Immune response in *Helicobacter pylori*-induced low-grade gastric-mucosa-associated lymphoid tissue (MALT) lymphoma

Rie Yamasaki,1 Kenji Yokota,2 Hiroyuki Okada,3 Shyunji Hayashi,4 Motowo Mizuno,3 Tadashi Yoshino,1 Yoshikazu Hirai,4 Daizou Saitou,5 Tadaatsu Akagi1 and Keiji Oguma2

1,2,3 Departments of Pathology,1, Bacteriology2 and Medicine and Medical Science,3, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700-8558, Japan
4 Department of Infection and Immunity, Jichi Medical School, 3311-1 Yakushiji, Minami-kawachi, Tochigi 326-0498, Japan
5 National Cancer Center, Central Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

We have reported previously that heat-shock protein 60 kDa (hsp60) of *Helicobacter pylori* is an important antigen in the pathogenesis of gastric mucosa-associated lymphoid tissue (MALT) lymphoma. In order to investigate associations with host immune reactions and hsp60 antigen, CD40 ligand (CD40L) expression and cytokine production were analysed following stimulation with hsp60. To provide a clear antigen-driven immune response, peripheral blood mononuclear cells (PBMC) from patients with low-grade MALT lymphoma and gastritis and those from healthy volunteers were stimulated with recombinant *H. pylori* hsp60 and *H. pylori* cell lysate in the presence of cytokines (IL4 and granulocyte-macrophage colony-stimulating factor). mRNA expression was also analysed by a cDNA microarray containing 1100 genes. Expression of CD40L on PBMCs of patients with MALT lymphoma was increased by cytokines or by combination with stimulation with hsp60 antigens. The production of IL4 in PBMC cultures was increased in patients with MALT lymphoma; however, production of IFN-γ was at low levels. DNA microarray analysis indicated increased levels of HLA-DR and integrin mRNAs. In cases of low-grade MALT lymphoma, adaptive immune responses against hsp60 may be enhanced by host factors, such as antigen presentation and T-cell activation, resulting in B-cell proliferation, which can be demonstrated during chronic *H. pylori* infection.

INTRODUCTION

A close link between *Helicobacter pylori* infection and the development of gastric mucosa-associated lymphoid tissue (MALT) lymphoma has been established in some studies. *H. pylori* infection has been confirmed in most cases of MALT lymphoma (Isaacson & Spencer, 1993; Parsonnet et al., 1994; Wotherspoon et al., 1991, 1993). Low-grade MALT lymphomas often regress following the eradication of *H. pylori* (Bayerdorffer et al., 1995), and they relapse with re-infection with *H. pylori* (Cammarota et al., 1995; Horstmann et al., 1994).

Heat-shock protein (hsp) is a candidate cross-reacting antigen in microbial infections. Our previous studies have shown that hsp60 is expressed in follicular dendritic cells (FDC) of gastric lymphoid tissues in patients with gastric MALT lymphoma (Kobayashi et al., 1998), and antibodies to human hsp60 have been detected in patients with MALT lymphoma (Kawahara et al., 1999). These results indicate that hsp60 may be associated with the host immune reaction in cases of *H. pylori* infection and, more specifically, with B-cell proliferation in the gastric mucosa of patients with MALT lymphoma. Some reports have indicated that the proliferation and differentiation of MALT lymphoma tumour cells depends on *H. pylori*-antigen-specific T cells, as well as on the co-stimulatory signal of the CD40 ligand (CD40L) on CD4+ helper T cells and on the cytokines associated with the T helper 2 (Th-2)-type response (Greiner et al., 1998; Hussell et al., 1993, 1996; Knorr et al., 1999).

Development of DNA microarrays or DNA chips has...
revolutionized the analysis of gene expression profiles. A DNA microarray can contain thousands of cDNAs or oligonucleotides and, with a single hybridization step, allows systematic comparison of the expression of corresponding genes among samples. The use of DNA microarrays has already allowed the identification of candidate mammalian genes related to carcinogenesis and cell differentiation (Duggan et al., 1999; Khan et al., 1999; Wang et al., 1999).

In the present study, we produced \textit{H. pylori} hsp60 as a glutathione S-transferase (GST)-fusion protein to investigate the immunological roles of hsp60 as a bacterial antigen. Peripheral blood mononuclear cells (PBMCs) were antigen-specific dendritic cells (DC) induced by hsp60 stimulation in the presence of cytokines [granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL4]. We measured the expression of CD40L on CD4+ cells and the production of Th-1 (IFN-γ) and Th-2 (IL4) cytokines from PBMCs induced by hsp60-driven immune responses via antigen-presenting cells (APC). To detect genes associated with the immune reaction in cases of MALT lymphoma, we also analysed mRNA expression using a cDNA microarray.

**METHODS**

**Patients.** PBMCs were obtained from 24 \textit{H. pylori}-positive patients, including 11 patients with low-grade MALT lymphoma (mean age 57 years; range 43–77 years) and 13 patients with gastritis (mean age 51; range 43–65). PBMCs were also obtained from 11 \textit{H. pylori}-negative healthy volunteers (mean age 45; range 29–55). For the microarray range 48–72) and five with gastritis (mean age 42; range 30–56). The five patients with gastritis showed high antibody titres without clinical symptoms. Informed consent was obtained from each of the patients and healthy volunteers.

Diagnosis was based on findings obtained upon endoscopic examination and on histological examination of biopsied gastric specimens. Diagnosis of low-grade MALT lymphoma was confirmed according to the criteria of the revised European–American lymphoma classification (Harris et al., 1994) and was based on the histopathology. Tissue specimens were examined by haematoxylin–eosin stain and immunohistochemistry using antibodies to CD3, CD79a (Dako) and cytokeratin AE1/AE3 (Boehringer Mannheim). All MALT lymphomas showed diffuse neoplastic infiltrates of centrocyte-like cells, lymphoepithelial lesions and an immunophenotype compatible with that of MALT lymphoma. Positive infection with \textit{H. pylori} was documented by culture, histopathology and the presence of serum antibodies to \textit{H. pylori}, as confirmed by ELISA. Negative \textit{H. pylori} infection of healthy controls was defined by seronegativity to \textit{H. pylori}.

**Cloning, expression and purification of recombinant protein.** The amino acid sequence of \textit{H. pylori} hsp60 was analysed by Genetix software and searched for T-cell epitopes and homology to human hsp60 (Fig. 1). Whole hsp60 (rHSPw) and two partial domains (rHSP2 and rHSP4-5) were selected. Recombinant proteins were expressed as GST-fusion proteins by a PCR cloning method. PCR primers were designed based on sequences published in GenBank. To facilitate cloning, restriction endonuclease cleavage sites were included in the primers. PCR primers 5’-GGAGGATCCCATGACGTTAGCCAAAGAGAAATAAAATTT and 5’-GGGCTCGAGCTAGCCATATGCCCTAATCC (rHSPw), 5’-TTTGGATCCGCCGTGAGTGACTCTTACATC and 5’-TTTGGATCCGAGAGGGTCGAGCTTCACTAA (rHSP2) and 5’-GTGCAGTCCTCACCGAAGATTTGGCTG and 5’-TTTCCTGAGCCACTTGGATCCGCTTGTAAC (rHSP4-5) were used to amplify parts of the ORF from purified \textit{H. pylori} ATCC 43504T genomic DNA. The PCR was performed in a 20 μl volume containing ExTaq polymerase (Takara), 5 mM sense and anti-sense oligonucleotide primers and 500 ng \textit{H. pylori} genomic DNA. The cycling conditions were 25

![Fig. 1. Expression design of recombinant hsp60. Amino acid sequences of \textit{H. pylori} and human hsp60 were analysed by Genetix software. Whole hsp60 and two partial regions of \textit{H. pylori} hsp60 were expressed as GST-fusion proteins and were designated rHSPw, rHSP2 and rHSP4-5. rHSPw contained the whole hsp60 (Met1–Met545) coding region, rHSP2 (Glu101–Ser200) contained a domain of the T-cell epitope cluster (Lys356–Thr372) and rHSP4-5 (Arg383–Gly435) contained a domain of the T-cell epitope cluster (Asp396–Gly412) and an upstream region (Ser352–Arg393) common to \textit{H. pylori} and human hsp60. Anti-human hsp60 mAb LK-2 reacts with both \textit{H. pylori} and human hsp60 (Kawahara et al., 1999). The epitope recognized by LK-2 is located between residues 383 and 419 of human hsp60 (corresponds to residues Gly356 to Gly392 of \textit{H. pylori} hsp60; shown by an arrow) (reported by Kobayashi et al., 1998). Asterisks (*) indicate residues that are identical in the human sequence. Boxes indicate T-cell epitopes.](https://example.com/)
cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. The amplicon was cut with BanHI and SalI restriction endonucleases and cloned into the vector pGEX-5X-3 (Amersham Pharmacia) by standard techniques; the resultant plasmid was transformed into Escherichia coli DH-5α. Recombinant protein was expressed at 25 °C in LB broth containing 2 % glucose and ampicillin (100 μg ml⁻¹) at an OD₆₀₀ of 0.6–0.8 and the mixture was incubated at 37 °C for 3 h. The culture was induced with 0-1 mM IPTG. Cells were harvested by centrifugation at 7000 g for 10 min and resuspended in ice-cold PBS. The bacterial suspension was frozen at −70 °C and thawed and cells were disrupted by sonication on ice for 2 min using a probe sonicator (Astrason) set to full power. Soluble fusion proteins expressed by the GST–hsp clones (rHSPw, rHSP2 and rHSP4-5) were cleaved with thrombin and purified by glutathione–Sepharose 4B (Amersham Pharmacia) affinity chromatography according to the manufacturer’s instructions.

**Culture of PBMCs.** PBMCs (1 × 10⁶ cells ml⁻¹) from patients and healthy volunteers were cultured in RPMI 1640 with 10 % fetal calf serum in flat-bottomed 24-well plates for 1 week in a 5 % CO₂ incubator. To provide a clear antigen-specific immune response to hsp60, the PBMCs were induced as APC stimulated by cytokines and recombinant hsp60s (Chen et al., 1998; Tsai et al., 1997; van den Biggaal et al., 2000). Cytokines were added at the onset of culture at the following final concentrations: 150 pg IL4 ml⁻¹ (R&D Systems) and 400 pg GM-CSF ml⁻¹ (Chemicon International). The initial amount of IL4 added (150 pg ml⁻¹) decreased and was undetectable within 72 h (data not shown). Recombinant H. pylori hsp60s (rHSP2, rHSP4-5 and rHSPw) and H. pylori lysate were then added to the culture at final concentrations of 10 μg ml⁻¹. To assess the proliferation activity of rHSPs, PBMCs from four normal healthy volunteers were stimulated with rHSP2, rHSP4-5, rHSPw and H. pylori cell lysate. Proliferation was assayed by a CellTitre (Promega).

**Flow cytometric analysis.** Cultured PBMCs were collected and incubated for 30 min at 4 °C with directly conjugated antibodies using both anti-CD4 (FITC-conjugated) and anti-CD40L (RPE-conjugated) (Dako). Cells were washed with PBS and fixed with 1 % paraformaldehyde in PBS. Expression of CD40L on CD4⁺ cells was analysed by FACS Calibur (Becton Dickinson). Each measurement analysed a sample containing 10 000 CD4⁺ cells.

**ELISA for cytokines.** After 1 week, levels of IL4 and IFN-γ in culture supernatants were measured using ELISA (BioSource International) following the manufacturer’s instructions.

**RNA preparation and cDNA microarray analysis.** For microarray analysis, PBMCs obtained from patients with MALT lymphoma and gastritis were cultured with rHSPw and GM-CSF and IL4 for 1 week. Cultured PBMCs were collected by centrifugation. Total RNA was extracted from the PBMC samples and amplified by the modified method of van Gelder et al. (1990). To obtain the expression profiles of lymphocytes from patients with MALT lymphoma and gastritis, biotin-labelled cRNA was synthesized from sample RNA (2 μg) using the ExpressionChip labelling system (Mergen). Samples were then allowed to hybridize with an oligonucleotide microarray (H02; Mergen) that contained oligonucleotides based on 1100 genes that primarily encoded cell-surface antigens, cytokines, growth factors, cell differentiation factors, extracellular matrices, interferons, membrane proteins and oncogenes (H02 ExpressionChip database; http://www.mergen-ltd.com/H02/H02finder.asp). Hybridized slides were then incubated with streptavidin, anti-streptavidin primary antibody and Cy3-conjugated secondary antibody (Mergen); incubation proceeded according to the manufacturer’s instructions. Detection of signals and statistical analysis of digitized data were respectively carried out with a GMS 418 array scanner (Affymetrix) and GeneSpring 3 2 2 software (Silicon Genetics). Some of the data were saved in a Microsoft Excel file and were analysed using StatView-J 4 11.

**Statistical analysis.** Statistical analysis was performed using Student’s unpaired t-test.

**RESULTS**

**Proliferation by rHSP60**

PBMCs from four healthy volunteers were stimulated with rHSP60 proteins and H. pylori cell lysate. Proliferation of PBMCs was effectively induced by rHSP2 and rHSPw (Fig. 2). However, only high concentrations of rHSP4-5 caused proliferation of PBMCs. H. pylori lysate did not induce proliferation of PBMCs.

**CD40L expression**

Expression of CD40L on CD4⁺ cells was detected by flow cytometry. PBMCs stimulated with cytokines and additional hsp60 antigens showed higher expression of CD40L in patients with MALT lymphoma. rHSP2 and rHSP4-5 significantly enhanced CD40L expression in patients with MALT lymphoma in comparison with healthy volunteers (Table 1). High percentages of CD40L-positive cells were observed after stimulation with cytokines and co-stimulation with hsp60 antigens from patients with MALT lymphoma; however, such stimulation did not induce CD40L-positive cells in patients with gastritis or in healthy volunteers.

**Cytokine production**

IL4 production by PBMCs was low in cells from patients with gastritis and in healthy controls; almost all cases examined remained under the detection limit (< 2·0 pg ml⁻¹). Cytokine and antigen stimulation induced high IL4 production

![Fig. 2.](http://jmm.sgmjournals.org)
from PBMCs of patients with MALT lymphoma. IL4 levels stimulated by cytokines and antigens in those with MALT lymphoma were significantly higher than levels in both healthy volunteers and gastritis patients (Fig. 3). Co-stimulation with hsp60 (rHSP2, rHSP4-5 and rHSPw) antigens did not enhance IL4 production. On the other hand, only *H. pylori* lysate stimulation effectively induced IL4 from PBMCs.

Levels of IFN-γ in different diseases were compared (Fig. 4). IFN-γ levels in gastritis were higher than those in MALT lymphoma. Specifically, significant production of IFN-γ was induced in PBMCs from patients with gastritis that had been stimulated by cytokine and rHSP2. Co-stimulation with rHSPs did not enhance IFN-γ production from PBMCs of patients with MALT lymphoma. On the other hand, co-stimulation with rHSPs enhanced IFN-γ production in PBMCs of patients with gastritis as well as in those of healthy volunteers.

### Up- or downregulation of gene expression in patients with MALT lymphoma analysed by microarray

mRNAs showing increased expression in patients with MALT lymphoma (significance, *P* < 0.01) following rHSPw stimulation are shown in Table 2. Expression of a member of the tumour necrosis factor (TNF) receptor family (the mRNA of TNFRSF1A) was increased in PBMCs from patients with MALT lymphoma. The levels of expression of mRNAs of members of the TNF family are shown in Fig. 5. TNF and lymphotoxin β mRNA expression was increased in patients with MALT lymphoma. Some Th-1- and Th-2-associated genes are shown in Fig. 6. Expression of IL4 mRNA in MALT lymphoma cases was higher than that of gastritis cases. mRNAs showing decreased expression in MALT lymphoma (significance, *P* < 0.01) are shown in Table 3.

### Table 1. Percentage of CD40L-positive cells in CD4⁺ PBMCs under various stimulation regimes

<table>
<thead>
<tr>
<th>Disease (n)</th>
<th>Control</th>
<th>Cytokines</th>
<th>Cytokines plus:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>rHSP2</td>
<td>rHSP4-5</td>
</tr>
<tr>
<td>MALT lymphoma</td>
<td>11-62 ± 3.39</td>
<td>14-46 ± 2.82*</td>
<td>15-68 ± 2.96*</td>
</tr>
<tr>
<td>Gastritis</td>
<td>10-86 ± 1.44</td>
<td>8-92 ± 1.19</td>
<td>11-36 ± 0.89</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>8-78 ± 1.65</td>
<td>8-21 ± 1.80</td>
<td>9-93 ± 1.21</td>
</tr>
</tbody>
</table>

*Statistically significantly different (*P* < 0.05) compared with equivalent stimulation of the healthy control group.

*Fig. 3.* Production of IL4 from PBMCs. PBMCs (1 × 10⁶ cells ml⁻¹) from patients and healthy volunteers were cultured in RPMI 1640 with added IL4 and GM-CSF. Recombinant *H. pylori* hsp60 (rHSP2, rHSP4-5 and rHSPw) and *H. pylori* lysate were then added to the culture at a final concentration of 10 μg ml⁻¹. The concentration of IL4 in culture supernatants of the PBMCs after 1 week was assayed by ELISA. IL4 production associated with gastritis and healthy volunteers was low (generally below the detection limit). IL4 production under various conditions of stimulation of MALT lymphoma cells (filled bars) was higher. Statistical significance: **, *P* < 0.05; *, *P* < 0.01.

*Fig. 4.* Production of IFN-γ from PBMCs. rHSP2 significantly induced IFN-γ production in patients with gastritis. IFN-γ production in MALT lymphoma (filled bars), gastritis (shaded bars) and healthy volunteers (open bars) was analysed with various stimulations. rHSP stimulation with cytokines significantly enhanced IFN-γ production in gastritis and healthy volunteers. Statistical significance: **, *P* < 0.01.
DISCUSSION

A large number of tumour-infiltrating T cells have been recognized in MALT lymphoma tissues (Lederman et al., 1992), and proliferation of low-grade MALT lymphoma is dependent on \textit{H. pylori}-specific tumour-infiltrating T cells rather than on \textit{H. pylori} itself (Hussell et al., 1993, 1996).

CD40L is a co-stimulatory molecule that is expressed on activated CD4$^+$ T cells and induces B-cell activation and differentiation by binding to CD40 on B cells (Lederman et al., 1992). It has been reported that tumour B cells of MALT lymphoma patients are positive for CD40 and that tumour-infiltrating T cells are positive for CD40L (Greiner et al., 1998; Koulis et al., 1997). The growth of MALT lymphoma requires CD40-mediated signalling by T/B cell interaction (Knorr et al., 1999). In the present study, low levels of CD40L expression were increased by rHSP60 stimulation in CD4$^+$ cells from patients with MALT lymphoma; however, rHSP2 and rHSP4-5 enhanced CD40L expression in the presence of cytokines. rHSPw and rHSP2 proliferated PBMCs from healthy volunteers; however, rHSP4-5 did not (Fig. 2).

rHSP4-5 contains amino acid sequences that are identical in \textit{H. pylori} and humans that are recognized by an anti-human hsp60 mAb (LK-2) (Fig. 1). Stimulation with hsp60, which has T-cell epitopes common to the human antigen, may not induce CD40L expression in normal immune reactions, but could have induced expression of CD40L in

| Table 2. Genes induced in MALT lymphoma following rHSPw stimulation |
|-------------------------|-----------------|-----------------|
| Gene                   | Accession no.   | Intensity (mean ± SD) |
|                        |                 | MALT lymphoma | Gastritis |
| HLA-DR α-chain         | M60334          | 4.20 ± 2.32    | 0.42 ± 0.10 |
| Granzyme B             | J04071          | 16.48 ± 7.96   | 2.06 ± 0.71 |
| Integrin β2            | M62880          | 13.61 ± 5.65   | 2.12 ± 0.42 |
| Apolipoprotein B        | J02610          | 1.61 ± 0.55    | 0.49 ± 0.17 |
| Regulator of G-protein signalling I | X73427 | 0.24 ± 0.07    | 0.03 ± 0.15 |
| TNFRSF1A               | L41690          | 24.63 ± 8.51   | 3.36 ± 0.85 |

**Fig. 5.** mRNA expression of the TNF family analysed by microarray. GenBank accession numbers of TNF, lymphotoxin α and lymphotoxin β sequences were respectively X01394, D12614 and L11015. The levels of mRNA of TNF and lymphotoxin-β in MALT lymphoma cases (filled bars) were significantly increased compared with gastritis cases (open bars). Statistical significance: *, P < 0.05.

**Fig. 6.** mRNA expression of Th-1- and Th-2-associated genes analysed by microarray. Th-2-associated IL4 mRNA (accession no. M13982) was increased in patients with MALT lymphoma (filled bars), but was not significantly greater than that in gastritis patients (open bars). IL10 mRNA (accession no. M57627) in gastritis samples was significantly increased compared with MALT lymphoma samples. IL12A mRNA (p35 gene; accession no. M65291) was significantly increased in gastritis. mRNA expression of IL2 (accession no. V00564) was not different in MALT lymphoma and gastritis. Statistical significance: *, P < 0.05.
lymphocytes from patients with MALT lymphoma, resulting in B-cell proliferation and autoimmunity against hsp60 in MALT lymphoma.

Growth of MALT lymphoma also requires Th-2-type cytokines (Greiner et al., 1998). MALT lymphoma-like lesions appeared in the gastric mucosa after long-term *Helicobacter felis* infection in aged BALB/c mice, which are genetically prone to high production of Th-2-type cytokines and the B cell response (Enno et al., 1995). High levels of Th-2-type cytokines, but only low levels of Th-1-type cytokines, were recognized in MALT lymphoma tissues in vivo (Knorr et al., 1999). In contrast, strong Th-1-type cytokine production and relatively low levels of Th-2-type cytokine production were observed in tumour-infiltrating T cells from two patients with gastric MALT lymphoma after *H. pylori* stimulation in vitro (Hauer et al., 1999). Our study revealed that PBMCs from patients with gastric MALT lymphoma produced high levels of IL4, but only low levels of IFN-γ. Induction with cytokines (GM-CSF and IL4) induced a high level of IL4 production, indicating that patients with MALT lymphoma are highly sensitive to these cytokines as a host factor. IL4 production by antigen stimulation with hsp60 was limited; however, *H. pylori* lysate stimulation significantly enhanced IL4 secretion. The Th-2 response in *H. pylori*-associated MALT lymphoma may depend primarily on host factors and may be enhanced by certain *H. pylori* antigens.

The local cytokine response in *H. pylori*-infected patients is upregulated in the Th-1-type, since IFN-γ but not IL4 is increased (Ahlstedt et al., 1999; Lindholm et al., 1998). *H. pylori*-infected IFN-γ gene-knockout mice showed no inflammatory symptoms and *H. pylori*-infected IL4 gene-knockout mice showed severe gastritis (Smythies et al., 2000). Expression of class II major histocompatibility complex (MHC) on gastric epithelial cells was induced by *H. pylori* infection, and IFN-γ also induced class II MHC expression (Engstrand et al., 1989). These observations indicate that IFN-γ might play an important role in induction of the type of gastric inflammation caused by *H. pylori*. In addition, our data suggest that hsp60 antigens induced IFN-γ production from lymphocytes of gastritis patients and healthy persons. rHSP2 was the most effective at inducing IFN-γ production in patients with gastritis. Recently, some reports have indicated that hsp60 is associated with activation of the NF-κB signalling pathway via toll-like receptor 2 (TLR2) and TLR4 (Ohashi et al., 2000; Vendruscolo et al., 2001). These results indicate that hsp60 may play important roles in *H. pylori*-associated inflammation.

Levels of some mRNAs were increased by stimulation with hsp60 in patients with MALT lymphoma. HLA class II expression is necessary for lymphocyte (T cell) proliferation. PBMCs stimulated by rHSPw showed enhanced mRNA for HLA-DR (α-chain) in cases of MALT lymphoma. This may be one of the reasons why anti-hsp60 antibody production occurs in patients with MALT lymphoma. Lymphocyte-homing receptors (α7β1 integrin) are closely associated with secondary intestinal spread in patients with MALT lymphoma (Dogan et al., 1997). Interestingly, hsp60 stimulation induced β7 integrin in PBMC mRNA from patients with MALT lymphoma. Intraepithelial and lamina propria lymphocytes in *H. pylori*-positive gastric mucosa expressed granzyme B at high levels (Oberhuber et al., 1998). The function of granzyme B involves CD8+ T-cell differentiation, specialization and regulation (Sandberg et al., 2001). Therefore, granzyme B function in patients with MALT lymphoma may be of particular importance; however, the details of this association remain unclear. The levels of expression of TNF mRNA and lymphotoxin-β mRNA in MALT lymphoma cases were significantly increased (P < 0.05) compared with those of gastritis cases (Fig. 6). Both lymphotoxin and TNF

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession no.</th>
<th>Intensity (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proprotein convertase subtilisin/kexin 5</td>
<td>U49114</td>
<td>0.03 ± 0.08</td>
</tr>
<tr>
<td>Apoptosis inhibitor 4 (survivin)</td>
<td>U75285</td>
<td>0.10 ± 0.05</td>
</tr>
<tr>
<td>Bullous pemphigoid antigen 1 (230/240 kDa)</td>
<td>M69225</td>
<td>0.01 ± 0.02</td>
</tr>
<tr>
<td>Aquaporin 6, kidney-specific</td>
<td>U48408</td>
<td>0.05 ± 0.06</td>
</tr>
<tr>
<td>IL13</td>
<td>L06801</td>
<td>0.03 ± 0.04</td>
</tr>
<tr>
<td>Regulator of G-protein signalling</td>
<td>AF030107</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>Proprotein convertase subtilisin/kexin 7</td>
<td>U33849</td>
<td>0.03 ± 0.05</td>
</tr>
<tr>
<td>Chloride channel 4</td>
<td>X77197</td>
<td>0.09 ± 0.15</td>
</tr>
<tr>
<td>TNF receptor superfamily, member</td>
<td>M33294</td>
<td>0.34 ± 0.13</td>
</tr>
<tr>
<td>RAB4 (member of RAS oncogene family)</td>
<td>NM004578</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>G antigen 7</td>
<td>AF058988</td>
<td>0.03 ± 0.05</td>
</tr>
<tr>
<td>Lymphocyte antigen 77</td>
<td>U19271</td>
<td>1.39 ± 0.36</td>
</tr>
<tr>
<td>Glucan (1,4-α), branching enzyme 1</td>
<td>L07956</td>
<td>2.16 ± 0.73</td>
</tr>
<tr>
<td>Cystatin A (stefin A)</td>
<td>X05978</td>
<td>4.75 ± 1.29</td>
</tr>
</tbody>
</table>
are associated with cell death; therefore, these molecules may play important roles in mucosal injury or regulation of immunological reaction in patients with MALT lymphoma.

In contrast, expression of several genes was increased by stimulation with hsp60 in patients with gastritis. Survivin is an anti-apoptosis gene found in half of all high-grade non-Hodgkin’s lymphoma cases, but it is not found in low-grade lymphoma cases (Ambrosini et al., 1997, 1998). A decrease in mRNA expression may be one of the features of MALT lymphoma. Cystatin A mRNA was augmented in controls. The cystatin family consists of endogenous cysteine protease inhibitors associated with antigen presentation (Katunuma et al., 1994). The role of cystatin A in H. pylori infection is still unclear; however, it is possible that low expression of cystatin A in MALT lymphoma cases may be associated with production of anti-hsp60 auto-antibodies via APCs. Lymphocyte antigen 75 has also been associated with antigen processing as an endocytic receptor (Jiang et al., 1995). Expression of antigen-processing-associated genes differs between gastritis and MALT lymphoma cases, and these genes may be involved in the development of MALT lymphoma.

A small increase in IL13 mRNA expression was observed in control samples (Table 3). IL13 and IL4 function (i.e. B-cell activation) is similar, but their signalling pathways are independent. IL4 production in culture supernatants was increased and mRNA expression was also increased in patients with MALT lymphoma (Figs 3 and 6). IL4 may play an important role in B-cell proliferation in patients with MALT lymphoma. IL12 induces a Th-1 reaction, but IL10 strongly inhibits Th-1 reactions. An increase in both cytokines was also observed in H. pylori-infected gastric mucosa (Hida et al., 1999), suggesting that H. pylori infection, by inducing a predominantly Th-1 reaction, may be controlled by IL10 production in patients with gastritis. IL2 is also a marker for the Th-1 reaction, whereas H. pylori antigen stimulation did not induce IL2 production from PBMCs (Meyer et al., 2000). IL2 may not be involved in the pathogenesis of MALT lymphoma and gastritis cases.

Adaptive immunity induced by H. pylori infection may progress along different pathways in various diseases. Here, bacterial hsp60 generally induced IFN-γ production; it is possible that bacterial hsp60 may sometimes contribute to partial protective immunity in patients with H. pylori infection. As a primary immune reaction, H. pylori infection can induce production of IFN-γ from lymphocytes or epithelial cells (Meyer et al., 2000; Yasumoto et al., 1992). IFN-γ stimulates macrophage activation, which, in turn, activates naïve T cells (Th-0 cells). T-cell responses progress to Th-1 and/or Th-2 differentiation, and adaptive immunity protects against microbial infections (Romagnani, 1996; Wang et al. 2001). During life-long infection with H. pylori, it becomes apparent that many T cells cannot easily be classified into Th-1 and Th-2 subsets in humans. Th-1 dominant immunity can sometimes lead to immune-mediated gastric damage in cases of H. pylori infection. As regards MALT lymphoma, we have previously reported that hsp60 is the immune-dominant antigen, and humoral immunity against hsp60 is strongly induced by H. pylori infection. Normal immunity may not progress to a strong Th-2 reaction because bacterial and human hsp60 share identical amino acid sequences. However, in patients with MALT lymphoma, H. pylori infection induces Th-2-dominant immunity against hsp60, caused by IL4 stimulation and a co-stimulatory signal through CD40L expression. A DNA chip study revealed certain genes that were associated with the immune system pathogenesis of H. pylori infection. Increased levels of mRNAs of HLA-DR and integrin may be associated with T-cell proliferation and lymphocyte infiltration in patients with MALT lymphoma, while granzyme B and the TNF family may possibly be associated with injury of the gastric mucosa. In low-grade MALT lymphoma, adaptive immunity against hsp60 may be enhanced by antigen presentation and by T-cell activation as a host factor, resulting in B-cell proliferation that can be demonstrated during chronic infection. The possibility exists that continuous B-cell proliferation, enhanced by host immune reactions, may lead to the growth of lymphoma cells. Therefore, gastric MALT lymphoma, which is induced by H. pylori infection, may develop by an antigen-driven immune response enhanced by host factors.

In conclusion, hsp60 is an important antigen in H. pylori infection. The antigen-driven immune response to hsp60 was closely associated with the pathogenesis of MALT lymphoma. Genes that are induced or reduced through stimulation by hsp60 may become useful for diagnosis of H. pylori-associated MALT lymphoma. Furthermore, we are continuing to study hsp60 and host immunity in H. pylori infection.

ACKNOWLEDGEMENTS
This work was supported by grant number 12670256 of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

REFERENCES

http://jmm.sgmjournals.org


