

# Immunogenetic analysis of gastric MALT lymphoma-like lesions induced by *Helicobacter pylori* infection in neonatally thymectomized mice

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**Most gastric mucosa-associated lymphoid tissue (MALT) lymphomas are caused by *Helicobacter pylori* (*H. pylori*) infection. We previously reported that acquired lymphoid follicles with germinal centers were induced by *H. pylori* infection in neonatally thymectomized (nTx) mice. In the present study, we developed gastric MALT lymphoma-like lesions in nTx mice by long-term *H. pylori* infection, and performed immunogenetic analyses. BALB/c mice were thymectomized on the 3rd day after birth. At 6 weeks of age, mice were orally infected with 10<sup>8</sup> *H. pylori* and serially killed 2, 4, 6, and 12 months later. Normal BALB/c and noninfected nTx mice served as controls. Follicle formation occurred after 2 months of *H. pylori* infection in the nTx mice. Follicle formation and infiltration of intraepithelial lymphocytes progressed in a time-dependent manner. Lymphoepithelial lesions, a characteristic feature of MALT lymphoma, also occurred in a time-dependent manner (100% at 12 months). Serum immunoelectrophoresis revealed a monoclonal band (M-protein) in 30% (3/10) of mice 6 months after infection. M-protein-positive mice had amplification of one or two IgM and/or IgG heavy-chain genes in the gastric B lymphocytes, as determined with polymerase chain reaction, suggesting mono- or oligoclonality. Overexpression of Bcl-X<sub>L</sub> protein was immunohistologically observed in the infiltrating B lymphocytes and in some follicular B lymphocytes in 80% (8/10) of the cases at 12 months. Thus, *H. pylori* infection is involved in the development of gastric MALT lymphoma-like lesions in nTx mice. Our mouse model is useful for clarifying the pathogenetic mechanism of gastric MALT lymphoma by *H. pylori* infection.**

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Accumulating evidence indicates that *Helicobacter pylori* (*H. pylori*) infection is involved in the pathogenesis of not only gastritis, peptic ulcers, and gastric cancer but also mucosa-associated lymphoid tissue (MALT)-type gastric B-cell lymphoma, so-called MALT lymphoma.<sup>1–4</sup> Immunologic interactions between the host and *H. pylori* seem to have critical roles in the development of follicular gastritis, which appears to be an important condition preceding gastric MALT lymphoma.<sup>5</sup> Recently,

an API2-MALT1 fusion transcript derived from t(11;18) was reported to be involved in the pathogenesis of *H. pylori*-uninfected gastric MALT lymphoma.<sup>6,7</sup> On the other hand, the pathogenesis of *H. pylori*-positive gastric MALT lymphoma without t(11;18) remains unclear.

BALB/c mice thymectomized on the 3rd day after birth spontaneously develop autoimmune gastritis. In neonatally thymectomized (nTx) BALB/c mice, which have a Th1-predominant reaction in the stomach, CD4<sup>+</sup> Th1 cells induce autoimmune gastritis, whereas lymphoid follicles never occur.<sup>8–11</sup> In previous studies, we demonstrated that infection of BALB/c nTx mice with mouse-adapted *H. pylori* permitted successful colonization of *H. pylori* in the corpus, leading to the formation of follicular gastritis 2 months after infection in association with

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appearance of a Th2-type reaction.<sup>5,12</sup> In the present study, therefore, we extended our previous findings and attempted to develop gastric MALT lymphoma-like lesions in nTx mice with long-term *H. pylori* infection, and performed immunogenetic analyses.

## Materials and methods

### Mice and Thymectomy

Male and female BALB/c Cr.Slc mice (Japan SLC, Shizuoka, Japan) were bred in our animal facility in Kyoto University under specific pathogen-free conditions. Mice were offered commercially available food pellets (F2; Funabashi Farm, Inc., Chiba, Japan) and drinking tap water. Neonatal thymectomy was performed on the 3rd day after birth under ether anesthesia as described previously.<sup>8–11</sup> We previously confirmed that there were no immunologic differences between male and female mice.<sup>8–11</sup> The mice were divided into four groups ( $n = 10$  in each group): (i) normal (non-nTx) mice without *H. pylori* infection, (ii) normal (non-nTx) mice with *H. pylori* infection, (iii) nTx mice without *H. pylori* infection, and (iv) nTx mice with *H. pylori* infection.

### Bacteria and Infection

*H. pylori* (TN2GF4) isolated from a patient with a duodenal ulcer was donated from M Nakao (Pharmaceutical Research Division, Takeda Chemical Industries, Ltd, Osaka, Japan). It was maintained in blood agar base no. 2 with horse serum (5%, vol/vol) containing amphotericin B at 2.5 mg/l, trimethoprim at 5 mg/l, polymyxin B at 1250 IU/l, and vancomycin at 10 mg/l. The plates were incubated in a microaerophilic atmosphere at 37°C for 48 h. The inoculated *H. pylori* strain, TN2GF4, was CagA and VacA positive as described elsewhere.<sup>13</sup> Both nTx and non-nTx mice at 6 weeks of age were either orally infected with  $10^8$  *H. pylori* organisms or given saline as described previously.<sup>12</sup> Mice were infected with *H. pylori* in an infection-controlled animal facility of Nippon Shinyaku Co. (Kyoto, Japan). The experiments were approved by the animal research committee of Kyoto University. Infected mice were killed 2, 4, 6, and 12 months later. Colonization of *H. pylori* was confirmed by PCR Southern blot analysis for the urease gene using DNA extracted from the corpus mucosa. The urease gene was amplified at all time points after infection.<sup>12</sup> Non-infected mice were killed at the same time. The sera and stomachs were stored at -80°C until use. We confirmed the abnormal hyperimmune status of nTx mice by measuring the serum autoantibody levels against the parietal cells by enzyme-linked immunosorbent assay immediately before infection (6 weeks of age) as described previously.<sup>12</sup>

### Histology and Immunohistochemistry

The stomach was removed from each mouse, fixed with 4% phosphate-buffered formaldehyde (pH 7.2), and prepared for histologic examination. The sections were stained with hematoxylin and eosin. Immunohistochemical staining with monoclonal antibodies (MAbs) was performed on freshly frozen sections using the avidin-biotin immunoperoxidase method as described previously.<sup>12</sup> Briefly, freshly frozen sections were fixed in acetone for 10 min, rinsed in phosphate-buffered saline (pH 7.2), and incubated with 10% normal rat or horse serum (as a blocking agent) for 20 min. The sections were incubated for 1 h with the primary MAbs, biotin-conjugated rat anti-mouse B220, CD4, CD8, and mouse anti-mouse Bcl-X MAbs (Becton & Dickinson, Franklin Lakes, NJ, USA). For staining with anti-Bcl-X MAb, we used a commercially available mouse immunoglobulin detection kit as the secondary antibody (Ab) (VECTOR MOM Immunodetection Kit, Vector Laboratories, Inc., Burlingame, CA, USA) to detect mouse primary Ab on mouse tissues. After incubation with the primary and secondary Abs, the sections were washed with phosphate-buffered saline and then incubated with avidin-biotin peroxidase complex (Vector Laboratories, Inc.) for 30 min. Sections washed with phosphate-buffered saline were reacted with a fresh mixture of 0.05% 3,3'-diaminobenzidine and 0.005% H<sub>2</sub>O<sub>2</sub> in Tris buffer (0.05 M, pH 7.6) and then washed with distilled water. The control samples were exposed to normal rat or mouse serum instead of the primary Abs. These control samples showed no staining. Nuclei were counterstained with methylgreen-pyronin staining.

### Serum Immunoelectrophoresis

To identify the monoclonality of serum immunoglobulin, immunoelectrophoresis was performed in each group 6 months after infection according to the method of Scheidegger.<sup>14</sup> It was performed in slab gels prepared using a mixture of agar (8 g/l) and agarose (4 g/l) in barbital buffer (0.06 M, pH 8.6) with antiserum to whole mouse serum (Dainippon Pharmaceutical Co., Ltd, Osaka, Japan).

### Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for Immunoglobulin Genes

To identify mono- or oligoclonality of the infiltrating gastric B lymphocytes, we performed RT-PCR for heavy-chain immunoglobulin genes using a commercially available kit (Ig-Prime Kits, Novagen, Madison, WI, USA) according to the manufacturer's instructions. In brief, total RNA was extracted from the murine stomach with the RNA extraction solution (ISOGEN, Nippon Gene, Tokyo, Japan), and then reverse-transcribed into complementary

DNA with the SuperScript Preamplification System (Gibco-BRL, Gaithersburg, MD, USA). The total RNA in the reaction mixture was heated at 42°C for 50 min and at 70°C for another 15 min and then chilled on ice. Six leader sequences were set up at the 5' side of mRNAs that are commonly used to amplify the mouse IgM and IgG heavy chains and two different primers of 3' side specific for IgM and IgG, respectively, were set in the constant region. The primer sequences used in this study are shown in Table 1. PCR was performed with a mixture of cDNAs, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 200 mM of each deoxynucleotide triphosphate (Perkin-Elmer, Branchburg, NJ, USA), 50 pM of each specific primer, and 1.0 U of *Taq* DNA polymerase (AmpliTaQ Gold; Perkin-Elmer). Amplification was performed with a thermal cycler (GeneAmp PCR System 9600R; Perkin-Elmer) for 35 cycles of 20 s at 95°C, 1 min at 55°C, and 1 min at 72°C. The final cycle induced an extension step for 10 min at 72°C. A 10-μl aliquot of each PCR product was electrophoresed on a 2.0% agarose gel containing ethidium bromide.

## Results

### Histopathology and Immunohistochemistry (Table 2)

Approximately 60% of the nTx mice developed severe inflammation in the gastric body, with no or weak inflammation in the antral mucosa at 6 weeks of age. nTx mice that developed inflammation were used for the following experiments.

Mononuclear cells infiltrated the lamina propria of the corpus mucosa 6 weeks after nTx in association with the production of autoantibodies against parietal cells (data not shown). As the inflammatory cells expanded along the glands, destruction of parietal cells and chief cells became prominent, resulting in glandular atrophy. There were no germinal centers observed, however, and the diffusely or massively infiltrating cells consisted mainly of lymphocytes and a few other mononuclear cells, including plasma cells and macrophages. These pathologic features remained unchanged throughout the experiment (Figure 1a). These observations are consistent with previous reports.<sup>8-12</sup>

After *H. pylori* infection, the gastric mucosa of the nTx mice was not significantly altered for the first 2 weeks. At 1 month (10 weeks of age) after infection,

**Table 2** Lymphoid follicles, LELs, and Bcl-X<sub>L</sub> expression in the stomach of *H. pylori*-infected nTx Mice (*n* = 10)

	2M	4M	6M	12M
Follicles <sup>a</sup>	6(60)	4(40)	8(80)	9(90)
LELs <sup>a</sup>	6(60)	8(80)	9(90)	10(100)
Bcl-X <sub>L</sub> <sup>b</sup>	0(0)	0(0)	2(20)	8(80)

<sup>a</sup>Follicles and LELs were examined histologically.

<sup>b</sup>Bcl-X<sub>L</sub> expression in B lymphocytes was observed immunohistologically.

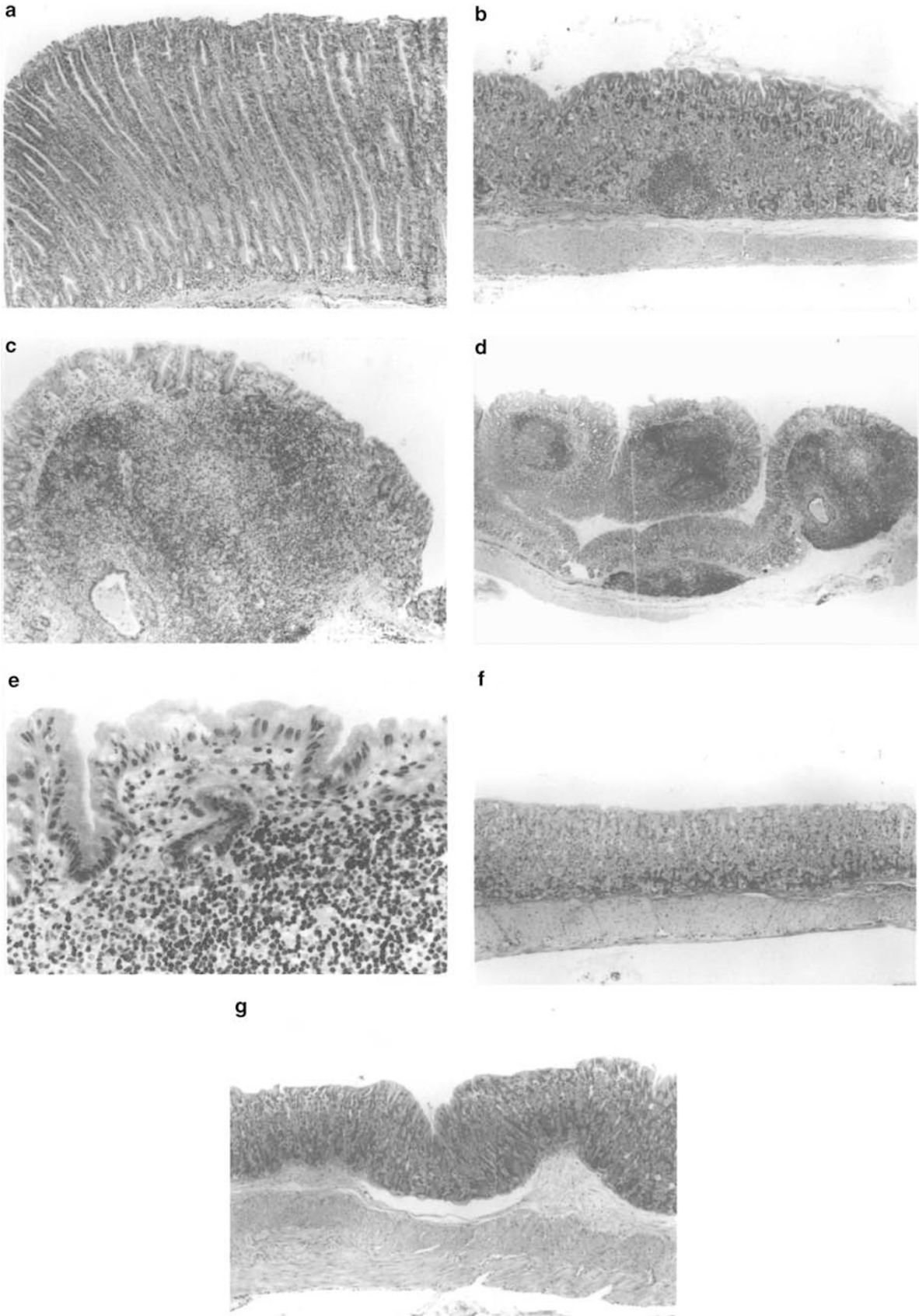
LEL: lymphoepithelial lesion; M: months after *H. pylori* infection; nTx: neonatally thymectomized.

Numbers of positive cases are shown. In all, 10 mice were studied for each time point. Values in parenthesis demonstrate percentages of positive cases.

**Table 1** RT-PCR primers for heavy-chain immunoglobulin genes

Name	Sequence (5'-3')
MuIgV <sub>H</sub> 5'-A	GGGAATTCATGRASTTSKGGYTMARCTKGRITTT
MuIgV <sub>H</sub> 5'-B	GGGAATTCATGRAATGSASCTGGGTYWTYCTCTT ACTAGTCGACATGGACTCCAGGCTCAATTTAGTTTTTCCT
MuIgV <sub>H</sub> 5'-C	ACTAGTCGACATGGCTGTCYTRGBGCTGYTCYTCTG ACTAGTCGACATGGVTTGGSTGTGGAMCTTGCAATTCCT ACTAGTCGACATGAAATGCAGCTGGRTYATSTTCTT
MuIgV <sub>H</sub> 5'-D	ACTAGTCGACATGGRCAGRCTTACWYTYTCATTCCT ACTAGTCGACATGATGGTGTAAAGTCTTCTGTACCT ACTAGTCGACATGGGATGGAGCTRTATCATSYTCTT
MuIgV <sub>H</sub> 5'-E	ACTAGTCGACATGAAGWTGTGGBTAACTGGRT ACTAGTCGACATGGRTGGASCKKIRTCITTTMTCT ACTAGTCGACATGAACTTYGGGYTSAGMITTGRITTT
MuIgV <sub>H</sub> 5'-F	ACTAGTCGACATGTACTTGGGACTGAGCTGTGTAT ACTAGTCGACATGAGAGTGTGATTCITTTTGTG ACTAGTCGACATGGATTTTGGGCTGATTTTTTTTATTG
MuIgMV <sub>H</sub> 3'-1 MuIgGV <sub>H</sub> 3'-2	CCCAAGCTTACGAGGGGGAAGACATTTGGGAA CCCAAGCTTCCAGGRCCARKGGATARACIGRTGG

I: inosine; R: A or G; Y: C or T; K: G or T; S: C or G; M: A or C; W: A or T; B: C or G or T.



however, clusters of mononuclear cells appeared at the bottom of the lamina propria in the gastric mucosa as reported previously.<sup>5</sup> There was a distinct change in lymphoid cell infiltration 2 months after infection (14 weeks of age, Figure 1b); unlike noninfected nTx mice, infected mice had lymphoid follicle development in the corpus mucosa. Follicle formation and infiltration of intraepithelial lymphocytes were enhanced in a time-dependent manner (follicles; 60% (6/10) at 2 months, 40% (4/10) at 4 months, 80% (8/10) at 6 months and 90% (9/10) at 12 months; Figure 1b–d), whereas no follicles formed in noninfected nTx mice throughout the experiment (Figure 1a). Lymphoepithelial lesions (LELs), a characteristic feature of human MALT lymphoma, were also observed in our model mice (LELs; 60% (6/10) at 2 months, 80% (8/10) at 4 months, 90% (9/10) at 6 months, and 100% (10/10) at 12 months; Figure 1e). On the other hand, there was little or no inflammation in the gastric mucosa of normal (non-nTx) mice irrespective of *H. pylori* infection (Figure 1f and g) throughout the experiment.

The phenotypes of the infiltrating mononuclear cells in the body mucosae of nTx mice noninfected or infected with *H. pylori* were analyzed by immunoperoxidase staining for CD4, CD8, and B220. A major portion of the lymphocytes infiltrating the gastric mucosa in both groups of mice for up to 12 months were CD4<sup>+</sup> T cells, whereas a small number of CD8<sup>+</sup> cells appeared in the later stages (data not shown). Although *H. pylori* infection did not significantly alter the distribution pattern of T cells, the most characteristic change was the formation of follicles with B cells (Figure 2a–c).

Staining for Bcl-X<sub>L</sub> protein was also performed in infected and noninfected nTx mice. Bcl-X<sub>L</sub> protein was overexpressed in the cytosol of the infiltrating B lymphocytes and in some follicular B lymphocytes at 6 and 12 months after infection (Bcl-X<sub>L</sub>; 20% (2/10) at 6 months and 80% (8/10) at 12 months; Figure 2d). In contrast, noninfected nTx mice did not show any Bcl-X<sub>L</sub> immunoreactivity (Figure 2e).

**Figure 1** Histology of gastric mucosa of *H. pylori*-infected nTx mice (hematoxylin and eosin staining). **(a)** The body mucosa of nTx mouse without *H. pylori* infection (2 months after saline administration instead of *H. pylori*), showing infiltration of mononuclear cells, gland atrophy characterized by the loss of parietal and chief cells, and hyperplasia of foveolar epithelium. Original magnification, × 100. **(b)** and **(c)** Follicle formation in the body mucosa at 2 and 12 months after *H. pylori* infection in nTx mice, respectively. Original magnification, × 100. **(d)** Massive follicles in the body mucosa at 12 months after *H. pylori* infection in nTx mouse. Original magnification, × 40. **(e)** Massive lymphoepithelial lesions in *H. pylori*-infected nTx mouse (12 months after infection). Original magnification, × 400. **(f)** and **(g)** Gastric mucosa of *H. pylori* noninfected and infected normal (non-nTx) mice (2 months after saline or *H. pylori* administration), respectively. No or very little lymphocyte infiltration was observed in both groups of mice. Original magnification, × 100.

## Immunoelectrophoresis

Serum immunoelectrophoresis revealed a monoclonal band (M-protein) in 30% (3/10) of nTx mice 6 months after infection. These serum M-protein-positive mice certainly indicated gastric MALT lymphoma-like lesions. M-protein in the  $\gamma$ -region was identified to be IgG type (Figure 3).

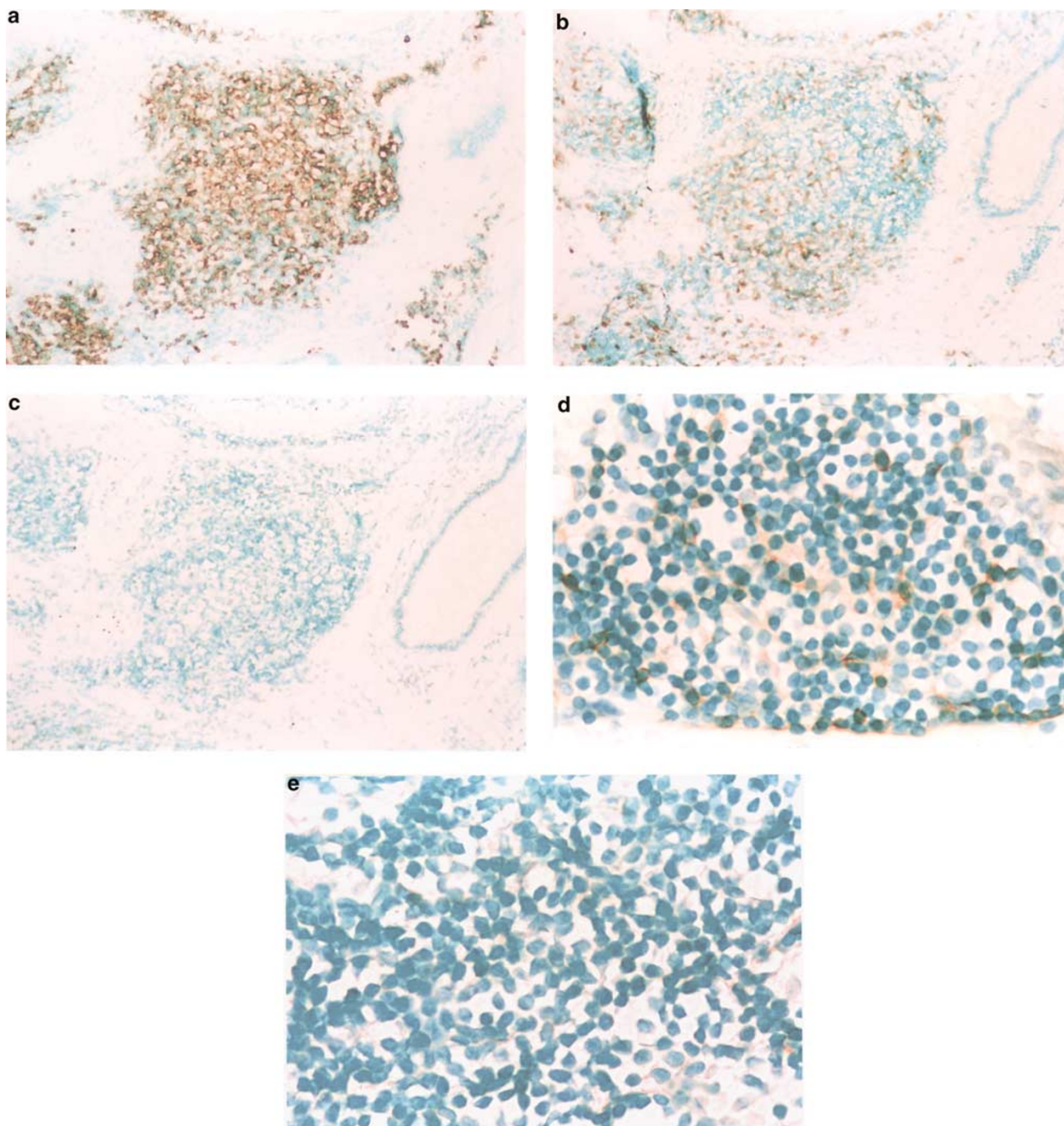
## Detection of Heavy-chain Immunoglobulin Genes by RT-PCR

M-protein-positive nTx mice 6 months after infection had amplification of one or two IgM and/or IgG heavy-chain genes of the gastric B lymphocytes, suggesting mono- or oligoclonality. In contrast, in noninfected nTx mice, all IgM and IgG primers tested were amplified equally, suggesting polyclonality (Figure 4).

## Discussion

Several investigators have attempted to establish animal models of gastric MALT lymphoma induced by *Helicobacter* species. Erdman *et al*<sup>15</sup> reported that *H. mustelae* might induce gastric MALT lymphoma in ferret. Enno *et al*<sup>16</sup> reported that long-term infection with *H. felis* in normal BALB/c mice occasionally induced MALT lymphoma-like lesions without the preceding follicular gastritis.<sup>17</sup> In their animal models, however, it took a long period of time to develop MALT lymphoma-like lesions, and moreover, the efficiency for developing the disease was unsatisfactory. In addition, precise genetic as well as immunologic analysis was not performed in the previous studies. Finally, the development of gastric MALT lymphoma-like lesions by *H. pylori* infection has not been reported. In previous studies, we succeeded in inducing lymphoid follicle formation in association with chronic gastritis by *H. pylori* infection in BALB/c mice that were thymectomized on the 3rd day after birth.<sup>5,12</sup> In the present study, we extended our previous findings and established a model of gastric MALT lymphoma-like lesions after long-term infection with *H. pylori* using the same animals.

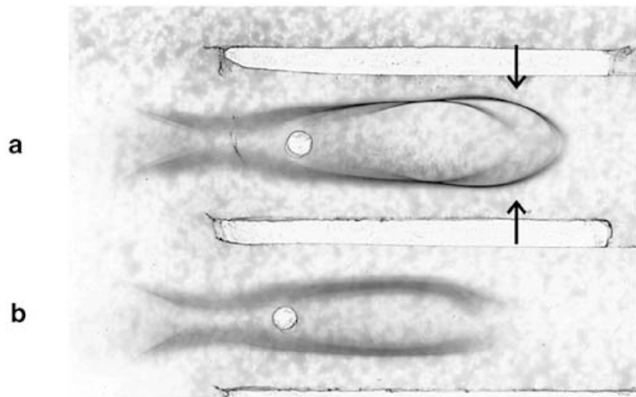
The diagnosis of human MALT lymphoma is based on pathologic criteria, which include reactive lymphoid follicles, LELs, centrocyte-like cells, and the same phenotype as marginal zone B cells (CD5<sup>-</sup>, CD10<sup>-</sup>, CD19<sup>+</sup>, CD20<sup>+</sup>). Genetic analysis such as detection of t(1;14), t(11;18), trisomy 3, and rearrangement of immunoglobulin heavy chains, and immunohistochemical analysis such as detection of restricted immunoglobulin light chains ( $\kappa$ ,  $\lambda$ ) and positive bcl-2 and negative cyclin D1 staining are also helpful for the diagnosis of human MALT lymphoma.<sup>18–20</sup> Our nTx BALB/c mice with chronic *H. pylori* infection for more than 6 months had reactive lymphoid follicles with massive LELs,



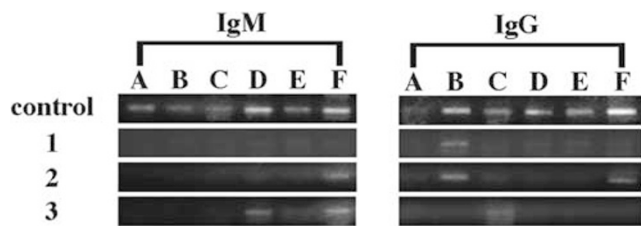
**Figure 2** Immunohistochemical characterization of infiltrating lymphocytes in the gastric mucosa of *H. pylori*-infected nTx mice 12 months after infection. Massive B220<sup>+</sup> cells (a), scattered CD4<sup>+</sup> cells (b), and no CD8<sup>+</sup> cells (c) were observed in lymphoid follicles. Original magnification,  $\times 200$ . Staining of Bcl-X<sub>L</sub> was observed in some follicular B lymphocytes 12 months after *H. pylori* infection (d), whereas no Bcl-X<sub>L</sub>-positive B lymphocytes were observed in noninfected nTx mouse (e). Original magnification,  $\times 1000$ .

mono- or oligoclonality of immunoglobulin heavy-chain genes, positive Bcl-X<sub>L</sub> staining, a Bcl-2 family protein in the lymphoid follicles, and M-proteinemia. These findings strongly suggest that gastric lesions induced by *H. pylori* infection in our mice are similar to human gastric MALT lymphoma.

The BALB/c mouse is a Th2-dominant strain for intracellular infections such as those by *Leishmania major*, whereas the C57BL/6J mouse is believed to be a Th1-dominant strain.<sup>21</sup> In BALB/c mice, nTx depletes CD25<sup>+</sup> regulatory T cells in the periphery, which results in the development of Th1-dominant autoimmune atrophic gastritis with the loss of



**Figure 3** Serum immunoelectrophoresis of nTx mouse 6 months after *H. pylori* infection (a) and that without infection (b). A monoclonal band (M-protein) was observed in 30% (3/10) of the infected mice. Arrows indicate monoclonal bands.



**Figure 4** RT-PCR of specific IgM and IgG heavy-chain genes of the gastric B lymphocytes in nTx mice with or without *H. pylori* infection (6 months). Control shows positive bands for all primers used, whereas M-protein-positive mice (1–3) had amplification of one or two IgM and/or IgG heavy-chain genes, suggesting mono- or oligoclonality. Control: a nTx mouse without infection. 1–3: M-protein-positive mice with *H. pylori* infection.

parietal and chief cells.<sup>22,23</sup> In BALB/c mice, development of gastric MALT lymphoma-like lesions by *H. pylori* infection was observed in nTx mice but not in non-nTx mice. Moreover, lymphoid follicle formation by *H. pylori* infection was never observed in C57BL/6J mice irrespective of nTx, although these mice developed severe active gastritis by *H. pylori* infection, which is believed to be a typical Th1-type inflammation (data not shown).<sup>24–26</sup> These data suggest that, in contrast to *H. pylori*-induced active gastritis, both Th1 and Th2 responses are necessary for the development of MALT lymphoma-like lesions by *H. pylori* infection. Supporting this idea, we observed, in addition to IFN- $\gamma$ , expression of IL-4, a representative Th2-type cytokine, in the gastric mucosa of only *H. pylori*-infected BALB/c mice with nTx that developed lymphoid follicles.<sup>5,12</sup> Taken together, our previous and present findings suggest that genetic background and the resulting specific immune responses to *H. pylori* have critical roles in the development of MALT lymphoma-like lesions.

Overexpression of *bcl-2* identified in human B-cell follicular lymphoma is associated with the

translocation of t(14;18), which results in the prolongation of cell survival by inhibition of apoptosis.<sup>27</sup> In addition, positive staining of Bcl-2 protein is observed in human MALT lymphoma.<sup>19,28,29</sup> In a preliminary study, however, we performed immunohistochemical staining for Bcl-2 protein in our mice, but did not detect positive staining in infiltrating B cells (data not shown). Therefore, in the present study, we studied *bcl-X<sub>L</sub>*, a *bcl-2* family gene that is essential for the development of B cells by providing a survival signal for the maintenance of pre-B cells that becomes negative as they mature.<sup>30–32</sup> Bcl-X<sub>L</sub> protein was not observed at 2 and 4 months after infection, but was clearly expressed in the MALT lymphoma-like lesions after 6 months. These data are compatible with the fact that in B-lymphocyte *bcl-X<sub>L</sub>* mRNA is upregulated by administration of *Helicobacter* antigen *in vitro*.<sup>17</sup> Thus, Bcl-X<sub>L</sub> might have an important role as an antiapoptotic molecule in the development of MALT lymphoma-like lesions in our mice.

In conclusion, our results demonstrated that gastric MALT lymphoma-like lesions developed in the gastric mucosa of nTx BALB/c mice after long-term *H. pylori* infection. This animal model appears to be useful for elucidating the mechanism for the development of gastric MALT lymphoma induced by *H. pylori* infection.

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