean ± SEM) and IL-10−/−/IgA++/+ (2.67 ± 0.17, mean ± SEM) mice, arguing against the idea that IgA exerts an anti-inflammatory effect in WT mice through reduction of complement deposition in the gastric mucosa (not shown). By contrast, the presence of complement receptors (CD21/CD35) was different between IgA-deficient or mice with normal IgA production (Fig. 7). Thus, H. pylori-infected naive or immunized IgA-deficient mice exhibited significantly increased levels of complement receptors in the gastric tissue as compared with IgA++/+ mice (1.15 ± 0.82 vs 0.67 ± 0.75 mean ± SD). Taken together, absence of IgA Abs in the context of an H. pylori infection resulted in increased gastritis with higher CD4+ T cell counts and more CD21/CD35-expressing cells in the gastric mucosa, while complement C3b deposition was unaffected compared with that seen in WT mice.

**Discussion**

The present study provides evidence that IgA Abs produced against H. pylori bacteria impair rather than promote host resistance against infection. Because IL-10 exerts potent anti-inflammatory effects and inhibits Th1-type immunity, mice deficient in IL-10 developed severe gastritis in response to H. pylori infection (20, 30, 34). By crossing IgA- and IL-10-gene knockout mice we succeeded in establishing a model that was sufficiently sensitive to detect whether IgA Abs could impact on gastric inflammation and bacterial colonization during an H. pylori infection (36, 37). Whereas studies in mUT mice provided the initial data that lead us to hypothesize that Abs may, in fact, counteract protection against infection by dampening inflammation and/or increasing bacterial colonization, the IgA−/−/IL-10−/− mouse model clearly shows that this effect is IgA-dependent and, thus, appears not to be a function of specific IgM or IgG Abs (14). This finding also suggests that prophylactic immunization that generates gastric IgA Abs might weaken rather than strengthen host resistance against infection. In addition, our study is the first to show that IL-10−/− mice develop stronger protection than WT mice following oral immunization. Thus, elicitation of IgA and IL-10 locally in the gastric mucosa appears to be a successful strategy used by H. pylori bacteria to evade host protection against infection (20, 21, 30, 31). In contrast, in the absence of IgA and IL-10, immunized mice developed the strongest protection as compared with that induced in WT mice. Immunized IgA−/−/IL-10−/− mice exhibited the most efficient IFN-γ production and infiltration of CD4+ T cells into the gastric mucosa, which agrees well with the notion that Th1 CD4+ T cells play a key role in host resistance against H. pylori infection (17, 18, 30, 35).

We found a positive correlation between the level of inflammation and the absence of IgA in our IL-10−/− model. The IgA−/−/IL-10−/− mice developed the most pronounced gastritis, strongest infiltration of CD4+ T cells, and lowest bacterial colonization as compared with IgA++/+/IL-10−/− mice. Immunization improved
resistance to infection even further in this model, which was evident already at 2 wk following a challenge infection and remained significant at 7 wk, when also unimmunized IgA<sup>−/−</sup>/IL-10<sup>−/−</sup> mice spontaneously developed protection. In contrast, WT (IL-10<sup>−/−</sup>/IL-10<sup>+/+</sup>) mice on the mixed C57BL/6 × 129Sv background, demonstrated no obvious difference between IgA-deficient and normal mice. A previous study using IgA<sup>−/−</sup> mice from the same original founders (37) reported even increased bacterial colonization in IgA<sup>−/−</sup> mice as compared with WT mice (15). This illustrates that resistance against bacterial colonization and gastritis may be multifactorial and governed by both genotype and phenotype. The former aspect was well-documented in previous studies demonstrating significant genetic differences in disease outcome between different mouse strains, C57BL/6 mice being the more sensitive and BALB/c the least sensitive strain with regard to gastric inflammation after infection (22–24). However, we could not demonstrate an effect of IgA on the inflammation and the bacterial colonization in WT mice on the mixed C57BL/6 × 129Sv (IgA<sup>+/+</sup> vs IgA<sup>−/−</sup>) genetic background (37), probably because multiple mechanisms could compensate host responses and influence the outcome of an H. pylori infection. Only when combining IgA deficiency with another host factor affecting the level of gastric inflammation, such as IL-10, was a model obtained that was sensitive enough to allow us to reveal the impact of IgA Abs on the host resistance against infection. To avoid influences of confounding genetic elements on gastric inflammation and bacterial colonization, we successfully derived the IgA<sup>−/−</sup>/IL-10<sup>−/−</sup> and IgA<sup>−/−</sup>/IL-10<sup>+/+</sup> mice from the same F<sub>1</sub> founders (37). In fact, in this way the IgA<sup>−/−</sup>/IL-10<sup>−/−</sup> and IgA<sup>−/−</sup>/IL-10<sup>+/+</sup> mice were probably genetically more closely related than IgA<sup>+/+</sup> and IgA<sup>−/−</sup> (C57BL/6 × 129Sv) or IL-10<sup>−/−</sup> and WT mice, respectively.

The reason why we observed an influence of lack of Abs on the degree of gastritis in μMT mice on a pure C57BL/6 background and not in IgA<sup>−/−</sup> mice on the mixed C57BL/6 × 129Sv (IgA<sup>+/+</sup> vs IgA<sup>−/−</sup>) genetic background may have to do with reduced susceptibility to development of gastric inflammation to H. pylori infection in the latter strain (14). It should be pointed out that we found an 18-fold difference in bacterial colonization following a challenge infection in immunized IL-10-deficient mice on a pure C57BL/6 as opposed to the mixed C57BL/6 × 129Sv background.
This clearly indicates genetic influences on resistance against infection other than IL-10, which also contributed to the altered immunopathology caused by the lack of IgA. Nevertheless, we believe that the contribution of IgA in reducing host inflammatory responses against infection is real, despite this evidence of undefined genetic differences between mice on the C57BL/6 or mixed C57BL/6/H11003/129Sv backgrounds. Indeed, presence of IgA significantly \((p < 0.05)\) weakened host resistance against \(H.\ pylori\) infection in both naive or well-immunized IL-10-deficient mice derived from the same F1 founders, albeit on the mixed C57BL/6 × 129Sv background. Moreover, significantly less gastritis, lower CD4+ T cell counts, less Th1 activity, and increased bacterial colonization were associated with the presence of IgA in our model. There is no reason to believe that undefined strain-dependent genetic elements influencing \(H.\ pylori\)-induced gastritis would segregate in a nonrandom fashion between F2 individuals, unless they are encoded on the same chromosome as IgA. To unravel such a possibility de novo generation of genetically defined double gene knockout mice, rather than inter/backcrossing of strains, would be required.

The IL-10−/− mouse model does not allow us to discriminate between a direct or indirect anti-inflammatory effect of IgA, the indirect effect functioning via mechanisms resulting in bacterial evasion and increased colonization. Whereas both possibilities are plausible, most earlier studies give support to the latter theory (44, 47–49). We and others have found no effect of Abs on the postimmunization gastritis phenomenon clearly indicating that IgA Abs per se are unlikely to dampen inflammation (14, 15). Of course, a direct neutralizing effect of Ab on the presence of proinflammatory factors released by the bacterium might contribute to reducing the

**FIGURE 6.** Complement C3b deposition in the gastric tissue at 7 wk after a primary infection with \(H.\ pylori\). C3b deposition in the gastric tissue was determined by immunohistochemistry using an anti-C3b mAb followed by anti-rat biotin-labeled Ab on frozen sections as described in Materials and Methods. \(H.\ pylori\)-infected gastric tissues exhibited equal C3b deposition in IgA+/+ /IL-10+/+ (D; score 3) and IgA−/− /IL-10−/− (E; score 3) mice. As negative controls, we used gastric tissue sections from naive C3-deficient mice labeled with rat anti-C3b mAb plus anti-rat biotin-labeled second Ab (A) or infected IgA+/+ /IL-10−/− mice labeled with anti-rat biotin only (B) or infected IgA−/− /IL-10−/− mice labeled with isotype control Ab (not shown). Naive IgA+/+ /IL-10−/− mice exhibited weak C3b deposition in the gastric mucosa (C; score 1). All sections are at the same magnification (×20). Bar, 100 μm.

**FIGURE 7.** Complement. C3b fragment, deposition (A) and complement receptor (B) expression in the gastric tissue at 7 wk after a primary infection with \(H.\ pylori\) in IgA+/+ /IL-10+/+ or IgA−/− /IL-10−/− mice. For detection of C3b fragment deposition or complement receptor expression, we labeled frozen sections with rat anti-C3b (A) or anti-CD21/CD35 (B) mAbs, respectively, followed by an anti-rat biotin-labeled second Ab. Microscopic analysis of visual fields used a scoring system based on labeling intensity and a scale from 0 to 4, as described in Materials and Methods. The bars represent mean ± SEM of pooled data from two to three experiments with 8–10 mice per group and experiment.
inflammation (47). Also, human IgA has been shown to down-regulate inflammation by suppressing cytokine production or reducing the oxidative burst in neutrophils and monocytes (50, 51). However, it is more likely that the presence of specific IgA Abs leads to bacterial evasion and thereby stronger colonization (44, 48, 49, 52). A mechanism by which this is possible has recently been demonstrated by Carlsson et al. (53), who showed that opsonizing IgA Abs and complement C4b-binding protein bound to the M protein on the surface of Streptococcus pyogenes could prevent phagocytosis. Specific IgA Abs may, thus, bind to the H. pylori bacteria in the gastric lumen and thereby allow the bacteria to evade immune recognition, although phagocytosis is unlikely to be the main mechanism for protection against H. pylori infection (44, 48, 49, 54). A deposition of IgA and IgG Abs on the bacteria below a critical threshold could, however, avoid activation of an inflammatory response and in this capacity also impair the elimination of the bacteria (49, 55). Furthermore, Abs could also facilitate bacterial adherence to the mucus layer making the gastric epithelium a more hospitable environment for the bacteria. Such an effect has been reported with Abs against the capsular polysaccharide of pneumococci, which exposed the ligand for phosphorylcholine, and in this way facilitated bacterial adherence to epithelial cells (52). Finally, an inhibiting effect of IgA Abs on complement activation could account for the reduced inflammatory response seen in the IL-10−/− mice. In fact, a recent report demonstrated that infection in IL-10−/− mice with H. felis resulted in increased levels of serum complement and when complement was depleted in these mice they exhibited decreased gastritis, which delayed the clearance of bacteria (31). It has also been shown that human IgA Abs can significantly inhibit the activation of complement stimulated by Ag-bound IgG Abs (44). However, we found that the level of complement fragment (C3b) deposition in the gastric mucosa in IL-10−/− mice was unaffected by the presence or absence of IgA, indicating that complement activation is unlikely to account for the difference in gastritis between IgA−/− and IgA−/− H. pylori-infected IL-10−/− mice. Moreover, we reported previously that μMT mice also have increased resistance against H. pylori infection associated with more severe gastritis (14). It remains to be investigated whether μMT mice had increased gastric complement deposition following infection. Such a finding would, however, argue against IgA Abs interfering with IgG-induced complement activation, as μMT mice also lack IgG Abs. Taken together, this would point to an anti-inflammatory mechanism of IgA not involving complement activation. The increased expression of CD21/CD35 in IgA−/− is rather to be taken as a sign of the aggravated gastric inflammation.

IL-10 has been shown to down-regulate host resistance to microbial infections (56, 57). Studies of infected IL-10−/− or WT mice depleted of IL-10 by specific Ab treatment have clearly demonstrated that elimination of a pathogen can be more effective when IL-10 is absent (58–60). Furthermore, pathogens, such as Yersinia bacteria, can induce IL-10 production to evade host protection (61). We found that infection of IL-10−/− mice with H. pylori resulted in at least 100-fold reduction in colonizing bacteria in the gastric tissue concomitant with development of severe gastric inflammation, which is in accord with previous reports (20, 30–32). We extended the information from previous studies by incorporating data on protection in well-immunized IL-10−/− mice and found that these mice were significantly better protected than WT mice, demonstrating that IL-10 counteracts the development of vaccine-induced protection against an H. pylori infection. However, somewhat at variance with our results, Panthel et al. (32) recently reported that IL-10−/− mice failed to develop protection following immunization using a similar immunization protocol with H. pylori lysate and CT adjuvant. We think this possible discrepancy easily is accounted for by the fact that these authors assessed protection at 21 wk postchallenge while we waited only 2 wk (32). At 21 wk postchallenge the IL-10−/− mice spontaneously have eliminated or dramatically reduced bacterial counts even without prior immunization, thus making the later time point difficult to use for the analysis of vaccine-induced protection (32). It is noteworthy to mention that the IL-10−/− mouse is one of very few experimental models that has been shown to spontaneously clear a primary infection with H. pylori bacteria (20, 21, 30).

Given that IgA Abs are not required for clearance of bacteria, nor are they required for prevention of bacterial colonization, their role in host resistance against an H. pylori infection awaits to be established. By dampening inflammation they indirectly appear to promote the presence of bacteria in the infected individual. Of course, there is undoubtedly a fine balance in host responses between those that lead to an effective clearance of bacteria and those that cause the immunopathology resulting from the H. pylori infection (14, 19). At present most experimental models indicate that postimmunization gastritis and clearance of bacteria are positively correlated, which would mean that IgA Ab production would be a disadvantage following specific vaccination. The emerging awareness of this possibility would argue in favor of the development of H. pylori-specific vaccines that primarily stimulate cell-mediated immunity and avoid elicitation of Ab responses, IgA Abs, in particular. Such vaccines may be constructed based on relevant peptide sequences, which usually are poorly recognized by Abs, and not from whole organisms, purified proteins, or DNA (62). Work in this area is largely missing today, but based on the present results, we think that vaccine development along these lines may prove fruitful.

Disclosures

The authors have no financial conflict of interest.

References


