

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

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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.

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## 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'Elisio *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;

Abbreviations: *cag* PAI, *cag* pathogenicity island; HSP, heat-shock protein; MALT, mucosa-associated lymphoid tissue.

Kawahara *et al.* 1999). Studies *in vitro* have indicated that HSP60 induces IL8 from gastric epithelial cells (Yamaguchi *et al.*, 1999) and IL6 from monocytes/macrophages (Gobert *et al.*, 2004). It has also been reported that the HSP60s of *Mycobacterium tuberculosis*, *Campylobacter rectus* and *Chlamydia pneumoniae* induce the production of IL8 by human monocytic cells, human gingival fibroblasts and human endothelial cells, respectively (Bulut *et al.*, 2002; Flohe *et al.*, 2003; Friedland *et al.*, 1993; Hinode *et al.*, 1998). Therefore, we speculated that *H. pylori* HSP60 might be involved in the activation of macrophages.

To investigate the mechanisms of macrophage activation associated with HSP60, we prepared whole HSP60 (rHSPw; Met<sup>1</sup>–Met<sup>545</sup>) and two partial domains (rHSP2, Glu<sup>101</sup>–Ser<sup>200</sup>, and rHSP4-5, Ile<sup>300</sup>–Gly<sup>435</sup>) as GST fusion proteins and six peptides of the rHSP4-5 domain. Two monoclonal antibodies (mAbs) were also prepared by immunizing BALB/c mice with rHSP4-5. It was found that IL8 was secreted from monocytic cells (U937 and NOMO-1) following treatment with rHSPw and rHSP4-5 as well as with *H. pylori* cells. IL8 induction was inhibited by one of the mAbs against HSP60.

## METHODS

**Bacterial strains and cell culture.** *H. pylori* ATCC 43504 cells were cultured on brain heart infusion (BHI) agar (Becton Dickinson) supplemented with 7% horse blood and incubated at 37 °C under microaerophilic conditions. After incubation for 5 days, bacteria were harvested in RPMI 1640 with 10% fetal calf serum (FCS) to an OD<sub>600</sub> of 1.0 corresponding to approximately  $1 \times 10^8$  c.f.u. ml<sup>-1</sup>. Intact and heat-killed (60 °C for 30 min) bacterial cells were used to stimulate U937 cells.

*H. pylori* strain SS-1, *Helicobacter felis* and *Helicobacter hepaticus* (kindly provided by A. Lee, University of New South Wales, Sydney, Australia) were cultured on BHI agar supplemented with 7% horse blood and incubated at 37 °C under microaerophilic conditions for 5 days. *Campylobacter jejuni* JCM 2013 were also cultured on BHI agar supplemented with 7% horse blood and incubated at 37 °C under microaerophilic conditions for 48 h. *Escherichia coli* NIH-JC2, *Salmonella enterica* serotype Enteritidis strain IID 604, *Salmonella enterica* serotype Typhi strain IID 907, *Pseudomonas aeruginosa* IFO 3455, *Proteus mirabilis* (clinical isolate) and *Citrobacter freundii* (clinical isolate) were cultured on BHI agar for 24 h. Bacterial cells were collected from the agar plates and washed in PBS three times. Cells were disrupted with an ultrasonic sonicator (Astrason) and centrifuged at 20 000 g for 20 min. Supernatants were subjected to a protein assay (Bio-Rad). The soluble cell lysates were used as antigens for ELISA and Western blotting.

U937 (human macrophage-like) and NOMO-1 (human monocytic cell-like) cell lines (Japanese Collection of Research Bioresources) were maintained in RPMI 1640 supplemented with 10% FCS, 50 U penicillin G sodium ml<sup>-1</sup> and 50 µg streptomycin sulfate ml<sup>-1</sup>. Cultures were maintained at 37 °C in an atmosphere of 5% CO<sub>2</sub> with 99% humidity.

**Cloning, expression and purification of recombinant protein.** The amino acid sequence of *H. pylori* HSP60 was analysed using Genetix software and a search was done for T-cell epitopes and homology to human HSP60. Whole HSP60 (rHSPw; Met<sup>1</sup>–Met<sup>545</sup>) and two partial domains (rHSP2, Glu<sup>101</sup>–Ser<sup>200</sup>, and rHSP4-5, Ile<sup>300</sup>–Gly<sup>435</sup>) were designed and expressed as GST fusion proteins (Yamasaki *et al.*, 2004). Soluble fusion proteins expressed by the GST–HSP clones

(rHSPw, rHSP2 and rHSP4-5) were purified by glutathione–Sephacrose 4B (Amersham Pharmacia) affinity chromatography according to the manufacturer's instructions. To exclude the effects of trace amounts of LPS in the recombinant protein preparations, the endotoxin activity of rHSP60 preparations was determined using a *Limulus* amoebocyte lysate assay kit (BioWhittaker) according to the manufacturer's instructions. The preparations of rHSP2, rHSP4-5 and rHSPw contained 20, 23, 30 endotoxin units (EU) (µg protein)<sup>-1</sup>, respectively.

**Production of mAbs.** mAbs against rHSP4-5 were prepared as previously reported with slight modifications (Oguma *et al.*, 1984). Female BALB/c mice (8 weeks old) were inoculated subcutaneously twice at 4-week intervals with 100 µg rHSP4-5 mixed with Freund's incomplete adjuvant. Four weeks after the second injection, a booster dose of 8 µg rHSP4-5 was injected intravenously. Three days later, the spleen was removed aseptically and dissociated into a single-cell suspension. The lymphocytes (10<sup>8</sup> cells) obtained were fused with P3 X63-Ag8-U1 myeloma cells (10<sup>7</sup> cells) using 50% polyethylene glycol 4000 (Merck). Fused cells were plated into 96-well tissue-culture plates (CellStar) and grown in HAT medium (RPMI 1640 containing 0.1 mM hypoxanthine, 0.4 µM aminopterin, 0.016 mM thymidine and 20% FCS) at 37 °C. When colonies became visible macroscopically, culture supernatants were screened for the production of anti-rHSP4-5 Abs by ELISA using plates coated with the recombinant proteins. Ab-producing hybridomas were subcloned in 96-well tissue-culture plates by limiting dilution. RPMI 1640 supplemented with 15% FCS and 5% Briclone hybridoma cloning medium (Dainippon Pharmaceutical Co.) was used throughout the experiment. Ab production was checked by ELISA and positive clones were expanded in large-scale cultures. The cells (>10<sup>7</sup>) were collected and injected intraperitoneally into BALB/c mice that had been pre-treated 7 days earlier with pristane (0.3 ml). Ascitic fluid was collected and the mAbs were purified by DE52 (Whatman) ion-exchange chromatography. The classes and subclasses of mAbs obtained were determined with a mouse monoclonal typing kit (mouse MonoAB ID/SP kit; The Binding Site).

**ELISA and Western blotting.** The reactivity of mAbs to the rHSP60 preparations, bacteria and human cells was checked by ELISA and Western blotting. One microgram of recombinant protein or 5 µg bacterial antigen in 100 µl coating buffer (pH 9.6) was coated on to the wells of a 96-well microtitre plate and left overnight at 4 °C. The wells were blocked using PBS containing 10% skimmed milk for 2 h at room temperature. Various concentrations of mAb were reacted for 2 h at room temperature and washed with PBS containing 0.05% Tween 20. Peroxidase-labelled rabbit anti-mouse IgG (Dako) was then added and the plates were incubated for 2 h at room temperature. After washing, the wells were reacted with ABTS tablets (Roche) as a substrate. The A<sub>405</sub> was measured on a model 680 ELISA plate reader (Bio-Rad).

Bacterial lysates were subjected to SDS-PAGE and transferred to PVDF membranes (Millipore). After reaction with mAbs, the reaction was detected using peroxidase-labelled rabbit anti-mouse IgG (Dako) as the secondary Ab and by ECL Western blotting detection reagents (Amersham Pharmacia Biotech). The luminol reaction was detected with an LAS-1000 Mini Bio-imaging Analyser System (Fuji Photo Film).

**IL8 production stimulated by bacteria and rHSP60 proteins.** U937 and NOMO-1 cells ( $5 \times 10^5$  cells ml<sup>-1</sup>) were co-incubated with different concentrations of intact (live) or heat-killed *H. pylori*, or with the rHSP60s, in 96-well plates for 8 h. The experiment was conducted in triplicate for each concentration. Heat-killed *H. pylori* was prepared by heating intact *H. pylori* at 60 °C for 30 min before stimulation. IL8 in cell-culture supernatants was collected by centrifugation and assayed by ELISA using a human IL8 ELISA development kit (Techne). The concentration of IL8 was determined using a standard curve obtained with recombinant IL8. The detection limit of the ELISA was 31 pg ml<sup>-1</sup>.

**Real-time PCR for IL8 mRNA.** Total RNA was isolated from U937 cells using RNazol-B (Tel-Test). After DNase I (Invitrogen) treatment, cDNA was prepared using ReverTra Dash (Takara). Quantitative PCR analysis was performed on a LightCycler using the LightCycler primer set for human IL8 and G6PDH. Real-time PCR was carried out in 24 µl consisting of 10 µl cDNA sample, 10 µl water, 2 µl LightCycler primer set and 2 µl LightCycler FastStart DNA master SybrGreen I. The reaction mixtures were loaded into capillary tubes and thermal cycling was carried out as follows: activation of hot-start *Taq* DNA polymerase at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 68 °C for 10 s and 72 °C for 16 s. The DNA concentration was calculated using the LightCycler software and the IL8 : G6PDH ratio was calculated.

**Inhibition of IL8 production by anti-HSP60 polyclonal and monoclonal Abs.** Inhibition of the IL8-inducing activity of *H. pylori* by both polyclonal and monoclonal Abs was observed. Intact or heat-killed *H. pylori* cells suspended in RPMI 1640 without antibiotics (OD<sub>600</sub> = 0.5) were incubated with an equal volume of IgG (100 µg ml<sup>-1</sup> in 10 mM PBS, pH 7.4) of anti-*H. pylori* polyclonal Ab (Yunoki *et al.*, 2000), anti-HSP60 mAbs (2E7 and 7B5) or control mAb (anti-type C botulinum toxin) for 30 min at 37 °C. The bacterial suspension (500 µl) was then added to 500 µl U937 or NOMO-1 cell culture (5.0 × 10<sup>5</sup> cells) and incubated for 5 h at 37 °C. The supernatants were used for the IL8 ELISA.

**Production of synthetic peptide and ELISA.** The domain of rHSP4-5 was divided into six fragments containing 20 or 23 amino acids (Table 1). Each peptide was synthesized on an Applied Biosystems model 430A synthesizer using *N*-hydroxybenzotriazole esters of tert-butoxycarbonyl amino acids in an *N*-methylpyrrolidone solvent coupling system as described by the manufacturer. For induction of IL8 from U937 cells, synthetic peptides were added to the cell culture at a concentration of 100 µg ml<sup>-1</sup>. ELISA was performed as described above after coating 96-well microtitre plates with the peptides using a Peptide Coating kit (Takara Bio).

**Statistical analysis.** Results are expressed as means ± SEM. Data were compared using Scheffe's method and differences were considered significant for values of *P* < 0.05.

## 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 × 10<sup>6</sup> c.f.u. ml<sup>-1</sup> induced significant levels of IL8 production. Low concentrations of heat-killed *H. pylori* induced low levels of IL8 production, but high concentrations (>5 × 10<sup>7</sup> c.f.u. ml<sup>-1</sup>) 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 µg rHSP4-5 or rHSPw ml<sup>-1</sup> 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 µg rHSPw or 50 µg rHSP4-5 ml<sup>-1</sup> 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)<sup>-1</sup> (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 × 10<sup>5</sup> cells ml<sup>-1</sup>) were stimulated with intact *H. pylori* (1 × 10<sup>6</sup> c.f.u. ml<sup>-1</sup>), heat-killed *H. pylori* (5 × 10<sup>6</sup> cell ml<sup>-1</sup>) or rHSPw (100 µg ml<sup>-1</sup>) 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).

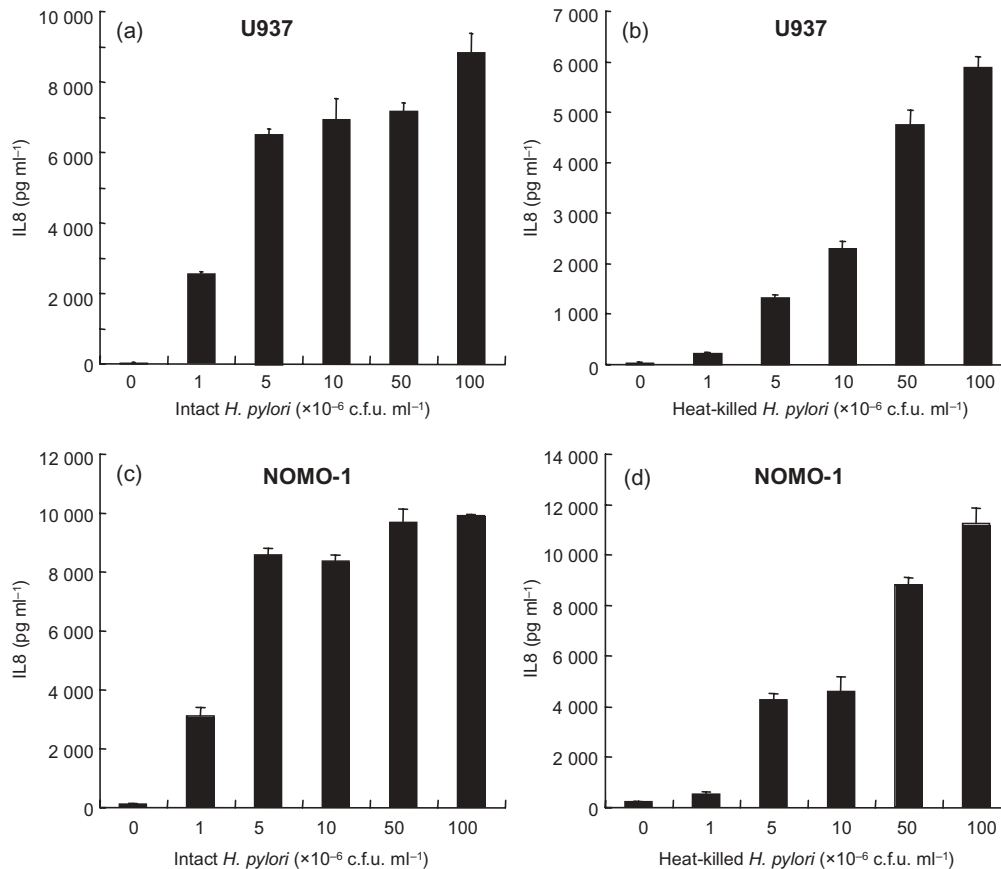
### Characterization of anti-rHSP4-5 mAbs

Two mAbs (2E7 and 7B5) were obtained. They were classified as IgG2b. The reaction of these mAbs to different rHSP60

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

Bold letters indicate amino acids identical to human HSP60. Positions are given relative to the N terminus.

Peptide	Sequence	Position	Molecular mass (Da)
A	VEFLG <b>KAK</b> IVIDKDN <b>T</b> TIVD	312–331	2218.54
B	GKGHSHD <b>V</b> KDRVA <b>Q</b> IK <b>T</b> QIA	332–351	2288.46
C	<b>S</b> TTSDYD <b>K</b> E <b>K</b> L <b>Q</b> ERLAKLSG	352–371	2269.47
D	G <b>V</b> AVIK <b>V</b> G <b>A</b> ASE <b>V</b> EM <b>K</b> E <b>K</b> KD	372–391	2088.42
E	<b>R</b> VDDALS <b>A</b> T <b>K</b> AA <b>V</b> EE <b>G</b> IVIG	392–411	2014.25
F	<b>G</b> GAAL <b>I</b> RA <b>A</b> Q <b>K</b> VHL <b>N</b> L <b>H</b> D <b>D</b> E <b>K</b> V <b>G</b>	412–434	2412.72



**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 (0–10<sup>8</sup> cells ml<sup>-1</sup>) 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  $\pm$  SEM of three wells.

preparations and bacterial cell lysates was 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).

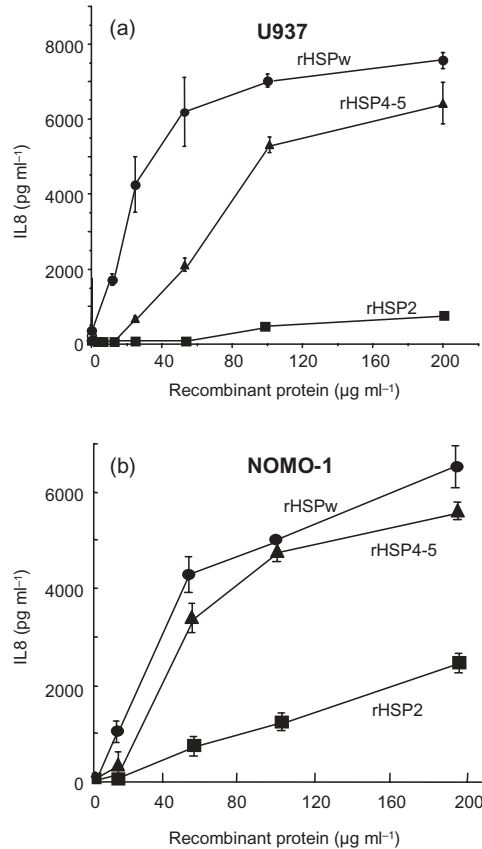
#### Anti-HSP60 mAb inhibits IL8 production by monocytic cells

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).

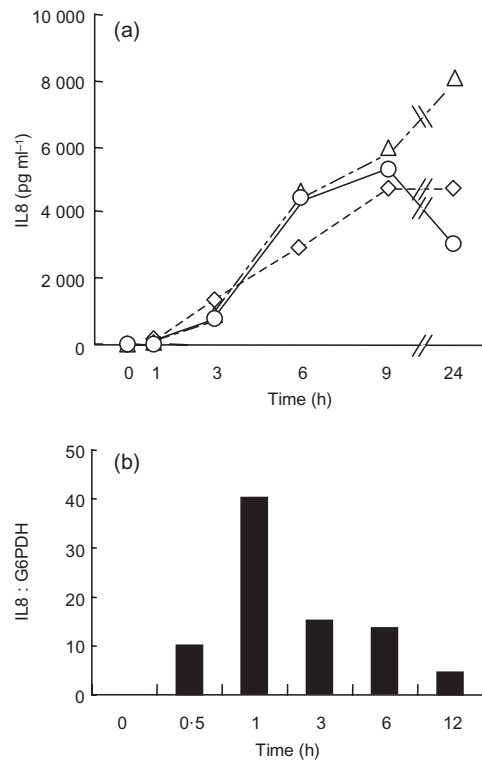


**Fig. 2.** Induction of IL8 stimulated by rHSP60. U937 (a) and NOMO-1 (b) cells ( $5 \times 10^5$  cells ml<sup>-1</sup>) were cultured in RPMI 1640 and stimulated with different concentrations of rHSP60 preparations. After stimulation for 12 h, the supernatant was collected and the production of IL8 was measured by ELISA. Data are expressed as means  $\pm$  SEM of three wells.

## 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 Ile<sup>300</sup>–Gly<sup>435</sup> 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*.

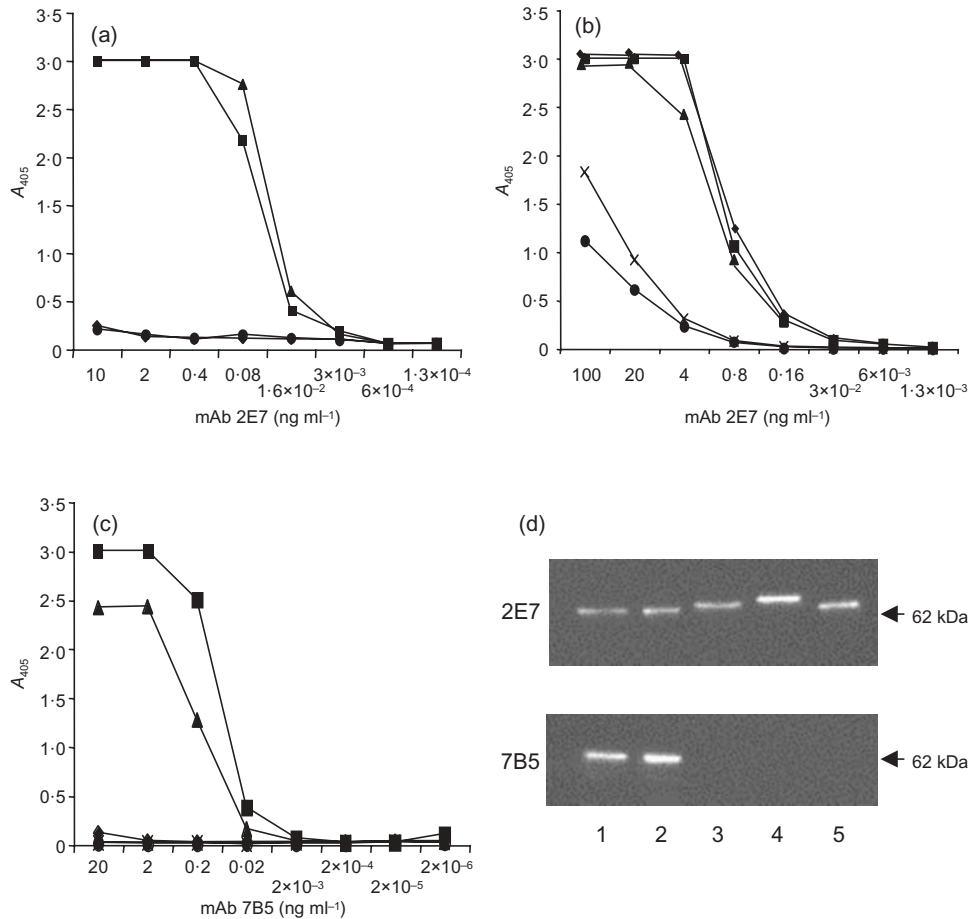
mAb 2E7 reacted with peptides A and D of rHSP4-5, whereas 7B5 reacted strongly with peptides E and F, but weakly with peptide A. It was thus concluded that the epitopes recognized by the two mAbs are different. It was not clear why the mAbs reacted with more than one peptide, but this may explain the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



**Fig. 3.** Induction of IL8 mRNA stimulated by rHSP60. (a) U937 cells were stimulated with  $2 \times 10^6$  cells of intact ( $\diamond$ ) or heat-killed ( $\circ$ ) *H. pylori* ml<sup>-1</sup> or 100 µg rHSPw ml<sup>-1</sup> ( $\triangle$ ). Samples were collected at 0, 1, 3, 6, 9 and 24 h and IL8 concentration was measured by ELISA. (b) mRNA expression was assayed using real-time PCR. U937 cells were stimulated with 100 µg rHSPw ml<sup>-1</sup> and collected at 0, 0.5, 1, 3, 6 and 12 h. The IL8 : G6PDH mRNA expression ratio at each time point was calculated.

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



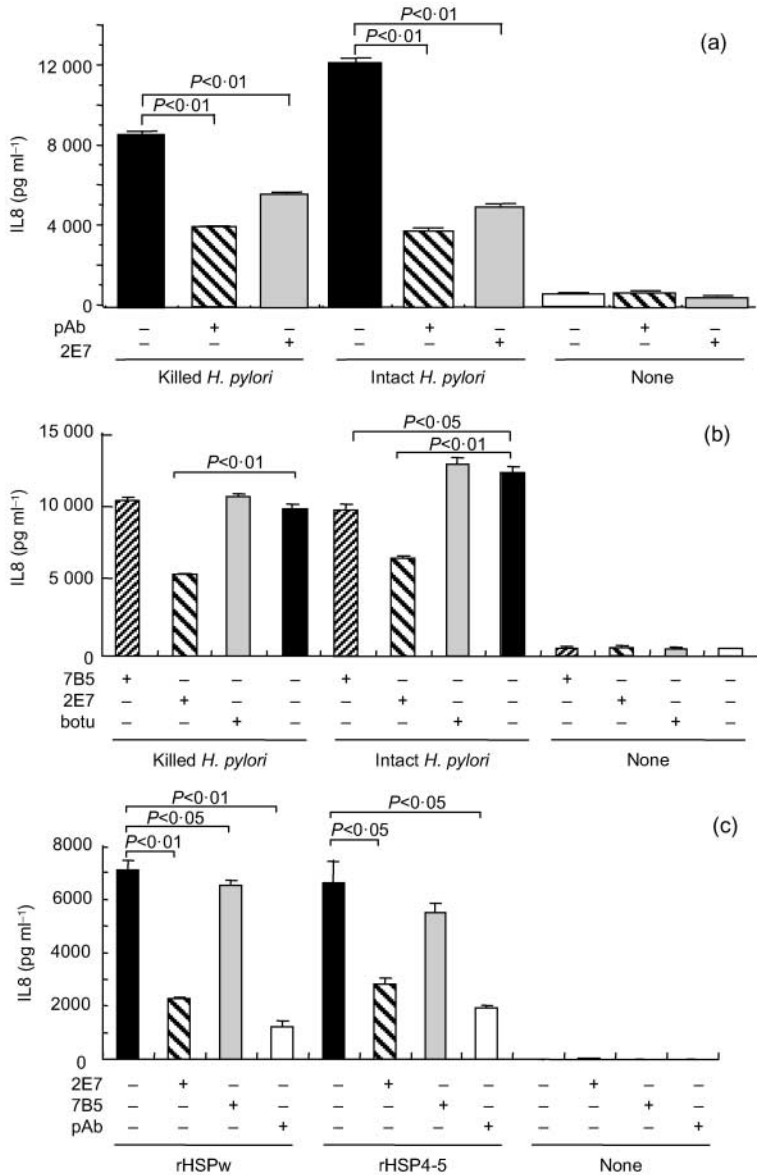
**Fig. 4.** Reactivity of mAbs to recombinant proteins and bacteria. (a) mAb 2E7 was diluted by 5-fold serial dilutions (from 10 to  $1.3 \times 10^{-4}$  ng ml $^{-1}$ ) and reacted with rHSP2 (◆), rHSP4-5 (▲), rHSPw (■) and human rHSP60 (●) by ELISA. (b, c) Different concentrations of 2E7 (b; 5-fold serial dilutions from 100 to  $1.3 \times 10^{-3}$  ng ml $^{-1}$ ) or 7B5 (c; 10-fold serial dilutions from 20 to  $2 \times 10^{-6}$  ng ml $^{-1}$ ) were reacted with *H. pylori* ATCC 43504 (▲) and SS-1 (■), *H. felis* (×), *Campylobacter jejuni* (●) and *H. hepaticus* (◆) antigens by ELISA. (d) The mAbs were reacted with bacterial antigens for Western blotting and the reaction was detected with a LAS-1000 Mini Bio-imaging Analyser system. Lanes: 1, *H. pylori* ATCC 43504; 2, *H. pylori* SS-1; 3, *H. felis*; 4, *Campylobacter jejuni*; 5, *H. hepaticus*.

sequence but also secondary protein structure or the three-dimensional structure of the protein on the bacterial surface may be important for IL8 production.

IL8 is a pro-inflammatory cytokine, the actions of which are reported to include neutrophil and T-lymphocyte chemotaxis, neutrophil activation and enhanced expression of neutrophil adhesion molecules (Sherry & Cerami, 1991). IL8 is released from gastric epithelial cells following stimulation with cag PAI and LPS of *H. pylori*. In this study, we demonstrated that IL8 was induced in U937 and NOMO-1 cells following stimulation not only with live *H. pylori* cells 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 within organized lymphoid tissue in the intestine (mostly located in the terminal ileum as Peyer's patches) (Wotherspoon, 1998). It seems that *H. pylori* is not able to survive in the human intestine. We have demonstrated here that heat-killed *H. pylori* induced IL8 production by mono-



**Fig. 5.** Anti-*H. pylori* and anti-HSP60 antibodies inhibit induction of IL8 secretion. Intact or heat-killed *H. pylori* was reacted with antibodies for 30 min in antibiotic-free RPMI 1640. U937 or NOMO-1 cells were then co-incubated with antibody-treated *H. pylori* and the supernatant was collected. IL8 levels in the supernatant were measured by ELISA. (a) Inhibition by anti-*H. pylori* polyclonal (pAb) and mAb 2E7 in U937 cells. (b) Inhibition by anti-HSP60 mAbs (2E7 and 7B5) and control mAb (botu) in NOMO-1 cells. (c) IL8 induction by rHSP4-5 and rHSPw was inhibited with polyclonal or monoclonal Abs in NOMO-1 cells. Results are shown as means  $\pm$  SEM ( $n = 3$ ).

**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  $\pm$  SEM.

Parameter	Peptide					
	A	B	C	D	E	F
Reactivity with 7B5 ( $A_{490}$ )	0.22	0.08	0.10	0.28	0.95	0.46
Reactivity with 2E7 ( $A_{490}$ )	0.19	0.09	0.13	0.22	0.17	0.18
IL8 production ( $\text{pg ml}^{-1}$ )	166 $\pm$ 14	130 $\pm$ 19	121 $\pm$ 16	108 $\pm$ 10	104 $\pm$ 11	97 $\pm$ 8

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

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.

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