Helicobacter pylori heat-shock protein 60 induces production of the pro-inflammatory cytokine IL8 in monocytic cells

Song-Nan Lin,¹ ² Kiyoshi Ayada,¹ Ying Zhao,¹ Kenji Yokota,¹ Ryuta Takenaka,³ Hiroyuki Okada,³ Rui Kan,¹ Shyunji Hayashi,⁴ Motowo Mizuno,⁵ Yoshikazu Hirai,⁴ Yoshihito Fujinami⁶ and Keiji Oguma¹

¹ ² Departments of Bacteriology¹, Dermatology² and Medicine and Medical Science³, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700-8558, Japan
³ Division of Bacteriology, Department of Infection and Immunity, Jichi Medical School, 3311-1 Yakushiji, Minami-Kawauchi-cho, Kouchi-Gun, Tochigi 329-0498, Japan
⁴ Gastroenterology, Hiroshima City Hospital, 7-33 Moto-cho, Naka-ku, Hiroshima 730-8518, Japan
⁵ National Research Institute of Police Science, 6-3-1 Kashiwanoha, Kashiwa 277-0882, Japan

Interleukin 8 (IL8) is usually produced in both epithelial and monocytic cells during bacterial infections, causing inflammation. Helicobacter pylori induces production of IL8 from gastric epithelial cells via its cag pathogenicity island (cag PAI) system, LPS and outer-membrane protein. In some bacteria, heat-shock protein 60 (HSP60) also elicits a strong pro-inflammatory response in cells of the innate immune system. Three recombinant H. pylori HSP60 (rHSP60) proteins of different sizes were produced and one of these was used to raise two monoclonal antibodies (2E7 and 7B5). IL8 production was found to be induced in cultured monocytic cells treated with H. pylori cells or rHSP60 proteins, as measured by ELISA, and the amount of IL8 produced was dose-dependent. Pre-incubation of H. pylori cells or rHSP60 preparations with the antibody 2E7 significantly inhibited IL8 production from monocytic cells. These results indicated that HSP60 is closely associated with IL8 production in monocytic cells.

INTRODUCTION

Chronic infection with Helicobacter pylori is recognized as the cause of chronic active gastritis, peptic ulcer diseases and mucosa-associated lymphoid tissue (MALT) lymphoma and is an important risk factor for the development of gastric carcinoma (Blaser, 1990; Parsonnet et al., 1991, 1994). The chronic phase of H. pylori infection associates adaptive lymphocyte immunity with innate immunity. Colonization of H. pylori in the mucosa leads to local infiltration of neutrophils, macrophages (Ernst & Gold, 2000) and T and B cells specific for H. pylori antigens (D’Elios et al., 1997; Di Tommaso et al., 1995). Chronic gastritis is initiated and maintained by cytokines that are secreted by gastric epithelial cells and macrophages. Interleukin 8 (IL8) is one of the principal mediators of the inflammatory response.

Initially, secretion of IL8 was reported from gastric epithelial cells co-cultured with live H. pylori (Crabtree et al., 1995) and it was later proposed that this reaction depended on the cag pathogenicity island (cag PAI) (Crabtree et al., 1999). Recently, it was also reported that bacterial surface molecules such as LPS and heat-shock protein 60 (HSP60) of H. pylori are closely associated with gastric inflammation. LPS induced secretion of IL8 via toll-like receptor 4 on host epithelial cells (Su et al., 2003). However, the biological activity of H. pylori LPS is lower than that of the LPS of other Gram-negative bacteria (Birkholz et al., 1993; Moran et al., 1992).

H. pylori seems to bind to gastric epithelial cells and mucin via HSP60 (Huesca et al., 1996), and adaptive immunity to HSP60 was induced in H. pylori-infected patients (Hayashi et al., 1998; Sharma et al., 1997). We reported previously that development of lymphoid tissue in patients with MALT lymphoma was associated with HSP60 (Kobayashi et al., 1998). Furthermore, we found that the IgG response to HSP60 was closely associated with gastric inflammation and the pathogenesis of MALT lymphoma (Ishii et al., 2001;
The image contains a page of a document discussing the cloning, expression, and purification of recombinant protein. The text begins with a study on the in vitro production of IL8 from gastric epithelial cells and the use of BHI agar supplemented with 7% horse blood and incubated at 37°C. The methods section describes bacterial strains and cell culture, bacterial preparation, and the purification of recombinant proteins using glutathione-Sepharose 4B. The study also mentions the production of mAbs against rHSP4-5 and their role in stimulating IL8 production. The methods include ELISA and Western blotting for the detection of antibodies. The text also discusses the production of IL8 using both bacterial and rHSP60 proteins and its detection using a standard curve. The document is attributed to S.-N. Lin and others.
**RESULTS**

Intact and heat-killed *H. pylori* and rHSP60s induce IL8 production in monocytic cells

IL8 production from U937 and NOMO-1 cells was analysed following treatment with various amounts of intact or heat-killed *H. pylori* or with the rHSP60 preparations. Intact or heat-killed *H. pylori* induced secretion of IL8 from U937 cells in a dose-dependent manner (Fig. 1a, b). Intact *H. pylori* stimulation using \( >1 \times 10^6 \) c.f.u. ml\(^{-1}\) induced significant levels of IL8 production. Low concentrations of heat-killed *H. pylori* induced low levels of IL8 production, but high concentrations (\( >5 \times 10^7 \) c.f.u. ml\(^{-1}\) ) of heat-killed *H. pylori* induced IL8 production at levels comparable with intact *H. pylori* stimulation. Production of IL8 from stimulated NOMO-1 cells showed the same trend (Fig. 1c, d).

Cells treated with 1–200 \( \mu \)g rHSP4-5 or rHSPw ml\(^{-1}\) also released IL8. rHSPw and rHSP4-5 preparations induced IL8 production from U937 and NOMO-1 cells in a dose-dependent manner (Fig. 2a, b). IL8 secretion was increased significantly by the addition of more than 25 \( \mu \)g rHSPw or 50 \( \mu \)g rHSP4-5 ml\(^{-1}\) in U937 cells. However, IL8 secretion induced by rHSP2 was lower than that of rHSPw and rHSP4-5. A polymyxin B (PMB)–agarose gel column (Pierce Biotechnology) was used to remove LPS and LPS-associated molecules in the preparation (Gao & Tsan, 2003). The concentration of LPS in the PMB–agarose column pass-through fractions was less than 10 EU (2 mg protein\(^{-1}\)) (Takenaka *et al.* 2004). IL8 secretion from U937 and NOMO-1 cells was not induced by stimulation with 10 EU *E. coli* LPS (Sigma), although LPS contamination is not associated with this experiment.

**mRNA expression stimulated by rHSP60**

U937 cells (5 \( \times \) 10\(^5\) cells ml\(^{-1}\)) were stimulated with intact *H. pylori* (1 \( \times \) 10\(^6\) c.f.u. ml\(^{-1}\)), heat-killed *H. pylori* (5 \( \times \) 10\(^6\) cell ml\(^{-1}\)) or rHSPw (100 \( \mu \)g ml\(^{-1}\) ) and the culture supernatant was collected at various times between 1 and 24 h. IL8 production induced by rHSPw increased from 1 to 9 h, reaching a plateau at 9 h (Fig. 3a). Real-time PCR was used for IL8 mRNA expression in U937 cells. Expression of IL8 mRNA was maximal at 1 h and continued for the following 12 h (Fig. 3b).

**Characterization of anti-rHSP4-5 mAbs**

Two mAbs (2E7 and 7B5) were obtained. They were classified as IgG2b. The reaction of these mAbs to different rHSP60 molecules in the preparation (Gao & Tsan, 2003). The concentration of LPS in the PMB–agarose column pass-through fractions was less than 10 EU (2 mg protein\(^{-1}\)) (Takenaka *et al.* 2004). IL8 secretion from U937 and NOMO-1 cells was not induced by stimulation with 10 EU *E. coli* LPS (Sigma), although LPS contamination is not associated with this experiment.

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**Table 1. Synthetic peptides of rHSP4-5**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Position</th>
<th>Molecular mass (Da)</th>
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<tbody>
<tr>
<td>A</td>
<td>VEFLGKAKIVDKDNTTIVD</td>
<td>312–331</td>
<td>2218-54</td>
</tr>
<tr>
<td>B</td>
<td>GKGHSYHKVDRVAQIKTQIA</td>
<td>332–351</td>
<td>2288-46</td>
</tr>
<tr>
<td>C</td>
<td>STTSDYDEKQLQERLAKSLG</td>
<td>352–371</td>
<td>2269-47</td>
</tr>
<tr>
<td>D</td>
<td>GVAEEKQGGAEEVEKKEKCD</td>
<td>372–391</td>
<td>2088-42</td>
</tr>
<tr>
<td>E</td>
<td>RVDDALATKAAVEEVEIG</td>
<td>392–411</td>
<td>2014-25</td>
</tr>
<tr>
<td>F</td>
<td>GGAALIRAAQKVHLNHDDEKVG</td>
<td>412–434</td>
<td>2412-72</td>
</tr>
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</table>
**Fig. 1.** Induction of IL8 from monocytic cells stimulated with intact or heat-killed *H. pylori*. U937 (a, b) and NOMO-1 (c, d) cells were stimulated with various amounts (0–10⁶ cells ml⁻¹) of intact (a, c) or heat-killed (b, d) cells. After stimulation for 8 h, the culture supernatant was collected and IL8 production was measured by ELISA. Data are expressed as means ± SEM of three wells.

**Preparations and bacterial cell lysates** were determined by ELISA. The mAbs reacted with rHSPw and rHSP4–5, but not with rHSP2 or human rHSP60 (Stressgen). Data for 2E7 are shown in Fig. 4(a); comparable results were obtained for 7B5. 2E7 and 7B5 did not react with *E. coli*, *S. Enteritidis*, *S. Typhi*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Citrobacter freundii* in ELISA and Western blotting (data not shown). mAb 2E7 reacted with *H. pylori*, *H. felis*, *Campylobacter jejuni* and *H. hepaticus* (Fig. 4b), whereas 7B5 reacted only with *H. pylori* (Fig. 4c). These results were also confirmed by Western blotting (Fig. 4d).

**Anti-HSP60 mAb inhibits IL8 production by monocytic cells**

Intact or heat-killed *H. pylori* cells were incubated with anti-*H. pylori* polyclonal IgG, anti-rHSP4–5 mAbs (2E7 and 7B5) or control mAb (anti-type C botulinum toxin) and then co-incubated with U937 or NOMO-1 cells. The concentration of IL8 in the supernatants was assayed after co-incubation for 8 h. Anti-*H. pylori* polyclonal Ab and anti-rHSP4–5 mAb (2E7) significantly inhibited IL8 production induced by both intact and heat-killed *H. pylori* (Fig. 5a). mAb 2E7 inhibited IL8 production stimulated by both intact and heat-killed *H. pylori*, but 7B5 inhibited IL8 production stimulated by only intact *H. pylori* (Fig. 5b). mAb 2E7 significantly inhibited IL8 production stimulated with rHSPw (68.2 % inhibition) and rHSP4–5 (57.4 % inhibition). Effective inhibition by 7B5 was not observed following stimulation with rHSP60w and rHSP4–5 (Fig. 5c).

**Reactivity of mAbs to synthetic peptides and peptide induction of IL8**

The reaction of synthetic peptides covering the rHSP4–5 domain (peptides A–F; Table 1) to the two mAbs was examined. mAb 7B5 reacted strongly with peptides E and F but weakly with peptide A, whereas 2E7 reacted strongly with peptides A and D (Table 2). The amino acid sequences of peptides A, B and F are not identical to that of human HSP60 (Table 1). In the induction using these six peptides, only peptide A induced production of IL8 significantly in U937 cells. Peptide B induced a lower level of IL8 production, whereas peptide F did not induce IL8 production (Table 2).
DISCUSSION

We investigated whether HSP60 induced the production of IL8 by monocytic cells. Induction of IL8 was observed following stimulation not only with rHSPw but also with rHSP4-5, indicating that the region Ile300-Gly435 of HSP60 is associated with induction of IL8 in monocytic cells. This was confirmed using two mAbs (2E7 and 7B5) prepared by immunizing mice with rHSP4-5. Both Abs reacted with rHSPw and rHSP4-5, but not with rHSP2 or human rHSP60, and significantly reduced the IL8-inducing activity. However, their inhibitory effects were different; 2E7 had a greater effect than 7B5 and inhibited the production of IL8 stimulated by both intact and heat-killed cells, while 7B5 only inhibited the production of IL8 induced by intact H. pylori.

mAb 2E7 reacted with peptides A and D of rHSP4-5, whereas 7B5 reacted strongly with peptides E and F, but weakly with peptide A. It was thus concluded that the epitopes recognized by the two mAbs are different. It was not clear why the mAbs reacted with more than one peptide, but this may explain the differences in their inhibitory effects on IL8 production.

Peptide A induced IL8 production in U937 cells compared with peptides B–F, although the level of IL8 was not very high. The amino acid sequences of peptides A, B and F are quite different from that of human HSP60, whereas peptides C, D and E possess similar amino acid sequences to human HSP60. Therefore, it was speculated that the amino acid sequence differences compared with human HSP60, specifically in peptide A, are important for IL8 induction. Monocytic cells may recognize a bacteria-specific sequence or particular conformation of this region and thus induce production of a pro-inflammatory cytokine.

mAb 7B5 reacted strongly with the synthetic peptides in ELISA. However, IL8 secretion was only weakly inhibited, or not inhibited, by 7B5 in heat-killed H. pylori or rHSP60 stimulations. In contrast, 2E7 had low reactivity to the peptides, but showed high levels of inhibition of IL8 production. Stimulation with the synthetic peptides induced low levels of IL8 production. In fact, recombinant proteins lost this activity if subjected to several freeze–thaw steps (data not shown), indicating that not only the amino acid
cells following stimulation not only with live demonstrated that IL8 was induced in U937 and NOMO-1 also flagella and the LPS, outer-membrane protein and LPS. Other reports have indicated that not only cag PAI, but also with heat-killed cells. This indicated that the production of IL8 is different from that associated with the Cag A and type IV secretion systems, which are formed only in living H. pylori cells. IL8 production was also induced by rHSP60 that had been passed through a column eliminating LPS. Other reports have indicated that not only cag PAI, but also flagella and the LPS, outer-membrane protein and HSP60 of H. pylori are associated with IL8 induction from epithelial cells or monocytes (Bhattacharyya et al., 2002; Cunningham et al., 2000; Lee et al., 2003; Yamaoka et al., 2000; Yamasaki et al., 2004). These non-cag antigens are located on the bacterial surface and have direct contact with host cells. The cag-independent pathway of cytokine induction also may involve inducing mucosal inflammation. Therefore, we concluded that HSP60 is an important molecule for the IL8-inducing system in H. pylori infection.

It is still not clear how the immune system recognizes H. pylori early in infection and reacts to the bacteria in the stomach, since MALT does not normally exist in the stomach. Usually, immune responses arise specifically from the stomach, since MALT does not normally exist in the stomach. It is still not clear how the immune system recognizes H. pylori early in infection and reacts to the bacteria in the stomach, since MALT does not normally exist in the stomach.
cytic cells through HSP60. This supports the hypothesis that a bacterial surface component such as HSP60 on H. pylori that has flowed from the stomach into the intestine may activate immune responses through lymphoid tissue such as Peyer’s patches. Some reports have indicated that cell-surface receptors are associated with the recognition of human HSP (Curry et al., 2003; MacDonald et al., 2002; Wang et al., 2002). The receptors on monocytic cells for H. pylori HSP60

**Table 2. Reactivity of synthetic peptides with mAbs 2E7 and 7B5, and peptide induction of IL8 in U937 cells**

Values for IL8 production are means ± SEM.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Reactivity with 7B5 (A490)</td>
<td>0.22</td>
</tr>
<tr>
<td>Reactivity with 2E7 (A490)</td>
<td>0.19</td>
</tr>
<tr>
<td>IL8 production (pg ml⁻¹)</td>
<td>166 ± 14</td>
</tr>
</tbody>
</table>
have still to be identified. We are now trying to identify the cell receptor for HSP60 and are also studying IL8 induction in gastric epithelial cells using H. pylori and rHSP60 preparations.

The Abs to specific sites recognizing HSP60 sequences of H. pylori that were different from human HSP60 inhibited IL8 production. Thus, we believe that specific domains of H. pylori HSP60 could be used as effective vaccines for anti-inflammatory therapy in the future.

**REFERENCES**


H. pylori HSP60 induces IL8 in monocytic cells.