Infiltration of CD8+ T cells containing RANTES/CCL5+ cytoplasmic granules in actively inflammatory lesions of human chronic gastritis

Noriko Ohtani[1,2,*], Haruo Ohtani[1,3], Takashi Nakayama[4], Hiroshi Naganuma[2], Eiichi Sato[1], Toshio Imai[5], Hiroshi Nagura[1] and Osamu Yoshie[4]

[1]Department of Pathology, Tohoku University Graduate School of Medicine, Japan; [2]Sendai City Hospital, Sendai, Japan; [3]Mito National Hospital, Mito, Japan; [4]Department of Microbiology, Kinki University School of Medicine, Osaka-Sayama, Japan and [5]Kan Research Institute, Kyoto, Japan

Chronic gastritis is frequently associated with infection of *Helicobacter pylori* and characterized by tissue infiltration of neutrophils, lymphocytes, and plasma cells. To address the mechanism of lymphocyte infiltration in chronic gastritis, we examined the expression of chemokines and their receptors using frozen sections of chronic gastritis, obtained from 23 patients who underwent gastrectomy for gastric cancer. By immuno histochemistry, lymphocytes in inflamed gastric mucosa expressed CCR5 abundantly, CXCR3 less frequently, and CCR4 sparsely. The numbers of CCR5+ cells, which were composed of mainly CD8+ and partly CD4+ T cells, were positively correlated with the degree of neutrophil infiltration, and decreased in areas with intestinal metaplasia or mucosal atrophy. RANTES/CCL5, one of the ligands of CCR5, was localized mainly in CD8+ and partly CD4+ T cells with a characteristic dotted pattern, and such lymphocytes were most densely distributed around the neck region of gastric glands. *In situ* hybridization confirmed the expression of CCL5 mRNA in these cells, and immunoelectron microscopy revealed localization of CCL5 in the membrane-bound granules, which most probably corresponded to the cytolytic granules of cytotoxic T cells. The numbers of CCL5+ lymphocytes showed a close correlation with the degree of neutrophil infiltration and markedly decreased in intestinal metaplasia. In conclusion, our data suggest that, together with neutrophils, CCL5+ T cells, presumably activated cytotoxic T cells, would play important roles in the active inflammatory process of chronic gastritis. Our data also suggest a self-recruiting mechanism involving CCR5 and CCL5 for tissue accumulation of such T cells.
Laboratory Investigation (2004) **84,** 368–375, advance online publication, 5 January 2004; doi:10.1038/labinvest.3700039

Keywords: gastritis; CCR5; RANTES/CCL5; Cytotoxic T cells; cellular immunity

Chronic gastritis is frequently associated with infection of *Helicobacter pylori* and characterized by tissue infiltration by neutrophils, plasma cells, and lymphocytes. Together with the virulence of *H. pylori*, such cellular infiltrate is considered to play major pathogenic roles in chronic gastritis, which eventually causes mucosal atrophy, intestinal metaplasia, and gastric cancer.[1–3] Chemokines are a group of small cytokines that play important roles in inflammatory and immunological responses by recruiting selective types of leukocytes.[4] Previous studies revealed the overexpression of CXC chemokines, Groα/CXCL1, and IL-8/CXCL8, and prominent infiltration of neutrophils expressing their receptors CXCR1 and CXCR2 in active gastritis.[5–8] Lymphocytes consisting of CD4+ and CD8+ T cells are also known to infiltrate in chronic gastritis.[9] However, roles of chemokines in lymphocyte infiltration in chronic gastritis remain to be elucidated.

It is known that memory/effector T cells are polarized into two major functional subsets; type 1 T cells (Th1/Tc1) that produce TNF-β and IFN-γ, and type 2 T cells (Th2/Tc2) that produce IL-4, IL-5, IL-6, IL-10, and IL-13.[10] Type 1 T cells are involved in

cell-mediated immunity and tissue-specific auto immune disorders, while type 2 T cells promote humoral immunity and allergic responses. Recent studies have shown that type 1 T cells preferentially express CCR5 and CXCR3, while type 2 T cells express CCR4 and possibly CCR3 and CCR8 as well (Yoshie *et al*[4]). CCR5 is the shared receptor for MIP-1α/CCL3, MIP-1β/CCL4, and RANTES/CCL5. It is also expressed by T cells upon antigenic stimulation in the presence of IL-12.[11] CXCR3 is the shared receptor of IP-10/CXCL10, MIG/CXCL9, and I-TAC/CXCL11, which are commonly inducible by IFN-γ.[12,13] CXCR3 is also expressed by activated T cells and NK cells.[14] In human inflamed, nonlymphoid tissues, CD4$^+$ T cells expressing CCR5 and CXCR3 accumulate with frequencies much higher than those in the peripheral blood, even without obvious specificity to the target organs.[4,15] CCR4 is the shared receptor for TARC/CCL17 and MDC/CCL22, and has been shown to be involved in various Th2-type immune responses in mice and humans.[4,16,17] To understand the mechanism of lymphocyte infiltration in chronic gastritis, especially in terms of the Th1 and Th2 balance, the present study was designed to analyze the *in situ* expression of CCR4, CCR5, CXCR3, and their corresponding chemokine ligands in chronic gastritis tissues. We report here that lymphocytes in chronic gastritis predominantly express CCR5, and a fraction of these cells further contain its ligand RANTES/CCL5 in their cytoplasmic granules. We discuss possible involvement of cytotoxic T cells in the pathogenesis of chronic gastritis.

Materials and methods

Patients

Tissue samples of chronic gastritis were obtained from 23 patients who underwent gastrectomy at Sendai City Hospital in 2000 and 2001 for the following diseases: 20 patients for gastric cancer (all adenocarcinoma), one for esophageal cancer (squamous cell carcinoma, TNM stage III), and two for chronic gastric ulcer. The age ranged from 32 to 83 years, with the average of 63.5 years. The male to female ratio was 14:9. The TNM stages of gastric cancer were I A in seven patients, I B in seven, II in three, III A in two and IV in one. Gastric ulcer was regarded as stage 0 for the statistical analysis. The present study was approved by the Ethical Committees of Tohoku University Graduate School of Medicine and Sendai City Hospital.

Tissue Processing

Immediately after surgical resection, fresh specimens were obtained from macroscopically non-neoplastic mucosa of the corpus and antrum. Specimens were fixed in periodate–4% paraformaldehyde–lysine for 4 h and frozen in all cases for immunohisto-

chemistry, and also fixed in 0.5% glutaraldehyde and 4% paraformaldehyde overnight and embedded in paraffin for *in situ* hybridization.[18] For RT-polymerase chain reaction (PCR), tissue specimens were snap frozen and kept at −80°C until RNA preparation.

RT-PCR

This was carried out as described previously[19] using six gastritis specimens from three patients with gastric cancer. Age and cancer stage of patients 1, 3, and 4 in Figure 1 were 64 years/stage IA, 36 years/ stage IV, and 81 years/stage IA, respectively. All were male Japanese. Considering the high prevalence of *H. pylori* infection in Japan, we purchased three so-called normal stomach RNA samples. Normal stomach 1, 2, and 3 in Figure 1 were 28-year-old male and 62-year-old male donors (Cell Applications, Inc., San Diego, CA, USA), and 52-year-old female donor (Stratagene, La Jolla, CA, USA), respectively. The primer for TARC/CCL17 was +5′-ACTGCTCCAGGGATGCCATCGTTTTT-3′ and −5′-ACAAGGGGATGGGATCTCCCTCACTG-3′. Other primers used were already described.[19,20] Amplification conditions were 35 cycles of
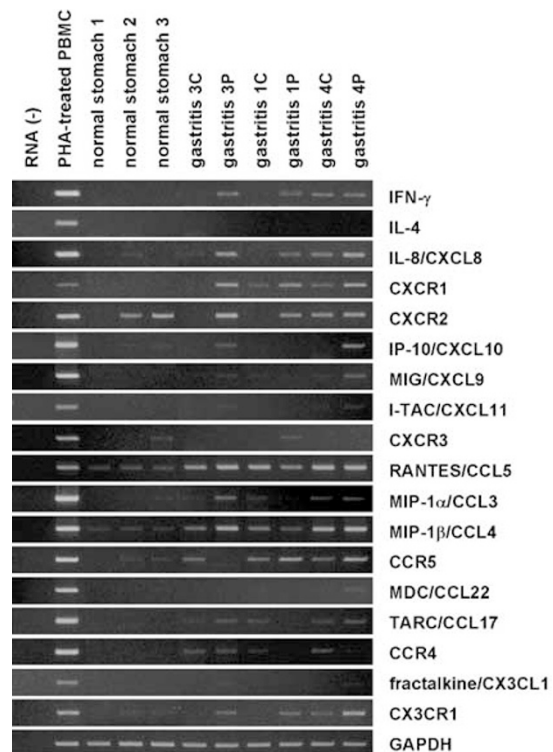
Figure 1 RT-PCR analysis. RT-PCR analysis was carried out for cytokines, chemokines, and chemokine receptors. Samples are as follows: RNA(−), no RNA (negative control), and PHA-treated PBMC, peripheral blood mononuclear cells treated with phytohemagglutinin for 3 days (positive control). C and P stand for corpus and pylorus of each patient. For details, see Materials and methods.

denaturation at 94°C for 30 s (5 min in the first cycle), annealing at 60°C for 30 s, and extension at 72°C for 30 s (5 min in the last cycle) for chemokines and chemokine receptors, and 27 cycles for GAPDH.

Antibodies and Immunohistochemistry

We used the following antibodies: anti-CCR4 (clone KM2160, mouse IgG1; applied at 1.14 μg/ml),[21] anti-CCR5 (clone 2D7, mouse IgG2a, PharMingen, San Diego, CA, USA; applied at 0.25 μg/ml), anti-CXCR3 (clone 49801.111, mouse IgG1, PharMingen; applied at 0.5 μg/ml), anti-RANTES/CCL5 (clone VL1, mouse IgG2b, Biosource International, Camarillo, CA, USA; applied at 2.0 μg/ml), anti-MIP-1α/CCL3 (clone 14215.41, mouse IgG2a, DAKO Japan, Kyoto, Japan; applied at 5 μg/ml), anti-MIP-1β/CCL4 (clone 24996.111, mouse IgG2b, DAKO Japan; applied at 5 μg/ml), anti-IP-10/CXCL10 (clone 33036.21, mouse IgG1, DAKO Japan; applied at 10 μg/ml), and rabbit polyclonal anti-human CX3CR-1 antibody applied at 0.2 μg/ml.[22] For double immunohistochemistry, we also used anti-CD4 (clone SK3, mouse IgG1, Becton-Dickinson, San Joe, CA, USA; applied at 1:200), anti-CD8 (clone SK1, mouse IgG1, Becton-Dickinson; applied at 1:100), anti-CD20 (clone L-27, mouse IgG1, Becton-Dickinson; applied at 1:200), anti-CD68 (clone EBM-11, mouse IgG1, DAKO Japan; applied at 1:15 000) and anti-CD-Lamp (clone 104.G4, mouse IgG1, ImmunoTech, Marseille, France; applied at 0.2 μg/ml). We also used rabbit polyclonal anti-*H. pylori* (DAKO Japan; applied at 1:500) and monoclonal antineutrophil elastase (NP-57, mouse IgG1, Dako Japan; applied at 1:500). We adopted the immunoperoxidase method using En-vision plus (Dako).[19] The primary and secondary antibodies were applied overnight and for 1 h, respectively. The chromogen and substrate were diaminobenzidine tetrahydrocloride and H_2O_2, respectively. For RANTES/CCL5 staining, we modified the method to enhance the permeability of antibodies into granules as already described.[20] The methods of double-labeling immunohistochemistry, the condition of control staining, and the chromogens used in RANTES-CD8 double staining were described elsewhere.[19,20]

Immunoelectron Microscopy

We adopted, pre-embedding immunoperoxidase method using PLP-fixed frozen sections.[18]

In situ Hybridization

The coding region of RANTES/CCL5 was amplified from PHA-treated peripheral blood leukocyte cDNA by PCR. Digoxigenin-labeled sense and antisense riboprobes were generated from the 276-bp fragment of RANTES/CCL5 cDNA subcloned into pCR script

SK+, and the *in situ* hybridization procedures were carried out as describe previously.[18] Briefly, the specimens were pretreated by microwave heating in the aqueous phase and proteinase K digestion. The probe concentration was 1 μg/ml. After washing, hybridized probes were visualized by alkaline phosphatase reaction according to the manufacturer's manual (Boehringer Manheim).

Assessments of Results

We scored the following variables into three grades as described in the updated Sydney classification:[23] *H. pylori*, neutrophils, mononuclear cells, atrophy of gastric glands, and intestinal metaplasia. Of these, grades 0–1 and 2–3 were classified into mild and severe, respectively. Intestinal metaplasia was evaluated by alcian blue-PAS stain, and *H. pylori* infection was evaluated by immunohistochemistry using anti-*H. pylori* antibody. For neutrophil assessment, immunohistochemistry for neutrophil elastase was adopted using alkaline phosphatase as labeling enzyme.

We counted the numbers of cells immunoreactive for CCR5, CXCR5, CCR4, and neutrophil elastase in the lamina propria of the pit areas using an ocular grid, 0.25×0.075 mm^2 in size, at ×400 magnification (0.0188 mm^2). The number of these cells was expressed as number per 0.19 mm^2, the whole area of a ×400 field. In nine specimens, we divided the specimens into two areas because of apparent histological heterogeneity. The total number of areas we examined amounted to 54. We measured seven to nine microscopic fields for each area, and the average numbers were calculated.

We adopted a different method to quantify immunoreactive cells for RANTES/CCL5: their numbers were counted using the whole area of a ×400 field (0.19 mm^2). A total of 62 microscopic fields were randomly chosen in the neck regions from 16 specimens obtained from eight patients. The degrees of neutrophil infiltration and intestinal metaplasia were judged by hematoxylin counterstaining in the same microscopic fields.

For statistical analysis, non parametric Friedman's test, Mann–Whitney's test, multiple comparison, and Spearman's test were used (SPSS 11.0, SPSS Japan, Tokyo).

Results

RT-PCR Analysis

To obtain an overall view, we carried out RT-PCR analysis (Figure 1). First, we corroborated the predominant expression of IFN-γ over IL-4 in gastritis tissues, supporting type 1-dominant immune responses in chronic gastritis.[9] IL-8/CXCL8 and its receptors, CXCR1 and CXCR2, were also expressed in gastritis tissues more apparently than in normal mucosa except two of three 'normal'

samples expressed CXCR2 signals (see Materials and methods for details). RANTES/CCL5, MIP-1α/CCL3, MIP-1β/CCL4, and their receptor CCR5 were generally more clearly expressed in gastritis tissues than in normal tissues. On the other hand, IP-10/CXCL10, MIG/CXCL9, I-TAC/CXCL11, and their shared receptor CXCR3 were generally inconspicuous in both gastritis and normal tissues. MDC/CCL22, TARC/CCL17, and their receptor CCR4 were also inconspicuously expressed in gastritis and normal tissues. The results suggested infiltration of neutrophils expressing CXCR1 and CXCR2 and other types of leukocytes expressing CCR5 in chronic gastritis. Notably, the signals of CX3CR1 were also frequently detected in gastritis tissues, suggesting infiltration of CX3CR1-expressing cytotoxic T cells,[22] although the expression of its ligand fractalkine/CX3CL1 was hardly detected.

Immunohistochemistry for Chemokine Receptors

First, we corroborated that CD3$^+$ T cells were quite infrequent in noninflamed gastric mucosal tissues in three cases, which were selected by screening of paraffin-embedded biopsy samples of 150 cases (data not shown). Next, we examined the immunohistochemical expression of CCR5, CXCR3, and CCR4 in chronic gastritis using 45 frozen sections obtained from 23 cases. All cases were infected with *H. pylori* as revealed by immunostaining (data not shown). CCR5$^+$ mononuclear cells were abundantly observed, which were mainly distributed in the lamina propria of all layers of the mucosa (Figure 2a). CXCR3$^+$ cells were less frequent and mostly confined to a deeper zone of the mucosa (Figure 2b). Cells expressing CCR4 were mostly absent in the inflamed mucosa (Figure 2c) and only seen in the peripheral T-cell zone of lymphoid follicles (data not shown). No CCR3$^+$ mononuclear cells were observed in inflamed mucosa (data not shown). Double staining performed in three representative cases revealed that CCR5$^+$ cells were composed of both CD8$^+$ and CD4$^+$ T cells, with the former predominant (Figures 2d and e). Macrophages or dendritic cells were negative for CCR5, CXCR3, or CCR4 (not shown). Immunohistochemistry for CX3CR1 performed in three representative gastritis cases showed that CX3CR1$^+$ mononuclear cells were detected in the lamina propria of the neck region (Figure 2f). As CX3CR1 is selectively expressed by perforin- and granzyme B-positive fully differentiated cytotoxic effector T cells,[22] this suggested infiltration of cytotoxic T cells in chronic gastritis. Negative controls showed no specific reactivity (Figure 2g).

Morphometric Analyses of Chemokine Receptors

Figure 3a shows a quantitative change of CCR5$^+$, CXCR3$^+$, and CCR4$^+$ mononuclear cells in chronic
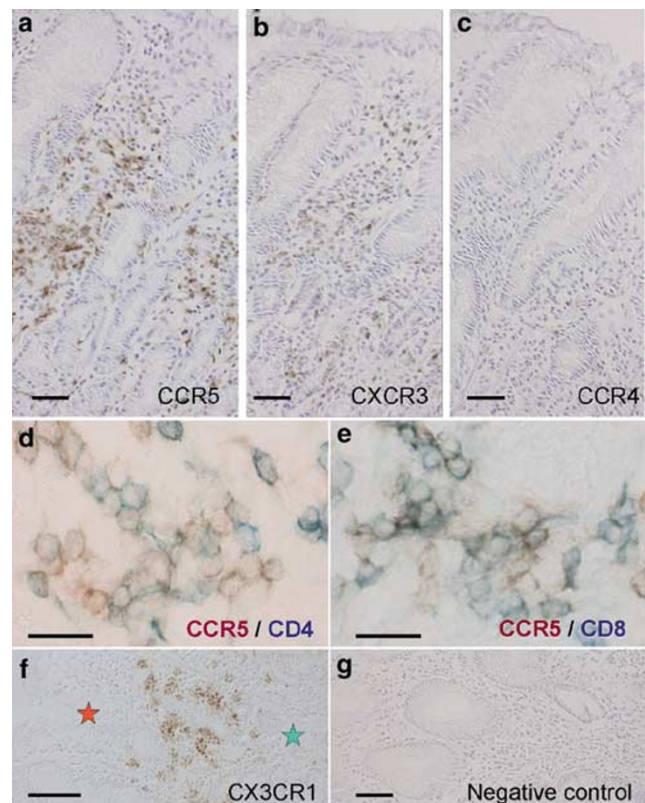

Figure 2 Immunohistochemistry for chemokine receptors. Low-power views of CCR5 (**a**), CXCR3 (**b**), and CCR4 (**c**), showing representative staining patterns (brown color). Hematoxylin counterstaining (**a–c**). Double staining for CCR5 and CD4 (**f**), and CCR5 and CD8 (**g**) (CCR5 in red, and CD4 or CD8 in blue). Representative results of three patients are shown. Localization of CX3CR1$^+$ cells (brown color) around the neck region (**f**), with red and green asterisks indicating the pit and gastric gland areas, respectively. Negative control with mouse IgG$_1$ (**g**). Negative controls with mouse IgG$_{2a}$, IgG$_{2b}$, and normal rabbit serum also showed no specific reactivity (not shown). Scale bars, 50 μm in **a–c**, **f**, and **g**, and 20 μm in **d** and **e**.

gastritis with CCR5$^+$ cells most abundant. The numbers of CCR5$^+$ cells were correlated with the degree of neutrophil infiltration (Figure 3b). The numbers of CCR5$^+$ cells were less abundant in the antrum than in the corpus ($P < 0.01$, Figure 3c), in severe intestinal metaplasia (15 areas) than in mild metaplasia (35 areas) ($P < 0.05$, Figure 3d), and in severe atrophy (25 areas) than in mild atrophy (12 areas) ($P < 0.01$, not shown). Since these patients underwent gastrectomy for gastric cancer, we explored a possible correlation with cancer stages. The numbers of CCR5$^+$ cells showed no significant correlation with cancer stage, patients' age, or gender (data not shown).

Immunohistochemistry for Chemokines

RANTES/CCL5 was clearly detected in infiltrating lymphocytes with a characteristic dotted pattern (Figure 4a). This pattern was quite similar to that of
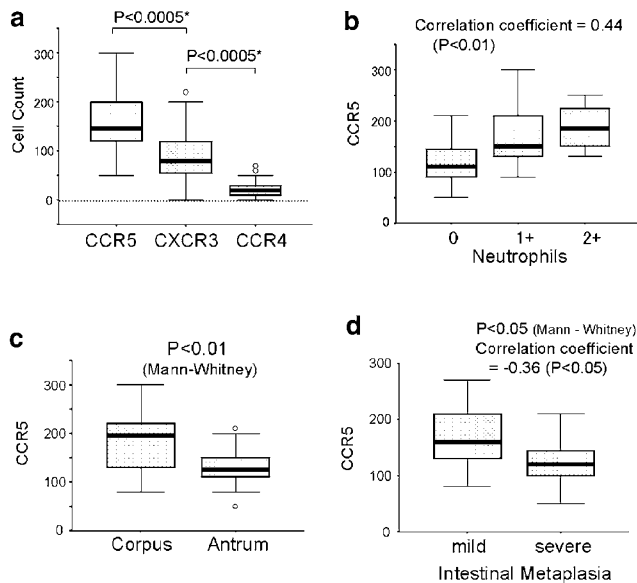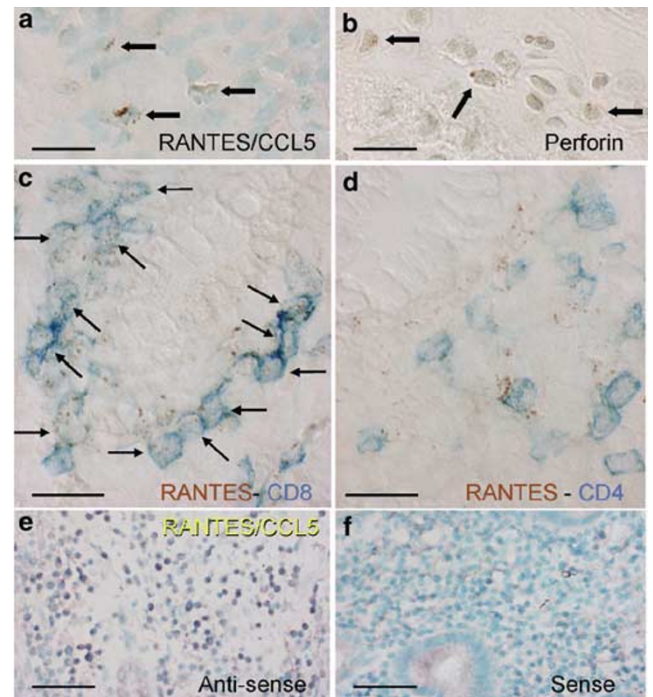
Figure 3 Quantitative analyses of chemokine receptor-positive cells in chronic gastritis. Vertical axis represents number of immunoreactive cells per 0.19 mm², the whole area of a ×400 field. All figures are expressed by Box–Whisker plot showing 75-, median-, and 25-percentile values. (a) Comparison among CCR5⁺, CXCR3⁺, and CCR4⁺ mononuclear cells. (b) Correlation between the number of CCR5⁺ cells and the degree of neutrophil infiltration. The numbers of areas showing neutrophil infiltration of 0, 1+, 2+, and 3+ were 12, 23, 4, and 0, respectively. (c) Difference of the number of CCR5⁺ cells between corpus and antrum. (d) Correlation between the number of CCR5⁺ cells and the degree of intestinal metaplasia. Correlation analyzed by Spearman's test (b, d).

Figure 4 Identification of RANTES/CCL5⁺ cells. Immunohistochemistry for RANTES (a) and perforin (b), both showing a dotted pattern in lymphocytes (brown color; arrows). Methygreen counterstaining with scale bar, 20 μm (a, b). Double immunohistochemitry for RANTES/CCL5 and CD8 (c), and RANTES/CCL5 and CD4 (d). Note that RANTES/CCL5⁺ granules (brown) were localized mainly in CD8⁺ T cells (blue) (arrows in c). Representative results from four patients are shown. No counterstaining with scale bar, 20 μm (c, d). In situ hybridization for RANTES/CCL5 (e) revealed that positive signals (dark purple) were seen in small mononuclear cells in the lamina propria. No definite signals were observed with sense probe (f). Methyl green counterstaining with scale bar, 50 μm (e, f).

perforin (Figure 4b), one of the contents of the cytolytic granule of cytotoxic T cells. Double staining performed in four representative cases consistently revealed that RANTES/CCL5⁺ cells were mostly CD8⁺ and partly CD4⁺ T cells (Figures 4c and d). These cells were concentrated near the gastric glands of the neck region (Figure 4c). CD14⁺ monocyte/macrophage-lineage cells or DC-lamp⁺ mature dendritic cells were negative for RANTES/CCL5 (data not shown). MIP-1α/CCL3 was positive in the same pattern as RANTES/CCL5, but only sparsely (data not shown). We were unable to demonstrate MIP-1β/CCL4, probably due to the limitation of the antibody used. Consistently with the results of RT-PCR analysis, no clear staining was observed for IP-10/CXCL10 or MIG/CXCL9 in gastritis tissues (data not shown).

In situ Hybridazation for RANTES/CCL5

Mononuclear infiltrate expressed mRNA for RANTES/CCL5 in chronic gastritis by in situ hybridization performed in four representative cases (Figure 4e). No such signals were detected with a sense probe (Figure 4f). Eck et al[7] reported that RANTES/CCL5 mRNA was positive in vascular endothelial cells in human gastritis tissues. In the

present study, we did not confirm positive signals for RANTES/CCL5 mRNA or its protein in this type of cells.

Immunoelectron Microscopy for RANTES/CCL5

Immunoelectron microscopy performed in two representative cases confirmed that RANTES/CCL5 was localized within membrane-bound granules that were sparsely distributed in the cytoplasm of lymphocytes (Figure 5). These granules, contrasted to abundant granules in NK cells, corresponded to cytolytic granules of cytotoxic T cells,[24] suggesting that cytotoxic T cells carry RANTES/CCL5⁺ granules in chronic gastritis.

Correlation of RANTES/CCL5+ Lymphocytes with Pathological Findings

To explore the significance of RANTES/CCL5⁺ lymphocytes in the pathogenesis of chronic gastritis, we quantified RANTES/CCL5⁺ lymphocytes in the
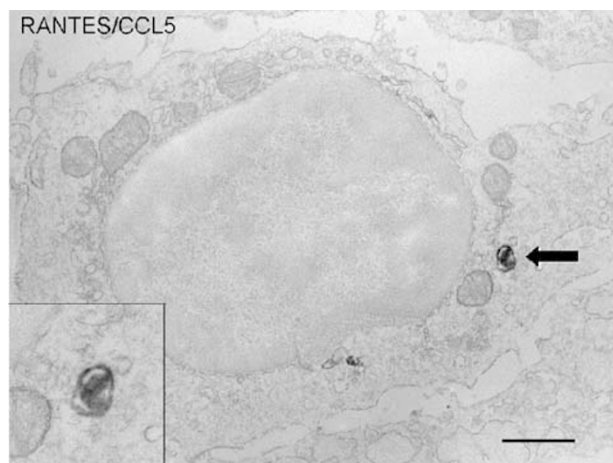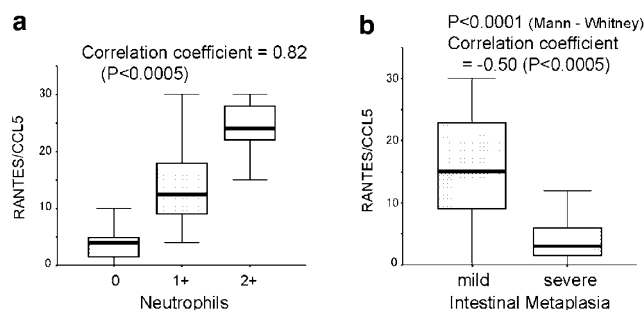
Figure 5 Immunoelectron microscopy for RANTES/CCL5 showing an immunoreactive granule in this lymphocyte (arrow) in black color by osmificated diaminobenzidine. Inset: a higher magnification of the granule. Scale bar, 1 μm.

Figure 6 Correlation of RANTES/CCL5$^+$ cells with the activity of gastritis. Morphometry was carried out as described in Materials and methods. Vertical axis represents the number of lymphocytes immunoreactive for RANTES/CCL5 per 0.19 mm^2, the whole area of a $\times$400 field. Data are expressed by Box–Whisker plot. The numbers of RANTES/CCL5$^+$ lymphocytes were correlated with the degree of neutrophil infiltration (**a**). The numbers of fields showing neutrophil infiltration of 0, 1+, 2+, and 3+ were 16, 30, 14, and 0, respectively. The numbers of RANTES/CCL5$^+$ lymphocytes were inversely correlated with the degree of intestinal metaplasia (**b**). The numbers of fields showing intestinal metaplasia scored mild and severe were 49 and 11, respectively. Correlation was analyzed by Spearman's test.

neck region of gastric glands. As shown in Figure 6a, a clear correlation was observed between the numbers of RANTES/CCL5$^+$ lymphocytes and the degree of neutrophil infiltration. The numbers of RANTES/CCL5$^+$ cells showed a marked decrease with the progress of intestinal metaplasia (Figure 6b). There were no significant differences in the numbers of RANTES/CCL5$^+$ lymphocytes between the corpus and antrum or between areas with mild atrophy and those with severe atrophy (data not shown). No significant correlation was found between the numbers of RANTES/CCL5$^+$ lymphocytes and cancer stage (data not shown).

Discussion

Chronic gastritis is characterized by tissue infiltration of neutrophils, lymphocytes, and plasma cells. It is now known that chemokine receptors not only explain the mechanism of tissue infiltration of leukocytes but also provide useful markers for the types of immune response including Th1/Th2 balance.[4] Here, we examined the *in situ* expression of Th1- and Th2-type chemokine receptors, namely, CCR5, CXCR3, and CCR4, and their respective ligands in chronic gastritis tissues. We have shown that CCR5 was abundantly expressed in lamina propria-infiltrating mononuclear cells, while CXCR3$^+$ cells were less frequent and CCR4$^+$ cells were sparse. This expression pattern is partly consistent with a dominance of type 1 immune response in human gastritis.[9] Of particular interest, we have further revealed that a fraction of infiltrating CD8$^+$ T cells and some CD4$^+$ T cells contain its ligand RANTES/CCL5 in their cytoplasmic granules, and that such cells were abundant around the neck region of gastric glands associated with neutrophil response. Even though we were unable to prove the

coexpression of CCR5 and RANTES/CCL5 in the same cells due to technical difficulties, our data suggest that a fraction of CCR5$^+$ T cells coexpress RANTES/CCL5. Previous studies documented the expression of RANTES/CCL5 in chronic gastritis with *H. pylori* infection.[6,7,25] Here, we have clarified its cellular and subcellular localization.

Previous *in vitro* studies using confocal laser microscopy have shown colocalization of RANTES/CCL5 with perforin in the cytolytic granules of CD8$^+$ cytotoxic T cells.[26,27] Our observation of the presence of RANTES/CCL5 in cytoplasmic granules of infiltrating CD8$^+$ T cells is consistent with these observations. Our findings are also consistent with a recent microarray study revealing that, in contrast to naïve CD8$^+$ T cells, effector CD8$^+$ T cells contain transcripts of CCR5, RANTES/CCL5, and MIP-1β/CCL4 at high levels.[28] Furthermore, Swanson *et al*[29] have demonstrated that memory T cells constitutively express RANTES/CCL5 mRNA but produce its protein only after stimulation via T-cell receptors. Therefore, RANTES/CCL5$^+$ CD8$^+$ T cells present in gastritis tissues are most probably memory CD8$^+$ T cells that have been stimulated with cognate antigens (activated cytotoxic T cells). Together with our similar observation in oral lichen planus,[20] our studies have demonstrated the presence of T cells carrying RANTES/CCL5$^+$ granules, presumably activated cytotoxic T cells, for the first time in human chronic inflammatory lesions. Owing to technical limitations, we were unable to test the possible presence of MIP-1α/CCL3 and MIP-1β/CCL4 in RANTES/CCL5$^+$ T cells.

Of particular interest, RANTES/CCL5$^+$ lymphocytes are enriched around the neck region of gastric glands, clearly correlated with neutrophil infiltration, and decreased in intestinal metaplasia.

374

Infiltration of neutrophils represents active inflammatory phase in chronic gastritis, while intestinal metaplasia represents one of the final stages of chronic gastritis after the interval of 10–20 years.[30] Thus, our data suggest that, together with neutrophils, RANTES/CCL5$^+$ cytotoxic T cells would play an important role for the tissue damage in active inflammatory processes of chronic gastritis. The close correlation between RANTES/CCL5$^+$ lymphocytes and neutrophils also suggests some functional interactions between cytotoxic T cells mediating cellular immunity and neutrophils. This notion may be consistent with a recent report showing that enhanced *Helicobactor*-specific Th1 immune response in IL-10$^{-/-}$ mice was diminished by the depletion of neutrophils.[31]

In conclusion, the present study has clarified an important aspect of active inflammatory processes in chronic gastritis by identifying tissue-infiltrating CD8$^+$ T cells bearing RANTES/CCL5$^+$ cytoplasmic granules. Elucidation of their roles in cellular immune responses in human chronic gastritis would be an interesting subject of future studies. As for the possible roles of RANTES/CCL5 released by T cells, it may enhance antigen-specific Th1 and CTL responses,[27] upregulate lysis of target cells and costimulatory function of antigen-presenting cells,[32] promote T-cell proliferation,[33,34] and further recruit CCR5$^+$ effector T cells (a self-recruiting mechanism). These possibilities remain to be analyzed.

References

1 Genta RM. *Helicobacter pylori*, inflammation, mucosal damage, and apoptosis: pathogenesis and definition of gastric atrophy. Gastroenterology 1997;113:S51–S55.

2 Genta RM. The immunobiology of Helicobacter pylori gastritis. Semin Gastrointest Dis 1997;8:2–11.

3 Graham DY. *Helicobacter pylori* infection in the pathogenesis of duodenal ulcer and gastric cancer: a model. Gastroenterology 1997;113:1983–1991.

4 Yoshie O, Imai T, Nomiyama H. Chemokines in immunity. Adv Immunol 2001;78:57–110.

5 Shimada T, Terano A. Chemokine expression in *Helicobacter pylori*-infected gastric mucosa. J Gastroenterol 1998;33:613–617.

6 Yamaoka Y, Kita M, Kodama T, *et al.* Chomokines in the gastric mucosa in *Helicobacter pylori* infection. Gut 1998;42:609–617.

7 Eck M, Schmausser B, Scheller K, *et al.* CXC chemokines Groα/IL-8 and IP-10/MIG in *Helicobacter pylori* gastritis. Clin Exp Immunol 2000;122:192–199.

8 Ohtani N, Ohtani H, Oki M, *et al.* CXC chemokine receptor 1 (CXCR1) is expressed by mainly neutrophils in inflamed gut and stomach tissues. Tohoku J Exp Med 2002;196:179–184.

9 Bamford KB, Fan X, Crowe SE, *et al.* Lymphocytes in the human gastric mucosa during *Helicobacter pylori* have a T helper cell 1 phenotype. Gastroenterology 1998;114:482–492.

10 Neurath MF, Finotto S, Glimcher LH. The role of TH1/ Th2 polarization in mucosal immunitiy. Nat Med 2002;8:567–573.

11 Iwasaki M, Mukai T, Gao P, *et al.* A critical role for IL-12 in CCR5 induction on T cell receptor-triggered mouse CD4(+) and CD8(+) T cells. Eur J Immunol 2001;31:2411–2420.

12 Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. Immunity 2000;12:121–127.

13 Sallusto F, Mackay CR, Lanzavecchia A. The role of chemokine receptors in primary, effector and memory immune responses. Annu Rev Immunol 2000;18: 593–620.

14 Qin S, Rottoman JB, Myers P, *et al.* The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. J Clin Invest 1998;101:746–754.

15 Kunkel EJ, Boisvert J, Murphy K, *et al.* Expression of the chemokine receptors CCR4, CCR5, and CXCR3 by humane tissue-infiltrating lymphocytes. Am J Pathol 2002;160:347–355.

16 Vestergaard C, Yoneyama H, Murai M, *et al.* Overproduction of Th2-specific chemokines in NC/Nga mice exhibiting atopic dermatitis-like lesions. J Clin Invest 1999;104:1097–1105.

17 Fujisawa T, Fujisawa R, Kato Y, *et al.* Presence of high contents of thymus and activation-regulated chemokine in platelets and elevated plasma levels of thymus and activation-regulated chemokine and macrophagederived chemokine in patients with atopic dermatitis. J Allergy Clin Immunol 2002;110:139–146.

18 Ohtani H, Motohashi H, Sato H, *et al.* Dual overexpression pattern of membrane-type metalloproteinase-1 in cancer and stromal cells in human gastrointestinal carcinoma revealed by *in situ* hybridization and immunoelectron microscopy. Int J Cancer 1996;68:565–570.

19 Katou F, Ohtani H, Nakayama T, *et al.* Macrophagederived chemokine (MDC/CCL22) and CCR4 are involved in the formation of T lymphocyte-dendritic cell clusters in human inflamed skin and secondary lymphoid tissue. Am J Pathol 2001;158: 1263–1270.

20 Iijima W, Ohtani H, Nakayama T, *et al.* Infiltrating CD8+ T-cells in oral lichen planus predominantly express CCR5 and CXCR3 and carry respective chemokine ligands RANTES/CCL5 and IP-10/CXCL10 in their cytolytic granules: a potential self-recruiting mechanism. Am J Pathol 2003;163:261–268.

21 Imai T, Nagira M, Takagi S, *et al.* Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activationregulated chemokine and macrophage-derived chemokine. Int Immunol 1999;11:81–88.

22 Nishimura M, Umehara H, Nakayama T, *et al.* Dual functions of fractalkine/CX3C ligand 1 in trafficking of perforin+/granzyme B+ cytotoxic effector lymphocytes that are defined by CX3CR1 expression. J Immunol 2002;168:6173–6180.

23 Dixon MF, Genta RM, Yardley JH, *et al.* Classification and grading of gastritis: the updated Sydney system. Am J Surg Pathol 1996;20:1161–1181.

24 Saiki Y, Ohtani H, Naito Y, *et al.* Immunophenotypical characterization of Epstein–Barr virus-associated gastric carcinoma: massive infiltration by proliferating CD8+ T-lymphocytes. Lab Invest 1996;75:67–76.

25 Kikuchi T, Kato K, Ohara S, *et al.* The relationship between persistent secretion of RANTES and residual infiltration of eosinophils and memory T lymphocytes

after *Helicobacter pylori* eradication. J Pathol 2000; 192:243–250.

26 Wagner L, Yang OO, Garcia-Zepeda EA, *et al.* Beta-chemokines are released from HIV-1-specific cytolytic T-cell granules complexed to proteoglycans. Nature 1998;391:908–911.

27 Kim JJ, Nottingham LK, Sin JI, *et al.* CD8 positive T-cells influence antigen-specific immune responses through the expression of chemokines. J Clin Invest 1998;102:1112–1124.

28 Kaech SM, Hemby S, Kersh E, *et al.* Molecular and functional profiling of memory CD8 T cell differentiation. Cell 2002;111:837–851.

29 Swanson BJ, Murakami M, Mitchell TC, *et al.* RANTES production by memory phenotype T-cells is controlled by a post-transcriptional, TCR-dependent process. Immunity 2002;17:605–615.

30 Vorobjova T, Faller G, Maaroos HI, *et al.* Significant increase in antigastric autoantibodies in a long-term follow-up study of *H. pylori* gastritis. Virchows Arch 2000;437:37–45.

31 Ismail HF, Fick P, Zhang J, *et al.* Depletion of neutrophils in IL-10$^{-/-}$ mice delays clearance of gastric *Helicobacter* infection and decreases the Th1 immune response to *Helicobacter*. J Immunol 2003;170: 3782–3789.

32 Taub DD, Ortaldo JR, Turcovski-Corrales SM, *et al.* Beta chemokines costimulate lymphocyte cytolysis, proliferation, and lymphokine production. J Leuko Biol 1996;59:81–89.

33 Taub DD, Turkovsky-Corrales SM, Key ML, *et al.* Chemokines and T lymphocyte activation: I. Beta chemokines costimulate human T lymphocyte activation *in vitro*. J Immunol 1996;156:2095–2103.

34 Lillard JW, Boyaka PN, Taub DD, *et al.* RANTES potentiates antigen-specific mucosal immune responses. J Immunol 2001;166:162–169.