Antibody-dependent macrophage-mediated activity against *Helicobacter pylori* in the absence of complement

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Helicobacter pylori is a Gram-negative bacterium, which chronically infects the stomach. Little is known about the immune mechanisms limiting the spread of infection and/or contributing to protection after experimental immunization. In this study, we investigated the hypothesis that specific antibodies and host cells cooperate in the immunity against *H. pylori*. Antibody-dependent cellular activity against *H. pylori* was assessed using specific immune serum, or purified IgG, in an *in vitro* assay, with peritoneal cells as effector cells. The natural antibacterial activity of peritoneal cells was significantly augmented by *H. pylori*-specific antibodies in a dose-dependent manner. A novel finding was that this killing effect did not require functional complement. Most of the bactericidal activity was associated with cells that were adherent, $DX5^-$, $CD19^-$, $CD11c^-$, Thy-1.2^-, $CD11b^+$ and $CD16/32^+$, indicating that the main effector population was represented by macrophages. Similar antibacterial killing was obtained with the macrophage cell line GG2EE. Cytochalasin D significantly impaired this antibacterial activity, suggesting that phagocytosis plays a major role in the antibody-mediated *H. pylori* killing.

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1 Introduction

Helicobacter pylori is a Gram-negative microaerophilic bacterium, which causes chronic gastritis and gastric ulcer and is associated with the development of gastric cancer [1, 2]. *H. pylori*-induced inflammation is characterized by accumulation of neutrophils in the gastric mucosa, followed by a marked infiltration of macrophages (Mϕ), as well as plasma cells and lymphocytes [1]. Despite the synthesis of specific antibodies and the recruitment of different cell populations to the gastric inflammatory areas, the infection remains life-long.

Studies in experimental models of infection with *H. pylori* have shown that immunization with selected antigens can induce protection both prophylactically and therapeutically [3]. The mechanisms by which protection is mediated are mostly unknown. An effector role for specific IgA [4] or IgG [5] has been postulated. However, in B cell knockout mice, vaccine-induced protection was equivalent to that observed in immunized wild-type mice, suggesting that antibodies play a minor role compared to cell-mediated immunity [6]. To better understand the immune mechanisms against *H. pylori*, we investigated the ability of peritoneal Mϕ to kill *in vitro* IgG-coated *H. pylori* bacteria in the absence of complement. The results indicate that antibody-dependent cellular activity (ADCA) may represent an important component of the effector arm of the immune response against *H. pylori.*

[I 22968]

Abbreviations: PC: Peritoneal cells **ADCA:** Antibody-dependent cellular activity

2 Results

2.1 ADCA against *H. pylori* by peritoneal cells

To evaluate the role of *H. pylori*-specific antibodies in enhancing the bacterial killing mediated by peritoneal cells (PC), an anti-serum specifically raised against *H. pylori* was used in the assay. The addition of this anti-serum significantly augmented the nonspecific antibacterial activity of PC (Fig. 1A). This activity was dose-dependent, since it increased with the E/T ratio. Similar results were also obtained with the *H. pylori* strains SS1

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and CCUG 17874 (data not shown), suggesting that the bacterial epitopes recognized by the immune serum were conserved.

2.2 Specificity of the antibody activity

To investigate the specificity of the antibody-dependent killing of *H. pylori*, different serum dilutions were tested. Fig. 1B shows a dose-dependent effect of the immune serum on the bactericidal activity of PC. Similar enhancing activity was observed, in a dose-dependent fashion, when affinity-purified IgG were utilized in the assay instead of the whole serum (Fig. 1C).

To further support the specificity of antibody-mediated activity on *H. pylori*, experiments were carried out with the antiserum pre-absorbed either with *H. pylori* or with an unrelated *Escherichia coli* strain. Pre-absorption of serum with an excess of *H. pylori* (Fig. 2A), but not with *E. coli* (Fig. 2B), completely abrogated the antibody-mediated effect on bacterial killing. The specificity of this activity was further demonstrated by the fact that an anti-*Vibrio cholerae* and an anti-group B streptococci-specific serum were totally unable to mediate this effect (Fig. 2C). Taken together, these data demonstrate the specificity of the antibodies on ADCA against *H. pylori*.

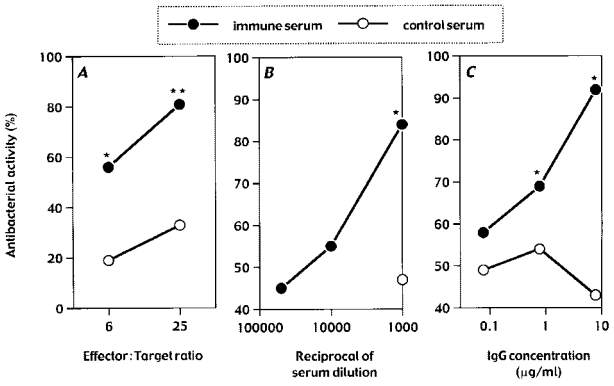

Fig. 1. Antibody-dependent activity by PC against *H. pylori*. Bacteria were pre-incubated with anti-*H. pylori* immune serum or with control serum and then co-cultured with effector cells at the indicated E/T ratios (A). (B, C) Dose-dependent effect of the immune serum (E/T=12;B) or purified IgG (E/T=25;C) on ADCA. Results are expressed as percentage of antibacterial activity. Asterisks indicate statistically significant differences between immune and control serum: **$p<0.01$; *$0.01 \leq p < 0.05$.

2.3 Characterization of the effector cells involved in the ADCA

To identify the cell population(s) mediating this antibacterial activity, PC were analyzed by flow cytometry. On the basis of cell size, it was possible to distinguish two major cell subsets, referred to as R1 and R2. R1 and R2 cells were sorted by fluorescence-activated cell sorter (FACS) and tested in the antibacterial assay, in comparison to the whole unfractionated PC preparation. Following enrichment of R1 cells, negligible antibody-dependent killing was observed (Fig. 3B). In contrast, most of the antibacterial activity was found in the R2 cell population (Fig. 3C) at levels comparable or even higher (at the E/T ratio of 1) than those obtained with the unfractionated PC (Fig. 3A).

The phenotypic analysis of the two populations revealed that R2 cells (which represented about 50–60% of the PC) were $DX5^-$, $CD19^-$, $CD11c^-$, Thy-1.2^-, $CD11b^+$ and $CD16/32^+$ (Fig. 3C), suggesting that the main effector cells belong to the Mϕ lineage. To further elucidate the role of Mϕ in the antibody-mediated bacterial killing, experiments were carried out using the murine Mϕ cell line GG2EE [7]. As shown in Table 1, GG2EE cells mediated very potent killing of *H. pylori* in the presence of specific anti-*H. pylori* antibodies with activities similar to those observed with PC.

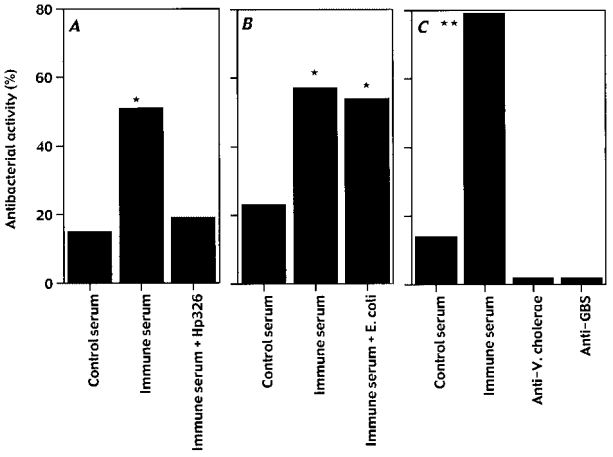

Fig. 2. H. pylori specificity of ADCA. The *H. pylori*-specific antiserum was pre-absorbed or not with an excess of *H. pylori* (A) or of *E. coli* microorganisms (B) and then incubated with *H. pylori.* In other experiments, an anti-*V. cholerae* or an anti-group B streptococci rabbit serum were used (C). Results are expressed as percentage of antibacterial activity. Asterisks indicate statistically significant differences between immune and control serum (A, B, and C) or between *E. coli*-pre-absorbed immune and control serum (B): **$p<0.01$; *$0.01 \leq p < 0.05$. E/T ratio=12:1.

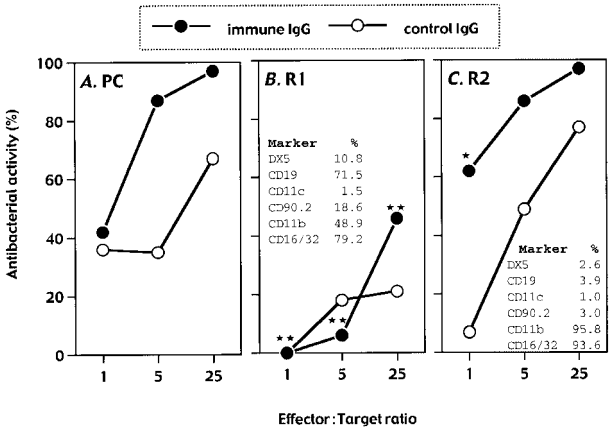

Fig. 3. ADCA of different PC subset populations. Unsorted PC (A) and cell subsets R1 (B) and R2 (C) were assayed for ADCA, using immune and control IgG. The IgG-specific antibacterial killing by R1 cells (B) was significantly lower than that observed with whole PC population (A); $^{**}p<0.01$. Killing with R2 cells at an E/T ratio of 1 was significantly higher than that observed with PC at the same E/T ratio; $^{*}0.01 \leq p < 0.05$. Phenotypic characteristics of the two populations are inserted in (B) and (C).

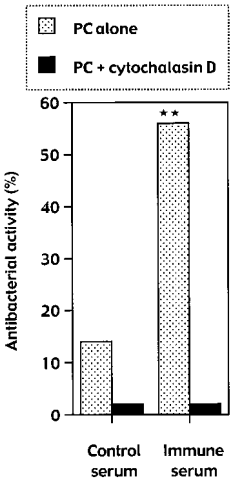

Fig. 4. Effect of cytochalasin D on ADCA. ADCA was carried out as described in the legend of Fig. 1, in the absence or in the presence of cytochalasin D at 5 μg/ml. Asterisks indicate statistically significant differences between immune and control serum: $^{**}p<0.01$. The results of a representative experiment at the E/T ratio of 1 are shown.

To investigate the possible mechanism(s) by which antibody-coated *H. pylori* was killed, experiments were carried out in the presence of the phagocytosis inhibitor cytochalasin D. Fig. 4 shows that cytochalasin D significantly reduced the bactericidal activity of PC, suggesting that phagocytosis plays a major role in the ADCA against *H. pylori*.

3 Discussion

In this study, we present evidence that mouse PC exert strong antibacterial activity against *H. pylori* in the presence of specific antibodies. The phenotypic characterization of the effector population showed that the cells involved in the *H. pylori* killing are Mϕ. A key finding of our work is that the ADCA observed occurs in the absence of complement, since all antibody preparations had been heat-treated before use. Tosi et al. [8] previously reported that sera containing IgG-specific antibodies from children with culture-proven *H. pylori* gastritis markedly enhanced phagocytosis of *H. pylori* by PMN leukocytes; however, the opsonic activity of such antibodies, unlike our results, was dependent on the presence of complement. The role of Mϕ in the antibacterial activity shown here was not restricted to the PC, since it was also observed with splenic Mϕ (data not shown) and with the Mϕ cell line GG2EE. It is reasonable to hypothesize that such an antibacterial mechanism could also be effective *in vivo*, through Mϕ directly or indirectly recruited by signals delivered by *H. pylori* [9, 10]. Recently, Allen et al. [11] have demonstrated that unopsonized CagA$^+$ *H. pylori* organisms avoid phagocytic killing by peritoneal Mϕ by delayed entry followed by homotypic phagosome fusion; as the authors speculate, this phenomenon may be altered *in vivo* by the presence of complement or specific IgG. The results of our experiments using cytochalasin D show, however, that the ADCA of PC require an active internalization via phagocytosis.

The role of specific antibodies in the immunity against *H. pylori* infection is still controversial. Studies on patients with chronic gastritis indicate that most of

Table 1. ADCA by GG2EE Mϕ cells against *H. pylori*[a)]

	Antibacterial activity (%)	
E/T ratio	Control serum	Immune serum
12	0	46 **
25	12	54 *
50	22	68 **

a) Bacteria were pre-incubated with anti-*H. pylori* immune serum or with control serum, and then co-cultured with GG2EE cells at the indicated E/T ratios. Asterisks indicate statistically significant differences between immune and control serum: $^{**}p<0.01$; $^{*}0.01 \leq p < 0.05$.

H. pylori-infected individuals develop strong antibody and cellular immune responses to the bacteria [12–14], although they fail to resolve their infection. Indeed, protection against experimental *H. pylori* infection can be achieved in mice by vaccination with urease in the absence of antibodies, but it requires MHC class II-restricted $CD4^+$ T cell response [6]. Similar results have also been obtained after therapeutic immunization [15]. On the other hand, a potential role for locally produced IgG in limiting the gastric colonization by *H. pylori* has been postulated in mice immunized with *H. pylori* antigens and protected against the infection [5]. The IgG-dependent Mϕ-mediated antibacterial activity shown in our model may represent one of the effector mechanisms by which mucosal antibodies and Mϕ limit *H. pylori* infection or contribute to protection following immunization. Work is now in progress to evaluate the ability of *H. pylori*-specific antibodies from other animal species to mediate ADCA.

In conclusion, the results presented here show that *H. pylori*-specific IgG are highly active in promoting *in vitro* a complement-independent killing of bacteria by MΦ. Further investigation is needed to identify the antigens recognized by these antibodies. This may shed light on the reciprocal interaction between this bacterium, which causes chronic infection, and the immune system that attempts to limit it.

4 Materials and methods

4.1 Bacterial strains

H. pylori type I strains SPM326 [16], SS1 [17] and CCUG 17874 (Culture Collection of the University of Gotheborg, Sweden) were used. Bacteria were grown for 24 h on Columbia agar plates as previously described [18]. *H. pylori* colonies were identified by morphology and by positive urease reaction.

4.2 Preparation of effector cells

Female 7–8-week-old C3H/HeN mice (Charles River, Italy) were injected i.p. with 1.5 ml of PBS. Twenty-four hours later, PC were collected after i.p. injection of 6 ml of cold RPMI 1640 medium. PC were washed and resuspended in RPMI 1640 with 10% heat-inactivated FBS and 2 mM L-glutamine without antibiotics. The GG2EE cell line was maintained *in vitro* as previously described [7].

4.3 Antisera

Antiserum against *H. pylori* strain SPM326 was raised in rabbits by multiple s.c. inoculations of 4×10^8 killed bacteria and Freund's adjuvant. A pool of sera from two immunized animals was prepared and shown to contain high titers of anti-*H. pylori* IgG antibodies, as measured by ELISA and Western blot. Preimmune serum from the same animals was used as control. Sera were heated at 56°C for 30 min, aliquoted and stored at –20°C until use. Purified IgG were obtained from heat-inactivated whole sera by affinity chromatography on HiTrap Protein G column (Amersham Pharmacia Biotech, Uppsala, Sweden). Pre-absorption of serum against either *H. pylori* SPM326 or *E. coli* DH10B strain (Gibco BRL, Life Technologies Ltd., Paisley, Scotland) was done by incubating the serum samples with 5×10^8 bacteria for 45 min on ice. At the end of the incubation the mixture was centrifuged for 10 min at 1,600×g and the serum harvested. In some experiments, polyclonal rabbit antisera raised against *V. cholerae* or group B streptococci (kindly provided by Drs. M. Giuliani and Dr. D. Maione, Chiron, Siena) were also used. All the hyperimmune rabbit sera employed had similar total Ig concentrations.

4.4 Bactericidal assay

A modification of the bactericidal assay reported by Nencioni et al. [19] was employed. Briefly, 10^4 bacteria were put into 5-ml tubes (Becton Dickinson & Co, Franklin Lakes, NJ) together with either appropriately diluted anti-*H. pylori* serum or normal serum (or IgG), centrifuged at 1,600×g for 10 min at 4°C and incubated for 20 min on ice. The effector cells were added to bacteria at different E/T ratios, and the tubes centrifuged at 350×g for 10 min at 4°C. The final volume of the mixture was 0.3 ml. Experimental and control tubes (which contained bacteria and serum, but not cells) were then incubated at 37°C for 2 h. At the end of the incubation, the pellets were vigorously resuspended; diluted aliquots were plated on Columbia agar plates. CFU were counted after 4–7 days. Two to three plates were prepared from each tube. The percentage of antibacterial activity was expressed as follows: % of bactericidal activity = 100–(100×no. CFU of experimental tubes/no. CFU of control tubes without cells).

4.5 Sorting and phenotypic characterization of effector cells

When PC were analyzed by FACS using the morphology parameters Forward Scatter (FSC) and Side Scatter (SSH), two major subsets of cells, namely R1 and R2, were visualized at a FSC threshold of 200. To separate the two populations, whole PC (2×10^7 cells/ml) were sorted in high-speed mode using a FACS Vantage SE (Becton Dickinson, San Jose, CA). Staining of the cells was performed in U-bottomed 96-well plates (Costar, Corning Inc., NY) according to standard procedures; cells were collected for the phenotypic analysis using CellQuest Software on an LSR Cytometer (Becton Dickinson). The following PE-conjugated mAb (PharMingen, BD Biosciences, San Jose, CA) were used: anti-mouse CD19 (clone 1D3), anti-mouse pan-NK

cells (clone DX5), anti-mouse CD90.2 (Thy-1.2; clone 53–2.1), anti-mouse CD11b (integrin α_M chain, Mac—1 α chain, clone M1/70), anti-mouse CD16/CD32 (Fcγ III/II receptor, clone 2.4G2), anti-mouse CD11c (integrin α_x chain, clone HL3).

4.6 Statistical analysis

Statistically significant differences between groups were determined using the unpaired Student's *t*-test, assuming significance if $p<0.05$.

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